M. Naeem · Tariq Aftab
M. Masroor A. Khan *Editors*

Catharanthus roseus

Current Research and Future Prospects

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Editors
M. Naeem
Plant Physiology Section
Department of Botany
Aligarh Muslim University
Aligarh, UP, India

Tariq Aftab
Plant Physiology Section
Department of Botany
Aligarh Muslim University
Aligarh, UP, India

M. Masroor A. Khan
Plant Physiology Section
Department of Botany
Aligarh Muslim University
Aligarh, UP, India

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Contributors

Harshita Abul Department of Pharmaceutics, College of Pharmacy, SGIT, Ghaziabad, U.P., India

Tariq Aftab Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Bilal Ahmad Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

M. Masidur Alam Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Department of Botany, Malda College, Malda, West Bengal, India

Akbar Ali Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Muzamil Ali Cellular Differentiation and Molecular Genetics Section, Department of Botany, Hamdard University, New Delhi, India

Petra Andrade-Hoyos Procesos Naturales, Ingeniería Forestal Comunitaria, Universidad Intercultural del Estado de Puebla, Calle Principal a Lipuntahuaca S/N; Lipuntahuaca, Huehuetla, Puebla, México

Matin ArefiFard Biological Department, Arak University-Islamic Azad University of Arak, Arak, Iran

S. Bala Abirami Department of Biotechnology, PRIST University, Thanjavur, Tamilnadu, India

Md. Abul Barkat Department of Pharmaceutics, Nanomedicine Research Lab, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India

Department of Pharmaceutics, Faculty of Pharmacy, Integral University, Kursi Road, Lucknow, India

Hebert Jair Barrales-Cureño Procesos Naturales, Ingeniería Forestal Comunitaria, Universidad Intercultural del Estado de Puebla, Calle Principal a Lipuntahuaca S/N; Lipuntahuaca, Huehuetla, Puebla, México

Ramesh Bellamkonda Department of Food Technology, Vikramasimhapuri University, Nellore, Andhra Pradesh, India

Sasi Bhusana Rao Bongu Department of Biochemistry, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India

Salvador Chávez-Salinas Departamento de Ingeniería en Biotecnología, Universidad Politécnica del Valle de Toluca, Santiaguito Tlalcilalcali, Almoloya de Juárez, México

Saralakumari Desireddy Department of Biochemistry, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India

V. Devi Rajeswari Department of Biomedical Sciences, School of Biosciences and Technology, VIT University, Vellore, Tamilnadu, India

R. Elangomathavan Department of Biotechnology, PRIST University, Thanjavur, Tamilnadu, India

Sudhakara Gujjala Department of Biochemistry, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India

Mohd. Idrees Department of Mathematics and Sciences, College of Arts and Applied Sciences, Dhofar University, Salalah, Sultanate of Oman

Hassan Jaleel Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Jagjit Kaur Department of Biotechnology, Maharishi Markandeshwar University, Mullana, Haryana, India

Amjad Khalil Department of Life Sciences, King Fahad University of Petroleum and Minerals, Dhahran, Saudi Arabia

M. Masroor A. Khan Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Shamshad Ahmad Khan Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune, Maharashtra, India

Kuldeep Kumar Department of Biotechnology, Multani Mal Modi College, Patiala, Punjab, India

Luis Germán López-Valdez Laboratorio de Productos Naturales, Área de Química, Departamento de Preparatoria Agrícola, Universidad Autónoma Chapingo, Texcoco Estado de, México

Alfonso Luna-Cruz Catedrático CONACYT, Facultad de Ciencias Agropecuarias, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, Mexico

Ajay Kumar Mathur Plant Biotechnology Division, Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, Uttar Pradesh, India

A. Mujib Cellular Differentiation and Molecular Genetics Section, Department of Botany, Hamdard University, New Delhi, India

M. Naeem Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Qifang Pan Plant Biotechnology Research Center, SJTU-Cornell Institute of Sustainable Agriculture and Biotechnology, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, People's Republic of China

Sanjeev Pandey Department of Botany, Banwarilal Bhalotia College, Asansol, West Bengal, India

Varsha Parasharami Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune, Maharashtra, India

Teena Pathak Department of Biotechnology, Multani Mal Modi College, Patiala, Punjab, India

S. Patharajan Department of Biotechnology, PRIST University, Thanjavur, Tamilnadu, India

Mallaiah Putakala Department of Biochemistry, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India

Md. Akhlaquer Rahman Department of Pharmaceutics, Faculty of Pharmacy, Integral University, Lucknow, India

S. Ramesh Department of Microbiology, PRIST University, Thanjavur, Tamilnadu, India

M.V. Rao Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Karuna Rasineni Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, USA

Cesar Reyes-Reyes Departamento de Ingeniería en Biotecnología, Universidad Politécnica del Valle de Toluca, Santiaguito Tlalcilalcali, Almoloya de Juárez, México

Yawar Sadiq Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Saisree Sagree Department of Biochemistry, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India

D. Sathya Prabhu Department of Biomedical Sciences, School of Biosciences and Technology, VIT University, Vellore, Tamilnadu, India

P. Senbagalakshmi Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

T. Senthil Kumar Department of Industry University Collaboration, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Asfia Shabbir Plant Physiology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Sreenivasa Reddy Singareddy Biochemistry Division, National Institution of Nutrition, Hyderabad, Telangana, India

Apoorva Singh Department of Biotechnology, Multani Mal Modi College, Patiala, Punjab, India

V. Subhashini Department of Environmental Sciences, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India

A.V.V.S. Swamy Department of Environmental Sciences, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India

Kexuan Tang Plant Biotechnology Research Center, SJTU-Cornell Institute of Sustainable Agriculture and Biotechnology, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, PR China

Dipti Tonk Cellular Differentiation and Molecular Genetics Section, Department of Botany, Hamdard University, New Delhi, India

Moin Uddin Botany Section, Women's College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Israr Ul Hassan Department of Mathematics and Sciences, College of Arts and Applied Sciences, Dhofar University, Salalah, Sultanate of Oman

Priyanka Verma Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Pune, Maharashtra, India

Nadia Zafar Cellular Differentiation and Molecular Genetics Section, Department of Botany, Hamdard University, New Delhi, India

