Sonia Malik   *Editor*

Production of Plant Derived Natural Compounds through Hairy Root Culture

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Affectionately dedicated to my dear father.

Preface

Natural compounds derived from plants are in high demand in the world market due to their lesser side effects and many other advantages. Biotechnology, by employing various in vitro techniques, including hairy root culture, provides an important alternative for stable and large-scale production of plant-derived natural compounds.

The purpose of this book is to provide the latest information about hairy root culture and its applications, with special emphasis on the potential of hairy roots for the production of bioactive compounds. Due to high growth rate as well as biochemical and genetic stability, it is quite possible to study the metabolic pathways related to the production of bioactive compounds using hairy root culture. The chapters will discuss about the feasibility/potential of hairy roots for plant-derived natural compounds. The advantages and difficulties of hairy roots for up-scaling studies in bioreactors are also included. Successful examples of hairy root culture of plant species producing bioactive compounds used in food, flavours, or pharmaceutical industries are also discussed. There are many applications of hairy root system ranging from phytoremediation to vaccine production and drug delivery, and many are yet to be explored. In spite of several successful reports of hairy root culture, there is still gap in the knowledge for up-scaling of this culture system for commercial utilization. This book will be the answer to all these questions and will be valuable to researchers as well as students working in the area of plant natural products, phytochemistry, plant tissue culture, medicines and drug discovery.

Sao Luis, Brazil   Sonia Malik

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Sonia Malik has obtained her doctoral degree in plant biotechnology from CSIR—Institute of Himalayan Bioresource Technology, Palampur, India, in July 2009. She did masters (Gold Medalist) in Honours Botany with specialization in plant biotechnology and tissue culture in 2001 from Guru Nanak Dev University, Amritsar, India. Her basic area of research is plant secondary metabolites and genetic manipulation, and she did work on several medicinal plants. After her post-doctoral studies at Brazil, Czech Republic and the United Kingdom, Dr. Malik has joined as a Professor of Biology at Biological and Health Sciences Center, Federal University of Maranhão, Sao Luis. At present she is an active member of post-graduate research programme at UFMA and actively involved in research, teaching and supervising research students at UG/PG levels. Dr. Malik has published more than 25 research articles in reputed journals. She has authored 7 book chapters and edited a book. She is an editorial board member and reviewer of many international scientific journals.

Chapter 1
The *Agrobacterium rhizogenes GALLS* Gene Provides an Alternative Method to Transform Plants

Walt Ream, Wei Wei, Maciej Maselko, and Larry Hodges

Abstract *Agrobacterium rhizogenes* and *A. tumefaciens* transfer DNA and effector proteins into plant cells, where transferred DNA (T-DNA) is inherited and expressed. Most transgenic plants are created using *A. tumefaciens*, but transformation by *A. rhizogenes* yields desirable single-copy transgenes more frequently than *A. tumefaciens* does. DNA transfer from both species resembles plasmid conjugation, but later events differ between species. Efficient transformation by *A. tumefaciens* requires single-strand DNA-binding protein VirE2, which *A. rhizogenes* lacks, so substrates for T-DNA integration differ greatly. In *A. rhizogenes*, the GALLS proteins substitute for (but do not resemble) VirE2. GALLS proteins occur in two forms: full-length (FL) and a more abundant C-terminal domain (CT). Both have protein-binding domains and type IV secretion signals. GALLS-FL has ATPase/ strand transferase and nuclear localization (NLS) domains, allowing it to enter the nucleus and bind VirD2, a pilot protein attached to single-stranded T-DNA (T-strands). GALLS-FL ATPase may pull T-strands into the nucleus. GALLS-CT stimulates an early step in gene transfer to plants; this effector protein alters host gene expression and stimulates T-DNA transfer, apparently by suppressing host defenses. These observations challenge the assumption that *A. rhizogenes* and *A. tumefaciens* transform plants and mitigate host defenses by the same mechanisms.

Keywords *Agrobacterium rhizogenes* • *GALLS* gene • Plant transformation • Single-copy transgenes

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Introduction

Overview *Agrobacterium tumefaciens* and *A. rhizogenes* cause crown gall and hairy root diseases by stable transfer of bacterial genes into plant cells (Chilton et al. 1977; White et al. 1985). Abnormal growth of plant tissue results from integration and expression of bacterial oncogenes located on the T-DNA region of tumor-inducing (Ti) or root-inducing (Ri) plasmids (Chilton et al. 1977, 1980; Willmitzer et al. 1980).

Some of the bacterial virulence (Vir) effector proteins that are exported to plants bind T-DNA. These proteins, together with host proteins, target T-DNA to the nucleus and maintain its integrity during integration into the genome. This makes *A. tumefaciens* a powerful tool to deliver genes to plants. Biologists created "disarmed" strains of *A. tumefaciens* that lack oncogenes but retain virulence (*vir*) genes needed to transfer T-DNA to plants (Gelvin 2003). This technology is widely used to create transgenic plants for research and biotechnology. Transgenes delivered via *A. tumefaciens* have lower copy numbers and undergo fewer rearrangements than do genes transferred by other methods.

Bacterial Effector Proteins Mediate Gene Transfer to Plants Gene transfer begins when T-DNA border sequences (Peralta et al. 1986; Peralta and Ream 1985; Shaw et al. 1984; Wang et al. 1984) are nicked by VirD1 and VirD2 (Wang et al. 1987; Yanofsky et al. 1986); VirD2 attaches to the 5′ end of the nicked strand (Durrenberger et al. 1989; Herrera-Estrella et al. 1988; Howard et al. 1989; Ward and Barnes 1988; Young and Nester 1988). VirD2 contains a secretion signal (Fig. 1.1) (Vergunst et al. 2005) and is transported into plant cells along with covalently attached single-stranded T-DNA (T-strands). Transport requires a type IV secretion system (T4SS) that includes 11 VirB proteins (Christie 1997; Sheng and Citovsky 1996; Winans et al. 1996) and VirD4, which couples the border-nicking DNA-protein complex to the secretion system (Albright et al. 1987; Jayaswal et al. 1987; Okamoto et al. 1991; Stachel et al. 1986). A nuclear localization sequence (NLS) in VirD2 (Fig. 1.1) binds host importin-α proteins, which mediate nuclear import (Ballas and Citovsky 1997; Bhattacharjee et al. 2008; Howard et al. 1992; Shurvinton et al. 1992; Tinland et al. 1992; Tzfira and Citovsky 2002).

Efficient Gene Transfer by *A. tumefaciens* Requires VirE2 Single-Stranded DNA-Binding Protein VirE2 and its secretory chaperone VirE1 are critical for pathogenesis; mutations in *virE2* reduce virulence to <1% of wild type (Christie et al. 1988; Citovsky et al. 1992; Duckely and Hohn 2003; Ward and Zambryski 2001). VirE2 contains two NLSs and a C-terminal signal for translocation into plant cells mediated by the VirB/D4 type IV secretion system (Fig. 1.1). VirE2 is required only in plant cells (Citovsky et al. 1992) and does not bind T-strands in *A. tumefaciens* (Cascales and Christie 2004); transgenic plants that produce VirE2 are fully susceptible to *virE2* mutants (Citovsky et al. 1992). VirE2 is the most abundant Vir protein (Engstrom et al. 1987); coating a T-strand completely requires one molecule of VirE2 for every 20 bases of single-stranded DNA (ssDNA) (Christie et al. 1988;
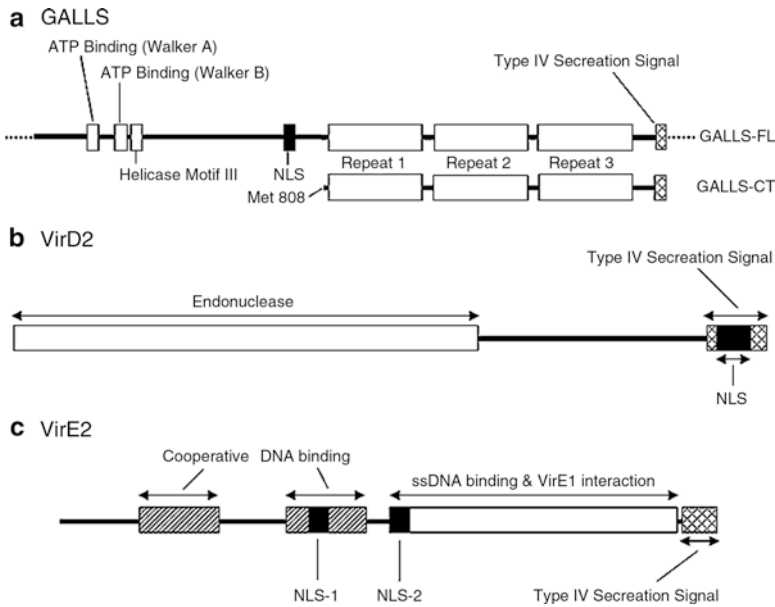
Fig. 1.1 Domains in the GALLS, VirD2, and VirE2 proteins. (**a**) GALLS-FL and GALLS-CT proteins. Boxes indicate the locations of the ATP binding sites (Walker A and B; [165]V**GVAGS**A**KTS**[174] and [235]**IVVIDE**MSM[243]), helicase motif III ([269]**KLICVGD**DR**QL**PP**V**GP**G**DLL[288]), nuclear localization signal ([705]**KRKR**AAA**K**EEIDS**RKK**MA**RH**[724]), GALLS repeats [amino acids 828–1093 (repeat 1); 1117–1382 (repeat 2); 1406–1671 (repeat 3)], and type IV secretion signal ([1743]PKAANDVD**R**LTRDFDE**R**I**R**V**R**GDGRGL[1769]; consensus sequence, **R**xxxxxxx**R**x**R**x**R**xx). Bold type indicates basic amino acids in the NLS or amino acids that match consensus sequences in the ATP binding sites, helicase motif III, and type IV secretion signal. The *GALLS* gene encodes a full-length protein (GALLS-FL; top bar) and a C-terminal domain (GALLS-CT; bottom bar), which is translated from an in-frame start codon (Met 808). (**b**) VirD2 functional domains. The N-terminal region contains the endonuclease domain (open box; amino acids 1–229). The NLS (black box; [396]**KR**P**R**D**RH**DGELGG**RKR**A**R**[413]) lies within the type IV secretion signal (hatched box; [395]PKRPRDRHDGELGGRKRA**R**GNR**R**DDGRGGT[424]). Arrows indicate the extent of each domain. (**c**) VirE2 functional domains. VirE2 contains two domains for cooperative binding to single-stranded DNA (ssDNA; forward hashed boxes) and two NLS sequences (black boxes; NLS-1, [205]**K**L**R**PED**R**YVQTE**K**YG**RR**[221], and NLS-2, [273]**KRR**YGGET**I**K**LKSK**[287]). NLS-1 lies within a cooperativity domain, and NLS-2 lies within a domain (amino acids 273–495) required for binding ssDNA and for interaction with VirE1, the secretory chaperone for VirE2. The type IV secretion signal occupies the C-terminus (hatched box; [504]FVRPEPASRPISDSRRIYES**R**P**R**SQSVNSF[533]). Adopted from Ream ([2009])

Citovsky et al. [1989]; Sen et al. [1989]). VirE2 protects T-strands from nuclease attack in plant cells (Gelvin [1998]; Rossi et al. [1996]; Yusibov et al. [1994]), and rare tumors induced by *virE2* mutants have severely truncated T-DNAs (Rossi et al. [1996]).

VirE2 may assist in nuclear import of T-strands (Gelvin [1998]; Rossi et al. [1996]; Yusibov et al. [1994]; Zupan et al. [1996]). Fluorescently labeled single-stranded DNA

(ssDNA) injected into plant cells remains in the cytoplasm, but ssDNA localizes at the nucleus when coinjected with VirE2 (Zupan et al. 1996). VirD2 alone mediates nuclear import of a covalently attached 25-nucleotide ssDNA molecule in permeabilized tobacco protoplasts, but nuclear import of a 250-nucleotide ssDNA also requires VirE2 (Ziemienowicz et al. 2001). VirE2 binds cooperatively to ssDNA (Christie et al. 1988; Citovsky et al. 1988, 1989; Das 1988; Gietl et al. 1987; Sen et al. 1989) exerting a force of 50 piconewtons on the DNA strand (Grange et al. 2008), which may pull T-strands into the nucleus (Fig. 1.2) (Ream 2009).
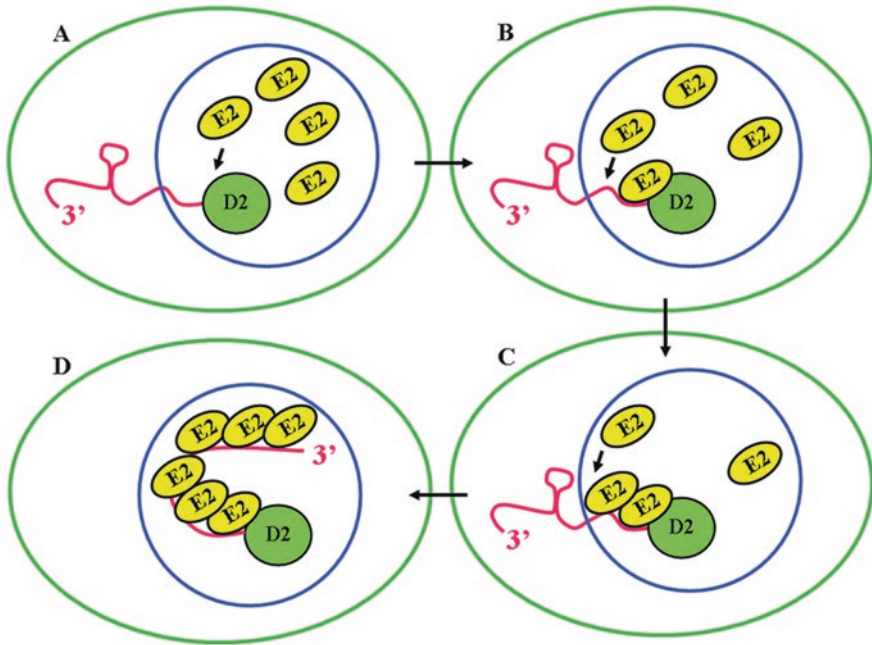
Fig. 1.2 Hypothetical model for VirE2-mediated nuclear import of T-strands. Circles represent the plant cell plasma (green) and nuclear (blue) membranes. Inside the nucleus, the solid green circle labeled "D2" depicts a molecule of VirD2 covalently attached to the 5′ end of the T-strand (red line). Yellow ovals labeled "E2" represent VirE2 proteins. (**a**) VirD2 enters the nucleus along with <250 nucleotides of attached T-strand DNA. VirE2 monomers enter the nucleus separately, and VirD2 recruits a VirE2 monomer to the 5′ end of the T-strand DNA. (**b**) The VirE2 monomer bound to VirD2 and the T-strand DNA recruits a second molecule of VirE2, pulling additional nucleotides of the T-strand DNA into the nucleus. (**c**) Cooperative binding of additional VirE2 molecules to the T-strand DNA in the 5′ to 3′ direction pulls more ssDNA into the nucleus. (**d**) Nuclear import is complete and the T-strand is fully coated with VirE2. Adopted from Ream (2009)

Plant Transformation by *Agrobacterium rhizogenes*

Gene Transfer to Plants by *A. rhizogenes* Requires the *GALLS* Gene This gene substitutes for *virE2*. Root-inducing (Ri) and tumor-inducing (Ti) plasmids share many similarities, including nearly identical organization of the *vir* operons (Moriguchi et al. 2001; Zhu et al. 2000). However, *virE1* and *virE2* are absent from the Ri plasmid and the genome of most *A. rhizogenes* strains (Hodges et al. 2004; Moriguchi et al. 2001). Although *A. rhizogenes* 1724 lacks *virE1* and *virE2* (Hodges et al. 2004; Moriguchi et al. 2001), it transfers T-DNA efficiently because a novel translocated protein (GALLS-FL) substitutes for VirE2, even though these proteins lack obvious similarities (Fig. 1.1) (Hodges et al. 2004, 2006, 2009).

The *GALLS* gene complements *virE2* mutations in *A. tumefaciens* and is essential for virulence in *A. rhizogenes* strains that lack VirE2 (Hodges et al. 2004). *GALLS* encodes two proteins: a low-abundance full-length protein (GALLS-FL) and an abundant C-terminal protein (GALLS-CT) translated from an alternative in-frame start codon (Hodges et al. 2009). Both GALLS proteins have type IV secretion signals at their C-termini (Fig. 1.1), which mediate transport to plant cells during infection (Hodges et al. 2006). GALLS-FL fully replaces VirE2 on some hosts; thus, GALLS-FL activity does not require GALLS-CT (Hodges et al. 2009). Full virulence on other hosts requires both GALLS proteins (Hodges et al. 2009). Mutations that abolish GALLS-CT synthesis without affecting GALLS-FL reduce virulence >100-fold on carrot roots and approximately fivefold on *A. thaliana* roots (Hodges et al. 2009).

GALLS-FL Contains a Predicted ATPase that May Transport T-Strand DNA into the Nucleus GALLS-FL enters the plant cell nucleus and binds the VirD2 pilot protein (Hodges et al. 2009), bringing GALLS-FL into close proximity with the 5′ end of T-strand DNA. The N-terminus of GALLS-FL resembles the strand transferase domain of *A. tumefaciens* TraA, which mobilizes ssDNA during conjugal transfer of the Ti plasmid to recipient bacterial cells (Farrand et al. 1996). This domain has ATP-binding and helicase motifs lacking in VirE2 and GALLS-CT (Fig. 1.1). Mutations in these motifs abolish the ability of GALLS-FL to substitute for VirE2 but do not destabilize the protein (Hodges et al. 2006). The predicted strand transferase of GALLS-FL may pull T-strand DNA into the nucleus (Fig. 1.3) (Hodges et al. 2009; Ream 2009).

GALLS-CT Stimulates Transformation GALLS-CT expressed in transgenic *A. thaliana* (Figs. 1.4 and 1.5b) stimulates transformation, indicating that this effector protein functions inside plant cells. Similarly, GALLS-CT transported from *Agrobacterium* into plant cells also stimulates transformation (Fig. 1.5c) (Hodges et al. 2009). Transfer of T-DNA with a *GUS*::intron (β-glucuronidase) gene from *Agrobacterium* into plant cells was monitored by assaying β-glucuronidase (GUS) enzyme activity; the intron prevents GUS expression in bacteria. Transformation
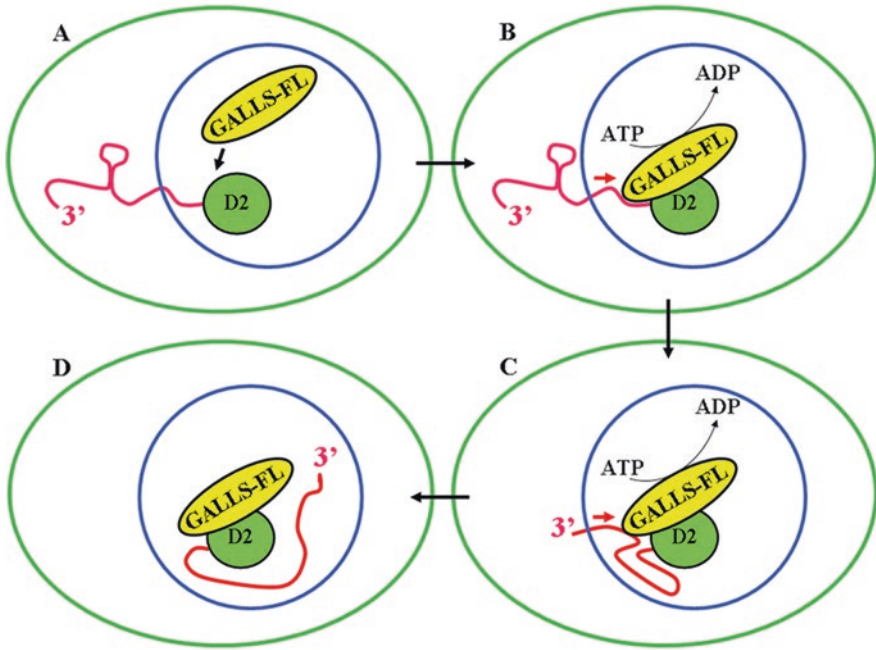
Fig. 1.3 Hypothetical model for GALLS-mediated nuclear import of T-strands. The green circle represents the plant cell plasma membrane, and the blue circle represents the nuclear membrane. Inside the nucleus, the solid green circle labeled "D2" depicts a molecule of VirD2 covalently attached to the 5′ end of the T-strand DNA (red line). The yellow oval labeled "GALLS-FL" represents GALLS-FL protein. (**a**) VirD2 enters the nucleus along with <250 nucleotides of attached T-strand DNA. GALLS-FL enters the nucleus separately, and VirD2 recruits GALLS-FL to the 5′ end of the T-strand DNA. (**b**) ATP-dependent translocation of GALLS-FL protein in the 5′ to 3′ direction along the T-strand DNA pulls additional nucleotides of T-strand into the nucleus. (**c**) GALLS-FL continues to pull the T-strand DNA into the nucleus, using its predicted helicase activity to disrupt any base pairs that form between complementary sequences in the T-strand. (**d**) Nuclear import of the T-strand DNA is complete. The T-strand DNA is not coated with bacterial effector proteins, but VirD2 and GALLS-FL may remain bound at the 5′ end prior to T-DNA integration. Adopted from Ream (2009)

(GUS activity) increased sixfold due to inducible expression of GALLS-CT in plant cells 24 h prior to infection (Fig. 1.5a, b). GALLS-CT increased transformation eightfold when produced in *Agrobacterium* (Fig. 1.5a, c) and 11-fold when expressed in both host and pathogen (Fig. 1.5a, d).

GALLS-CT Expressed in Transgenic *A. thaliana* Represses Defense Genes To identify host genes that respond to GALLS-CT, we compared the transcriptomes of the parental ecotype (Ws) and a transgenic line that expresses *GALLS-CT* from an estradiol-regulated promoter (Fig. 1.5b); we compared transcriptomes before and after estradiol treatment (Table 1.1). We prepared RNA from three biological replicates containing roots from 20 plants per sample. The transgenic line expressed
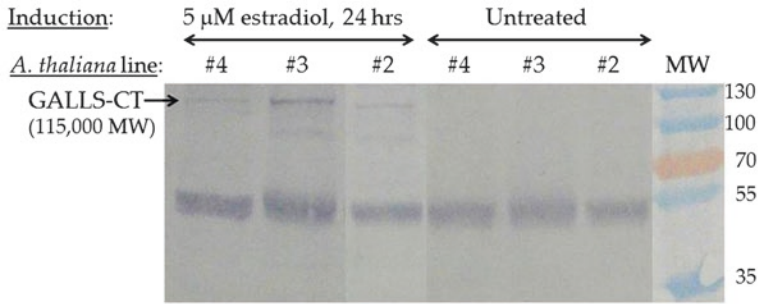
Fig. 1.4 Inducible GALLS-CT expression in transgenic *A. thaliana*. Plants were grown with or without 5-μM β-estradiol for 24 h. Proteins extracted from 30-mg leaf tissue were separated by SDS-PAGE; blots were probed with polyclonal anti-GALLS serum. *MW* molecular weight (in thousands)
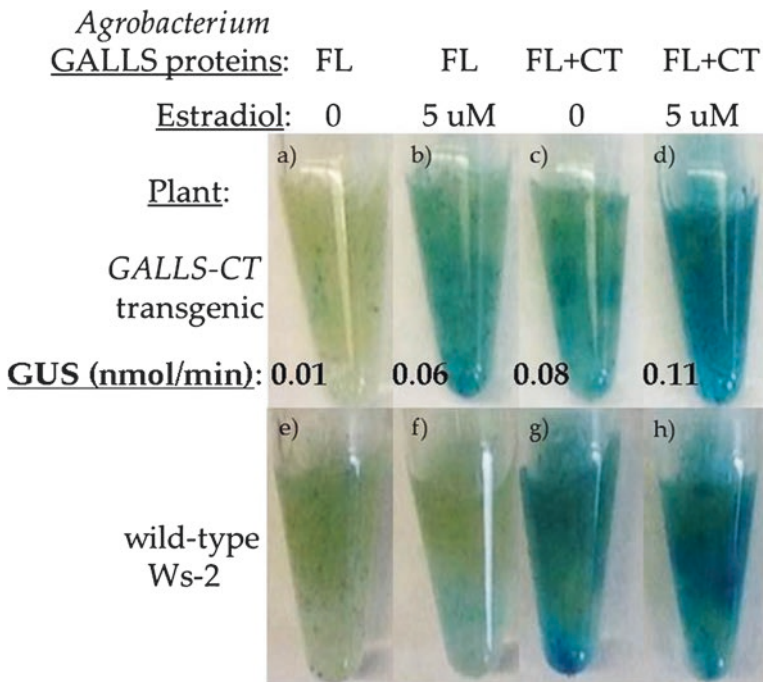
Fig. 1.5 GALLS-CT stimulates transformation. Transgenic *A. thaliana* roots expressing *GALLS-CT* from an estradiol-inducible promoter, along with wild-type controls, were infected with *Agrobacterium* that contained *GUS*::intron T-DNA and expressed either GALLS-FL or GALLS-FL + CT. Treated roots were incubated with 5-μM estradiol for 24 h prior to infection. Six days after infection, roots were stained with X-glucuronide. GUS activity in samples **a–d** (normalized to tissue weight) was also assayed by cleavage of *p*-nitrophenyl β-D-glucuronide, measured by increases in OD at 405 nm over three time points (reported as nmol/min)

Table 1.1 Genes affected by GALLS-CT expressed in transgenic *A. thaliana*

		Reads/kilobase transcript/10^6 mapped reads			
		Line/induction			
Gene	Function	Ws-Un	Ws-Ind	GALLS-Un	GALLS-Ind
GALLS	Bacterial effector protein	0.0	0.0	2.8	99.6
PR1	Pathogenesis-Related (PR) protein	71.4	33.3	46.5	3.8
PDF1.2b	PR protein; defensin	12.2	11.1	13.5	1.6
MIR472	Silence disease resistance genes	0.0	0.0	0.0	13.9
CML10	Calmodulin-Like; stress response	17.5	20.2	50.2	3.2
DMT2	DNA Methyltransferase; *Agro.* transf.	194.7	241.8	174.1	624.0
MIR397	Silence laccase genes	0.0	0.0	0.0	114.9
LAC2	Laccase; oxidize phenols, lignin	0.5	0.4	0.4	0.0
LCR25	Low MW Cys-Rich; Pollen Coat Prot.	0.0	0.0	0.0	5.1
LCR67	Low MW Cys-Rich; defensin; PCP	0.0	0.0	0.0	4.1
LCR76	Low MW Cys-Rich; Pollen Coat Prot.	0.0	0.0	0.0	8.1

full-length *GALLS-CT* mRNA (Table 1.1) and protein (Fig. 1.4) upon estradiol treatment. Plants expressing GALLS-CT showed significantly lower expression of defense genes, including *PR1* and *PDF1.2b* (Table 1.1). *PR1* (pathogenesis related 1) is a marker of the systemic acquired resistance (SAR) response (Durrant and Dong 2004; Mukhtar et al. 2009; Van Loon and Pieterse 2006), and *PDF1.2b* encodes a plant defensin that inhibits bacterial infections (Hiruma et al. 2011; Sels et al. 2008).

Plants expressing GALLS-CT contained microRNA miR472, which silences 19 NBS-LRR disease resistance genes in *A. thaliana* (Boccara et al. 2014). Control plants lacked miR472 RNA (Table 1.1), consistent with low levels of miR472 in uninfected *A. thaliana* (Boccara et al. 2014; Shivaprasad et al. 2012; Zhai et al. 2011). The miR472 precursor and primary transcripts contain a polyA tract, explaining its presence in the polyA-enriched RNA used for RNA Seq. NBS-LRR proteins detect pathogens and initiate a hypersensitive response (Belkhadir et al. 2004; Blume et al. 2000; Grant et al. 2000; Jabs et al. 1997; Kim et al. 2002; Nimchuk et al. 2003; Romeis et al. 2000, 2001; Xu and Heath 1998; Zimmermann et al. 1997). GALLS-CT may stimulate transformation by suppressing these host defense responses.

Cytoplasmic free calcium, together with calmodulins and calcium-dependent protein kinases, activate plant defense responses (Blume et al. 2000; Grant et al. 2000; Jabs et al. 1997; Kim et al. 2002; Romeis et al. 2000, 2001; Xu and Heath 1998; Zimmermann et al. 1997). *CML10* encodes a calmodulin-like protein expressed in response to stress. GALLS-CT reduced *CML10* mRNA 5- to 16-fold (Table 1.1), which may compromise the plant's response to *Agrobacterium* infection.

GALLS-CT Modulates Other Genes that Affect Transformation Efficiency Efficient *Agrobacterium*-mediated transformation of *A. thaliana* roots requires at least 24 chromatin-related proteins, including DNA methyltransferase 2 (DMT2); silencing *DMT2* reduces transformation efficiency (Crane and Gelvin 2007). *DMT2* mRNA levels increased 2.6- to 3.6-fold upon GALLS-CT expression (Table 1.1). Statistical analysis using DE Seq indicated that increased transcription of *DMT2* in plants expressing GALLS-CT is significant and may stimulate transformation.

Phenolic compounds released by wounded plant cells attract *Agrobacterium* cells and induce expression of their virulence genes (Stachel et al. 1985). These compounds act as chemical defenses and as lignin precursors for cell wall repair. Plants expressing GALLS-CT contained miR397, which silenced *LAC2* (laccase 2) (Table 1.1), an enzyme that oxidizes phenols and lignin. Control plants lacked miR397 RNA but contained *LAC2* mRNA (Table 1.1). Loss of laccase 2 may alter lignin metabolism or other phenolic compounds in a way that stimulates *Agrobacterium* infection.

GALLS-CT expressed in transgenic *A. thaliana* roots triggered expression of three genes encoding *l*ow-molecular-weight *c*ysteine-*r*ich proteins (*LCR25*, *LCR67*, *LCR76*; Table 1.1). These proteins belong to the *p*ollen *c*oat *p*rotein family of secreted proteins responsible for male self-incompatibility in *Brassica* species. Uninfected plants normally express these genes in seeds but not in roots (Table 1.1), so their expression in roots containing GALLS-CT protein is notable. LCR67 is a defensin; LCR25 and LCR76 resemble LCR67 and may be defensins also.

Some host defense proteins promote transformation by *Agrobacterium*. For example, *A. tumefaciens* infection triggers phosphorylation and subsequent nuclear localization of a host transcription factor (VIP1) that induces expression of host defense genes, including *PR1* (Djamei et al. 2007). Overexpression of VIP1 stimulates VirE2-dependent transformation, apparently by binding VirE2 and facilitating its nuclear import [the "Trojan horse" model (Djamei et al. 2007)]. Similarly, LCR proteins expressed in response to GALLS-CT may stimulate *Agrobacterium*-mediated transformation, despite their ability to antagonize infection by other pathogens. Alternatively, these LCR proteins may diminish the transformation-stimulating activity of GALLS-CT.

GALLS Protein Is Superior to VirE2 for Plant Transformation Efficient delivery of single-copy transgenes is desirable for plant science and biotechnology. Using otherwise isogenic strains, GALLS-mediated transformation of *A. thaliana* by flower dip yielded 12/34 (35%) single-copy transgenes compared to VirE2-mediated transformation, which gave 6/50 (12%) single-copy events (Fig. 1.6). Although both GALLS- and VirE2-mediated transformation yielded single-copy and multi-copy events, GALLS-mediated transformation increased the proportion of single-copy events approximately threefold. This suggests that GALLS and VirE2 affect T-DNA integration differently, probably by providing different substrates for integration.
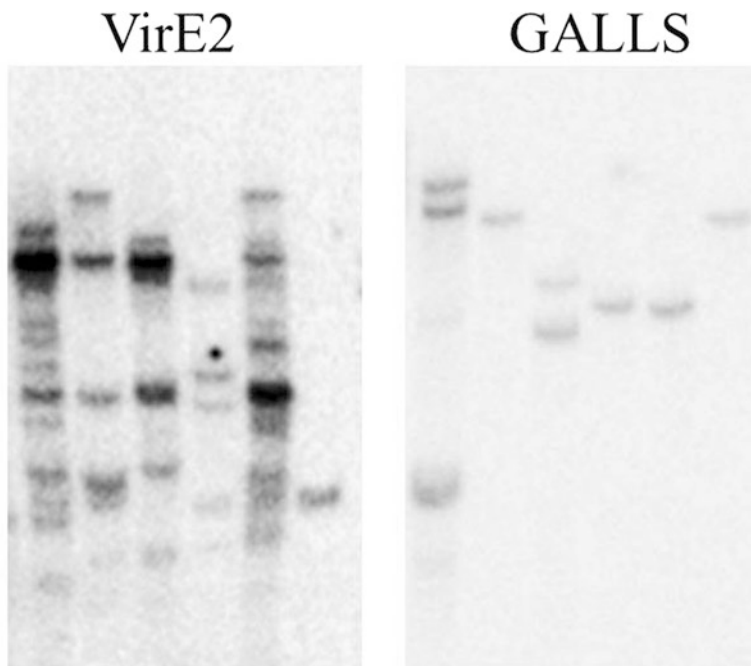
Fig. 1.6 Transformation mediated by GALLS yields single-copy transgenes more frequently than do VirE2-mediated events. The figure shows representative VirE2-mediated events (left panel) and GALLS-mediated events (right panel). Genomic DNA extracted from independent kanamycin-resistant transgenic *A. thaliana* Col-0 lines was digested with *Eco*RI prior to agarose gel electrophoresis and Southern hybridization. T-DNAs were derived from pCAMBIA2300. This T-DNA region contains a single *Eco*RI site (near the right border); T-DNA sequences left of this restriction site were labeled with ^{32}P and used to probe Southern blots. Thus, each band represents a separate copy of the T-DNA

Integration of T-DNAs may occur via multiple pathways that lead to very different outcomes: single-copy or low-copy events that yield (mostly) intact T-DNAs or high-copy events that yield many truncated T-DNAs (Fig. 1.6). In transgenic lines containing multiple copies of the T-DNA, many of the bands are less intense than are bands corresponding to full-length T-DNAs (Fig. 1.6), indicating that these lines contain multiple truncated copies of the T-DNA. PCR-based methods often used to detect transgenes cannot detect truncated copies of the T-DNA that lack one (or both) primer binding sites; therefore, studies that use PCR-based methods usually underestimate transgene copy number.

GALLS-Mediated Transformation Events Yield Intact T-DNAs Many models posit that VirE2 protects T-strand DNA from nuclease attack in vivo, as it does in vitro (Rossi et al. 1996). Rare T-DNAs produced in the absence of VirE2 (and GALLS) are severely truncated (Rossi et al. 1996); the resulting transgenes have truncations that appear to arise from exonuclease attack on the 3′ end of unprotected

T-strand DNA (Rossi et al. 1996). Because of its low abundance, GALLS-FL protein likely cannot coat T-strand DNA, raising the possibility that T-strand DNA may be subject to increased nuclease attack during GALLS-mediated transformation. VirE2-based hypotheses predict that transgenes delivered via the GALLS pathway (in the absence of VirE2) should be rare and truncated at their 3′ ends, as they are in the absence of both VirE2 and GALLS. In contrast to this prediction, GALLS and VirE2 promote transformation with approximately equal efficiency when expressed in otherwise isogenic strains. We sequenced junctions of T-DNA with host DNA in transgenic *A. thaliana* lines. The junction sequences from GALLS-mediated events looked similar to those reported for VirE2-mediated events: three T-DNA left ends were intact, another three had short (1–25 bp) deletions, and two had longer (124 and 257 bp) deletions. We sequenced three right T-DNA ends: one was intact, and the others had 10- and 32-bp deletions. The sporadic presence of novel "filler" sequences between T-DNA and host DNA also resembled VirE2-mediated events: four lacked filler sequences, whereas five others had short (5–42-bp fillers).

The junctions produced by GALLS-mediated transformation events are typical of those produced by VirE2-mediated transformation, which have been thoroughly characterized (Kleinboelting et al. 2015). The question we asked was whether T-DNAs produced by GALLS-mediated transformation would be severely truncated due to the absence of VirE2. Our results clearly show the T-DNAs that result from GALLS-mediated events are not severely truncated.

An Alternative Model for Protection of T-Strands The activities of VirE2 and GALLS-FL each prevent extensive degradation of T-strand DNA by host nucleases. Earlier, we proposed that VirE2 and GALLS-FL each facilitate nuclear import of T-strand DNA by different mechanisms (Figs. 1.2 and 1.3). In the absence of either VirE2 or GALLS-FL, T-strand DNA may be truncated because it cannot enter the nucleus efficiently in the absence of VirE2 or GALLS-FL, so the host may destroy the "stalled" DNA. We propose that cooperative binding of nuclear-targeted VirE2 may generate sufficient force to pull T-strand DNA into the nucleus, allowing the T-DNA to integrate before it is degraded. We hypothesize that GALLS-FL may substitute for VirE2 by using the nuclear-targeted GALLS-FL ATPase/strand transferase to pull T-strands into the nucleus, obviating the need for VirE2.

Summary

The ability of the GALLS proteins to substitute for VirE2 during *Agrobacterium*-mediated transformation of plants provides a valuable opportunity to understand this process. For example, the ability of an abundant ssDNA-binding protein to protect T-strand DNA from nuclease attack appeared to be crucial for transformation. However, in the presence of the GALLS proteins, T-strand DNA can survive (and integrate) without VirE2's protection. Instead, perhaps nuclear import of T-strand DNA is the critical step promoted equally well (in different ways) by VirE2

and GALLS-FL. Similarly, VirE2 appears to dampen host defense responses through its interaction with VIP1 (a host transcription factor), thereby increasing susceptibility to transformation. However, models for *Agrobacterium*-mediated transformation must account for the fact that the GALLS-FL and GALLS-CT proteins mediate efficient transformation, apparently without binding VIP1. GALLS-CT may modulate host defenses too, albeit in different ways than VirE2 does. The ability of bacterial effector proteins to weaken host defenses may be another critical step in transformation. Finally, substrates for T-DNA integration probably are fundamentally different in the GALLS and VirE2 pathways, and models for this process must take both systems into account.

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Chapter 2
Hairy Root Composite Plant Systems in Root-Microbe Interaction Research

Senthil Subramanian

Abstract Plant-associated microbes are key determinants of plant health and productivity. Research on model plant species has helped discover fundamental plant mechanisms that determine the outcomes of these microbial interactions. However, the species-specific nature of several key plant-microbe interactions necessitates research in non-model plant species. A major bottleneck for research on non-model species is the lack of efficient transformation methods. *Agrobacterium rhizogenes*-mediated hairy root composite plant system is a transformative tool that has enabled a multitude of transgenic approaches to be efficiently used in non-model species. This chapter provides a snapshot of research using key examples to highlight how the tool had helped advance the frontier of root-microbe interaction research focusing on arbuscular mycorrhizal symbiosis, nodulation, pathogen responses, and microbiome research. Limitations of and recent developments in hairy root composite plant systems are also discussed.

Keywords *Agrobacterium rhizogenes* • Arbuscular mycorrhizal symbiosis • Nodulation • Nematode • Microbiome • Symbiosis • Hormone

Introduction

The key influence of plant-associated microbes on plant growth, health, and yield has drawn increasing interest toward plant-microbe interaction research (Busby et al. 2017). A largely underexplored subtopic is root-associated microbes and their interactions with plants. In the early 1900s, classical microbiology studies pioneered by Lorenz Hiltner determined that the highest microbial density and

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diversity in soils occur very close to plant roots (Hinsinger and Marschner 2006). For example, root surface scrapings had multiple-fold more colony-forming units compared to soil samples 0.5 cm away from the roots (Clark 1940). Carbon-rich energy sources provided by the plant are the key drivers of such microbial enrichment. Indeed, plants release on average 10–15% (Jones et al. 2009; Dennis et al. 2010) of their photosynthetic assimilates into the rhizosphere. Such deposition does not appear to be a random release of carbon by the plant but an active recruitment of specific microbes for colonization and/or rhizosphere presence. Increasing evidence indicates that the plant species influences the composition of root microbial communities (Mougel et al. 2006; Weisskopf et al. 2006; Micallef et al. 2009). Indeed, an intricate coevolution of plants and rhizosphere microbial communities was suggested by the observation that resident plants or their root exudates are capable of maintaining the biomass and diversity of soil fungal communities to a much greater extent than nonresident/introduced plants (Broeckling et al. 2008). When *Arabidopsis* plants or root exudates were present, fungal communities in soils with a history of growing *Arabidopsis thaliana* showed increased biomass and diversity. This increase was not observed when a "nonresident" plant species (*Medicago truncatula*) or its root exudates were present in the same soil. Additional experiments over three generations indicated that resident plants or their root exudates are capable of maintaining the biomass and diversity of soil fungal communities to a much greater extent than nonresident plants (Broeckling et al. 2008). Similarly, invasive weeds might manipulate native rhizosphere microbial communities to their advantage, perhaps an evolutionary advantage enabling survival and dominance in new environments (e.g., Inderjit et al. 2006). However, pathogenic microorganisms appear to have evolved to utilize these plant "recruitment" signals to identify and colonize their hosts. For example, soybean roots release isoflavonoids, a group of specialized metabolites, to potentiate nitrogen-fixing rhizobia bacteria for colonization and phytoalexins to protect against pathogens (reviewed by Paiva 2000). Zoospores of the root rot pathogen *Phytophthora sojae* are chemotactic to isoflavonoids (Morris and Ward 1992) suggesting that the pathogen might use these molecules to find their host.

This intricate coevolution between the plant and associated microbial communities including pathogenic and symbiotic organisms warrants specific research on the plant species of interest. In other words, plant-microbe interaction studies using model organisms such as *A. thaliana* can address a number of fundamental questions intrinsic to the plant, but not the unique research needs for the majority of plant-microbe interactions that are species-specific. Key examples include symbiotic nodulation and AM fungal interactions as well as a number of species-specific plant-pathogen interactions. In addition, the composition of rhizodeposits varies substantially among different plant species including unique and species-specific rhizodeposit compounds (e.g., isoflavonoids that are legume-specific) requiring research on specific plant species to understand their influence on root-microbe interactions.

Need for an Efficient Transformation System in Non-model Plant Species

A number of plant species suffer from the lack of certain advantages that most model plant species have. The most crucial of these advantages are (a) the availability of genetic and genomic datasets and (b) the ability to efficiently generate transgenic lines for various functional experiments. Key examples of the approaches enabled by transgenic technology include the loss or gain of function assays, genetic complementation, evaluation of marker gene expression, cellular localization of proteins and tagging of organelles and compartments, and in vivo biomolecular interaction studies. Advances in high-throughput sequencing technologies have enabled the acquisition of transcriptomic, if not genomic, sequence information for a number of non-model plant species. The number of plant species with genome/chromosome assemblies in NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/browse/) has grown from just a single species (*A. thaliana*) to 102 entries in 2017. On the other hand, the number of transformable plant species has been largely limited to specific plant families. Among the different methods used to transfer foreign genes into the plant, the utilization of a disarmed strain of *Agrobacterium tumefaciens* is the most efficient and predictable. The protocols for *Agrobacterium*-mediated transformation of most plant species involve tedious procedures such as tissue culture and regeneration with a few exceptions such as *Arabidopsis* (Clough and Bent 1998), flax (Bastaki and Cullis 2014), *Setaria viridis* (Saha and Blumwald 2016), and Brassicales such as canola (Lu and Kang 2008), for which a simple floral dip method has been developed. Genetic transformation protocols for a number of other plant species utilize a plant tissue culture phase which requires specialized infrastructure and trained personnel (Anami et al. 2013). They also suffer from poor efficiency and thus do not allow high-throughput plant production required for most functional genomic research programs. While transient approaches such as leaf infiltration and protoplast transformation have been used for gene expression assays, they suffer from the lack of cellular context and are not suitable for biological processes lasting several days such as many root-microbe interaction studies.

Hairy Root Composite Plants as a Complementary Solution to Stable Plant Transformation

The lack of an effective transformation method is a major bottleneck for research in a number of plant species. However, this is being addressed in part by the use of *A. rhizogenes,* a close relative of *A. tumefaciens* and a naturally occurring pathogen of plants (Riker et al. 1930). Both organisms are capable of transferring T-DNAs into the plant. While *A. tumefaciens* infection results in largely unstructured galls, *A. rhizogenes* infection results in neoplastic, transformed "hairy" roots that closely resemble wild-type plant roots in cellular organization. *A. rhizogenes* possesses

root-inducing (Ri) plasmid-containing root locus (*rol*) genes *rolA*, *rolB*, and *rolC*. T-DNA derived from this plasmid is integrated into the plant genomic DNA (Chilton et al. 1982), and infected plant cells form a mass of undifferentiated cells which subsequently give rise to a "hairy root." Hairy roots have been generated from a broad range of diverse dicotyledonous plant families and even some gymnosperms.

The ability of hairy roots to grow in the absence of exogenously supplied plant hormones (unlike organ culture system) has been exploited to generate hairy root cultures, where these roots grow and branch profusely under axenic in vitro conditions. Hairy root cultures have been utilized for stable transgenic plant generation, secondary metabolite production, and root-microbe interaction studies (Georgiev et al. 2012). However, the need for in vitro maintenance and propagation conditions does not make hairy root cultures as suitable systems for a number of research questions especially root-microbe interactions involving intricate and complex signaling that occurs throughout the entire plant. For example, systemic signaling plays a key role in plant responses to nitrate (Zhang et al. 1999), autoregulation of nodulation (Delves et al. 1986), and phosphorus uptake regulation (Doerner 2008). The absence of stem tissues in hairy root cultures would make it virtually impossible to perform these studies.

Hairy root composite plants where hairy roots are induced from and left attached to shoot cuttings can address some of the concerns surrounding hairy root cultures. Both in vitro and "ex vitro" methods have been developed for the generation of composite plants consisting of a wild-type shoot with transgenic roots (Hansen et al. 1989; Collier et al. 2005). These methods are transformative especially for root-microbe interaction studies due to the reduced time required to generate transgenic plant tissues and the ability to be maintained independent of tissue culture. Research on two key plant-microbe interaction processes, nodulation and endomycorrhizal symbiosis, in legumes benefitted significantly from the adoption of composite hairy root composite plant systems for research (Boisson-Dernier et al. 2001).

An added advantage of the hairy root composite plant system is the adaptability of transformation vectors for use in stable transgenic plant generation. Therefore, candidate gene constructs can be efficiently screened by generating composite plants and can subsequently be moved directly into *A. tumefaciens* for a stable transgenic plant generation. In fact, methods for direct regeneration of stable transgenic plants from transformed hairy roots have also been developed (e.g., *M. truncatula;* Crane et al. 2006). This chapter presents a summary of plant-microbe interaction research enabled by the use of hairy root composite plant systems, highlighting key applications and discussing future potential. The goal here is not to present a comprehensive overview of discoveries made using hairy root composite plants but rather provide a snapshot using key examples of studies on arbuscular mycorrhizal (AM) symbiosis, legume nodulation, pathogenic interactions, and microbiome discovery.

Hairy Root Composite Plant Systems in Root-Microbe Interaction Research

Arbuscular Mycorrhizal Symbiosis

AM symbiosis is the most widespread association between plants and fungi (reviewed by Parniske 2008). Approximately 65% of all land plant species are capable of establishing a mutualistic interaction with the exclusively subterranean fungi of the phylum *Glomeromycota*. AM interactions appear to have evolved ~450 million years ago and might have played a key role in land colonization by plants. AM fungi are obligate biotrophs that depend entirely on carbon provided by a host plant for fungal metabolism and reproduction. The obligate symbiotic nature of these fungi necessitates cocultivation of the fungus with the plant both for maintenance and evaluation of plant-AM fungal interaction for research. The key plant rhizosphere signal that potentiates AM fungal colonization is the plant hormone strigolactone. AM fungi respond by producing chitin oligomers which are perceived by specific plant receptors. A signal transduction pathway including the receptor-like kinase SYMRK/DMI2, nuclear envelope-localized cation channels, the nuclear membrane calcium pump MCA8, and components of the nucleopore complex initiates calcium oscillations in the nucleus. These oscillations are decoded by a nuclear localized calcium and calmodulin dependent kinase CCamK/DMI3 together with its interacting partner IPD3/CYCLOPS. Subsequent activation of several transcription factors including NSP1, NSP2, and RAM1 activates gene expression and prepares the plant cells for colonization through the formation of the pre-penetration apparatus. Upon reaching primed cortical cells, AM hyphae form highly branched structures named arbuscules that facilitate the exchange of nutrients (reviewed by Gobbato 2015).

Root organ culture methods were instrumental in "clean" inoculum production for AM fungal research. While it was possible to get AM fungi to colonize excised and cultured roots, the maintenance of the coculture was not optimal as the roots detached from the plant require specific nutrient conditions and microenviornments for continued growth and branching. The ability of hairy roots to grow and branch in the absence of exogenous hormones was exploited by Mugnier and Mosses (1987) for cocultivation of AM fungi with plant roots. This hairy root culture system has been successfully used to advance research on plant-AM fungal interactions including studies on nutrient exchange, gene discovery, and functional analysis. However, it suffers from the fact that these roots are detached from the plant and may not truly reflect native physiological conditions. Composite plants on the other hand more closely reflect native conditions and have been successfully used for gene regulation assays (promoter element discovery), protein localization, and loss of function assays in the plant host or AM fungi.

Gene Regulation and Intracellular Markers

One of the most transformational uses of hairy root composite plants in AM symbiosis research is the development of transgenic root systems expressing marker genes. This enabled the identification of plant responses at the earliest stages of mycorrhizal colonization. *MtENOD11/12* genes are expressed in arbuscule-containing cortical cells of plants colonized by *Glomus* (Journet et al. 2001). Therefore, hairy root explants expressing the *pMtENOD11-gusA* fusion were generated and used to easily identify sites of AM fungal hyphal penetration in epidermal and cortical cells (Chabaud et al. 2002). Another landmark study was evaluated in vivo cellular dynamics within *M. truncatula* root epidermal cells using green fluorescent protein labeling of the microtubular cytoskeleton, actin filaments, and ER (Genre et al. 2005). Real-time imaging coupled with GFP tagging of cytoskeletal/ER components has revealed a complex multistep host response that precedes fungal entry. The plant cell synthesizes a transcellular apoplastic compartment that separates the penetrating hypha from the host cytoplasm. This novel structure comprising microtubules, microfilaments, and ER is assembled within a column of cytoplasm created during the progressive migration of the nucleus across the epidermal cell and defines the future path taken by the infection hyphae. An added resource developed later was a set of fluorescent protein fusions that label the nucleus, endoplasmic reticulum, Golgi apparatus, *trans*-Golgi network, plasma membrane, apoplast, late endosome/multivesicular bodies, transitory late endosome/tonoplast, tonoplast, plastids, mitochondria, peroxisomes, autophagosomes, plasmodesmata, actin, microtubules, periarbuscular membrane, and periarbuscular apoplastic space (Ivanov and Harrison 2014). These markers were expressed from the constitutive *AtUBQ10* promoter or the AM symbiosis-specific *MtBCP1* promoter to enable tracking of these cellular organelles/structures during AM fungal symbiosis or other processes. These resources have been validated in *M. truncatula* using hairy root composite plant system and should be easily adaptable for use in other species. The use of these markers to investigate AM symbiosis revealed that root cells undergo major cellular alterations in the nuclei, cytoskeleton, tonoplast, and plastids to accommodate their fungal endosymbiont. Other key examples of gene regulation discoveries made using hairy root composite plants include detailed promoter analysis studies of the *VfLb29* leghemoglobin gene promoter using a transcription fusion in transgenic *Vicia faba* and *M. truncatula* roots (Vieweg et al. 2004; Genre et al. 2005) and localization of phosphate transporter genes to periarbuscular membranes in soybean (Tamura et al. 2012) and *M. truncatula* (Pumplin and Harrison 2009).

Gene Function Discovery

Hairy root composite plant systems also enabled gene function discovery through loss or gain of function assays. An example of a comprehensive use of hairy root composite plants for gene function discovery in AM symbiosis is the use of RNAi, transcriptional fusions, and translational fusions to evaluate the role of *Vapyrin*, an

AM fungi-responsive gene in *M. truncatula* (Pumplin et al. 2010). Evaluation of AM fungal colonization in Vapyrin RNAi roots demonstrated that it is essential for arbuscule formation and efficient for epidermal penetration by AM fungi; promoter-GUS fusions showed that *Vapyrin* is induced transiently in the epidermis coincident with hyphal penetration and then in the cortex during arbuscule formation. Translational fusions demonstrated that the Vapyrin protein is cytoplasmic and that it accumulates in small puncta that move through the cytoplasm in cells containing AM fungal hyphae. An example of the use of gene-specific silencing in hairy roots to evaluate the function of closely related genes came from soybean (Indrasumunar et al. 2015). A leucine-rich repeat (LRR) receptor kinase (SymRK; also termed NORK) is required by legumes to establish a root endosymbiosis with Rhizobium bacteria as well as mycorrhizal fungi. Soybean has duplicated SymRK homeologues, but no mutants for these genes are available in soybean. Specific *GmSymRKβ* gene silencing resulted in a larger reduction of nodulation and mycorrhizal infection compared to that of *GmSymRKα*, suggesting it has the major activity of the duplicated gene pair. Other key examples include the discovery of the role in AM symbiosis for an ubiquitin-like protein that interacts with the symbiotic CCaMK (Kang et al. 2011), a carotenoid cleavage dioxygenase (Floss and Walter 2009), and a phosphate transporter (Maeda et al. 2006) in *Lotus japonicus*.

While the above studies are examples of reverse genetics to evaluate signaling components, such an approach has been used for metabolic engineering to determine the roles of specific enzymes/metabolites in AM fungal symbiosis. Colonization by AM fungi induces the accumulation of certain apocarotenoids in *M. truncatula*. Two isoforms of 1-deoxy-D-xylulose 5-phosphate synthase (DXS1 and DXS2) are crucial for this metabolic pathway, but only one of the isoforms (DXS2) is associated with AM fungal symbiosis. Specific silencing of MtDXS2 revealed that downstream isoprenoid products of this gene are crucial to sustain mycorrhizal functionality at later stages of the symbiosis (Floss et al. 2008).

Hormone Responses

The ability of hairy roots to grow independent of exogenous hormones is primarily due to their capacity to synthesize auxin and cytokinin (Cardarelli et al. 1987). While the levels of auxin in hairy roots are comparable to that in wild-type roots, they were reported to be more sensitive to auxin (Shen et al. 1988). This typically raises a concern about the suitability of hairy root composite plants to study hormone biology. However, a number of studies have successfully demonstrated that with appropriate controls, they can be used for hormone biology studies. Indeed, the role of auxin in the symbiotic interaction between *M. truncatula* and the AM fungus *Glomus intraradices* (recently named as *Rhizophagus irregularis*) was evaluated recently through the use of the synthetic auxin response marker DR5:GUS and alteration of auxin perception (Etemadi et al. 2014). DR5:GUS was preferentially expressed in root cells containing arbuscules suggesting that auxin activity might be crucial for cellular colonization of AM fungi and/or arbuscule formation. In

agreement, downregulation of auxin receptor genes through overexpression of microRNA393 (miR393) led to underdeveloped arbuscules in three different plant species.

During the establishment of AM symbiosis, an endogenous increase in jasmonic acid (JA) occurs. Enhanced expression of two full-length cDNAs coding for the JA-biosynthetic enzyme allene oxide cyclase from *M. truncatula* was observed during mycorrhization with *G. intraradices*. Antisense-mediated suppression of MtAOC expression in hairy roots resulted in lower JA levels and a remarkable delay in colonization with *G. intraradices* (Isayenkov et al. 2005). These roots had decreased number of arbuscules, but their structure was not altered indicating a crucial role for JA in the establishment of AM symbiosis.

Another interesting example is the manipulation of reactive oxygen species (ROS) generated by respiratory burst oxidative homologs (*Rboh*s) in common bean. Downregulation of *RbohB* in *Phaseolus vulgaris* was shown to suppress ROS production and abolish *Rhizobium* infection thread (IT) progression but also to enhance AM fungal colonization. On the other hand, overexpression of *RbohB* significantly enhanced ROS production, enhanced nitrogen fixation, and delayed nodule senescence but impaired AM fungal colonization (Arthikala et al. 2014, 2015).

