

## *Agrobacterium* in the Genomics Age

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Members of the genus *Agrobacterium* cause the neoplastic diseases crown gall, hairy root, and cane gall on numerous plant species. Extensive genetic analyses conducted in the 1980s identified key bacterial genes involved in virulence. During the past decade, however, genomic technologies have revealed numerous additional bacterial genes that more subtly influence transformation. The results of these genomic analyses allowed scientists to develop a more integrated view of how *Agrobacterium* interacts with host plants. In a similar manner, genomic technologies have identified numerous plant genes important for *Agrobacterium*-mediated genetic transformation. Knowledge of these genes and their roles in transformation has revealed how *Agrobacterium* manipulates its hosts to increase the probability of a successful transformation outcome. In this article, I review our current knowledge of *Agrobacterium*-plant interactions and how genomic and proteomic technologies have increased our understanding of this unique plant-microbe interaction.

*Agrobacterium* species are phytopathogens that cause a variety of neoplastic diseases, including crown gall (*Agrobacterium tumefaciens* and *Agrobacterium vitis*), hairy root (*Agrobacterium rhizogenes*), and cane gall (*Agrobacterium rubi*). Virulent strains of *Agrobacterium* contain tumor-inducing (Ti) or root-inducing (Ri) plasmids. During infection, enzymes encoded by plasmid-localized virulence (*vir*) genes process the T-DNA region of these plasmids. The resulting single-strand DNA (T-strand) linked to VirD2 protein exits the bacterium via a type IV protein secretion system and enters the plant cell. Within the plant, T-strands likely form complexes with other secreted virulence effector proteins, including VirE2, VirE3, VirD5, and VirF, and supercomplexes with plant proteins as they traverse the cytoplasm and target the nucleus. Once inside the nucleus, T-strands integrate randomly into the plant genome and express T-DNA-encoded transgenes. Two classes of T-DNA genes mediate the pathology of *Agrobacterium* infection. The first group, the oncogenes, either effect phytohormone production (*iaa* and *ipt*; Akiyoshi et al., 1984; Schroder et al., 1984), sensitize the plant to endogenous hormone levels (*rol* and other genes of pRi, *gene5* and *gene6* of pTi; Shen et al., 1988; Spanier et al., 1989; Tinland et al., 1990;

Korber et al., 1991), or may be involved in chromatin remodeling (*gene6b*; Terakura et al., 2007). Expression of these genes results in tumorigenic or rhizogenic growth. A second set of genes directs the synthesis of various low  $M_r$  compounds, opines, that can serve as energy sources for the inciting bacterial strain and can perhaps affect virulence (Veluthambi et al., 1989). For reviews, the reader should see Gelvin (2000, 2003), Tzfira and Citovsky (2001, 2003), McCullen and Binns (2006), and Citovsky et al. (2007). In addition, the reader is directed to an excellent new book on *Agrobacterium* biology (Tzfira and Citovsky, 2008).

Most plant biologists, however, best know *Agrobacterium* as an agent of horizontal gene transfer that plays an essential role in basic scientific research and in agricultural biotechnology. In the 1980s, scientists learned to disarm (delete the oncogenes and, usually, the opine synthase genes) virulent *Agrobacterium* strains such that tissues infected by the bacteria could regenerate into normal plants (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983). Substituting genes of interest for oncogenes and opine synthase genes resulted in plants expressing these novel transgenes and, thus, novel phenotypes. Although transgene substitution for oncogenes within T-DNA was initially conducted in cis (i.e. novel transgenes were placed within T-DNA of Ti-plasmids; Caplan et al., 1983; Fraley et al., 1985), the development of binary systems, in which T-DNA and virulence helper plasmids were separated into two different replicons (de Framond et al., 1983; Hoekema et al., 1983), greatly increased the utility of *Agrobacterium* as a vehicle for gene transfer in plant biology laboratories.

Throughout its development as a gene jockeying tool, genomic studies on *Agrobacterium* and its plant hosts guided scientists in basic science and agricultural biotechnology developments. In this article, I review some of the key genomic methodologies and findings that have contributed to our knowledge of how *Agrobacterium* works and will contribute in the future better to utilize *Agrobacterium*'s amazing gene transfer abilities in the laboratory and in the agricultural biotechnology industry.

### GENOMICS OF AGROBACTERIUM

#### Whole-Genome Mutagenesis

Although not frequently considered genomics, important early studies on *A. tumefaciens* and *A. rhizogenes* utilized whole-genome mutagenesis and mass

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phenotypic screening to define *Agrobacterium* genes important for transformation (i.e. T-DNA and Vir protein transfer) and tumorigenesis. Transposon mutagenesis was generally the method of choice because of the relatively random integration pattern of transposons in the bacterial genome and because the positions of transposon insertions could easily be determined by restriction endonuclease mapping. Thus, scientists localized genes involved in opine catabolism to a specific region of the Ti-plasmid and identified genes involved in crown gall tumorigenesis in the T-DNA, in regions of the Ti-plasmid not within the T-DNA (later to be identified as the virulence region), and in the bacterial chromosome (chromosomal virulence [*chv*] genes; Garfinkel and Nester, 1980; Holsters et al., 1980; Ooms et al., 1980; De Greve et al., 1981). More directed mutagenesis studies identified opine synthase genes and oncogenes in T-DNA regions of Ti- and Ri-plasmids and specific virulence genes within the *vir* gene region (Garfinkel et al., 1981; Leemans et al., 1981; Ooms et al., 1981; Ream et al., 1983; Inze et al., 1984; White et al., 1985; Stachel and Nester, 1986; Stachel and Zambryski, 1986). These studies involved testing of hundreds or thousands of individually mutagenized *Agrobacterium* strains for virulence, opine catabolism and synthesis, and tumor morphology and may thus be categorized as early *Agrobacterium* genomic studies.