About the Editors

M. Naeem is an Assistant Professor in the Department of Botany at Aligarh Muslim University, India. For more than a decade, he has devoted his research to improving the yield and quality of commercially important medicinal and aromatic plants (MAPs). His research focuses on escalating the production of MAPs and their active principles using a novel and safe technique involving radiation-processed polysaccharides as well as the application of potent PGRs. To date, he has successfully run three major research projects as Principal Investigator, two of which were sanctioned by the Department of Science & Technology, New Delhi. He also completed another intriguing research project on *Catharanthus roseus* awarded by CSTUP, Lucknow. Dr. Naeem has published seven books and more than 80 research papers in reputable national and international journals. He has also participated in various national and international conferences and acquired life memberships to various scientific bodies in India and abroad. Based on his research contributions, Dr. Naeem has been awarded a Research Associateship from the Council of Scientific & Industrial Research, New Delhi; a Young Scientist Award (2011) from the State Government of Uttar Pradesh; a Fast Track Young Scientist Award from the Department of Science & Technology, India; a Young Scientist of the Year Award (2015) from the Scientific and Environmental Research Institute, Kolkata; and a Rashtriya Gaurav Award (2016) from the International Friendship Society, New Delhi.

Tariq Aftab received his Ph.D. in the Department of Botany at Aligarh Muslim University, India, and is currently an Assistant Professor there. He is the recipient of a prestigious Leibniz-DAAD fellowship from Germany, a Raman Fellowship from the Government of India, and Young Scientist Awards from the State Government of Uttar Pradesh and Government of India. He is also a member of various scientific associations from India and abroad. He has worked as Visiting Scientist at IPK, Gaterleben, Germany, and in the Department of Plant Biology, Michigan State University, USA. He has published research articles in several peer-reviewed national and international journals and is the lead editor of the book *Artemisia annua:* Pharmacology and Biotechnology. His research interests include physiological, proteomic, and molecular studies on medicinal and aromatic plants.

M. Masroor A. Khan is a Professor in the Department of Botany at Aligarh Muslim University, India. After completing his Ph.D., he worked as a postdoctoral fellow at the Ohio State University, USA (1987–1988). Then he worked as Pool Scientist for the Council of Scientific and Industrial Research and as a Research Associate for CSIR and the University Grants Commission before he joined AMU as an Assistant Professor. In his 30 years of teaching experience at the university level, he has guided 8 Ph.D., 2 M.Phil., and 27 M.Sc. students. For the convenience of his students, he has also developed study materials and launched more than 100 class notes and PowerPoint presentations on his website. Prof. Khan has run six research projects sponsored by national and international funding agencies and has published about 120 research papers in reputable journals and books. In his research, Prof. Khan is working to promote the productivity and active ingredients of medicinal and aromatic plants using different strategies such as the application of mineral nutrition, PGRs, and nanoparticles. Prof. Khan has contributed greatly toward the establishment of Radiation-Processed Polysaccharides (RPPs) as plant growth promoters. In collaboration with the Bhabha Atomic Research Centre, Mumbai, he is researching the appropriate doses of RPPs that would help farmers increase the productivity of medicinal and aromatic plants and their active constituents. Another field of his research is nanoparticles, some of which he has found can enhance the essential oil production of some aromatic plants. He has also filed a patent in this regard.

Strategies for Enhancing Alkaloids Yield in *Catharanthus roseus* Via Metabolic Engineering Approaches

Kexuan Tang and Qifang Pan

Abstract As the only source for the low-abundance antitumor agents vinblastine and vincristine, *Catharanthus roseus* is highly valued for its diversity of more than 130 monoterpenoid indole alkaloids (MIAs) and has been studied extensively as a model for medicinal plants improvement. However, the low yield increases the cost and limits the industrial production of these valuable MIAs in medical use. The biosynthesis of these MIAs is a complex multistep enzymatic network that is tightly regulated by developmental and environmental factors. Many genes encoding constitutive structural biosynthetic enzymes, transcription factors, and transporters involved in these pathways have been cloned and characterized. To improve the MIA production, a couple of approaches have been carried on the plants, hairy roots, and cell culture of *C. roseus*, as well as on heterogeneous plant (like *Nicotiana benthamiana*), including abiotic and biotic methods. The main strategies for enhancing alkaloids yield is to genetically modify the MIA pathway and enhance the metabolic flux to MIA production via metabolic engineering strategies. Here, we will review the past decades' efforts on the MIA production.

Keywords Alkaloids • *Catharanthus roseus* • Genes • MIAs pathways

1 Introduction

Monoterpenoid indole alkaloids (MIAs) are important alkaloids for their medicinal bioactivities of highly value. *Catharanthus roseus* is the main and natural source to produce these valuable MIAs, including the antitumor drugs vinblastine and

K. Tang (✉) • Q. Pan
Plant Biotechnology Research Center, SJTU-Cornell Institute of Sustainable Agriculture and Biotechnology, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, PR China
e-mail: kxtang@sjtu.edu.cn; panqf@sjtu.edu.cn

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vincristine. The trace amount of useful MIAs in *C. roseus* forces great efforts to improve their production via various methods. Metabolic engineering is an approach to modify metabolic pathways and metabolites production via gene transfer technology. With the increasing knowledge of the MIA pathway and its biosynthetic genes, metabolic engineering is widely performed on MIA biosynthesis in *C. roseus* to boost the yields of targeted MIAs and their analogs.

1.1 Natural Compound-Targeted Engineering Strategies

Different types of genes cloned from the MIA pathway have been overexpressed in the cells, hairy roots, and plants of *C. roseus*, which showed different effects on MIA biosynthesis as well as at the level of the full metabolome (Table 1).

1.1.1 Structural Genes

MIA biosynthesis in *C. roseus* is a complex pathway including more than 35 coordinately regulated enzymatic steps producing at least 35 known intermediates (Fig. 1). Up to now, 30 structural genes of the key biosynthetic steps from MIA pathways have been cloned and identified. They are the first target genes used in metabolic engineering of *C. roseus*.