Host-Induced Gene Silencing

The most exciting application of hairy root composite plant systems in AM fungal research is the use of host-induced gene silencing (HIGS), which causes RNA interference in the fungus using a transgenic host plant (Helber et al. 2011). The lack of suitable methods to transform AM fungi makes HIGS an excellent approach for loss of function studies in the fungus. HIGS involves the transformation of the host plant with a silencing construct targeted to a fungal gene, and this method was used to successfully knock down the expression of the target gene in the AM fungus, *Rhizophagus irregularis* (formerly *Glomus intraradices*). Eleven AM fungal genes predicted to encode secreted proteins were inducible both by treatment with the plant hormone and strigolactone and during symbiosis. An RNAi construct designed to specifically target one of these genes (SIS1) was expressed in hairy roots of *M. truncatula*, resulting in significant suppression of SIS1 expression in the intraradical mycelium indicating successful HIGS. Silencing of SIS1 led to reduced colonization and formation of stunted arbuscules suggesting a crucial role for this AM fungal gene in symbiosis (Tsuzuki et al. 2016).

Nodulation

Root nodules are specialized nitrogen-fixing structures in roots of leguminous plants. They result from a well-coordinated symbiotic association between plants and rhizobia bacteria. Nodules are classified into two major types based on

meristem persistency: indeterminate and determinate (reviewed by Hirsch 1992). Indeterminate nodules are oblong and possess a persistent nodule meristem analogous to lateral roots. Examples of plants that form indeterminate nodules include temperate legumes, viz., pea, *M. truncatula*, and clover. In contrast, determinate nodules are spherical and lack a nodule meristem. Examples of plants producing determinate nodules include tropical legumes, viz., soybean, common bean, and *L. japonicus*. Despite these differences, most of the signaling elements identified so far are conserved between the two types of nodules.

The interaction between the symbiotic partners starts with the exchange of chemical signals. Legumes release specific flavonoids (a group of small phenolic compounds) as signal molecules into the soil. Perception of these compounds by compatible rhizobia bacteria leads to the production of specific lipochitooligosaccharide (LCO) bacterial signals (reviewed by Cooper 2007). LCOs from compatible rhizobia are in turn perceived by the host legumes through a receptor complex, comprised of a leucine-rich repeat receptor (e.g., *MtDMI2/LjSymRK*) and LysM receptor kinases (e.g., *LjNFR1, MtLYK3, LjNFR5, MtNFP*). A signal transduction pathway that includes an E3 ubiquitin ligase (e.g., MtPUB1), membrane microdomain-associated proteins (e.g., *MtFLOT2, MtSYMREM1*), cation channels (e.g., *LjCASTOR* and *LjPOLLUX/MtDMI1*), and nucleoporins (e.g., *LjNUP85* and *LjNUP133)* transduces the signal to the nucleus in the form of organized calcium spikes. Decoding of these signals by a CaCMK (MtDMI3/LjCCaMK) and a nuclear-localized coil-coil protein (MtIPD3/LjCYCLOPS) leads to activation of relevant transcription factors and gene expression. Within hours of LCO perception, the root hairs are deformed, and transcription of nodulation-specific genes begins in the root cells. Transcriptional regulation of these genes is mediated by transcription factors that belong to NIN, GRAS, NF-YA, and ERF families. Cells within the pericycle and cortical layers of the root initiate processes for cell division by ~24 h after LCO perception. By 48 h, the root hairs curl tightly to entrap the bacteria, and "infection threads" formed through invagination of the infected root hairs subsequently transport the bacteria to the dividing cortical cells. Bacteria colonize these nodule primordia cells and differentiate into membrane-enclosed bacteroids, and a mature nitrogen-fixing nodule forms in 2–3-week period (reviewed by Oldroyd 2013; Kang et al. 2016).

Gene Regulation and Cellular Localization of Gene Products

Rhizobial inoculation of hairy root cultures does not result in nodule formation. Nevertheless, hairy root composite plants can nodulate successfully and efficiently as wild-type plants (Kang et al. 2016). They have been widely used for several key discoveries in nodulation research. One of the earliest and most frequent uses for hairy root composite plants in nodulation research is to evaluate gene regulation and determine sites of expression of nodule-specific and/or nodulation-associated genes. A number of researchers generated series of 5′ deletions of promoters and evaluated transcriptional GUS fusions in hairy root composite plant with the goal of

discovering *cis* elements that confer nodule-specific gene expression. Examples include the evaluation of *P. vulgaris* glutamine synthetase (Shen et al. 1992); evaluation of rice *ENOD40* in soybean (Kouchi et al. 1999); discovery of cross-species regulation of a nodule-specific cysteine protease (Vincent et al. 2000); transcriptional regulation of *V. faba ENOD12* (Frühling et al. 2000), a soybean *PEPCase* (Nakagawa et al. 2003), and *L. japonicus ENOD40* promoters (Gronlund et al. 2005); and conservation of autoregulation gene expression between soybean and Lotus (Nontachaiyapoom et al. 2007) and the *ENOD8* esterase in *M. truncatula* (Coque et al. 2008). The use of translational fusions for cellular localization of proteins involved in nodule development has only been minimally utilized compared to AM fungal symbiosis. The most likely reason is the relatively difficult microscopy procedures required for proper visualization of fusion protein constructs in nodule cells. Nevertheless, hairy root composite plants have been used to discover that a membrane microdomain protein, GmFWL1, localizes to the tip of the soybean root hair cells in response to rhizobial inoculation (Qiao et al. 2017) and that the Rho-like GTPase, LjROP6, localizes to the plasma membrane and cytoplasm during Lotus nodule development (Ke et al. 2012).

Gene Function Discovery

One of the unique uses of hairy root composite plant systems in nodulation research is to evaluate species-specific roles of lectins in determining host specificity. Legume lectin stimulates infection of roots by rhizobia. Interestingly, introduction of the pea lectin gene into white clover hairy roots enables heterologous infection and nodulation by the pea symbiont *R. leguminosarum* biovar *viciae* (Diaz et al. 1989; van Eijsden et al. 1995). Pea lectin-transformed red clover hairy roots form nodule primordium-like structures after inoculation with pea-, alfalfa-, and Lotus-specific rhizobia, which normally do not nodulate red clover. Even exogenous application of lipochitin oligosaccharides derived from a broad range of rhizobia was active resulting in induction of cortical cell divisions and cell expansion. These indicated a broadened response to oligochitin signals in the transformed roots.

While forward genetic studies have contributed significantly to our knowledge on nodulation signaling and development, duplications in legume genomes (Young and Bharti 2012) have posed issues with the use of such an approach. Reverse genetic approaches where candidate genes were specifically selected based on their expression pattern and/or putative annotation have also equally contributed to the discovery of a number of genes involved in nodulation signaling and development. These studies relied heavily on hairy root composite plant technology. Indeed, even for characterization of genes discovered via forward genetics, hairy root composite plant technology was utilized for complementation, cellular localization, and/or expression assays.

One of the earliest studies used antisense suppression of *nodulin-35*, encoding a nodule-specific uricase from *Vigna aconitifolia* (moth bean) to determine that a

reduction in ureide biosynthesis limits the availability of symbiotically fixed nitrogen to the plant (Lee et al. 1993). This crucial experiment led to the conclusion that ureide-producing legumes developmentally control nitrogen assimilation. Similarly, antisense suppression of two small GTP-binding proteins whose cellular function is vesicular transport revealed their role in the biogenesis of the peribacaeroid membrane (Cheon et al. 1993). Antisense nodules were smaller in size and showed lower nitrogenase activity. Coupled with other observations, they appear to play specific roles in the expansion of infected cells and bacteroid release. The results from this study revealed the crucial role of peribacteroid membrane in nodulation and nitrogen fixation. More recent examples where RNAi was successfully used in hairy root composite plants to determine gene function during nodule development include the discovery of roles of a phosphate transporter (Maeda et al. 2006) and a novel RING finger protein (Shimomura et al. 2006) in *L. japonicus*, an apyrase (Govindarajulu et al. 2009), a membrane microdomain protein FWL1 (Libault et al. 2010), and a lipoxygenase (Hayashi et al. 2008) in soybean. Hairy root composite plant technique has enabled discoveries in non-model species with limited genetic resources. The role of a nodule-specific cysteine protease gene in nodule senescence was discovered in the green manure legume *Astragalus sinicus* via RNAi in hairy roots (Li et al. 2008). Knockdown of a translationally controlled tumor protein (TCTP) from the woody leguminous tree *Robinia pseudoacacia* which was upregulated in the infected roots resulted in the impaired development of both nodule and root hair. Subsequent analyses revealed potential involvement of this protein in symbiotic cell differentiation and in preventing premature aging of the young nodules (Chou et al. 2016). The roles of small CLE peptides in systemic inhibition of nodulation via the nodulation autoregulation pathway were determined through overexpression of these peptides in wild-type and autoregulation mutants (Okamoto et al. 2009; Mortier et al. 2010; Lim et al. 2011).

Conserved biochemical function of the key nodulation signaling transcription factor NSP1 was identified through complementation experiments in hairy roots (Heckmann et al. 2006). Close similarities in rhizobial response phenotypes enabled the cloning of NSP1 in *L. japonicus* based on the preexisting knowledge in *M. truncatula*. In hairy root transformations, LjNSP1 and MtNSP1 complemented both *Mtnsp1-1* and *Ljnsp1-1* mutants, indicating an evolutionarily conserved biochemical function. The nodule autoregulation receptor kinase (GmNARK of soybean and HAR1 of *L. japonicus*) is essential for the systemic autoregulation. The expression patterns of a 1.7-kb *GmNARKpr::GUS* in soybean hairy roots and a 2.0-kb *LjHAR1pr::GUS* construct in stable transgenic *L. japonicus* plants were strikingly similar and localized to living cells within vascular bundles, especially phloem cells in leaves, stems, roots, and nodules. Interestingly, the same expression pattern was detected in transgenic *L. japonicus* plants carrying the *GmNARKpr::GUS* construct (Nontachaiyapoom et al. 2007). The ability of promoters from orthologous genes from soybean and *L. japonicus* to interchangeably drive phloem-specific expression suggested high evolutionary conservation of gene regulation and function.

Metabolic Engineering and Hormone Biology

The earliest signals during the establishment of symbiosis between the plant and rhizobia bacteria are the release of flavonoids by the plant roots. These compounds had also been hypothesized to play a role in regulating auxin transport during nodule development, but genetic evidence was not available. In order to evaluate the roles of flavonoids in nodule development, RNA interference in hairy root composite plants was employed to silence key enzymes involved in flavonoid biosynthesis in *M. truncatula* and soybean. Flavonoid-deficient *Medicago* roots showed increased auxin transport and were unable to initiate nodules providing the first genetic evidence for the indispensable role of flavonoids in nodulation (Wasson et al. 2006). Subsequently, key enzymes associated with specific branches of flavonoid biosynthesis were silenced to reveal that flavones and flavonols (or related compounds) have distinct, critical roles during nodulation (Zhang et al. 2009). Flavones are essential as Nod gene inducers to stimulate Nod factor production once the bacteria enter the roots, while flavonols (or related compounds) are essential, most likely, as auxin transport regulators in *M. truncatula*. Indeed, PIN auxin transporters are expressed in *Medicago* nodules, and silencing their expression results in reduced nodule formation (Huo et al. 2006). RNAi silencing of isoflavone biosynthesis in soybean also led to increased auxin transport and reduced nodulation. However, a genistein-hypersensitive *B. japonicum* mutant that can synthesize the Nod signal in the presence of very low levels of isoflavone *nod* gene inducers was able to successfully nodulate these roots (Subramanian et al. 2006). Thus, flavonoid-mediated modulation of local auxin transport at the site of rhizobial infection is crucial during indeterminate nodulation of Medicago, but not during determinate nodulation of soybean (Subramanian et al. 2007). Hairy root composite plants were crucial tools in these studies that discovered distinct key roles for flavonoids during nodulation. Recently, RNAi in hairy roots and chemical supplementation were used to demonstrate the crucial role of flavonoids in actinorhizal nodulation of *Casuarina glauca* (Abdel-Lateif et al. 2013) underscoring the use of the technique in non-model species.

Hairy root composite plants were used to evaluate auxin activity and the role of auxin sensitivity during nodule development and discover key regulatory mechanisms by which hormone balance is achieved during nodule development. Auxin-responsive marker gene expression has been observed during both determinate and indeterminate nodule initiation (Mathesius et al. 1998; Boot et al. 1999; Pacios-Bras et al. 2003; Takanashi et al. 2011), suggesting that auxin might play a key role in nodule initiation. It was subsequently shown using stable and/or hairy root transgenic plants that auxin activity is very low during nodule formation and is suppressed in the nodule infection zone during post-initiation stages of determinate nodule development (Suzaki et al. 2012; Turner et al. 2013). Enhanced sensitivity to auxin in the nodule primordium was associated with reduced sensitivity to cytokinin and resulted in reduced nodule formation (Turner et al. 2013). In agreement, exogenous auxin inhibited nodule formation in *M. truncatula* (van Noorden et al. 2006),

and conversely resistance to auxin resulted in enhanced nodule development in this species (Kuppusamy et al. 2009). A central player in regulating auxin sensitivity in soybean nodules was discovered to be microRNA160 that negatively regulates a set of repressor auxin response factor transcription factors. Hairy root composite plant system was utilized to localize miR160 activity through a fluorescence sensor and to evaluate the role of miR160 in dictating hormone sensitivities and nodule development through loss and gain of function assays and hormone rescue experiments. Results from these experiments indicated that the miR160-ARF10 signaling module dictates developmental stage-specific sensitivities to auxin and cytokinin and directs proper nodule development (Nizampatnam et al. 2015). Similar experiments demonstrated a role for miR167-ARF8 module in dictating auxin sensitivity during nodule development as well (Wang et al. 2015). These discoveries would not have been possible or would have been significantly delayed if not for the availability of hairy root composite plant technologies. This effective transformation tool was employed for a wide range of transgenic manipulations including loss or gain of gene and/or microRNA expression to alter auxin signaling, localization of microRNA activity, quantitative evaluation of marker gene expression, and co-expression of marker gene and gene modification constructs.

Other key examples where hairy root composite plants were used to discover/ demonstrate the roles of hormones during nodulation include RNAi silencing of the cytokinin receptor homolog cytokinin response 1 in *M. truncatula* to demonstrate a key role for the hormone in nodulation (Gonzalez-Rizzo et al. 2006), the expression of a dominant negative abscisic acid (ABA) signaling component to identify a negative role for ABA in *Medicago* nodulation (Ding et al. 2008), the expression of a salicylate hydroxylase to reduce endogenous salicylic acid levels to discover a negative role for this hormone in rhizobial infection and nodulation in *Medicago* and *Lotus* (Stacey et al. 2006), and the expression of cytokinin oxidase to reduced cytokinin levels and demonstrate opposite roles for the hormone in lateral root formation and nematode and rhizobial symbiosis in *Lotus* (Lohar et al. 2004). While not exhaustive, the above list provides a snapshot of the range of different approaches enabled by the use of hairy root composite plant technology to discover the roles of hormones during nodule development. It is worth noting that in the majority of these studies, most hormone-associated phenotypes have been reproducible in hairy roots albeit their slightly altered sensitivity to auxin and cytokinin. For example, the expression of the synthetic auxin marker DR5 in soybean hairy roots (Turner et al. 2013) closely resembles that in root tips and nodules of stable transgenic *L. japonicus* (Suzaki et al. 2012). Reduction in cytokinin levels by the expression of a cytokinin oxidase in *Lotus* hairy roots resulted in increased lateral root numbers as observed in stable transgenic *Arabidopsis* (Lohar et al. 2004). Therefore, despite slightly altered sensitivity to plant hormones, hairy roots appear to be useful tools in studying hormone biology.

Resources for Nodulation Studies in Non-model Plant Species

A number of protocols for hairy root composite plant generation as well as inoculation with AM fungi or rhizobia have been developed for different non-model species. The system developed for peanuts (*Arachis*) is crucial as it enables experiments to evaluate the nonclassical features associated with its nodulation (Sinharoy et al. 2009). Similarly, hairy root generation methods were developed for *Discaria trinervis*, an actinorhizal plant (belonging to the Rosales order). *Frankia* is able to efficiently nodulate *D. trinervis* transgenic roots and nitrogen fixation rates, and feedback inhibition of nodule formation by nitrogen was similar in control and composite plants (Imanishi et al. 2011). In fact, the *MtENOD11*, a marker for early infection-related symbiotic events in model legumes, was expressed in infection zones in root cortex and in the parenchyma of the developing nodule in *D. trinervis* transgenic roots. The unique intercellular infection described in this species can be studied in detail because of hairy root composite plant technology. However, it should be noted that in *Elaeagnus angustifolia* (Russian olive), hairy roots produced unusual pseudoactinorhizal structures that appeared similar to those produced by Frankia (Berg et al. 1992). Therefore, careful experimental design, e.g., uninoculated controls, and thorough evaluation might be needed when adopting hairy root composite plant systems for new plant species. Pea is recalcitrant to transformation and grows poorly on plates, and these qualities have hampered molecular research despite the availability of a number of mutants. A transformation technique using hairy roots and methods for rhizobial inoculation to study early cellular events giving rise to nodule primordia was developed for pea to overcome these challenges (Clemow et al. 2011). Other resources developed for model organisms but can be easily adapted for use in non-model organisms include (a) the plant transformation vector, pHairyRed, that enables high-throughput, nondestructive selection of hairy roots carrying the construct of interest (Lin et al. 2011) and (b) an RNAi system for analyses of gene function by reverse genetics (Sinharoy et al. 2015). A vector system, based on copper-controllable gene expression that provides control over place as well as time of expression of an introduced gene, is an excellent tool that enables functional studies in nodules without pleiotropic effects on root or plant growth (Mett et al. 1996). This system allowed nodule-specific conditional (copper-induced) expression of antisense constructs of aspartate aminotransferase-P2 in transgenic *L. corniculatus* plants. When expression was induced by the addition of copper ions to the plant nutrient solution, aspartate aminotransferase-P2 activity declined dramatically, and a decrease of up to 90% was observed in nodule asparagine concentration. One can envision modification of such a system for use with other inducible expression constructs.

Plant-Pathogen Interactions

Plants are under constant threat from pathogenic microorganisms that attack them, causing detrimental effects on plant health. In agriculture, this results in significant yield losses. Plants deploy pattern recognition receptors to detect microbe- or pathogen-associated molecular signatures. Successful pathogens can evade detection by secreting effector proteins that mask these molecular signatures or inhibit relevant plant signaling. Plants also possess other receptors that act largely inside the cell to detect these effectors. Successful recognition of the pathogen results in rapid and massive transcriptional reprogramming involving a number of plant transcription factors and context-specific co-regulators that are crucial for host immunity (Birkenbihl et al. 2017; Tang et al. 2017).

The majority of plant-pathogen interaction studies have been performed on nontransgenic plants' probability due to the availability of genetic variation, i.e., resistant vs. susceptible genotypes. Basic research on the discovery and understanding of mechanisms of plant resistance to pathogens has heavily utilized transgenic technology on model organisms with the assumption that they might be well conserved across species. However, hairy root composite technology has been used to study a number of species-specific pathogenic interactions on non-model species. Notable are mechanisms of plant resistance against nematodes and the roles of species-specific phytoalexin molecules in plant defense.

Plant-Nematode Interaction

A number of plant parasitic nematodes are obligate pathogens that typically require a plant host to complete their lifecycle. The ability of hairy roots to grow under axenic conditions attracted the interest of researchers as the roots can be used as a vehicle to propagate such nematodes. For example, reproduction of *Meloidogyne javanica* was compared on hairy root cultures from different plant species (Verdejo et al. 1988). While some plant species yielded more females and eggs than others, those roots that grew at moderate rates and produced many secondary roots supported the highest reproduction. The reared nematodes completed their life cycles on new transformed root cultures or greenhouse tomato plants suggesting that no alterations in pathogen biology occurred due to growth on transgenic hairy roots. Similarly, while certain nematodes have broad host specificity enabling research on model plant species, some require the use of native hosts. A gene that confers resistance to root-knot nematode was cloned from the myrobalan plum (*Prunus cerasifera*). This gene confers complete-spectrum, heat-stable, and high-level resistance to the nematode. Hairy root composite plant system was used to determine that this gene can confer the same level of resistance in a complementation assay (Claverie et al. 2011). Other examples include the overexpression of salicylic acid methyltransferase in susceptible backgrounds to confer resistance against soybean cyst nematode (Lin et al. 2013), expression of a nematode gene encoding the secreted

fatty acid- and retinol-binding protein in tomato hairy roots to identify its role in parasitism (Iberkleid et al. 2015), and overexpression of a terpene synthase gene to enhance resistance against soybean cyst nematode (SCN) (Lin et al. 2017). Even in cases where map-based cloning was used to identify candidate genes, hairy root systems were instrumental in confirming function. An excellent example is the discovery of copy number variation of three different genes in the *rhg1-b* locus of soybean, each of which contributes to resistance (Cook et al. 2012). Overexpression of the individual genes in the susceptible cultivar roots was ineffective, but overexpression of the genes together conferred enhanced SCN resistance. The experiments required the evaluation of a number of different plant transformation constructs which required a quick and efficient transformation system such as hairy roots.

Phytoalexins

Phytoalexins are low molecular mass secondary metabolites with antimicrobial activity produced by plants. Their production and/or release is typically induced in response to pathogen attack. A number of phytoalexins are of species-specific nature, and genetic studies to determine their function often necessitate the use of transgenic technology in the native species. Pisatin is an isoflavonoid phytoalexin synthesized by pea (*Pisum sativum* L.). In a pioneering study, hairy roots with reduced pisatin levels were generated by suppression of two key biosynthesis pathway genes, isoflavone reductase (IFR) which catalyzes an intermediate step in pisatin biosynthesis and (+)6a-hydroxymaackiain 3-*O*-methyltransferase which catalyzes the final step, and expression of a fungal gene encoding pisatin demethylating activity (Wu and VanEtten 2004). Hairy roots with reduced pisatin content were more susceptible, underscoring the hypothesis that phytoalexin production is a disease-resistance mechanism. Isoflavonoid derivatives have phytoalexin activity in soybean and a number of other legumes. Silencing of the key isoflavonoid biosynthesis enzyme, isoflavone synthase (IFS), or the 5′-deoxyisoflavonoid branch pathway enzyme, chalcone reductase (CHR), led to the breakdown of resistance against the root rot pathogen, *Phytophthora sojae*. Loss of resistance was accompanied by suppression of hypersensitive response (HR) cell death and suppression of cell death-associated activation of hydrogen peroxide and peroxidase. Results from these studies indicated that 5-deoxyisoflavonoids play a critical role in the establishment of cell death and race-specific resistance (Subramanian et al. 2005; Graham et al. 2007). In another study, soybean hairy root lines with suppressed expression of chalcone synthase 6 or isoflavone synthase 2 had significantly lower levels of isoflavones and their derivative coumestrol (Lozovaya et al. 2007). These roots failed to induce the production of soybean phytoalexin glyceollin, in response to the fungal pathogen *Fusarium solani* f. sp. *glycines* that causes soybean sudden death syndrome. In agreement, there was very high fungal growth on these roots indicating the importance of phytoalexin synthesis in root resistance to pathogens. Soybean hairy roots transformed with the resveratrol synthase and resveratrol oxymethyl transferase genes accumulated glycoside conjugates of the stilbenic compound

resveratrol and the related compound pterostilbene, which are normally not synthesized by soybean plants. Interestingly, the accumulation of these compounds increased their resistance to the soybean pathogen *Rhizoctonia solani* (Zernova et al. 2014). Another key example for the use of hairy root composite plants in phytoalexin research is the discovery of ATP-binding cassette (ABC) transporter located in the plasma membrane as the likely transporter of isoflavonoid phytoalexins (Banasiak et al. 2013).

A variety of novel approaches in plant-pathogen interaction research were possible because of the availability of hairy root composite plant systems. Examples include the use of *Medicago* hairy roots as a model to study the smut pathogen *Ustilago maydis* (Mazaheri-Naeini et al. 2015), discovery of the role in virulence of the *Phytophthora* effector PSR2 in soybean (Xiong et al. 2014), and evaluating pathogen-induced expression of the soybean GmaPPO12 promoter including the identification of potential regions crucial for induction (Chai et al. 2013). Another interesting approach is the expression of pathogen elicitors in plants to determine their role in pathogenesis. Such an approach has also been used in hairy root systems to induce the production and secretion of high-value plant metabolites. For example, the expression of oomycetal proteinaceous elicitor, β-cryptogein, in *Coleus blumei* hairy roots under the control of alcohol-inducible promoter caused significant decrease of soluble phenolics and rosmarinic acid in hairy root lines and increase of phenolics, rosmarinic acid, and caffeic acid in the culture medium (Vukovic et al. 2013). These data suggest that β-cryptogein might be a potential regulatory factor for phenolic secretion from the roots and can be utilized in commercial production systems for efficient extrusion into the culture medium.

Microbiome Research

Intricate coevolution does not appear to be limited to the plant host and specific microbes. Plant growth, development, and health are also influenced by interactions among members of the microbial communities and simultaneous interaction of the plant with multiple members of the community (Tkacz and Poole 2015). The influence of plant genotype and the environment on the composition and diversity of rhizosphere microbiota is subject of wide interest (Gottel et al. 2011; Bulgarelli et al. 2012; Peiffer et al. 2013; Philippot et al. 2013). In particular, how the microbiome composition is influenced by specific rhizodeposit compounds and/or rhizodeposition machinery has also been investigated (Walker et al. 2003; Bais et al. 2006). The availability of genetic mutants impaired in biosynthesis and transport of specific rhizodeposit compounds are crucial for the success of these studies (Badri et al. 2008, 2009). A number of species-specific compounds are likely to attract specific microbes that have the capacity to metabolize them as carbon source, or might serve as signal molecules to specific rhizosphere microbes (e.g., isoflavonoids in soybean). A major bottleneck in evaluating the roles of these compounds is the lack of a comprehensive collection of genetic mutants in all plant species. Hairy

root composite plants offer great potential for these studies as transgenic can be used to alter the activities of specific host genes and evaluate their roles in microbiome composition and activity.

Recently RNAi in hairy root composite plants was used to silence the biosynthesis of isoflavonoids in soybean with the goal of evaluating their role in shaping the rhizosphere microbiome (White et al. 2015, 2017). The results from these studies showed that hairy root transformation itself influenced the bacterial community structure. The most abundant phyla in proximal soils of untransformed roots were *Proteobacteria* (~79%) and *Bacteroidetes* (~8–11%) in agreement with a number of other plants. Interestingly, the abundance of *Proteobacteria* was much lower (~56–60%), whereas that of *Bacteroidetes* was higher (~16–22%) in proximal soils of hairy roots. This indicated that the hairy root transformation influenced rhizosphere bacterial communities even at the phylum level. Subsequent evaluation revealed that hairy root transformation impacted numerous bacterial families that were otherwise unaffected in proximal soils of untransformed soybean roots when compared to that of the bulk soil. However, the majority of the families (~74%) that were differentially abundant in the untransformed roots vs. bulk soil showed similar trends of differential abundance in hairy roots suggesting that these families can be successfully studied using hairy roots. Notable exceptions included *Sphingomonadaceae* which were enriched in untransformed roots but unaltered in hairy roots and *Acidobacteriaceae* whose abundance was reduced in untransformed roots but unaltered in hairy roots. Another study sought to obtain knowledge on microbiome of specific organic growing media that inhibit hairy root induction (Grunert et al. 2014a). The goal in this study was to utilize the knowledge to control hairy roots as it is a major disease in tomato. A comparison of the microbiomes of organic and inorganic growing media indicated potential competitive interactions of specific microbial families with *A. rhizogenes* (Grunert et al. 2014b) providing promising potential for disease suppression.

Recent Developments and Future Prospects

A number of root-microbe interactions result in distinct cell-type-specific responses. For example, localized responses occur in cells colonized by the microbe that are distinct from those in adjacent non-colonized cells. In root nodules, there are specific zones and cell types with distinct biological functions and gene expression patterns. Therefore, profiling gene expression and other molecular signatures at cell-type resolution has very high potential to better inform us about plant responses during their interaction with microbes. Cell-type-specific profiling is enabled by two recent methods, isolation of nuclei tagged in specific cell types (INTACT; Deal and Henikoff 2011) and translating ribosome affinity purification (TRAP; Mustroph et al. 2013; Reynoso et al. 2015). Both techniques require a well-characterized cell-type-specific promoter. With the availability of a number of promoters that respond specifically to specific microbes in colonized cells, the application of these methods

in root-microbe interactions is an exciting possibility using hairy root composite plants. In cells expressing the INTACT construct, nuclear envelope is tagged with a biotin ligase recognition sequence, and the co-expressed biotin ligase biotinylates them. This enables isolation of those tagged nuclei using streptavidin affinity purification. Therefore, if the construct were to be driven using a promoter that is specifically expressed in arbuscule-containing cells, nuclei from those cells can be isolated and molecular signatures evaluated. Similarly, cells expressing the TRAP construct have ribosomes assembled with a larger subunit protein that carries a FLAG peptide tag. These ribosomes (along with bound RNAs) can be affinity purified using an anti-FLAG antibody.

A suite of promoters that mark cell- or tissue-specific expression were developed for root development research in tomato (*Solanum lycopersicum*) using the hairy root composite plant system (Ron et al. 2014). These include promoters that drive expression in the stele, endodermis, QC and initials, phloem, maturing xylem, meristematic cortex cells, meristematic, elongating and mature cortex cells, lateral root cap and epidermal cells, and the tomato root meristem. Remarkably, the authors stated that the gene expression of reporters was indistinguishable in plants transformed by *A. tumefaciens* (when available) as compared with *A. rhizogenes*. This suggested that hairy roots are not only anatomically similar to wild-type roots but also have similar cell-type identity and developmental signaling pathways. Therefore, the use of hairy root composite plants to evaluate cell-type-specific responses to microbe interactions is very promising.

Another exciting development is the use of genome editing tools in hairy root composite plants. RNA-guided genome editing using the bacterial type II CRISPR/ Cas9 system enables precise, site-specific deletions in the DNA (reviewed by Bortesi and Fischer 2015). A single-guide RNA that contains a guide sequence region of 19–22 bp that matches the target DNA sequence to be mutated guides the nuclease to the sequence-specific position on the DNA for cleavage. Sequence-specific guide RNAs can be designed against genes of interest to generate specific deletions and thus effectively generate null alleles. Hairy root composite plants have been used primarily to evaluate the efficacy of various guide RNAs and promoters to drive the components prior to investing time in generating stable transgenic plants (Jacobs et al. 2015; Michno et al. 2015; Jacobs and Martin 2016). However, currently the method suffers from relatively poor mutagenic efficiency. For example, when a sgRNA targeting SYMRK locus was expressed in *L. japonicus* hairy roots, only about 35% mutagenic efficiency was observed. The authors also evaluated two sgRNAs targeting three homologous leghemoglobin loci to obtain multigene knockouts. Only 20 out of 70 hairy root transgenic plants exhibited white nodules, with at least two leghemoglobin genes disrupted in each plant (Wang et al. 2016). Nevertheless, with improved efficiencies using more efficient nucleases (e.g., Murovec et al. 2017), and multiple guide RNAs, it might be possible to utilize CRISPR gene editing in hairy root composite plants for root-microbe interaction research.

Limitations and Considerations

Research summarized above provides a snapshot of the broad applications that have been enabled in root-microbe research because of hairy root composite plant systems. It should, however, be noted that the majority of monocotyledonous species are not or poorly amenable to *A. rhizogenes*-mediated hairy root transformation (Porter and Flores 1991). Therefore, the majority of these approaches are limited to dicotyledonous species. As discussed with some examples above, the method produces composite plants with transgenic roots and untransformed shoots. Therefore, shoots still have a "wild-type" phenotype with regard to the activity of the gene target when used for loss or gain of function assays. For example, if a particular compound is transported from the shoot to the root, silencing biosynthesis in the root alone may not be effective. The majority of procedures used for hairy root composite plant generation initiate multiple independent transgenic roots from the shoot explant. In many protocols, the gene cassette of interest is carried on a separate binary vector and not on the Ri plasmid. Therefore, only a portion of the hairy roots (typically 20–60%) carry the gene cassette of interest. This necessitates the use of a "visible" selectable markers (e.g., constitutively expressed fluorescent proteins) to enable separation of "non-transgenic" roots from roots useful for experimental procedures. Finally, each hairy root is an independent transgenic event, and therefore some level of variation is expected from root to root even in the same hairy root composite plant. Nevertheless, highly efficient procedures enable generation of a large number of hairy root composite plants in a small space within a relatively short period of time which can easily overcome a number of these limitations.

Conclusions

Hairy root composite plants have been and will continue to be a transformative tool in root-microbe interaction research. The tool has enabled the implementation of a variety of transgenic research approaches in multiple plant species that do not have effective transformation systems. Of particular note are legumes that are agriculturally significant due to their high protein grains and symbiotic nitrogen fixation capacity. The ability to monitor hormone and microbe-responsive marker genes, fluorescently tag cellular compartments and organelles, localize proteins of interest, and manipulate gene and microRNA activities and complementation assays for confirmation of gene function and determination of evolutionary conservation are key applications that have pushed the frontier of knowledge on legume-microbe interactions. Limitations of hairy root composite plant systems can be easily overcome with careful experiment design taking into consideration specific limitations such as the need for a visible selection marker, root-to-root variability, and an altered microbiome. Recent developments such as conservation of cell-type-specific markers and

the application of genome-editing tools are exciting opportunities that would enhance the utility of hairy root composite plants for discoveries in root-microbe interaction research.

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Chapter 3
Production and Biosynthesis of Bioactive Stilbenoids in Hairy Root Cultures

Tianhong Yang, Lingling Fang, and Fabricio Medina-Bolivar

Abstract Stilbenoids are plant defense phenolic compounds that exhibit numerous biological activities with potential applications in human health. These compounds are present in non-taxonomically related plants species, such as grape and peanut. As many other defense compounds, most stilbenoids are either absent or accumulate in very low levels in non-stressed plants, thereby, highlighting the need to develop a sustainable system for their production. In order to address this issue, hairy root cultures of stilbenoid-producing species, including grapevine (*Vitis vinifera*), muscadine (*V. rotundifolia*), and peanut (*Arachis hypogaea*), have been established via infection of plant tissues with *Agrobacterium rhizogenes*. Several elicitation treatments have been explored in order to increase the levels of stilbenoids in these cultures. Among these treatments, the co-treatment with methyl jasmonate and methyl-β-cyclodextrin has been the most effective in providing sustainable and high levels of stilbenoids. Different types of stilbenoids have been identified in hairy roots of different plant species. For instance, prenylated stilbenoids are found in elicitor-treated peanut hairy root cultures but not in hairy root cultures of muscadine or grapevine. In addition to providing a platform for stilbenoid production, hairy roots are also being explored to study the biosynthesis of these bioactive compounds. The present chapter provides the status of production and biosynthesis of stilbenoids in grapevine, muscadine, and peanut hairy root cultures.

Keywords *Agrobacterium rhizogenes* • Cyclodextrin • Elicitation • Grape • Grapevine • Hairy root • Muscadine • Peanut • Stilbene • Stilbenoid

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Introduction

Plants defend themselves against pathogen attack through various mechanisms, such as induction of defense-related proteins, accumulation of active oxygen species, promotion of the hypersensitive response, and production of low molecular mass secondary metabolites, which are synthesized de novo after stress and known as phytoalexins (Ahuja et al. 2012). Based on their chemical structure, phytoalexins cover diverse classes of molecules including flavonoids, isoflavonoids, stilbenoids, and sequiterpenes. The stilbenoids can be found in a limited number of taxonomically non-related plant species, including economically important crops such as grapevine (*Vitis vinifera*), peanut (*Arachis hypogaea*), and blueberry (*Vaccinium* spp.) (Flamini et al. 2013; Lyons et al. 2003; Rimando et al. 2004; Sales and Resurreccion 2014; Shen et al. 2009). In addition to their role as phytoalexins (Ahuja et al. 2012; Hasan et al. 2012), stilbenoids also provide protection against oxidative and other abiotic stresses such as UV radiation (Chong et al. 2009). Recently, stilbenoids have received tremendous interest not only for their *in planta* function as phytoalexins but also for their wide variety of biological activities in mammalian cells, which have potential benefits on human health (Baur and Sinclair 2006).

Biological Activities of Stilbenoids

As a most notable member in the stilbenoid family, resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) has a wide range of biological activities which may have impact on human health, including antioxidant, anti-inflammatory, cardioprotective, antiviral, anticancer, and antiaging properties as demonstrated in in vitro and animal studies (Baur and Sinclair 2006; Tomé-Carneiro et al. 2013). The number of preclinical and clinical trials of resveratrol on various human diseases is growing gradually (Tomé-Carneiro et al. 2013). Despite the many effects observed in vitro, the poor bioavailability of resveratrol in vivo due to its rapid absorption and metabolism leading to various metabolites such as resveratrol glucuronides and sulfates has been a classical drawback for this molecule (Vitaglione et al. 2005).

In addition to resveratrol, several in vitro studies have focused on the biological activities of natural resveratrol analogs, such as the prenylated stilbenoids. Among these, the prenylated stilbenoid arachidin-1 exhibited equivalent or even better antioxidant activity in a pork oil system than butylated hydroxytoluene (BHT), which is primarily used as an antioxidant food additive (Chang et al. 2006). Similarly, arachidin-1 isolated from peanut hairy root culture prevented the oxidation of human low-density lipoproteins in a thiobarbituric acid reactive substance (TBARS) assay (Abbott et al. 2010). Another prenylated stilbenoid, arachidin-2, showed a very potent inhibition of reactive oxygen species (ROS) generation in human promyelocytic leukemia (HL-60) cells when compared to other peanut phytoalexins (Sobolev et al. 2011).

The anti-inflammatory activity of prenylated stilbenoids has been mainly tested on mouse macrophage cells. Prenylated stilbenoids arachidin-1, arachidin-2, and arachidin-3 reduced the production of prostaglandin E2 (PGE2) and the level of nitric oxide in lipopolysaccharide-induced RAW 264.7 macrophages (Chang et al. 2006; Djoko et al. 2007; Sobolev et al. 2011). Arachidin-1 also inhibited the expression level of COX-2 protein, which has been linked to the production of inflammatory mediators and is commonly used as a marker for anti-inflammatory activity of natural compounds (Djoko et al. 2007).

Studies on anticancer activity have shown that arachidin-1 exhibits high efficacy in inducing programmed cell death in HL-60 cells, with an approximately fourfold lower EC_{50} than resveratrol (Huang et al. 2010). Importantly, this prenylated stilbenoid had no effect on nonproliferating cells. In another cytotoxicity study, arachidin-1 also exhibited moderate cytotoxicity to other four cancer cells lines (SK-MEL, KB, BT-549, and SK-OV-3) (Sobolev et al. 2011).

Arachidin-1 purified from black skin peanut seeds has shown higher anti-adipogenic activity than resveratrol and arachidin-3 by inhibiting adipocyte differentiation in 3T3-L1 cells. This anti-adipogenic activity had no cytotoxicity on the differentiating pre-adipocytes, and therefore it was suggested that arachidin-1 should be further explored in anti-obesity strategies (Liu et al. 2013). More recently, arachidin-1 and arachidin-3 isolated from peanut hairy root cultures exhibited anti-viral activity in rotavirus-infected HT29.f8 cells by inhibiting rotavirus replication (Ball et al. 2015). This antiviral activity was not observed with either of their non-prenylated analogs, piceatannol or resveratrol, suggesting that the antiviral mechanism might depend on the prenyl moiety of arachidin-1 and arachidin-3.

In order to identify a potential molecular target for different peanut prenylated stilbenoids, a type I and type II cannabinoid receptor (CNR1 and CNR2) binding study of arachidin-1 and arachidin-3 was performed, and the result demonstrated that these compounds modulate both cannabinoid receptors in vitro (Brents et al. 2012). Molecular modeling studies with CNR2 indicated that the lipophilic side chain (3-methyl-1-butenyl group or 3-methyl-2-butenyl) in the structure of the arachidins improved their binding affinity to the cannabinoid receptor (Brents et al. 2012). Moreover, in the kinetic study of eight human recombinant UDP-glucuronosyltransferase (UGTs) using peanut phytoalexins as substrates, arachidin-3 and arachidin-1 were metabolized to fewer glucuronidated products and exhibited slower metabolism than their non-prenylated analogs, resveratrol and piceatannol, indicating that the prenylated side chain prevents the formation of additional glucuronidated products and might enhance the metabolic stability and bioavailability of these stilbenoids (Brents et al. 2012).

Hairy Root Cultures of *Vitis* Species

Hairy roots are naturally induced by *Agrobacterium rhizogenes* on plants by transferring the T-DNA regions of the Ri-plasmid from the bacterium to the plant genome. When the T-DNA-encoded genes involved in auxin biosynthesis and
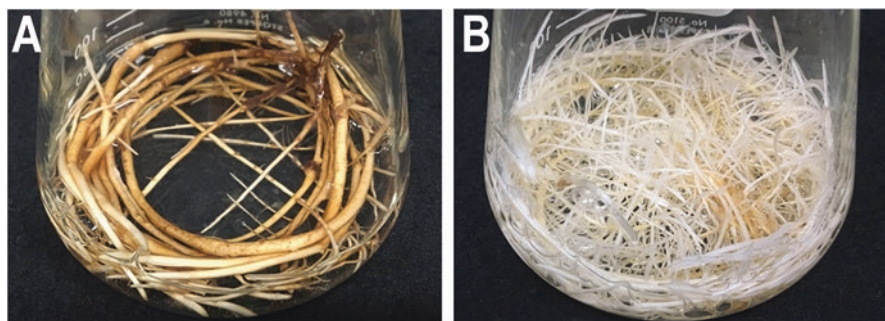
Fig. 3.1 Phenotypes of muscadine and peanut hairy root cultured in a 250 mL Erlenmeyer flasks. (**a**) Hairy root culture of muscadine (*Vitis rotundifolia*) cv. Noble line Nob-2Cot. (**b**) Hairy root culture of peanut (*Arachis hypogaea*) cv. Hull line 3A

sensitivity are integrated into the plant genome and expressed, the transformed plant cells can differentiate into "immortalized" roots that can be maintained in hormone-free medium. Moreover, as differentiated tissues, hairy roots can reproduce the entire biosynthetic potential of the parental plant. With advantages of high growth rate and genetic stability, the hairy root culture system has been used as a tool to investigate the production and biosynthesis of plant specialized metabolites under controlled conditions.

The first study describing the stilbenoid profile in a *Vitis* species hairy root culture has been carried out in muscadine grape (*Vitis rotundifolia*; herein referred as muscadine) (Nopo-Olazabal et al. 2013). Two hairy root lines, Fry-3A and Nob-2Cot, were produced via *A. rhizogenes*-mediated transformation of muscadine cvs. Fry and Noble, respectively (Fig. 3.1a). The accumulation of four stilbenoids, resveratrol, piceatannol, ε-viniferin, and piceid, in these two hairy root culture lines was studied upon treatment of cultures with the stress hormone methyl jasmonate (MeJA). The levels of stilbenoids were determined by high-performance liquid chromatography. Later, the effects of MeJA and hydrogen peroxide on the accumulation of stilbenoids in cultures of line Fry-3A were further determined along a time course from 0 to 96 h (Nopo-Olazabal et al. 2014).

Hairy root cultures of another *Vitis* species, the common grape vine (*V. vinifera*; herein referred as grapevine) of cv. Pinot Noir 40024, have been established as well, via infection of *A. rhizogenes* ATCC 15834 (Tisserant et al. 2016). Elicitation of hairy roots with MeJA and methyl-β-cyclodextrin (MCD) led to an induction of resveratrol, piceid, ε-viniferin, and δ-viniferin in the medium. Moreover, ^{13}C NMR-based dereplication method and liquid chromatography coupled to mass spectrometry (LC-MS) were used to identify other oligostilbenes, including pallidol, maackin, scirpusin A, and vitisin B, in medium extracts of cultures elicited with MeJA and MCD (Fig. 3.2) (Tisserant et al. 2016).
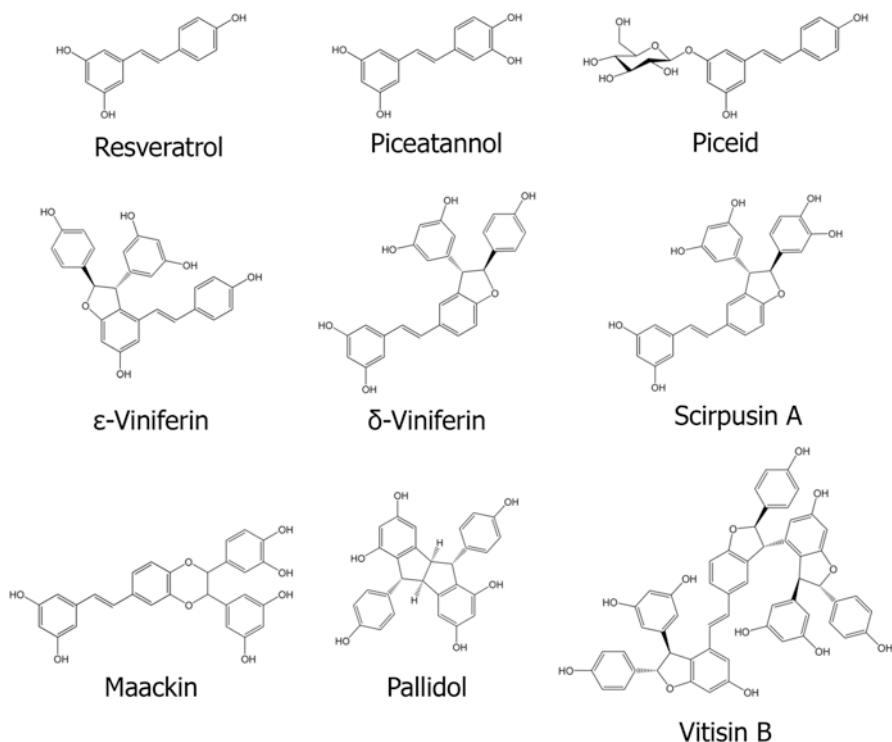
Fig. 3.2 Stilbenoids identified in grapevine and muscadine hairy root cultures

Growth Patterns of *Vitis* Hairy Root Cultures

Muscadine hairy root lines Fry-3A and Nob-2Cot lines were grown in 250 mL Erlenmeyer flasks with 50 mL of BDS (modified B5 medium; Dunstan and Short 1977) medium containing 3% sucrose, while the hairy root line of grapevine was cultured in 100 mL flasks with 20 mL of half strength SH (Schenk and Hildebrandt 1972) medium with 2% sucrose (Nopo-Olazabal et al. 2013; Tisserant et al. 2016). The scale of culture, culture medium, and number of root tips for subculture varied among these cultures; however, all shared a similar growth pattern in liquid medium. For instance, muscadine line Fry-3A had no lag phase after subculture and reached the stationary phase at day 24 with a specific growth rate (μ) of 0.065 day^{-1} (doubling time T_d = 10.7 days) and 0.082 day^{-1} (doubling time T_d = 8.4 days) in BDS medium and BDS medium containing growth regulators, respectively (Nopo-Olazabal et al. 2013). The hairy root line of grapevine also showed no apparent lag phase and reached the stationary phase at day 35 with a growth rate of 0.046 day^{-1} (doubling time T_d = 14.9 days) in half strength SH medium (Tisserant et al. 2016). In comparison, grapevine cell suspensions reached the stationary phase after 14 days of culture (Bru et al. 2006).

Constitutive Stilbene Production in *Vitis* Hairy Roots

Resveratrol and other stilbene derivatives in *Vitis* species play a role as phytoanticipins and are stored in the root as well as in other tissues at basal concentrations (VanEtten et al. 1995). As a highly differentiated tissue, the hairy roots of *Vitis* species also inherit this feature from their parental plant. About 20 μg g^{-1} DW of resveratrol, 40 μg g^{-1} DW of piceid, and 86 μg g^{-1} DW of ε-viniferin were observed in the tissue of muscadine Fry-3A and Nob-2Cot hairy root lines after 21 days of culture without elicitation (Nopo-Olazabal et al. 2013). Similarly, hairy root cultures of grapevine accumulated per flask about 175 and 217 μg of stilbenes, which included resveratrol, piceid, ε-viniferin, and δ-viniferin, at 25 and 35 days of culture, respectively (Tisserant et al. 2016). Interestingly, most of the constitutive stilbenes in hairy root cultures of *Vitis* species were accumulated inside the root tissue, while only between 1 and 5% of the total amount of stilbenes were secreted into the culture medium.

Induction of Stilbenoid Production in Hairy Roots of *Vitis* Species in Response to Various Elicitors

The biosynthesis of stilbenoids in *Vitis* is highly induced by environmental stress. As an oxidative stress agent, hydrogen peroxide has been used as elicitor to induce the production of stilbenoids in muscadine Fry-3A hairy root line (Nopo-Olazabal et al. 2014). After hydrogen peroxide treatment, the level of intracellular stilbenoids increased slightly from the basal level. The main stilbenoids stored in the root were piceid and ε-viniferin, while resveratrol was mainly secreted into the medium with a maximum yield of 72.7 μg g^{-1} DW after 10 mM hydrogen peroxide treatment.

As the most common signaling molecule used for the elicitation of grape cell suspensions (Lijavetzky et al. 2008; Belchí-Navarro et al. 2012), MeJA has also been applied to hairy root cultures of grapevine and muscadine to stimulate stilbenoid production. Upon MeJA treatment, the maximum yield of intracellular resveratrol in muscadine Fry-3A hairy root line was 106 μg g^{-1} DW, a sevenfold increase when compared to the non-elicited control, while 48.5 μg g^{-1} DW of resveratrol was secreted into the culture medium (Nopo-Olazabal et al. 2014). The intracellular levels of piceid and ε-viniferin showed no significant increase when compared to the non-elicited control, and about 3.9 μg g^{-1} DW of piceid and 11.4 μg g^{-1} DW of ε-viniferin were found in the medium upon treatment with MeJA (Nopo-Olazabal et al. 2014). The hairy roots of grapevine also responded to MeJA treatment. Total stilbenoids, including resveratrol, piceid, ε-viniferin, and δ-viniferin, increased from a basal level of approximately 1400–2730 μg g^{-1} DW in the hairy root tissue after MeJA treatment (Tisserant et al. 2016). Under the same treatment, total stilbenoids in the medium of the grapevine hairy root culture showed about eightfold

increase when compared to the non-elicited group, reaching 19 mg/L (Tisserant et al. 2016). In general, the production of intracellular and extracellular stilbenoids in both grapevine (*V. vinifera)* and muscadine (*V. rotundifolia)* hairy root cultures was induced by MeJA, and about 20% of total stilbenoids were secreted into the medium. Resveratrol was the main form of stilbenoid secreted into the culture medium, while most of the piceid and viniferins remained intracellularly.

Cyclodextrins (CD) are molecules derived from starch with hydrophobic inner and hydrophilic outer surfaces. They have been used in food, pharmaceutical, drug delivery, and chemical industries. The beta form of cyclodextrin (β-CD) consists of seven sugar molecules bound together in a ring and can form 1:1 inclusion complexes with resveratrol (López-Nicolás et al. 2006) and piceatannol (Matencio et al. 2016), resulting in an increase in the solubility of these compounds in solution. The methylated form of β-CD (MCD) has been used as an approach to increase the production of stilbenoid in grape cell suspension cultures (Belchí-Navarro et al. 2012; Bru et al. 2006; Lijavetzky et al. 2008), as well as in the grape hairy root cultures. Upon treatment of 30–70 mM MCD, grapevine hairy root cultures produced 5500 µg g^{-1} DW of intracellular stilbenoids and 91 mg/L of secreted stilbenoids, which represented 4.5- and 920-fold increases when compared to the non-elicited controls, respectively (Tisserant et al. 2016).

It has been reported that MCD and MeJA have a synergistic effect on the biosynthesis and production of stilbenoids in both grapevine cell suspension culture (Belchí-Navarro et al. 2012) and peanut hairy root cultures (Yang et al. 2015). A similar effect has also been observed in grapevine hairy root culture. Upon co-treatment with MeJA and MCD, hairy root cultures of grape produced about 7000 µg g^{-1} DW of intracellular stilbenoids and secreted 165 mg/L of stilbenoids into the medium (Tisserant et al. 2016). By increasing their solubility, the use of MCD led to an extremely high amount of secreted stilbenoids (more than 90% of total stilbenoids) in grapevine hairy root cultures when compared to non-MCD treatment (less than 20%) and control group (only 1–5%).

Functional Characterization of Genes in Grapevine Hairy Root Culture

Other than a production system for stilbenoids, grapevine hairy roots have been used for functional characterization of transcriptional factors which regulate phenolic biosynthetic pathways in grapevine. For instance, to study the regulation of two MYB transcription factors in the proanthocyanidin pathway, *VvMybPA1* or *VvMybPA2* was ectopically expressed in grapevine hairy roots, resulting in a significant increase of proanthocyanidin in the root tissue (Terrier et al. 2008). The ectopic expression of *MYB15* resulted in a fivefold increase in the accumulation of resveratrol glucoside, piceid, and elevated levels of phenylalanine ammonia-lyase (PAL) and stilbene synthase (STS) expression in grapevine hairy roots (Holl et al. 2013).

More recently, to elucidate the *in planta* role of shikimate dehydrogenase which is involved in grape gallic acid biosynthesis, *VvSDH3* was overexpressed in grapevine hairy root, and this approach led to enhanced contents of aromatic amino acids and hydroxycinnamates (Bontpart et al. 2016).

Hairy Root Culture of Peanut

Peanut (*Arachis hypogaea*), a species from the Fabaceae (Leguminosae) family, is capable of producing resveratrol and other polyphenolic compounds as phytoalexins. More than 30 stilbenoid derivatives have been described in peanut tissue upon biotic or abiotic stress (Potrebko and Resurreccion 2009; Sobolev et al. 2010; Sobolev 2013; Sobolev et al. 2016; Wu et al. 2011). Interestingly, the majority of these stilbenoids are prenylated and contain 3-methyl-1-butenyl, 3-methyl-2-butenyl, and other moieties which are derived from a five-carbon side chain (Fig. 3.3) (Aguamah et al. 1981; Cooksey et al. 1988; Sobolev et al. 2006). In addition to peanut, prenylated stilbenoids can be also found in *Macaranga mappa* (Van Der Kaaden et al. 2001) and *M. alnifolia* (Yoder et al. 2007) (Euphorbiaceae family), *Artocarpus integer* (Boonlaksiri et al. 2000) and *A. gomezianus* (Hakim et al. 2002) (Moraceae family), *Carex distachya* (Fiorentino et al. 2006) (Cyperaceae family), and *Lonchocarpus chiricanus* (Ioset et al. 2001) (Leguminosae family), but these compounds are rare in other stilbenoid-producing species, such as *V. vinifera* or *V. rotundifolia*. Interestingly, other legume species such as *Sophora flavescens* and *Glycine max* that do not produce stilbenoids produce prenylated flavonoids and prenylated isoflavonoids (Yazaki et al. 2009).