Rong et al. (1990) conducted a second type of genetic screening, using the promoter-less *lacZ*-containing transposon MuDI-1681, to identify plant-inducible *Agrobacterium* genes on the chromosome of *A. tumefaciens* A136 (C58 chromosomal background lacking a Ti-plasmid). These authors assayed several thousand randomly mutagenized *Agrobacterium* strains for induced gene expression on plates containing carrot root extract and X-gal. Insertion of the transposon into the *picA* gene revealed that this gene was >10-fold inducible by the root extract. The *picA* gene, currently identified as encoding a polygalacturonase-like protein (Atu3129), was the first identified plant-inducible *Agrobacterium* chromosomal gene.

### *Agrobacterium* Whole-Genome Sequencing

Scientists had sequenced large portions of the *Agrobacterium* genome, including entire Ti-plasmids, by the late 1990s and the following years (Barker et al., 1983; Gielen et al., 1984; Slightom et al., 1986; Thompson et al., 1988; Ward et al., 1988; Rogowsky et al., 1990; Suzuki et al., 2000; Moriguchi et al., 2001; Oger et al., NC\_010929; Kalogeroki and Winans, NC\_002377). Generation of a complete nucleotide sequence of the nopaline-type strain *A. tumefaciens* C58 (Goodner et al., 2001; Wood et al., 2001), from which many *Agrobacterium* strains commonly used for plant genetic engineering are derived [e.g. GV3010::pMP90, C58-Z707, NT1(pKPSF2), EHA101/105, AGL-0/-1; see Lee and Gelvin (2008) for characteristics of these strains], opened the door for more extensive analyses of this

important phytopathogen. *A. tumefaciens* C58 contains four replicons: a circular and a linear chromosome and two plasmids (pTiC58 and pAtC58). The genome contains approximately 30 insertion sequence elements and encodes an unusually large number of transporters (at least 153) and two-component regulatory systems (at least 25). Recently, Ulker et al. (2008) described the surprising observation that *Agrobacterium* can transfer its chromosomal DNA to plants. Interestingly, particular insertion sequence elements and transporter gene sequences are hot spots for chromosomal DNA transfer. The preferential appearance of these chromosomal sequences associated with T-DNA in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) T-DNA/plant DNA junctions suggests multiple mechanisms for chromosomal DNA mobilization during T-DNA transfer (Gelvin, 2008).

The complete genome sequence and annotation of *A. tumefaciens* C58 is posted on <http://depts.washington.edu/agro/>. In addition to this biovar I *A. tumefaciens* strain, DNA sequence analysis of the *Agrobacterium radiobacter* biovar II strain K84 and the *A. vitis* biovar III strain S4 has recently appeared (Slater et al., 2009). *A. radiobacter* K84 is an especially important strain because it and its derivatives are widely used as biocontrol agents against tumorigenic *Agrobacterium* strains (Kerr and Panagopoulos, 1977; Jones et al., 1988). Comparative analysis of the sequences of the three *Agrobacterium* strains and several other species of the family *Rhizobiaceae* indicate a complex genome evolution, including the migration of gene blocks among replicons within and between species. Sequencing of other *Agrobacterium* strains (the biovar III strain *A. vitis* F5R19 and the biovar II strain *A. rhizogenes* A4) is in progress.

### *Agrobacterium* Transcriptional Profiling

Based upon the *A. tumefaciens* C58 sequence, scientists have generated microarrays to probe the response of bacterial genes to environmental and chemical conditions important for *Agrobacterium* virulence and plant defense.

The first such study investigated genes on the octopine-type Ti-plasmid pTiA6 and the nopaline-type Ti-plasmid pTiC58. Cho and Winans (2005) incubated bacteria individually containing these Ti-plasmids with acetosyringone (AS), a potent inducer of the *vir* gene regulon synthesized by wounded plant cells (Stachel et al., 1985; Stachel and Nester, 1986; Stachel and Zambryski, 1986). They used RNA extracted from induced and noninduced cells as probes of microarrays containing all Ti-plasmid genes. As expected, they observed an increase in all previously identified *vir* genes, along with several other Ti-plasmid genes previously not identified as part of the *vir* regulon. Most interestingly, they noted an increase in expression of all Ti-plasmid-encoded genes, suggesting that AS induction of the *vir* regulon increases the copy number of the Ti-plasmid relative to that of the bac-

terial chromosomes. Veluthambi et al. (1988) previously observed a similar increase in Ti-plasmid copy number in bacteria cocultivated with plant cells. Further investigation by Cho and Winans (2005) demonstrated that the *repABC* operon, essential for replication of these Ti-plasmids, was induced by AS. Induction was under the control of the two-component VirA/VirG regulatory system also responsible for *vir* gene induction. Thus, when *Agrobacterium* is in the environment of a wounded plant cell, the Ti-plasmid overreplicates, perhaps increasing the probability of T-DNA transfer to the plant.

The plant wound environment in which *Agrobacterium* effects horizontal gene transfer is acidic (Fierer and Jackson, 2006), and the bacterium must maintain pH homeostasis. An acidic environment is also essential for efficient *vir* gene induction (Stachel et al., 1986), and two promoters regulate *virG*, one of which is acid inducible (Mantis and Winans, 1992; Chang and Winans, 1996). In addition, two chromosomal genes important for *vir* gene induction and transformation, *chvG* and *chvI*, are acid inducible (Charles and Nester, 1993; Li et al., 2002). With these facts in mind, Yuan et al. (2008a) conducted a microarray-based, whole-genome transcriptional profiling study of all *Agrobacterium* genes responding to acidic conditions. These authors identified 152 acid-responsive genes. These included previously identified acid-induced genes, genes involved in cell envelope synthesis, genes involved in exopolysaccharide (succinoglycan) synthesis and metabolism, several newly recognized acid-inducible *vir* genes (*virE0*, *virE1*, *virH1*, and *virH2*), and genes encoding a recently described type VI secretion system (Wu et al., 2008). Acidic conditions repressed a number of genes, including some involved in motility, chemotaxis, and cellular metabolism.