Genes from the Tryptophan Pathway

The expression of a more tryptophan inhibition-resistant Arabidopsis *ASα* enzyme coupled with a glucocorticoid-inducible promoter in *C. roseus* hairy roots dramatically increased tryptophan and tryptamine yields but not of MIAs, except lochnericine, after induction with 3 μM dexamethasone (Hughes et al. 2004a). Transgenic hairy roots expressing both *ASα* and *ASβ* subunits produced more tryptamine and showed a greater resistance to feedback inhibition of AS activity by tryptophan than those only expressing *ASα* (Hong et al. 2006). When fed with the terpenoid precursors 1-deoxy-D-xylulose, loganin, and secologanin, respectively, hairy roots overexpressing *ASα* or *Asβ* could increase the levels of hörhammericine, catharanthine, ajmalicine, lochneriricine, and tabersonine (Peebles et al. 2006). As a side effect, the metabolic flux into the flavonoid pathway was also transiently increased when the *AS* overexpressing hairy roots were induced by 0.2 μM dexamethasone, which caused increases of catechin and naringin in hairy roots (Chung et al. 2007). This might be due to the induction of the phenylalanine/tyrosine pathway by tryptophan (Verpoorte and Alfermann 2000). TDC overexpression in *C. roseus* transgenic calli results in increased tryptamine levels but not in increased MIA production (Goddijn et al. 1995), neither in *C. roseus* cell cultures (Whitmer et al. 2002b). On the contrary, no increase of tryptamine

Table 1 Overexpression of genes involved in MIA biosynthesis in the cell cultures, hairy roots, and plants of *Catharanthus roseus*

Varieties	Genes	Metabolites significantly affected in levels	References
Hairy roots	*GmMYBZ2*	Catharanthine	Zhao et al. (2001)
	HMGR	Campesterol, serpentine, ajmalicine, catharanthine	Ayora-talavera et al. (2002)
	ASα	Tryptophan, tryptamine, lochnericine	Hughes et al. (2004a)
	TDC	Serpentine	Hughes et al. (2004b)
	ASα+ TDC	Tryptamine	
	ASα	Tryptamine	Hong et al. (2006)
	ASα +ASβ	Tryptophan, tryptamine	
	ASα +ASβ+TDC	Tryptamine	
	ASαβ	Naringin, catechin, salicylic acid	Chung et al. (2007)
	DAT	Hörhammericine	Magnotta et al. (2007)
	ORCA2	Catharanthine, vindoline	Liu et al. (2011)
	G8O(G10H)	Catharanthine	Wang et al. (2010)
	G8O+ORCA3	Catharanthine	
Cell cultures	*ORCA3*	Serpentine, ajmalicine, tabersonine, hörhammericine	Peebles et al. (2009)
	DXS	Ajmalicine, serpentine, lochnericine, tabersonine, hörhammericine	Peebles et al. (2010)
	G8O	–	
	Asα	Tryptophan, tryptamine, lochnericine, tabersonine, hörhammericine	
	DXS +G8O	Ajmalicine, tabersonine, lochnericine, hörhammericine	
	DXS+ Asα	tryptamine, tabersonine, lochnericine, hörhammericine, tryptophan	
	TDC	Tryptamine	Canel et al. (1998); Whitmer et al. (2002b)
	STR	Strictosidine, ajmalicine, catharanthine, serpentine, tabersonine	Canel et al. (1998); Whitmer et al. (2002a)
	PRX1	Ajmalicine, serpentine, H_2O_2	Jaqqi et al. (2011)
	ORCA3	Tryptophan, tryptamine	Van der Fits and Memelink (2000)
	CYP76B6	10-hydroxy geraniol	Collu et al. (2001)
	CjMDR1	Ajmalicine, tetrahydroalstonine	Pomahacova et al. (2009)

(continued)

Table 1 (continued)

Varieties	Genes	Metabolites significantly affected in levels	References
Plant	*DAT*	Vindoline	Wang et al. (2012)
	ORCA3	Vindoline, catharanthine	Pan et al. (2012)
	ORCA3 + *G10H(G8O)*	Strictosidine, vindoline, catharanthine	

but a 129% increase of serpentine was noted on induction of 3 μM dexamethasone in hairy roots overexpressing *TDC* (Hughes et al. 2004b). Expressing *TDC* from *C. roseus* in cell cultures or plants of *Nicotiana tabacum* resulted in the formation of tryptamine up to 10 μg/g FW and 18–66 μg/g FW, respectively (Hallard et al. 1997). When co-overexpressing *AS* and *TDC* in hairy roots, an enhanced ability to produce tryptamine was observed, but only a transiently increased accumulation of tabersonine and lochnericine among all measured alkaloids (Hughes et al. 2004b; Hong et al. 2006). To study the effect of introducing MIA alkaloid biosynthetic genes in a plant normally only producing secologanin, Hallard and coworkers (Hallard 2000) introduced both the *TDC* and *STR* into *Weigelia* hairy roots. Compared to normal roots no more secologanin could be observed, whereas tryptamine, ajmalicine, and serpentine could be detected in the hairy roots. This confirmed the presence of a glucosidase able to hydrolyze strictosidine. Though the alkaloid levels were very low, it shows that MIAs can also be made in non-alkaloid-producing plants. That means alternative crops for making MIA. In that context also the production of strictosidine was achieved in yeast cells in which STR and SGD are overexpressed and which are fed with secologanin and tryptamine (Geerlings et al. 2001). The cells could produce 3 g/L of strictosidine in 3 days, many times more than ever achieved in plant cell cultures. As STR was mainly excreted to the medium, whereas SGD was in the cells, grinding the whole culture resulted in the production of cathenamine. The transgenic yeast cells could be grown on the juice pressed out of the berries of *Symphoricarpus albus* rich in sugar and secologanin from which strictosidine was made after feeding tryptamine (Geerlings et al. 2001).

