Transfer of T-DNA and Vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and suppresses host defense gene expression

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Summary

*Agrobacterium tumefaciens* is a plant pathogen that incites crown gall tumors by transferring to and expressing a portion of a resident plasmid in plant cells. Currently, little is known about the host response to *Agrobacterium* infection. Using suppressive subtractive hybridization and DNA macroarrays, we identified numerous plant genes that are differentially expressed during early stages of *Agrobacterium*-mediated transformation. Expression profiling indicates that *Agrobacterium* infection induces plant genes necessary for the transformation process while simultaneously repressing host defense response genes, thus indicating successful utilization of existing host cellular machinery for genetic transformation purposes. A comparison of plant responses to different strains of *Agrobacterium* indicates that transfer of both T-DNA and Vir proteins modulates the expression of host genes during the transformation process.

Keywords: *Agrobacterium*, transformation, host response, BY-2 cell suspension cultures, macroarrays, expression profiling.

Introduction

*Agrobacterium tumefaciens* is the only known organism capable of interkingdom DNA transfer, transforming not only plants (often resulting in the formation of crown gall tumors; Anderson and Moore, 1979; DeCleene and DeLey, 1976) but also fungi and human cells (de Groot et al., 1998; Kunik et al., 2001; Piers et al., 1996; Rho et al., 2001). Scientists have used this ability of *Agrobacterium* for generating transgenic plants in a method commonly known as *Agrobacterium*-mediated transformation. During the transformation process, the transferred (T)-DNA complex, containing a single-stranded T-strand DNA molecule that was processed from the tumor-inducing (Ti)-plasmid and VirD2 protein, transfers from *Agrobacterium* to plant cells, along with VirE2 and VirF proteins. VirD2 is attached covalently to the 5′ end of the T-strand (Durrenberger et al., 1989; Herrera-Estrella et al., 1988; Howard et al., 1989; Young and Nester, 1988) and may serve as a pilot protein to guide the T-strand through the VirB/VirD4 channel comprising a bacterial type IV protein secretion system (Christie and Vogel, 2000; Gelvin, 2000; Tzfira et al., 2000; Zupon et al., 2000). VirE2, a single-stranded DNA-binding protein, coats the T-strand and may protect it from nucleolytic degradation that can occur in the plant cytoplasm and perhaps in the nucleus (reviewed in Binns, 2002; Gelvin, 2000; Tzfira and Citovsky, 2002; Zupon et al., 2000). Several decades of intensive study has resulted in a relatively detailed understanding of the functions of *Agrobacterium* chromosomal virulence (chv) and Ti-plasmid-encoded virulence (vir) genes that participate in the various stages of the *Agrobacterium*-plant interaction. However, the molecular components underlying the events occurring in host cells during T-complex transfer and T-DNA integration into the host genome are largely unknown. There are at least four steps in which plant factors participate during the *Agrobacterium*-mediated transformation process: bacterial attachment to the plant cell surface, transfer of T-DNA from
bacteria to plant cells across the plant cell wall and membrane, nuclear transport of the T-complex, and stable integration of T-DNA into the plant genome (Gelvin, 2000; Sheng and Citovsky, 1996; Zupan and Zambryski, 1995, 1997). These events may involve direct interactions between plant proteins and Agrobacterium virulence (Vir) proteins that are exported to the plant and accompany the T-DNA on its journey through the plant cell to the nucleus (de la Cruz and Lanka, 1998; Gelvin, 2000; Sheng and Citovsky, 1996). Plant proteins involved in these infection-related events are also most likely involved in basic cellular processes such as cell wall biosynthesis, nuclear protein and nucleic acid trafficking, and DNA repair and recombination.