Salicylic acid (SA) is a major signaling molecule that is important for plant defense responses. Although induction of SA and downstream plant defense genes by bacterial elicitation is well studied, fewer reports have investigated the effect of plant-derived SA on pathogen gene expression. Two groups used microarray analysis to investigate the effect of SA on the accumulation of *Agrobacterium* transcripts. Yuan et al. (2007) showed that SA, at concentrations that do not influence bacterial growth (2–8  $\mu\text{M}$ ), inhibits *vir* gene expression in acidified medium containing AS. At higher concentrations (>10  $\mu\text{M}$ ), SA inhibits bacterial growth in acidic medium. Transcriptional profiling of RNA from bacteria incubated for 6 h with AS and 6  $\mu\text{M}$  SA indicated that expression of Ti-plasmid-localized *vir* genes and the *repABC* genes was repressed. However, SA induced a number of *Agrobacterium* genes, including *attKLM*, which encodes a quorum degradation system. Because plants deficient in SA production are hypersusceptible to *Agrobacterium* transformation, whereas elicitation with SA decreased virulence (Yuan et al., 2007; Anand et al., 2008; Veena and S.B. Gelvin, unpublished data), these data suggest that the plant signaling molecule SA may inhibit transformation by

shutting down *vir* gene expression and consequently T-DNA transfer. Anand et al. (2008) noted another effect of SA on *Agrobacterium*. Bacteria treated with 100  $\mu\text{M}$  SA did not efficiently attach to plant cells. Thus, bacterial attachment may be yet another process that is disrupted by this plant hormone.

To explore further the effects of plant-released signal molecules on *Agrobacterium* gene expression, Yuan et al. (2008b) incubated bacteria with physiological levels of SA, indole-3-acetic acid (IAA), and  $\gamma$ -amino butyric acid (GABA) that do not inhibit *Agrobacterium* growth. Previous data had indicated that each of these compounds affect *Agrobacterium* virulence (Chevrot et al., 2006; Liu and Nester, 2006; Yuan et al., 2007; Anand et al., 2008). Incubation of *Agrobacterium* at acid pH with each of these compounds, followed by microarray analysis, revealed 100 to 200 genes for each treatment whose expression was modulated. In some instances, different compounds affected the same genes, whereas numerous *Agrobacterium* genes showed differential regulation by one compound only. IAA inhibited expression of the entire Ti-plasmid-localized *vir* regulon but did not have appreciable effects on expression of chromosomal virulence genes. Thus, the effect of IAA on *vir* regulon induction was similar to that of SA. However, the effects of SA and GABA on *Agrobacterium* gene expression were generally very different. In a most interesting exception, SA and GABA both induced the *attKLM* operon, which is involved in destroying the quorum sensing homoserine lactone that serves as a signaling molecule between *Agrobacterium* cells. In addition, seven genes were coregulated by IAA, SA, and GABA. Most of these were transporters, and mutation of some of these resulted in altered AttM lactonase activity. Taken together, these data suggest that at later times during *Agrobacterium* infection, plant signal molecules shut down *vir* gene expression (which is no longer needed once infection has been established) and may destroy quorum sensing signals.

### *Agrobacterium* Proteomics

Engstrom et al. (1987) conducted the first proteomic study of *Agrobacterium*. Using one-dimensional SDS-PAGE, they identified 10 to 15 protein bands that appeared in various *Agrobacterium* strains following incubation with the *vir* gene inducer AS. Several of these bands corresponded to VirB membrane proteins comprising the type IV secretion system that transfers T-DNA and virulence effector proteins to plants. They also identified VirF and VirE2, two proteins of the *vir* regulon. In addition, they detected a number of other AS-induced proteins encoded by the Ti-plasmid or by the *Agrobacterium* chromosome. Similarly, Rong et al. (1990) detected by one-dimensional SDS-PAGE 10 plant-induced *Agrobacterium* chromosomal protein bands.

Rosen et al. (2004) made the first attempt at experimentally defining the *Agrobacterium* proteome. Using two-dimensional gel electrophoresis, they detected ap-

proximately 300 proteins from exponentially growing bacteria. Interestingly, approximately 10% of the proteins were represented by multiple spots on the gel. The authors suggested that a high level of protein modification of the proteome occurs. Similar studies by this group (Rosen et al., 2001, 2002) investigated stress (high temperature, oxidative, and mild acid conditions) and heat shock-induced proteins of *Agrobacterium*. This group also identified proteins induced when the bacteria were incubated with and bound to cut tomato (*Solanum lycopersicum*) root segments, simulating plant infection conditions (Rosen et al., 2003). As controls, they examined, by two-dimensional gel electrophoresis, proteins from unbound bacteria and bacteria not incubated with root segments. Incubation of bacteria with roots induced approximately 30 proteins, regardless of whether the bacteria bound to the root segments or not. Although incubation with root segments induced ChvE, AttK, and AttM (all proteins involved in virulence), their experiments detected no induced Ti-plasmid-encoded virulence proteins. Because, for example, VirE2 is a major virulence protein induced by phenolic molecules such as AS (Engstrom et al., 1987; Lai et al., 2006), the results of this study indicate either that *vir* gene induction did not efficiently occur or that it occurred in only a small percentage of the bacteria.