Fig. 1 (Continued) 4-diphosphate synthase, *IDI* isopentenyl diphosphate isomerase, *GPPS* GPP synthase, *GES* geraniol synthase, *G8O* geraniol 8-oxidase, *8-HGO* 8-hydroxygeraniol oxidoreductase, *IS* iridoid synthase, *IO* iridoid oxidase, *7-DLGT* 7-deoxyloganetic acid-*O*-glucosyltransferase, *7DLH* 7-deoxyloganic acid hydroxylase, *LAMT* loganic acid-O-methyltransferase, *SLS* secologanin synthase, *AS* anthranilate synthase, *TDC* tryptophan decarboxylase, *STR* strictosidine synthase, *SGD*, strictosidine-β-D-glucosidase, *T16H*, tabersonine 16-hydroxylase, *16OMT* O-methyltransferase, *NMT N*-methyltransferase, *D4H* desacetoxyvindoline-4-hydroxylase, *DAT*, deacetylvindoline-4-*O*-acetyltransferase, *Prx1* α-3′,4′-anhydrovinbastine synthase (AVLBS)

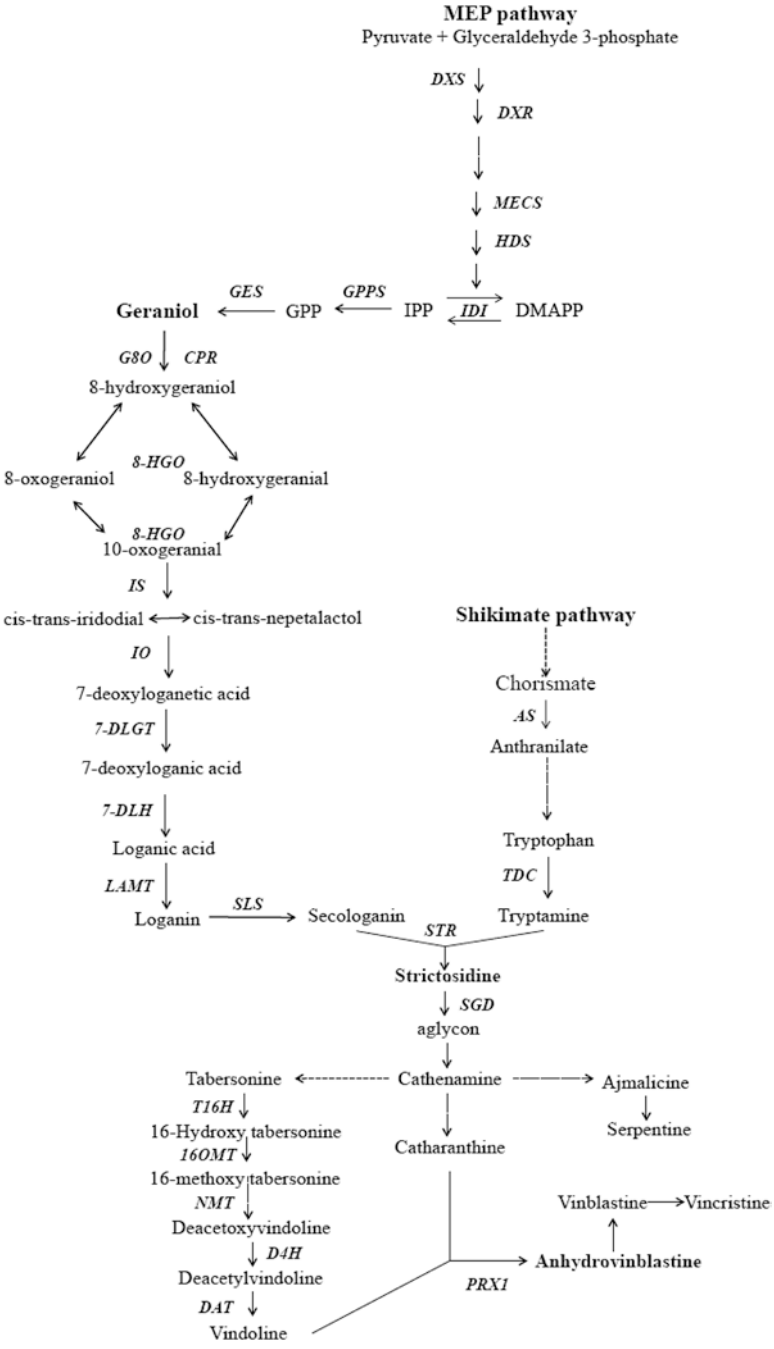

Fig. 1 The scheme of MIA biosynthesis pathways in *Catharanthus roseus*. *DXS* 1-deoxy-D-xylulose 5-phosphate synthase, *DXR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *MECS* 2-Cmethyl-D-erythritol 2,4-cyclodiphosphate synthase, *HDS* 1-hydroxy-2-methyl-2-butenyl

Genes from the MEP and Iridoid Pathways

In the MEP pathway, the genes encoding *DXS*, *DXR*, 2-C-methyl-Derythritol-2,4-cyclodiphosphate synthase (*MECS*), hydroxymethylbutenyl-4-diphosphate (*HDS*), and *IDI1* have been cloned and characterized from *C. roseus* (Chahed et al. 2000; Veau et al. 2000; Guirimand et al. 2012). *DXS* overexpression resulted in a significant increase in ajmalicine, serpentine, and lochnericine but a significant decrease in tabersonine and hörhammericine in *C. roseus* hairy roots. In fact, overexpression of *DXS* and *DXR* has been found to increase terpenoid production in several plants. For example, *DXS* overexpression enhances the production of various isoprenoids in *Arabidopsis* (Estevez et al. 2001), and *DXR* overexpression increases essential oil yield in peppermint, carotenoid accumulation in ripening tomatoes (Mahmoud & Croteau 2001), and various isoprenoids in tobacco leaves (Hasunuma et al. 2008). There is no information about the use of *MECS* and *IDI1* in metabolic engineering on MIA production.