Despite the diverse bioactivities of prenylated stilbenoids demonstrated in in vitro studies, in vivo studies have not been conducted due to their limited availability (Chang et al. 2006; Liu et al. 2013; Sobolev et al. 2011; Sobolev 2013). As phytoalexins, peanut stilbenoids are normally produced upon biotic or abiotic stress, and many studies have used fungus (*Aspergillus* and *Rhizopus*) to induce the biosynthesis of prenylated stilbenoids in peanut seeds (Aisyah et al. 2015; Cooksey et al. 1988; Sobolev 2008; Sobolev et al. 2009, 2010, 2016). However, when peanut seeds are co-cultured with fungi, toxic compounds such as aflatoxins are produced by *Aspergillus* species, resulting in a potentially negative effect to human health. Moreover, *A. flavus* and *R. oligosporus* can modify and metabolize prenylated stilbenoids by glycosylation, sulfation, and hydroxylation (Aisyah et al. 2015). This complex profile of stilbenoids in fungal-challenged peanut seeds makes it difficult for distinguishing peanut stilbenoid derivatives produced by the peanut seed itself or from fungal stilbenoid metabolism and presents a challenge for the identification of peanut stilbenoids and elucidating their biosynthesis. As an axenic elicitor-controlled bioproduction platform, the hairy root culture of peanut offers tremendous advantages when compared to fungal-challenged peanut seeds, and therefore hairy roots are being used for producing peanut stilbenoids and elucidating their biosynthetic pathway.
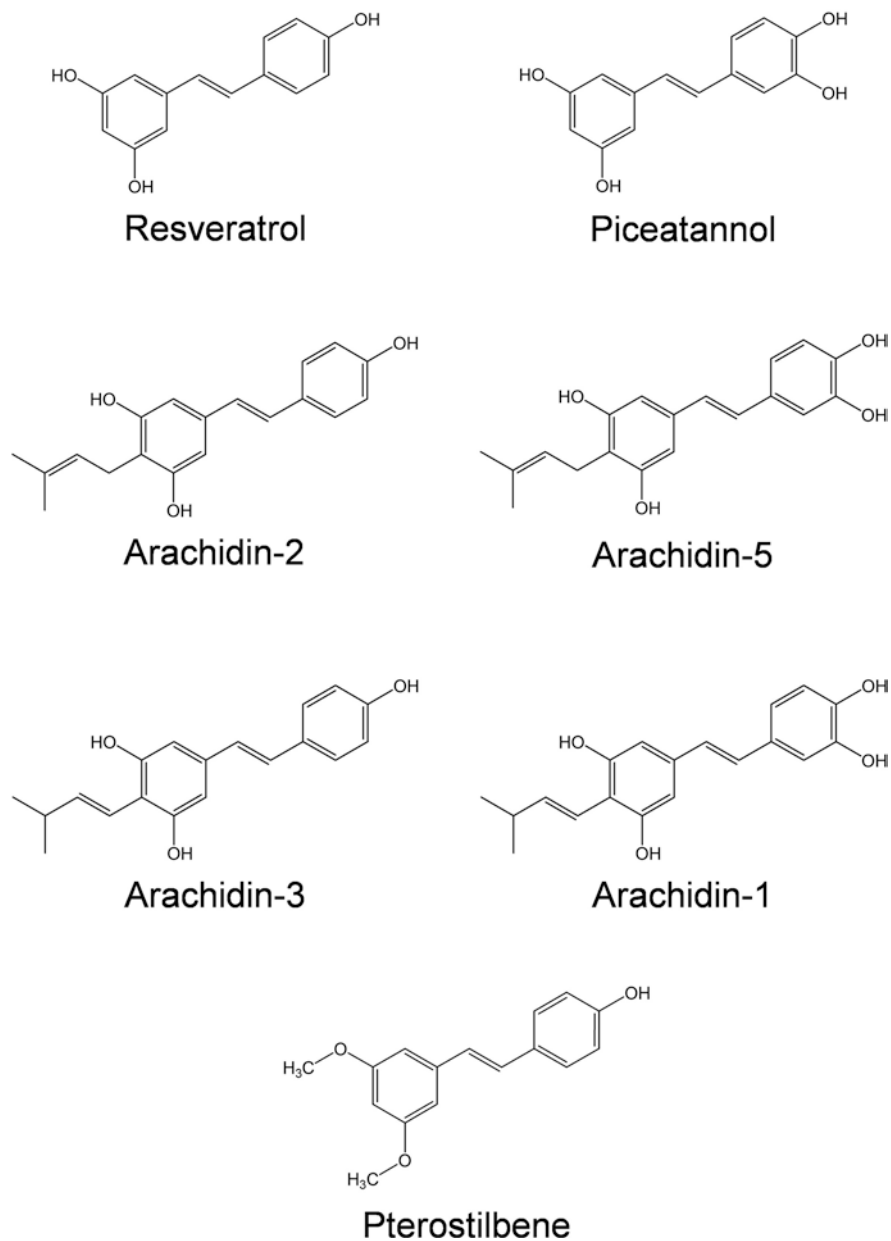
Fig. 3.3   Stilbenoids identified in peanut hairy root cultures

The first use of a peanut hairy root culture for stilbenoid production was reported in 2007 (Medina-Bolivar et al. 2007). Three peanut hairy root lines were obtained from peanut cv. Andru II via infection of *A. rhizogenes* ATCC 15834, and line 2 with the highest response to sodium acetate elicitor treatment was selected for further elicitation analysis. Upon 10.2 mM sodium acetate treatment, peanut hairy root line 2 produced resveratrol and pterostilbene. A hairy root culture of another peanut cultivar, i.e., Hull, was established later. In this study, cotyledonary leaves generated from in vitro seedings of peanut cv. Hull were inoculated with *A. rhizogenes* ATCC 15834, and hairy root line 3 was selected for its vigorous growth (Condori et al. 2010) (Fig. 3.1b). Under selected elicitor treatments, hairy root cultures of cv. Hull line 3 produced and secreted into the culture medium resveratrol, piceatannol, and their prenylated analogs arachidin-1 and arachidin-3 which were also described in fungal-challenged peanut seeds (Fig. 3.3) (Condori et al. 2010; Yang et al. 2015). Moreover, other two prenylated analogs, arachidin-2 and arachidin-5, were identified in the culture medium upon co-treatment of MeJA with MCD (Fig. 3.3) (Yang et al. 2016).

Growth of Peanut Hairy Root Culture

Peanut hairy root lines Andru II line 2 and Hull line 3 were grown in 250 mL Erlenmeyer flasks with 50 mL of medium at 28 °C and continuous darkness (Medina-Bolivar et al. 2007; Condori et al. 2010; Yang et al. 2015). An exponential growth of Andru II line 2 in B5 medium with 2% sucrose was observed from day 9 (~0.3 g of fresh weight) to day 21 (~5.1 g of fresh weight) with specific growth rate (μ) of 0.236 day^{-1} (Medina-Bolivar et al. 2007). The medium used for growing peanut hairy roots of Hull line 3 was a modified MS (MSV) medium with 3% sucrose instead of B5 medium with 2% sucrose due to a higher biomass accumulation (Condori et al. 2010; Yang et al. 2015). Hull line 3 hairy root culture exhibited a shorter exponential growth phase (days 3–12), but a longer stationary phase (days 12–18) in MSV medium (specific growth rate (μ) of 0.318 day^{-1}), when compared to the roots grown in B5 medium (0.258 day^{-1} specific growth rate (μ)) (Condori et al. 2010). Both peanut hairy root lines have higher specific growth rates than that of grapevine and muscadine hairy root cultures. The vigorous growth characteristics of the peanut hairy root cultures facilitated a time for elicitation at day 12 for Andru II line 2 and day 9 for Hull line 3. This time was earlier, when compared to that at day 18 and day 21 of grapevine or muscadine hairy root lines, resulting in a shorter cycle for biomass growth and elicitation. Moreover, peanut hairy roots of cv. Hull line 3 have been scaled up from 250 mL flask culture containing 50 mL of culture medium to 5 L airlift balloon-type bioreactors containing 4 L of culture medium (Sivakumar et al. 2011) and to 1, 4, and 20 L mist bioreactors (Sivakumar et al. 2010). The specific growth rates (μ) observed in 20 L mist reactors were 0.147 day^{-1} with a biomass yield of 7.77 g DWL^{-1}, which might support elicitation at larger scale in future studies.

Intracellular Stilbenoids in Peanut Hairy Roots

In contrast to grapevine and muscadine hairy root cultures which exhibit basal production and accumulation of stilbenoids inside root tissue, the hairy root cultures of peanut only produce stilbenoids upon elicitor treatment, and most of stilbenoids are secreted into the culture medium. Only trace amounts of resveratrol have been detected in ethyl acetate extracts of the medium and root tissue of non-elicited peanut hairy root cultures of cv. Andru II line 2 (Medina-Bolivar et al. 2007). Similarly, resveratrol, arachidin-1, or arachidin-3 has not been detected in the root tissue of peanut hairy root cultures of cv. Hull line 3, and only trace amounts of resveratrol (less than 3 μg g^{-1} DW) have been detected in the medium after incubation for 6 h with fresh MSV medium (Condori et al. 2010). In contrast to the high ratio of intracellular stilbenoids to extracellular stilbenoids in non-elicited or elicited grapevine hairy root (excluding MCD treatment), non-elicited peanut hairy root culture exhibited a very low ratio of intracellular stilbenoids to extracellular stilbenoids. Even upon sodium acetate elicitation, 99% of resveratrol produced by peanut hairy roots of cv. Andru II line 2 and over 90% of resveratrol, arachidin-1, and arachidin-3 in peanut hairy root culture of cv. Hull line 3 were secreted into the medium (Condori et al. 2010; Medina-Bolivar et al. 2007).

Upon treatment with sodium acetate, hydrogen peroxide, or MeJA alone, the levels of secreted stilbenoids in peanut hairy root cultures of cv. Hull line 3 decreased to barely detectable levels after reaching their maximum levels (Yang et al. 2015). Moreover, it has been suggested that exogenous resveratrol fed to the culture medium of non-elicited peanut hairy root cultures could be uptaken and further degraded by the root tissue, via a series of enzymatic reactions (Yang et al. 2015, 2016). This constitutive catabolism of stilbenoids may protect peanut hairy roots from potential toxic effects of stilbenoids accumulated at high levels within the cell and could also explain why only trace amounts of stilbenoids are found inside the root tissue as well.

Secretion of Stilbenoids in Peanut Hairy Root Cultures in Response to Elicitors

Stilbenoids have been barely or not detected in the medium of non-elicited peanut hairy root cultures. To induce the biosynthesis of stilbenoids, various elicitors have been tested, and ethyl acetate has been used as a solvent to extract them from the culture medium (Condori et al. 2010; Medina-Bolivar et al. 2007; Yang et al. 2015). After 24 h of sodium acetate treatment, a 60-fold increase in resveratrol levels was observed in peanut hairy root cultures of cv. Andru II line 2 (Medina-Bolivar et al. 2007). The levels of resveratrol, piceatannol, arachidin-1, and arachidin-3 in the culture medium of peanut hairy root of cv. Hull line 3 induced by various elicitors were determined along a time course from 0 to 96 h (Yang et al. 2015). The levels

of resveratrol and piceatannol were around 178 and 19.5 µg g^{-1} DW, respectively, after 18-h treatment of 10 mM H$_2$O$_2$, while the extracellular prenylated stilbenoids, arachidin-1 and arachidin-3, accumulated to 90 and 59 µg g^{-1} DW after 42- and 36-h treatment with 10.2 mM of sodium acetate, respectively (Yang et al. 2015). As mentioned earlier, after the stilbenoids reached their maximum level in the culture medium, their levels decreased to undetectable levels possibly due to uptake and catabolism by enzymes in the hairy root tissue.

To increase the solubility of stilbenoids and potentially prevent these compounds from being uptaken back by the root tissue, methyl-β-cyclodextrin (MCD) alone or combined with MeJA has been applied to peanut hairy root cultures of cv. Hull line 3 (Yang et al. 2015). Under 9 g/L MCD treatment, the levels of prenylated stilbenoids, arachidin-1 and arachidin-3, in the medium increased dramatically and remained at very high levels even up to 96 h, whereas the levels of resveratrol and piceatannol were not sustained after 72 h. Meanwhile, the levels of secreted stilbenoids, including resveratrol and piceatannol, were maintained at 3800 and 460 µg g^{-1} DW, respectively, upon co-treatment with MCD and MeJA for 96 h. The maximum yields of prenylated stilbenoids, arachidin-1 and arachidin-3, were around 7000 and 13700 µg g^{-1} DW, which were about 110- and 370-fold higher than that observed with sodium acetate treatment. Upon the MCD and MeJA co-treatment, another two prenylated stilbenoids, arachidin-2 and arachidin-5, accumulated up to 1400 and 2750 µg g^{-1} DW in the medium of peanut hairy root of Hull 3 line (Yang et al. 2016). Moreover, a synergistic effect between MCD and MeJA on the expression of resveratrol synthase gene, a key gene involved in stilbenoid biosynthesis, might be the potential explanation for the high yield of stilbenoids in peanut hairy roots upon this combined two-elicitor treatment (Yang et al. 2015).

Biosynthesis of Prenylated Stilbenoids in Peanut Hairy Root Culture

The most characterized member in the stilbenoid family, resveratrol, is produced via the phenylpropanoid and acetate pathway and shares most of their biosynthetic pathway with chalcones (Fig. 3.4). In its biosynthesis, phenylalanine ammonia-lyase (PAL), the first enzyme in the phenylpropanoid pathway, converts phenylalanine to cinnamic acid. The latter is further oxidized to *p*-coumaric acid by cinnamate 4-hydroxylase (C4H). 4-Coumarate-CoA ligase (4CL) is the enzyme responsible for the transformation of *p*-coumaric acid into 4-coumaroyl-CoA. At the end, resveratrol synthase (RS) condensates three molecules of malonyl-CoA which are synthetized via the acetate pathway and one *p*-coumaroyl-CoA to form resveratrol. Interestingly, resveratrol synthase and chalcone synthase are closely related and both of them use 4-coumaroyl-CoA and perform three condensation reactions with malonyl-CoA to form a linear tetraketide which later is folded into new ring systems. The difference between these two enzymes is a special property of stilbene
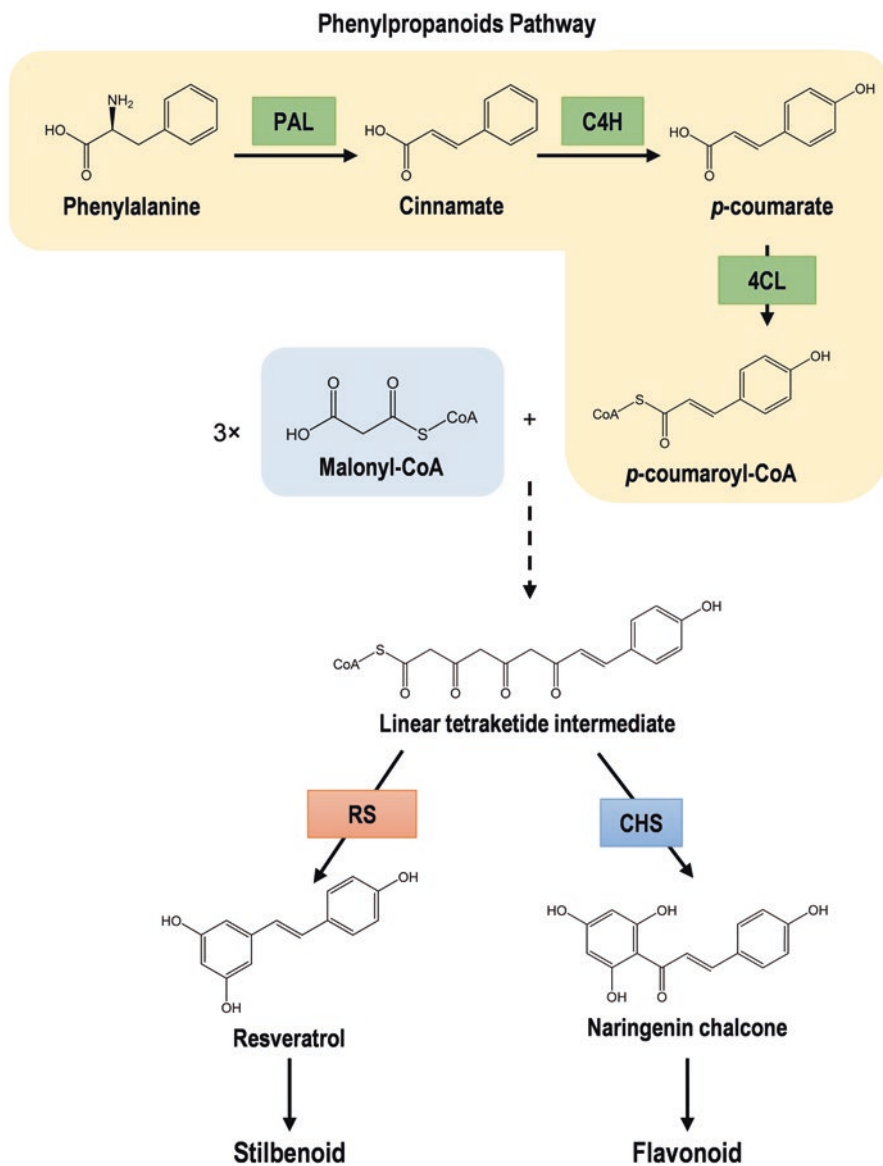
Fig. 3.4 Biosynthetic pathway of resveratrol and chalcone. *PAL* phenylalanine ammonia-lyase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumarate-CoA ligase, *CHS* chalcone synthase, *RS* resveratrol synthase

synthases which looses the terminal carboxyl group as CO_2 resulting in a release of four molecules of CO_2 during each reaction compared to three molecules of CO_2 released from the chalcone synthase reaction. Actually, from an evolutionary point of view, stilbene synthase might have evolved from chalcone synthase via gene duplication and mutations to new and improved functions (Tropf et al. 1994). Interestingly, chalcone synthase can be modified into a resveratrol synthase by mutagenesis of a few key amino acids (Flombaum and Santos 2005). Although the enzymes involved in resveratrol biosynthesis have been elucidated (Chong et al. 2009), the majority of diverse enzymes in the downstream steps (beyond resveratrol) which lead to the different prenylated stilbenoids in peanut have not been described.

As a sustainable and controlled system for prenylated stilbenoid production, hairy roots of peanut have been leveraged for elucidating the biosynthesis of prenylated stilbenoids in this species. In the biosynthesis of peanut prenylated stilbenoids, dimethylallyl diphosphate (DMAPP) derived from isoprenoid biosynthesis via isomerization of isopentenyl diphosphate (IPP) by isopentenyl diphosphate isomerase (Ramos-Valdivia et al. 1997) provides the prenyl donor for the prenylation of stilbenoids. Two distinct routes are known for the biosynthesis of DMAPP, the mevalonic acid (MVA) pathway which occurs in the cytosol and the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway occurring in the plastid (Fig. 3.5) (Lohr et al. 2012). To determine the biosynthetic origin of DMAPP, distinct metabolic inhibitors, mevastatin and clomazone, were applied to the elicitor-treated peanut hairy root cultures to inhibit the activity of key rate-limiting enzymes involved in the biosynthesis of DMAPP by either the MVA or MEP pathway (Fig. 3.5). Briefly, mevastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), or clomazone, a herbicide that inhibits 1-deoxy-D-xylulose-5-phosphate synthase (DXS), was applied to block either the MVA or MEP pathway (Fig. 3.5). Upon treatment with these inhibitors, mevastatin had no effect on the level of extracellular stilbenoids in elicited peanut hairy root cultures, while clomazone showed a significant inhibition on the yield of prenylated stilbenoids, along with higher yield of resveratrol when compared with the non-inhibitor group (Yang et al. 2016). These results demonstrated that the prenyl chain of the prenylated stilbenoids is synthesized through the MEP pathway in plastids and that the prenylated stilbenoids may derive from resveratrol.

The key reaction involved in the biosynthesis of prenylated stilbenoids is prenylation, which is catalyzed by aromatic prenyltransferase allowing a 5-carbon (dimethylallyl) prenyl chain to be attached to the aromatic backbone of the stilbene. This prenylation plays an important role in diversification of stilbenoids in peanut and links the shikimate and acetate pathways synthesizing the aromatic moiety, with the isoprenoid pathway which provides the prenyl moiety (Yazaki et al. 2009). Taking advantage of the peanut hairy root bioproduction system, the first stilbenoid-specific prenyltransferase activity was characterized from the microsomal fraction of elicited peanut hairy roots (Yang et al. 2016). This membrane-bound prenyltransferase transfers a dimethylallyl group to the 4 position of resveratrol to form arachidin-2. It also prenylates piceatannol to arachidin-5 and pinosylvin to
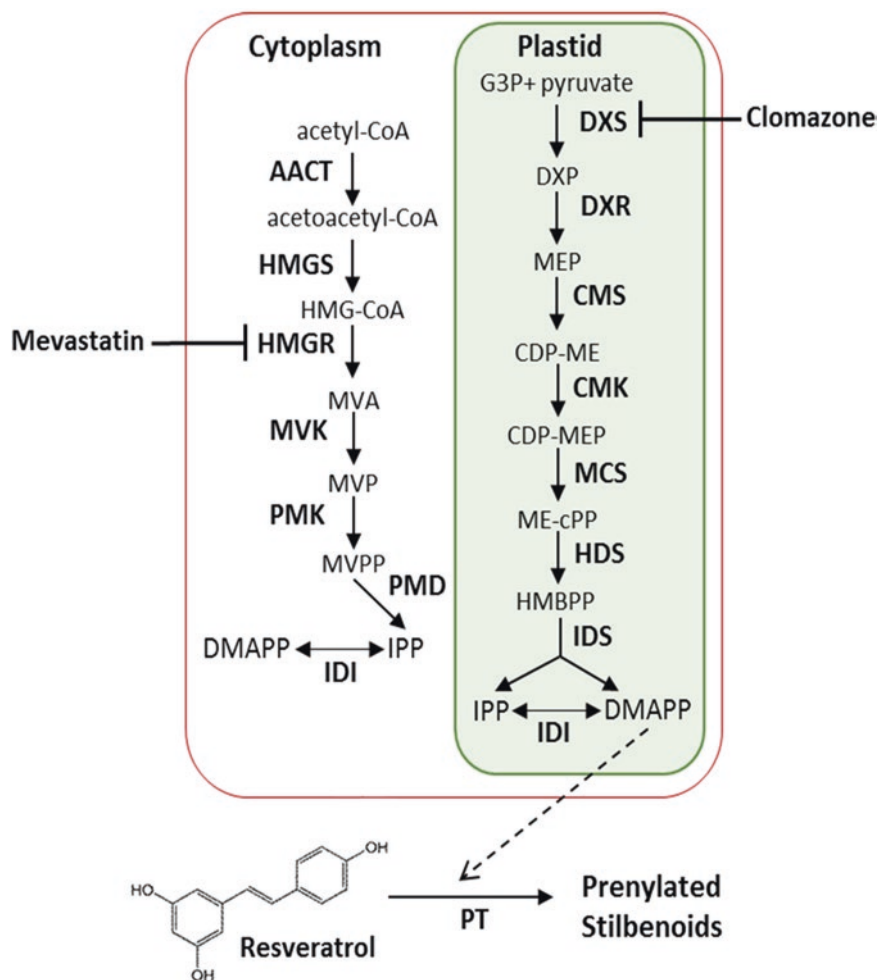
Fig. 3.5 Proposed biosynthetic origins of prenylated stilbenoids in peanut. The building block of the isoprenoids can be produced via the MVA pathway (cytoplasm) or MEP (plastid). These metabolic pathways can be inhibited by mevastatin (cytoplasm) and clomazone (plastid). *CDP-ME* 4-diphosphocytidyl-2-C-methylerythritol, *CDP-MEP* 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, *CoA* coenzyme A, *DMAPP* dimethylallyl pyrophosphate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *G3P* glyceraldehyde 3-phosphate, *HMBPP* 4-hydroxy-3-methyl-but-2-enyl pyrophosphate, *HMG-CoA* 3-hydroxy-3-methylglutaryl-CoA, *IPP* isopentenyl pyrophosphate, *ME-cPP* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, *MEP* 2-C-methylerythritol 4-phosphate, *MVA* mevalonate, *MVP* mevalonate-5-phosphate, *MVPP* mevalonate-5-pyrophosphate. Enzyme abbreviations are *AACT* acetoacetyl-CoA thiolase, *CMK* 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, *CMS* 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, *DXR* 1-deoxy-D-xylulose 5-phosphate reductase, *DXS* 1-deoxy-D-xylulose 5-phosphate synthase, *HDS* 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, *HMGR* HMG-CoA reductase, *HMGS* HMG-CoA synthase, *IDI* isopentenyl diphosphate:dimethylallyl diphosphate isomerase, *IDS* isopentenyl diphosphate:dimethylallyl diphosphate synthase, *MCS* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *MVK* mevalonate kinase, *PMD* pyrophosphomevalonate decarboxylase, *PMK* 5-phosphomevalonate kinase, *PT* prenyltransferase
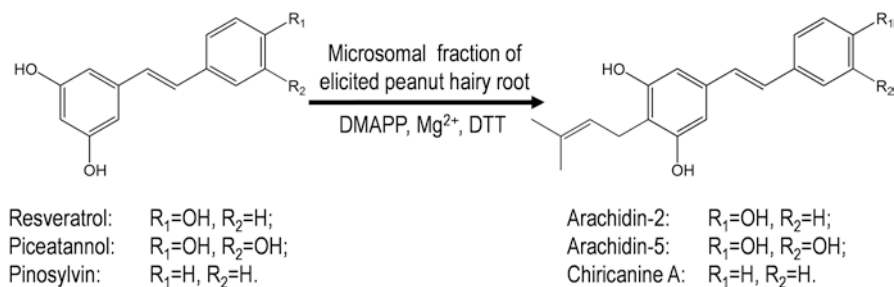
Fig. 3.6 Substrate specificity of resveratrol prenyltransferase in a microsomal fraction of elicited peanut hairy root

chiricanine A (Fig. 3.6). Moreover, the microsomal fraction isolated from elicited peanut hairy root does not prenylate flavanone, flavone, or isoflavone backbones, even though it shares several common features with flavonoid-specific prenyltransferases, such as requiring a divalent cation as cofactor and a basic buffer for optimal reaction rate. These features confirm that this enzyme is a stilbenoid-specific prenyltransferase (Yang et al. 2016).

Conclusions

Hairy roots of grapevine, muscadine, and peanut have been developed as sustainable bioproduction systems for stilbenoids. These cultures produce common stilbenoids such as resveratrol and its hydroxylated analog piceatannol. However, prenylated stilbenoids have been detected in peanut hairy root cultures only. Several elicitors have been tested to induce the biosynthesis of stilbenoids, and the co-treatment with MeJA and methyl-β-cyclodextrin (MCD) has been the most effective. Interestingly, the majority of stilbenoids are secreted into the medium of peanut hairy root cultures, whereas muscadine and grapevine hairy root cultures accumulate stilbenoids in the root tissue and medium. The high level of stilbenoids that accumulate in the medium of peanut hairy root cultures co-treated with MeJA and MCD has opened the possibility to purify high quantities of prenylated stilbenoids which are being used in different bioassays. Future studies to scale-up the production of stilbenoids in hairy root cultures should be underway. The elicitor-controlled and reproducible production of stilbenoids in hairy root cultures has also led to studies focused on their biosynthesis. Particularly, in peanut, the first stilbenoid-specific prenyltransferase enzyme activity was characterized using hairy roots, and most likely the diverse genes encoding this and other stilbenoid-modifying enzymes will be identified using this biological system. Recently, the bioconversion of resveratrol fed to transgenic tobacco hairy roots expressing a human CYP450 hydroxylase and a grapevine resveratrol methyltransferase led to the production of the

pharmacologically active stilbenoid, pterostilbene (Hidalgo et al. 2017). The application of hairy roots for production and discovery of known and novel stilbenoids is endless and will continue to provide opportunities to identify and produce novel bioactive compounds with applications in human health.

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Chapter 4
Hairy Root Cultures of *Rhodiola rosea* to Increase Valuable Bioactive Compounds

Henrik Lütken, Natalia Meropi-Antypa, Oliver Kemp, Josefine Nymark Hegelund, and Renate Müller

Abstract *Rhodiola rosea*, commonly known as roseroot, is an arctic and alpine plant distributed on the northern hemisphere. The plant has for long been used ethnobotanically as a means of increasing endurance and as a general cure against several diseases. Nowadays, the medicinal properties of roseroot have been characterized, and some of its important bioactive compounds are salidroside and the rosavinoids rosavin, rosarin, and rosin. The primary effect of the plant has been described as adaptogenic, i.e., providing a nonspecific broad-range response. Recently, *R. rosea* has increased in popularity which has led to overexploitation in nature, and new bio-sustainable production methods are needed for future production. Transformation with the soil bacterium *Agrobacterium rhizogenes* is a promising strategy to increase the natural content of bioactive compounds within plants. The increase of the bioactive compounds is caused by the *root oncogenic loci* (*rol*) genes, present on the transfer DNA within the bacterial plasmid. The *rol* genes are integrated in the plant host genome during transformation, causing formation of hairy roots. Other species in the *Rhodiola* genus have been successfully transformed by *A. rhizogenes*. However, several optimizations in terms of selection of superior plant lines, explant for transformation, and tissue culture are needed in order for *R. rosea* to serve as a platform for the production of bioactive compounds in hairy root cultures. Once established, several further measures could be taken to increase the content of bioactive compounds further, in that respect genome editing via the CRISPR/Cas9 system is emerging as a powerful beacon.

Keywords *Agrobacterium rhizogenes* • Arctic • Medicinal plants • Roseroot • Transformation

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Introduction

Rhodiola rosea L. (roseroot, golden root, or arctic root) (Fig. 4.1) from the stonecrop family (Crassulaceae) has traditionally been used in folk medicine on the northern hemisphere. *R. rosea* was applied for enhancement of endurance and general vigor, as well as for alleviating symptoms as fatigue, depression, infections, and anemia by people in Russia and northern Europe (Brown et al. 2002; Yousef et al. 2006). In Siberia, *R. rosea* is still offered to newlyweds to boost fertility, and it has traditionally been used by the Mongolians against cancer (Ishaque et al. 2012; Saratikov and Krasnov 1987). One of the earliest records of using *R. rosea* can be found in Dioscorides' *De Materia Medica* under the name *rodia riza*, later named *R. rosea* by the Swedish botanist Carl Linnaeus due to the roselike fragrance of the freshly cut root (Brown et al. 2002).

The current book chapter focuses on *R. rosea* with emphasis of generating hairy root cultures to increase valuable bioactive compounds. Firstly, the morphological variation of the plant is described, followed by its geographical distribution to illustrate the high degree of genetic diversity within the species. These sections are succeeded by description of the ethnobotanical aspects of *Rhodiola* leading up to the next section covering the medicinal properties of *R. rosea*. The second part covers the approaches toward hairy root cultures of *R. rosea*. Here the current challenges are reviewed with emphasis on tissue culture as a prerequisite for transformation with wild-type *Agrobacterium rhizogenes*. The latter part comprises the utility of the soil bacterium *A. rhizogenes*. Herein the effect of the *root oncogenic loci* (*rol*) genes from *A. rhizogenes* is discussed in terms of effect on production of bioactive compounds, and the current status for *A. rhizogenes*-mediated transformation of the *Rhodiola* genus is reviewed. Finally, future perspectives for increasing bioactive compounds of hairy root cultures of *R. rosea* are discussed.
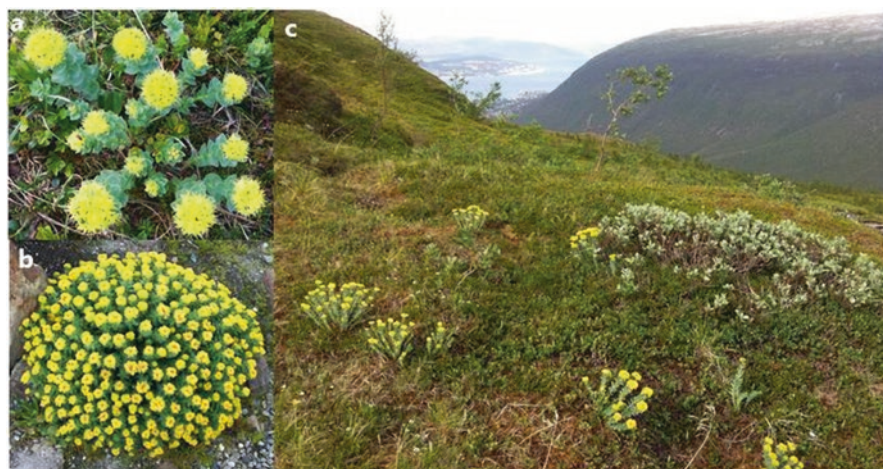
Fig. 4.1 Roseroot (*Rhodiola rosea* L.) habitus and plant community. (**a**) Wild specimen. (**b**) Older domesticated specimen. (**c**) *R. rosea* in subarctic habitat (All photographs are taken in Tromsø, Troms Fylke, Norway, by Henrik Lütken)

Plant Morphology

The morphological characteristics of *R. rosea* include alternate, lanceolate, pointed, dentate, fleshy leaves (Fig. 4.1a). Although most studies state *R. rosea* as dioecious, monoecious plant with hermaphroditic flowers occur (Cuerrier et al. 2014a). The petals are elliptical, sometimes absent, and extend beyond the calyx (Chartier 2004). The flowers are approximately 5–8 mm in diameter, greenish or yellowish for the females and reddish for the males, and are organized in tight corymbs containing up to 150 flowers (Chartier 2004; Cuerrier et al. 2014a). The wind-dispersed seeds emerge from mature follicles. The dehiscence of the follicles can be reversible and is dependent on the surrounding humidity. Each fruit contains one to two oblong, dark brown seeds of approximately 0.5–1.0 mm. Flowering occurs from April to August. The rhizomes are thick, branched, and with a scaly surface and a roselike smell; their average weight spans from 70 g up to 3.5 kg. Multiple shoots can stem from the same rhizome (Tasheva and Kosturkova 2012) (Fig. 4.1b). However, *Rhodiola* is extremely polymorphic throughout its distribution range, with most variable characteristics being the flower color, leaf margin dentation, and plant size and habit (Spongberg 1978).

Geographical Distribution

R. rosea has an amphi-Atlantic distribution of temperate and subarctic regions of the northern hemisphere (Fig. 4.1c). It is commonly found in Asia, Europe (Scandinavia, United Kingdom, most central European mountains, the Balkans), Iceland, Greenland, and Arctic, with the eastern coastal regions of North America being its distribution limit (Cuerrier et al. 2014a; Grech-Baran et al. 2015; Guest and Allen 2014; Panossian et al. 2010).

  R. rosea usually grows on grassy or rocky slopes on elevations up to 2280 m (Tasheva and Kosturkova 2012), as well as in tundra and along running water. However, it is tolerant to poor substrates but can grow well in richer ones (Cuerrier et al. 2014a). Most botanists agree on the southern Siberian mountains as the origin of the species and the area where the plant is still most commonly found within Russia. The migration of *R. rosea* was later directed toward the surrounding mountain ranges and the Arctic (Kozyrenko et al. 2011).

Ethnobotany of Rhodiola *spp.*

In addition to *R. rosea*, various species belonging to the *Rhodiola* genus have been traditionally used by the indigenous people of Europe and Asia through the years (Cuerrier et al. 2014b; Zhang et al. 2014b). It is estimated that at least 20 *Rhodiola* species are part of Asian folk medicine, including *R. alterna*, *R. crenulata*, *R. kirilowii*, and *R. sachalinensis* among others. China is one of the regions with the highest

diversity for the *Rhodiola* genus, where numerous species commonly known as "Hongjingtian" are part of the traditional Chinese, Nepalese, and Tibetan medicinal practices. A plethora of members of the genus are used as Hongjingtian in China, including *R. crenulata*, *R. rosea*, *R. sachalinensis*, *R. himalensis*, *R. serrata*, *R. fastigiata*, and *R. kirilowii* (Liu et al. 2013). Conversely, the underground parts of *R. crenulata* (the only *Rhodiola* included in the official Chinese pharmacopoeia) are extensively administered to treat numerous ailments such as asthma, apoplexy, and thoracic obstruction (Cuerrier et al. 2014b; Zhang et al. 2015a). *R. algida*, a species endemic to the Qinghai-Tibetan Plateau, has been used to alleviate altitude sickness and fatigue (Zhang et al. 2014a). *R. yunnanensis*, known as "mingleshi" or "haisainai" throughout different regions, is applied for treating wounds, fractures, and infections (Cuerrier et al. 2014b).

Outside China, *R. quadrifida*, *R. imbricata*, and *R. himalensis* are known by the people of Mongolia, India, and central Nepal accordingly as a means of moderating a number of conditions including fatigue, gastrointestinal disorders, or fevers (Cuerrier et al. 2014b). In the Pamir Mountains of Tajikistan and Afghanistan, the indigenous people use *R. gelida*, locally known as "zarchoy," as an energy enhancer (Kassam et al. 2010). In addition, many ethnic groups in Russia and in Siberia have applied *R. rosea* to increase endurance and for treating conditions spanning from impotence to depression, schizophrenia, asthenia, and fatigue to flu, gastrointestinal disorders, cancer, and infections (Khanum et al. 2005). For example, the people of Altai apply the aerial parts of the plant as a stimulant and performance enhancer, while the subterranean part was used against diseases (Perinskaya and Sakanyan 2014). Furthermore, indigenous populations of North America, Alaska, and Greenland value highly the benefits of *R. integrifolia* and *R. rosea* both as a nutrient-rich food source as well as for their medicinal properties (Cuerrier et al. 2014b).

In Europe, documented evidence regarding the medicinal use of *Rhodiola* spp. exists exclusively for *R. rosea*, as it is the only species found there. Linnaeus described the plant as an astringent, as well as a treatment for hernias, hysteria, and headaches. *R. rosea* was included in the first Swedish pharmacopoeia in 1755 and was characterized as a plant with anti-inflammatory and stimulatory effects by German researchers (Brown et al. 2002). In Norway, *R. rosea* is regionally known under a plethora of different names, including søstergras ("sisters grass"), systerøter ("sisters roots"), or stubberod ("stump root"). For Norway's northern regions, names such as kalvegras ("calf grass"), kalvedans ("calf dance"), or kalverot ("calf root") have led to the assumption that the plant was fed to cattle potentially as a vitamin C source against scurvy. This particular effect of *R. rosea* was also mentioned in the writings of Norwegian bishop and botanist Johan Ernst Gunnerus and Danish writer Henrik Pontoppidan, who wrote that "the root heals scurvy" and that it is "a splendid force against scurvy." Further documented applications include the use of *R. rosea* as a poultice for treating swollen limbs, urinary disorders, and eye diseases (Alm 2004).

Medicinal Properties

Today, *R. rosea* is still a popular medicinal plant for which several studies have confirmed adaptogenic, psychostimulant, cardioprotective, antioxidant, antidepressant, and anticarcinogenic properties (Brown et al. 2002; Grech-Baran et al. 2015; Panossian et al. 2010). According to Panossian (1999), there are three criteria that define an adaptogen:

- The production of a nonspecific response in an organism, i.e., a broad-spectrum resistance against biotic and abiotic stresses.
- A normalizing effect on physiology, by counteracting or preventing disturbances caused by stressors.
- The normal functioning of the organism must remain undisturbed by the broad range of therapeutic effects caused by the adaptogen (Panossian et al. 2010).

The positive effects related to *R. rosea* are attributed to the presence of numerous bioactive compounds in the root and rhizomes of the plant; the most important are salidroside and its aglycon tyrosol and the cinnamyl alcohol glycosides rosavin, rosarin, and rosin (Fig. 4.2) (Elameen et al. 2010; Ganzera et al. 2001; Perinskaya and Sakanyan 2014). Several products containing *R. rosea* extracts are available on the market, such as *R. rosea* extract SHR-5, which is
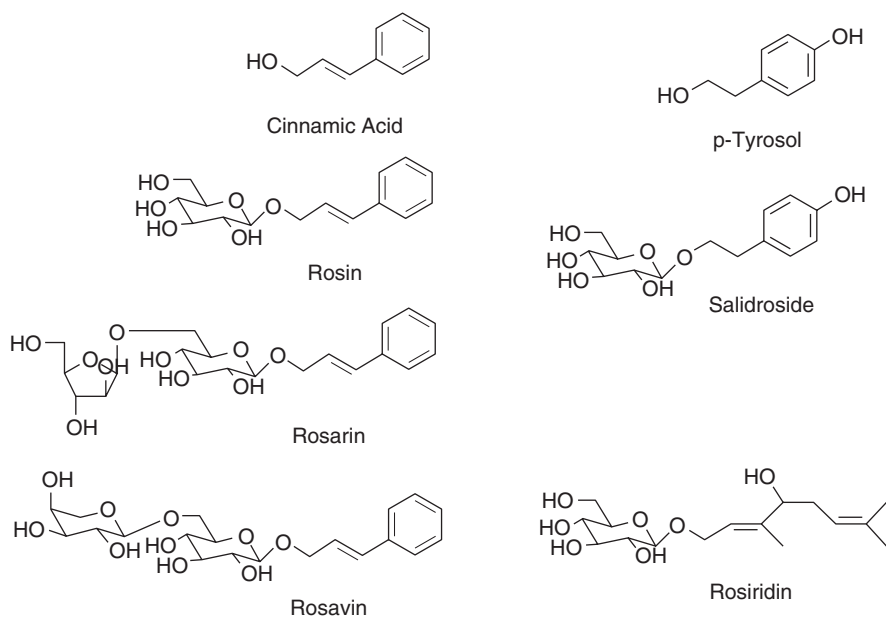
Fig. 4.2 Main bioactive compounds in *R. rosea*

Table 4.1 Bioactive compounds in *Rhodiola* spp.

Rhodiola species	Compound
R. crenulata	**Salidroside and tyrosol**, rutin, picein, lotaustralin, rodiocyanoside A, crenulatin, rhodionin, rhodiosin, daucosterol, beta-sitosterol, hydroxycinnamic, and gallic acid
R. kirilowii	**Salidroside and tyrosol**, **rosin**, **rosavin**, **rosarin**, cinnamyl alcohol, tannins, daucosterol, beta-sitosterol, hydroxycinnamic, gallic acid, chlorogenic acid, and lotaustralin
R. quadrifida	**Salidroside and tyrosol**, **rosin**, **rosavin**, **rosarin**, cinnamyl alcohol, rhodiooctanoside, rhodiolin, mongrhoside, rhodiocyanosides A and B, rhodioflavonoside, rhodiooctanoside, tricetin, l-rhamnopyranoside, and chlorogenic acid
R. rosea	**Salidroside and tyrosol**, **rosin**, **rosavin**, **rosarin**, cinnamyl alcohol, lotaustralin, rodiocyanoside A, rosiridol, rosaridin, daucosterol, beta-sitosterol, rhodiolosid A–C, gallic, hydroxycinnamic acid, acetylrodalgin, and tricin
R. sachalinensis	**Salidroside and tyrosol**, **rosarin**, **rosavin**, cinnamyl alcohol, multiflorin B, tricetin, afzelin, kaempferol, rhodionin, rhodiosin, lotaustralin, hetorodendrin, and glucopyranoside

Compounds of key importance are highlighted in bold

being sold in tablet form in Sweden since 1985 (Panossian et al. 2010). In Denmark, *R. rosea* is a registered medical product in the category of botanical drugs (Brown et al. 2002).

As previously mentioned, several species of *Rhodiola* have been reported to contain numerous bioactive compounds in their roots and rhizomes (Table 4.1). Some authors stated that the compounds rosavin (Ishaque et al. 2012), rosarin, and rosin are specific to *R. rosea* (Brown et al. 2002; Panossian et al. 2010; Vouillamoz et al. 2012); however, the presence of these compounds has now also been reported in other species of the genus (Grech-Baran et al. 2015; Peschel et al. 2013) (Table 4.1).

For many years the extracts of *R. rosea* have been extensively studied by East European groups (Furmanowa et al. 1995; Saratikov and Krasnov 1987). The bioactive compounds found in the rhizome of *R. rosea* are divided in several groups: phenylethanoids (salidroside, tyrosol), phenylpropanoids (rosin, rosarin, rosavin), flavonoids (rodiolin, rodionin, rodiosin), phenolic acids (chlorogenic, hydroxycinnamic, gallic), cyanoglycosides, monoterpenoids (rosiridol, rosaridin), and triterpenes (daucosterol, beta-sitosterol) (Brown et al. 2002; Ma et al. 2012) (Table 4.1). Essential oil extracts have been reported to contain 75 different compounds, mostly belonging to the volatile groups of monoterpene hydrocarbons (25.4%), and aliphatic and monoterpene alcohols (37.5%), constituting more than 86% of the total oil. The main compound responsible for the roselike scent of the rhizomes has been identified as geraniol (Rohloff 2002).

According to Ganzera et al. (2001), the monoterpene rosiridin is also used as a *R. rosea* marker in addition to salidroside, rosavin, rosarin, and rosin to achieve the optimum basis for extract identification. Notably, many commercially available *R. rosea* extracts are

standardized for rosavinoids and salidroside in a 3:1 ratio as they naturally occur in the plant (Brown et al. 2002; Ganzera et al. 2001). However, most products lack further information regarding the specific composition of the extracts, raising questions about their quality. Based on Ganzera's study, rosiridin was the dominant compound, while rosarin and rosin were present in comparable ratios yet low concentrations (Ganzera et al. 2001).

Toward Hairy Root Cultures of *Rhodiola rosea*

Current Challenges

The wild populations of *R. rosea* have been affected by the increasing trend in medicinal herb use, as the excessive harvesting has led to a significant decrease in population size (Tasheva and Kosturkova 2012). The wild populations of *R. rosea* are today included in the list of endangered species in many countries including Russia, Great Britain, Czech Republic, Bosnia and Herzegovina, and Slovakia, and harvesting is now forbidden in several places, e.g., in Bulgaria (Grech-Baran et al. 2015). Domestication attempts have been performed in Sweden, Finland, Norway, Poland, Italy, and Switzerland, mainly focusing on agrobiological observations and quality analyses of the endemic populations. However, the present cultivation areas are still limited to approximately 8–12 ha Europe-wide, emphasizing multiple limiting factors of *R. rosea*'s cultivation, such as 4–6-year long life cycle, the need of special machinery, and the high production costs in small-scale farming (Galambosi 2014). These limitations, coupled with high demands of water supply and low seed germination (Platikanov and Evstatieva 2008), also make potential restoration of natural populations difficult. These aspects are reflected in current prices of salidroside and rosavin with the market prices of USD 350–500 and 1200–1500, per 100 mg, respectively (Sigma-Aldrich, ChemFaces). Hence, alternative platforms are critical for maintaining wild populations of *R. rosea*.

Due to the increasing market demands (Peschel et al. 2013), further research should aim at discovering sustainable ways for the exploitation of *R. rosea*. The application of cultivation techniques and biotechnological methods is a promising way to help in the restoration of the wild populations of *R. rosea* as well as for producing high-quality plants in terms of bioactive compound yield (Tasheva and Kosturkova 2012). Furthermore, the advent of recent advances in scientific areas such as metabolic engineering, biochemical profiling, and pharmaceutical screening widens the possibilities of better exploitation of plant-derived bioactive compounds (Chandra et al. 2013). In addition, with respect to the slow-growing nature of *R. rosea*, the establishment of a faster-growing and less demanding model *Rhodiola* species could prove an exceptionally valuable tool for further research.

Plant Tissue Culture of R. rosea

Currently, the demand for medicinal plant products by the industry and the consumers has led to the need for their rapid and economical production. However, conventional medicinal plant preparations suffer various limitations due to contamination by microorganisms or environmental pollutants, the seasonality of bioactive compounds, and quality losses in processing and storage (Joshee et al. 2013). Moreover, seed-grown plants are usually highly heterozygous, demonstrating variations in growth and yield that lead to poor quality products deemed unsuited for commercial release (Yadav et al. 2012).

Consequently, biotechnological methods have become crucial for the multiplication and enhancement of medicinal plants (Tripathi and Tripathi 2003). Plant tissue culture involves the growing and multiplication of cells, tissues, and organs on solid or liquid media under sterile conditions in a controlled environment (Yadav et al. 2012) and can be broadly divided into three categories. Micropropagation is the most common approach, through which organized meristems (i.e., shoot tips, axillary buds) are isolated and induced to grow into whole plants. The second approach involves the formation of adventitious shoots on leaf, root, and stem segments or on callus. Lastly in the third approach, cells and callus cultures are induced for somatic embryogenesis (Rout et al. 2000).

Among the several factors that affect the success of tissue cultures, the relative concentrations of growth regulators in the growth medium are prevalent. Different auxins/cytokinins ratios can lead to the induction of calli, root, or shoot development, whereas gibberellins are mostly responsible for organ growth but not organ initiation (Rout et al. 2000). As reviewed by Yadav et al. (2012), growth effects of changes in the ratio between the two hormones have been reported for various medicinal plants.

Studies regarding in vitro cultures of *R. rosea* first appeared approximately 30 years ago. The first advances were made by Russian groups, mostly focusing on the establishment of the cultures, i.e., explant types, nutrient media, growth regulators, sterilization, callus induction, organogenesis, and regeneration. However, the aim for in vitro production of *R. rosea* secondary metabolites was present from very early on (György et al. 2004). In more recent studies, Furmanowa et al. (1995) presented the results of a very detailed investigation on *R. rosea* in vitro cultures. The explants originated either from shoot tips or from leaf segments and were grown in three different nutrient media with various combinations of growth regulators. Among the most promising results were shoot tips grown in Nitsch and Nitsch medium supplemented with 0.1 mg/L kinetin and 0.1 mg/L indole-3-acetic acid (IAA), which developed shoots after 8 weeks and leaves after 12 weeks in culture. Callus with differentiating capacity was achieved with various media; however, optimal growth was observed on Murashige and Skoog (MS) media with 6-benzyladenine (BA) and 1-naphthaleneacetic acid (NAA) or BA and indole-3-butyric acid (IBA) (Furmanowa et al. 1995).

György et al. (2004) established callus cultures from leaves of *R. rosea* seedlings. The leaves were grown in MS media enriched with 1.5 mg/L BA and 0.5 mg/L NAA. The calli were further cultivated as aggregates in suspension culture aiming

at enhancing the production of cinnamyl glycosides by addition of various concentrations of their main precursor, cinnamyl alcohol. The experiment resulted in three-to sixfold increase in rosin content; however, only traces of rosavin were detected. Interestingly, when György et al. (2005) supplemented the callus aggregate cultures with glucose as an alternative carbon source, the production rate of cinnamyl alcohol was almost doubled (György et al. 2004, 2005).

Tasheva and Kosturkova (2010) investigated numerous parameters for organogenesis induction in Bulgarian *R. rosea* mostly regarding the effect of 24 MS modifications on different explant types (rhizome buds, lead nodes, stem and radix segments) and seed germination rates. In the case of explants originating from wild-grown plants, the results were highly variable. Apical bud explants formed calli with 100% response in an MS modification containing low IAA and BA concentrations; however, no further progress could be made. Plant regeneration from wild-grown explants was only possible using rhizome buds grown in media modifications containing 2 mg/L zeatin and 0.2 mg/L IAA; however, all other explant types, i.e., rhizomes, stem segments, and leaf nodes, did not respond to any of the treatments. When in vitro grown seedlings, germinated in response to GA₃, were used as explant source, both stem segments with leaf node and apical buds showed promising results in terms of organogenesis rates, reaching up to 85% and 82%, respectively, in zeatin-containing media. In terms of rhizogenesis of regenerants, highest rates were achieved in the presence of two media containing IAA, IBA, and gibberellic acid (GA₃), while media containing NAA were the only ones inducing callus formation. Finally, promising results reported in this study showed successful adaptation of in vitro grown regenerants to high mountain conditions, where more than 70% of the planted *R. rosea* survived winter (Tasheva and Kosturkova 2010).

Natural Transformation with Agrobacterium rhizogenes

A promising strategy for increasing the bioactive compounds in *R. rosea* is transformation with the soil bacterium *Agrobacterium rhizogenes*. Plants derived from transformations using unmodified/wild-type bacterial strains without using recombinant nucleic acids are according to present regulations in the European Union not classified as GMO (Christensen et al. 2010; Christensen et al. 2008; European Union 2001). Similarly, in Japan transformants derived from wild-type *A. rhizogenes*-mediated transformation are free from the legal controls of GMOs (Mishiba et al. 2006). As several studies have shown natural occurrence of gene transfer from bacteria to plants, there is scientific support for the process to be labeled as non-GMO. For example, the *root oncogenic loci* (*rol*) genes from *A. rhizogenes* have been naturally transferred to plants, during evolution as *rol* gene homologs have been detected in several plant species including, e.g., tobacco (*Nicotiana tabacum*), *Linaria vulgaris*, and sweet potato (*Convolvulus ipomoea*) (Intrieri and Buiatti 2001; Kyndt et al. 2015; Matveeva et al. 2012). Collectively, this makes transformation with wild-type *A. rhizogenes* and its *rol* genes to a

promising tool in molecular plant breeding for increasing the content of valuable bioactive compounds (see below). Although the legislation on this transformation process appears relatively straightforward, it is still important to bear in mind that the plants created using this strategy are developed by biotechnological methods. Also, how plants produced using these methods are perceived by consumers and producers is based on matters of individual views. Conversely, an extensive experiment in Europe demonstrated that informing consumers on the benefits of food biotechnology products on health or sustainability was insufficient to achieve a positive shift in consumer opinion and preference toward the products (Lucht 2015).

Agrobacterium rhizogenes and Its Root Oncogenic Loci Genes

The *Agrobacterium* genus comprises gram-negative soil bacteria many of which cause several diseases in plants. Four disease-causing species of *Agrobacterium* are recognized, *A. rhizogenes*, *A. rubi*, *A. tumefaciens*, and *A. vitis*, along with *A. radiobacter* which is the species most often isolated from soil but is nonpathogenic (Kim 2000; Matthysse 2006). *A. rhizogenes* is responsible for the development of hairy root disease by infecting and inserting specific genes into the plant host's genome from wounded sites (White et al. 1985). When *A. rhizogenes* infects a plant cell and transfers the T-DNA, hairy root growth occurs at the infection site which provides a marker-free method for identifying putative transformants. The successful production of transgenic plants using *A. rhizogenes* without antibiotic markers has previously been described for *Petunia hybrida* by deploying the *rol*ABC cluster as a phenotypic marker (Khan et al. 2010). The disease is caused by the insertion of specific genes located on the transfer DNA (T-DNA), into the host plants genome. The T-DNA is contained on the virulence plasmid called the root-inducing (Ri) plasmid, which is comparable to the tumor-inducing (Ti) plasmid found in *A. tumefaciens* (Huffman et al. 1984). In agropine strains of *A. rhizogenes*, two T-DNA regions, i.e., left (T_L) and right (T_R) T-DNA region, occur. The two regions are separated by a 15Kb spacer region which is not integrated; as a result the two T-DNA parts can be independently transferred to the nuclear genome of infected plant cells (Fig. 4.3) (Chandra 2012; Vilaine and Casse-Delbart 1987). During transformation with wild-type *A. rhizogenes*, T_L-DNA always seems to be present upon successful transformation often accompanied by T_R; however, T_R has not been shown to be present without T_L (Alpizar et al. 2008; Hegelund et al. 2017). The T_L contains 18 open reading frames (ORFs) including the important four *root oncogenic loci* (*rol*) genes, *rol*A, *rol*B, *rol*C, and *rol*D (Fig. 4.3), which have a fundamental role in the induction of hairy root formation (Nemoto et al. 2009; Slightom et al. 1986). Transgenic plants transformed with the four *rol* genes without the remaining T_L-DNA display the full hairy root phenotype (Casanova et al. 2005; Christey 2001). Other genes on the T_L-DNA generally enhance the effects of the *rol* genes (Britton et al. 2008; Ozyigit et al. 2013).