More recently, Lai et al. (2006) investigated *Agrobacterium* proteins induced by the phenolic *vir* regulon inducer AS. Using two-dimensional gel electrophoresis coupled with mass spectrometry, they identified 11 AS-induced proteins. Nine of these proteins were well-known Ti-plasmid-encoded Vir proteins (VirE2, several VirB proteins, Tzs, VirH1, and VirK), thus verifying their *vir* regulon induction conditions. In addition, they identified two proteins encoded by chromosomal genes, HspL (a small heat shock protein) and Y4mC (a protein of unknown function). Reverse transcription-PCR analysis indicated that transcripts of the genes encoding these proteins also were AS inducible and that induction was dependent upon the two-component sensing system VirA/VirG that mediates induction of the *vir* regulon. All *vir* regulon genes previously identified contain a *vir* box in their promoter regions (Das et al., 1986). The *y4mC* gene promoter similarly contains a *vir* box, but, interestingly, the *hspL* promoter does not. Thus, *hspL* activation by AS may be an indirect consequence of expression of Vir proteins. In addition, Wu et al. (2008) analyzed proteins secreted by *Agrobacterium* into the medium. They identified 12 proteins, including VirB1\* (a cleaved fragment of VirB1 protein) and Hcp (hemolysin-coregulated protein). Hcp is secreted by a newly discovered type VI secretion system.

#### GENOMICS OF PLANT GENES IMPORTANT FOR AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION

Scientists have used a variety of genomic techniques to investigate plant genes important for *Agrobacterium*-

mediated transformation. These include forward genetic screens to identify mutant plants with altered transformation susceptibility, yeast two-hybrid studies to detect plant proteins that interact with Virulence effector proteins, and transcriptional profiling to discover plant genes whose expression is altered following *Agrobacterium* infection. In addition, reverse genetic analyses have been used to probe the importance of candidate genes in the transformation process.

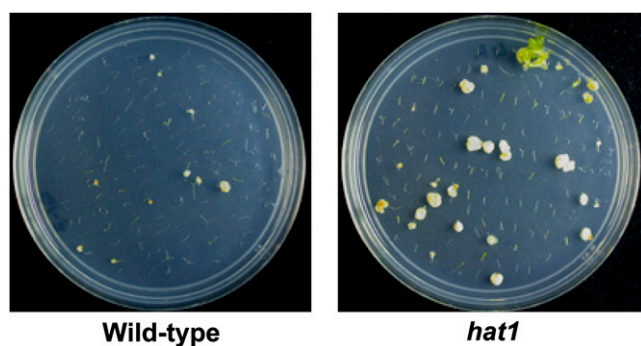
#### Forward Genetic Screens for Plant Mutants with Altered Transformation Characteristics

Plant species, and even different cultivars/genotypes of the same species, are notoriously varied in their transformation susceptibility (DeCleene and DeLey, 1976; Anderson and Moore, 1979; Conner and Commisse, 1992; van Wordragen and Dons, 1992; Bliss et al., 1999; Pena and Seguin, 2001; Somers et al., 2003; Shrawat and Lorz, 2006). In addition, *Agrobacterium* can transform *Streptomyces*, yeast, and other fungal species (Bundock et al., 1995, 2002; Piers et al., 1996; de Groot et al., 1998; Abuodeh et al., 2000; Kelly and Kado, 2002; Roberts et al., 2003; Schrammeijer et al., 2003; van Attikum and Hooykaas, 2003; Michielse et al., 2004), HeLa cells (Kunik et al., 2001), and sea urchin embryos (Bulgakov et al., 2006). Thus, *Agrobacterium* is incredibly promiscuous in its ability to mediate horizontal gene flow among numerous species of different phylogenetic kingdoms. A genetic basis for susceptibility to *Agrobacterium* exists in many crop species (Owens and Cress, 1984; Szegedi and Kozma, 1984; Smarrelli et al., 1986; Robbs et al., 1991; Bailey et al., 1994; Mauro et al., 1995), and Nam et al. (1997) also described a genetic basis for various degrees of susceptibility among approximately 40 *Arabidopsis* ecotypes.

Large-scale forward genetic screening of approximately 20,000 T-DNA mutagenized *Arabidopsis* lines resulted in the first identification of plant genes involved in *Agrobacterium*-mediated transformation (Nam et al., 1999; Zhu et al., 2003b). These forward genetic analyses revealed >120 genes encoding proteins involved in transformation and, because the screen was not saturating (e.g. no gene was discovered more than once), the authors suggested that >200 *Arabidopsis* genes likely influence plant transformation susceptibility (Zhu et al., 2003b). The authors termed mutants with greatly decreased susceptibility to transformation *rat* (for resistant to *Agrobacterium* transformation) mutants and the corresponding mutant genes, *rat* genes. The identified genes represent most of the proposed transformation events that occur in the plant (bacterial attachment/biofilm formation, T-DNA and Virulence protein transfer to the plant, cytoplasmic trafficking and targeting of the proposed T-complex to the nucleus, virulence protein removal from the T-strand, T-DNA integration into the plant genome, and transgene expression).