At the edge of primary and secondary metabolism, G8O as gatekeeper could be a carbon flux controlling step for the iridoid pathway. The encoding gene was overexpressed in the hairy roots of *C. roseus*, which resulted in a higher accumulation of catharanthine (0.063–0.107% of dry weight) than in the wild-type lines (0.019 and 0.029%) (Wang et al. 2010). When co-overexpressing *DXS* and *G8O*, the hairy roots showed a significant increase in ajmalicine by 16%, lochnericine by 31%, and tabersonine by 13% (Peebles et al. 2010). Considering their location with respect to the IPP/DMAPP branching point for terpenoid classes, it is conceivable that the overexpression of one downstream structural gene alone is unable to effect the channeling of the flux at this upstream branch point while the overexpression together with an upstream gene of the IPP/DMAPP branch point may affect the carbon fluxes by a push and pull effect toward the MIA iridoid precursor. An increased production of IPP/DMAPP and *G8O* overexpression may increase the flux in the monoterpenoid branch and from there into MIA. *DXS* and *ASα* co-overexpression displayed a significant increase in hörhammericine by 30%, lochnericine by 27%, and tabersonine by 34% in hairy roots (Peebless et al. 2010). Recent discoveries of IDI1, GPPS, GES, and iridoid synthase encoding genes provide new possibilities for the regulation and improvement of MIA production.

Genes from the Alkaloids Pathway

Cultures of *STR* transgenic cells consistently showed tenfold higher STR activity than wild-type cultures, which favored the biosynthetic flow through the pathway. Two such lines accumulated over 200 mg/L of strictosidine and strictosidine-derived MIAs, including ajmalicine, catharanthine, serpentine, and tabersonine, while maintaining wild-type levels of TDC activity (Canel et al. 1998). Whitmer et al. (2002a, b) showed that in *C. roseus* cell lines overexpressing TDC or STR have an overcapacity of indole alkaloid biosynthesis enzyme activities, as feeding of loganin resulted in a large increase of alkaloid production whereas the combination of

loganin and tryptamine feeding even further increased the level of alkaloids. Apparently, the iridoid pathway is the most limiting step, but when that limitation is overcome the tryptophan pathway becomes limiting. Overcoming one limiting step immediately shows what the next limiting step is. A single structural gene overexpression will thus always have only a limited effect on the overall flux in a pathway. On the other hand, it shows that probably many biosynthetic steps are already present, and only the enzymatic machinery has to be started up by increasing the amount of the limiting substrate by feeding or genetic modification. The elucidation of the full iridoid pathway as described above is thus a major breakthrough opening new possibilities to explore for increasing MIA production.

The key gene *DAT* for the vindoline biosynthesis was introduced into *C. roseus* plants by *Agrobacterium tumefaciens*, which resulted in an increase of vindoline level in the leaves (Wang et al. 2012). However, overexpression of *DAT* in hairy roots altered their MIA profile and accumulated more hörhammericine compared to control lines (Magnotta et al. 2007). Comparative analysis revealed that MIA pathway genes have elevated expression levels in *CrPrx* overexpression transgenic hairy roots, whereas they had a significant reduction in their transcript level in *CrPrx-RNAi* transgenic hairy roots (Jaggi et al. 2011). Alkaloid analysis showed higher levels of ajmalicine and serpentine in these peroxidase overexpressing cell lines. All these transgenic lines produced higher amounts of H_2O_2 (Jaggi et al. 2011). The oxidative burst or H_2O_2 production is closely related to indole alkaloid production (Zhao et al. 2001). In leaves of *C. roseus*, PRX together with phenolic compounds were suggested to represent an important sink of excess H_2O_2, diffusing from the chloroplast under high light exposure (Ferreres et al. 2011). These results indicate a role of the *CrPrx* gene in the regulation of MIA pathway and other metabolic pathways, thus affecting the production of specific alkaloids. In order to study the role of CrPrx and CrPrx1 in plants, these two peroxidases were expressed in *Nicotiana tabacum* (Kumar et al. 2011). The transformed plants exhibited increased peroxidase activity. Increased oxidative stress tolerance was also observed in transgenics when treated with H_2O_2 under strong light conditions. However, differential tolerance to salt and dehydration stress was observed during germination of T1 transgenic seeds. Under these forms of stress, the seed germination of *CrPrx*-transformed plants and wild-type plants was clearly suppressed, whereas *CrPrx1* transgenic lines showed improved germination. *CrPrx*-transformed lines exhibited better cold tolerance than *CrPrx1*-transformed lines. These results indicate that vacuolar peroxidases play an important role in salt and dehydration stress, while cell wall-targeted peroxidases render cold stress tolerance.

1.1.2 Transporter Genes

Since MIA biosynthesis involves at least four different cell types and in each of them at least five different subcellular compartments, the trafficking of pathway intermediates from one to another compartment requires an efficient transport system. Previous research also suggested that transport is one of the potential factors in

regulation of MIA biosynthesis. However, the knowledge about MIA membrane transport mechanisms is still very limited.

Transport has basically two aspects, a physicochemical and a biochemical one. In cells and in an organism diffusion will always take place. Concentration gradients make molecules to diffuse in a liquid phase. Moreover, molecules will equilibrate between the aqueous phase and lipid phase (membrane). Mass transfer factors determine the rate of the uptake in a lipophilic membrane from water and the release again to water, i.e., they affect the transport rate through a membrane. That allows calculations of the rate of diffusion of compounds between cells and cellular compartments. The complexity of this system is further increased by the pH, making that acids and bases at different pH have different solubility in the liquid phases. For example, an alkaloid in acidic conditions is poorly soluble in a lipid phase, but at basic conditions it is better lipid soluble. So at a high ratio of protonated to non-protonated alkaloids, which is at acidic conditions, transport will be slow through a membrane, at higher pH it will be the opposite. Modeling uptake in *C. roseus* vacuoles using these physical–chemical processes resulted in an ion-trap model for alkaloid uptake in vacuoles that fitted reasonably well the experimental results using isolated vacuoles. The lower pH in the vacuole than in the cytosol causes preferred accumulation of alkaloids in the vacuole if compared with the cytosol as the uptake rate on the more basic cytosolic site of the membrane is faster than on the more acidic vacuolar side. This physicochemical process requires ATP for maintaining the low vacuolar pH, so depletion of ATP will inhibit uptake, similar as in case of ABC transporters (Blom et al. 1991). On the other hand, Deus-Neumann and Zenk (1984) reported uptake kinetics for active transport for some indole alkaloids. Ajmalicine, catharanthine, and vindoline showed different rates, and all were ATP dependent. From this it was hypothesized that the vacuolar transport occurred via selective transporter proteins. Roytrakul (2004) reported a detailed study on the uptake of several *C. roseus* alkaloids and secologanin in isolated vacuoles. By adding inhibitors of the various classes of transport proteins, for each individual compound quite a different and complex picture came out. For each single compound, different transporter seems to be involved (Roytrakul and Verpoorte 2007).