The T_R carries several genes responsible for opine synthesis (Christey 2001) and auxin synthesis (Gaudin et al. 1993; Huffman et al. 1984; White et al. 1985) and a *rol*B homolog (Fig. 4.3) (Lemcke and Schmülling 1998). However, it has been demonstrated that the *aux*1 and *aux*2 genes also can support the hairy root growth. The virulence genes required for the processing and subsequent transfer of the T-DNA into the host plant genome are located on the Ri plasmid, outside the T-DNA (Fig. 4.3), and thus are not inserted into the host plant's genome (Gelvin 2003; White et al. 1985). For ornamental plants, it has been shown that the degree of the Ri phenotype in the regenerated plants often varies among plant species, different strains of *A. rhizogenes* due to dissimilar interaction of bacterial genes and plant genes (Lütken et al. 2012). Moreover, it is dependent on the actual transformation event, i.e., copy number of inserted T-DNAs, the insertion site of the T-DNA, and transcriptional inactivation of T-DNA genes (Christensen and Müller 2009). Moreover, the phenotypic expression might be due to differences in T-DNA copy numbers integrated into the plant genome, the T-DNA integration site, and even the length of the insert (Tepfer 1984, 1990). Additionally, it has been assumed that T-DNA gene products do not function in the same way in all host plants (Porter 1991). The abovementioned aspects are all important in terms of assessing the potential effects of hairy root cultures derived from *A. rhizogenes*-transformed *Rhodiola*.
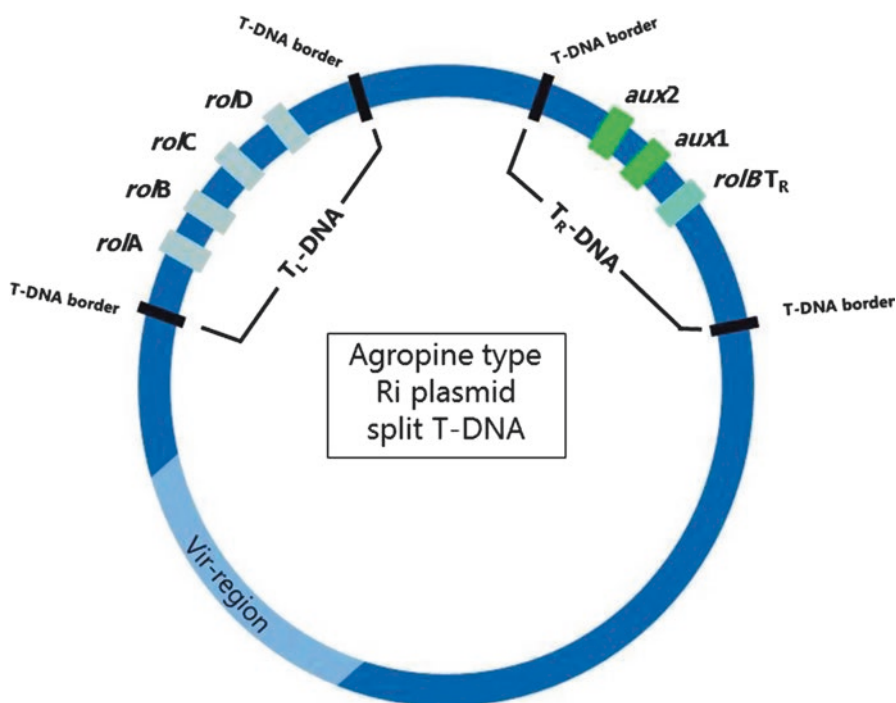
Fig. 4.3 Simplified illustration of the agropine-type root-inducing (R_i) plasmid. Positions of the *rol* and *aux* genes are shown on the T_L-DNA and T_R-DNA, respectively

Effect of rol Genes on Bioactive Compounds

In planta expression of the *rol* genes, alone or combined, leads to profound metabolic alterations with changes in the secondary metabolism of hairy root cultures of the transformed plants (Bulgakov 2008). This often leads to increased secondary metabolite quantities with high value to the medicinal industry (Canter et al. 2005). Moreover, hairy roots often grown faster and are considered to be genetically stable (Zhou et al. 2007). Hence, the hairy root cultures represent a promising material for the production of secondary metabolites of pharmaceutical value. It has been reported that *Vitis amurensis* plant cells transformed with the *rol*B gene had a 100-fold increased production of resveratrol and *rol*C-transformed root cultures of *Atropa belladonna* had the production of hyoscyamine and scopolamine increased up to 12-fold. *Panax ginseng* root cultures transformed with *rol*C displayed a production of ginsenosides of more than 6% dry weight, which is close to the maximum biosynthesis capacity (Bulgakov 2008). The specific functions of the *rol* genes are discussed below in relation to the effect of increasing the bioactive compounds in plants.

rolA

The gene sequences of *rol*A range between 279 and 423 bp and encode for proteins with a molecular mass of approximately 11 kDa (Nilsson and Olsson 1997). The rolA protein is probably a member of DNA-binding proteins as it has a high isoelectric point and can interact with nucleic acids supporting a function as transcription factor (Levesque et al. 1988; Veena and Taylor 2007). Moreover, the rolA protein is not associated with the membrane which is supported by a role as a transcription factor (Vilaine et al. 1998). Two elements are present in the promoter region of *rol*A: one is auxin regulated the other is induced by light (Carneiro and Vilaine 1993). Expression of *rol*A leads to changes in the hormone levels of abscisic acid, gibberellin, auxin, and the cytokinins isopentenyladenosine, dihydrozeatin riboside, and zeatin riboside (Dehio et al. 1993). The biochemical function of rolA is generally unknown. However, the presence of rolA has been reported to stimulate the production of secondary metabolites in transformed root lines of tobacco (Palazón et al. 1997) and transformed callus of *Rubia cordifolia* (Shkryl et al. 2008). An interesting biotechnological peculiarity of rolA is that when the gene was expressed in *R. cordifolia* calli, it ensured remarkably stable levels of anthraquinones and *rol*A expression simultaneously provided conditions for vigorous callus growth (Bulgakov 2008).

rolB

The *rol*B gene sequence ranges between 765 and 840 bp translating into a 254–279 aa protein with a molecular weight of 30 kDa (Filippini et al. 1996). The promoter region of *rol*B consists of five domains, A–E. It has been shown that domain B is important because it induces *rol*B by auxin stimuli (Capone et al. 1994). The rolB

protein is located in the plasma membrane, and it has been shown to exhibit tyrosine phosphatase activity (Filippini et al. 1996). The rolB protein contains a CX5R motif, and this motif is considered to be responsible for rolB function as a tyrosine phosphatase (Fauman and Saper 1996; Lemcke and Schmülling 1998). According to Nilsson and Olsson (1997), *rol*B is the only *rol* gene responsible for hairy root formation. However, Trovato et al. (2001) suggested that the functional ornithine cyclodeaminase encoded by rolD (see below) might also lead to induction of hairy roots (Nilsson and Olsson 1997; Trovato et al. 2001). The root-inducing function of rolB is supported by mutation studies; when specific amino acids were changed or parts deleted in the rolB protein, the root formation declined (Moriuchi et al. 2004). In *Arabidopsis thaliana*, expression of *rol*B showed dwarfing and premature necrosis of rosette leaves, changed leaf and flower morphology, and development of an enhanced number of inflorescences for each rosette area compared with wild type (Kodahl et al. 2016). The rolB protein is known to stimulate the production of new meristems which, depending on the local hormone composition, will differentiate into specific organs (Altamura 2004). Because roots in general are the most frequent adventitious organ to be produced, rolB plants produce an abundance of roots. The produced roots are fast growing, highly branched and grow in a non-geotropic manner (Altamura 2004). The way in which rolB protein most likely influences organogenesis is by altering the auxin perception (Maurel et al. 1994). Among the four *rol* genes, *rol*B is the most powerful inducer of secondary metabolism and is also the most powerful suppressor of cell growth (Bulgakov 2008). In the study by Shkryl et al. (2008), using transformed root callus tissue of *Rubia cordifolia*, *rol*B was found to be the most powerful inducer of secondary metabolites compared to *rol*A and *rol*C. In *R. cordifolia* anthraquinone levels increased approximately threefold when *rol*B was overexpressed (OE) (Bulgakov et al. 2003). When applying increasing concentrations of serine/threonine protein phosphatase inhibitors, in a related experiment, the *rol*B-OE anthraquinone content increased further. Bulgakov et al. (2003) suggested that this was due to different sensitivity of the phosphatases involved in regulating the anthraquinone production. Moreover, in *R. cordifolia* transformed with *rol*B, the total peroxidase activity was enhanced between 23 and 86 times. Furthermore, the level of *rol*B expression influenced expression of the main peroxidase gene transcript (Shkryl et al. 2013). The most prominent example of the effectiveness of rolB transformation was demonstrated for *Vitis amurensis* cells, where transformation led to more than a 100-fold increase in resveratrol production (Kiselev et al. 2007). However, growth suppression is a limiting factor for the practical application of rolB-transformed cells since it has been documented to exhibit a growth-inhibiting effect on the calli (Bulgakov 2008).

rolC

The *rol*C gene sequences comprise 537–543 bp ORFs coding for 178–180 aa proteins with a molecular mass of approximately 20 kDa. The *rol*C protein is located in the cytosol (Estruch et al. 1991). The promoter of *rol*C contains a myb response constituent similar to the sequence found in a *Hordeum vulgare myb* gene. myb is a

transcription factor, and *rol*C gene expression may hence be modulated by myb genes (Hu et al. 2003). Moreover, the *rol*C gene is activated by sucrose, and the activation of *rol*C proceeds due to a sucrose-responsive, cis-alternate region of the promoter (Yokoyama et al. 1994). Furthermore, insertion of *rol*C in transgenic plants led to improved sucrose assimilation and preservation (Mohajjel-Shoja et al. 2010). The rolC protein functions as a β-glucosidase and leads to the synthesis of cytokinins (Estruch et al. 1991). It has also been found that expression of *rol*C leads to stunted growth, increased number of side shoots, and moreover decreases in flower size and male fertility. These morphological changes are thought to be a product of cytokinin activity (Schmülling et al. 1988). In transgenic *rol*C plants, impaired production of cytokinins to approximately 20% of the non-transgenic plants has been observed. The concentration of one gibberellic acid precursor increased five to six times, while another gibberellic acid precursor decreased to approximately 30% compared to the non-transgenic plants. This demonstrates an amendment of the IAA/cytokinin equivalence (Nilsson et al. 1993). Through studies comprising transformed plants and plant cell cultures, it has been shown that *rol*C gene is capable of stimulating the production of tropane alkaloids (Bonhomme et al. 2000), pyridine alkaloids (Palazón et al. 1998b), indole alkaloids (Palazón et al. 1998a), ginsenosides (Bulgakov et al. 1998), and anthraquinones (Bulgakov 2008; Bulgakov et al. 2002, 2003; Shkryl et al. 2008). A positive correlation has been demonstrated between changes in *rol*C expression and the growth capacity and nicotine production of the transgenic roots of tobacco plants. Similar to rolA and rolB, rolC has been shown to stimulate the production of secondary metabolites in transformed root lines and calli of *R. cordifolia* (Shkryl et al. 2008). Specifically, the expression of *rol*C led to stable increase in the anthraquinone content with an approximate twofold change between the transformed and non-transformed cells. In conclusion, it is believed that rolC signals for the activation of secondary metabolic processes. It is likely that rolC may confer a wider spectrum of defense reactions in addition to secondary metabolite stimulation (Bulgakov 2008).

rolD

The *rol*D gene is only found in agropine types of *A. rhizogenes* and contains 1032 bp encoding a 344-amino acid protein (Mauro et al. 1996). This gene is the least studied of the *rol* genes. Nonetheless, rolD is the only rol proteins with a known biochemical function. *rol*D encodes a functional ornithine cyclodeaminase producing proline by reducing ornithine (Trovato et al. 2001). Moreover, proline is involved in osmotolerance regulation (Nanjo et al. 1999), and hence the bioactive compounds will increase the cell size due to water uptake. Production of proline will decrease the water uptake and stabilize the cell size. In this way rolD only has an indirect function in the production of secondary metabolites. Similar to *rol*B, the expression of *rol*D is induced by auxin; however, the underlying molecular mechanism is not known (Mauro et al. 1996). The major

morphological effects of *rol*D expression may be maintenance of hairy root growth and abundant flowering of transformed plants (Trovato et al. 2001). In *rol*D-overexpressing plants, there is enhanced production of the defense response pathogenesis-related protein (PR-1) (Bettini et al. 2003). This is priming the cell against pathogens. The effects of rolD on secondary metabolite production are not known (Bulgakov 2008).

Agrobacterium rhizogenes-Mediated Transformation of Rhodiola

The first report on transformation of *Rhodiola* appears to be for *R. sachalinensis* (Zhou et al. 2007). In this study the agropine A4 strain of *A. rhizogenes* was used, and emphasis was made on determining the optimal concentration for *A. rhizogenes*, where it was found that OD600 = 0.5 provided the highest infection rate of approximately 70%. Moreover, the best time for incubation of plant material in the bacterial solution was found to be 20 min. Furthermore, the optimal time for cocultivation was found to be 3 days. The molecular analysis confirmed the presence of *rol*C, which was also supported by Southern blot. Interestingly, the study also evaluated the effect of various biological elicitors and chemical precursors and their effect on biomass production of the hairy roots as well as the content of salidroside. It was found that optimal concentrations for the elicitors and the precursors were 0.05 mg/L and 1 mmol/L, respectively (Zhou et al. 2007). Another study by Zych et al. (2008) aimed at determining the optimal *A. rhizogenes* strains for transformation of *R. kirilowii* and evaluated three different strains (ATCC 15834, LBA 9402 and NCIB 8196). It was found that only strain LBA 9402, in combination with the T-DNA transfer stimulating compound acetosyringone, led to hairy root formation with approximately 95% of the infected explants producing hairy roots. Molecular analysis confirmed the presence of T_L and T_R T-DNA parts, represented by *rol*B and *aux*1, respectively. For *R. rosea* a comprehensive study was made by Tasheva and Kosturkova (2012) to elucidate factors important for transformation; moreover, three approaches for *A. rhizogenes*-mediated transformation were compared targeting application of bacterial solutions in droplets, injuring explants and submersion of bacterial cultures, and finally injection of plantlets. None of the approaches seemed to be successful as hairy roots were generally not formed and necrosis of plant tissue was frequently observed (Tasheva and Kosturkova 2012). In a preliminary study by Himmelboe et al. (2015), *R. rosea* was similarly subjected to transformation by *A. rhizogenes*. Approximately 15% of inoculated leaf explants formed hairy roots, and no hairy roots were observed in control. However, the hairy roots did not grow sufficiently to facilitate confirmation by PCR. Collectively, further research is needed both in terms of optimization of the in vitro growth media as well as elucidation of the plant-bacterium interactions to facilitate proliferous hairy root cultures of *R. rosea*.

Upscaling of Hairy Root Cultures to Industrial Production

In 2011 the global market of drugs derived from plants had a value of USD 21.4 billion and was forecasted to USD 22.1 billion in 2012. Due to an annual growth rate of 3.7% in the global market, it is predicted to be $26.6 billion in 2017 (Lawson 2013). As previously mentioned *Rhodiola* transformed with part of the T-DNA from *A. rhizogenes* could lead to enhanced production of bioactive compounds compared to wild plants (Kiselev et al. 2007). Many different medicinal compounds can be produced by the hairy root cultures. However, the contents of medicinal secondary metabolites from industrially produced plants are often low. Consequently, enhancing the yield of the transformed root capabilities to produce bioactive compounds is of outmost importance (Oksman-Caldentey and Hiltunen 1996). According to Giri and Nasaru (2000), the central constriction for utilization of hairy root cultures in the industry is the upscaling to an industrial level from laboratory conditions. Specifically, the main bottleneck for commercial exploitation of HR cultures is the development and scaling up in adequate bioreactors that allow optimal biomass growth and efficient metabolite extractions (Stiles and Liu 2013). Nevertheless, progress has been made on commercialization of hairy root products. ROOTec Bioactives Ltd., founded in 2005 in Switzerland, currently produces phytochemicals from hairy roots induced from 17 plant species in bioreactors. The constrictions arise due to the complex nature of hairy roots (Giri and Narasu 2000); hairy root cultures originating from transformation by *A. rhizogenes* have increased auxin sensitivity or enhanced auxin production due to the presence of the *aux* genes on the T_R-DNA (Giri and Narasu 2000; Spanò et al. 1988). Many factors influence the growth and production of secondary metabolites in hairy root culture, such as the addition of hormones in different concentrations, the quantity of sucrose in the medium, the nitrogen supplied, and the amount of different ions such as Mg^{2+} and K^+ (Dell et al. 1989; Nin et al. 1997; Rhodes et al. 1994). Substantial efforts in terms of selection of superior clones, media composition, and methods to elicitate the bioactive compounds further as well as efficient methods to extract the compounds will be of pivotal importance for economically viable production.

Currently, applied research on hairy roots is ongoing at several universities, e.g., VTT Technical Research Centre of Finland; Amiens University, France; and Arkansas Biosciences Institute in the USA. Moreover, commercial production of hairy roots for cultivation of bioactive compounds is taking place at several commercial companies throughout the world, e.g., Green2Chem in Belgium; Root Lines Technology SAS, France; RootBioTec in Switzerland; and The Good Scents Company, WI, USA. These strategies cover several plant species and methods to produce and secrete valuable bioactive compounds from hairy root cultures.

Future Perspectives

Selection of Superior Clones

Arctic plants like *Rhodiola* are known to produce phenolic compounds as defense mechanism against free radicals caused by high light and cold temperatures, as well as flavonoids for UV protection (Cavaliere 2009). Moreover, in a study comparing arctic and temperate *Oxytropis* spp. grown in controlled environments that mimicked arctic and temperate conditions, significant differences were reported at the transcriptional level. Genes related to cold acclimation were twice as expressed when temperate species were grown in arctic conditions compared to their arctic counterparts, supporting that gene expression in stress-adapted plant species is less responsive to UV elicitation (Archambault and Strömvik 2011). Furthermore, the content of the bioactive compounds varies significantly in *R. rosea*, e.g., rosavin and salidroside range from 2.90 to 85.95 mg g^{-1} FW and 0.03 to 12.85 mg g^{-1} FW, respectively, in wild-type *R. rosea* plants. Moreover, plants collected at higher latitudes presented higher contents compared to the low-latitude counterparts (Elameen et al. 2010). Hence, it is expected that *R. rosea* of arctic (or alpine) origin represent the most promising starting point in the establishment of future breeding programs. Moreover, modern analytical chemistry and computational data analysis allow for high-throughput and simultaneous metabolite analysis. By combining data sets from samples of different experimental parameters and setups, it is possible to extract information about single-metabolite properties from highly heterogenic mixes, such as those found in *R. rosea*. Through metabolomics it is possible to identify genetic and environmental factors correlating with compounds of interest. These methods are therefore important tools in the further studies on *R. rosea* with the aim of selecting superior material for commercial production in terms of entire plants and/or hairy root cultures derived from *A. rhizogenes*-mediated transformation.

Genome Editing to Enhance the Content of Bioactive Compounds

In recent years, the genome-editing tool clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) has emerged as a novel beacon for precise genome editing in plants. It is now possible to precisely alter the genomes of plants in realistic timeframes (Bortesi and Fischer 2015). This system relies on sequence-specific nucleases (SSN) that can be engineered to target almost any target of interest. The Cas9 DNA nuclease derived from *Streptococcus pyogenes* together with a guiding RNA molecule constitutes a singular complex necessary for catalyzing the double-stranded break (DSB) of a target DNA sequence. The targeting is conferred by sequence homology in a 17–21 bp region of the guiding RNA molecule (gRNA). This allows for a versatile system easy to design. By introducing DSBs into a desired sequence, it is then possible to modify this sequence through either homology-directed repair (HDR) or nonhomologous end joining (NHEJ). The

ubiquitous repair mechanism NHEJ repairs DSBs but often results in small insertions or deletions (indels) that may result in a knockout mutation (Belhaj et al. 2013). The CRISPR/Cas9 system could be applied to target genes associated with key steps within relevant biosynthesis pathways in *R. rosea* and infer knockout mutations. A previous study showed that downregulation of these repressors resulted in significant increases of production of polyphenols in the model plant *Arabidopsis thaliana* (Zhang et al. 2015b). Recent studies have proposed that the beneficial effects of roseroot are due to a plethora of secondary metabolites rather than few specific compounds (Gramsbergen et al. 2012). The phenylpropanoid pathway is involved in early committed steps of many polyphenols, such as rosavinoids and the flavonoids. Phenylalanine ammonia-lyase (PAL) isozymes catalyze the first step in the phenylpropanoid pathway where phenylalanine undergoes deamination (Fraser and Chapple 2011). These isozymes are regulated by Kelch repeat F-box (KFB) proteins that regulate the turnover of PAL isozymes for proteolytic degradation (Zhang et al. 2013). These regulators therefore serve as interesting targets for bioengineering of the phenylpropanoid pathway. Furthermore, these regulators contain conserved motifs that may serve as targets for homology-based gene fishing, thereby enabling identification of target genes (Zhang et al. 2013). More specifically, over 90 genes with *KFB* homology have been identified in Arabidopsis, including genes encoding Arabidopsis proteins involved in circadian control and in PAL ubiquitination and proteolysis (Zhang et al. 2015b). It can therefore be hypothesized that the most effective strategy for bioengineering is to target early rate-limiting steps thereby directing carbons into the biosynthesis pathways that are common for many bioactive compounds. By combining the knowledge from studies conducted in *A. thaliana* with state-of-the-art precise genome-editing method CRISPR/Cas9, the biosynthesis of beneficial compounds in *R. rosea* could be effectively enhanced. This approach may increase the potential of *R. rosea* as a medicinal plant and prove to be an applicable strategy for other beneficial plants. Furthermore, the genome-editing approach could potentially be combined with hairy root cultures, derived from *A. rhizogenes* transformation to further increase bioactive compounds.

Conclusions

R. rosea is an arctic and alpine plant naturally distributed from Siberia in the east over Northwestern Europe as well as European mountains to Northeastern parts of the USA and Canada in the west. The species is morphologically highly diverse and is found as both female and male plants. Traditionally, *Rhodiola* has been used as a health-stimulating plant, primarily in arctic areas. Later, its bioactive compounds and their properties were characterized. Important compounds include rosavinoids and salidroside, and the mode of action of the combined bioactive compounds comprises adaptogenic responses, alleviation of winter depression, and anticarcinogenic properties. Its medicinal properties have led to overexploitation of natural populations of the plant; hence, novel approaches for bio-sustainable production are

needed. Biotechnological platforms appear promising in that respect: however, further studies comprising tissue culture are needed to support the biotechnological approaches. A promising tool for increasing the content of the bioactive compounds in *R. rosea* is via *Agrobacterium rhizogenes*-mediated transformation. Through this process *root oncogenic loci* (*rol*) genes are transferred and integrated into the plant genome leading to formation of hairy roots. Studies have shown that the *rol* genes can increase the content of bioactive compounds significantly in the hairy root cultures. Moreover, the hairy root cultures can serve as a primary indicator for successful transformation, thereby circumventing the use of antibiotic selection markers and their negative effects on cell differentiation and proliferation. Another advantage is that the use of unmodified *A. rhizogenes* strains is characterized as non-GMO method, e.g., in Europe. Further optimization and upscaling are however needed if the hairy root cultures are to be used commercially. Moreover, selection of superior clones is also of pivotal importance. The recent advent of genome-editing techniques provides a novel approach for further increasing the content of bioactive compounds by allowing, e.g., knockout of negative regulators in the pathway of the synthesis of the bioactive compounds.

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Chapter 5
Production of Indole Alkaloids in *Catharanthus roseus* L. Hairy Root Cultures

Moemen S. Hanafy, Mohamed A. Matter, and M.R. Rady

Abstract Hairy root cultures of *Catharanthus roseus* L. induced by *Agrobacterium rhizogenes* have been studied extensively by several research groups for the production of the valuable indole alkaloids. Due to the pharmaceutical importance and the low content of indole alkaloids in this plant, *C. roseus* became one of the best-studied medicinal plants. Various biotechnological approaches, such as pathway engineering, precursor feeding and scaling up in bioreactors, etc., have been explored to improve the production of secondary metabolites from this plant species. The hairy roots proved to be a sustainable source for the economic mass in vitro production of indole alkaloids such as pharmaceutically valuable anticancer alkaloids: vinblastine and vincristine. This chapter provides a comprehensive account of the hairy root cultures of *C. roseus* L. and various biotechnological methods used to elevate the production of pharmaceutically important indole alkaloids. The chapter also indicates how biotechnological endeavors might improve the future progress of research for production of alkaloids using *C. roseus* L. hairy roots.

Keywords *Agrobacterium rhizogenes* • Bioreactor • Genetic transformation • Indole alkaloids • Madagascar periwinkle

Abbreviations

AP2	APETALA2
AS	Anthranilate synthase
CrBPF1	*C. roseus* box P-binding factor 1 homologue
CrMYC	*C. roseus* MYC transcription factor
CrPrx	Apoplastic peroxidase gene
CrWRKY	*C. roseus* WRKY transcription factor

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DAT	Deacetylvindoline 4-O-acetyltransferase
DXS	1-Deoxy-D-xylulose synthase
G10H	Geraniol 10-hydroxylase
GmMYBZ2	Soybean transcription factor MYBZ2
JA	Jasmonic acid
MeJA	Methyl jasmonate
ORCA	Octadecanoid-responsive *Catharanthus* AP2-domain
STR	Strictosidine synthase
TDC	Tryptophan decarboxylase
TIAs	Terpenoid indole alkaloid
ZCT	Zinc-finger *Catharanthus* transcription factor

Introduction

Plants are well-known source for medicine for thousands of years. According to the World Health Organization (WHO), millions of people still relay on folk medicine which is based on traditional remedies such as herbs for their medical treatments. Moreover, the active ingredients of many drugs are plant-derived pharmaceuticals. One of the most important plants used in the folk medicine is *Catharanthus roseus* L. (commonly known Madagascar periwinkle). This plant is a tropical/subtropical perennial herb and considered to be one of the richest natural sources of valuable secondary metabolites. It belongs to family Apocynaceae and contains more than 130 indole alkaloids (He et al. 2015) such as vindoline, ajmalicine, serpentine, catharanthine, vinblastine, and vincristine, which have been used in the treatment of blood cancer, hypertension, and Hodgkin's disease (Hanafy et al. 2016; Lounasmaa and Galambos 1989; Verma et al. 2014, 2015). Vinblastine and vincristine are the most expensive because of their extremely low abundance in the plant: 500 kg of dried *C. roseus* leaves is needed to produce just 1 g of vinblastine (Misra et al. 1996; Noble 1990), making their extraction very expensive. Moreover, the chemical synthesis of these alkaloids still remains infeasible due to their complex structures and chiral centers, and host systems such as bacteria or yeast cannot be used due to the complex and incomplete TIAs biosynthetic pathway (Hughes and Shanks 2002; Verpoorte et al. 1999). Due to the importance of the alkaloids of *C. roseus*, this plant has been used by several research groups as an experimental system for plant metabolic engineering (Hughes et al. 2004b).

Generally, the production level of the secondary metabolites in nature is quite low and dependent on environmental condition, the physiological and developmental stage of the plant (Bruni and Sacchetti 2009). High accumulation levels of these secondary metabolites normally happened when the plant's defense system is activated in response to biotic or abiotic stresses (Gray et al. 2003; Sirvent et al. 2003).

Biotechnological tools have been emerged as a sustainable system for the production of valuable secondary metabolites from medicinal plants regardless the seasonal and environmental conditions. This system has been employed since the end of the 1960s to produce useful secondary metabolites using plant cell and tissue culture technology through stress factors, bioregulators, light, plant growth regulators, and synthetic precursors (Bourgaud et al. 2001; Zhao et al. 2001a, b) and by osmotic pressure adjustment of the culture medium, light irradiation, and addition of different carbon sources (Iwase et al. 2005). The advantage of this system is the indefinite growth of the calli, and its growth can be easily maintained. However, to our best knowledge, there are few cases that can be used at commercial scale such as shikonin (Fujita et al. 1981), tuberose polysaccharides (Honda et al. 1996), and paclitaxel production using tissue culture techniques; for review see Malik et al. (2016) and Zhong (2002). The main disadvantage of this system is the instability of the production of the secondary metabolites using calli culture during the induction of callus from explants using growth regulators (DiCosmo and Misawa 1995). Even with the establishment of cell lines with high accumulation of secondary metabolites, these cell lines might lose their productivity during subculturing (Deus-Neumann and Zenk 1984) due to some genetic or epigenetic changes (Kaeppler and Phillips 1993; Phillips et al. 1994). Therefore, the production of useful secondary metabolites using callus culture is a risky technology (Iwase et al. 2005). Thus, it is advantageous to focus on enhancement of the accumulation of useful secondary metabolites using an alternative system by employing genetically stable cells instated of callus. Therefore, hairy root culture system is a better and recommended platform for in vitro production of secondary metabolites due to their higher level of cellular differentiation and improved genetic or biochemical stability in culture (Christey 2001; Rijhwani and Shanks 1998). In this respect, Tian (2015) reported that secondary metabolites of 155 plant species of 41 families have been produced by hairy root cultures after transformation by *Agrobacterium rhizogenes* strains of diverse host ranges and virulence levels. Thus, hairy root system presents a feasible technology with greater accessibility and affordability for producing valuable secondary metabolites (Gaines 2004). Many research reports have been published on the application of hairy root system to metabolite or therapeutic protein production, biotransformation of core skeletons of secondary metabolites into novel compounds, gene discovery and metabolic pathway characterization, and phytoremediation; for review see Banerjee et al. (2012), Bourgaud et al. (2001), Georgiev et al. (2007), Guillon et al. (2008), Ono and Tian (2011), and Tian (2015). This chapter focuses specifically on the production of different classes of indole alkaloids in hairy roots of *C. roseus* L. Various biotechnological approaches such as callus, suspension, shoot, and hairy root have been used to enhance the production of TIAs of *C. roseus*. In order to have a complete picture about the production of the TIAs from hairy root cultures of *C. roseus*, in the following parts, the major TIAs in *C. roseus* and different strategies for the production of these valuable secondary metabolites will be presented.

Major *C. roseus* Alkaloids

The genus *Catharanthus* has been examined for the presence of TIAs (Heijden et al. 2004; Noble 1990; Svoboda 1966). Out of more than 130 alkaloids, vindoline, ajmalicine (an alkaloid found in the roots), serpentine, catharanthine, vinblastine, and vincristine are the most potent alkaloids present in *C. roseus* plant (Fig. 5.1). Indole alkaloids are a class of alkaloids containing a structural moiety of indole; many indole alkaloids also include isoprene groups and are thus called terpene indole or secologanin tryptamine alkaloids. Indole alkaloid containing more than 4100 known different compounds is one of the largest classes of alkaloids (Seigler 2001).

The biosynthetic pathway of the *Catharanthus* TIAs is a very complex pathway. However, at present, much is known about the biosynthesis of the alkaloids, and a few enzymes have been isolated, and several genes are now available for metabolic engineering (Fig. 5.2), allowing the development of transgenic cells, organs, and plants with modified alkaloid profiles. The early stages of alkaloid biosynthesis in *C. roseus* involve the formation of secologanin derived from the terpenoid (isopren-
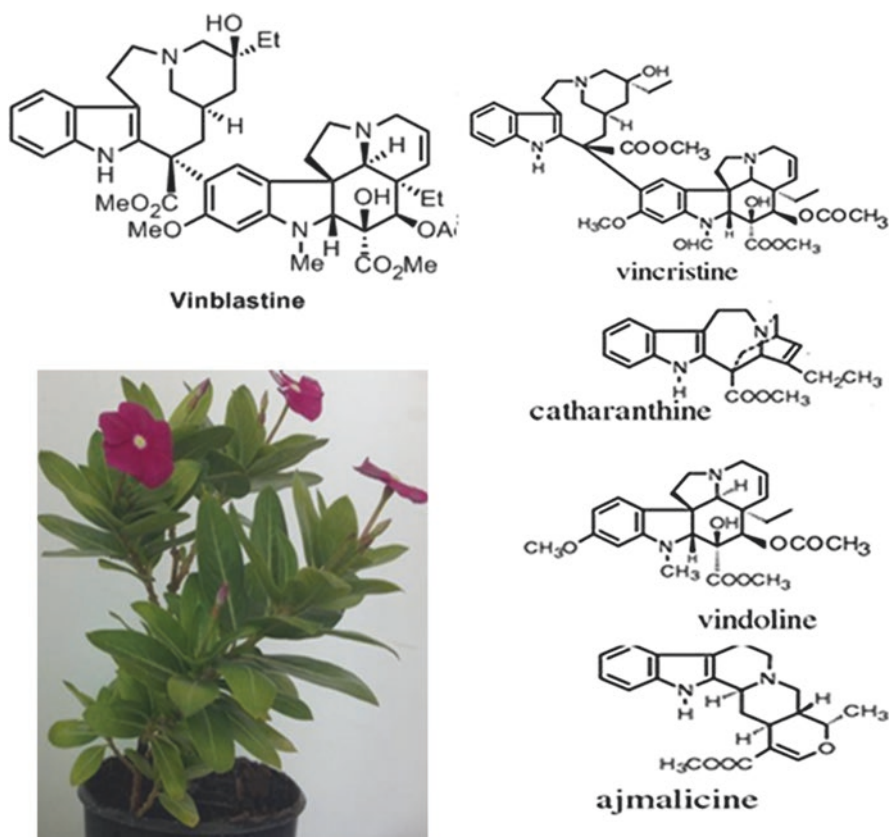
Fig. 5.1 *C. roseus* plant and the structure of various TIAs it produces
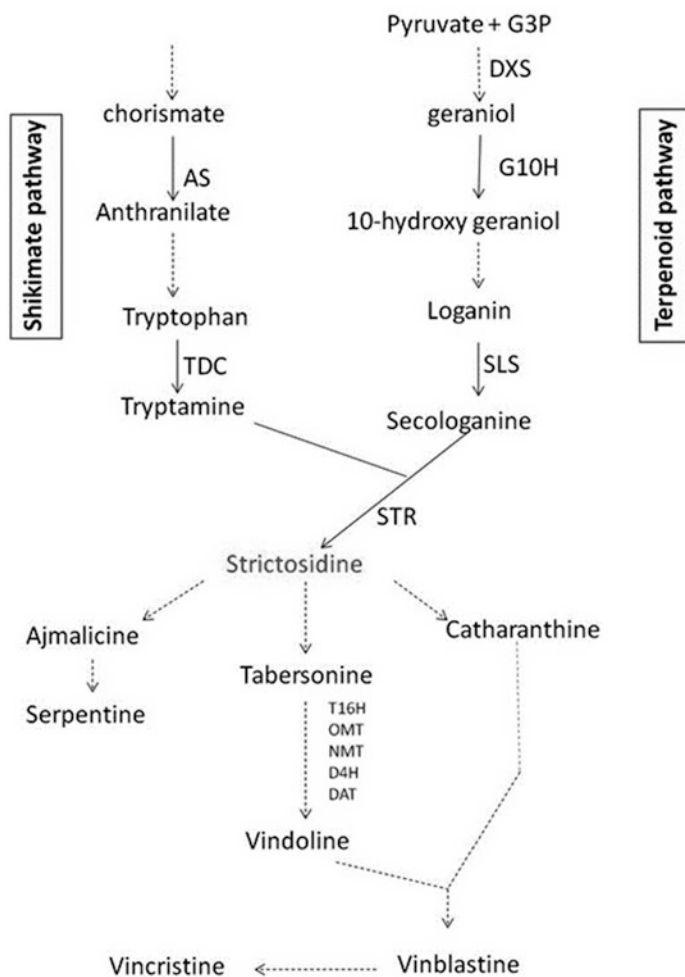
Fig. 5.2 Terpenoid indole alkaloids (TIAs) biosynthesis in *C. roseus*, including enzymes. Dashed lines indicate several enzymatic steps. *DXS* 1-deoxy-D-xylulose-synthase, *G3P* glyceraldehyde 3-phosphate, *G10H* geraniol 10-hydroxylase, *SLS* secologanin synthase, *AS* anthranilate synthase, *TDC* tryptophan decarboxylase, *STR* strictosidine synthase, *T16H* tabersonine 16-hydroxylase, *OMT* O-methyltransferase, *NMT* N-methyltransferase, *D4H* desacetoxyvindoline 4-hydroxylase, *DAT* deacetylvindoline 4-O-acetyltransferase. Adapted from Hanafy et al. (2016), Meijer (1993), Rizvi et al. (2016), Tikhomiroff and Jolicoeur (2002), and Wang et al. (2012)

oid) biosynthesis and its condensation with tryptamine to produce the central intermediate strictosidine, the common precursor for the monoterpenoid indole alkaloids, Fig. 5.2 (El-Sayed and Verpoorte 2007).

A variety of drugs such as Velsar®, Velban®, Velbe® (vinblastine sulfate), etc. contain vinblastine as a major component. Also, many commercially available drugs such as Oncovin®, Vincasar PFS®, Vincrex®, and Marqibo® (vincristine sulfate) contain vincristine as a major component. These drugs are recommended for the treatment of

blood cancer and hypertension. These drugs are produced by international companies such as Eli Lilly and Company, Teva Pharmaceutical Industries, Bristol-Myers Squibb Pharmaceutical, Spectrum Pharmaceuticals, AdvaCare Pharma, Talon Therapeutics, Fresenius Kabi, Adria Laboratories, and Hospira, Inc. The consumption of vinblastine and vincristine worldwide in 2005 was estimated with total value of US$ 150–300 million (Barkat et al. 2017). These important anticancer compounds, i.e., vinblastine and vincristine, were derived from the coupling of catharanthine with vindoline. Catharanthine is produced almost exclusively in the wax exudates on the leaf surface, whereas vindoline is accumulated in specialized internal leaf cells, suggesting a hypothesis of the involvement of transport processes for their coupling to take place (Roepke et al. 2010). Another major alkaloid is ajmalicine or raubasine which have a broad application in the treatment of circulatory diseases, especially in the relief of obstruction of normal cerebral blood flow.

Production of TIAs from *C. roseus* Using Cell and Tissue Culture

Cell and Callus Cultures

Callus is an undifferentiated tissue which represents a potential good source of secondary metabolites and other natural products. It is also uses as a source of fast growing suspension culture of single cells or small clusters of cells when cultured in liquid culture medium. Callus culture facilitated the optimization of alkaloid production, whereas media composition was effective for the callus induction so as to enhance the alkaloid production and conservation of threatened genotype (Salma et al. 2008). In this category, rapid accumulation of indole alkaloids was observed when cell cultures of *C. roseus* were treated with *Pythium aphanidermatum* (Eilert et al. 1987). Alkaloid formation was preceded by increasing in the activities of the enzymes tryptophan decarboxylase and strictosidine synthase. A two-stage process for enhanced ajmalicine production in elicited *C. roseus* cell cultures was developed in shake flasks and a bioreactor (Zhao et al. 2000). The results showed that using combined elicitor treatment of an *A. niger* mycelium and tetramethylammonium bromide yields the highest content of ajmalicine in the nutrient medium and in the elicited cells grown in 1000-ml flasks.

The effects of nitrogen sources on primary and secondary metabolism were studied in *C. roseus* calli throughout a 20-day culture cycle (Vazquez-Flota et al. 2000). The results showed that no quantitative changes were observed in alkaloid metabolism since the levels of total alkaloids were similar in calli cultured on the different nitrogen sources. The activity of tryptophan decarboxylase (TDC), which catalyzes the first committed step in indole alkaloid biosynthesis, remained unaffected.

Attempts to increase the yield of alkaloids in cell suspension culture of *C. roseus* by supplying precursors or intermediate compounds are found to be effective in many cases. Supplementation of precursors, organic compounds, and multiple feedings of loganin increased the biosynthesis of indole alkaloids in *C. roseus* cell cultures. Feeding

stemmadenine to *C. roseus* cell suspension culture resulted in the accumulation of catharanthine, tabersonine, and condylocarpine (El-Sayed et al. 2004). They concluded that condylocarpine is not an intermediate in the pathway to catharanthine or tabersonine when it is fed to the cultures. The results support the hypothesis that stemmadenine is an intermediate in the pathway to catharanthine and tabersonine.

The relationship between hypoxia and ajmalicine production in cell suspension culture of *C. roseus* was investigated (Senoussi et al. 2007). The results showed that the lack of oxygenation in cells provokes a very strong inhibition in accumulation of the alkaloids. This can be explained by the absence of some downstream enzymes involved in the biosynthesis chain of alkaloids or by the blockage of the upstream terpenes to the production stage. Moreover, the present study showed that the addition of the loganin in the fourth day, in the cell culture medium subjected to hypoxia, restored the alkaloid production. Also, addition of BAP increased the ajmalicine production. Therefore it could be suggested that the BAP can without doubt decrease the effects of the hypoxia and increases the ajmalicine production. Growth and alkaloid accumulation by a *C. roseus* cell suspension cultures fed with alkaloid precursors tryptamine and loganin was studied by El-Sayed and Verpoorte (2002). They reported that feeding the cells with tryptamine and loganin resulted in high accumulation of strictosidine, reaching a maximum at the third day after feeding. Just feeding the cells with precursors, an increase of sixfold in ajmalicine was detected. Moreover, the effect of feeding precursors on growth varies depending on the cell line and the type of precursor used. In another study, effects of auxin and cytokinin on vincristine production by callus cultures of *C. roseus* L. were investigated (Kalidass et al. 2009). It was found that the concentrations of the growth regulators alpha-naphthalene acetic acid (NAA) and kinetin played a critical role in the production of vincristine. A new method to enhance ajmalicine production in *C. roseus* cell cultures based on the joint use of β-cyclodextrins and methyl jasmonate (MeJA), when accompanied by a short exposure to UV, enhanced extracellular ajmalicine accumulation to 1040 mg/l in suspension-cultured cells of *C. roseus* (Almagro et al. 2011a).

Cell selection methods provide a promising way for producing cell lines yielding the increased levels of alkaloids, for example, cell line screening of *C. roseus* for high yield production of ajmalicine was conducted by Xiang et al. (2011). The content of ajmalicine reached 2.578 and 0.895 mg/g DW in callus and in cell suspension culture, respectively. The cell suspension culture treated with methyl jasmonate (MeJA) harvested a drastic increase of ajmalicine yield (2.2 mg/g DW in cell and 2 mg/l in culture medium). Manipulation of physical aspects and nutritional elements in a culture is the most fundamental approach for optimization of culture productivity. The effect of growth regulators on alkaloid content in callus culture of *C. roseus* was reported by Verma et al. (2012). The results showed that the highest enhancement of total alkaloid content was found in 0.50 mg/l of 2,4-D and 1.0 mg/l of BA, compared with other combinations. Their findings indicated that in addition to plant growth regulators and strength of the MS media, various carbon sources and their concentrations had a significant influence on leaf callus growth and total alkaloid content. It was found that half-strength MS basal medium supplemented with 2,4-D and BA (0.5 mg/l and 1.0 mg/l, respectively) and 6% sucrose was best for

biomass production of leaf callus and enhancement of alkaloid accumulation in *C. roseus*. The stress response after jasmonic acid (JA) treatment was studied in cell suspension cultures of *C. roseus* (Goldhaber-Pasillas et al. 2014). They concluded that the effect of JA on the primary and secondary metabolism was based on changes in profiles of fatty acids and terpenoid indole alkaloids.

Cambial meristematic cells from *C. roseus* grown in the bioreactor showed higher yields of vindoline, catharanthine, and ajmalicine than those cultured in flasks. These cultures were treated with 10 mM β-cyclodextrin and 150 μM MeJA and gave 799, 654, and 426% higher- vindoline, catharanthine and ajmalicine, respectively, than yields obtained from control cultures without elicitors (Zhou et al. 2015).

Recently, Pandiangan et al. (2015) found that addition of tryptophan as a precursor in *C. roseus* cell culture increased catharanthine content of aggregate cells after 14 days of culture and has optimum content (150 mg/l) upon treatment with tryptophan. However, vinblastine content was detected after three weeks of culturing as a result of tryptophan precursor treatment. Biotransformation is an area of biotechnology that has gained considerable attention. It is the ability of plant cell culture to catalyze the conversion of readily available on inexpensive precursor into a more valuable final product.

Organ and Shoot Cultures

Due to problems of production instability, the production of plant secondary metabolites using dedifferentiated cells (callus) is not always feasible on an industrial scale. To propose an alternative methodology, which does not use dedifferentiated cells, another system for producing useful secondary metabolites using shoot cultures was developed. In vitro propagation of plants holds tremendous potential for the production of high-quality plant-based medicines, and the same can be achieved through micropropagation. Plant regeneration from shoot, stem, and meristems has yielded encouraging results in *C. roseus*. An attempt was made to device an economical alternative for the production of medicinally important indole alkaloids in tissue culture of *C. roseus*. For instance, photoautotrophic shoot cultures were established in liquid medium with cotton fiber as a supporting agent in an indigenously designed culture vessel. Autotrophic cultures, which have the potential of a cost-effective system, produce 10% more total alkaloid as compared to mixotrophic cultures (Mitra et al. 1997).

Iwase et al. (2005) developed a novel system for producing ajmalicine and serpentine using suspension culture of the intact plant leaves of *C. roseus* in phytohormone-free MS liquid medium. The results showed that leaves separated from intact plants were able to survive in liquid culture conditions. Adjustment of the osmotic pressure in the medium, light irradiation and addition of glucose were effective to promote the production of TIAs such as ajmalicine (Aj) and serpentine (Sp). By feeding glucose (10 g/l) on day 10 of the culture period, Aj was produced at a concentration of about 18 mg/l, and Sp was produced at a concentration about 11-fold that of the control. Under continuous light conditions, the total amount of TIAs was increased about 2.8-fold compared to that in the control. In a recent study,

alkaloids were extracted from *C. roseus* plants cultivated conventionally and plants derived from in vitro cultures (Wesołowska et al. 2016). Gas chromatography-mass spectrometry (GC-MS) analysis revealed that the propagation method had a significant effect on the percentage content of alkaloids in *C. roseus* herb. Plants derived from in vitro cultures were richer in vindorosine and vindoline, while conventionally cultivated in tetrahydroalstonine and ajmalicine.

Transgenic Cell Cultures

Four distinct transformed cell lines of *C. roseus* were obtained with *A. tumefaciens* strains A281 and BO542 and *A. rhizogenes* strain K599 (O'Keefe et al. 1997). The preliminary data on the alkaloid profiles was determined by 2D TLC and HPLC/MS. Stable vindoline production was demonstrated in the undifferentiated suspension cell line strains CR-BO542, CR-K599, and CR-A281S (shooty teratomas). Active vindoline biosynthesis in the suspension cell lines was further confirmed by measuring the activity of acetyl-coenzyme-A-deacetylvindoline-O-acetyltransferase, one of the final enzymes in the biosynthetic pathway of vindoline, in cell-free extracts.

To obtain more insight into the regulation of terpenoid indole alkaloid biosynthesis in *C. roseus* cell cultures, a transgenic cell line overexpressing TDC, and thus having a high level of tryptamine, was fed with various amounts of precursors (tryptophan, tryptamine, loganin, and secologanin) in different time schedules and analyzed for TIAs production (Whitmer et al. 2002). It was found, alkaloid accumulation by line T22 was enhanced by addition of loganin or secologanin; however, the secologanin feeding was less effective. Tryptamine or tryptophan alone had no effect on TIAs accumulation. The overexpression of TDC causes this cell line to produce quite large quantities of alkaloids after feeding loganin or secologanin. However, in combination with tryptophan or tryptamine, feeding of these precursors resulted in an even further increase of alkaloid accumulation, and under optimal conditions line T22 accumulated around 1200 μmol/l of TIAs, whereas the control cultures accumulated less than 10 μmol/l TIAs.

The productivity of several transgenic cell lines of *C. roseus* was monitored over a period of 30 months (Whitmer et al. 2003). The transgenic cultures were obtained by *Agrobacterium*-mediated transformation of leaf explants with constructs containing recombinant versions of the endogenous Str and Tdc genes, which, respectively, encode strictosidine synthase (STR) and TDC. The activities of STR and TDC varied greatly over time, occasionally falling to levels not significantly different from those of non-transgenic cultures. However, the authors concluded that, despite maintaining the cell lines of the transgenic character, the cell lines gradually lost the ability to accumulate terpenoid indole alkaloids (TIAs). The diversity of alkaloids produced was also negatively affected by long-term subculture.

In conclusion, results from these studies have begun to form a picture of the complex network that leads to the formation of the terpenoid indole alkaloids. Even though there are still many unknown compounds and uncharacterized enzymes within this pathway (Heijden et al. 2004; Hughes and Shanks 2002). Research efforts might

focus on metabolic engineering of the known enzymes and compounds in order to further characterize this complex network. Precursor feeding studies in particular can help identify limiting enzymes in the pathway in order to focus future research efforts on these limiting enzymes (Peebles et al. 2006). According to literature, it was reported that unorganized plant tissue cultures are in most cases unable to produce secondary metabolites at the same levels as the intact plant. So, the hairy root system based on inoculation with *A. rhizogenes* (a gram-negative soil bacterium) has become popular in the two last decades as a method of producing alkaloids from *C. roseus.*

Strategies for Improving TIAs Production by Hairy Root Cultures

A. rhizogenes, a soil bacterium, has the ability to infect wounded plants and plays a crucial role in the induction of hairy roots by integrating a DNA segment into the host plant genome (Chilton et al. 1982). These hairy roots can be isolated from the infected explants and cultured in liquid culture indefinitely. The bacteria can be easily removed from the culture by antibiotic treatment. The advantages of hairy roots can be summarized as (1) genotypic and phenotypic stability over long-term culture, (2) high growth rate without exogenous supply of plant growth regulators, and (3) they produce high levels of secondary metabolites (Srivastava and Srivastava 2007). The application of hairy roots as a robust platform system to produce different classes of valuable secondary metabolite or therapeutic protein, biotransformation, gene discovery, and metabolic pathway characterization has been reviewed (Georgiev et al. 2007; Giri and Narasu 2000; Guillon et al. 2006a, b, 2008; Ono and Tian 2011; Shimomura et al. 1991; Tian 2015). To sum up, the majority of reported hairy root induction experiments, irrespective of bacterial strain; and the emergence of roots at infection sites of leaves were observed within 14–18 days of infection. These reports indicate the high susceptibility of *C. roseus* for different *A. rhizogenes* strains. Figure 5.3 shows schematic diagram of the production of transgenic hairy roots of *C. roseus* and different stages of development of hairy roots of *C. roseus*.

According to databases (SCOPUS) as on April 2017, there are about 3393 articles on hairy roots, and 698 limits to *C. roseus*, of which 582 are research reports and 60 review articles, 26 book chapters, and the rest for conference papers and short surveys (source: http://www.scopus.com). The yield of Madagascar periwinkle TIAs such as vincristine and vinblastine from whole plants is extremely low (about 0.0002%), and this makes their extraction very expensive (Tian 2015). Due to this reason, finding out alternative sources and strategies for production of the TIAs in higher amounts is a long-held goal of many research group, reviewed by Almagro et al. (2015).

Hairy root cultures of *C. roseus* have been established to enhance the production of TIAs including vindoline (Bhadra et al. 1993), catharanthine (Islas et al. 1994; Vazquez-Flota et al. 1994), and vincristine and vinblastine (Hanafy et al. 2016). Furthermore, various abiotic, chemical, and biotic factors were applied to the hairy roots for alkaloids yield enhancement (Bhadra et al. 1993; Shanks et al. 1998; Vazquez-Flota et al. 1994)
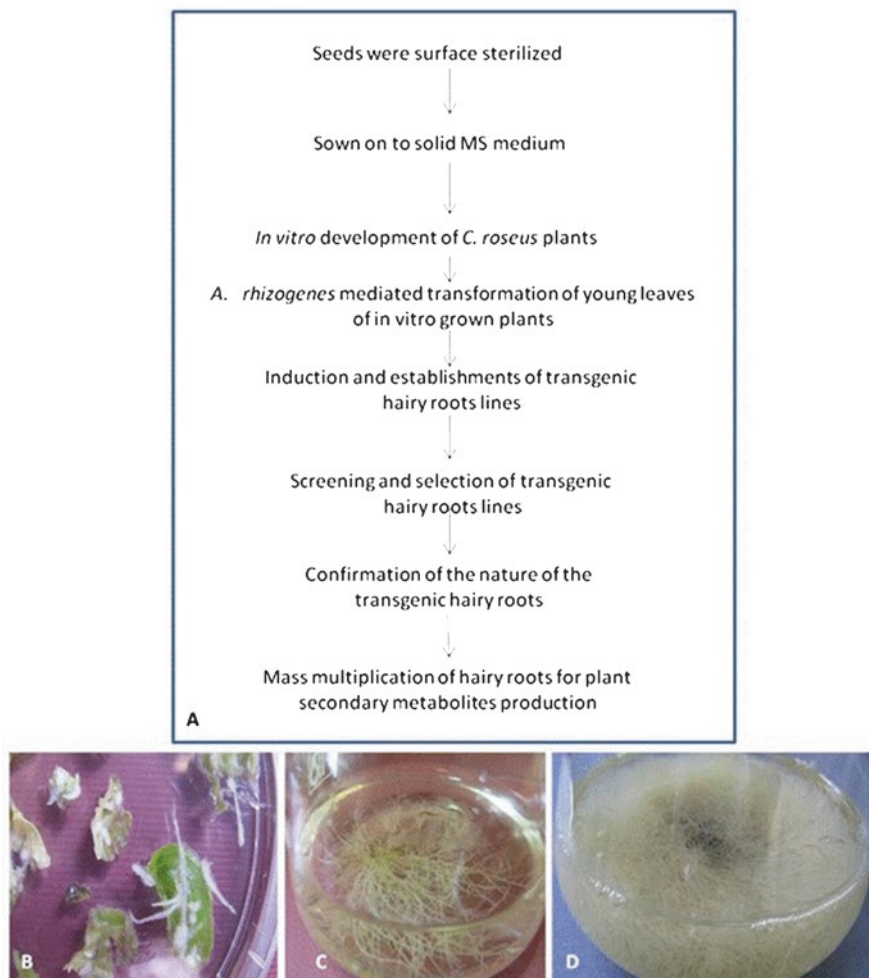
Fig. 5.3 Flowchart of the production of transgenic hairy roots of *C. roseus* (**a**) and different stages of development of the transgenic hairy root culture: (**b**) induction of hairy root by *A. rhizogenes* after 18 days of infection, (**c**) hairy roots in liquid medium after 30 days of inculcation, and (**d**) mass production of hairy roots for plant secondary metabolite production (unpublished data)

as well as by elicitor treatments combined with the adsorption of the alkaloids released in the medium on XAD resins (Sim et al. 1994). The cell suspension/hairy root interchange system is of interest because it combines high biomass (in the form of cells) and high alkaloid production (in the form of hairy roots) as described by Jung et al. (1995). Moreover, overexpression of specific key genes in periwinkle may consider a promising strategy to improve the alkaloid production or create new pathways for the synthesis of new pharmaceutical products (Garnier et al. 1999).

Hairy root culture is a promising strategy for *C. roseus* TIAs production, as they have been shown to produce target secondary compounds in higher amounts. For example, the vindoline content in two clones of hairy root cultures of *C. roseus* was found with

levels higher than the amounts reported in cell cultures (0.04–0.08% DW) (Bhadra et al. 1993). The production of ajmalicine, serpentine, and catharanthine has been repeatedly reported by different authors, sometimes reaching levels superior to non-transformed root cultures (around 0.2–0.4% DW) (Bhadra et al. 1993; Jung et al. 1995).

Data presented in Table 5.1 summarizes the major research works dealing with the use of hairy root cultures of *C. roseus* as a platform for production of TIAs. The published reports demonstrated that production and cultivation of hairy roots of *C. roseus* has been performed in vitro as an alternative platform to produce these products. Batra et al. (2004) established 250 independent hairy root clones after the infection of the leaf explants by *A. rhizogenes* agropine-type A4 strain. These clones were evaluated for growth, morphology, number of integration of Ri T-DNA genes, and alkaloid contents. The accumulation of considerable amounts of root-specific alkaloids ajmalicine and serpentine was observed in the presence of both the T(L)-DNA and T(R)-DNA genes (C1) and the T(L)-DNA gene (C3) alone. The possible role of the T(L)-DNA and T(R)-DNA genes on growth and alkaloid accumulation in these root clones was also described.

Elicitors and Precursor Feeding

An elicitor is a substance (natural or synthetic) that can initiate or improve biosynthesis of specific compounds when introduced in small concentration to a living cell system, usually resulting in the accumulation of secondary metabolites (Radman et al. 2003). Feeding with specific precursors has been proved in many cases to be a successful strategy to increase the levels of TIAs such as geraniol, 10-hydroxygeraniol, secologanin, tryptophan, loganin, and tryptamine which have been the precursors most extensively added to the culture media (Almagro et al. 2015; Morgan and Shanks 2000). On the other side, elicitation is one of the most effective strategies to improve the production of secondary metabolites in plant platforms (Patel and Krishnamurthy 2013). TIAs production in *C. roseus* is induced by either abiotic (heavy metal ions and UV light) and biotic elicitors (yeast, fungal extracts, cyclodextrins, and signaling molecules like nitric oxide and jasmonates), or culture condition (culture media stress and composition, pH, temperature, and oxygenation) (Almagro et al. 2015; Heijden et al. 2004; Mujib et al. 2012).

Many research efforts have been focused on optimization of the TIAs production by optimization of the hairy root culture condition and addition of elicitors. For example, TIAs production is enhanced with low levels of phosphate, nitrate, and ammonium (Schlatmann et al. 1992). Sucrose as a carbon source does not affect TIAs production but improves the growth of the hairy root (Mujib et al. 2012). Transgenic hairy roots were established from different explants of *C. roseus* through the infection with *A. rhizogenes* (Sun and Zeng 2005). The MS culture medium was contained sucrose as carbon and lactalbumin as nitrogen source, in which, elevated level of total alkaloids was observed as compared to other explants and callus tissues. TIAs production is maximized in hairy root cultures at 25 °C (Bailey and Nicholson 1990).