Examples of plant proteins identified in these initial genetic screens and mediating transformation include those involved in cell wall structure and biosynthesis (Rat1 and Rat4, and arabinogalactan and cellulose synthase-like [CslA9] proteins, respectively; Zhu et al., 2003a; Gaspar et al., 2004), cytoskeleton proteins potentially involved in cytoplasmic trafficking of T-complex components (actins and a kinesin; Zhu et al., 2003b), importin  $\alpha$  and  $\beta$  proteins that may mediate nuclear targeting of T-complex components (Ballas and Citovsky, 1997; Bakó et al., 2003; Bhattacharjee et al., 2008), chromatin proteins such as various histones, histone acetyltransferases, histone deacetylases, and histone chaperones that may facilitate T-DNA integration into the plant genome (Nam et al., 1999; Mysore et al., 2000; Yi et al., 2002, 2006; Tian et al., 2003; Zhu et al., 2003b; Gelvin and Kim, 2007), and histone proteins that can increase transgene expression (G. Tenea and S.B. Gelvin, unpublished data). The nature of these *rat* genes has stimulated reverse genetic experiments to determine the potential roles of candidate genes in the transformation process (see below).

Recently, the Gelvin laboratory further identified several *Arabidopsis* mutants that are hypersusceptible to *Agrobacterium* transformation (*hat* mutants and, therefore, *hat* genes; Fig. 1; N. Sardesai and S.B. Gelvin, unpublished data). *Arabidopsis* lines containing T-DNA activation tags (Weigel et al., 2000) provide a resource for overexpressed genes that may influence transformation susceptibility. When roots of these mutagenized plants were assayed at low bacterial inoculum conditions ( $10^2$ - to  $10^3$ -fold lower than that usually used to screen for *rat* mutants), we identified seven independent lines that displayed increased



**Figure 1.** Activation tagging identifies *Arabidopsis* mutants that are hypersusceptible to *Agrobacterium* transformation (*hat* mutants). Root segments of wild-type (ecotype Wassilewskija) and T-DNA activation-tagged mutants (Weigel et al., 2000) were inoculated with the tumorigenic strain *A. tumefaciens* A208 at low inoculum density ( $10^6$  colony forming units/mL). After 2 d of cocultivation, the root segments were transferred to Murashige and Skoog medium lacking phytohormones and tumors were allowed to develop (Zhu et al., 2003b). The plates were photographed after 4 weeks. Note the larger and more numerous tumors formed on root segments of the *hat1* mutant line, compared to the tumors formed on wild-type roots. The *hat1* mutant has a T-DNA activation tag inserted into a cellulose synthase-like gene. Expression of a neighboring UGT gene is greatly enhanced in the *hat1* mutant.

levels of transformation relative to that of wild-type control plants. T-DNA/plant DNA junction sequences from five *hat* mutants identified several new genes involved in transformation susceptibility, including a cellulose synthase-like protein (CslB5), a potassium transporter family protein (two independent T-DNA insertion lines), a UDP-glucosyltransferase (UGT), and a myb transcription factor (MTF).

Overexpression of the UGT cDNA in wild-type plants confirmed that this gene is a *hat* gene. Interestingly, metabolic profiling of roots from UGT overexpressing plants indicated alterations in the levels of key defense compounds, and microarray analyses of these plants revealed decreased expression of most genes in the phenylpropanoid biosynthetic and SA signaling pathways (N. Sardesai, A. Perera, R. Doerge, and S.B. Gelvin, unpublished data). These results further indicate that plant defense response signaling pathways are involved in susceptibility to *Agrobacterium*-mediated transformation (see the discussion of transcriptional profiling below).

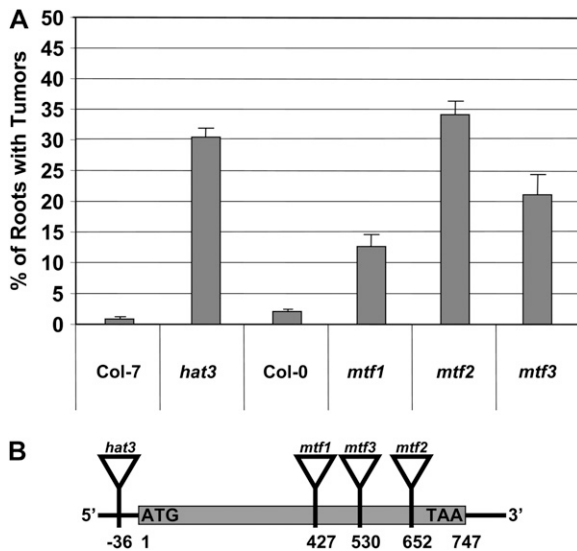
The *hat3* mutant has a T-DNA activation tag inserted into the 5' untranslated region of an MTF gene. Although we could not isolate any homozygous *hat3* mutants (suggesting that this MTF is essential for normal plant growth and development), heterozygous *hat3* mutants are approximately 10-fold more susceptible to *Agrobacterium*-mediated transformation than are wild-type control plants (Fig. 2A; N. Sardesai and S.B. Gelvin, unpublished data). Three additional independent T-DNA insertions in this gene are also *hat* mutants (Fig. 2B), indicating that this MTF is a negative regulator of *Agrobacterium*-mediated transformation. Microarray analysis of RNA isolated from roots of *mtf* mutant plants indicated that a WRKY transcription factor gene was expressed to a lower level in the mutant. A homozygous T-DNA insertion into this WRKY transcription factor gene also resulted in a *hat* phenotype. This WRKY transcription factor is involved in regulating plant defense responses, once again implicating plant defense responses as a component of transformation susceptibility.

As an alternative to screening T-DNA insertion mutants for *hat* and *rat* phenotypes, Anand et al. (2007b) used virus-induced gene silencing to investigate *Nicotiana benthamiana* genes important for *Agrobacterium*-mediated transformation. The authors identified 21 genes whose expression, when lowered, resulted in an altered crown gall phenotype. Proteins encoded by these genes include a nodulin-like protein,  $\alpha$ -expansin, VIP1, importin- $\alpha$ , and histones H2A and H3.