The uptake into the vacuole is thus dependent on a combination of factors, first of all there is the bidirectional diffusion-driven transport. On top of that, there are multidrug-resistant associated proteins (MRP, inhibited by glibenclamide) and ATP Binding Cassette (ABC, inhibited by *ortho*-vanadate) type of transporters involved in uptake. Whereas multidrug resistant (MDR) (P-glycoproteins, inhibited by cyclosporine A and verapamil) and MDR coupled with proton symport, are responsible for extrusion. To further complicate the transport system, glutathione was found to cis-activate the MRP transport of ajmalicine into the vacuole (Roytrakul 2004; Roytrakul and Verpoorte 2007). Considering the multicompartment system involved in the MIA biosynthesis, it is clear that with the already very complex transport system into vacuoles, the model for a single-cell or multi-cell system is impossible to describe. The need for sufficient energy and co-factors in the different compartments add further to this complexity. In an attempt to calculate the rate of transport between cells by using the various available data on uptake of compounds and a

number of assumptions based on observations from other plants, it became clear that at least diffusion alone would result in a biosynthetic rate more or less of what is found in the plant (Supandi et al. 2009, unpublished results). It means that the selective transport might play a role in accumulating compounds in certain cells and in some of the specific biosynthetic steps, e.g., by accumulating certain compounds in a vacuole, where they are oxidized to yield serpentine or dimeric alkaloids. In case of serpentine, this anhydronium compound is much more polar than ajmalicine from which it is formed by oxidation, thus becomes trapped into the vacuole. The fact that tobacco vacuoles excrete strictosidine, whereas *C. roseus* vacuoles store it (Hallard et al. 1997) shows at least that every plant species will have different transport systems with different selectivity. Considering that the MIAs are confined to certain cell types may also in part be due to specific transport systems in the cellular membrane(s). It means that introduction of a novel pathway in a plant may be hampered by lack of transport of intermediates.

The example of *CjMDR1,* an ABC transporter gene specific for berberine transport originally isolated from *Coptis japonica*, shows the problems one may encounter in genetically modifying transport. This gene was expressed in *C. roseus* cell cultures (Pomahacova et al. 2009). The endogenous alkaloids, ajmalicine and tetrahydroalstonine, were accumulated significantly more in *C. roseus* cells expressing *CjMDR1* in comparison with control lines after feeding these alkaloids, but transport of other alkaloids was not affected, and even no effect at all on berberine transport into the cells was observed.

A unique catharanthine ABC-transporter (*CrTPT2*) belonging to the pleiotropic drug resistance (PDR) family has been cloned and functionally characterized. It is expressed predominantly in the epidermis of young leaves (Yu and De Luca 2013). Further analysis suggested that CrTPT2 may be specific to MIA-producing plant species, where it mediates secretion of alkaloids to the leaf surface. *CrTPT2* gene expression is induced under the treatment with catharanthine, and its silencing redistributes catharanthine into the leave, causing an increase of dimeric alkaloid levels in the leaves.

Recently, strong support for active MIAs uptake by *C. roseus* mesophyll vacuoles through a specific H (+) antiport system was reported (Carqueijeiro et al. 2013). The vacuolar transport mechanism of the main MIAs accumulated in *C. roseus* leaves, vindoline, catharanthine, and α-3′,4′-anhydrovinblastine was characterized using a tonoplast vesicle system. Vindoline uptake was ATP dependent, and this transport activity was strongly inhibited by NH_4^+ and carbonyl cyanide m-chlorophenyl hydrazine and was insensitive to the ATP-binding cassette (ABC) transporter inhibitor vanadate. Spectrofluorimetric assays with a pH-sensitive fluorescent probe showed that vindoline and other MIAs indeed were able to dissipate an H^+ pre-established gradient across the tonoplast by either vacuolar H^+-ATPase or vacuolar H^+-diphosphatase. Though it was claimed that this system would be responsible for the MIA transport instead of an ion-trap mechanism or ABC transporters, it seems unlikely, as at least physicochemical-based transport will always occur and the various previous reports found alkaloid specificity for the uptake into the vacuole.

1.1.3 Transcription Factors

Transcription factors (TFs) are sequence-specific-DNA-binding proteins that interact with the promoter regions of target genes and modulate the rate of mRNA synthesis by RNA polymerase II (Gantet and Memelink 2002). They usually control the expression of more than one gene vital for normal development and functional physiology in plants. Several TFs have been found to be involved in the regulation of secondary metabolism. In *C. roseus*, MIA biosynthesis is related with plant defense and controlled by a number of signals including developmental cues, light, and biotic and abiotic stress. Regulation of MIA biosynthetic genes is coordinated by several types of TFs (Fig. 1).