Table 5.1 Major studies dealing with elevation of the TIAs by elicitation in hairy root cultures of *C. roseus*

Elicitors	Changing in indole alkaloids	References
Sucrose, phosphate, nitrate, and ammonia concentrations	Specific production of alkaloids was highest at the lowest levels of all the nutrients studied	Toivonen et al. (1991)
Monosaccharides	Twofold increase in catharanthine content upon fructose supplementation	Jung et al. (1992)
Low temperature (19.5 °C)	Total TIAs was increased with decreasing temperature (2.56 mg/g DW). Due to the growth retardancy at low temperatures, the best volumetric productivity was obtained at 24 °C (0.98 mg l⁻¹d⁻¹)	Toivonen et al. (1992)
Macerozyme, Aspergillus culture homogenate, MeJA	High accumulation of ajmalicine	Vazquez-Flota et al. (1994)
Permeabilizing agent (dimethyl sulfoxide) and a fungal elicitor (*Penicillium* sp. and *A. niger* KCTC 123) to provide physical and biochemical stress, respectively, together with in situ adsorption using Amberlite resin XAD-7	XAD-7 greatly enhanced the release of catharanthine and ajmalicine from hairy root cultures, with an increase in total production. Catharanthine production was increased to 21.8 mg/l in the presence of the resin from 15.3 mg/l in its absence	Sim et al. (1994)
	Dimethyl sulfoxide (0.5% v/v) treatment with in situ adsorption using XAD-7 resin was found appropriate for releasing indole alkaloids from hairy roots without affecting cell viability	
	0.01 g/l *A. niger* homogenate enhanced catharanthine production by 2.5 times. Ajmalicine was not greatly affected	
	0.01 g/l *Penicillium* sp. enhanced significantly the production and secretion of catharanthine and ajmalicine	
	Combining in situ adsorption sequentially with these techniques, the release ratio of catharanthine and ajmalicine was enhanced up to 20 and 70%, respectively, which was 3.4 and 2 times higher than that obtained with in situ adsorption by XAD-7 alone	
Pectinase and jasmonic acid	An increase of 150% in tabersonine-specific yield was observed upon addition of 72 units of pectinase. JA addition caused an increase in the specific yields of ajmalicine (80%), serpentine (60%), lochnericine (150%), and hörhammericine (500%) in dosage studies	Rijhwani and Shanks (1998)

(continued)

Table 5.1 (continued)

Elicitors	Changing in indole alkaloids	References
Oxygenase inhibitors	1-Aminobenzotriazole (ABT) inhibited hörhammericine formation; clotrimazole (CLOT) inhibited the accumulation of lochnericine. The use of JA in combination with these inhibitors suggested an inducible cytochrome P-450 enzyme was responsible for the formation of hörhammericine. The inhibitor study also revealed that both lochnericine and hörhammericine are "turned over" in hairy root cultures	Morgan and Shanks (1999)
Feeding with Geraniol, 10-hydroxygeraniol, or loganin	1.5-Fold increase in tabersonine (1.4 mg/g DW)	Morgan and Shanks (2000)
MES-buffering in medium	Tabersonine synthesis enhanced in MES-buffered medium but had a negative effect on lochnericine accumulation	Morgan et al. (2000)
Ca^{2+} Antagonists (verapamil, $CdCl_2$, EGTA)	25% enhancement in the total alkaloid content by verapamil and $CdCl_2$ feeding that blocked the Ca^{2+} flux across the plasma membrane. Secretion into the medium also enhanced ten times. 90% of the total alkaloid secretion stimulated by the specific Ca^{2+}chelator, EGTA	Moreno-Valenzuela et al. (2003)
Methyl jasmonate	Tabersonine was converted to lochnericine due to 7-epoxidase activation	Rodriguez et al. (2003)
MS medium plus sucrose as carbon material and lactalbumin as nitron material	Contents of total alkaloids in hairy roots were higher than in explants and calli	Sun and Zeng (2005)
Light 280–315 nm at 9000 $\mu W/cm^2$	Increase the production of total terpenoid indole alkaloids, lochnericine, strictosidine, and ajmalicine and a decrease of hörhammericine	Binder et al. (2009)
Jasmonic acid (JA)	Increase in TIAs metabolites and transcripts of pathway genes	Peebles et al. (2009)
Elicitation with MeJA (250 μM)	Sevenfold increase in ajmalicine (6.34 mg/g DW), 2.9-fold increase in serpentine (1.71 mg/g DW), threefold increase in ajmaline (12 mg/g DW), threefold increase in catharanthine (4.34 mg/g DW)	Ruiz-May et al. (2009)
Elicitation with sodium nitroprusside (0.1 mM)	Significant increase in serpentine, catharanthine, ajmalicine, lochnericine, and tabersonine production	Li et al. (2011)

Abiotic stress by mannitol, sodium chloride, potassium chloride, cadmium chloride, and PVP K-30	Ajmalicine production accumulation was significantly increased to 2.53 mg/g DW (182% increase than blank control) and by KCl to 4.09 mg/g DW (227% increase). The maximum secretion of ajmalicine in the medium for mannitol, cadmium chloride, PVP, and NaCl was 5.4 mg/l, 1.74 mg/l, 2.192 mg/l, and 2.02 mg/l, respectively, as opposed to 1.32 mg/l in blank control	Thakore et al. (2012)
Statistically optimized medium was treated with permeabilizing agents (DMSO, Triton X-100, n-hexadecane, and Tween 80)	TritonX-100 (0.1% v/v) and n-hexadecane (2%v/v) led to 98% increase in the specific yield of ajmalicine in the roots	Thakore et al. (2013)
MS, medium strength	Production of vincristine and catharanthine of up to 442, 0.7 ng/mg FW, respectively	Hanafy et al. (2016)
Hairy root culture was attempted in several bioreactor configurations	Ajmalicine production (34 ± 2.3 mg/l) was better in modified bubble column bioreactor with polyurethane foam (PUF) support as compared to shake flask	Thakore et al. (2017)

Over 25% increase of total alkaloid content in hairy roots of *C. roseus* was noticed by Moreno-Valenzuela et al. (2003) when the produced hairy roots were treated with antagonists, like verapamil and CdCl2 that block the Ca^{2+} flux across the plasma membrane. Moreover their secretion increased in the culture medium by ten times. *C. roseus* hairy roots exhibit normal growth processes and optimal TIAs levels at an initial pH of 5.5–6.0 and under good aeration conditions (Lee-Parsons 2007; Mujib et al. 2012). Also, MS media strength has an effect on the TIAs accumulation (Hanafy et al. 2016). On the other side, elicitors are considered to be one of the most powerful strategies to enhance the biosynthesis of the secondary metabolites in medicinal plants (Almagro et al. 2015).

Among the elicitors used, *C. roseus* hairy root cultures treated with MeJA have been extensively studied and used to significantly increase TIAs; for review see Almagro et al. (2015). In this context, elicitation of *C. roseus* hairy roots with MeJA resulted in high content of ajmalicine and catharanthine (Vazquez-Flota et al. 1994) and a sevenfold increase in ajmalicine, 2.9-fold increase in serpentine, and threefold increase in catharanthine (Ruiz-May et al. 2009). The study of Palazón et al. (1998) indicated that vindoline and catharanthine producing hairy roots were thin and slow in growth. Moreover, their study demonstrated that early subculturing induced fast growth of hairy tissues that accumulated enhanced level of lochnericine. They proved a correlation between the expression of the *rol C* gene (one of the genes on the T-DNA of the *A. rhizogenes* and is involved in the induction and development of the roots) and TIAs alkaloid accumulation in *C. roseus* hairy roots. In order to improve the biomass of the hairy roots of *C. roseus* that contained elevated concentrations of the TIAs, various bioreactors/fermenters were tested (Nuutila et al. 1994). Table 5.1 presents the major research reports dealing with the influence of elicitation, precursor feeding on the production of TIAs in the *C. roseus* hairy roots.

TIAs Biosynthetic Pathway Engineering

Overexpression of Genes That Regulate TIAs Biosynthesis in Hairy Roots

Engineering the biosynthetic pathway of the secondary metabolites represents the introduction and expression of foreign key genes into the host genome for alteration in cell metabolism leading to an increased flux of target compounds (the metabolite of interest or any conversion product) in a biosynthetic pathway (Mehrotra et al. 2010; Peebles et al. 2007; Zhou et al. 2011). Thus, metabolic engineering of TIAs can be effectively applied to elevate the accumulation of the valuable TIAs in the transgenic hairy roots. The recent understanding of TIAs biosynthetic pathway as described above and cloning of various key genes have promoted pathway engineering in *C. roseus* hairy roots.

Several efforts have been made by various research groups to engineer *C. roseus* hairy roots in order to elevate the accumulation of the TIAs such as vinblastine and vincristine by overexpression of key metabolic and regulatory genes. The initial

studies have been focused on overexpressing key genes encoding key enzymes in the first steps of the shikimate (indole) or terpenoid branch (Fig. 5.2). The TIAs biosynthesis is a complex process begins with the combination of tryptamine and secologanin. These two precursors are derived from two different pathways (Fig. 5.2). A continuous supply of tryptamine is necessary for operation of the pathway. This tryptamine is formed by the conversion of tryptophan through the activity of TDC. This step is rate-limiting as the accumulation of tryptophan results in its feedback inhibition of anthranilate synthase (AS) activity (Poulsen et al. 1993; Singh et al. 1991). AS catalyzes the first committed step of tryptophan synthesis by converting chorismate to anthranilate. Plant AS is composed of two subunits, ASα and ASβ (Poulsen et al. 1993). ASα subunit catalyzes the conversion of chorismate to anthranilate with ammonia as the amino donor and is susceptible to feedback inhibition. However, the ASβ is responsible for transferring an amino group from glutamine to the ASα subunit. Therefore, ASβ is also playing a vital role in tryptophan biosynthesis (Hong et al. 2006). A mutation in anthranilate synthase alpha-subunit (ASα) gene yields an enzyme that is feedback resistant to tryptophan has been isolated from Arabidopsis thaliana, cloned, and characterized extensively (Li and Last 1996). A 300-fold increase in tryptophan, a tenfold increase in tryptamine concentration, and a twofold increase of lochnericine as a result of overexpression of a feedback-insensitive ASα in *C. roseus* hairy roots was previously reported (Hughes et al. 2004b). Overexpression of both ASβ and feedback-insensitive ASα in *C. roseus* hairy roots resulted in elevation of the content of tryptophan and tryptamine as a result of 4.5-fold higher resistance to tryptophan feedback inhibition (Hong et al. 2006). The higher resistance to tryptophan resulted in sixfold enhancement in tryptamine as compared to hairy roots expression ASα alone. Engineering the TIAs pathway in *C. roseus* hairy roots through heterologous overexpression of tryptophan feedback resistant anthranilate synthase holoenzyme (AS alpha and beta) has been attempted by another research group (Chung et al. 2007). The transgenic hairy roots were analyzed, and it was noted that the uninduced and induced AS alpha and beta hairy roots accumulated up to 1.2 and 4.5 mg/g DW, respectively, over a period of 72 h (Chung et al. 2007).

The overexpression of TDC caused no significant enhancement in tryptamine concentration, but 129% increase in the serpentine was achieved in *C. roseus* hairy roots (Hughes et al. 2004b). However, when both genes (TDC and feedback insensitive ASα) were co-expressed, a sixfold increase in tryptamine was reported (Hughes et al. 2004a). In another approach, expression of TDC together with mutant ASα and ASβ gave a synergistic effect and significantly increased tryptamine levels further than expressing just mutant ASα or ASβ (Hong et al. 2006). Therefore, it could be concluded that, manipulations of single or multiple genes in the indole (shikimate) pathway would increase the levels of tryptamine, but do not lead to significant increases in downstream TIAs (Rizvi et al. 2016).

Engineering of downstream genes of the TIAs biosynthesis *C. roseus* hairy roots was a target of many scientists to increase TIAs content. In this context, the expression of the terminal step of vindoline biosynthesis, deacetylvindoline 4-O-acetyltransferase (DAT), in *C. roseus* hairy root cultures was reported

(Magnotta et al. 2007). The study showed that several hairy root lines expressed high levels of DAT enzyme activity compared to control hairy root cultures. The DAT expression lines accumulated altered alkaloid profile with respect to hörhammericine accumulation, but levels of lochnericine and tabersonine were unaltered. The expression profiles of one early and two late TIAs biosynthetic pathway genes, namely, strictosidine synthase, desacetoxyvindoline 4-hydroxylase, and deacetylvindoline 4-O-acetyltransferase, were studied in *C. roseus* (Dutta et al. 2005). The study revealed a positive correlation between transcript abundance and accumulation of related alkaloids in the different transgenic hairy root lines of the genetic resources. Moreover, the authors noted TAI biosynthetic pathway is differentially regulated in response to different abiotic stresses (Dutta et al. 2005, 2007). Semiquantitative RT-PCR analysis of TIAs and related primary pathway genes in response to dehydration, low temperature, UV light, salinity, and wounding revealed their negative regulation in response to low temperature. HPLC analysis confirmed the notion that TIAs biosynthetic pathway is negatively controlled by low-temperature stress. Moreover, the impact of both overexpression and suppression of a peroxidase gene, CrPrx in *C. roseus* transgenic hairy root lines has been reported (Jaggi et al. 2011). Expression analysis by real-time PCR in 35S-CrPrx and CrPrx-RNAi transgenic hairy root lines showed differential transcript profile for peroxidases as well as for genes and regulators involved in MIA (monoterpenoid indole alkaloid) pathway of *C. roseus*. Metabolite analysis detected higher levels of ajmalicine and serpentine accumulation in overexpressed lines. Also, it was observed that all overexpressed transgenic lines produced more amount of H_2O_2. The obtained results concluded a role of *CrPrx* gene in the regulation of MIA pathway genes and regulators, thus affecting the production of specific alkaloids (Jaggi et al. 2011).

Overexpressing several key genes in the upper part of the TIAs pathway has been carried out (Peebles et al. 2011) in order to increase flux toward downstream metabolites within hairy root cultures. Transgenic hairy root lines were recovered with inducible overexpression of 1-deoxy-D-xylulose synthase (DXS), a key enzyme in the terpenoid pathway, or geraniol 10-hydroxylase (G10H), a limiting enzyme in the production of TIAs. Also they constructed hairy root lines with inducible expression of DXS and ASα or DXS and G10H. Transgenic hairy roots overexpressing DXS resulted in a significant increase in ajmalicine by 67%, serpentine by 26%, and lochnericine by 49% and a significant decrease in tabersonine by 66% and hörhammericine by 54%. Co-overexpression of DXS and G10H caused a significant increase in ajmalicine by 16%, lochnericine by 31%, and tabersonine by 13%. Likewise, DXS and ASα overexpression displayed a significant increase in hörhammericine by 30%, lochnericine by 27%, and tabersonine by 34%. These results point to the need for overexpressing multiple genes within the pathway to increase the flux toward vinblastine and vincristine. Table 5.2 presents the major studies dealing with elevation of the TIAs by overexpression of genes/transcription factors involved in TIAs biosynthesis in hairy roots of *C. roseus.*

Table 5.2 Major studies dealing with elevation of the TIAs by overexpression of genes/transcription factors involved in TIAs biosynthesis in hairy roots of *C. roseus*

Key gene/transcription factor	Changing in indole alkaloids	References
Truncated hamster 3-hydroxy-3-methylglutaryl-CoA Reductase (HMGR)	Five to seven times more serpentine was accumulated than the control, but a low level of ajmalicine and no accumulation of catharanthine were reported	Ayora-Talavera et al. (2002)
TDC/ASα	Tryptamine content was elevated sixfold in transgenic line overexpressed TDC and ASα	Hughes et al. (2004a)
Overexpression of deacetylvindoline 4-O-acetyltransferase (DAT)	Fourfold increase in hörhammericine (0.16 mg/g DW)	Magnotta et al. (2007)
G10H/ORCA 3	Hairy root clones expressing the *G10H* gene alone or along with transcription factor ORCA3 showed 6.5-fold enhancement as compared with non-transgenic lines (control)	Wang et al. (2010)
Constitutive overexpression of CrORCA3 (octadecanoid-responsive *Catharanthus* AP2/ERF domain), MeJA, and sodium nitroprusside (SNP)	ORCA3 overexpression led to a slight decrease of the accumulation of catharanthine, while MeJA treatment caused a large increase in the catharanthine concentration. SNP treatment alone or SNP in combination with MeJA treatment caused a dramatic decrease of the catharanthine concentration	Zhou et al. (2010)
Apoplastic CrPrx	Three- and fivefold increase in ajmalicine and serpentine, respectively, was observed in transgenic hairy roots	Jaggi et al. (2011)
Over expression of 1-deoxy-D-xylulose synthase (DXS)	A significant increase in ajmalicine by 67%, serpentine by 26%, and lochnericine by 49% and a significant decrease in tabersonine by 66% and hörhammericine by 54%	Peebles et al. (2011)
Overexpression of anthranilate synthase α subunit (ASα)	1.25-Fold increase in lochnericine (2.5 mg/g DW) and a significant decrease in tabersonine and hörhammericine. The changes in terpenoid indole alkaloid concentrations after overexpressing *ASα* were tracked over 11 years. The major alkaloid levels in induced and control roots at 11 years are comparable with the metabolite levels at 5 years	Peebles et al. 2011; Sun et al. (2017)
Overexpression of geraniol 10-hydroxylase (G10H)/DXS	A significant increase in ajmalicine by 16%, lochnericine by 31%, and tabersonine by 13%	Peebles et al. (2011)

(continued)

Table 5.2 (continued)

Key gene/transcription factor	Changing in indole alkaloids	References
Overexpression of ASα/DXS	A significant increase in hörhammericine by 30%, lochnericine by 27%, and tabersonine by 34%	Peebles et al. (2011)
Overexpression of transcription factor CrWRKY1	Threefold increase in serpentine (0.291 mg/g DW) and tenfold increase in ajmalicine (0.015 mg/g DW). Significant decreases of catharanthine and tabersonine	Suttipanta et al. (2011)
Overexpression of transcription factor ORCA3	2.49-Fold increase in catharanthine (5.6 mg/g DW) and 2.4-fold increase in vindoline. Vinblastine could not be detected	Tang et al. (2011)
Overexpression of transcription factor ORCA2	2.03-Fold increase in catharanthine (4.8 mg/g DW) and 3.67-fold increase in vindoline. Vinblastine could not be detected	Liu et al. (2011)
ORCA 3	The content of catharanthine and vindoline in the transgenic hairy root lines was increased 2.49-fold and 4.2-fold in comparison to the control hairy root lines, respectively. Vinblastine could not be detected	Tang et al. (2011)
GmMYBZ2	Catharanthine content was decreased in transgenic hairy root lines	Zhou et al. (2011)
ORCA 2	Catharanthine and vindoline contents were increased as a result of the overexpression of the transcription factor ORCA 2	Li et al. (2013)
DXR/STR/MECS	Co-overexpression of DXR and STR or MECS and STR had higher levels of ajmalicine than those with overexpression of a single gene alone such as DXR, MECS, and STR	Chang et al. (2014)
Co-expression of ORCA3 (octadecanoid-responsive *Catharanthus* AP2-domain proteins) and strictosidine beta-glucosidase (SDG) genes	Significant increase in serpentine by 44%, ajmalicine by 32%, catharanthine by 38%, tabersonine by 40%, lochnericine by 60%, and hörhammericine by 56%. The total alkaloid was increased significantly by 47%	Sun and Peebles (2016)

Overexpression of Transcription Factors Which Regulate TIAs Biosynthesis

Transcription factors are promising metabolic engineering targets due to their ability to regulate multiple biosynthetic pathway genes (Memelink and Gantet 2007). These transcription factors bind to a specific DNA sequence and interact with the promoter of the target genes to regulate the rate of initiation of mRNA synthesis (Gantet and Memelink 2002). Therefore, higher level of TIAs can be achieved by increasing the expression of several transcription factors. Most of the genes encoding important enzymes that regulate the TIAs biosynthetic pathway are tightly controlled by specific transcription factors together with developmental and environmental factors. Thus, much research has been dedicated to understand the regulation of TIAs and manipulate the regulatory pathway to increase TIAs biosynthesis.

In *C. roseus*, only a few transcription factors have been isolated and characterized, highlighting the octadecanoid-responsive *Catharanthus* AP2/ERF-domain proteins such as ORCA2 and ORCA3, CrBPF1, CrWRKY1, CrMYC1, and CrMYC2 (Almagro et al. 2011b, 2014, 2015; Goldhaber-Pasillas et al. 2014; Ruiz-May et al. 2009; Wilson and Roberts 2014). These transcription factors respond to JA, MeJA, and/or elicitors. Several attempts have been focused on engineering the TIAs biosynthetic pathway by overexpression of the transcription factors that regulate TIAs biosynthesis in *C. roseus* especially with hairy root cultures which are considered as powerful system for TIAs production (Table 5.2). A *C. roseus* WRKY transcription factor, CrWRKY1, is preferentially expressed in roots and induced by the phytohormones jasmonate, gibberellic acid, and ethylene (Suttipanta et al. 2011). The overexpression of CrWRKY1 in *C. roseus* hairy roots upregulated several key TIAs pathway genes, especially TDC, as well as the transcriptional repressors ZCT1 (for zinc-finger *C. roseus* transcription factor 1), ZCT2, and ZCT3. However, CrWRKY1 overexpression repressed the transcriptional activators ORCA2, ORCA3, and CrMYC2. Overexpression of a dominant-repressive form of CrWRKY1, created by fusing the SRDX repressor domain to CrWRKY1, resulted in the downregulation of TDC and ZCTs but the upregulation of *ORCA3* and *CrMYC2*. CrWRKY1 bound to the W box elements of the *TDC* promoter in electrophoretic mobility shift, yeast one-hybrid, and *C. roseus* protoplast assays. Upregulation of *TDC* increased TDC activity, tryptamine concentration, and resistance to 4-methyl tryptophan inhibition of CrWRKY1 hairy roots. Compared to control roots, CrWRKY1 hairy roots accumulated variable but significant increase of serpentine (from 131.5 ± 23.7 to 291.5 ± 73.2 μg g^{-1}) and ajmalicine (from 12.8 ± 1.4 to 15.4 ± 1.6 μg g^{-1}). The preferential expression of CrWRKY1 in roots and its interaction with transcription factors including ORCA3, CrMYC2, and ZCTs may play a key role in determining the root-specific accumulation of serpentine and ajmalicine in *C. roseus* plants (Suttipanta et al. 2011).

The overexpression of ORCA3 in hairy root cultures increased the content of catharanthine and vindoline up to 2.49-fold and 4.2-fold in comparison with the control cultures, respectively (Tang et al. 2011). However, vinblastine content could

not be detected in both transgenic and control hairy root cultures. Liu et al. (2011) also observed that the overexpression of ORCA2 in *C. roseus* hairy root cultures provoked an increase of catharanthine and vindoline in the transgenic hairy root extracts up to 2.03-fold and 3.67-fold in comparison to the control lines, respectively. Likewise, the combined overexpression of geraniol 10-hydroxylase (G10H) and ORCA3 in the transgenic hairy roots increased the accumulation of catharanthine up to 6.5-fold compared to control roots (Wang et al. 2010). In a recent report, Rizvi et al. (2016) attempted to elevate the accumulation of TIAs in *C. roseus* hairy roots through silencing the transcriptional repressor ZCT1 gene. Their results indicated that the transgenic hairy roots showed reduced expression of ZCT1 and ZCT2 genes. However, the expression of the third ZCT3 gene remained unchanged and no elevation in TIAs content. They suggested that silencing of all three ZCTs may be required to relieve their repression on TIAs biosynthesis.

Conclusion and Future Aspects

In a plant such as *C. roseus* where preserving the natural resources is highly demanded, utilization of transgenic hairy roots can serve as an alternative platform for the production of medicinally important TIAs. To exploit these hairy roots for TIAs production, different approaches, such as scale-up and productivity enhancement strategies, have been tried. Although hairy roots can be grown in laboratory bioreactors, more efforts toward simplifying the process and industrial upscaling of technology at bioreactor level in order to make it feasible at a larger scale with low costs and labor inputs are highly required in this direction. More biogenic studies might be done for obtaining more information about enzyme activities in TIAs biosynthesis which are still not fully unclear. Engineering several genes and transcription factors may be necessary for significant increases in downstream TIAs production.

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Chapter 6

Enhancement of Medicinally Important Bioactive Compounds in Hairy Root Cultures of *Glycyrrhiza*, *Rauwolfia*, and *Solanum* Through In Vitro Stress Application

Mrinalini Srivastava and Pratibha Misra

Abstract Enhancement of secondary metabolites through elicitation in hairy root culture is a very effective method which is broadly used to simulate the stress responses in plants. Elicitors are compounds that induce plants to produce secondary metabolites at elevated levels and reduce the processing time required to achieve high product concentrations. Hairy root cultures are considered as an excellent alternative for the supply of pharmaceutically important secondary metabolites/bioactives, due to their inherent genetic and biochemical stability. Plant-based secondary metabolites are well accepted in India as well as other countries to cure even the serious medical problems. In this chapter, three medicinally important plants are discussed in which stress-based elicitation of secondary metabolites has been achieved in hairy root cultures. These three plants contain important secondary metabolites in their different parts. Glycyrrhizin found in *Glycyrrhiza glabra* plant is used as antiulcer, immunomodulatory, antiallergic, and anti-inflammatory. Glycyrrhizin is also effective against HIV and severe acute respiratory syndrome (SARS)-like viruses. In *Solanum* plant, steroidal glycoalkaloids contain pharmaceutically important secondary metabolites. Solasodine, a major alkaloid of *Solanum* plant, is used as a contraceptive in different parts of the world. Ajmaline and ajmalicine are important root-specific indole alkaloids of *Rauwolfia serpentina*. Ajmalicine is useful in circulatory disorders, while ajmaline is principally known for its antiarrhythmic and antihypertensive activities. The main objective of this chapter is to provide knowledge in these plants regarding elicitation-based enhancement of valuable secondary metabolites in the form of research studies conducted till date (as per author's knowledge).

Keywords Elicitors • Hairy root cultures • Medicinal plants • Secondary metabolites • Stress

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Introduction

Plants synthesize a large range of natural products also known as secondary metabolites; some of them are incredibly important for the survival of mankind. Some secondary metabolites, such as essential oils and saponins, were produced by plants during long-term coevolution as a mechanism of defense response against pathogens (Du Fall and Solomon 2011; War et al. 2012). The productions of enormous varieties of secondary metabolites in plants have a direct impact on plant fitness. These secondary metabolites are being used for various purposes, such as food ingredients, medical drugs, and starting materials for chemical industry, etc. (Zhao et al. 2013). Consequently, pathogens and constituents of microbial cells, mainly the polysaccharide fractions or carbohydrates, can be used to stimulate the production of secondary metabolites in plant cell and tissue cultures (Zhao et al. 2005). Due to tremendous medicinal properties, the demand for medicinal plants is constantly increasing due to which some of them are under threatened category also (Muthukumar et al. 2004).

In recent years, all over the world, people are more aware and attentive toward natural medicines or plant extracts. According to a survey, 40% or more of the pharmaceuticals prescribed in Western countries are derived from natural resources. In Ayurveda, which was recognized as the original traditional system of Indian medicine, a remarkable and significant medical importance of a number of plant species has been well described (Gantait et al. 2014).

The majority of world's populations rely on plants for medicines including life-saving drugs since thousands of years (Tripathi and Tripathi 2003). Plants have been used in medicines either directly as traditional medicines or through formulations which are prepared and dispensed by traditional medical practitioners. About one-quarter of approved drugs contain plant extracts or active ingredients obtained from plant substances. Paclitaxel and vinblastine, most valuable anticancer agents, are derived solely from plant sources. Analgesic aspirin was also originally derived from species of *Salix* and *Spiraea* (Roberts 1988).

Plant secondary metabolite production was strongly influenced by various environmental factors such as humidity, light intensity, temperature, minerals, water, CO_2, etc. Climate change is causing an evident impact on plant vegetation including medicinal plants (Mishra 2016).

Plant cell culture techniques were used as an excellent alternative to study and produce plant secondary metabolites (Vanisree et al. 2004). It has emerged as a promising substitute to produce difficult-to-extract secondary metabolites. But the production of plant secondary metabolites through plant cell culture technology is suffering from many biotechnological and biological restrictions. Low yield of secondary metabolites in plant cell cultures is the major obstacle. In this context, the use of biotic and abiotic elicitors to improve the yield of bioactive compounds served as the best possible strategy (Tiwari and Rana 2015).

Techniques of plant tissue culture have been considered as a science mainly due to the requirement of specific environmental conditions for each medicinal plant.

Plant tissue culture is being used as a valuable tool to synthesize the same sort of chemicals as that from the natural plant. Besides this, some novel compounds are also synthesized via biotransformations (Moyo et al. 2011; Ahmad et al. 2013).

Agrobacterium rhizogenes-mediated transformations were broadly studied in medicinal plants. These modified root cultures are blessed with unique properties along with general advantages of in vitro cultures (Hussain et al. 2012). Hairy roots provide an excellent alternative due to the constant high production of secondary metabolites and maintenance of their stable nature for a longer period along with hormone-free growth over cell suspension cultures. Hairy roots synthesize bioactive compounds similar or even in higher amounts as compared to normal root cultures (Agostini et al. 2013). The hairy root cultures of many plant species have been established for the production of secondary metabolites (Zhao et al. 2014).

Accumulation of secondary metabolites in in vitro culture is directly or indirectly affected by elicitation. The mode of action of elicitation is simply based on the natural accumulation of secondary metabolites in plants as defense response, and elicitors are considered as substances or agents to induce plant defense responses (Zhou and Wu 2006). Elicitation can also be utilized to identify genes involved in the biosynthesis of bioactive secondary metabolites (Zhao et al. 2005). To increase the yield of effective compounds, various elicitors have been used with the hope of improving plant secondary metabolites (Li et al. 2011).

Elicitors are categorized under two categories, i.e., biotic (plant or pathogen origin) and abiotic (chemical, mineral, and physical agents). These elicitors are commonly used to increase the growth rate and production of secondary metabolites in hairy root systems (Soleimani et al. 2012). Elicitors are substances that cause the accumulation of phytoalexins in plants as well as induce the pathways related to defense response resulting in the synthesis of secondary metabolites in plants (Nourozi et al. 2014).

There are several important medicinal plants where elicitation strategy was used in hairy root cultures. *Momordica charantia* (Cucurbitaceae) is such plant where the role of jasmonic acid (JA) and salicylic acid (SA) was investigated on phenolic compound production and biomass accumulation in hairy root cultures. Hairy root cultures elicitated with JA and SA enhanced the production of phenolic compounds, flavonoid contents, and total phenolics significantly in comparison to non-elicited hairy root cultures (Chung et al. 2016), although only after SA treatment increase in biomass was reported in hairy root cultures.

Hairy root cultures of *Isatis tinctoria* were established for flavonoid (FL) production. Eight bioactive flavonoids, such as neohesperidin, rutin, buddleoside, quercetin, liquiritigenin, kaempferol, isoliquiritigenin, and isorhamnetin, were determined by LC-MS/MS (Gai et al. 2015). *A. rhizogenes* (ATCC15834)-mediated transformation in *Prunella vulgaris* produced 15–30 times more rosmarinic acid (RA) in comparison to intact plants. Further enhancement in RA content was achieved through elicitation by ethephon and SA (Ru et al. 2016). Hairy root cultures of *Valeriana officinalis* were treated with two to six times higher concentration of magnesium and calcium than in normal Murashige and Skoog's (MS) medium from 3 to 7 days. The uppermost amount of valerenic acid in hairy root culture was

7.9 times higher than the control (Torkamani et al. 2014). Vazquez-Flota et al. (2004) conducted an experiment in transformed root cultures of *Catharanthus roseus*. Many factors such as the concentration of medium, addition of biotic elicitors, and hydrolytic enzymes were used as strategies for enhancing the yield of alkaloids. Noteworthy results were obtained when sucrose concentration was enhanced from 3 to 4.5%. Treatment of *Aspergillus* and macerozyme increased the accumulation of ajmalicine although the addition of methyl jasmonate (MJ) was proved beneficial for increasing the yield of both ajmalicine and catharanthine (Vazquez-Flota et al. 1994).

This chapter is an effort to review all the studies on elicitation strategies for enhancing the content of secondary metabolites in hairy root cultures of three essential medicinal plants *Glycyrrhiza*, *Rauwolfia*, and *Solanum* spp.

Rauwolfia spp.

Rauwolfia genus is primarily known for its bioactive terpenoid indole alkaloids (TIA), such as reserpine, ajmaline, ajmalicine, yohimbine, serpentine, vomiline, etc. (Joshi and Kumar 2000). These alkaloids are mainly present in root bark of the plant (Mehrotra et al. 2013). *R. serpentina* L. is an evergreen shrub having woody, glabrous, and perennial habit with a maximum height of 60 cm. The leaves of the plants are elliptic to lanceolate or obovate in shape in whorls of three; roots are tuberous with pale brown cork (Deshmukh et al. 2012). This plant is commonly known as "Sarpagandha," "Chandrabhaga," snakeroot plant, Chotachand, Chandrika, Harkaya, etc. (Mallick et al. 2012). It is a tropical plant belonging to family Apocynaceae. The roots and leaves of *R. serpentina* are of medicinal importance and catch the attention in the field of medicine because of the presence of secondary metabolites (Poonam and Mishra 2013). In India, it has been used as a part of the Ayurvedic system for curing various ailments (Pant and Joshi 2008). The roots of *R. serpentina* are used as a medication for treating insomnia, hypertension, mental agitation, gastrointestinal disorders, epilepsy, excitement, traumas, anxiety, excitement, sedative insomnia, schizophrenia, and insanity (Meena et al. 2009; Poonam and Mishra 2013). According to Rajendran and Agarwal (2007), fruits and seeds of this plant are used by ethnic tribes of Virudhunagar district of Tamil Nadu, India, for its medicinal or ethnobotanical properties. *R. serpentina* plant has been considered under endangered category by the International Union for the Conservation of Nature and Natural Resources (IUCN) (Shetty et al. 2014). This plant contains abundant medicinal properties and strongly suffered from habitat distortion (Mehrotra et al. 2015).

Hairy root cultures of *R. serpentina* plants proved to be a very advantageous alternate method among all conservational strategies. Ajmaline and ajmalicine are important root-specific indole alkaloids of *R. serpentina*. Ajmaline is principally known for its antiarrhythmic and antihypertensive activities, while ajmalicine is useful in circulatory disorders (Srivastava et al. 2006).

In a study, hypocotyl segments of *R. micrantha* were used for hairy root induction with the help of *A. rhizogenes* strain ATCC 15834. Half-strength MS medium in combination with 0.2 mg indole 3-butyric acid L^{-1} and 0.1 mg α-naphthaleneacetic acid L^{-1} showed enhancement in ajmaline and ajmalicine concentration in comparison to roots grown in an auxin-free medium. In this report, production of ajmaline and ajmalicine was reported for the first time in hairy root cultures of *R. micrantha* (Sudha et al. 2003).

A study was conducted to evaluate the effect of three elicitors, methyl salicylate, SA, and acetylsalicylic acid, on phenolic content of *R. serpentina.* The content of caftaric acid, caffeic acid, chlorogenic acids, and cichoric acid along with rutin (flavonoid) was investigated in shoots and roots of the plant. In the shoot of the plant, the content of the cichoric acid was significantly (0.05%) enhanced through the application of all elicitors. Salicylic acid was proved as the most effective elicitor at 10 M concentration. For chlorogenic acid, caftaric acid, and rutin, methyl salicylate at the concentration of 10 M proved to be best among all tested elicitors in shoots as well as in roots of the plants. All elicitors significantly increased the cichoric acid content, but best result was obtained for 1000 M salicylic acid (Nair et al. 2013).

Estimation of bioactive compounds is very necessary for the valuation of herbal drugs. Many natural factors such as climate, altitude, rainfall, etc. are responsible for the growth of plants, and these conditions also affect the contents of plant metabolites. Variation in geographical areas alters the level of secondary metabolites, and this information could be used for generating the special conditions to enhance the yield of bioactive compounds. Keeping this in mind, four different parts of Southern India were used to collect *Rauwolfia* samples for analyzing the reserpine level. Considerable variation in the concentration of reserpine has been recorded (Kumar et al. 2010).

Shetty et al. (2014) developed an efficient and reproducible method for the induction of callus and hairy roots from in vitro as well as in vivo explants of *R. serpentina.* Hairy roots were used for the large-scale production of secondary metabolites. Thin-layer chromatography results showed that the reserpine was present in in vivo and in vitro explants and callus and it can be enhanced through various methods. Further, hairy roots were induced from leaf explants, and these hairy roots would be utilized for large-scale production of secondary metabolites (Shetty et al. 2014).

In *R. serpentina*, the presence of two nitrogen-containing compounds, vomilenine and reserpine, was first time examined through DART technique. For identification the intact hairy roots were analyzed by holding them in the gap between the DART source and the mass spectrometer. The confirmation of the structures of the identified compounds was made through their accurate molecular formula determinations (Madhusudanan et al. 2008).

Harisaranraj et al. (2009) concentrated on the production of reserpine by elicitating embryogenic suspension cultures of *R. serpentina* through different concentrations (50–500 μM) of MJ. Reserpine content significantly increased by the effect of MJ, although the fresh weight, dry weight, and growth ratio of embryos were

significantly decreased by increasing MJ concentrations. The highest yield was 7.3-fold enhancements.

Nurcahyani and Anggarwulan (2008) studied the effect of Cu^{2+} on callus growth and reserpine synthesis of in vitro cultures of *R. serpentina*. The increase in reserpine production was observed at 5 and 10 μM concentration of Cu^{2+} after 15 days of elicitation, although decrease in concentration was reported at higher concentration of Cu^{2+}.

An efficient transformation system for *R. serpentina* was established with *A. rhizogenes* strain LBA 9402. The transformed root lines showed significant differences in their reserpine content (Ray et al. 2014).

There is the constant global demand of terpenoid indole alkaloids as the natural sources of these alkaloids were unable to fulfill such a huge demand. In this regard, hairy root cultures of *R. serpentina* provide an ultimate alternative source for the production of these alkaloids (Goel et al. 2010). Benjamin et al. (1993) also reported hairy roots induction with the use of *A. rhizogenes* strain ATCC 15834 from the leaf explants of *R. serpentina* (Goel et al. 2010; Liu et al. 2012; Sudha et al. 2003). Different *A. rhizogenes* strains, i.e., SV4, LBA9402, and SV2, were evaluated for their transformation ability. Among them SV2 strain was found more competent for the induction of hairy roots in leaf explants of *R. serpentina* (Sarma et al. 1997). ATCC 15834 strain of *Agrobacterium* was also exploited for their transformation ability regarding induction of hairy roots in *R. micrantha* (Sudha et al. 2003). Madhusudanan et al. (2008) reported induction of hairy roots in leaf explants of *R. serpentina* using A4 strain. A novel compound, 3-epi-a-yohimbine, was reported from these hairy roots. Plantlet formation from high reserpine yielding hairy root lines of *R. serpentina* has also been reported (Mehrotra et al. 2013). The effect of biotic and abiotic elicitors on the production of important metabolites in hairy root cultures of *R. serpentina* was analyzed. Ajmalicine production could be stimulated up to 14.8-fold at 100 mM concentration of NaCl after 1 week of treatment. However, ajmaline concentration could only be increased up to 2.9-fold at 100 mg L^{-1} dose of mannan after 1 week of treatment (Srivastava et al. 2016).

Solanum spp.

Solanum khasianum was originated from India; stem and leaves of this bushy annual or short-lived perennial plant are packed with spines. Steroidal glycoalkaloids are abundant in *Solanum* genus. These alkaloids are used as the precursor for the synthesis of steroidal drugs. Solasodine is the aglycone moiety of glycoalkaloids. Solasodine can be easily converted to 16-dehydropregnenolone, which is a key intermediate in the synthesis of steroidal drugs, such as progesterone and cortisone. Due to the presence of these valuable alkaloids, in the traditional system of medicine, *Solanum* species are used for the treatment of several diseases as in liver diseases, in asthma, and in different kinds of inflammation (Patel and Krishnamurthy 2013).

Shilpha et al. (2015) used leaves of *S. trilobatum* L. for the establishment of hairy root cultures of the infection of *A. rhizogenes*. Various strains, like MTCC 2364, MTCC 532, and ATCC along with A4, were used in this study. An elicitation strategy was performed for enhancing the solasodine level in *S. trilobatum*. A hairy root line elicited with methyl jasmonate (4 M) for 2 weeks, a 1.9-fold and 6.5-fold enhanced production of solasodine (9.33 ± 0.04 mg g^{-1} DW) was obtained in comparison to unelicited and nontransformed normal roots, respectively. Real-time PCR analysis was also performed for monitoring the expression level of HMG-CoA reductase during the first 2 weeks of elicitation. A significant enhancement was also observed in the total flavonoids (521.09 mg g^{-1} DE), total phenolics (150.42 mg g^{-1} DE), and radical scavenging activity (83.3%) at 4 M MJ concentration in comparison with control (Shilpha et al. 2015).

Jain and Singh (2015) evaluated *S. melongena* (L.), a medicinally important plant of Solanaceae family, for its efficiency for induction of hairy roots using various strains of *A. rhizogenes*, such as LBA 9402, NCIM 5140, MTCC 532, A4, and R1022. Hypocotyl explants infected with NCIM 5140 strain proved as most effective regarding hairy root induction. Acetosyringone with 100 μM in cocultivation media enhanced the incidence of hairy root induction within a short period. A study was performed for demonstrating the effect of different elicitors, viz., SA, yeast extract (YE), and pectin, on solasodine production in *S. melongena* (L.) hairy root cultures. Pectin with 1% concentration was observed to be the most efficient elicitor to enhance the solasodine production from 6.5 to 151.23 μg g^{-1} DW, i.e., 23-fold over hairy root control (μg g^{-1} DW) and 88-fold in comparison to field control (1.71 μg g^{-1} DW) (Jain and Singh 2015).

Hairy root cultures of *S. aculeatissimum* were developed through *A. rhizogenes* strain 15834. Production of steroidal saponin along with root growth was investigated under various culture conditions. Gamborg's B5 medium was found suitable for growth and steroidal saponin production. Growth and steroidal saponin production were enhanced when 100 μg L^{-1} auxin was added to the medium. The addition of 2,4-D inhibited growth, although production of steroidal saponin was highest with NAA (Ikenaga et al. 1995).

The effect of various elicitors was examined on solasodine production in hairy root cultures of *S. elaeagnifolium* Cav. Chitosan, hemicellulase, H_2O_2, Ag-NO_3, *Hormonema* sp., and *Pythium* sp.'s homogenates were used as elicitors, but no effect on solasodine production was observed. Homogenates of *Sclerotinia sclerotiorum* reduced the solasodine production about 30% in comparison to the control. This activity could be attributed to the fact that *S. sclerotiorum* elicitation induced the sesquiterpene biosynthesis instead of alkaloid production (Parsons et al. 2006).

In hairy root cultures of *S. khasianum*, the effects of biotic and abiotic elicitors were observed on the production of important metabolites. Solasodine content could be enhanced up to 4.0-fold and 3.6-fold at 100 mM and 200 mM NaCl, respectively, after 6 days of treatments (Srivastava et al. 2016).

In *S. khasianum*, increased proportion of CO_2 in the medium enhanced the growth and secondary metabolite production in hairy root cultures. Light and temperature play a role in the determination of growth and secondary metabolite

production in these hairy root cultures. These factors also control the appearance of green color in hairy roots (Jacob and Malpathak 2004). Transgenic hairy root cultures were also established for *S. khasianum* plant. Single-chain variable fragment (scFv) protein against solamargine was expressed in transgenic hairy roots. Results showed that the concentration of solasodine glycoside could be increased to 2.3-fold in transgenic hairy roots than nontransgenic hairy roots. It may be concluded that scFv expression in transgenic lines might have stimulated biosynthesis pathways. Plantlet regenerated from these hairy roots and fruits obtained from these transgenic plants also contained twofold higher solasodine glycoside content in comparison to plants generated by nontransgenic hairy roots (Putalun 2011).

For the enhancement of solasodine concentration, Quadri and Giulietti (1993) observed the effect of a fungal elicitor obtained from *Alternaria* sp. on suspension culture and entrapped cells of *Solanum elaeagnifolium* Cav. Fourteen-day-old cultures were used for elicitation of 1% FW/V autoclaved homogenates. In suspension culture, 0.9–1.5 mg g^{-1} DW (65%) increase and in entrapped cells 0.75–1.4 mg g^{-1} DW (about 95%) enhancement were observed. The maximum accumulation was obtained after 72 h of elicitation.

In this study, the combination of water stress and infection of plant-parasitic nematodes was studied on the nutritional quality of tomatoes (Atkinson et al. 2011). The level of phenolic compounds, carotenoids, and sugar in fruits was estimated along with physiological responses after plant encountered with one or both the stresses. The amount of carotene and carotenoid lycopene was found lesser in water-stressed tomatoes but showed diverse responses after combined stress. Nematode stress was responsible for enhancement in flavonoid level, albeit with reduced yield, although the level of chlorogenic acid was positively affected by water stress, nematodes, and combined stress. Combined stress also enhanced sugar level. These results enlightened the utility of the combination of stress.

An (2014) observed the effects of yeast extract (YE) and MeJA on the growth and solasodine production of *S. hainanense* cells. The results showed that various concentrations of MeJA (50–250 μM) and YE (1–4 g L^{-1}) have different eliciting influences. The increase in solasodine concentration was observed through elicitation of 3 g L^{-1} of YE and 50 μM of MeJA at the initial stage of cell culture where the increase was 1.9- and 1.3-fold, respectively, over the non-elicited cells. According to this study, YE (biotic elicitor) was found more successful in enhancing solasodine production than MeJA (abiotic elicitor).

Glycyrrhiza spp.

G. glabra L. plant belongs to Fabaceae family and is the inhabitant of Central and Southwest Asia. Cultivation of this plant occurred in Northern India, Italy, France, the UK, the USA, Russia, Spain, Germany, and China (Parvaiz et al. 2014). *G. glabra* is commonly known as Jothi-madh, Mulhatti (Hindi), Yashtimadhu, Madhuka (Sanskrit), licorice, liquorice, sweetwood (English), Jashtimadhu, Jaishbomodhu

(Bengali), Yastimadhuka, atimaddhura (Kannada), Jethimadhu (Gujarat), Iratimadhuram (Malayalam), Jatimadhu (Oriya), Jeshtamadha (Marathi), Athimaduram (Tamil) and Atimadhuranu, and Yashtimadhukam (Telugu) (Jatav et al. 2011). It is a perennial shrub, with hardy habit and height up to 2.5 m. The leaves are alternate, compound, and imparipinnate. Leaves have four to seven pairs of leaflets oblong, lanceolate, and elliptical in shape. The flowers are narrow lavender to violet in color. The fruit is up to 1.5-cm-long compressed legume or pod. Seeds are brown in color and reniform in shape (Jatav et al. 2011).

G. glabra plant is blessed with many medicinal properties. This plant is used in the treatment of dyspepsia, gastric ulcers, fevers, liver ailments, asthma, bronchitis, sore throats, Addison's disease, and rheumatoid arthritis. It is also useful as an antitussive, expectorant, and laxative. In ancient times, this plant was also suggested in cases of women sterility. Licorice root is considered under top five herbs, which are recommended for the treatment of fatigue. This herb decreases temptation for sugars and increases cortisol activity in the human body. Glycyrrhizin is present in a very high amount in licorice roots. Besides glycyrrhizin, some other triterpene saponins are also present. Saponins are used for various purposes as foaming and detergent and also as emulsifying and sweetening agents (Nasrollahi et al. 2014). Although licorice roots contain many beneficial properties, some side effects are also associated, due to high doses and prolonged use of this, such as hypokalemia, hypertension, mineralocorticoid effects, myoglobinuria, lethargy, quadriplegia, etc. (Nasrollahi et al. 2014).

The roots of licorice contain a large amount of glycyrrhizin (up to 15%) and oleanane-type triterpene saponins. These saponins are used in various foods and industrial, cosmetic, and pharmaceutical applications. Saponins are commercially used in food industry as foaming, detergent, emulsifying, wetting, and sweetening agents (Hostettmann and Marston 2005; Shibata 2000). These compounds are also utilized in cleansing and personal care sectors and also as ingredients in the cosmetics such as shampoos, shower gels, hair conditioners, liquid soaps, lotions, baby care products, toothpastes, and mouthwashes. The pharmacological properties of triterpenes have been broadly studied which showed that these compounds have significant medicinal properties. Besides this, they also showed involvement in plant defense responses. Glycyrrhizin is also efficient against several viruses, such as HIV (Ito et al. 1987, 1988) and severe acute respiratory syndrome (SARS caused by coronavirus-like viruses) (Cinatl et al. 2003).

The use of licorice is more than 4000 years old. It is considered under important medicinal plants mentioned in Assyrian herbal (2000 BC). Licorice is used as laxative, demulcent, antitussive, expectorant, and sweetener from traditional Siddha system of medicine. It is used for curing acute respiratory problems, gastritis, gastric ulcers, inflammatory conditions in general, and adrenal exhaustion. Compounds found in licorice roots possess both estrogenic and antiestrogenic activity, and due to these properties, this important herb is used for treating the female hormonal problems (Jatav et al. 2011).

A study in *G. inflata* hairy roots was performed using different elicitors, such as chitosan and MeJA, for enhancing the glycyrrhizin contents. Chitosan did not

significantly alter the content; however, on increasing the duration, the content of glycyrrhizin decreased. 100 μM MeJA after 5 days of treatment enhanced glycyrrhizin content 5.7 times higher than the control. Further increase in duration decreases glycyrrhizin content (Vasconsuelo and Boland 2007). Yeast extract treatment proved as effective for enhancing the glycyrrhizin yield but lesser effective in comparison to MeJA (Wongwicha et al. 2011).

The effect of MeJA, chitosan, and yeast extract on *G. inflata* (Batal) hairy root cultures was monitored. MeJA at 100 μM concentration was the most effective for increasing the glycyrrhizin production up to 108.9 ± 1.15 μg g^{-1} DW after 5 days of elicitation (Putalun et al. 2011).

An investigation was performed for induction of hairy roots in *G. glabra* using strain K599 in leaf explants, and a comparative study was also done to analyze its growth kinetics at shake flask and bioreactor level. Four different basal media were used in this study, MS basal semisolid, NB, B5, and WP medium. Among them, maximum TF frequency was observed on MS basal semisolid medium, although WP medium could not induce hairy root formation. NB modified medium supported best hairy root growth. Approximately 20 times enhancement in root biomass was reported after 45 days of culture, after increasing this culture period browning of roots started. Under the same set of conditions, normal roots exhibited only twofold increase in biomass in shake flask cultures (Mehrotra et al. 2008).

Licoagrochalcone A and licoagrocarpin, two new prenylated flavonoids, were isolated from the hairy root cultures of *G. glabra*. By spectroscopic evidence, the structures of the new compounds were elucidated as 3-prenyl-2′,4,4′-trihydroxychalcone and (6a*R*, 11a*R*)-4-prenyl-3-hydroxy-9-methoxypterocarpan, respectively (Asada et al. 1998).

Flavonoids are economically important compounds, but its amounts are insufficient in hairy roots. To conquer this difficulty, the combination of transgenic approach and elicitation techniques was used to increase the flavonoid production. cDNA encoding chalcone isomerase (chi) gene was overexpressed in hairy roots of *G. uralensis* Fisch. Subsequently, transgenic and wild cultures were elicited with PEG 8000 (2%) and yeast extract (YE) (0.1%), and the combination of these two elicitors is also used. Total flavonoids were extracted and measured. The obtained results demonstrated that the highest flavonoid was obtained in double-treated transgenic hairy roots (2.838 g 100 g^{-1} DW). The amount of flavonoid in wild-type hairy roots and the untreated transgenic hairy roots was 0.842 and 1.394 (g 100 g^{-1} DW), respectively. The enhanced accumulation of flavonoids was also correlated with the elevated level of chi transcripts and CHI activity; it confirmed the key role of chi in the flavonoid biosynthesis. This research verified that the combination of PEG8000-YE elicitation with metabolic engineering was an effective strategy to enhance the flavonoid production in hairy roots of *G. uralensis* Fisch (Zhang et al. 2009).

In transformed *A. precatorius* cell suspension cultures, twofold increase in glycyrrhizin yield was obtained against untransformed cultures. To improve the yield of glycyrrhizin, some fungal elicitors prepared from *Aspergillus niger* and *Rhizopus stolonifer* were tested at different concentrations in transformed cell suspension

cultures of *A. precatorius*. The maximum enhancement of 4.9- and 3.8-fold in glycyrrhizin contents was obtained with *A. niger* (7.5% v/v) and *R. stolonifer* (5.0% v/v), respectively, on the fifth day after elicitor treatment (Karwasara et al. 2011). In seedlings of *G. uralensis* growth, lipid peroxidation, osmolyte concentration, antioxidant metabolism, and Si content were examined under control conditions as well as salt and drought stress conditions [100 mM NaCl with 0, 10, and 20% of PEG-6000 (polyethylene glycol-6000)] with or without 1 mM Si. The addition of Si markedly affected the *G. uralensis* growth in a combination of NaCl and PEG treatment. The addition of Si improved germination index, germination rate, seedling vitality index, and biomass under control and NaCl treatment. Si also increased radicle length under control, NaCl, and combination of NaCl and PEG treatment (NaCl-10% PEG), while under NaCl-20% PEG combination, it decreased some parameters such as radicle length, seedling vitality index, and germination parameters. The salt and drought stress-induced oxidative stress were modulated by Si application (Zhang et al. 2017).

Two *A. rhizogenes* strains MTCC 2364 and MTCC 532 were evaluated in terms of a number of hairy roots, transformation frequency, and glycyrrhizin production in *Abrus precatorius*. After elicitation with methyl jasmonate, maximum glycyrrhizin production (2.5-fold) was found in hairy roots transformed with strain 532 (Sajjalaguddam and Paladugu 2016).

A study was performed by Li et al. (2016) on a 1-year-old *G. uralensis* Fisch. ex DC (Fabaceae), treating it with three different exogenous phytohormones, like auxin (indole-3-acetic acid), gibberellins, and MeJA in the months of June and July. Control plants were treated with water. The glycyrrhizic acid content of roots was significantly increased in the plants which were treated in the month of June. The increase also occurred in the plants which were treated in July, but the effect was lesser in comparison to the plants treated in June. Auxin at 40 mg L^{-1} and gibberellin at 40 mg L^{-1} concentration significantly enhanced the accumulation of glycyrrhizic acid in *G. uralensis* roots. Methyl jasmonate at 100 and 25 mg L^{-1} in June and July, respectively, also significantly promoted glycyrrhizic acid content. Major active compositions, such as isoliquiritin, liquiritin, liquiritinapioside, and isoliquiritinapioside, were found positively correlated with glycyrrhizic acid content (Li et al. 2016).

Shabani et al. (2009) conducted an experiment in *G. glabra* and reported the maximum yield of glycyrrhizin which occurred at 2 mM MeJA concentration after 24-h treatment, in the form of 3.8-fold increase as compared to the control.

Ahmed and Baig (2014) observed the effect of diverse biotic elicitors such as fungal extract prepared from *Aspergillus niger* and *Penicillium notatum* on cell cultures of *Psoralea corylifolia* L. Besides this, yeast extract and chitosan were also studied. Ninefold enhancements were reported in psoralen concentration in treated cells over control one after *A. niger* elicitation. Elicitation with *P. notatum* yeast extract and chitosan caused four- to sevenfold higher psoralen productions in contrast to control cells. Above all, extract of *A. niger* at 1.0% v/v increased the highest accumulation of psoralen in the cultured cells, i.e., 9850 μg g^{-1} DCW.

The effect of various arbuscular mycorrhizal (AM) fungi was observed on the growth and development of *G. glabra* (licorice). Several species of AM, such as *Glomus intraradices* and *Glomus mosseae*, and a mixture of fungi (*G. intraradices*, *G. cladoideum*, *G. mosseae*, *G. caledonium*, *G. microagregatum*, and *G. etunicatum*) were used in the study. Licorice growth rates were determined by measuring the colonization rate of the plants by the fungi, dry plant biomass, phosphorus concentration, and concentration of secondary metabolites. The results of this study showed that the AM fungi enhanced the leaf and root biomass, phosphorus content, and secondary metabolite content of plants (Liu et al. 2014).

For addressing the difference between secondary metabolite content of closely related plant species and their hybrids, a study was conducted between three *Glycyrrhiza* species (*G. uralensis*, *G. glabra*, and *G. inflata*). The *Glycyrrhiza* species (genotypes) for 95 batches of samples were identified by DNA bar codes of the internal transcribed spacer and trnV-ndhC regions. The chemotypes were revealed by LC/UV- or LC/MS/MS-based quantitative analysis of 151 bioactive secondary metabolites, including 17 flavonoid glycosides, 24 saponins, and 110 free phenolic compounds. For the 76 homozygous samples, the three *Glycyrrhiza* species showed significant biosynthetic preferences, especially in coumarins, chalcones, isoflavones, and flavonols. In total, 27 species-specific chemical markers were discovered. The 19 hybrid samples indicated that hybridization could remarkably alter the chemical composition and that the male parent contributed more to the offspring than the female parent did. This is hitherto the largest-scale targeted secondary metabolomics study of medicinal plants and the first report on uniparental inheritance in plant secondary metabolism. The results are valuable for biosynthesis, inheritance, and quality control studies of licorice and other medicinal plants (Song et al. 2017).

Conclusions

The combination of hairy root culture and elicitation in medicinal plants is the very promising strategy for enhancing the yield of secondary metabolites, due to the high demand of bioactive compounds and their poor yield in natural sources. Significant enhancement (up to commercial level) was obtained by implementation of these techniques. Study of these fruitful topics increases the knowledge of elicitation or stress-related parameters. Readers could utilize these techniques to improve the yield of other commercially important secondary metabolites in different plants.

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Chapter 7
An Efficient Hairy Root System for Withanolide Production in *Withania somnifera* (L.) Dunal

Appakan Shajahan, Chandrasekaran Thilip, Kunnampalli Faizal, Valiyaparambath Musfir Mehaboob, Palusamy Raja, Abubakker Aslam, and Krishnan Kathiravan

Abstract *Withania somnifera* (L.) Dunal is one of the most important medicinal plant belonging to the family Solanaceae. Its root have been used as a drug since ancient times, and various pharmacological effects have been attributed to the occurrence of secondary compounds like withaferin-A and withanolide-A. Recently, huge interests are generated for production of these bioactive compounds through *Agrobacterium rhizogenes*-mediated hairy root culture techniques. The present review explores the culture conditions for efficient *Agrobacterium*-mediated hairy root culture system of *W. somnifera* for withanolide production. The hairy root induction is influenced by several factors like bacterial stain, type of explant, and cocultivation methods. The transformation efficiency could be enhanced by the addition of acetosyringone and SAAT treatments during cocultivation. Recent studies have also shown positive correlations of elicitors and biosynthetic pathway genes on withanolide production in hairy root culture of *W. somnifera*.