Identification of *rat* and *hat* mutants emphasizes the utility of large-scale forward genetic screens to understand the plant contribution to the *Agrobacterium*-mediated transformation process.

#### Yeast Two-Hybrid Screening for Plant Proteins That Interact with Virulence Effector Proteins

*A. tumefaciens* transfers at least five Virulence effector proteins to plants (VirD2 attached to the T-strand,



**Figure 2.** An MTF negatively affects transformation susceptibility. *A*, Transformation efficiency of the *hat3* (MTF) mutant and its wild-type control (ecotype Columbia-7 [Col-7]) and three independent T-DNA insertion mutants in the MTF gene (*mtf1*, -2, and -3) and its wild-type control (ecotype Columbia-0 [Col-0]). Root segments were inoculated with the tumorigenic strain *A. tumefaciens* A208 at low inoculum density ( $10^5$  colony forming units/mL). After 2 d of cocultivation, the root segments were transferred to Murashige and Skoog medium lacking phytohormones and tumors were allowed to develop (Zhu et al., 2003b). The plates were photographed after 4 weeks. Note the more numerous tumors formed on root segments of MTF mutant lines compared to the tumors formed on wild-type roots. *B*, Map of the MTF gene mutated in the *hat3* mutant. Numbers below the bar indicate nucleotides (+1 is the start site of translation). *hat3*, *mtf1*, *mtf2*, and *mtf3* indicate the positions of three independent T-DNA insertions into the gene.

VirD5, VirE2, VirE3, and VirF; Otten et al., 1984; Stahl et al., 1998; Vergunst et al., 2000, 2003, 2005; Schrammeijer et al., 2003). In addition, *A. rhizogenes* transfers GALLS-FL (full-length) and GALLS-CT (C-terminal) to plant cells (Hodges et al., 2006). Several laboratories have used yeast two-hybrid systems to search for plant proteins that interact with these effector proteins or with other proteins that appear on the bacterial surface. The rationale for these experiments is that if a plant protein interacts with an *Agrobacterium* protein, it is likely that this plant protein is involved in the transformation process.

VirB2 is the major constituent protein of the *Agrobacterium* T-pilus (Lai and Kado, 1998). The T-pilus is an important bacterial structure that may come into contact with the plant during T-DNA and Vir protein transfer. Although proteins on the plant cell surface had previously been implicated in bacterial adhesion (Neff and Binns, 1985; Gurlitz et al., 1987; Neff et al., 1987; Wagner and Matthysse, 1992; Swart et al., 1994; Clauce-Coupel et al., 2008), no previously identified plant surface protein directly influenced bacterial virulence. Hwang and Gelvin (2004) used the processed form of VirB2 (Lai and Kado, 1998) as a bait protein to screen in yeast for Arabidopsis VirB2 interacting pro-

teins. In addition to a RAB8 GTPase, they identified three reticulon domain proteins termed BTI1, -2, and -3 (for VirB2 Interacting proteins 1, 2, and 3). Decreasing expression of the Arabidopsis *BTI* genes by T-DNA mutagenesis or RNA interference (RNAi) resulted in reduced susceptibility to *Agrobacterium*-mediated transformation, whereas overexpression of *BTI1* made the plant hypersusceptible to transformation. As would be expected of a protein that interacts with the T-pilus, the BTI proteins localize to the plant surface. Although more experiments need to be conducted, the BTI proteins may serve as receptors for VirB2 protein on the T-pilus.

In addition to VirB2, the role of VirB5 (a minor T-pilus constituent) needs further exploration. In animal pathogens that have type IV secretion systems, VirB5 orthologs, such as CagL, may serve as specialized adhesins that interacts with human integrin  $\beta$ 1 and fibronectin during bacterial/animal cell contact (Backert et al., 2008). It would be interesting to determine, using yeast two-hybrid systems, whether *Agrobacterium* VirB5 interacts with a specific plant surface protein.

VirD2 is the pilot protein that guides the T-strand through the type IV secretion system into the plant cell, through the plant cytoplasm, and into the nucleus. VirD2 may also influence T-DNA integration into the plant genome (Tinland et al., 1995; Mysore et al., 1998). It is therefore likely that VirD2 interacts with plant proteins during this journey, and yeast two-hybrid analyses have identified a number of these proteins. The first of these was the nuclear transfer importin  $\alpha$  protein AtKAP $\alpha$  (Ballas and Citovsky, 1997), now known as IMP $\alpha$ -1 (Bhattacharjee et al., 2008). The Arabidopsis genome encodes nine importin  $\alpha$  proteins, and VirD2 interacts in yeast with all tested importin  $\alpha$  isoforms (Ballas and Citovsky, 1997; Bakó et al., 2003; Bhattacharjee et al., 2008). Additionally, bimolecular fluorescence complementation studies in planta indicated that each of these isoforms interacts with VirD2 and localizes the complex to the nucleus (Bhattacharjee et al., 2008).

Yeast two-hybrid screening additionally identified several other plant proteins that interact with VirD2. These include several cyclophilins (Deng et al., 1998; Bakó et al., 2003), the kinase CAK2Ms (Bakó et al., 2003), and a protein phosphatase PP2C (Tao et al., 2004). Interaction with these latter two proteins suggested that VirD2 may be a phosphoprotein. Bakó et al. (2003) confirmed this hypothesis, and Tao et al. (2004) showed that PP2C can regulate nuclear entry of VirD2.

