Sonia Malik *Editor*

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Springer

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Editor
Sonia Malik
Biological and Health Sciences Center
Federal University of Maranhao
Sao Luis, MA, Brazil

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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

Acknowledgements

Foremost, I would like to thank the Almighty without whom nothing is possible. I would like to express my sincere gratitude to my parents and grandparents for their unconditional love, encouragement and unwavering support throughout my life. I would not be where I am today without their countless sacrifices and blessings. They have given me the best gift of all, higher education and the freedom of choice. I owe my success to them. My special words of thanks go to my husband Dr. Surender Kumar Sharma for his eternal support and understanding of my goals and aspirations. I also thank my wonderful son Aadit for always making me smile and understanding me when I was working on this book instead of playing with him. He is the best child a mother could hope for. I express my heartfelt regard to my mother-in-law and father-in-law for their love and moral support. I owe a special thanks to my brothers and their families for their love and affection.

I would also like to acknowledge the Springer team for their help in editing this book, timely suggestions, technical support and friendly reminders about the deadlines. Last but not least, I acknowledge all the authors for their contributions and without them there would be no book at all.

Contents

Contributors

Abubakker Aslam Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India

Lizbeth Castro-Concha Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Yucatán, Mexico

María de Lourdes Miranda-Ham Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Yucatán, Mexico

Kunnampalli Faizal Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India

Lingling Fang Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

Moemen S. Hanafy Plant Biotechnology Department, National Research Centre (NRC), Cairo, Egypt

Josefine Nymark Hegelund Section for Crop Sciences, Department of Plant and Environmental Sciences, University of Copenhagen, Taastrup, Denmark

Larry Hodges Department of Microbiology, Oregon State University, Corvallis, OR, USA

Gwi-Taek Jeong Department of Biotechnology, Pukyong National University, Busan, Republic of Korea

Krishnan Kathiravan Department of Biotechnology, University of Madras, Chennai, Tamil Nadu, India

Oliver Kemp Section for Crop Sciences, Department of Plant and Environmental Sciences, University of Copenhagen, Taastrup, Denmark

Mahesh Kumar Natural Product Biotechnology Group, Agricultural and Food Engineering Department, Indian Institute of Technology Kharagpur, Kharagpur, India

Henrik Lütken Section for Crop Sciences, Department of Plant and Environmental Sciences, University of Copenhagen, Taastrup, Denmark

Renate Müller Section for Crop Sciences, Department of Plant and Environmental Sciences, University of Copenhagen, Taastrup, Denmark

Maciej Maselko Department of Microbiology, Oregon State University, Corvallis, OR, USA

Mohamed A. Matter Plant Biotechnology Department, National Research Centre (NRC), Cairo, Egypt

Fabricio Medina-Bolivar Arkansas Biosciences Institute, Department of Biological Sciences, Arkansas State University, Jonesboro, AR, USA

Valiyaparambath Musfir Mehaboob Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India

Natalia Meropi-Antypa Section for Crop Sciences, Department of Plant and Environmental Sciences, University of Copenhagen, Taastrup, Denmark

Pratibha Misra CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh, India

Adinpunya Mitra Natural Product Biotechnology Group, Agricultural and Food Engineering Department, Indian Institute of Technology Kharagpur, Kharagpur, India

Hosakatte Niranjana Murthy Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju, Republic of Korea

Department of Botany, Karnatak University, Dharwad, India

Kee Yoeup Paek Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju, Republic of Korea

Don-Hee Park Department of Biotechnology and Bioengineering, Interdisciplinary Program of Graduate School for Bioenergy and Biomaterials, Chonnam National University, Gwangju, Republic of Korea

So Young Park Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju, Republic of Korea

M.R. Rady Plant Biotechnology Department, National Research Centre (NRC), Cairo, Egypt

Palusamy Raja Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India

Walt Ream Department of Microbiology, Oregon State University, Corvallis, OR, USA

Appakan Shajahan Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India

Mrinalini Srivastava CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh, India

Senthil Subramanian Department of Agronomy, Horticulture, and Plant Science, and Department of Biology and Microbiology, South Dakota State University, Brookings, SD, USA

Yahaira Tamayo-Ordoñez Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Yucatán, Mexico