Keywords Ashwagandha • *Agrobacterium rhizogenes* • Transformation • Acetosyringone • SAAT (sonication-assisted *Agrobacterium*-mediated genetic transformation) • Elicitors

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Introduction

Withania somnifera (L.) Dunal (Solanaceae), also known as ashwagandha, winter cherry, or Indian ginseng, is an important ancient ayurvedic drug of Indian traditional medicine system. This plant is a source of extremely important pharmacological steroidal lactones known as withanolides (Chakraborty et al. 2013). It can be effectively used against mental diseases, asthma, inflammation, arthritis, rheumatism, tuberculosis, and many more diseases, including cancer (Pati et al. 2008). Withanolide-A and withaferin-A (Fig. 7.1) are the important withanolides present in this plant and formed by mevalonate and downstream of triterpenoid pathway through cyclization of 2,3-oxidosqualene to cycloartenol (Sabir et al. 2012). Withanolide-A can be used for the treatment of neural degeneration diseases such as Parkinson's and Alzheimer's diseases, convulsions, and cognitive function impairment (Nagella and Murthy 2011), while withaferin-A is known to possess angiogenesis inhibition and therapeutic action against carcinogenesis (Mohan et al. 2004; Sabir et al. 2011). This important medicinal plant has now become rare since it is not widely available in wild condition (Sivanesan 2007).

Hairy roots have been accepted as a promising system for synthesis of valuable bioactive compounds under in vitro condition in the absence of expensive growth hormones (Giri and Narasu 2000; Hu and Du 2006). This system possesses several genuine properties that make their use in metabolic engineering, such as fast growth, short doubling time, ease of maintenance, and ability to synthesize a range of metabolic compounds (Swain et al. 2012). The development of *Agrobacterium rhizogenes*-mediated hairy root cultures offers a remarkable potential for large-scale production of withanolides from *W. somnifera* (Bandyopadhyay et al. 2007; Saravanakumar et al. 2012; Sivanandhan et al. 2012, 2013). Hairy root culture systems include components such as explant; *A. rhizogenes* strain; infection methods, including physical and chemical method of enhancement; and finally incubation conditions. Variations in these components are the key in standardization of protocol
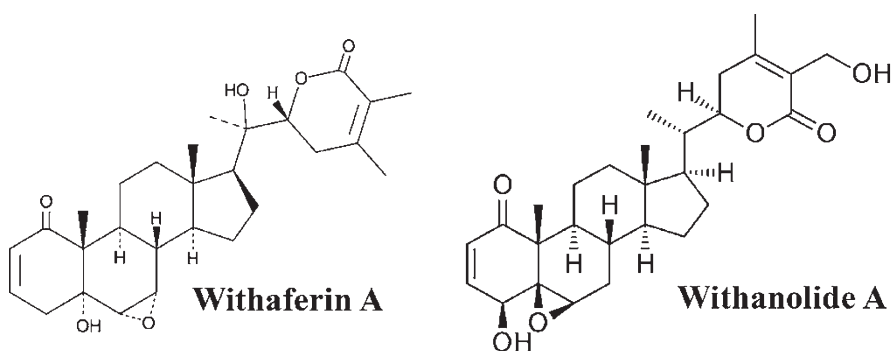

Fig. 7.1 Chemical structure of withaferin-A and withanolide-A

for transformation. These components are interdependent in effecting the overall result, that is, the production of hairy root clones with higher amount of targeted phytochemicals.

Efficiency of *A. rhizogenes* Strains on Hairy Root Induction

The interaction between host plant and bacteria results exchange of food materials and informational signals that cause several morphogenetic changes. These signals can be small molecules or even genetic information. During infection, *A. rhizogenes* transmits DNA (T-DNA) into the plant genome, carrying instructions that will lead to the formation of a transformed root. Transformed roots are capable of accelerated elongation and branching in in vitro; therefore, they are used in the production of root-specific secondary metabolites (Tepfer et al. 1989). The differential efficiency of various *A. rhizogenes* strains in promoting the induction, growth, and secondary metabolite production of hairy roots in *W. somnifera* also has been reported by many workers (Pawar and Maheshwari 2004; Murthy et al. 2008; Saravanakumar et al. 2012; Sivanandhan et al. 2014; Thilip et al. 2015) (Table 7.1). The ability of *A. rhizogenes* strains varied greatly in their ability to induce hairy roots and frequency of transformation. *A. rhizogenes* strain R1000 has the highest induction frequency (88%) when compared to all other strains reported so far.

Effect of Explant on Transformation

The influence of explants on transformation during hairy root induction of *W. somnifera* was documented by several workers (Pawar and Maheshwari 2004; Murthy et al. 2008; Saravanakumar et al. 2012; Sivanandhan et al. 2014; Thilip et al. 2015). They used leaf, cotyledon, petiole, and internode segments as explants. Among these various explants, leaf explants produced higher transformation frequency when infected with *A. rhizogenes* strain R1000 (Fig. 7.2a), in the order of 88% (Sivanandhan et al. 2014) and 50.6% (Thilip et al. 2015). Most of hairy roots emerged from the wounds, which are sites of infection by *A. rhizogenes*. It was hypothesized that the cells contain high level of auxin and sucrose to be an ideal target for infection and hairy root induction (Nilsson and Olsson 1997). This may be due to variation in the stage of cell cycle and metabolic activities of various cells in the explants adjacent to a wound (Potrykus 1990). Usually phloem cells contain high amount of sucrose and IAA; therefore, they are the target sites for *A. rhizogenes*. These bacterial strains show varied preference for hairy root induction depending on the histology of explants. However, the overall response depends upon the virulence of *A. rhizogenes* strain and its interaction with the plant species and tissue type. Most of the authors reported that leaf explants showed the best response not only for hairy root induction but also adventitious root formation without *A. rhizogenes* infection (Rafi et al. 2010).

Table 7.1 *A. rhizogenes*-mediated hairy root culture in *W. somnifera* for withanolide production

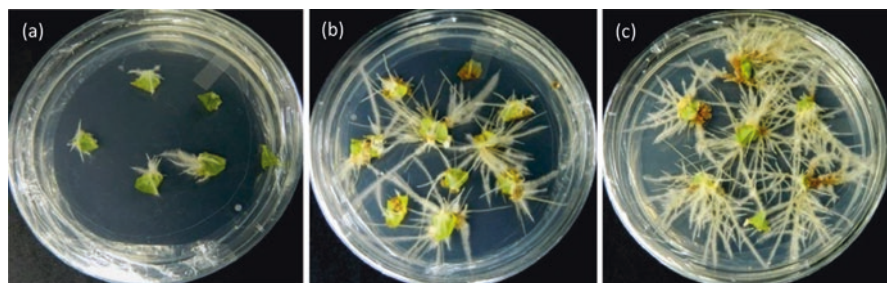| Strain	Explants	Infection method	Transformation efficiency	Confirmation of transgenic status	Withanolide content	Authors' names
MTCC2364 MTCC532	Leaf	Co-cultivation period—24 h Cefotaxime—250 mg l–1	20%	–	–	Pawar and Maheswari (2004)
R1601	Leaf	Co-cultivation period—2 days Cefotaxime—400 mg l–1 and kanamycin sulfate—100 mg l–1	40.3%	*npt* II, *rol* B genes (PCR, Southern hybridization)	Withanolide-A (157.4 µg g l–1 DW)	Murthy et al. (2008)
	Cotyledon		3.33%			
R1000	Petiole	Co-cultivation period—2 days AS—100 µM Cefotaxime—250 mg l–1	64%	*rol* C gene (PCR)	Withaferin-A (72.3 mg g l–1 DW)	Saravanakumar et al. (2012)
	Leaf		42.5%			
	Internode		37.7%			
R1000	Leaf	Co-cultivation period—5 days AS—100 µM Cefotaxime—300 mg l–1	88%	–		Sivanandhan et al. (2014)
	Cotyledon		64%			
A4	Leaf		79%			
	Cotyledon		38%			
R1000 MTCC2364 MTCC532	Leaf (Middle portion)	Co-cultivation period—2 days AS—100 µM Cefotaxime—200 mg l–1 Heat treatment—41 °C for 5 min Sonication—15 s	50.6%	*rol* B gene (PCR)	Withaferin-A (6.17 mg g l–1 DW) Withanolide-A (3.82 mg g l–1 DW)	Thilip et al. (2015)
			29.3%			
			18.6%			

Fig. 7.2 (**a**) Effect of leaf explants on hairy root induction. (**b**) Hairy root proliferation after 3 weeks in the heat-treated cultures (41 °C for 5 min). (**c**) Hairy root proliferation after 3 weeks upon sonication (15 s) and heat treatment (41 °C for 5 min)

Cocultivation and Effect of Acetosyringone on Hairy Root Induction

Cocultivation time is the period between dipping of explants in bacterial culture and control of bacterial growth. Prolonged cocultivation causes overgrowth of the bacteria around the explant, which lead to necrosis of tissue. Shorter cocultivation may result in unsuccessful gene transfer from bacterial strain to explant. So, the transformation efficiency may differ by the different duration of cocultivation. Recent studies reveal that the phenolic substances such as acetosyringone enhance transformation efficiency in *W. somnifera* (Saravanakumar et al. 2012; Sivanandhan et al. 2014). The transfer of T-DNA from *A. rhizogenes* to host tissue is mediated by *vir* genes present in the bacteria. An expression of *vir* genes is greatly enhanced by acetosyringone through the signals from the wounds of host tissues (Pitzschke and Hirt 2010). In *W. somnifera*, cocultivation period of 2 days and 100 μM acetosyringone showed better transformation efficiency (Pawar and Maheshwari 2004; Murthy et al. 2008; Saravanakumar et al. 2012; Thilip et al. 2015) than control (Table 7.1).

Effect of Heat Treatment and Sonication

Heat treatment is a common approach to enhance *Agrobacterium*-mediated transformation efficiency in several species (Hiei et al. 2006), and it was also reported in *W. somnifera* (Thilip et al. 2015). The leaf explants of *W. somnifera* were infected with *A. rhizogenes* at various temperatures, viz., 39, 41, 43, and 45 °C for duration of 3, 5, 7, or 10 min. Treatment of 41 °C for 5 min (Figs. 7.2b and 7.3a) showed higher percentage of (76.0%) transformation (Thilip et al. 2015).

Sonication-assisted *Agrobacterium*-mediated genetic transformation (SAAT) is also an effective method to transfer foreign genes into recalcitrant target plants (Trick and Finer 1997). This has been used to enhance *Agrobacterium*-mediated
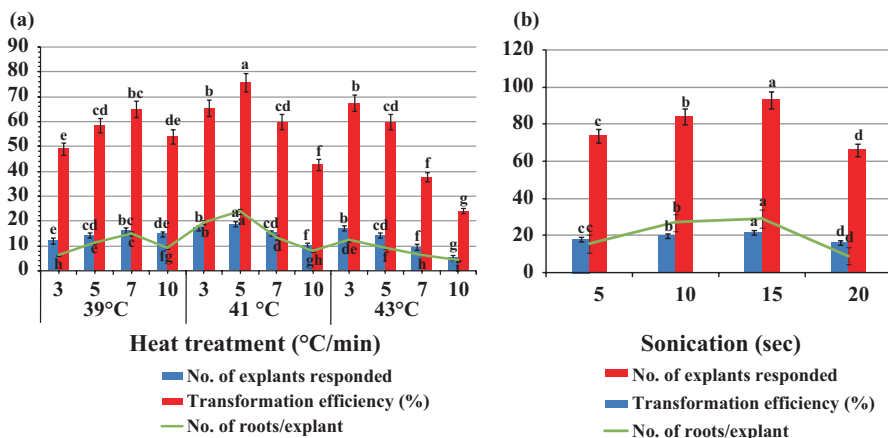
Fig. 7.3 (**a**) Influence of heat treatment on transformation efficiency. (**b**) Influence of sonication on hairy root induction

transformation of many different plant species like soybean, loblolly pine, black locust, flax, and citrus (Santarem et al. 1998; Tang et al. 2001; Zaragoza et al. 2004; Beranova et al. 2008; de Oliveira et al. 2009). The combined effect of heat and ultrasound sonication showed a further enhancement in the transformation efficiency of *A. rhizogenes* with *W. somnifera*. Among the various treatments, 15 s sonication combined with heat treatment (41 °C for 5 min) proved to be the best and produced 93.3% of transformation efficiency (Figs. 7.2c and 7.3b) after 3 weeks (Thilip et al. 2015). Sonication treatment above 15 s exhibited lower percentages of transformation efficiency, and this might be because of tissue damage due to prolonged sonication. The enhanced transformation rates using sonication-assisted *Agrobacterium*-mediated genetic transformation (SAAT) probably resulted from sonication-induced "micro-wounding" that helped in the secretion of more acetosyringone like phenolic signal compounds from the host tissue to activate *vir* gene expression (Santarem et al. 1998). Wound-induced production of signal polyphenolic compounds enhances the access to putative cell wall binding factor to the *Agrobacterium* during infection (Stachel et al. 1985). This combined SAAT system (Fig. 7.4) can be used for hairy root production on a large scale at a faster rate compared to previous methods of hairy root induction in *W. somnifera*.

Confirmation of Transgenic Status and Detection of Withanolides

In the recent studies, polymerase chain reaction (PCR) analysis (Saravanakumar et al. 2012; Thilip et al. 2015) was carried out to determine the integration of the transgene in the hairy roots of *W. somnifera*. They reported that the PCR results of
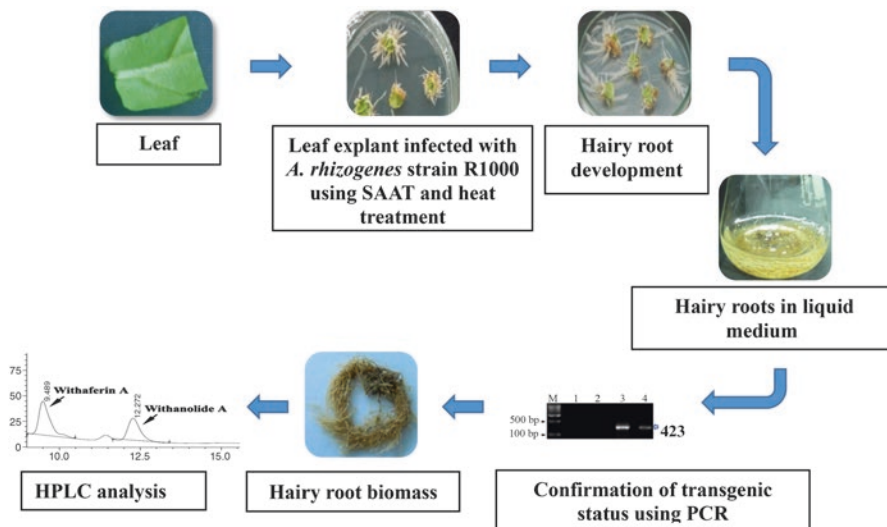
Fig. 7.4 Schematic representation of withanolide production in hairy root cultures of *W. somnifera*

all the hairy root lines contained 423 bp *rol* B gene fragment and 557 bp *rol* C gene fragment, which were the parts of T-DNA of *Ri* plasmid. High-performance liquid chromatography (HPLC) analysis was used to assess the production of withaferin-A (Saravanakumar et al. 2012; Thilip et al. 2015) and withanolide-A (Murthy et al. 2008; Thilip et al. 2015) in the hairy root culture (Fig. 7.5).

Effect of Elicitors and Biosynthetic Genes on Withanolide Production

The production and accumulation of valuable secondary metabolites into hairy root cultures can be enhanced by using suitable elicitors in the medium (Radman et al. 2003). Supplementation of hairy root culture of *W. somnifera* with MeJ (methyl jasmonate) and SA (salicylic acid) showed severalfold more withanolide production than the control. Sivanandhan et al. (2013) observed that the separate treatment of 15 µM MeJ and 150 µM SA exhibits highest production of withaferin-A (57.46 and 70.72 mg/g DW) and withanolide-A (114.38 and 132.44 mg/g DW). Co-transformation of biosynthetic genes or transcription factors, along with the hairy root system, is an another exciting approach for withanolide production in *W. somnifera*. Sil et al. (2015) were successfully transformed squalene synthase SS1 gene from *Arabidopsis thaliana* to *W. coagulans.* These co-transformed hairy roots were produced increased rate of withanolide-A as compared to in vitro grown roots. Soni et al. (2011) also co-transformed β-cryptogein gene (which is responsible for

Fig. 7.5 HPLC analysis of withanolide production in hairy root cultures. (**a**) Standard withaferin-A. (**b**) Standard withanolide-A. (**c**) Withanolides (withaferin-A and withanolide-A) in hairy root culture

fungal elicitor protein) along with a hairy root culture of *W. somnifera*. These β-cryptogein co-transformed roots did not directly enhanced withanolide production, but they enhance the activity of PAL (phenylalanine), an enzyme involved in the phenylpropanoid pathway.

Conclusion

This review has provided a comprehensive detail about *A. rhizogenes*-mediated hairy root induction in *W. somnifera* and in vitro produ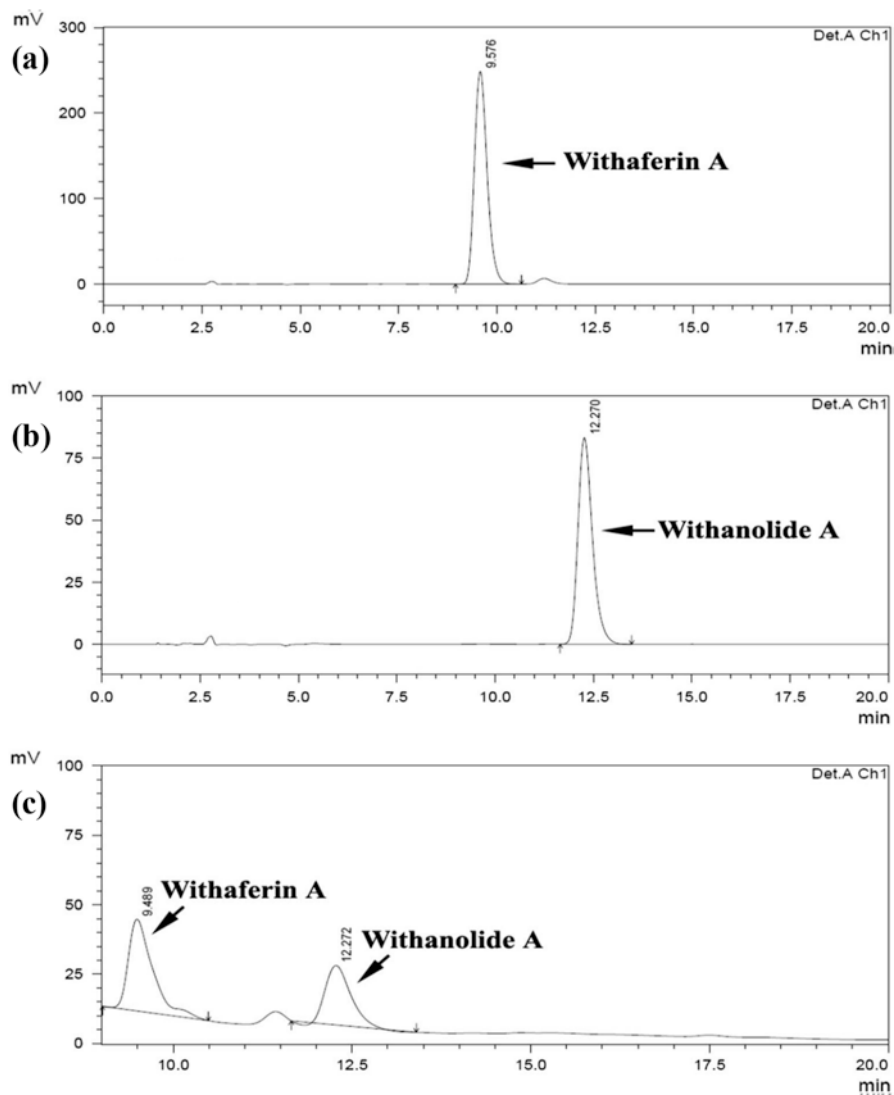ction of withanolides. These recent developments represent that the in vitro production of withanolides from *W. somnifera* through hairy root culture could be enhanced by using efficient cocultivation methods and culture conditions. Application of elicitors like MeJ and SA and co-transformed hairy roots with biosynthetic genes shows promising prospects toward withanolide production using hairy root culture technology.

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Chapter 8
Hairy Root Culture of *Nicotiana tabacum* (Tobacco) as a Platform for Gene Manipulation of Secondary Metabolism

Mahesh Kumar and Adinpunya Mitra

Abstract *Nicotiana tabacum* (tobacco) has been regarded as a model system for plant tissue culture and genetic engineering research because of its versatility in tissue culture and molecular biology. Hairy root cultures (HRCs), which are a result of *Agrobacterium rhizogenes* (a Gram-negative soil bacterium) infection, offer potential for high production of valuable secondary metabolites and have been well established in tobacco. Furthermore, the genetically engineered HRCs are becoming attractive choice for mass production of desired phytochemicals. In addition, genetic manipulation will further enhance the secondary metabolite synthesized by normal hairy root. Several studies on HRCs have demonstrated the production of plant secondary metabolites through genetic engineering approach. Tobacco is a well-researched system in all aspects being it genetic makeup, physiology (including secondary metabolism), or biochemistry, which will make the work easy to genetically manipulate its hairy root culture. In this chapter, we present a number of studies on genetically manipulated HRCs of tobacco in which the genetic manipulation leads to alteration in secondary metabolism.

Keywords *Nicotiana tabacum* • *Agrobacterium rhizogenes* • Transgenic root culture • Secondary metabolites

Introduction

Plants produce large and diverse groups of compounds with low molecular weight which do not participate in primary physiological functions, such as photosynthesis, respiration, reproduction, growth, etc., but are important for the survival of the plants in their environment (Walton and Brown 1999). These compounds are called secondary metabolites, also described as natural compounds. Plants utilize these

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compounds mainly as defense molecules against pathogens, mechanical injury, and herbivores. These compounds also function in the plant, as attractants for the pollinators, signal molecules, etc. Throughout the human history, secondary metabolites have been used for various purposes as medicine, insecticides, cosmetics, flavors, and fragrances. These phytochemicals are synthesized in the plants in small amounts and often accumulated in specialized tissues; as a result their isolation and purification are difficult (Kim et al. 2002). Also, in several cases, organic synthesis of these chemicals is costly, and extraction from plants has been the main approach to obtain these important phytochemicals in a reasonable cost. Furthermore, growing the plant to maturation takes longer time depending on the species, and quantity of these phytochemicals depends on various other factors such as pathogens and environmental factors. These challenges led the scientists to use tissue culture methodology for obtaining the valuable secondary metabolites.

Among the number of tissue culture systems, hairy root culture (HRC) is found to be the most promising system for mass production of secondary metabolites because of its numerous advantages over the other tissue culture systems, e.g., faster growth and higher accumulation of secondary metabolite, no requirement of any growth hormone in the culture medium, genetically stable, etc. Hairy roots are disease manifestation exhibited by plant. According to initial discovery in the past, hairy root syndrome is dicotyledonous plant's root disease caused by *Agrobacterium rhizogenes* bacterium. This syndrome/disease was there in the nature but has come to the limelight in the early twentieth century after *A. rhizogenes* discovery as causative agent of a disease characterized by neoplastic outgrowth of fine roots at the site of infection (Riker et al. 1930; Hildebrand 1934). *A. rhizogenes* is a rod-shaped soil bacterial pathogen which comes under the family *Rhizobiaceae* and genus *Agrobacterium* (Tzfira and Citovsky 2000). This phenotype (hairy root) is a result of T-DNA (transfer DNA) integration from the bacterial Ri-plasmid (root-inducing plasmid) into the plant genome. Agropine-type Ri-plasmid has split T-DNA, i.e., two independent sequences (T_L-DNA and T_R-DNA), while single T-DNA in all the other types of Ri-plasmid (Veena and Taylor 2007) carry *rol* genes and *aux* genes responsible for hairy root phenotype and auxin biosynthesis, respectively (Guillon et al. 2006b). Extensive studies have been performed on mechanism of T-DNA transfer in *A. tumefaciens*. The infection and transfer of T-DNA in *A. rhizogenes* are assumed to be similar as it is observed in the case of *A. tumefaciens* (Veena and Taylor 2007). The entire transformation process can be summarized in five steps: (1) releasing of phenolic compounds (particularly acetosyringone) upon injury of roots, sensed by *A. rhizogenes* bacteria, triggering attachment of the bacteria to the roots, (2) T-DNA processing inside bacterial cells and formation of T complex, (3) transferring T complexes from the *Agrobacteria* to the host plant genome, (4) integration and expression of T-DNA in the plant genome, and (5) formation of hairy roots (Georgiev et al. 2012). Many studies have been performed on plant infection by *A. tumefaciens* and *A. rhizogenes* and mechanism of T-DNA transfer (especially in *A. tumefaciens*); many reviews are available related to them (Georgiev et al. 2012; Chandra 2012; Lee and Gelvin 2008; White and Winans 2007; Veena and

Taylor 2007; Tzfira and Citovsky 2006; McCullen and Binns 2006; Tzfira et al. 2004; Sevón and Oksman-Caldentey 2002; Tzfira and Citovsky 2002; Ziemienowicz 2002; Zhu et al. 2000; Zupan et al. 2000; Zupan and Zambryski 1995; Costantino et al. 1994; Winans 1992; Gelvin 2003; Guillon et al. 2006a).

The mechanism of hairy root disease has been exploited judiciously to develop valuable biotechnological tool for gene transformation in the plant. Also, this has been used to establish an evolutionary root culture system in the field of plant tissue culture known as HRC. HRCs have already been established successfully in more than 450 different plant species, including dicots (natural hosts of *Agrobacterium*), monocots, and some gymnosperms, and are continuously being induced in new plant species (Veena and Taylor 2007; Ono and Tian 2011). The mechanism of hairy root disease is further used to develop *A. rhizogenes*-mediated transformation of desired gene into HRCs and useful to perform metabolic engineering and medical molecular pharming in the HRCs. These genetically manipulated HRCs can be termed as transgenic hairy root cultures (THRCs). In recent past, studies on HRC metabolic engineering were increased immensely. Many studies on hairy root-based metabolic engineering have been performed with diverse plant species for enhancing and/or altering the secondary metabolite production. In addition, researchers have also been succeeded to produce new secondary metabolites in HRCs which is alien to the parent HRC by introducing new set of gene(s).

Nicotiana tabacum (tobacco) is a member of Solanaceae family and regarded as model system in plant research because it is an exceptionally versatile system for tissue culture and genetic engineering. There is a long list of studies on plant tissue culture and molecular biology which have originated from the research associated with tobacco. Tobacco HRC has been established in the 1980s (Spanó et al. 1981, 1982; Costantino et al. 1984), and numerous studies have been performed since then in relation to secondary metabolite production. Researchers have used the tobacco HRC as a platform to manipulate its secondary metabolism or introduce new diversion in the pathway through hairy root-based metabolic engineering. This will lead to higher accumulation of valuable secondary metabolites of tobacco and/or new valuable phytochemical (which are not produced in tobacco, naturally) production. Also, there have been studies where these HRCs used to utilize substrates and produced chemicals of high value. In this chapter, we will discuss a number of studies on genetically engineered HRCs of tobacco leading to secondary metabolite production, aiming to establish HRC of tobacco as a platform for gene manipulation of secondary metabolism.

Transgenic Hairy Root Culture of Tobacco and Secondary Metabolite Production

The production of plant secondary metabolites has been getting tremendous attention from recent past due to their beneficial properties in food, cosmetic, and pharmaceutical industries. Many new compounds with greater bioactivity have been

identified, and search process is still continued. Beneficial properties of secondary metabolites for mankind raised the demands which lead to development of various approaches/methods including THRC to enhance production of the metabolites. As stated previously, THRC is a genetically engineered/manipulated HRC developed by *A. rhizogenes*-based transformation. Scientists are now using binary vectors in place of original Ri-plasmid for genetic transformation. Binary vectors earlier made were Ti-plasmid based, but later Ri-based binary vectors were made to utilize in genetic transformation in hairy root (for detail about binary vectors, see review in Lee and Gelvin 2008; Murai 2013).

Induction of HRC does not require any sophisticated apparatus and can easily be performed in a simple microbiology laboratory (Georgiev et al. 2008; Dandekar and Fisk 2005; Hamill and Lidgett 1997). If one has the transgenic *A. rhizogenes* strain (strain containing binary vector with desired gene), establishment of THRC can easily be done same as HRC generation, using general laboratory protocol which includes seven steps: (1) growing the *A. rhizogenes* strain, (2) preparation of explant and infection with the agrobacterial culture, (3) cocultivation, (4) selection and hairy root induction, (5) subcloning the hairy root, (6) confirming integration and expression of genes (*rol* genes and gene of interest), and (7) confirmation of successful transformation.

Before the infection to the explant, agrobacterial culture has been added with acetosyringone (a phenolic compound) to activate the bacteria for infection. The confirmation of successful genetic transformation can be done in several ways. In the past, the production of opines was used to confirm successful genetic transformation (Georgiev et al. 2008). However, disappearance of opine production was reported in many cases after several cell division cycles (Flores et al. 1987; Sevón and Oksman-Caldentey 2002; Georgiev et al. 2008), which made this process nonreliable. Other way is to directly confirm the gene of interest or the T-DNA genes either by polymerase chain reaction (PCR) or southern blot hybridization (Xie et al. 2001; Le Flem-Bonhomme et al. 2004; Georgiev et al. 2008; Sil et al. 2015; Kumar et al. 2016). The confirmation can also easily be done using PCR or northern blot hybridization, if the engineered agrobacterial strains will be harboring the reporter gene β-glucuronidase (GUS) (Cseke et al. 2007; Georgiev et al. 2008). The green fluorescent protein (GFP) marker gene can also be used for the confirmation (Merritt et al. 1999; Georgiev et al. 2008). Western blotting can also be used to prove successful transformation by confirming the integration and expression of desired gene (Tepfer et al. 1998; Vuković et al. 2013). During three decades, the transgenic hairy root of tobacco (*N. tabacum*) was utilized for a number of valuable purposes, for example, production of desired biologically active secondary metabolic substances, regeneration of transgenic plant, and medical molecular pharming, i.e., production of antibodies, enzymes, etc. In this chapter, we are only dealing with altered production of secondary metabolites upon gene manipulation in hairy root of tobacco (*N. tabacum*) which has been done through different approaches and will be presented further in the chapter as case studies.

Case Study 1: Increased Production of Cadaverine and Anabasine Alkaloids in Hairy Roots of *N. tabacum* by Introducing Bacterial Lysine Decarboxylase (*ldc*) Gene (Fecker et al. 1993)

In this study, researchers have introduced two direct repeats of a bacterial lysine decarboxylase (*ldc*) gene under the control of cauliflower mosaic virus (CaMV) 35S promoter in several hairy root cultures of *N. tabacum* varieties and produced several transgenic lines; some possess higher lysine decarboxylase (LDC) enzyme activity, while others have lower LDC activity (Fecker et al. 1993). The agrobacterial strain used for the transformation is C58C1 pRiA4b (for details, see Jouanin et al. 1986). The gene *ldc* encodes for LDC enzyme responsible for conversion of lysine to cadaverine which finally leads to the formation of anabasine (Fecker et al. 1993) either via diamine oxidase (DAO) enzyme-involved reaction step (in *N. glauca*) (Fecker et al. 1993) or via *N*-methylputrescine oxidase (MPO) enzyme (Watson et al. 1990). *N. tabacum* varieties normally contain low anabasine (nicotine/anabasine ratio ca. 50:1), but the transgenic lines of their hairy root (*ldc*-transformed hairy roots of *N. tabacum* varieties) showed enhanced production of cadaverine as well as anabasine but reduction in nicotine content. They explained that the reduction of nicotine content may be due to competition of cadaverine with *N*-methylputrescine for the enzyme MPO as evident from Watson et al. findings (Watson et al. 1990). They further advocated that cadaverine-derived precursor which competes with putrescine-derived precursor for nicotinic acid also resulted in reduced nicotine production. The transgenic lines with higher LDC activity showed increment in cadaverine level from ca. 50 µg (control culture) to about 700 µg/g dry mass, and some of the produced cadaverine was used for the synthesis of anabasine, as represented by a threefold increase of this alkaloid (Fecker et al. 1993). Transgenic lines with lower LDC activity have cadaverine, and anabasine amounts were comparatively lesser and sometimes hardly different from control culture (Fecker et al. 1993). They also did lysine feeding experiment and found that lines even with low LDH activity showed increased cadaverine and anabasine levels (Fecker et al. 1993). Thus the production of two alkaloids (cadaverine and anabasine) was increased by expressing bacterial *ldc* gene using tobacco hairy root as experimental system. This study further suggested that LDC protein targeted to the compartment of lysine biosynthesis will be important for better substrate supply and improve biosynthesis of both the alkaloids because lysine biosynthesis can be assumed to happen in the plastid of hairy roots. As earlier studies on transgenic potato and transgenic tobacco plant expressing bacterial dihydrodipicolinate synthase indicated biosynthesis of lysine in the chloroplast but not in cytoplasm (Shaul and Galili 1991; Perl et al. 1992), they argue that in hairy root cells, lysine biosynthesis also functioned in plastid of nongreen tissue (Fecker et al. 1993).

Case Study 2: Increase Level of Anatabine Alkaloid Production in Hairy Roots of *N. tabacum* by Downregulating *PMT* Gene Using Antisense Approach (Chintapakorn and Hamill 2003)

Nicotiana species are known for biosynthesis of pyridine alkaloids (Dawson 1962), with either nicotine or its derivatives nornicotine as dominant component in most cases while anabasine as major alkaloids in some species (Saitoh et al. 1985; Sisson and Severson 1990; Chintapakorn and Hamill 2003). Anatabine is present as a minor constituent of alkaloid fractions in all the *Nicotiana* species analyzed in detail including *N. tabacum* (Saitoh et al. 1985; Hamill et al. 1986; Parr and Hamill 1987; Sisson and Severson 1990; Chintapakorn and Hamill 2003). In *N. tabacum*, the synthesis of nicotine occurs exclusively in roots (Dawson 1941, 1942a, 1962; Baldwin 1989; Chintapakorn and Hamill 2003), and PMT is the key enzymes of the biosynthesis pathway (Wagner et al. 1986c; Feth et al. 1986; Feth and Wagner 1989; Chintapakorn and Hamill 2003). PMT activity ensures the adequate supply of 1-methyl-Δ^1-pyrrolinium cation required for nicotine biosynthesis (Chintapakorn and Hamill 2003). Another substrate required for nicotine biosynthesis is nicotinic acid which is synthesized as a part of pyridine nucleotide cycle (Dawson 1962; Mann and Byerrum 1974; Leete 1983; Wagner et al. 1986a, b; Chintapakorn and Hamill 2003). QPT is the key enzyme for nicotinic acid formation (Wagner and Wagner 1985; Wagner et al. 1986a, b; Sinclair et al. 2000; Chintapakorn and Hamill 2003). Anatabine biosynthesis does not need PMT enzyme but requires QPT as anatabine biosynthesis is thought to proceed via nicotinic acid (Leete and Slattery 1976; Leete 1979; Chintapakorn and Hamill 2003). It can be assumed that downregulation of *PMT* gene might resulted to reduction in nicotine synthesis and increment in anatabine production as higher concentration of nicotinic acid available to be utilized for the biosynthesis of anatabine (Fig. 8.1).

Antisense-mediated approach has been used here to downregulate the activity of PMT in hairy root of *N. tabacum* to visualize its effect on alkaloid profile (Chintapakorn and Hamill 2003). Transformed plant lines were also established by regeneration from transformed hairy roots and analyzed for *PMT* downregulation and its effect on alkaloid biosynthesis. The antisense-*PMT*-transgenic hairy root culture was established using *A. rhizogenes* strain LBA 9402 harboring pYC3JR plasmid. This plasmid was generated using *PMT* coding sequence (Hibi et al. 1994) and binary vector pFIH10 (Hamill et al. 1987) by Chintapakorn (2002), in which *PMT* coding sequence is in antisense orientation under control of CaMV 35S promoter containing duplicated upstream sequence (Kay et al. 1987). The control transformed root lines were generated using either *A. rhizogenes* strain LBA 9402 alone or having pBI121 (CaMV 35S-GUS; Jefferson et al. 1987). The produced transgenic hairy roots showed reduced PMT activity along with a decrement in nicotine content relative to controls (Chintapakorn and Hamill 2003). The antisense-*PMT*-transformed hairy roots and leaf tissues of regenerated transgenic plant showed a significant increment in anatabine content compared to controls
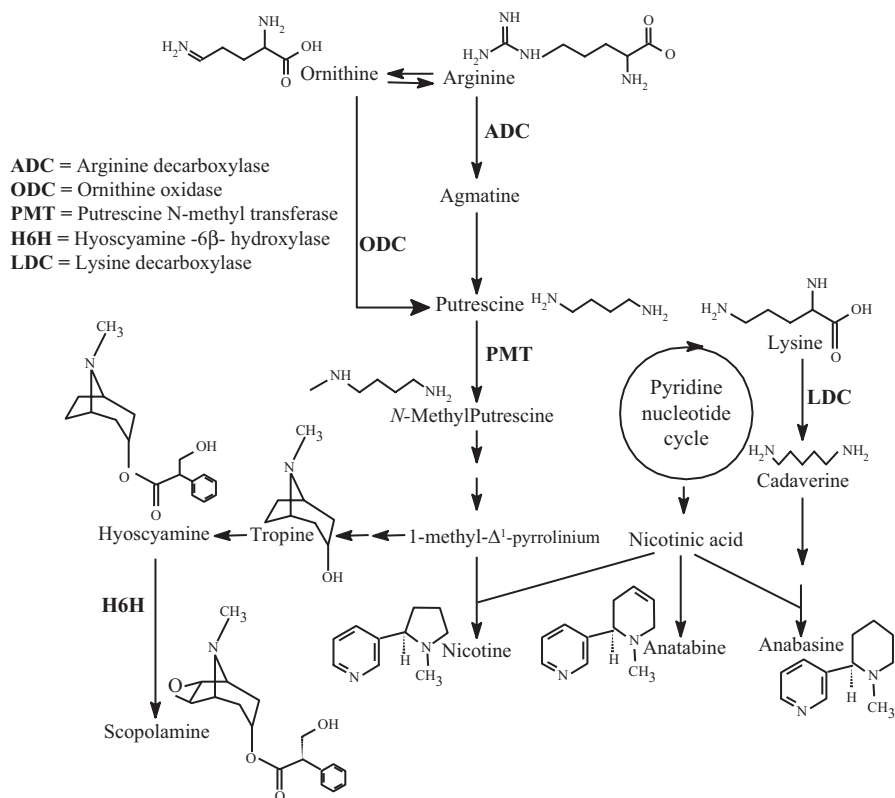
Fig. 8.1 Schematic diagram of pyridine and tropane alkaloid pathway (adopted from Chintapakorn and Hamill 2003, note: one arrow may represent more than one reaction)

(Chintapakorn and Hamill 2003). There has been little or no effect on transcript levels of other genes encoding enzymes involved in alkaloid metabolism, including quinolinic acid phosphoribosyltransferase (QPT) upon antisense-*PMT* manipulation as indicated by *Northern* hybridization experiment (Chintapakorn and Hamill 2003). QPT performs a key role in regulation of nicotinic acid biosynthesis which is necessary for both nicotine and anatabine generations. Chintapakorn and Hamill (2003) suggested that enhanced levels of anatabine in the transgenic lines were the results of relative oversupply of nicotinic acid which was used directly to synthesize anatabine due to lack of adequate levels of 1-methyl-Δ^1-pyrrolinium cation (ultimate product of PMT activity). Furthermore, accumulation of nicotinic acid in vivo is toxic to *Nicotiana* root (Robins et al. 1987); possibly, conversion of excess nicotinic acid to anatabine is a detoxification mechanism that helps to maintain normal growth patterns even if synthesis of the 1-methyl-Δ^1-pyrrolinium cation does not remain in balance with nicotinic acid synthesis. For supporting this suggestion, they presented a study on *N. rustica* and *N. alata*, where high production of anatabine was observed, which was ensued from millimolar inoculation of nicotinic acid in

medium of the cultures (Robins et al. 1987; Friesen et al. 1992). Also, inhibition of *N*-methyl-pyrrolinium in *N. rustica* root cultures by adding 1 mM *N*-propyl putrescine resulted in reduced nicotine content along with enhanced anatabine content (Boswell et al. 1999).

Case Study 3: Enhanced Secretion of Tropane Alkaloids in Hairy Roots of *N. tabacum* by Overexpressing *h6h* Gene from *H. niger* with Exogenous Supply of Hyoscyamine into the Medium (Häkkinen et al. 2005)

Tropane alkaloids are pharmaceutically important because of their anticholinergic properties (Mateus et al. 1999; Häkkinen et al. 2005). These alkaloids include mainly hyoscyamine and scopolamine; the latter is more valuable and is preferred for its higher physiological activity and fewer side effects (Häkkinen et al. 2005). Scopolamine is synthesized from hyoscyamine in a two-step process catalyzed by hyoscyamine-6-hydroxylase (H6H). In the first step, hyoscyamine is hydroxylated to 6-hydroxyhyoscyamine and then converted to scopolamine in the second step through epoxidation (Hashimoto and Yamada 1986; Häkkinen et al. 2005). This enzyme is localized in the pericycle of the root, which is the site for biosynthesis and accumulation of tropane alkaloids (Hashimoto and Yamada 1986; Matsuda et al. 1991; Häkkinen et al. 2005). Further, Hashimoto et al. (1993) showed that *h6h* gene overexpression in hairy roots of hyoscyamine-producing plants led to enhanced scopolamine/hyoscyamine ratio (Hashimoto et al. 1993; Häkkinen et al. 2005). *Atropa belladonna* showed almost complete conversion of hyoscyamine to scopolamine upon expression of *h6h* gene from *Hyoscyamus niger* (Yun et al. 1992; Häkkinen et al. 2005). Later, Jouhikainen et al. (1999) reported that overexpression of *h6h* gene in *H. muticus* hairy root resulted to increment in scopolamine content while the hyoscyamine content remain unchanged (Jouhikainen et al. 1999; Häkkinen et al. 2005). In 2004 another report on overexpression of genes was published where researchers overexpressed *pmt* and *h6h* genes in *H. niger* hairy root and found increased scopolamine production (Zhang et al. 2004; Häkkinen et al. 2005). Hain et al. (1990) expressed a foreign gene code for stilbene synthase (isolated from groundnut) in *N. tabacum* that resulted in the synthesis of resveratrol (Hain et al. 1990; Häkkinen et al. 2005). Rocha et al. (2002) expressed *h6h* and tropinone reductase (*trI*) genes (isolated from *H. niger*) together in *N. tabacum* plant followed by hyoscyamine and tropinone feeding to its detached leaves. They observed elevation (3–13-fold) in nicotine content along with the direct product of these enzymes (Rocha et al. 2002). Additionally, the nicotine and tropane alkaloids are synthesized in roots, so it is better to study the effect of exogenously applied substrate along with related gene expression in the hairy root culture system, which leads to generation of this study (Häkkinen et al. 2005).

In this work, they used *N. tabacum* hairy roots to assess bioconversion of exogenously applied hyoscyamine to scopolamine by overexpressing *h6h* gene from *H. niger* and compared it with *h6h* overexpressing *H. muticus* hairy roots (naturally synthesize hyoscyamine) along with overall alkaloid accumulation in both hairy roots that were examined (Häkkinen et al. 2005). Transgenic hairy roots were generated using *A. rhizogenes* LBA9402 pLAL21 carrying the *35S-h6h* and the *nptII* gene as a marker, constructed by Jouhikainen et al. (1999) and Häkkinen et al. (2005). They reported that the transgenic *N. tabacum* hairy root carrying *35S-h6h* transgene showed more efficient uptake of hyoscyamine from culture medium with higher rate of bioconversion to scopolamine in comparison to *h6h*-overexpressing *H. muticus* hairy roots. Also, scopolamine secretion into the culture medium was found to be very efficient (85% of the total scopolamine). In addition, nicotine alkaloid production was also enhanced.

Case Study 4: Minor Alteration in Alkaloid Profile of Tobacco Hairy Root upon Antisense-Mediated Reduction in Arginine Decarboxylase (ADC) Activity (Chintapakorn and Hamill 2007)

Putrescine is an important intermediate in nicotine biosynthesis (Mizusaki et al. 1971) which is known to provide pyrrolidine ring of the nicotine (Saunders and Bush 1979). Putrescine can be synthesized from amino acid ornithine and/or arginine, catalyzed by ornithine decarboxylase (ODC) and/or arginine decarboxylase (ADC), respectively (Flores and Filner 1985). A number of reports suggested the importance of both ODC and ADC together for sufficient supply of putrescine for nicotine biosynthesis, and both were upregulated in response to chemical signals arising from damaged shoots to produce enhanced nicotine (Hibi et al. 1994; Baldwin et al. 1994, 1997; Zhang and Baldwin 1997; Chintapakorn and Hamill 2007). Although, earlier study suggested ADC-mediated route to putrescine is operated during nicotine biosynthesis in non-wounded plants (Yoshida and Mitake 1966), it remains unclear whether the ADC-mediated route to putrescine is preferentially operated for nicotine synthesis under non-stressful condition in hairy root or plants. To address this question, Chintapakorn and Hamill (2007) conducted the study on ADC-antisense-transformed hairy root of tobacco with the aim to analyze changes in alkaloid profile. They also performed the analysis of alkaloid profile in ADC-antisense-transformed hairy root-regenerated transgenic tobacco plant (Chintapakorn and Hamill 2007). They established the transgenic ADC-antisense hairy root line by applying similar approach as performed by Chintapakorn and Hamill (2003). For controls, hairy roots were generated using *A. rhizogenes* LBA 9402 only or *A. rhizogenes* LBA 9402 consisting pBI 121 (Jefferson et al. 1987). Significantly reduced level of ADC transcript and ADC activity was observed in transgenic hairy root, while other genes including ODC remain unaltered upon

reduced ADC expression (Chintapakorn and Hamill 2007). Nicotine concentration was found to be comparable in antisense-ADC and control hairy roots throughout most of their respective culture cycle except at the latter stage of growth (Chintapakorn and Hamill 2007). Nicotine content was observed about 20% lower in antisense-ADC-transformed hairy root compared to control at the latter stage of growth (Chintapakorn and Hamill 2007). The contents of anatabine which is not derived from putrescine were slightly raised in antisense-ADC-transformed hairy roots. Chintapakorn and Hamill (2007) concluded from this study on antisense-ADC-transformed hairy root that putrescine plays a role but not of prime importance for nicotine biosynthesis in hairy root of tobacco.

Case Study 5: Enhanced Secretion of Nicotine in Cultured Medium Through Reducing the Expression of Nicotine Uptake Permease (*NUP1*) Gene in Hairy Root of *N. tabacum* (Hildreth et al. 2011; Zhao et al. 2013)

N. tabacum produces nicotine as major alkaloid (Saitoh et al. 1985; Hildreth et al. 2011; Zhao et al. 2013) which is synthesized in root specifically root tips (Dawson 1942a, b; Dawson and Solt 1959; Solt 1957; Hildreth et al. 2011; Zhao et al. 2013). Also, growth of roots is an absolute requirement for net nicotine production (Baldwin 1988; Dawson 1942b; Zhao et al. 2013). Nicotine is a known natural insecticide (Kircher and Lieberman 1967; Richardson and Busbey 1937; Smith and Goodhue 1943; Zhao et al. 2013) and potent anti-inflammatory agent (Isman 2006; Mabley et al. 2011; Zhao et al. 2013). NUP1 is a plasma membrane-localized nicotine-specific uptake transporter, mostly expressed in root tips, where nicotine biosynthesis is also localized (Zhao et al. 2013).

The study reporting enhanced accumulation of nicotine in culture medium by reducing the expression of *NUP1* gene in *N. tabacum* hairy root was performed by two groups of researchers. The first group is Hildreth et al.; they performed the study in 2011, and the other group is Zhao et al.; they carried out the study in 2013.

Hildreth et al. (2011) apart from *NUP1-RNAi*-transformed tobacco plant (generated by *A. tumefaciens*-based transformation using RNAi vector pHANNIBAL having 448 bp PCR fragment derived from *NUP1*) also worked with *NUP1-RNAi*-transformed hairy root of tobacco which is generated by *A. rhizogenes* carrying similar RNAi vector as in the case of *NUP1-RNAi*-transformed plant (Hildreth et al. 2011). They advocated that NUP1 affect nicotine alkaloid metabolism and localization and root growth in tobacco (Hildreth et al. 2011). They observed that reduction in NUP1 mRNA in *NUP1-RNAi*-transformed hairy roots of *N. tabacum* resulted in reduced total nicotine levels and enhanced accumulation of nicotine in culture medium (Hildreth et al. 2011).

The usefulness of hairy root cultures to produce phytochemicals depends on both the yields of desired phytochemicals and the cost associated with the bioprocessing to isolate the desired chemicals (Zhao et al. 2013). Phytochemical accumulated in

the culture media needs less effort in bioprocessing and so less cost compared to phytochemicals accumulated in tissues (Zhao et al. 2013). Keeping this in mind, Zhao et al. (2013) performed the study using a genetic-oriented approach, i.e., *NUP1-RNAi* transformation approach (this approached was used previously by Hildreth et al. (2011)), associated with environmental interaction (aeration level and media pH) to maximize nicotine yield in the culture media. They created a new wild-type (Xanthi-105) and a new T3 generation homozygous *NUP1*-reduced expression (T13-8-101) (Hildreth et al. 2011) hairy root line of *N. tabacum* by inoculating sterile leaves, respectively, with *A. rhizogenes* ATCC15384 (Zhao et al. 2013). This study assessed the levels of nicotine release in the media by wild-type and *NUP1*-reduced expression hairy roots upon various treatments including different aeration conditions and pH change to find culture conditions that raised nicotine accumulation in culture media (Zhao et al. 2013). This study revealed that the NUP1-reduced expression line grows faster and utilizes less oxygen and more importantly releases more nicotine in media (Zhao et al. 2013). Media basification which was found to be associated with hairy root growth led to reduction of nicotine in media, but condition was reversed (i.e., increment of nicotine levels in media) when media pH was decreased (Zhao et al. 2013). Furthermore, kinetic study revealed that stimulation in branching of tobacco hairy root significantly improved nicotine accumulation in media (Zhao et al. 2013).

Case Study 6: Production of Geraniol (The First Committed Step in Terpenoid Indole Alkaloid Biosynthesis Pathway) in Hairy Root of *N. tabacum* by Expressing Geraniol Synthase Gene Isolated from *Valeriana officinalis* L. (Ritala et al. 2014)

Terpenoid indole alkaloids (TIAs) are one of the major alkaloids isolated from plants. The first committed step of TIA biosynthesis pathway is geraniol formation (Simkin et al. 2013; Ritala et al. 2014). Geraniol is an acyclic monoterpene alcohol synthesized in a single step from geranyl diphosphate (GPP), catalyzed by enzyme geraniol synthase (Iijima et al. 2004; Yang et al. 2005; Ritala et al. 2014). Geraniol is a valuable compound for pharmaceutical, fragrance, and cosmetic industries (Carnesecchi et al. 2001; Chen et al. 2006; Chen and Viljoen 2010). Heterologous geraniol synthesis has been achieved previously by expressing *VoGES* gene (geraniol synthase gene isolated from *Valeriana officinalis* L.) in tobacco (*N. tabacum* L. Samsun NN) (Dong et al. 2013). Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) analyses of leaf extracts have shown formation of free geraniol in the plant's aerial parts. However LC-MS study revealed conversion of geraniol to conjugated glycosides by endogenous tobacco enzymes (Dong et al. 2013). Hairy roots are known to contain plastids and also synthesized plastidial isoprenoids including monoterpenes (Lourenco et al. 1999) and diterpenes (Zhi and Alfermann 1993). As the synthesis of geraniol occurs in plastids/chloroplast, it is interesting to see how tobacco hairy roots would perform. Taking this in

account, Ritala et al. (2014) carried out the study on hairy root of *N. tabacum* expressing *VoGES* gene to produce geraniol and its derivatives. They also studied a range of scale-up system in order to evaluate tobacco hairy root as platform for geraniol production for commercial application.

They established *VoGES*-transformed hairy roots of *N. tabacum* (cv. Petit Havana SR1) which is initiated by infecting the sterile leaves with *A. rhizogenes* strain LBA9402 harboring pBIN2.4VoGES1 vector (Ritala et al. 2014). This vector had a geraniol synthase gene isolated from *V. officinalis* augmented with an artificial plastid targeting signal under the control of CaMV 35S promoter. Wild-type hairy roots (WT) were generated by *A. rhizogenes* LBA9402 (wild strain) and control hairy root clones by transformation with *A. rhizogenes* LBA9402 containing empty vector (BIN) (Ritala et al. 2014). They observed transgenic-*VoGES*-hairy roots accumulated geraniol, while WT and control were devoid of this (Ritala et al. 2014). The GC-MS analysis presented that the accumulation of free geraniol in 20 *VoGES*-expressing hairy root clones was an average 13.7 µg/g DW (dry weight) and maximum 31.3 µg/g DW (Ritala et al. 2014). More detail analysis revealed that geraniol derivative was present in six major glycoside forms which resulted to total geraniol contents up to 204.3 µg/g DW following deglycosylation (Ritala et al. 2014). Moreover, accumulation of tobacco alkaloids such as nicotine, nornicotine, anabasine, and antalline was not significantly different between transgenic and control hairy roots, suggesting *VoGES* overexpression did not affect other alkaloid pathways (Ritala et al. 2014). This was well supported by fluxomic studies done previously which showed that basic metabolism of tobacco hairy root was resistant to perturbation (Masakapalli et al. 2014).

Case Study 7: Expressing an Elicitor Gene (*Cryptogein* Gene) in Hairy Root of *N. tabacum*

Elicitor treatment is known for enhancement of a particular or range of phytochemicals in plant and hairy roots, so if successful expression of gene producing a particular elicitor can be done, it might produce same results as exogenous application of elicitor. Cryptogein is an established proteinaceous elicitor secreted by phytopathogenic fungus *Phytophthora cryptogea* (Milat et al. 1991). Exogenous application of cryptogein on tobacco (*N. tabacum*) resulted in capsidiol production and enhanced accumulation of many phenylpropanoids (Milat et al. 1991; Amelot et al. 2011, 2012). Cryptogein was also expressed internally in various plant species and hairy roots and produced alteration in secondary metabolite pathway with enhancement of important phytochemicals (Chaudhuri et al. 2009; Majumdar et al. 2012; Vuković et al. 2013; Sil et al. 2014; Kumar et al. 2016). Endogenous expression of cryptogein in tobacco hairy roots and its effect on secondary metabolism are going on in author's laboratory, and initial results showed overall increment in total phenolic compounds (author's unpublished data). These THRCs were generated through
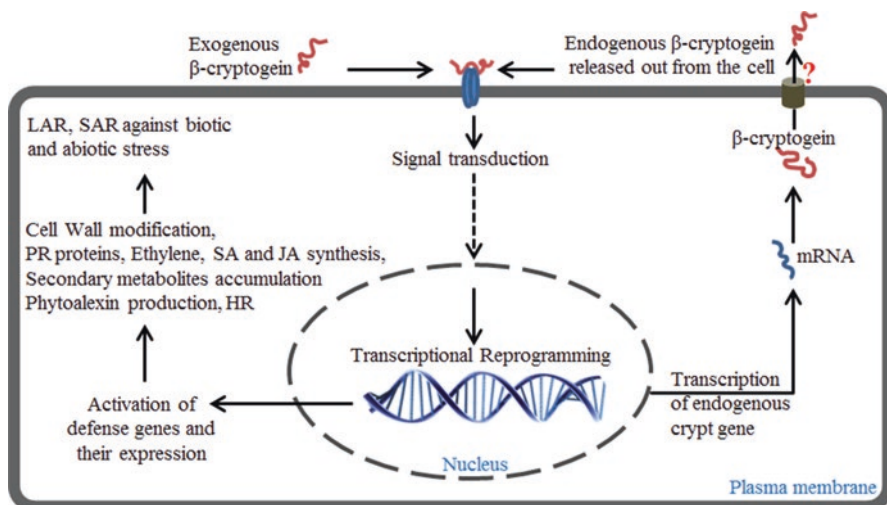
Fig. 8.2 Diagrammatic representation of β-cryptogein action when expressed endogenously in a plant cell

infection of in vitro grown tobacco plant with *A. rhizogenes* strain LBA9402-cryptogein containing pBIN19 vector harboring synthetic β-cryptogein under the control of constitutive promoter CaMV 35S (Kumar et al. 2016). The regenerated plantlets from these transgenic hairy roots have shown increment in chlorogenic acid (soluble phenolic compound) and various other wall-bound phenolic compounds with upregulation of gene involved in phenylpropanoid pathway (Kumar et al. 2016). The mechanism of β-cryptogein action has been shown diagrammatically in Fig. 8.2 based on a number of studies carried out in the field of β-cryptogein-plant interaction.