The single-strand DNA binding protein VirE2 is important for transformation. *Agrobacterium* strains mutant for *virE2* are highly attenuated in virulence (Stachel and Nester, 1986). VirE2 plays numerous important roles within the plant cell (Citovsky et al., 1992; Ward and Zambryski, 2001) and therefore likely interacts with numerous plant proteins. Yeast two-hybrid analyses have confirmed these interactions. VirE2 interacts with numerous importin  $\alpha$  isoforms; however, only interaction with IMP $\alpha$ -4 results in nu-

clear localization (Bhattacharjee et al., 2008; Lee et al., 2008). VirE2 also interacts in yeast with the VirE2 interacting proteins VIP1 and VIP2 (Tzfira et al., 2001; Anand et al., 2007a). Interaction of VirE2 with these proteins likely contributes to nuclear targeting and genomic integration of T-strands (Tzfira et al., 2001; Citovsky et al., 2004; Li et al., 2005; Loyter et al., 2005; Anand et al., 2007a; Bhattacharjee et al., 2008; Lacroix et al., 2008).

VirF is a nonessential virulence protein for infection of most plant species. However, it is required for efficient transformation of a few species (Melchers et al., 1990; Regensburg-Tuink and Hooykaas, 1993). Schrammeijer et al. (2001) screened for VirF interacting proteins in yeast and identified a plant Skp1 ortholog. Skp1 (ASK1) is a component of the SCF ubiquitin ligase complex that identifies and marks proteins for degradation via the 26S proteasome. Indeed, experiments in both yeast and in planta indicated the importance of VirF in proteolysis of VirE2, suggesting that VirF plays a role in stripping VirE2 from T-strands prior to integration (Tzfira et al., 2004).

VirE3 is a nuclear-localized *Agrobacterium* effector protein that may serve as a plant transcription factor (Schrammeijer et al., 2003; Garcia-Rodriguez et al., 2006). VirE3 may also substitute for plant-encoded VIP1 when this latter protein is limiting (Lacroix et al., 2005). In yeast, VirE3 interacts with several importin  $\alpha$  isoforms, with pCsn5-1 (also known as AJH1), a component of the COP9 signalosome involved in protein degradation, and with pBrp, a plant transcriptional activator. An intriguing potential function for VirE3 may be as a molecular bridge to transport plant transcription factors to the nucleus where they may activate plant genes involved in tumorigenesis or transformation (Garcia-Rodriguez et al., 2006).

GALLS-FL and GALLS-CT are two effector proteins encoded by some *A. rhizogenes* Ri-plasmids (Hodges et al., 2006). Although these proteins do not share sequence homology with *A. tumefaciens* VirE2, they can substitute for this essential *A. tumefaciens* virulence effector protein (Hodges et al., 2004, 2009). Recent yeast two-hybrid analysis using GALLS-FL as the bait identified a specific interacting plant protein (GALLS interacting protein [GIP]; Y. Wang and S.B. Gelvin, unpublished data). Bimolecular fluorescence complementation experiments confirmed GALLS-FL and GALLS-CT interaction with GIP in planta (L.-Y. Lee and S.B. Gelvin, unpublished data). GIP is encoded by one of an eight-member multigene family whose functions are unknown. Research in this author's laboratory is aimed at defining the role of GIP in both *A. rhizogenes*- and *A. tumefaciens*-mediated plant transformation.

### Host Transcriptional Profiling and *Agrobacterium* Infection

Several recent studies have investigated host transcriptional responses to *Agrobacterium* infection or to

crown gall tumorigenesis. Veena et al. (2003) used suppressive subtractive hybridization and DNA microarrays to investigate the transcriptional response of tobacco BY-2 cells to infection by several nontumorigenic *Agrobacterium* strains. The authors used nontumorigenic strains to avoid complications resulting from phytohormone overproduction by expression of oncogenes and studied the initial plant response by limiting sampling to times <36 h after infection. The results of these experiments indicated that *Agrobacterium* exquisitely manipulates expression of the plant genome to facilitate transformation: plant genes important for transformation, such as those encoding histone proteins, were induced by the bacterium, whereas expression of genes involved in host defense responses was suppressed. Interestingly, Anand et al. (2007a) later showed that expression of numerous Arabidopsis histone genes was higher in wild-type Arabidopsis plants than in *vip2* (VirE2 interacting protein 2) mutant plants. Arabidopsis *vip2* mutant plants are highly recalcitrant to *Agrobacterium*-mediated transformation. These authors suggested that VIP2, a putative transcription factor, may play a role in maintaining high-level expression of histone genes important for transformation.

Ditt et al. (2001) used a disarmed *Agrobacterium* strain to infect *Ageratum* cell cultures. Using cDNA/amplified fragment length polymorphism analyses of RNA extracted at relatively long times after infection (48 h), the authors identified a few genes whose expression was either repressed or induced by cocultivation with *Agrobacterium*. Whereas expression of most of the identified genes was similarly affected by cocultivation with *Escherichia coli*, expression of two genes (encoding a nodulin-like protein and a lectin-like protein kinase) was specifically induced by *Agrobacterium* infection.

Ditt et al. (2006) also used Arabidopsis Affymetrix ATH1 microarrays to investigate host gene expression changes following infection of Arabidopsis suspension cell cultures with a tumorigenic *Agrobacterium* strain. Interestingly, the authors were only able to detect transcriptional changes 48 h after infection. In contrast to the results of Veena et al. (2003), the study of Ditt et al. (2006) indicated that *Agrobacterium* infection induced, rather than repressed, defense gene expression and that infection repressed expression of genes encoding proteins involved in cell proliferation. This latter observation was rather surprising considering that growth of the cell cultures was not slowed by bacterial infection. The seemingly opposing results of Ditt et al. (2006) and those of Veena et al. (2003) may be explained by the different plant culture systems used (tobacco and Arabidopsis) and the fact that one group used disarmed strains, whereas the other used tumorigenic strains that would result in the overproduction of phytohormones by the host and, eventually, production of tumors. Differential gene expression occurs in Arabidopsis crown gall tumors (Deeken et al., 2006). The expression of numerous genes, in-

cluding those involved in cell wall biosynthesis, Suc degradation, transport, and glycolysis, is up-regulated in tumors, whereas expression of genes involved in photosynthesis, nitrogen metabolism, lipid metabolism, and amino acid synthesis is down-regulated. Differential gene expression in crown gall tumors correlated with altered solute profiles, leading the authors to speculate that metabolism in mature crown gall tumors occurs mainly anaerobically (Deeken et al., 2006).