ORCAs

The best-known TFs regulating MIA biosynthesis are the jasmonates-responsive ORCAs (octadecanoid-responsive *Catharanthus* AP2-domain proteins) from the plant-specific AP2/ERF (APETALA2/ethylene-responsive factor) family, i.e., ORCA2 and ORCA3, for which the regulation mechanism of the MIA biosynthetic genes in *C. roseus* is well established. *ORCAs* expression is induced by jasmonates (van der Fits and Memelink 2001), which is a major and essential signaling pathway to induce MIA biosynthesis. Jasmonates are first converted to the bioactive jasmonate isoleucine derivative (JA-Ile). Perception of JA-Ile by CrCOl1 causes the degradation of the CrJAZ proteins, derepressing the CrMYC2 protein. CrMYC2 then activates the expression of *ORCAs*, which in its turn activate the expression of MIA biosynthetic genes through binding to the JERE (jasmonate and elicitor-responsive element) in the promoter of targeted genes (Menke et al. 1999; van der Fits and Memelink 2000; Zhang et al. 2011). Ectopic expression of *ORCA3* in cell cultures of *C. roseus* increased the expression of the MIA biosynthetic genes *TDC*, *STR*, *CPR,* and *D4H*, as well as two genes encoding primary metabolic enzymes (*AS* and *DXS*) (van der Fits and Memelink 2000). This indicates that ORCA3 is a central regulator of MIA biosynthesis and positively regulates the biosynthesis of MIAs and their precursors. Nevertheless, ORCA3 does not regulate the expression of *G8O* and *DAT*. Overexpression of *ORCA3* caused an increase of ajmalicine and serpentine but a decrease in tabersonine, lochnericine, and hörhammericine in hairy roots (Peebles et al. 2009). When *ORCA3* combined with *G8O* were overexpressed in hairy roots, alkaloid accumulation level analyses showed that all transgenic clones accumulated more catharanthine, with the highest accumulation level 6.5-fold more than that of the non-expression clone (Wang et al. 2010). ORCA2 from *C. roseus* was demonstrated to regulate the expressions of *STR, TDC,* and *SGD* gene, but has no effect on the CYP-related reductase (*CPR*), which is regulated by ORCA 3 (Menke et al. 1999; Li et al. 2013). Transgenic hairy root cultures overexpressing *ORCA2* showed an average content of catharanthine that was increased up to 2.03 in comparison to the control lines, respectively. However, vinblastine could not be detected in the transgenic and control hairy root cultures by HPLC (Liu et al. 2011). Transgenic

C. roseus plants overexpressing ORCA3 alone (OR lines), or co-overexpressing G10H and ORCA3 (GO lines) were obtained by genetic modification (Pan et al. 2012). It was found that ORCA3 and G10H overexpression significantly increased the accumulation of strictosidine, vindoline, catharanthine, and ajmalicine but had limited effects on anhydrovinblastine and vinblastine levels.

ZCTs and BPF

The zinc finger-binding proteins ZCT1, ZCT2, and ZCT3 (members of the transcription factor IIIA-type zinc finger family) were found to bind to the promoters of *STR* and *TDC*. This interaction repressed the activity of *STR* and *TDC*. The binding of the ZCTs to the *STR* promoter has been suggested to counteract the activation of *STR* by ORCA2 or ORCA3 (Pauw et al. 2004).

Using an enhancer domain of the *STR* promoter as bait in a yeast one-hybrid screen resulted in the isolation of *CrBPF1*, a periwinkle homolog of the MYB-like transcription-factor BPF1 from parsley (van der Fits et al. 2000). *CrBPF1* expression is induced by elicitors but not jasmonates, which indicates that elicitors induce *STR* expression in periwinkle cells via jasmonic-acid-dependent and -independent pathways.

Sequence analysis of the *STR* and *TDC* promoters shows that they contain a G-box or G-box-like binding site. Two G-box-binding factors, CrGBF1 and CrGBF2, were subsequently identified in *C. roseus* and shown to repress the transcription of *STR* by binding to the G-box sequence (Siberil et al. 2001).

WRKYs

A *C. roseus* WRKY transcription factor, CrWRKY1, is preferentially expressed in roots and induced by the phytohormones jasmonate, gibberellic acid, and ethylene (Suttipantaa et al. 2011). Overexpression of *CrWRKY1* in *C. roseus* hairy roots upregulated several key MIA pathway genes, especially *TDC*, as well as transcriptional repressors *ZCT1*, *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 down-regulation of *TDC* and *ZCTs* but up-regulation of *ORCA3* and *CrMYC2*. CrWRKY1 binds to the W-box elements of the *TDC* promoter in the electrophoretic mobility shift, yeast one-hybrid and *C. roseus* protoplast assays. Up-regulation 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 up to threefold higher levels of serpentine. 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 in *C. roseus* plants.

Other TFs

The root-specific MADS-box transcription factor Agamous-like 12 (Agl12) from *Arabidopsis thaliana* was expressed on the differentiation of suspension cells from *C. roseus* (Montiel et al. 2007). The expression of Agl12 is sufficient to promote an organization of suspension cells into globular parenchyma-like aggregates but is insufficient by itself to induce complete morphological root differentiation. Agl12 expression selectively increases the expression of genes encoding enzymes involved in the early biosynthetic steps of the terpenoid precursor of the alkaloids. The transgenic cell lines expressing Agl12 produced significant amounts of ajmalicine, which indicates that TFs involved in tissue or organ differentiation may constitute new metabolic engineering tools to produce specific valuable MIAs. Murata and De Luca (2005) reported that ORCA3 and an AP2/ERF type of transcription factors were expressed in all four cell types (epidermis, IPAP, laticifers, and idioblast cells).

Although different types of TFs have been reported to interact with the genes in the MIA pathway, regulation of the key enzyme genes involved in its branches still remains unclear and need to be figured out, such as the iridoid pathway, vindoline pathway, and bisindole alkaloids pathway.

1.2 Unnatural Compound-Targeted Engineering Strategies

Approaches to generate new-to-nature compounds from plant-based pathways are also developed on *C. roseus*, which modifies the structure of a natural product to improve the biological activity of the compound. Replacement of an endogenous starting material with an unnatural compound is a strategy that has been broadly applied in prokaryotic biosynthetic pathways (O'Connor 2012). Now, genetic manipulation is performed on the MIA pathway combined with precursor-directed biosynthesis and engineered enzymes to produce various unnatural products in *C. roseus*.

RNA-mediated suppression of tryptamine biosynthesis in *C. roseus* hairy root culture eliminates the production of monoterpenoid indole alkaloids derived from tryptamine and secologanin. But when an unnatural tryptamine analog, 5-fluorotryptamine 1a, was fed to both wild-type and silenced cultures, a variety of novel fluorinated alkaloids, such as fluoro-ajmalicine, fluoro-tabersonine, and fluoro-serpentine, were produced and not contaminated with the natural alkaloid counterparts in silenced lines (Runguphan et al. 2009). The flux of the unnatural substrate could be enhanced to the downstream alkaloids through some branches of the pathway when the natural, endogenous substrate is limited or unavailable. Targeted silencing of substrate biosynthesis combined with precursors feeding programs a plant alkaloid pathway to more effectively produce desirable novel products, which opens new areas of combining synthesis and biosynthesis to increase chemodiversity.