Conclusion and Future Perspectives

Tobacco is mainly known for production of alkaloid group of secondary metabolite especially nicotine and other minor alkaloids such as nornicotine, anabasine, anatabine, and cadaverine. Case studies discussed in this chapter prove that tobacco hairy root cultures are used for gene manipulation in alkaloid metabolism. In addition, geraniol (naturally absent in tobacco) has been successfully produced by them through genetic manipulation and they can serve as platform for its commercial production. Furthermore, expression of *cryptogein* gene in hairy root of tobacco showed promising initial results toward accumulation of phenolics. These *crypt*-transformed hairy roots can be utilized to enhance production of secondary metabolites; however, in-depth study is required.

Tuan et al. (2014) reported that overexpression of *Arabidopsis thaliana* transcription factor *AtPAP1* has enhanced chlorogenic acid content in hairy roots of *Platycodon grandiflorum* (natural producer of chlorogenic acid) (Tuan et al. 2014). Previously, enhancement of rutin was observed in *Fagopyrum esculentum* hairy root upon expression of *AtMYB12* (an *A. thaliana* transcription factor) (Park et al. 2012). So it is interesting to see if these transcription factors could work in tobacco hairy root for elevated production of chlorogenic acid and rutin for commercial utilization as chlorogenic acid and rutin are phenolic compounds of great value as antioxidants. In addition, all these transgenic tobacco HRCs can be tested for better growth in bioreactor level so as to utilize these roots for commercial production of valuable phytochemicals.

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Chapter 9

Synthesis of Benzylisoquinoline Alkaloids and Other Tyrosine-Derived Metabolites in Hairy Root Cultures

Felipe Vázquez-Flota, María de Lourdes Miranda-Ham, Lizbeth Castro-Concha, and Yahaira Tamayo-Ordoñez

Abstract Tyrosine is the starting unit of a wide range of specialized metabolites in plants, including the pharmaceutically important benzylisoquinoline alkaloids (BIAs). Although in vitro cell suspension cultures from some BIA-producing species have been obtained, there are only a few documented cases for the establishment of hairy root cultures, even though this system has been intensively used in other alkaloid-producing plants. Hairy root cultures have been generated from three members of *Papaver* genus, including *P. somniferum*, *P. orientale*, and *P. bracteatum*. Morphine- and morphinan-type alkaloids have been identified in all of them, suggesting that the biosynthetic capacity is maintained in all cases. Sanguinarine and other benzophenanthridine-type alkaloids were also identified in these cultures, as well as in hairy roots from *Eschscholzia californica* (Papaveraceae). Only one report of berberine-producing hairy root was found in an extensive literature search, although attempts on probable transformation of recalcitrant species were detected. The use of *Beta vulgaris* hairy root cultures for the synthesis of betalains, pigments derived from tyrosine, was also included.

Keywords Alkaloids • Betalains • Hairy roots • Papaveraceae • Tyrosine

Introduction

Alkaloids are low-molecular-weight, nitrogenous cyclic compounds with a limited taxonomical distribution. Nitrogen in alkaloids is in an oxidized state, and most of the time, it comes directly from amino acids (Roberts and Wink 1998). Although also found in bacteria, fungi, and some animals, plants represent the main source for alkaloids. Alkaloids display toxic effects and belong to the chemical weaponry

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against pathogens and herbivores. Mechanisms for alkaloid toxicity frequently involve interference of cell receptors or critical cell events, such as division. Therefore, relatively low doses are required to achieve maximum effects. Low alkaloid concentrations in plant tissues are a reflection of the strict regulatory mechanisms controlling their synthesis, both in time and space, as a strategy to avoid damages to tissues where they are produced. However, the precise location of alkaloids in specific cell types allows them to reach the concentrations required to exert their effects (Beaudoin and Facchini 2014).

Alkaloids, and plants bearing them, have been historically used as remedies, stimulants, and poisons. In fact, the use of opium poppy (*Papaver somniferum*) for its known morphine-based properties as narcotic and painkiller is among the oldest documented case for the utilization of medicinal plants. Although there are some claims about opium usage during the Neolithic Age in Eastern Europe, solid historical evidences support its consumption by Sumerians, more than 6000 years ago (Wink 1998a). Today, alkaloids are used in several pharmaceutical formulations. Some examples include the cytotoxic agents' vinblastine and vincristine from the Madagascar periwinkle (*Catharanthus roseus*), the anticholinergic tropane alkaloids from *Atropa belladonna* and other solanaceous plants, and the bronchodilator ephedrine from *Ephedra*, among many others (Schmeller and Wink 1998).

Alkaloids comprise a diverse group of secondary metabolites. Around 30,000 of them have been already described; most are derived from a few amino acids, such as arginine, ornithine, lysine, tryptophan, and tyrosine. Tyrosine gives origin to the benzylisoquinoline alkaloids (BIAs), which represent one of the most diverse groups. Over 2500 BIAs, distributed among plants from the superorders Magnoliflorae and Ranunculiflorae, have been described. Many of these alkaloids have pharmaceutical importance; hence, plants producing them have been the subject of intense research and manipulation toward yield improvement. In vitro culture systems have been very useful as a platform to conduct fundamental studies, as well as to implement commercial production strategies.

In here, we present selected cases of the use of root cultures from different plant species producing BIAs of pharmaceutical importance. Conditions for the establishment of cultures, including genetic transformation with *Agrobacterium rhizogenes*, are discussed, as well as the strategies used to increase alkaloid production, such as modification of media composition and culture settings, application of elicitors of secondary metabolism, and genetic manipulation of the biosynthetic pathways. Targets were focused on morphine, widely used for treating chronic pain; berberine, currently used as an insulin sensitizer in type 2 diabetes patients; and sanguinarine, with antimicrobial and antiretroviral properties (Rubio-Piña and Vázquez-Flota 2013).

An Overview of the BIA Biosynthetic Pathway

BIA is on the most diverse class of alkaloids. They are formed from tyrosine and can be grouped in different families, depending on described internal molecular arrangements. Three of these families are morphinan-, protoberberine-, and

benzophenanthridine-type alkaloids, which include several pharmaceutically important compounds (Liscombe et al. 2005). The synthesis of all BIAs shares a set of common reactions and intermediaries, diverging in specific metabolic branches that lead to the formation of each one of them, at some critical points. The different BIAs families show a taxonomically associated distribution to a few related families or genera within the same family (Liscombe et al. 2005).

Synthesis of BIAs starts with two units of tyrosine: one of them is deaminated by the action of aromatic monoamine oxidase (MAO), whereas the other is hydroxylated and decarboxylated, involving the participation of a tyrosine hydroxylase and tyrosine/DOPA decarboxylase (TyDC). The resulting products: 4-hydroxyphenylacetaldehyde (4-HPA) and L-3,4-dihydroxyphenylethylamine (dopamine), respectively, are condensed forming *S*-norcoclaurine by norcoclaurine synthase (NCS). After two *O*- and one *N*-methylation, this trihydroxylated intermediary is converted to *S*-reticuline (Fig. 9.1). Reticuline is the last common intermediary in the synthesis of the different BIAs families, representing a major branching point in the synthesis of either the morphinan- or the protoberberine/benzophenanthridine-type alkaloids (Fig. 9.1; Hagel and Facchini 2013).

The committed synthesis of protoberberines and benzophenanthridines starts with the formation of an intramolecular carbon-carbon bond between the *N*-methyl group and the phenolic ring of *S*-reticuline, carried out by the berberine bridge enzyme (BBE). This reaction results in *S*-scoulerine, which represents the last common intermediary in the synthesis of both berberine (protoberberines) and sanguinarine (benzophenanthridines). This compound is either used by the *S*-scoulerine *O*-methyltransferase (SOMT) for berberine synthesis or by *S*-cheilanthifoline synthase (CheSyn) for sanguinarine synthesis (Fig. 9.1; Hagel and Facchini 2013). The formation of a 2–3 methyldioxy bridge (O-CH$_2$-O), catalyzed by tetrahydroprotoberberine oxidase (STOX), is the last step in berberine biosynthesis (Fig. 9.1), whereas the formation of an additional 9–10 methylendioxy bridge and further oxidation is required for sanguinarine synthesis (Fig. 9.1).

Morphine biosynthesis is restricted to a few species within in the *Papaver* genus, mainly *P. somniferum*. It involves the transformation of *S*-reticuline to salutaridinol in four enzymatic steps: one oxidation and three NADPH-dependent reductions (Fig. 9.1). Salutaridinol is then *O*-acetylated by salutaridinol 7-*O*-acetyltransferase (SalAT), and the resulting product spontaneously cyclizes into thebaine, which is demethylated to codeinone by thebaine demethylase (T6ODM). Reduction of thebaine by codeinone reductase (COR) yields codeine, which after demethylation by codeine demethylase (CODM) produces morphine (Fig. 9.1; Beaudoin and Facchini 2014). An alternative minor route, initiating in thebaine and bypassing codeine formation, has also been described in *P. somniferum* (Beaudoin and Facchini 2014).

As mentioned, these pathways are lengthy and frequently function as metabolic lattices rather than linearly. Moreover, the whole process is subjected to complex regulatory mechanisms, restricting alkaloid synthesis to well-defined conditions and with enzymes displaying specific spatial distribution in tissues, cell types, and subcellular organelles (Beaudoin and Facchini 2014). In addition, tyrosine is one of the scarcest amino acids and its synthesis is strictly controlled. In this way, genetic
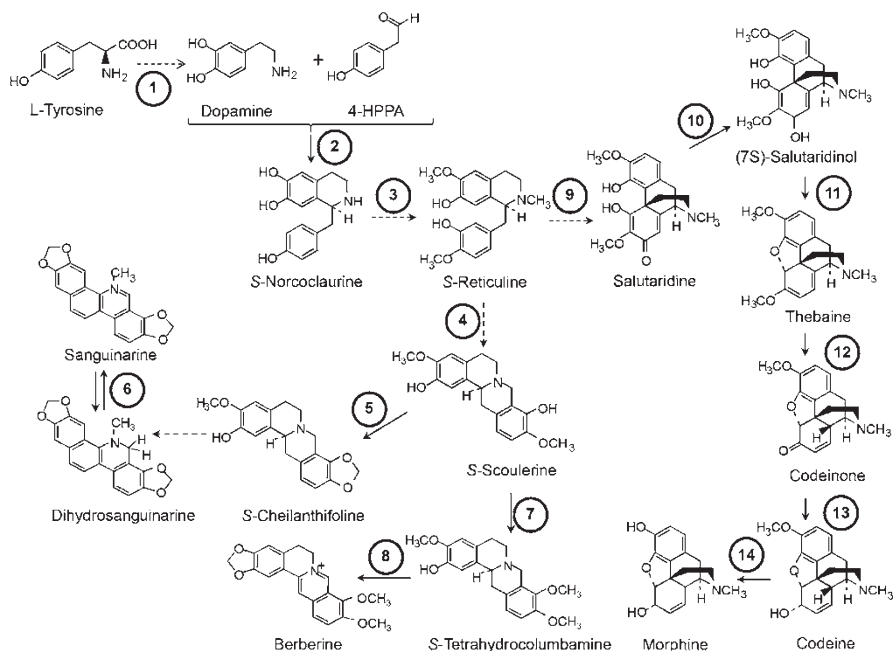
Fig. 9.1 A condensed view of the biosynthetic pathway of the main types of benzylisoquinoline alkaloids. *4-HPPA* 4-hydroxyphenylacetaldehyde, (1) *TyDC* tyrosine/DOPA decarboxylase, (2) *NCS* norcoclaurine synthase, (3) *4-OMT* 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase, (4) *BBE* berberine bridge enzyme, (5) *CheSyn* cheilanthifoline synthase, (6) *DBOX* dihydroxyben-zophenanthridine oxidase, (7) *SOMT* scoulerine *O*-methyltransferase, (8) *STOX* *S*-tetrahydroprotoberberine oxidase, (9) *SalSyn* salutaridine synthase, (10) *SalR* salutaridine reductase, (11) *SalAT* salutaridinol 7-*O*-acetyltransferase, (12) *T6ODM* thebaine 6-*O*-demethylase, (13) *COR* codeinone reductase, and (14) *CODM* codeine *O*-demethylase. Dashed lines represent multiple steps. Enzymes numbered in multistep conversions indicate their participation, among others. In reactions catalyzed by 11 and 12, spontaneous reactions are also involved

manipulation of BIAs biosynthesis at the whole plant level could be very complicated. However, in vitro cell and organ culture have provided useful tools for the identification and isolation of some of the enzymes involved and their corresponding genes.

Morphine-Type Alkaloids

Although some other plants from the Papaveraceae, Menispermaceae, and Berberidaceae families can produced the five-ring morphinan-type alkaloids, morphine and the closely related alkaloids codeine and thebaine are restricted to a few species within the *Papaver* genus, mainly *P. somniferum* (opium poppy; Liscombe et al. 2005). Noscapine and papaverine are other pharmaceutically important

morphinan-type alkaloids found in the poppy family. These alkaloids are commercially obtained from opium, the dry latex harvested from unripe capsules (fruits) corresponding to the cytoplasm of laticifer cells (Beaudoin and Facchini 2014).

Morphine acts by binding to the opioid receptors located in the central nervous system, occupying the sites for endorphin neurotransmitters, and inducing, in this way, a comfortable deep sleep, hallucinations, and analgesic effects. At higher doses, morphine can provoke respiratory failure caused by smooth muscle paralysis (Wink 1998b). As mentioned above, *P. somniferum* is the main source for morphine. Codeine is a minor opium alkaloid, which is obtained through the methylation of morphine, although it is preferred to prepare it from thebaine (see Fig. 9.1), which is abundant in *P. bracteatum* latex. Thebaine does not possess any medicinal, non-stimulatory effects, and cultivation of *P. bracteatum* does not have the legal constrains of *P. somniferum*. Codeine has a similar action mechanism to that of morphine and can also be used as painkiller, although its main use, in lower doses, is as a cough suppressant. Codeine displays affinity to receptors located at the cough center, in the medulla oblongata at the brain stem (Schmeller and Wink 1998). Noscapine is a phthalide isoquinoline alkaloid (also known as narcotine), which is also obtained from the opium poppy. It has been also used as a cough suppressant, with a similar action to codeine, but without any sedative or analgesic effects. However, recently it has been shown that noscapine displays good anticancer effects toward melanoma, gliomas, and breast, ovarian, colon, and lung cancer. Noscapine suppresses cell proliferation in tumoral cell lines by inhibiting protein kinases' activities of certain epidermal growth factor receptors (EGFR) (He et al. 2016). Papaverine is another alkaloid found in opium. It belongs to the benzylisoquinoline class and can also be found in the *Chelidonium* genus (Papaveraceae). This alkaloid has vasodilator and smooth muscle relaxant activities due to its inhibitory effects on phosphodiesterase activity. It is commonly used in the treatment of visceral and vascular spasms, and, until before the development of sildenafil and related drugs, it was also prescribed in the treatment of erectile dysfunction (Liu and Couldwell 2005).

Interest in *Papaver* root cultures rose since in vitro cell suspensions and callus cultures did not present morphine accumulation, even though they readily produced a number of other BIAs, namely, sanguinarine (Nessler 1990). Nevertheless, upon cell reorganization into structured organs, morphine accumulation could be detected again (Yoshimatsu and Shimonura 1992; Kassem and Jacquin 2001). Morphinan-type alkaloids and papaverine were detected in roots regenerated from non-transformed calli induced from hypocotyls (Laurain-Matter et al. 1999).

Hairy roots have been obtained from three species of the *Papaver* genus: *P. somniferum* (the opium poppy), *P. bracteatum* (Persian poppy), and *P. orientale* (Oriental poppy).

The first report of *P. somniferum* hairy roots described the use of *A. rhizogenes* strain R1000 on 5-day-old hypocotyls. Although these cultures were able to grow in the absence of growth regulators, addition of indole-3-acetic acid (1 mg L^{-1}) greatly improved their growth rate. *P. somniferum* hairy roots were highly branched, lacked geotropism, and, anatomically, only differed from normal plant roots by

presenting a less compact arrangement of epidermal cells and an excess of root hairs. This maintenance of the organ structure was relevant, since cell organization is critical for morphine synthesis. In fact, alkaloid profiles in the hairy roots were similar to those of non-transgenic root cultures, predominantly finding morphine and noscapine and other minor alkaloids in similar relative abundances (Park and Facchini 2000a). It should be mentioned that other *A. rhizogenes* strains (13333 and R1200rolD) also induced hairy roots, and, though cultures were established, no data on their alkaloid profiles were reported. Remarkably, strains 15834 and C58C1 were also able to induce root formation after their inoculation on hypocotyls, and calli formed after a few weeks in culture (Park and Facchini 2000a). *P. somniferum* hairy roots derived from hypocotyls infected with *A. rhizogenes* hypervirulent strain LBA 9402 showed an increase of 40% from total alkaloids found in roots from 3-month-old plantlets (Le Flem-Bonhomme et al. 2004). This was apparently due to higher codeine accumulation, since morphine remained around 0.25% dry weight (DW) in both untransformed and hairy roots, while codeine was almost fourfold higher (0.18% DW) in the hairy root line compared to the untransformed ones. However, morphine was recovered in the liquid medium in comparable amounts to those inside the root tissues (Le Flem-Bonhomme et al. 2004). This suggests that biosynthetic capacity for morphinan-type alkaloids can be naturally increased in hairy roots, although there are no reports of excretion of morphine and other morphinan-type alkaloids by roots under natural conditions. Other root cultures were also obtained using *A. rhizogenes* strain 15834 and even *A. tumefaciens* GV 31010 (pMP90RK). Nevertheless, though all those lines produced alkaloids, the precise alkaloid blend recovered was not described (Le Flem-Bonhomme et al. 2004).

Hairy roots cultures have been also obtained from *P. bracteatum* (the Persian or Iranian poppy), which accumulate mainly thebaine. Since thebaine can be easily transformed in morphine (see Fig. 9.1), this plant was considered as a replacement for opium poppy in an attempt to reduce illegal trade (Seddigh et al. 1982). Hairy roots were obtained by infecting 7-day-old hypocotyls with *A. rhizogenes* strain R15834. As previously reported, these root lines required auxins (Naphthalene acetic acid, 1 mg L^{-1}) to induce a faster growth rate (Rostampour et al. 2009). Alkaloid profiles were comparable to those of untransformed roots cultures, showing the presence of noscapine and morphine, although thebaine contents were not reported (Rostampour et al. 2009). Interestingly, lower transformation efficiency in comparison to *P. somniferum* was observed (50% vs 90% for *P. bracteatum* and *P. somniferum*, respectively). It has been described that there is a general lower efficiency observed for perennial species, for Persian poppy is a perennial one, in comparison to annual herbaceous (Patel et al. 2013). Transformation could be improved as well as growth rate by modifying infection and culture conditions (Sharafi et al. 2013b). In fact, the use of acetosyringone, a phenolic compound released by injured cells in response to *Agrobacterium*, increased infection rates when explants from radicles, hypocotyls, and cotyledons were employed, regardless of the infective strain (A4, ATCC15834, LBA9402, MSU440, and A13). Moreover, when arginine was incorporated and NH_4NO_3 eliminated from the media during cocultivation, infection effi-

ciencies improved (Sharafi et al. 2013b). Interestingly, hypocotyls explants infected with the LBA9402 strain generated hairy root lines that showed the most vigorous growth (Sharafi et al. 2013b). Thebaine was the main morphinan-type alkaloid detected, in values around 1% DW, which was comparable to those in untransformed root cultures, with barely detectable amounts of codeine (Sharafi et al. 2013a).

The Oriental poppy (*P. orientale*) is also used as source for thebaine and oripavine (which is considered a starting compound for thebaine synthesis), but not for morphine or codeine. Hairy root cultures were obtained by infection of 28-day-old *P. orientale* hypocotyls with *A. rhizogenes* strain ATCC15834, which resulted to be the best combination among other tissues (cotyledons and shoots) and strains (C58C1, GM, and R1000) tested, followed by infection with C58C1 also on hypocotyls (Hashemi and Naghavi 2016). It is noteworthy to point out that alkaloid profile analysis revealed the presence of codeine and morphine in values around 0.08–0.1% DW, in addition to thebaine, which was slightly higher (ca. 1.2% DW; Hashemi and Naghavi 2016). This suggests that the transformation process by itself promoted the activation of the metabolic branch involved in the formation of these alkaloids from thebaine (Fig. 9.1).

Attempts to increase the contents of morphinan alkaloids in poppy plants through genetic engineering had been envisioned, since biosynthetic genes have been isolated (Allen et al. 2007; Larkin et al. 2007). In *P. bracteatum* hairy roots overexpressing a foreign *P. somniferum SalAT* gene (*PsSalAT*), which is involved in the formation of thebaine from salutaridinol (Fig. 9.1), increased thebaine accumulation by around 40%, in comparison to normal hairy roots. Interestingly, codeine and morphine, which are derived from thebaine, remained only slightly above detection limits (Sharafi et al. 2013b).

P. bracteatum hairy roots overexpressing *P. somniferum CodR* gene (*PsCodR*) were obtained (Sharafi et al. 2013b). As mentioned above, lack of codeine and morphine in *P. bracteatum* is due to the absence of codeinone reductase activity, encoded by *CodR* (Beaudoin and Facchini 2014; see Fig. 9.1). Interestingly, *PsCodR* transgenic hairy root lines constitutively expressed the foreign gene and accumulated low, but detectable amounts of codeine (around 0.1% DW) and up to 0.25% DW morphine, which was absent in the wild-type and normal hairy roots (Sharafi et al. 2013b). Such an increase in morphine accumulation coincided with diminished thebaine accumulation and was higher than values detected in Persian poppy capsules (Sharafi et al. 2013a, b).

Elicitors have been used to promote morphinan-type alkaloid accumulation in *Papaver* hairy roots. Exposure of *P. orientale* hairy roots to chemical inducers of secondary metabolism, such as salicylic acid (SA) and methyl jasmonate (MeJa), in doses of 100 μM each, increased in a time-dependent manner the accumulation of thebaine, codeine, and morphine, for up to 48 h (Hashemi and Naghavi 2016). MeJa induced a better response, since morphine reached a sixfold increase over the control, unexposed cultures after 48 h, whereas codeine almost tripled control values after 2 h of exposure. In roots exposed to SA, maximal morphine and codeine accumulations also occurred at 48 and 24 h of exposure, representing increases of three-

and twofold, respectively (Hashemi and Naghavi 2016). These increments in alkaloid accumulation were preceded by expression of late genes in morphinan-type alkaloid synthesis, such as salutaridine synthase (SalSyn) and salutaridine reductase (SalR), involved in salutaridinol formation; salutaridinol 7-*O*-acetyltransferase (SalAT), involved in thebaine formation; thebaine 6-demethylase (T6ODM), participating in codeine and morphinone formation; and codeine demethylase (CODM) and codeine reductase (COR), involved in morphine and codeine synthesis (Hashemi and Naghavi 2016; see Fig. 9.1).

Benzophenanthridine Alkaloids

Benzophenanthridine alkaloids (BPA) are four-ring compounds, formed by a benzene ring fused to a phenanthridine residue (see Fig. 9.1). This class of quaternary alkaloids is mainly distributed in the Caprifoliaceae, Fumariaceae, Meliaceae, Papaveraceae, and Rutaceae families. Sanguinarine and chelerythrine, two of the best studied BPA, display a wide variety of physiological activities (Rubio-Piña and Vázquez-Flota 2013). Sanguinarine has been included as an antiplaque agent in some mouthwashes and toothpastes and used as expectorant. However, given its toxic effects and linkage to leukoplakia, sanguinarine was declared unsafe by FDA, and hence, its use drastically reduced (Dvorak et al. 2006). However, recently it has been employed in food supplements for livestock (swine, poultry, and cattle) since it stimulates weight gain (Robbins et al. 2013). Moreover, at submicromolar doses (<1 μM), sanguinarine displays an effective antiproliferative activity, with no toxic side effects observed in hepatic tumoral cell lines (Dvorak et al. 2006). Interestingly, antiretroviral activity also has been described for this alkaloid (Rubio-Piña and Vázquez-Flota 2013), as well as antituberculosis effects (Dvorak et al. 2006). Chelerythrine has similar medicinal properties as sanguinarine (Dvorak et al. 2006). Cell targets for most BPA are similar, due to their similar structural features and chemical behavior. These include ionic interactions with both nucleophilic and anionic moieties of amino acid in polypeptide chains, intercalation in DNA, and inhibition of protein kinase C, as well of those Ca^{2+}-, AMPc-, and phospholipid-dependent protein kinases (Wang et al. 1997). Ionic interactions of BPA are rather unspecific and can bind to membrane proteins of different cell structures, causing their inactivation. BPA also can interfere with cytochrome-P450-dependent enzymes (Zdarilová et al. 2006).

P. somniferum hairy roots, derived from hypocotyls infected with *A. rhizogenes* R1000 (Park and Facchini 2000a) and LBA9402 (Le Flem-Bonhomme et al. 2004), accumulated sanguinarine in a similar relative abundance to non-transformed root cultures, as well as to *Eschscholzia californica* (California poppy) hairy roots (Park and Facchini 2000b; MacLeod and Facchini 2006). Interestingly, *E. californica* hairy roots also maintained their anatomical features, just as those derived from *P. somniferum* (Park and Facchini 2000b). Furthermore, LBA9402 hairy roots excreted sanguinarine to the medium, and this feature was markedly increased in hairy roots (0.3% DW) compared to the untransformed roots where it was only detected in trace

amounts (Le Flem-Bonhomme et al. 2004). This could have been a reflection of a defensive reaction, since BPA are induced in response to different environmental stresses, such as bacterial infection (Le Flem-Bonhomme et al. 2004).

A similar sanguinarine pattern accumulation was also recorded for *A. rhizogenes* R15834 *P. bracteatum* hypocotyl-derived hairy roots mentioned lines above (Rostampour et al. 2009), but if sanguinarine was excreted to the external medium, it was not mentioned.

Hairy roots of the African tree *Zanthoxylum zanthoxyloides* (syn. *Fagara zanthoxyloides*) (Rutaceae), an endangered species, have been obtained by infecting the central vein of leaves with *A. rhizogenes* strain LBA9402 (Etsè et al. 2014). The root bark of this tree is widely used by local population of West Africa as a remedy against different diseases, such as ascariasis, sickle cell anemia, and sore throats (Oni 1993). *Z. zanthoxyloides* roots produce chelerythrine, a BFA, and skimmianine, a furanoquinoline alkaloid (Adeniyi and Odumosu 2008). Up to nine hairy root lines were obtained and all of them accumulated chelerythrine and skimmianine. Chelerythrine was found in the 1% DW rank, whereas skimmianine accumulated to around 0.05% DW (Etsè et al. 2014). Some derivatives of chelerythrine were also detected.

Attempts to produce hairy roots from *Argemone mexicana*, a member of the Papavereace family, which accumulates both benzophenanthridine and protoberberine alkaloids have failed, even though different explants, including mature stems and leaves, as well as hypocotyls, cotyledons, and radicles from developing seedlings, have been used in combination with different *A. rhizogenes* stains, such as ATCC15834, ATCC15834-PTDT, K599, and K599-PTDT. It is noteworthy to mention that other species of the same genus (*A. pleicantha*) have also been recalcitrant to *A. rhizogenes* transformation. Transient *A. tumefaciens* transformation has been achieved in hypocotyls and radicles of *A. mexicana* seedlings (Godoy-Hernández et al. 2008). Marked increases in alkaloid contents (sanguinarine and berberine) have been repetitively detected in response to *Agrobacterium* infection. It is not clear if the accumulated alkaloid amounts have reached the lethal threshold, since it has been noticed that *Agrobacterium* strains are sensitive to both sanguinarine and berberine under in vitro conditions. Both alkaloids display antimicrobial activity against soil pathogens (Rubio-Piña and Vázquez-Flota 2013). Moreover, a poor in vitro rhizogenic response has been observed for this plant (Xool-Tamayo et al. 2017). Untransformed roots, obtained by exscinding the complete root system from in vitro plantlets, showed a slow growth rate, even in the presence of different auxins. Nevertheless, alkaloid patterns were similar to those from roots of intact plants, since both sanguinarine and berberine were detected in comparable values (around 1% DW each; Xool-Tamayo et al. 2017).

Protoberberine Alkaloids

Protoberberine alkaloids (PBA) are quaternary tetracyclic compounds, formed by a tetrahydrophenanthridine bond to a benzene ring (Fig. 9.1). PBA share most of their biosynthetic pathways with BPA (Fig. 9.1) and are distributed in similar plants

families, including Papaveraceae, Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae, Annonaceae, Magoliaceae, and Convolvulaceae (Grycová et al. 2007). However, PBA coincide with BPA in only a few species (Rubio-Piña and Vázquez-Flota 2013). Berberine is the most common PBA, and it can be found in plants from the *Berberis*, *Coptis*, *Hydrastis*, and *Thalictrum* genera, accumulating mainly in roots, rhizomes, and stems and, less frequently, in leaves and fruits (Grycová et al. 2007).

Berberine displays an intense yellow color, and it is used as a fabric dye and for histological staining. Plants producing berberine have been used in traditional Chinese medicine for the treatment of gastroenteritis and other stomach illnesses. This alkaloid also has antiamebic properties and a powerful antimicrobial activity. It has been included in different agrochemical products to control diverse plagues (Grycová et al. 2007; Rubio-Piña and Vázquez-Flota 2013). Berberine and other PBA show a wide range of biological activities, including inhibition of DNA and protein synthesis, reduction of membrane permeability, and uncoupling of oxidative phosphorylation (Grycová et al. 2007). Berberine pharmacological applications range from possible uses as antiretroviral, antibiotic, antifungal, and cytotoxic agent, since it can bind to neuroreceptors, intercalate in DNA, and inhibit reverse transcriptase and DNA topoisomerase and telomerase activities (Chen et al. 2004). Recently, supplements of berberine have demonstrated to be efficient as insulin sensitizer in diabetes type 2 patients (Yin et al. 2008) for it increases glucose uptake by a different mechanism from that of insulin, which is independent of phospholipid-dependent protein kinases but activated by AMP-dependent protein kinases (Zhou et al. 2007). Moreover, berberine can also control hyperlipidemia in experimental animals maintained on high-fat diets by increasing transcript levels of the low-density lipoprotein (LDL) receptor and repressing 3-hydroxy-3-methyl-glutaryl-CoA reductase gene expression. Interestingly, animals under berberine treatments did not develop atherosclerotic lesions in the aorta and other major arteries (Chang et al. 2012). However, some caution in its usage is still recommended, since some mild toxicity has been observed, mainly due to its inhibitory action on liver cytochrome-P450-dependent enzymes, which could interfere or reduce the elimination of different drug-derived metabolites (Lan et al. 2015).

Although several suspension cell lines of berberine-producing species have been reported, including *Coptis japonica*, *Berberis vulgaris*, and *Thalictrum flavum*, among others (Roberts et al. 2010), there are few reports on PBA producing hairy root cultures, and this might be related to the fact that high amounts of berberine can be recovered from undifferentiated cultures (Roberts et al. 2010).

Hairy roots from *Berberis aristata* DC (Berberidaceae; common name: Indian berberry), which is considered an endangered species, were established (Brijwal and Tamta 2015). Roots accumulate high berberine contents and are commonly used in the treatment of diabetes, eye and ear infections, malarial fever, and gastrointestinal disorders, among other diseases (Kala 2002). Calli derived from in vitro plants' leaflets and nodal stem segments were infected with *A. rhizogenes* MTCC532 and MTCC2364 strains. Best infection frequency was found using strain 532 on

callus tissues and incorporation of 100 μM acetosyringone improved tissue transformation (Brijwal and Tamta 2015). Unfortunately, alkaloid contents were not reported.

Tinospora cordifolia (Menispermaceae, common name: heart-leaved moonseed) is an herbaceous plant that accumulates berberine and other PBA; it has been used as a medicinal plant in India and other countries for the treatment of digestive ailments (Verma et al. 2006). Hairy roots were obtained by infecting in vitro shoots with *A. rhizogenes* strain 2402. An external supply of tyrosine was required to reach berberine values of 0.34% DW, which could be increased up to 0.47% DW by exposing the hairy roots to MeJa. These values were only comparable to those from intact roots (Verma et al. 2006).

Attempts to produce hairy roots from *Cissampelos pareira* (Menispermaceae, common name: velvet plant) through infection of leaves with different *A. rhizogenes* strains have not been too successful (Shad and Deepa 2015, 2016). This plant is used in traditional Indian medicine to treat different ailments, such as heart complaints, diarrhea, and fungal infections. Leaves from mature trees were infected using different *A. rhizogenes* strains (A4, MSU440, K599, NCIM 5140, and 9402) in the presence of acetosyringone. However, no hairy roots were obtained, and only callus formation was observed when infecting with MSU440 strain and, to a lesser extent, with A4 strain (Shad and Deepa 2016). Low amounts of berberine (around 0.05 and 0.06% DW) were recovered from these cultures (Shad and Deepa 2016).

Berberine has not been detected in any of the hairy roots obtained from the different *Papaver* species mentioned above or in the *E. californica* cultures.

Other Tyrosine-Derived Metabolites

Betalains

Betalains are water-soluble nitrogenous pigments derived from tyrosine. They are manly synthesized by plants belonging to the order Caryophyllales, with the exception of the Caryophyllaceae and Molluginaceae, where color is due to anthocyanins (Castellanos-Santiago and Yahia 2008). Betalains and anthocyanins are two different families of pigments that are not found together in the same plant. There are two types of betalains: betaxanthins, which present a yellow coloration and the red-purple betacyanins. Betalains provide an attractive appearance to flowers and fruits pointing out the importance of color as an attractant in the pollination process and seed dispersion (Schaefer et al. 2004). Betalains present a strong antioxidant and free radicals scavenger activities (Gandía-Herrero et al. 2012). Recent studies have revealed that these pigments can be active in a dose-dependent fashion in the inhibition of cancer cell growth and proliferation (Sreekanth et al. 2007). When introduced to the diet of mice, they have demonstrated a strong health-promoting potential by inhibiting tumor formation (Lu et al. 2009).

The biosynthetic pathway of these pigments begins with the hydroxylation of L-tyrosine to L-dopa (Fischer and Dreiding 1972), through the action of tyrosinase. This enzyme has been isolated from *Portulacca grandiflora*, *Beta vulgaris*, and *Suaeda salsa* (Gandía-Herrero et al. 2004; Steiner et al. 1999; Wang et al. 2007). Some of the initial and late reactions in the pathway are enzymatically catalyzed, but some of the intermediate steps proceed spontaneously (cyclizations, aldimine formation, etc.). Early reactions are catalyzed by tyrosinase and the dopa 4,5- and 2,3-dioxygenase, whereas the late ones by glucosyl-, hydroxycinnamoyl-, and malonyltransferases (Strack et al. 2003).

Natural sources for betalains are the red beetroot (*Beta vulgaris* L. ssp. vulgaris), colored Swiss chard (*Beta vulgaris* L. ssp. cicla), grain or leafy amaranth (*Amaranthus* sp.), and cactus fruits, especially of the *Opuntia* and *Hylocereus* genera (Cai et al. 1998; Stintzing et al. 2002; Vaillant et al. 2005). The major commercial sources for betalains are the juice concentrates or powders of red beetroot that contain betanin (red pigment) and vulgaxanthin I (yellow pigment). Given the high content of betanin in red beetroot, there is a limitation in color variability, along with an undesirable earthy-like flavor, resulting from the presence of geosmin and some pyrazines (Azeredo 2009). Juice concentrates may also contain high concentrations of soil-borne microbes that can potentially contaminate food products. Moreover, there is also the fact of standardizing the addition of these pigments in terms of the differing metabolite content from batch to batch, due to cultivation conditions (Stintzing and Carle 2007). Hence the application of in vitro systems for betalain production may overcome these problems.

Beet cell cultures have been used for producing betalains (Akita et al. 2000; Leathers et al. 1992), based on the fact that it would be easier to control the quality and availability of pigments from external factors. However, these cultures could not compete with those pigments from the beetroot, which is cheap and abundant (0.5 g betanin per kg root; Gasztonyi et al. 2001). Another approach is to obtain them from hairy root cultures. The pioneering work of Hamill et al. (1986) showed the potential of these cultures, obtained through transformation of young plantlets with *A. rhizogenes* strain LBA 9402, to produce specialized metabolites. They found that 17-day-old *B. vulgaris* hairy root cultures produced similar quantities of betalains compared to the true roots of plants, with similar betacyanin/betaxanthin ratios. Several studies have dealt with the improvement of yields by modifying the medium constituents (Taya et al. 1994), the type and age of the inoculum (Pavlov et al. 2003), or even pH, to enhance betalain release to the medium (Mundokan et al. 1998). Given the excellent results obtained in betalain yield and growth from hairy root cultures, the next step was to scale-up the process. The use of air bubble column reactor with a fed-batch mode has proved to be quite successful for betalain production ($29.1 \, \text{mg g}^{-1}$ DW) and also an amenable system to manipulate the betacyanins/betaxanthins ratios in the cultures (Pavlov et al. 2007). Other reactors have been also used for the production of betalains with promising results. The use of temporary immersion RITA™ system has yielded 0.188% DW betalains (0.096 and 0.092% DW betacyanins and betaxanthins, respectively), which is comparable to the results obtained with the bubble column reactor (Pavlov and Bley 2006).

It is noteworthy to mention that red beet hairy root cultures have not only been envisioned as a source for betalains but also of other health-promoting compounds, such as antioxidants and phenolics (Georgiev et al. 2010; Pavlov et al. 2002). Betalain extracts obtained from hairy root cultures of red beetroot *B. vulgaris* cv. Detroit Dark Red, obtained by infecting young leaves with *A. rhizogenes* strain ATCC 15834 (Pavlov et al. 2002), presented higher antioxidant activity than extracts obtained from mature beetroots: sixfold higher 2,2-dyphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability (90.7% inhibition, $EC_{50} = 0.11$ mg vs 14.2% inhibition, $EC_{50} = 0.70$ mg) and 3.2-fold higher oxygen radical absorbance capacity (4.1 mM TE g^{-1} dry extract vs 1.25 mM TE g^{-1} dry extract). There were also important increases (20-fold) of total phenolic compounds, such as 4-hydrobenzoic acid, caffeic acid, catechin, epicatechin, and rutin (Georgiev et al. 2010).

Salidroside

Salidroside is a glucoside of tyrosol accumulated in members of the *Rhodiola* genus, such as *R. crenulata* and *R. rosea*, perennial herbaceous Crassulaceace plants growing on the Tibet plateau, above 5000 m ASL. These plants have antidepressive, antifatigue, and anxiolytic activities that have been attributed to the presence of salidroside (Lan et al. 2013). Hairy roots were obtained from *R. crenulata* leaves infected with *A. tumefaciens* C59C1, carrying both empty and the endogenous *TyDC* gene vectors (Lan et al. 2013). Both wild-type and transgenic roots produced salidrosides, but a noteworthy increase was noticed in those overexpressing TyDC. Moreover, in both cases, treatments with inducers of secondary metabolism, such as SA and MeJa, activated the synthesis of these chemicals, as revealed by the accumulation of early and late biosynthetic genes (Lan et al. 2013).

Conclusions

Hairy roots are a suitable model to produce valuable fine chemicals, alkaloids included. The advantages of using them for such a purpose are many, such as obtaining yields comparable to those of field plants, high growth rates, and genetic stability (Gandhi et al. 2015; Guillon et al. 2006; Loyola-Vargas and Miranda-Ham 1995; Sevón and Oksman-Caldentey 2002). Moreover, hairy root cultures are not classified as genetically modified organisms, as defined by the European Parliament Directive 2001/18/EC, hence are not subject of EU regulations regarding GMOs (OJ, L106, 17.4.2001, p. 1–38) (Georgiev et al. 2010). Interestingly, transformation by itself may increase alkaloid production in some cases (Le Flem-Bonhomme et al. 2004), and this has been related to the effects on *rolC* expression, which is involved in hairy root induction (Sevón and Oksman-Caldentey 2002; Sharma et al. 2013). There are also downsides related to loss of possible participation of aerial tissues,

recalcitrance to transformation, and costs derived from equipment required for massive culture (Guillon et al. 2006; Sevón and Oksman-Caldentey 2002). One common problem associated with scaling-up hairy root cultures has been the availability of bioreactors with the capacity to hold considerable volumes, since tissue integrity should be preserved as well as enabling a homogeneous distribution of oxygen and nutrients to support root growth. The use of low-shear reactors either pneumatically stirred or of the trickle-bed type has allowed the maintenance of hairy root cultures of different alkaloid-producing species in the rank of 10 L. Moreover, some estimations suggest that hairy roots cultures of *Hyoscyamus muticus* could be scaled up to a 10 m³ rank, maintaining hyoscyamine yields around 35 mg L^{-1}, which are similar to the ones obtained in batch cultures (Georgiev et al. 2013; Ramakrishnan and Curtis 2004). As a lower cost alternative, the use of multiple 1 L disposable plastic reactors has been proposed (Georgiev et al. 2013).

There are plenty of reports of hairy roots from species producing pharmaceutically important alkaloids. For example, *Catharanthus roseus* (Apocynaceae, common name: Madagascar periwinkle), which produces monoterpenoid indole alkaloids, has been intensively used both as a model for the study of the biosynthetic pathway, as well as a platform for production with commercial goals (Guillon et al. 2008; Magnotta et al. 2007; Rizvi et al. 2016; Zhao et al. 2013). This has been also the case of plants producing tropane and nicotine alkaloids, including different species in the *Datura*, *Hyoscyamus*, *Atropa*, and *Nicotiana* genera (Häkkinen et al. 2005; Moyano et al. 2002; Zhang et al. 2004). However, there is a limited number of reports of hairy root cultures of BIA-producing plants. This could be a reflection of the high performance of some undifferentiated cell cultures (Roberts et al. 2010). As an alternative, antimicrobial activity ascribed to BPA (sanguinarine) and PBA (berberine), which are induced in response to bacterial infection, might have hampered successful attempts of inducing hairy root cultures (Shad and Deepa 2016). However, hairy roots from *Papaver* species have demonstrated to be an amenable system for the study of morphinan-type alkaloids and have even been employed in their production (Hashemi and Naghavi 2016). Interestingly, endophytes isolated from different *P. somniferum* tissues, roots included, were able to modify alkaloid profiles of such tissues, and even those in distant organs (Pandey et al. 2016). Since most of the anatomical and physiological features of *A. rhizogenes* transformed roots remain unaltered, a co-culture system could be envisioned to open new venues in the understanding of these highly appreciated pharmaceutical compounds.

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Chapter 10
Mass Production of Transformed Hairy Root for Secondary Metabolites: A Case Study of *Panax ginseng* Hairy Roots

Gwi-Taek Jeong and Don-Hee Park

Abstract Ginseng plant (*Panax ginseng* C.A. Meyer) is a famous oriental medicinal plant, and it has many bioactive compounds useful for human. In this work, we reorganized the published papers on the development of large-scale culture system and secondary metabolite production of transformed hairy roots induced from *P. ginseng*. Hairy roots showed an active branching and fast growth pattern in plant growth regulator-free medium. Hairy roots has a short lag period of 4 days, and then the exponential growth phase continued from 4 to 45 days in the shake flask cultures. During the exponential growth phase, secondary metabolites of hairy roots are lowly accumulated due to relation with non-growth-associated mechanism. To develop large-scale culture system, several kinds of bioreactor systems were applied to cultivate hairy roots. In 20 L air bubble bioreactor, hairy roots have grown 18-fold on a dry weight basis and growth rate of 0.34 day^{-1} with a 0.2% (w/v) inoculum during 36 days. The introduction of several elicitors stimulates the formation of secondary metabolites and can reduce the cultivation time. Overall, hairy root culture systems are useful and available to mass production of plant-derived products.

Keywords Hairy root • *Panax ginseng* • Bioreactor • Elicitor • Growth condition

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Introduction

Both plants and plant-derived products are reliable source of valuable compounds. Over 100,000 compound-derived plants are currently discovered and isolated directly from plant resources; also over 4000 new chemicals have been found every year (Alfermann and Petersen 1995; Banthorpe 1994; Jeong and Park 2006a; Lee et al. 2004; Verpoorte et al. 1999). These compounds are known to be available in the production of valuable components (Alfermann and Petersen 1995; Jeong and Park 2006a; Lee et al. 2004; Verpoorte et al. 1999). Many plant-derived products can be obtainable by direct extraction from plant resources. By the way, the large-scale production of useful compound from field-grown or original habitats has been limited due to various laborious environmental problems such as low growth rates, limited areas, climate dependency, plant pests and diseases, and high labor dependency (Canto-Canché and Loyola-Vargas 1999; Cho et al. 2003; Jeong and Park 2005a; Park et al. 2003). Due to biosynthetically totipotent plant cells, plant cell and tissue are capable of producing the entire range of chemicals derived from parent plant (Ramachandra and Ravishankar 2002). Plant cell and tissue cultures are attractive alternatives in terms of secondary metabolite production (Jeong and Park 2006a; Ramachandra and Ravishankar 2002). In plant cell/tissue, the cell growth and metabolite production are very complex according to their culture condition (Verpoorte and Alfermann 2000). In spite of the enormous potential of valuable plant products, the large-scale production of useful metabolites from plant culture in bioreactors is hard to commercialize (Giria and Narasu 2000; Jeong and Park 2005a; Lee et al. 2004; Mavituna 1992).

Hairy roots are induced by the genetic modification in hormone metabolism of plant cells transformed with *Ri*-plasmid of *Rhizobium rhizogenes*, the pathogenic soil bacterium (Hamill and Lidgett 1997; Hooykaas and Schilperoort 1992; Jeong et al. 2002a, 2007). Hairy roots are characterized by definite growth patterns and inherent genetic stability (Hamill and Lidgett 1997). These hairy roots are considered as potential resources of plant root-derived secondary metabolites, in fields of pharmaceutical, cosmetic, medical, and fine chemical fields for commercial applications (Jeong and Park 2005a; Uozumi and Kobayashi 1994). Hairy root culture has some advantages compared to cell culture such as high and stable growth, as well as secondary metabolite production, non-requirement of hormones, and inherent genetic stability (Giria and Narasu 2000; Nilsson and Olsson 1997).

Hairy root culture in large-scale bioreactor systems is more complex due to its unique growth characteristics that occurred by heterogeneous, entangled, and structured nature of fibrous roots (Bais et al. 2002; Giria and Narasu 2000; Jeong and Park 2005a; Jeong et al. 2003, 2004; Liu et al. 2003; Nobuyuki and Takesh 1994). Although, many attempts of hairy root culture system are performed using bioreactors for large-scale applications, by the way, it is still under development. However, plant cell and tissue cultures remain a promising and available goal (Bourgaud et al. 2001; Jeong and Park 2005a).

This work focused on the review and reconstitution of published papers (Jeong et al. 2002a, b, 2003, 2004, 2005, 2007; Jeong and Park 2005a, b, 2006a, b) on the development of large-scale culture system and secondary metabolite production of transformed hairy roots induced from *P. ginseng* C.A. Meyer, which is well known for its benefits for human health.

Panax ginseng C.A. Meyer

Panax ginseng C.A. Meyer is one of the famous oriental medicinal plants distributed in the Korean Peninsula (Jeong et al. 2002a; Nam 1996). Ginseng plants possess many bioactive compounds useful for human health (Jung et al. 1996; Wu and Zhong 1999). Also, lots of pharmacological and biochemical researches have been performed to reveal the ginseng plant's efficiency. Ginseng's major compounds relevant to pharmaceutical effects have been known as ginsenosides, polysaccharides, phenolic compounds, antioxidants, peptides, fatty acids, alcohols, and vitamins (Jeong and Park 2006a; Wu and Zhong 1999). Recently, the non-saponin components of ginseng have been greatly focused for their anticancer, antidiabetic, and immunomodulatory effects (Fuzzati et al. 1999; Jeong and Park 2006a; Jung et al. 2002).

P. ginseng Hairy Root Induction and Cultivation

Induction of P. ginseng Hairy Roots

P. ginseng hairy roots induced by *R. rhizogenes* strain KCTC 2744 from 5-year-old, field-grown ginseng roots. After 7 weeks of infection with *R. rhizogenes*, hairy roots were induced from the surface of ginseng root sections. Induced hairy roots were transferred to fresh hormone-free 1/2 Murashige and Skoog (MS) medium with cefotaxime as antibacterial agent and inoculated at 23 °C under dark conditions. Induced hairy roots showed an active branching and fast growth pattern in hormone-free medium (Jeong et al. 2002a).

Cultivation of Hairy Roots

In plant cell/tissue cultures, cell growth and secondary metabolite production are affected by nutritional and environmental conditions and cultivation system. In order to obtain a high productivity, the culture conditions should be optimized (Bhadra and Shanks 1995; Jeong et al. 2004; Kanokwaree and Doran 1997). *P. ginseng* hairy roots were cultivated on various culture conditions in 250 mL shake

Fig. 10.1 Photos of growing root segments of *Panax ginseng* hairy root in solid culture for 10 days. (**a**) End part and (**b**) center part (*Source*: Jeong et al. 2004 Appl Biochem Biotech 113–116:1193–1203)

flask. It showed an active branching and faster growth than original roots in hormone-free medium. In the basic culture conditions of 1/2 MS medium with 3% sucrose, pH 5.8 at 23°C, hairy roots showed good growth. Based on 1/2 MS basal medium, optimal concentration of nitrogen and phosphate are 30 mM and 0.62 mM, respectively. Also the optimized inoculum amount was 0.4% (w/v). Moreover, the final cell amount is not proportional to the increase of inoculum amount. In secondary metabolite production, the contents of crude saponin and polysaccharides increased at high sucrose concentration (Jeong et al. 2002a).

The growth morphology and branching pattern of hairy roots may be an important parameter for affecting the performance of large-scale cultivation system (Falk and Doran 1996; Jeong et al. 2004; Kanokwaree and Doran 1997). The complex physical structure of hairy roots caused hard handling problems for preparation and inoculation of inoculum in large-scale cultivation. Also, their complex branching pattern can influence the growth and metabolite formation in hairy root culture system. Moreover, it is not simply related to the inoculum conditions (Woo et al. 1997). Bhadra and Shanks (1995) suggest the concept of physiological diversity of hairy root tip, which is the process of cell division, elongation, and maturation regions, for growing of hairy roots. The physiological and physical status of inoculum was influenced on the growth and metabolite formation in large-scale cultivation (Jeong et al. 2004).

In shake flask cultures, hairy root growth was dependent on the inoculum conditions such as the status (length, part, and age) of root tips and inoculum size in 250 mL culture flask (Jeong et al. 2004). In inoculating part of hairy roots, end parts (root tips with apical meristem) were grown to 1.6-fold than that of center parts. Root tips showed increased length with lateral root formation. The center part of root tip mainly formed lateral roots without increasing root length (Fig. 10.1). In the case of inoculation with different lengths (5–25 mm) of root tips, 5-mm-long root tips showed the highest growth than others. In pre-cultured age of inoculant, the

highest growth was achieved at 10-day age. With respect to inoculum size, the highest growth was obtained at 0.4% (w/v) inoculum size. Also, in 1 L-scale bioreactor cultures, the growth rate sharply increased at 0.2% (w/v) inoculum size during long periods (Jeong et al. 2004).

Growth Characteristics and Nutrient Consumption

In order to evaluate the potential of hairy root cultures, quantitative information regarding hairy root growth kinetics, nutrient uptake, and biomass and product yields are required (Jeong and Park 2006a; Kwok and Doran 1995). For cell growth and metabolite formation in hairy root culture, the different composition of inorganic nutrients and carbon sources are required.

In case of growth profiles of *P. ginseng* hairy roots in the shake flask cultures (Fig. 10.2), hairy roots appeared a short lag period for 4 days, and the exponential growth phase continued from 4 to 45 days. During the exponential growth phase, 0.355 g-dw g-cells^{-1} day^{-1} of growth rate appeared. Finally, the maximum biomass amount of 11.4 g-dw L^{-1} was obtained at the 59th day. The medium conductivity showed countertrend pattern to the cell growth. The decline of conductivity reflects the degree of consumption of inorganic nutrients by hairy roots (Jeong and Park 2006a; Uozumi and Kobayashi 1994). In monitoring of sugar concentration, the decrease of sugar concentration occurred inversely with increases of biomass like conductivity change. Also, the phase of decline of biomass growth subsequently occurred in the exhaustion stage of the carbon source (Jeong and Park 2006a; Rho and Andre 1991). The mono-sugar concentrations such as glucose and fructose were increased over the initial 20 days and subsequently decreased, whereas sucrose was decreased and almost consumed after about 25 days. It means that hairy roots consumed glucose and fructose produced by sucrose hydrolysis (Jeong and Park 2006a).

Generally, plant cell media contain two kinds of nitrogen source such as ammonium and nitrate. In monitoring of nitrogen source uptake, ammonium ions were continuously consumed and exhausted to 20 days, whereas nitrate was consumed within 40 days. It is known that ammonium ions are easily taken up more rapidly than nitrate ions by plant cells (Rho and Andre 1991; Verpoorte and Alfermann 2000). Furthermore, biomass yields depend on the consumption of ammonium and nitrate ions (Verpoorte and Alfermann 2000). The phosphate ions are nearly consumed within 16 days; moreover, the increase of biomass was maintained until exhaustion of the nitrogen and phosphate ions (Jeong and Park 2006a).

In the case of contents of secondary metabolites in *P. ginseng* hairy root cultures (Fig. 10.3), the highest total ginseng saponin (92.09 mg g^{-1}) and phenolic contents (16.29 mg g^{-1}) were obtained at the 59th and 47th days in 250 mL shake flask-scale cultures, respectively. During the exponential growth phase, secondary metabolites of hairy roots are lowly accumulated. It shows that the production of secondary metabolites in *P. ginseng* hairy roots is related with non-growth-associated process (Jeong and Park 2006a).
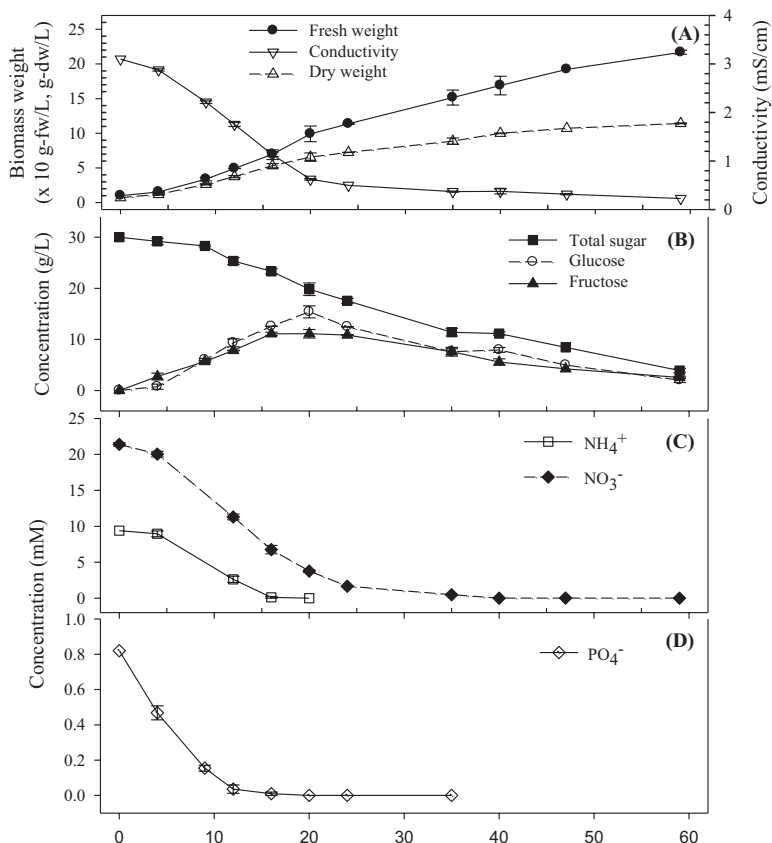
Fig. 10.2 Time course of biomass growth profiles and consumption of major medium components of *Panax ginseng* hairy roots in 250 mL flask cultures. (**a**) Biomass growth and conductivity, (**b**) sugars, (**c**) ammonium and nitrate, and (**d**) phosphate (*Source*: Jeong and Park (2006) Biotechnol Bioprocess Eng 11:43–47)

Estimation of Hairy Root Weight Using Medium Components

During the cultivation of hairy root in bioreactors, biomass weight cannot be measured directly for analysis. To obtain the biomass information, indirect measurement will be introduced. Generally, during the hairy root culture, cell growth and consumption of medium component occurred simultaneously. Medium conductivity has been introduced as a useful tool for the indirect measurement of biomass amount in hairy root culture (Ballica et al. 2001; Jeong and Park 2006a; Rho and Andre 1991; Taya et al. 1989a).