In addition to examining host transcriptional responses following *Agrobacterium*-mediated transformation or the development of crown gall tumors, Kim et al. (2007) used Arabidopsis Affymetrix ATH1 microarrays and custom macroarrays to investigate the transcriptional and methylation status of host T-DNA integration sites. The results of these assays indicated that, in the absence of selection, T-DNA target sites were not preferentially transcribed to a greater extent than were Arabidopsis genes in general and that T-DNA integration occurred without regard to the methylation status of the target DNA.

#### Reverse Genetic Screening for Genes Required for *Agrobacterium*-Mediated Transformation

A large number of studies have employed reverse genetic strategies to determine the role of candidate genes in the transformation process. Candidate genes include those identified by yeast two-hybrid and transcriptional profiling analyses, as well as additional members of multigene families when one family member clearly plays a role in virulence. Gene/expression disruption techniques have included T-DNA insertional mutagenesis and RNAi and antisense inhibition of gene expression. Overexpression of several plant genes has also resulted in a hat phenotype (Mysore et al., 2000; Tzfira et al., 2002; Hwang and Gelvin, 2004; Yi et al., 2006; G. Tenea, J. Spantzel, and S.B. Gelvin, unpublished data). Some genes confirmed as *rat* genes by these studies include those encoding BTI proteins (Hwang and Gelvin, 2004), various importin  $\alpha$  family members (Bhattacharjee et al., 2008), VIP1 (Tzfira et al., 2001, 2002; Li et al., 2005), VIP2 (Anand et al., 2007a), Ku80 (West et al., 2002; Friesner and Britt, 2003; Li et al., 2005), DNA ligase IV (Friesner and Britt, 2003; van Attikum et al., 2003), SGA1 (G. Tenea and S.B. Gelvin, unpublished data), and various histones (Mysore et al., 2000; Yi et al., 2006; Anand et al., 2007b; G. Tenea and S.B. Gelvin, unpublished data). In addition, mutation of several genes involved in plant defense responses and signal transduction results in altered susceptibility to *Agrobacterium*-mediated transformation (Veena, N. Sardesai, and S.B. Gelvin, unpublished data).

Crane and Gelvin (2007) conducted a large-scale reverse genetic screen for Arabidopsis *rat* mutants. Using 340 independent mutant lines containing RNAi constructions targeted against 109 chromatin genes, they identified 24 genes important, to various extents,

for transformation. These genes encoded histone acetyltransferases, histone deacetylases, chromatin remodeling proteins, DNA methyltransferases, global transcription factors, histone H1, nucleosome assembly factors, SET domain proteins, and antisilencing group proteins. Some of these genes, such as *HDT19*, were previously implicated in the transformation process (Tian et al., 2003). Most interesting were three genes whose expression is important for T-DNA integration: *HDT1*, *HDT2*, and *SGA1*. *HDT1* and *HDT2* encode histone deacetylases, whereas *SGA* encodes a histone H3 chaperone/chromatin assembly protein also known as ASF1 in yeast and animals.

#### CONCLUSIONS AND PROSPECTIVE

In addition to its long-described history as a plant pathogen (Smith and Townsend, 1907), *Agrobacterium* is a natural genetic engineer that scientists have used for gene transfer experiments for the past 25 years. Whole-genome saturation mutagenesis studies in the early 1980s defined many *Agrobacterium* genes important for transformation, but only during the past decade have scientists applied modern genomic technologies to unravel the full complement of bacterial and host proteins important for transformation. In the near future, advances in molecular biology combined with novel imaging (e.g. Lee et al., 2008) and genetic (e.g. House et al., 2004) techniques will give scientists a considerably more refined view of bacterial and host proteins involved in transformation. This knowledge will likely result in improved transformation technologies, both to increase our ability to control *Agrobacterium* host range and to improve the quality (e.g. single-copy T-DNA insertions that result in predictable and stable transgene expression) of transformation events.

Numerous important questions need to be answered to understand *Agrobacterium*-mediated plant genetic transformation more fully. Many of these questions beg genome-wide answers: (1) What roles do plant defense responses, and *Agrobacterium*'s ability to overcome these responses, play in transformation? (2) How does the transferred VirD2/T-strand assemble with other virulence effector proteins and host proteins to traverse the plant cytoplasm and nucleus? (3) What roles do plant proteins play in T-strand targeting to plant chromatin and in T-DNA integration into the genome? Can we manipulate *Agrobacterium* for gene targeting (site-directed integration) purposes? (4) How can we best manipulate both the bacterium and the host to obtain high-quality transformation events? (5) How does *Agrobacterium* manipulate host metabolism for its advantage? (6) How does *Agrobacterium* interact with other organisms in the rhizosphere? (7) To what extent do the lessons we have learned about transformation using laboratory conditions apply to transformation in nature? (8) Has horizontal gene transfer effected by *Agrobacterium* species influenced plant



evolution? These and other questions will likely be answered using genomic, proteomic, and metabolomic approaches.

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