A mutant strictosidine synthase gene with reengineered substrate specificity was transformed into *Catharanthus roseus*. The resulting transgenic plant cell culture produced a variety of unnatural alkaloid compounds when cocultured with simple, achiral, commercially available precursors that the reengineered enzyme was designed to accept (Runguphan and O'Connor 2009). This work demonstrates the power of engineering new structures of complex alkaloidal natural products in plant cultures.

Another example is to validate the function of the engineered flavin-dependent halogenase RebH. In vivo, the tryptamine-specific RebH mutant (Y455W) was transformed into the alkaloid-producing plant *C. roseus*, and the de novo production of the halogenated alkaloid 12-chloro-19, 20-dihydroakuammicine was observed. The resulting tissue cultures accumulated substantial levels of 7-chlorotryptophan while wild-type (WT) RebH has been integrated into periwinkle metabolism previously. By installing chlorine onto tryptamine, the RebH Y455W mutant circumvents the bottleneck that tryptophan decarboxylase accepts 7-chlorotryptophan at only 3% of the efficiency of the native substrate tryptophan. In comparison with cultures harboring RebH and WT RebF, tissue cultures containing mutant RebH Y455W and RebF also accumulate microgram per gram fresh-weight quantities of 12-chloro-19,20-dihydroakuammicine but, in contrast, do not accumulate 7-chlorotryptophan, demonstrating the selectivity and potential utility of this mutant in metabolic engineering applications (Glenn et al. 2011).

The development of approaches to generate new-to-nature compounds from *C. roseus* MIA pathway will produce a number of MIA analogs which have to improve or alter biological activity, and will further enhance our ability to hijack the downstream MIA pathways (O'Connor 2012).

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In Vitro Biotechnological Production and Pharmacological Studies of Antileukemic Alkaloids of *Catharanthus roseus*

Hebert Jair Barrales-Cureño, Petra Andrade-Hoyos, Alfonso Luna-Cruz, Cesar Reyes-Reyes, Salvador Chávez-Salinas, and Luis Germán López-Valdez

Abstract Different techniques of in vitro cultures of the medicinal plant *Catharanthus roseus* are available. In this regard, the plant is a source of important secondary metabolites that are compounds widely used in pharmacology. For instance, vinblastine and vincristine are alkaloids employed in the treatment of leukemia. This chapter discusses the techniques mostly used in the field of modern biotechnology, such as the in vitro culture of callus and suspension cells, as well as those related to organs, roots, and seedlings. Similarly, the chapter encompasses the types of explant cultures used, induction rates, and the culture environment, jointly with hormones and concentration employed. Also discussed is the level of production

H.J. Barrales-Cureño (✉) • P. Andrade-Hoyos
Procesos Naturales, Ingeniería Forestal Comunitaria,
Universidad Intercultural del Estado de Puebla, Calle Principal a Lipuntahuaca S/N;
Lipuntahuaca, Huehuetla, Puebla, C.P. 73475, México
e-mail: hebert.jair@uiep.edu.mx

A. Luna-Cruz
Catedrático CONACYT, Facultad de Ciencias Agropecuarias, Universidad Michoacana de San Nicolás de Hidalgo, Avenida Francisco J. Múgica S/N. Ciudad Universitaria, CP. 58030, Morelia, Michoacán, Mexico

C. Reyes-Reyes • S. Chávez-Salinas
Departamento de Ingeniería en Biotecnología, Universidad Politécnica del Valle de Toluca, Carretera Toluca-Almoloya de Juárez Km. 5.6 C.P. 50904, Santiaguito Tlalcilalcali, Almoloya de Juárez, Mexico
e-mail: hebert.jair@uiep.edu.mx

L.G. López-Valdez
Laboratorio de Productos Naturales, Área de Química, Departamento de Preparatoria Agrícola, Universidad Autónoma Chapingo, AP74 Oficina de correos Chapingo, Km 38.5, Carretera México-Texcoco, Texcoco Estado de, 56230, México

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of each category of alkaloids according to the type of in vitro culture. Similarly, new metabolites obtained from suspension cell cultures of *Catharanthus roseus*, along with major pharmacological studies recently conducted, are contained in the chapter.

Keywords *Catharanthus roseus* • Callus culture • Root cultures • Cell suspension cultures • Antileukemic alkaloids

1 Introduction

Biotechnology of plant in vitro cultures represents a successful tool in the production of callus and cell cultures that have the capacity to produce secondary metabolites, compounds of great interest in the pharmaceutical and medical fields (Barrales-Cureño and Ramírez 2013). Alkaloids vincristine and vinblastine, elements produced from *Catharanthus roseus,* are a good example of this. Vinblastine and vincristine are potent mitotic inhibitors that are used in chemotherapy for leukemia. They are complex, chemically synthesized structures, similar to other drugs used in the fight against cancer, such as Taxol (Barrales-Cureño and Soto 2012; Barrales-Cureño et al. 2012, 2015, 2016). In that regard, biotechnological approaches represent the best way in obtaining these compounds.

Recently, the production of vinblastine and vincristine has been induced and research carried out as it has never been over the plant in vitro cultures by means of hormone combination of auxins and cytokinins (Villa-Ruano et al. 2011). Cell potency represents the basis of in vitro culture, a term defined as the potential capacity of a single plant cell to regenerate into a whole plant. Several in vitro techniques, such as micropropagation of adventitious meristems or organs, including tissues and cell cultures, provide a large amount of material of *Catharanthus roseus* that is used in the isolation of dimeric and indole mono-type alkaloids with multitherapeutic properties. In this regard, research has demonstrated that *Catharanthus roseus* have the potential to regenerate through somatic organogenesis during the induction of friable calluses. Likewise, in vitro cultures of multiple shoots can be induced directly. The great pharmacological importance of terpene indole alkaloid, associated with low content in plants (approximately 0.0005% of dry weight), stimulates intensive research regarding metabolic routes of the alkaloids occurring in various studies over in vitro culture. These allow determining the concentrations that occur in in vitro callus and cell suspension cultures.

In this chapter, the importance of different types and conditions of in vitro cultures in the production of vinblastine and vincristine as antileukemic alkaloids is highlighted, as well as that of other related metabolites including the main medical applications of *Catharanthus roseus* species.