In case of *P. ginseng* hairy root culture (Fig. 10.4a), medium conductivity was correlated with biomass weight as presented: $X = -0.063 + 0.260C$ ($R^2 = 0.982$), where X is dry weight of hairy roots ($\times 10$ g-dw L^{-1}) and C is medium conductivity (mS cm^{-1})
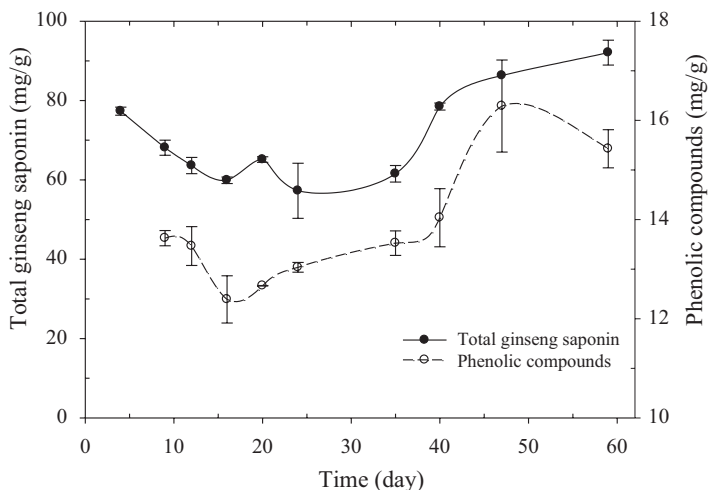
Fig. 10.3 Biosynthesis of secondary metabolites of hairy roots during cultivation in flask cultures (*Source*: Jeong and Park (2006) Biotechnol Bioprocess Eng 11:43–47)
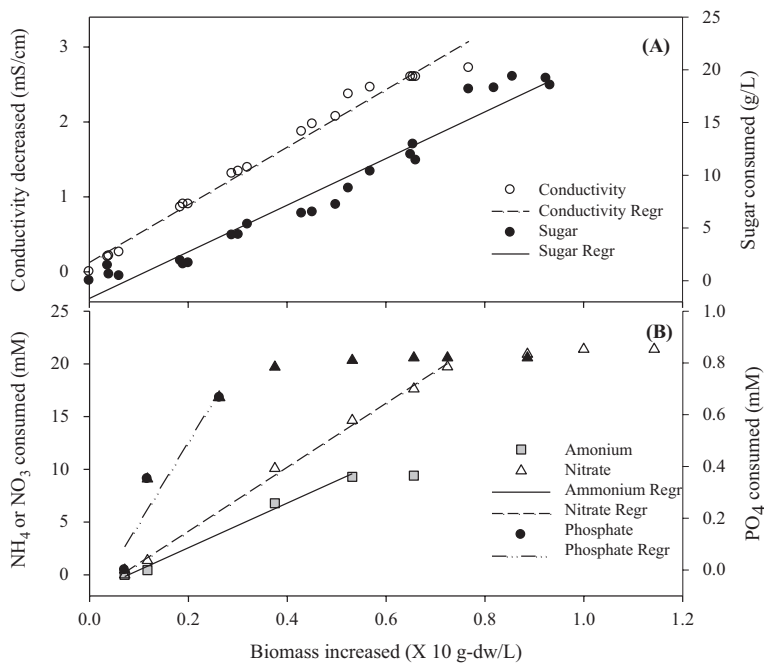
Fig. 10.4 Linear relationship between biomass growth and nutrient consumption by the *Panax ginseng* hairy roots in the 250 mL flask cultures. (**a**) Conductivity and sugar and (**b**) ammonium, nitrate, and phosphate (*Source*: Jeong and Park (2006) Biotechnol Bioprocess Eng 11:43–47)
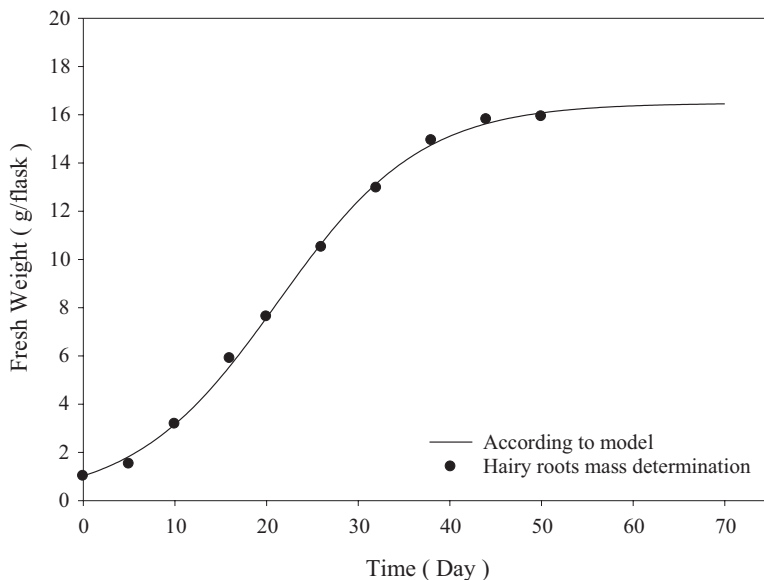
Fig. 10.5 Growth kinetics of *Panax ginseng* hairy roots in flask cultures (*Source*: Jeong et al. (2002b) Appl Biochem Biotechnol 98–100:1115–1127)

(Jeong and Park 2006a). However, this evaluation method was limited by the differences in the changes of conductivity and growth rate at later culture period. Also, total sugar consumed in medium was closely related with biomass increase in hairy root culture as presented: $X = 0.076 + 0.045C$ ($R^2 = 0.956$), where X is dry weight of hairy roots ($\times 10$ g-dw L^{-1}) and C is the total sugar consumed (g L^{-1}) (Fig. 10.4b) (Jeong and Park 2006a). The relationships between the consumption of ammonia/nitrate ions and increase of biomass weight in hairy root cultures were observed. The apparent biomass yields were calculated from a relationship of biomass increase versus substrate consumption (sugar, ammonium, nitrate, and phosphate) in *P. ginseng* hairy root cultures (Fig. 10.4b). The apparent biomass yields based on nutrient uptake are conductivity [2.60 g-DW L^{-1} mS^{-1}], carbon [0.45 g-DW g-sugar^{-1}], ammonium [0.47 g-DW L^{-1} mM NH_4^{-1}], nitrate [0.33 g-DW L^{-1} mM NO_3^{-1}], and phosphate [3.17 g-DW L^{-1} mM PO_4^{-1}] in the 250 mL flask cultures (Jeong and Park 2006a).

Growth Kinetics of **P. ginseng** *Hairy Roots*

The growth of *P. ginseng* hairy roots appeared as a typical sigmoid-shaped curve (Fig. 10.5). Generally, logistic model is known as the useful tool for estimating of maximal biomass (Edwards and Willce 1968; Jeong et al. 2002b). The growth kinetics of *P. ginseng* hairy roots in flask culture was described according to a logistic model (Edwards and Willce 1968) as follows: $X = X_m / \{1 + \exp(b - kt)\}$, where X is fresh weight of hairy roots (g), X_m is the maximal fresh weight of hairy roots (here

16.48 g), t is culture period (days), and b and k are model constants. In 250 mL flask-scale cultivation condition, model constant b and k are estimated as 2.716 and 0.128, respectively. Also, the doubling time at exponential growth phase was calculated as 6.5 days (specific growth rate 0.15 day^{-1}) (Jeong et al. 2002b).

Mass Production of Hairy Roots in Bioreactors

For the mass production of hairy roots, several bioreactors were introduced. By the way, culture of hairy roots in bioreactors is a complex task due to continuous growth and unique configuration, such as heterogeneous, structured, and entangled nature of fibrous roots (Giria and Narasu 2000; Jeong et al. 2002b, 2003; Taya et al. 1989b). Also, hairy roots tend to distribute unevenly and form highly dense and tangled root clumps. It caused the flow resistance and limitation of mass transfer of nutrients in bioreactor (Yu and Doran 1994). To scale-up bioreactor for hairy root culture, the simultaneous supply of nutrients from both liquid and gas phases are necessary. In bioreactor design for hairy root cultures, several factors such as the culture conditions (growth characteristics, nutrient and oxygen requirements, temperature) and the mass flow restriction by root clumps should be considered (Jeong et al. 2002b; Taya et al. 1989a; Yu and Doran 1994).

Air Bubble Four-Stage Column Bioreactor

In air bubble column reactor, like airlift bioreactor, air bubbles generate less shear stress, so that it is available for heterogeneous, structured, and entangled nature of hairy roots (Jeong et al. 2002b; Sajc et al. 2000; Taya et al. 1989b). In the air bubble column bioreactor, hairy roots presented closely packed hairy roots at each stage of the column. During 32 days, hairy roots increased about 36 times and showed 1.112 g-dw g-cells^{-1} day^{-1} of growth rate, which is about 3.02-folds higher than that of flask culture (Table 10.1). These enhanced results can be obtained by the blocking of hairy root floating on the upper side of reactor by rising of air bubble during culture periods (Jeong et al. 2002b).

Table 10.1 Comparison of growth and secondary metabolite content on various bioreactors

Bioreactor type	Culture time (days)	Growth ratio (times)	Growth rate (fw (dw) g g^{-1} d^{-1})	Polysaccharide (g g^{-1})
250 mL flask	38	14.93	0.393 (0.368)	0.191
Air bubble column type	42	51.680	1.230 (1.112)	0.106
Modified stirred type	32	36.290	1.134 (1.198)	0.096

Source: Jeong et al. (2002a) Appl Biochem Biotechnol 98–100:1115–1127

Modified Stirred Bioreactor (Frame-Fixing Type)

In stirred bioreactor, hairy roots contact with impeller, which cause the damage and shear stress of hairy roots. To overcome this stress, in the case of frame-fixing type bioreactor, cultivation space of hairy roots is separated from agitator and impeller by application of stainless steel mesh frame (Jeong et al. 2002b). *P. ginseng* hairy roots in the frame-fixing type stirred bioreactor have grown about 52-folds (1.198 g-dw g-cells^{-1} day^{-1} of growth rate) after 42 days; also, it is 3.26-fold higher than that of flask cultures (Table 10.1). In this type of bioreactor, several problems such as mass transfer limitation by formed root clumps and damage of frame-out-growing roots by rotation of impeller occurred at later stages during culture period (Jeong et al. 2002b).

Stirred Bioreactor

In this stirred bioreactor, mixing was conducted using magnetic stirrer, and aeration was performed from the bottom of bioreactor. During 27 days, *P. ginseng* hairy root increased about 24-fold of initial inoculum (Table 10.2). Also, 0.85 g-dw g-cell^{-1} day^{-1} of growth rate was obtained. It is higher about 2.65-fold than that of 250 mL flask culture (Jeong et al. 2003).

Bubble Column Bioreactors

In air bubble bioreactor, aeration and mixing of medium occur simultaneously by air supply. Like an airlift bioreactor, the ascending air bubbles generate less shear stress. Air bubble bioreactors require low initial equipment and maintenance cost; also it has low contamination possibility due to simple structure (Giria and Narasu 2000; Jeong et al. 2003).

In 5 L-scale air bubble bioreactor (Table 10.2), *P. ginseng* hairy roots have grown about 55-fold with growth rate of 1.38 day^{-1} during 39 days. The polysaccharide content (0.11 g g-dw^{-1}) was lower than that of flask culture. During the later periods, hairy root was floating on the upper part in bioreactor. It caused limited use of culture space. Also, hairy roots construct the root ball/mat due to meristem-dependent growth pattern. It caused the mass transfer limitation of nutrients and oxygen to core part of root mat, which caused the death of hairy roots in core part (Jeong et al. 2003).

In 19 L-scale air bubble bioreactor (Table 10.2), *P. ginseng* hairy roots have grown about 38-fold with growth rate of 0.96 day^{-1} after 40 days (2.6-fold higher than flask culture). It means that mass cultivation of hairy roots could be possible in air bubble bioreactors. However, the polysaccharide content (0.13 g g-dw^{-1}) was lower than that of natural ginseng roots (Jeong et al. 2002a, 2003).

Table 10.2 Comparison of growth kinetics and metabolite production of *Panax ginseng* hairy roots in bioreactors

Bioreactor type	Culture time (day)	Aspect ratio (height/diameter)	Working volume (L)	Growth ratio (fold)	Growth rate (day^{-1})	Intracellular polysaccharide ($g\ g^{-1}$)	Carbon yield (g-dw g-sugar^{-1})
Static culture	28	–	0.05	5.8	0.21	N.T.	N.T.
Shake-flask culture	48	–	0.2	16.5	0.34	0.19	0.19
Stirred type	27	1.43	0.8	24	0.85	N.T.	0.19
Bubble column type	25	7.14	2.6	11	0.44	0.11	0.13
	39	1.41	4.0	55	1.38	0.11	0.22
	40	1.48	17.0	38	0.96	0.13	0.18

N.T. not tested

Source: Jeong et al. (2003) *Appl Biochem Biotechnol* 105–108:493–503

Table 10.3 Comparison of growth kinetics and metabolite productions for *Panax ginseng* hairy roots in bioreactors

Bioreactor type	Working volume (mL)	Inoculum size (g-fw)	Culture time (day)	Growth rate (day^{-1})	Total ginseng saponin (mg g^{-1})	Acidic polysaccharide (mg g^{-1})	Phenolic compound (mg g^{-1})
Air bubble	800	3.2	36	0.451	–	–	–
	2000	8	17	0.349	81.3	119.3	14.1
	4000	16	35	0.534	91.6	348.3	15.6
	10,000	20	37	0.414	76.3	294.3	12
	16,000	32	36	0.371	85.8	254.9	11.5
20 L horizontal bubble	16,000	32	35	0.407	87.3	270.4	14.7
	16,000	64	41	0.181	68.1	202	15.2

Source: Jeong and Park (2005b) *Biotechnol Bioprocess Eng* 10(6):528–534

Table 10.2 compared the growth kinetics and metabolite content of *P. ginseng* hairy roots in several bioreactors. Generally, the growth rates of several bioreactors were higher than that of shake flask culture. The contents of intracellular polysaccharide of hairy roots cultured on bioreactors were 0.11–0.13 g g-dw^{-1}, which are lower values than that of natural *P. ginseng* roots (0.45–0.79 g g-dw^{-1}) (Jeong et al. 2002a). However, carbon yield was 0.13–0.19 g-dw/g-sugar, which did not indicate closely the linear correlation to the cell growth rate in several-scale bioreactors (Jeong et al. 2003).

Air Bubble Bioreactors

Table 10.3 compared the growth kinetics and metabolite productions in air bubble bioreactors. In a 1 L-scale air bubble bioreactor (1.4 height/diameter ratio), *P. ginseng* hairy roots grew 16.3-fold, and growth rate of 0.458 day^{-1} was observed. In 5 L air bubble bioreactor, *P. ginseng* hairy roots have grown to 299 g-fw with 0.4% (w/v) inoculum in 35 days. The growth rate was 0.534 day^{-1}. Also, the contents of total ginseng saponin, acidic polysaccharides, and phenolic compounds were achieved to 91.6 mg g^{-1}, 348.3 mg g^{-1}, and 15.6 mg g^{-1}, respectively. In a 12 L bioreactor (0.83 height/diameter ratio), hairy roots have grown to 15.3-folds and growth rate of 0.534 day^{-1} with 0.2% (w/v) inoculum in 37 days. Also, the contents of total ginseng saponin, acidic polysaccharides, and phenolic compounds were achieved to 76.3 mg g^{-1}, 294.3, and 12.0 mg g^{-1}, respectively (Jeong and Park 2005a).

In 20 L air bubble bioreactor, hairy roots have grown 18-fold on a dry weight basis and growth rate of 0.34 day^{-1} with a 0.2% (w/v) inoculum in 36 days (Fig. 10.6). The medium pH has consistently gone down until 12 days but subsequently increased. It is known that the consumption of ammonium ions by plant cell induces a drop of pH during initial cell growth stage, after pH rise with the subse-
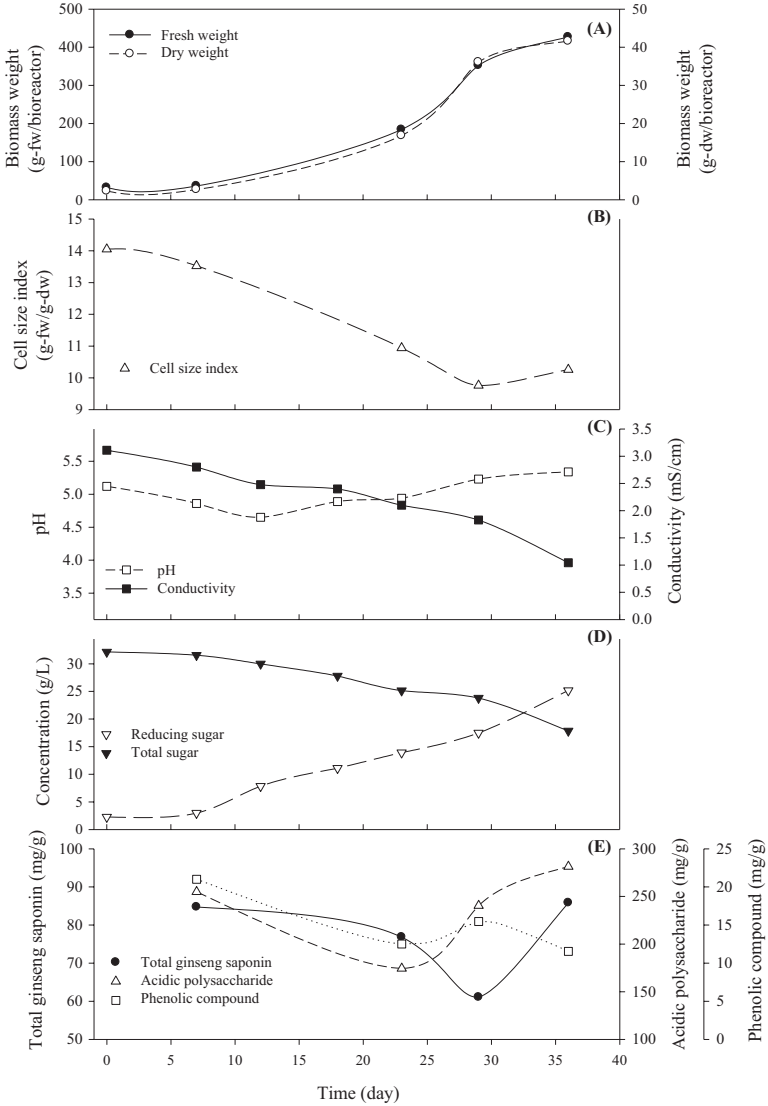
Fig. 10.6 Time course of the growth, nutrient consumption, and metabolite production of hairy roots in a 20 L air bubble bioreactor, with a 0.2% inoculum (*Source*: Jeong and Park (2005a) Biotechnol Bioprocess Eng 10(6):528–534)

quent consumption of nitrate ions (Jeong and Park 2005a, 2006a; Rho and Andre 1991; Verpoorte and Alfermann 2000). The medium conductivity decreased inversely with the increase in biomass during cultivation. Also, total sugar concentration was diminished inversely to growing hairy roots. The content of total ginseng saponin of the hairy roots decreased at 28 days. This is because secondary

metabolites are generally produced during the stationary or non-growth phase (Jeong and Park 2005a). In the case of various inoculum sizes in 20 L-scale air bubble bioreactor, the highest growth rate was obtained to 0.1% (w/v). Also, the final weight of hairy roots did not have linear relation with increase of inoculum size (Jeong and Park 2005a).

Enhancement of Secondary Metabolites by Elicitation

Generally, plants generate secondary metabolites as a defense mechanism against stress by pathogens attack and environmental condition. Elicitors are applied to stimulate the formation of secondary metabolites, which reduced the cultivation time to obtain high-value products (Ramachandra and Ravishankar 2002). The elicitation mechanisms are different and complex due to little knowledge about biosynthetic pathways of plant-derived secondary metabolites. Therefore, these approaches tend to be empirical progress (Bhagwath and Hjorts 2000; Jeong and Park 2005b, 2006b; Jeong et al. 2005). Several elicitors have been applied to stimulate cell metabolism to enhance the secondary metabolites in plant cell culture (Akimoto et al. 1999; Jeong et al. 2005). Elicitors are divided into two groups: abiotic and biotic. Abiotic elicitor is of non-biological origin, such as heavy metals, UV light, salts, and chemicals. On the other hand, biotic elicitor is of biological origin, such as polysaccharides, glycoproteins, enzymes, chitosan and its oligomers, bacteria, and yeast (Bhagwath and Hjorts 2000). The efficiency of elicitation depends on several conditions such as elicitor kind and amount and time and period of elicitation (Bhagwath and Hjorts 2000; Jeong and Park 2005b; Jeong et al. 2005).

The exogenous supply of salicylic acid (SA), which is naturally occurred in many plants, influences various physiological and biochemical reactions in plant metabolisms. SA and acetylsalicylic acid (ASA, chemical derivate of SA) have been reported to enhance the metabolite productivity in plant cell/tissue cultures (Lee et al. 2001). By SA elicitation during *P. ginseng* hairy root culture, the accumulation of total ginseng saponin enhanced slightly at lower dosages (0.1–0.5 mM). Also, the elicitation of ASA enhanced about 1.1-fold total ginseng saponin content, i.e., from 0.1 to 1.0 mM (Jeong et al. 2005).

Several abiotic elicitors (tannic acid, selenium, nickel, and sodium chloride) are introduced to enhance the ginseng saponin biosynthesis in *P. ginseng* hairy root culture (Jeong and Park 2006b). Tannic acid, a commercial form of tannin, is an acid-like substance, called a polyphenol (Seigler 1995). In case of tannic acid elicitor, cell growth was profoundly inhibited, whereas the increase of ginseng saponin content did not occur.

Selenium is not an essential nutrient; also, its effects on growth and biosynthesis of plant have yet to be properly known (Jeong and Park 2006b; Lopez-Bucio et al. 2003). In case of selenium as an elicitor, the content and productivity of ginseng saponin were enhanced about 1.31-fold and 1.33-fold at 0.5 mM, respectively.

Nickel (Ni) is an essential minor element, and a little information has been reported regarding its stimulatory effects on plant growth (Gerendas et al. 1999; Witte et al. 2002). The elicitation of nickel (20 μM NiSO₄) enhanced the content and productivity of ginseng saponin to about 1.20-fold and 1.2-fold, respectively; moreover, it did not inhibit cell growth (Jeong and Park 2006b). Salinity may affect a variety of metabolic processes in plant growth and development (Arshi et al. 2002). In the effect of salinity (0.1% (w/v) sodium chloride), the content and productivity of ginseng saponin were enhanced about 1.15-fold and 1.13-fold, respectively (Jeong and Park 2006b).

Several biotic elicitors (chitosan, glucosamine, yeast, and bacteria elicitors) are introduced to enhance the ginseng saponin biosynthesis in *P. ginseng* hairy root culture (Jeong et al. 2005; Jeong and Park 2005b). Chitosan and its oligomer, which is components of mycelium cell wall, are known to be potent signaling molecules and reported to be effective and strong elicitors in several plant in vitro cultures (Akimoto et al. 1999; Jeong et al. 2003; Sudha and Ravishankar 2002). In the application of the chitosan elicitor and glucosamine, *P. ginseng* hairy root growth caused a slight decrease; however, the contents of ginseng saponin were slightly stimulated with the increase of elicitor amount (Jeong and Park 2005b). With the elicitations of yeast elicitor and bacterial elicitor, *P. ginseng* hairy roots stimulated the formation of total ginseng saponin to about 1.17 times and about 1.23 times, respectively (Jeong et al. 2005). The bacterial elicitor has some advantageous treatment compare to yeast elicitor. The preparing of bacterial elicitor is easier than that of yeast elicitor due to complex purification step for preparation (Jeong et al. 2005). These elicitation results represented that the productivity of secondary metabolites in *P. ginseng* hairy roots can be increased via the application of several elicitation methodologies (Akimoto et al. 1999; Jeong et al. 2003, 2005; Jeong and Park 2005b, 2006b; Sudha and Ravishankar 2002).

Effect of Plant Growth Regulator and Reversible Morphological Change in *P. ginseng* Hairy Roots

Plant growth regulators play a definitive role in plant cell growth and secondary metabolite biosynthesis. The types and concentrations of auxin and/or cytokinin have significant effects on both cell growth and metabolite formation in plants (Ramachandra and Ravishankar 2002). It is known that morphological alterations of plant cell occurred by the response of exogenously added plant growth regulators, in in vitro cultures (Toyoda et al. 1991).

In case of *P. ginseng* hairy root culture, several plant growth regulators have been observed to do alternations in morphology, cell growth, and secondary metabolite formation. Benzylamino purine and kinetin in *P. ginseng* hairy root culture enhanced the cell growth and phenolic compound synthesis. α-Naphthalene acetic acid and 2,4-dichlorophenoxyacetic acid profoundly inhibited the cell growth. However,
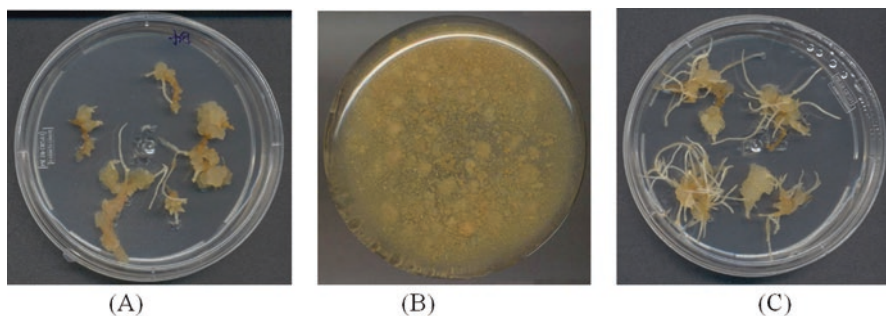
Fig. 10.7 Photographs showing morphology changes due to plant growth regulators. (**a**) Callus induction from *P. ginseng* hairy roots, (**b**) suspension culture of callus induced from *P. ginseng* hairy roots, and (**c**) hairy root regeneration from callus induced from hairy roots (*Source*: Jeong et al. (2007) *Biotechnol Bioprocess Eng* 12:86–91)

indole-3-acetic acid slightly enhanced growth at low concentration range. The addition of auxin did not enhance the accumulation of total phenolic compounds in contrast to gibberellic acid and cytokinins (Jeong et al. 2007).

As a result of the effects of auxin and cytokinin on the growth and morphological changes of hairy roots, callus formation was observed by addition of cytokinin (Fig. 10.7a). Hairy roots considerably altered to callus, which has an easily distinguishable morphology from original hairy roots. Also, high concentrations of benzylamino purine significantly formed callus growth. Callus cells derived from hairy roots were cultivated in suspended cultures with liquid medium containing hormone (Fig. 10.7b). The regenerated *P. ginseng* hairy roots retained the characteristics of the original hairy roots such as active growth with extensive lateral branching in hormone-free medium (Fig. 10.7c). The hairy roots regenerated from callus represented a stable phenotype of hairy roots. From these regeneration of hairy roots, cell aggregates (callus) could be applied to seeding system due to advantages with regard to inoculation in large-scale hairy root culture systems (Jeong et al. 2007).

Conclusions

This work reviewed the development of large-scale culture system and secondary metabolite production of *P. ginseng* hairy roots. In plant hairy root cultures, cell growth and secondary metabolite production are affected by nutritional and culture conditions and cultivation system. In large-scale bioreactor design, the growth properties and nutrient utilization of hairy roots and mass transfer and heat transfer of bioreactor should be considered. Finally, the introduction of elicitation method has enhanced secondary metabolite formation, and it can increase productivity. In conclusion, hairy root culture systems are useful and available to mass production of plant-derived products.

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Chapter 11
Production of Ginsenosides by Hairy Root Cultures of *Panax ginseng*

Hosakatte Niranjana Murthy, So Young Park, and Kee Yoeup Paek

Abstract *Panax ginseng* C. A. Meyer, commonly known as ginseng, is a popular medicinal plant, used as a traditional medicine in many countries. Ginsenosides are triterpene compounds which have multiple biological and pharmaceutical applications including neuroprotection, anticancer, antidiabetic, hepatoprotective, and immunomodulatory activities. Cultivation of ginseng till harvest of matured roots takes 5–7 years, whereas wild ginseng is rare and a highly expensive commodity. Therefore, many researchers studied biotechnological means especially cell and organ cultures for the production of ginsenosides. Transformed hairy roots were induced in ginseng, and they were cultured in vitro for biomass and secondary metabolite production. Various chemical and physical parameters have been worked out for optimal biomass and ginsenoside accumulation. Several researchers have tested bioreactor system for cultivation of ginseng hairy roots to produce ginsenosides. This review highlights the recent progress in the hairy root induction, selection of elite clones, establishment of suspension culture, strategies adopted for biomass, and bioactive compound production.

Keywords Biomass • Elicitor • Saponins • Secondary metabolite • Suspension cultures

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Introduction

Panax ginseng C. A. Meyer, commonly known as "ginseng," is a medicinal plant which is cultivated in Korea, China, Japan, and other Southeast Asian countries and has been used as an herbal medicine in these countries for thousands of years. These days it is also popular as a nutraceutical or as a functional food in the rest of the world especially in North America and Europe (Murthy et al. 2014a). It exhibits pharmacological effect including antifatigue, anticancer, antidiabetic, cardioprotective, hepatoprotective, immunomodulatory, and antioxidant properties (Briskin 2000; Park et al. 2005; Murthy et al. 2014b, c). The biologically active components of ginseng are triterpenoid saponins, known as ginsenosides. Ginsenosides are classified into three groups based on their structure, namely, the Rb group (panaxadiols, including Rb_1, Rb_2, Rc, Rd, and so forth), Rg group (panaxatriols, including Rg_1, Re, Rf, Rg_2, and so forth), and Ro group (oleanolic acid) (Fig. 1; Park et al. 2005). Ginseng roots which are naturally available are very scarce; the current ginseng supply depends almost exclusively on field cultivation which is time-consuming and labor-intensive. Agricultural production of ginseng roots requires 5–7 years from seed planting to mature root harvesting during which the plant growth is highly susceptible to a number of environmental factors, pathogens, and pests (Proctor 1996). Therefore, biotechnological methods such as cell, tissue, and organ cultures have been exploited for ginseng biomass and its bioactive constituent production (Wu and Zhong 1999; Murthy et al. 2016, 2014a, d; Thanh et al. 2014a, b). Many investigators have studied the production of ginsenosides using callus tissue, cell suspension cultures, but the productivity obtained has remained low because of low growth rates (Furuya et al. 1983; Mathur et al. 1994). Additionally, the induction and establishment of hairy roots after the infection of *Panax ginseng* roots in *Agrobacterium rhizogenes* have been successfully performed (Inomata et al. 1993; Mallol et al. 2001; Sivakumar et al. 2005; Yoshikawa and Furuya 1987). There are various reports on ginseng hairy root cultures focusing on the increase of biomass growth and ginsenoside productivity by the optimization of culture medium and environment (Kim et al. 2013; Palazon et al. 2003a, b; Sivakumar et al. 2005; Yu et al. 2000, 2005). It is also possible to enhance the productivity of ginsenosides by application of bioreactor technologies (Choi et al. 2005; Sivakumar et al. 2005; Yu et al. 2000, 2005; Palazon et al. 2003b; Jeong and Park 2005, 2006; Jeong et al. 2003). This review summarizes the methods of ginseng hairy root cultures for the production of biomass and ginsenosides.

Induction of Hairy Roots in *Panax ginseng*

Hairy roots from ginseng were induced from root-derived callus following infection with *Agrobacterium rhizogenes* strain A4 (Yoshikawa and Furuya 1987), from root discs using *A. rhizogenes* strain A4 (Hwang et al. 1999; Mallol et al. 2001), R-1000 (Woo et al. 2004), KCTC-2703 (Yu et al. 2000), and from petiole segments with *A. rhizogenes* strain 15834 (Yoshimatsu et al. 1996). Root discs developed both callus tissues and hairy roots after 4–8 weeks of infection with *A. rhizogenes* strain A4. Various hairy root lines were recognized with respect to morphology and growth

(Mallol et al. 2001): HR-M line was with primary roots, extensive lateral branching, and a profusion of root hairs, C-M line showed callus-like appearance with very thick primary roots and several secondary roots, and T-M line was long and thin without branching points. Hairy roots lines were maintained in Schenk and Hilderbrandt (1972) liquid medium and kept in a rotary shaker at 100 rpm, 26 °C in the dark by subculturing them once after 2 weeks. They could able to maintain these three root phenotypes over successive subcultures for 2 years. Hairy root lines showed differential growth rate (final fresh weight/fresh inoculum weight)—highest growth values were 7.9, 7.1 and 3.2, respectively, by C-M, HR-M, and T-M roots, and growth rate was 1.5 with respect to non-transformed roots. Hairy roots were found to be superior in growth and accumulation of biomass in suspension cultures, and such reports are already on records in hairy root cultures of cucumber (Amselem and Tepfer 1992), rosy periwinkle (Palazon et al. 1998), jimsonweed, and tobacco (Moyano et al. 1999).

Selection of Clones/Lines for Ginsenoside Accumulation

Of the three hairy root lines selected by Mallol et al. (2001), HR-M root lines achieved the highest ginsenoside content (5.4 mg/g DW), whereas C-M line accumulated 4.8 mg/g DW and non-transformed roots possessed 4.5 mg/g DW. Both Rb and Rg group ginsenosides were produced by these hairy root lines; Rg group ginsenosides were always higher than that of the Rb group. The ginsenoside pattern also varied depending on the root phenotypes. The main ginsenosides found in T-M, HR-M, and C-M root lines were the Re ginsenosides followed by the Rg_1. In T-M root the Re and Rg_1 represented 57.7 and 16.8%, respectively, of the total ginsenoside contents; in HR-M and C-M root lines, the Re constituted 40.1 and 38.1%, and the ginsenoside Rg_1 represented 28.5 and 27.9%, respectively, of the total. With respect to ginsenoside yield (after 4 weeks of suspension culture), Mallol et al. (2001) observed highest yield of 7.29 mg/l by HR-M root lines, followed by C-M root lines (71.6 mg/l). The lowest ginsenoside yield of transformed roots phenotypes was with T-M root lines (24.8 mg/l). In concurrence with these observations, Woo et al. (2004) and Yu et al. (2000) also selected hairy root lines producing total ginsenoside contents 4–5 times higher than that of a common hairy root population.

Ginseng Hairy Root Culture: Optimization of Factors Influencing Biomass and Metabolite Production

Effect of Growth Regulators on Biomass and Metabolite Production

Yu et al. (2000) used hormone-free Murashige and Skoog (1962) liquid medium for establishment of hairy root suspension cultures; whereas, Mallol et al. (2001) have used SH liquid medium for multiplication and growth of ginseng hairy roots.

The ginseng hairy root cultures have been thus established with the hairy root lines selected by various laboratories. These hairy roots were stable in culture, and the growth rate was much faster than that of non-transformed roots in hormone-free medium. The hairy roots grew rapidly in a hormone-free medium and showed biphasic growth: rapid growth during first 2 weeks and slower growth thereafter (Yu et al. 2000). Thus ginseng hairy root suspension cultures need 4–6 weeks depending on the hairy root clones/lines to achieve growth, biomass, and metabolite accumulation. The 0.5 g fresh weight of ginseng hairy roots increased ca. 100-fold after 6 weeks of culture in hormone-free medium. Hwang et al. (1999) studied the effect of exogenous auxins including indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), and 2, 4-dichlorophenoxy acetic acid (2, 4-D) on growth and ginsenoside accumulation, and they reported that IAA at 0.5 mg/l was most effective hormone for promotion of growth, multiplication, and accumulation of ginsenosides. The increase of root growth in ginseng was the result of an increase in branching and root elongation. Similarly, in case of *Rubia tinctorum*, hairy roots cultured in presence of IAA showed the maximal growth rate and the highest alkaloid production. In contrast, Inomata et al. (1993) showed that the addition of IBA with ginseng hairy root cultures increased the growth ratio and ginsenoside accumulation. Another study demonstrated that the exogenous IAA had no great effect on hairy root growth or the production of alkaloids (Rhodes et al. 1994). Hwang et al. (1999) showed accumulation of higher levels of ginsenosides when 0.5 mg/l gibberellic acid (GA) was added to the medium. However, in the hairy root cultures of *Artemisia annua*, GA greatly stimulated the increase of fresh weight, mainly due to the enhanced branching but inhibited the production of alkaloids (Liu et al. 1997). Hwang et al. (1999) also studied the effect of putrescine, spermidine, and spermine on growth and ginsenoside content, and these hormones did not promote growth of hairy roots or accumulation of ginsenosides. In case of the roots of *Duboisia myoporoides*, the addition of putrescine and spermidine led to the increased production of scopolamine (Yoshika et al. 1989). Therefore, the differential effect of phytohormones on growth and secondary metabolite accumulation is thought to be related to the genotype and physicochemical characteristics of the explants.

Effect of Medium Salt Strength on Biomass and Metabolite Production

The culture medium and salt strength influence the growth, physiology, and metabolism of in vitro cultured explants (Murthy et al. 2014e). Therefore, the selection appropriate medium and salt concentration of culture medium are very much essential. Sivakumar et al. (2005) studied the effect of 0.50, 0.75, 1.0, and 1.50 salt strength of MS medium on hairy root growth and ginsenoside productivity. Salt strength of 0.75 was optimal for ginseng hairy root growth as compared to other

Table 11.1 Optimization of salt strength of Murashige and Skoog medium for ginseng hairy root growth and ginsenoside production

Salt strength	Biomass		Percentage DW	Growth rate	Ginsenoside (mg/g)	Ginsenoside yield (mg/l)
	Fresh weight (g)	Dry weight (g)				
0.50	15.6 ± 0.43	1.22 ± 0.02	7.82	5.73	13.20 ± 0.25	161.04
0.75	18.7 ± 0.30	1.37 ± 0.01	7.33	6.43	9.78 ± 0.30	133.99
1.00	16.9 ± 0.12	1.20 ± 0.01	7.10	5.63	6.82 ± 0.81	81.84
1.50	14.2 ± 0.50	1.04 ± 0.2	7.32	4.88	5.65 ± 1.12	58.76

Data collected after 5 weeks culture using 400 ml of conical flask containing 100 ml of MS medium

Growth rate was calculated from increase in dry weight. Values are quotients of dry weight after cultivation and dry weight of the inoculum

Mean values are with standard error of three replicates

media (growth rate 6.43; Table 11.1), whereas, 0.5 strength MS medium was responsible for maximum ginsenoside production (161.04 mg/l; Table 11.1). A full strength MS medium was suitable for cell suspension cultures of *Gymnema sylvestre* for biomass accumulation and gymnemic acid production (Nagella et al. 2011). Similarly, a full strength MS medium was appropriate for growth of cells and secondary metabolite accumulation in *Withania somnifera* (Praveen and Murthy 2010).

Effect of Carbohydrates on Biomass and Metabolite Production

Sucrose, glucose, fructose, glucose + fructose, and sucrose + glucose were tested on ginseng hairy root growth and ginsenoside productivity by Sivakumar et al. (2005), and they found sucrose as a suitable carbohydrate. They also verified the effect of different concentrations of sucrose in range 1–9% and reported that hairy root growth, biomass accumulation increased with increase in sucrose concentration, whereas low sucrose concentration such as 2% was suitable for ginsenoside production (Table 11.2). The maximum ginsenoside content (8.01 mg/g DW) and ginsenoside productivity were obtained with the 2% sucrose. Sucrose at 1–3% was favorable for both Rg and Rb group of ginsenosides. Among various ginsenosides, the contents of Rg_1, Rb_1, Rb_2, and Rd decreased compared to other ginsenosides (Table 11.3). Based on such results, they followed two-stage culture system for ginseng hairy root culture; during the growth stage, higher sucrose concentration was used, while during the ginsenoside production stage, a relatively lower concentration of sucrose (2%) was maintained. The level of sucrose has been shown to affect the growth, development, and metabolism of transformed roots (Wang and Weathers 2007). For instance, 3% sucrose was found to be optimal for biomass accumulation, and 4% sucrose favored the production withanolide A in the tested concentrations (1–8%) with hairy root cultures of *Withania somnifera* (Praveen and Murthy 2012). Similarly, 4–6% of

Table 11.2 Effect of sucrose concentration in Murashige and Skoog medium on ginseng hairy root growth and ginsenoside production

| Sucrose (%) | Biomass | | Percentage dry weight | Growth rate | Ginsenosides (mg/g dry weight) | | | Ginsenoside yield (mg/l) |
	Fresh weight (g)	Dry weight (g)			Rg	Rb	Total	
1	15.40 ± 0.04	0.69 ± 0.01	4.51	4.14	3.00	4.13	4.13	49.20
2	21.50 ± 0.50	1.30 ± 0.22	5.59	7.74	2.80	5.22	5.22	104.13
3	23.29 ± 0.40	1.31 ± 0.01	5.63	7.80	3.20	5.30	5.30	85.54
5	22.50 ± 0.03	1.83 ± 0.01	8.15	10.92	1.70	3.28	3.28	91.13
7	21.10 ± 0.80	1.90 ± 0.05	8.60	11.31	1.12	1.56	1.56	50.73
9	19.80 ± 0.05	2.09 ± 0.06	10.56	12.44	1.02	0.74	0.74	36.99

Data collected after 5 weeks culture using 400 ml of conical flask containing 100 ml of MS medium
Growth rate was calculated from increase in dry weight. Values are quotients of dry weight after cultivation and dry weight of the inoculum
Mean values are with standard error of three replicates

Table 11.3 Effect of sucrose concentration supplemented to Murashige and Skoog medium on accumulation of ginsenosides in ginseng hairy roots

| Sucrose (%) | Ginsenosides (mg/g dry weight) | | | | | | | Ratio of Rb/Rg |
| | Rg group | | | Rb group | | | | |
	Rg_1	Re	Rf	Rb_1	Rc	Rb_2	Rd	
1	2.54	0.32	0.12	2.27	0.62	0.77	0.47	1.38
2	2.50	0.21	0.08	2.24	0.77	1.09	1.06	1.86
3	2.29	0.19	0.05	1.30	0.54	1.18	1.01	1.66
5	1.53	0.13	0.04	1.09	0.61	0.91	0.67	1.93
7	0.99	0.09	0.04	0.59	0.30	0.44	0.23	1.39
9	–	0.08	0.05	0.14	0.31	0.03	0.27	0.73

Data collected after 5 weeks culture using 400 ml of conical flask containing 100 ml of MS medium
Mean values are with standard error of three replicates

sucrose was found to be optimal for accumulation of steroidal alkaloids in hairy root cultures of *Solanum aviculare* (Yu et al. 1996), and 3% of sucrose was beneficial for accumulation of gymnemic acid with hairy root cultures of *Gymnema sylvestre* (Nagella et al. 2011), respectively.

Effect of pH on Biomass and Metabolite Production

The hydrogen ion concentration of the culture medium is also one of the factors influencing the growth of cultured cells and organs and productivity of secondary metabolites (Murthy et al. 2014e). The medium pH is usually set at 5.6, and extreme pH values are avoided. The concentration of hydrogen ions in the medium changes

Table 11.4   Influence of medium pH on hairy root growth and ginsenoside production

pH	Biomass dry weight (g)	Growth rate	Ginsenosides (mg/g dry weight)	Ginsenoside yield (mg/l)
3.0	1.07 ± 0.03	6.37	15.6 ± 0.98	166.92
4.0	1.14 ± 0.08	6.77	17.0 ± 2.26	193.80
5.0	1.18 ± 0.02	7.01	18.5 ± 0.35	218.30
6.0	1.25 ± 0.01	7.44	19.1 ± 0.56	238.85
6.5	1.21 ± 0.02	7.22	19.8 ± 0.98	239.68
7.0	1.02 ± 0.02	6.01	13.2 ± 1.10	134.64

Data collected after 5 weeks culture using 400 ml of conical flask containing 100 ml of MS medium

Growth rate was calculated from increase in dry weight. Values are quotients of dry weight after cultivation and dry weight of the inoculum

Mean values are with standard error of three replicates

during culture period; this is due to uptake of nutrients by the cultured explants or the accumulation of metabolites (McDonald and Jackman 1989). Sivakumar et al. (2005) studied the effects of initial medium pH on ginseng hairy root growth and ginsenoside production in MS medium, and they reported that the maximum growth rate (7.44) and optimal ginsenoside productivity (239.68 mg/l) were obtained at 6.0 and 6.5, respectively. They have observed the inhibition of hairy root growth and ginsenoside production when initial pH was below 4.0 or above 7.0 (Table 11.4). In *Withania somnifera* hairy root cultures, the initial medium pH 5.8 favored biomass accumulation (12.1 g/l DW), and a medium pH of 6.0 favored accumulation of withanolide A in roots (13.84 mg/g DW; Praveen and Murthy 2012). In hairy root cultures of *Tagetes patula*, a medium pH of 5.7 was suitable for the growth and accumulation of thiophene (Mukundan and Hjortso 1991).

Effect of Temperature and Light on Biomass and Metabolite Production

Temperature and light are the bioprocess parameters affecting suspension cultures (Murthy et al. 2014e). It has been shown that the optimal temperature treatment of suspension cultures is necessary for accumulation of biomass and production of metabolites (ten Hoopen et al. 2002; Zhong and Yoshida 1993). The stimulatory effect of light on biomass growth and formation of secondary metabolites was shown in red beet (*Beta vulgaris*; Shin et al. 2004) and Chinese basil (*Ocimum basilicum*; Zhong et al. 1991). Whereas, light has an inhibitory effect on metabolite accumulation in purple gromwell (*Lithospermum erythrorhizon*; Tabata et al. 1974). Yu et al. (2005) verified the effect of temperature on ginseng hairy root growth under different temperature regimes 13/20, 20/13, 25/25, and 30/25 °C day and night cycles and obtained highest hairy root growth with cultures incubated at

Table 11.5 Effect of incubation temperature (with 16 h/8 h day/night cycles) on growth and ginsenoside production of ginseng hairy roots cultivated in 5 l bioreactors containing 4 l of medium for 4 weeks

Growth temperature (°C)	Biomass		Growth rate	Ginsenoside (mg/g dry weight)	Ginsenoside yield (mg/l)
	Fresh weight (g)	Dry weight (g)			
13/20	431 ± 1.0	28 ± 1.0	19.7	4.5 ± 0.1	31.5 ± 1.5
20/13	892 ± 0.9	65 ± 0.8	45.8	8.2 ± 0.1	133.9 ± 0.9
25/25	889 ± 0.6	51 ± 0.7	35.9	10.5 ± 0.1	133.4 ± 1.2
30/25	764 ± 0.8	64 ± 0.9	45.1	6.4 ± 0.1	71.6 ± 0.5

Mean values are with standard error of three replicates
Growth rate was calculated from increase in dry weight. Values are quotients of dry weight after cultivation and dry weight of the inoculum

Table 11.6 Effect of light quality on growth and ginsenoside production of ginseng hairy roots cultivated in 5 l bioreactors containing 4 l of medium for 4 weeks

Light source	Biomass		Growth rate	Ginsenosides (mg/g dry weight)			Ginsenoside yield (mg/l)
	Fresh weight (g)	Dry weight (g)		Rg	Rb	Rb/Rg	
Dark	270 ± 1.0	24 ± 0.6	11.4	2.8 ± 0.2	4.5 ± 0.2	1.6 ± 0.1	27.8 ± 1.0
Fluorescent light	226 ± 0.8	21 ± 0.6	10.1	5.3 ± 0.1	3.7 ± 0.7	0.7 ± 0.1	30.2 ± 0.9
Metal halide light	193 ± 1.1	19 ± 0.3	8.9	3.5 ± 0.4	3.4 ± 0.3	0.9 ± 0.2	23.3 ± 0.2
Blue light	236 ± 0.2	24 ± 0.9	11.3	3.8 ± 0.4	3.9 ± 0.5	1.0 ± 0.1	26.6 ± 0.4
Red light	284 ± 0.9	25 ± 1.0	11.6	3.1 ± 0.8	4.1 ± 0.7	1.3 ± 0.1	20.9 ± 0.4
Blue plus red lights	183 ± 0.9	21 ± 0.9	10.1	3.4 ± 0.1	2.9 ± 0.2	0.8 ± 0.2	24.2 ± 0.7

Mean values are with standard error of three replicates
Growth rate was calculated from increase in dry weight. Values are quotients of dry weight after cultivation and dry weight of the inoculum

20/13 °C, whereas highest ginsenoside accumulation was with cultures incubated at 25/25 °C (10.5 mg/g DW; Table 11.5). Likewise, Yu et al. (2005) tested the effect of fluorescent light, metal halide light, blue light, red light, and blue plus red light on growth and accumulation of ginsenosides in ginseng hairy root cultures and showed optimal growth of ginseng hairy roots with red light treatment (Table 11.6). They observed positive effect of fluorescent light on accumulation of ginsenosides (9.0 mg/g DW) compared to dark treatment (8.4 mg/g DW; Table 11.6). They also detected differential accumulation of Rb and Rg groups of ginsenosides with dark or light treatments. Rb group ginsenosides were highest in the dark-grown cultures (4.5 mg/g DW), and the production of Rg group ginsenosides was optimal in the light-grown cultures (fluorescent light, 5.3 mg/g DW). Therefore, control of ginsenoside accumulation in hairy root cultures is possible with varied light and dark treatments.

Effect of Elicitors on Biomass and Metabolite Production

Hairy root cultures have been established in various laboratories for the production of ginsenosides; however, the ginsenoside content in these hairy root lines was consistently low. Therefore, elicitation treatment of hairy root cultures has been widely studied for the overproduction of ginsenosides. Yu et al. (2000) tested effect of jasmonic acid as an elicitor in the range of 1.0–5.0 mg/l. Jasmonic acid strongly inhibited ginseng hairy root growth and biomass accumulation; however, it strongly improved ginsenoside production (Table 11.7). They have reported fourfold increment in ginsenoside content (58.65 mg/g DW), when compared to control (15.85 mg/g DW). Among ginsenosides, the Rb group showed an increase, while the Rg group was stable (Table 11.8). Rb_1 and Rb_2 ginsenosides increased 4.6 and 7.7 times, respectively, whereas other ginsenosides increased marginally as compared to control (Table 11.8). Based on their results, Yu et al. (2000) suggested two-stage culture system for ginseng hairy root culture. In the first stage a medium

Table 11.7 Effect of jasmonic acid on growth and ginsenoside production of ginseng hairy roots after 5 weeks of culture

| Jasmonic acid (mg/l) | Biomass | | | Ginsenosides (mg/g dry weight) | | | Ratio of Rb/ Rg | Ginsenoside yield (mg/l) |
	Fresh weight (g)	Dry weight (g)	Growth rate	Rb	Rg	Total		
0.0	30.2a	1.52a	7.12	10.31d	5.51a	15.85d	1.92d	240.92d
1.0	24.5b	1.31b	6.12	30.08c	5.87a	35.98c	5.14c	471.34c
2.0	20.0c	1.08c	5.04	41.59b	6.05a	47.69b	7.24b	515.05b
5.0	14.1d	0.86d	4.04	59.98a	5.60a	58.65a	9.28a	504.39a

Mean values followed by different letters within a column are significantly different at $P \leq 0.05$ by Duncan's multiple range test. Each treatment was repeated three times
Growth rate was calculated from increase in dry weight. Values are quotients of dry weight after cultivation and dry weight of the inoculum

Table 11.8 Effect of jasmonic acid on production of different ginsenosides in ginseng hairy roots after 5 weeks of culture

| Jasmonic acid (mg/l) | Ginsenosides (mg/g dry weight) | | | | | | | |
| | Rg group | | | Rb group | | | | |
	Rg_1	Re	Rf	Rb_1	Rc	Rb_2	Rd	Total
0.0	1.63ab	3.02a	0.87a	7.23d	1.20b	1.13d	0.11c	15.85d
1.0	1.85a	3.49a	0.52a	18.99c	5.56a	3.34c	2.3b	35.98c
2.0	1.73ab	3.69a	0.63a	24.09b	7.26a	4.62b	4.6a	47.69b
5.0	1.34b	3.74a	0.61a	33.70a	6.19a	8.80a	4.3a	58.65a

Mean values followed by different letters within a column are significantly different at $P \leq 0.05$ by Duncan's multiple range test. Each treatment was repeated three times

without elicitor facilitates the growth of hairy roots, while in the second stage the hairy root biomass would be transferred to fresh medium containing jasmonic acid, which triggers the accumulation of ginsenoside content. Palazon et al. (2003a) followed such suggestions and established ginseng hairy root cultures using selected clones, namely, C-M, HR-M, and T-M. They introduced methyl jasmonate (22.4 mg/l) as elicitor during progressive declaration growth phase, i.e., on day 25 of culture and obtained positive response. The root lines C-M, HR-M, and T-M accumulated 2, 1.8, and 4 times higher ginsenoside content compared to control cultures with elicitor treatment. Kim et al. (2013) reported the accumulation of novel ginsenoside such as Rg_3, with methyl jasmonate treatment of ginseng hairy root culture, and this metabolite is not present naturally in ginseng. Choi et al. (2005) and Kim et al. (2009) utilized the ginseng hairy root cultures which were treated with methyl jasmonate for the analysis of gene transcripts and to identify genes involved in biosynthesis of ginsenosides. Liang et al. (2015) evaluated the effect of Tween 80 permeabilization on ginsenoside secretion in *Panax ginseng* hairy root cultures and reported that with the use of 1.2% (w/v) Tween 80 for 25 days; approximately 76% of the total ginsenosides was released into the surrounding medium. Recently, Zhang et al. (2015) cloned α-L-rhamnosidase gene from *Bifidobacterium breve* into ginseng hairy roots for the enhanced accumulation of Rg_1 ginsenoside in the hairy roots. Ge et al. (2014) tested the efficacy of ginseng hairy roots in biotransformation and produced novel alkaloidal glycosides using tetrahydroprotoberberines as substrates. Similarly, Chen et al. (2008) used hairy roots of ginseng for regioselective glycosylation of hydroxybenzoic acids into their glycosides and glycosyl esters.

Establishment of Hairy Root Suspension Cultures in Bioreactors

Jeong et al. (2003) tested the growth characteristics of ginseng hairy roots in various bioreactors such as stirred bioreactors (1-l capacity with 800 ml working volume) and bubble column bioreactors (3-l, 5-l and 19-l capacity with 2.5-l, 4-l and 17-l working volume, respectively) and obtained hairy root growth of about 55-fold of inoculum after 39 days in 5-l bioreactor and 38-fold of inoculum after 40 days in a 19-l bioreactor. Palazon et al. (2003b) tested the effect of three variables, namely, the bioreactor system (2-l wave or 3-l spray reactor), medium exchange, and culture period of ginseng hairy roots (line T12), for the production of ginsenosides. Among the rectors, the wave bioreactor found to be more efficient in promoting hairy root growth. In wave reactor with medium exchange every 14 days over a culture period of 56 days, there was 28-fold increment of inoculum, giving a root biomass of 284.9 g/l and a ginsenoside content 145.6 mg/l. Yu et al. (2003) tested 10 l drum-type airlift bioreactors containing 8 l of working capacity with

aeration rate of 0.1 vvm (air volume/medium volume/minute) for cultivation of ginseng hairy roots and obtained fresh biomass 1670 g of fresh biomass (40 g of initial inoculum) and 109 g of dry biomass with growth yield of 76.8. The total ginsenoside content was 14.65 mg/g DW with ginsenoside productivity of 199.6 mg/l. Thus cultivation of ginseng hairy roots in airlift bioreactors is highly promising for the production of ginsenosides.

Conclusion and Future Perspectives

Hairy root cultures of ginseng have demonstrated great promise in terms of biomass accumulation and production of ginsenosides. Research developments on ginseng hairy root cultures have demonstrated that selection of superior clones/lines, establishment of suspension culture, optimization medium ingredients, culture conditions, and elicitation have been worked out successfully. Even bioreactor cultures have been initiated; however, important parameters such as selection of suitable bioreactor type, inoculum density, agitation/aeration, nutrient feeding, and precursor feeding have not been worked out, and future research efforts should be focused in these areas. Assessment of ginsenoside biosynthetic pathway and application of metabolic engineering are also desirable to obtain useful metabolites from ginseng hairy root cultures.

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