Chandrasekaran Thilip Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, Tamil Nadu, India

Felipe Vázquez-Flota Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Yucatán, Mexico

Wei Wei Department of Microbiology, Oregon State University, Corvallis, OR, USA

Tianhong Yang Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR, USA

About the Author

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

W. Ream (✉) • W. Wei • M. Maselko • L. Hodges
Department of Microbiology, Oregon State University, Corvallis, OR, USA
e-mail: reaml@science.oregonstate.edu

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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;

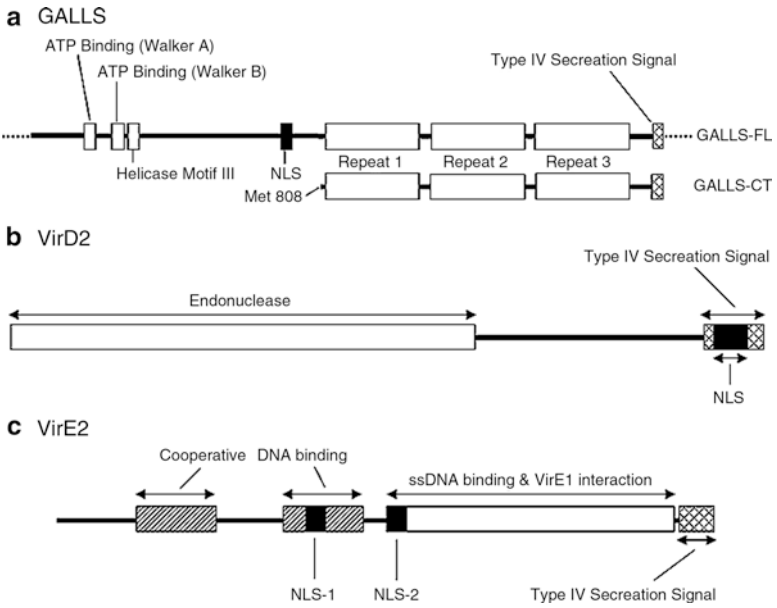

Fig. 1.1 Domains in the GALLS, VirD2, and VirE2 proteins. (**a**) GALLS-FL and GALLS-CT proteins. Boxes indicate the locations of the ATP binding sites (Walker A and B; [165]V**G**VA**GS**A**KT**S[174] and [235]**IVVIDE**MSM[243]), helicase motif III ([269]**KLI**C**VGD**DR**QL**P**PV**GP**G**DLL[288]), 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**RIR**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]**KL**R**P**ED**R**YVQTE**K**YG**RR**[221], and NLS-2, [273]**KRR**YGGETI**KL**K**SK**[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**RP**R**S**QSVNSF[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).

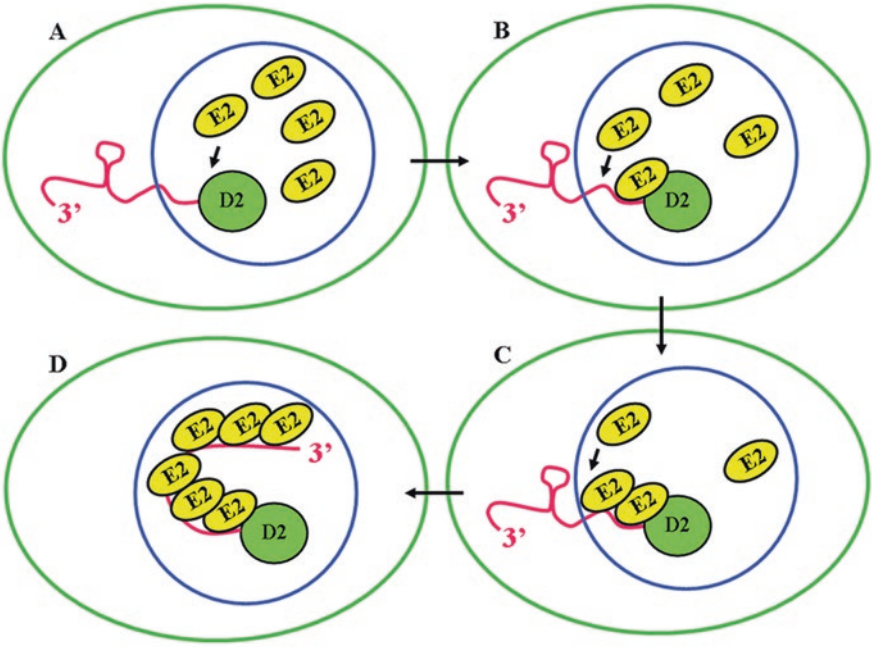

Fig. 1.2 Hypothetical model for VirE2-mediated nuclear import of T-strands. Circles represent the plant cell plasma (green) and nuclear (blue) membranes. Inside the nucleus, the solid green circle labeled "D2" depicts a molecule of VirD2 covalently attached to the 5′ end of the T-strand (red line). Yellow 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

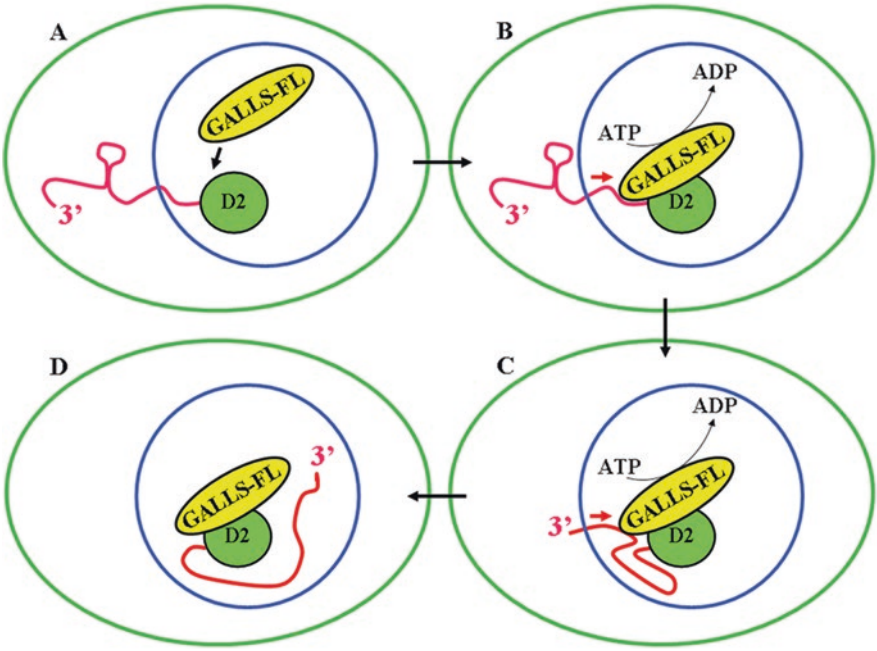

Fig. 1.3 Hypothetical model for GALLS-mediated nuclear import of T-strands. The green circle represents the plant cell plasma membrane, and the blue circle represents the nuclear membrane. Inside the nucleus, the solid green circle labeled "D2" depicts a molecule of VirD2 covalently attached to the 5′ 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

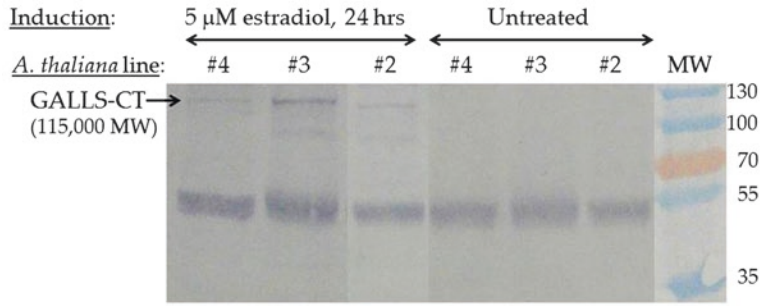

Fig. 1.4 Inducible GALLS-CT expression in transgenic *A. thaliana*. Plants were grown with or without 5-µM β-estradiol for 24 h. Proteins extracted from 30-mg leaf tissue were separated by SDS-PAGE; blots were probed with polyclonal anti-GALLS serum. *MW* molecular weight (in thousands)

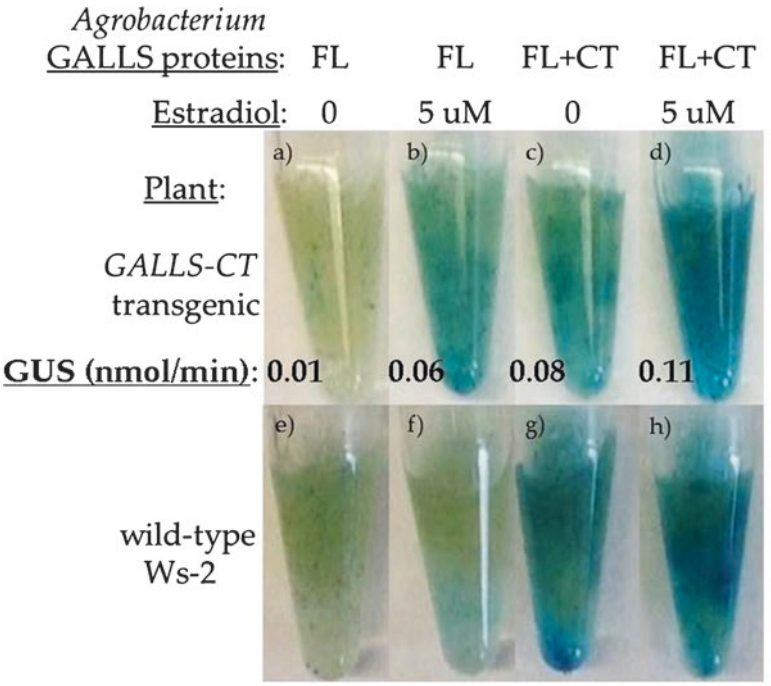

Fig. 1.5 GALLS-CT stimulates transformation. Transgenic *A. thaliana* roots expressing *GALLS-CT* from an estradiol-inducible promoter, along with wild-type controls, were infected with *Agrobacterium* that contained *GUS*::intron T-DNA and expressed either GALLS-FL or GALLS-FL + CT. Treated roots were 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.

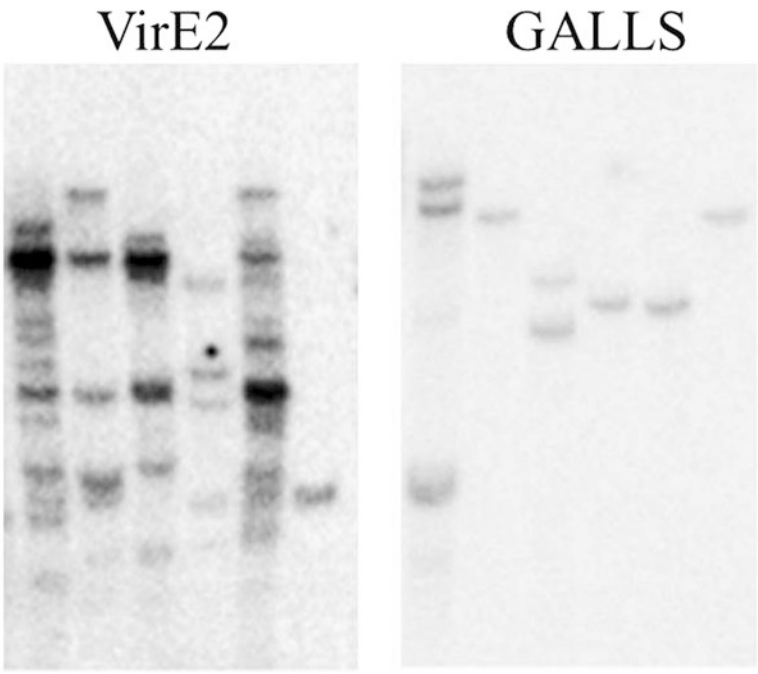

Fig. 1.6 Transformation mediated by GALLS yields single-copy transgenes more frequently than do VirE2-mediated events. The figure shows representative VirE2-mediated events (left panel) and GALLS-mediated events (right panel). Genomic DNA extracted from independent kanamycin-resistant transgenic *A. 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