The identification and characterization of Arabidopsis thaliana resistant to Agrobacterium transformation (rat) mutants (Nam et al., 1999), several ecotypes of Arabidopsis that are recalcitrant to Agrobacterium-mediated transformation (Nam et al., 1997), a genetic basis for susceptibility to crown gall disease in some plant species (Bailey et al., 1994; Mauro et al., 1995; Owens and Cress, 1984; Robbs et al., 1991; Smarrelli et al., 1986; Szegedi and Kozma, 1984), and an increase in plant cell susceptibility to transformation following phytohormone treatment (Bartel et al., 1993; Chateau et al., 2000) have provided evidence for the involvement of plant factors in the transformation process. Yeast two-hybrid screens have identified several plant proteins, including VLP1, VLP2, a karyopherin–α, a cyclophilin, a Skp1 homolog, and a type 2C protein phosphatase, that interact with VirD2, VirE2, or VirF (Ballas and Citovsky, 1997; Deng et al., 1998; Schrammeijer et al., 2001; Tzifa et al., 2001; Y. Tao, P. Rao and S.B. Gelvin, submitted). In addition, a plant DNA ligase may affect T-DNA integration (Ziemienowicz et al., 2000).

Screening for differentially expressed genes is one approach to unravel the molecular basis of a biological system. In this study, we have used suppressive subtractive hybridization and macroarray techniques to identify plant genes that are differentially regulated during various stages of Agrobacterium-mediated transformation. Our aim was both to identify plant genes that are differentially expressed during the transformation process and to determine which transferred factors (T-DNA and Vir proteins) are responsible for this differential expression. The results of our experiments indicate that Agrobacterium infection leads to differential expression of numerous plant genes. Detailed expression profiling of these genes indicates that Agrobacterium infection results in an early induction of defense genes. This induction represents a general response to all strains of Agrobacterium tested. These defense genes are, however, repressed during later stages of Agrobacterium-mediated transformation following infection by transfer-competent, but not by transfer-deficient, bacterial strains. In addition, at later stages of the transformation process, infection by transfer-competent strains results in high-level expression of various plant genes that are important for cell growth or T-DNA integration. We conclude that the transfer of T-DNA and Vir proteins modulates the expression of host genes required for the success of transformation. The identification and molecular characterization of these genes provides a better understanding of the plant response to Agrobacterium-mediated transformation. Such information may help to develop methods to enhance the frequency of transformation of recalcitrant plant species and varieties. Conversely, these results may also be used to design strategies to prevent crown gall disease in various plants susceptible to Agrobacterium infection.

**Results**

**Experimental design**

Table 1 shows the characteristics of the five Agrobacterium strains used in this study. To avoid potential responses to

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Relevant characteristics</th>
<th>Antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>At542</td>
<td>EHA105; disarmed pTiBo542 in A136</td>
<td>Non-oncogenic, super-virulent; transfers Vir proteins from pTiBo542</td>
<td>rif</td>
</tr>
<tr>
<td>At793</td>
<td>pBISN1 in A136</td>
<td>Non-oncogenic, avirulent; lacks a Ti-plasmid; cannot transfer T-DNA or Vir proteins</td>
<td>kan, rif</td>
</tr>
<tr>
<td>At804</td>
<td>At542 + pBISN1</td>
<td>Non-oncogenic, super-virulent; transfers T-DNA from pBISN1, and Vir proteins</td>
<td>kan, rif</td>
</tr>
<tr>
<td>At1221</td>
<td>At804 + pUCD3960</td>
<td>Non-oncogenic, avirulent, expresses osa; transfers T-DNA from pBISN1, but cannot transfer VirE2</td>
<td>car, kan, rif, spe</td>
</tr>
<tr>
<td>At1222</td>
<td>At804 + pUCD5533</td>
<td>Non-oncogenic, super-virulent; transfers T-DNA from pBISN1, and Vir proteins; empty vector control for At1221</td>
<td>car, kan, rif, spe</td>
</tr>
</tbody>
</table>

*a* All strains lack oncogenes within their T-DNA; super-virulence refers to the potential to transform many plant species at high frequency. *b* car, carbenicillin; kan, kanamycin; rif, rifampicin; spe, spectinomycin.
phytohormones produced by transformed plant cells infected with tumorogenic *Agrobacterium* strains, we used only ‘disarmed’ strains lacking oncogenes within the T-DNA. In addition, these strains are not known to contain phytohormone biosynthetic genes elsewhere on the Ti-plasmid. *A. tumefaciens* EHA105 contains a disarmed ‘super-virulent’ Ti-plasmid derived from pTiBo542; this strain is highly transformation proficient, but non-tumorogenic, on many plant species (Hood et al., 1993) including tobacco. We introduced the T-DNA binary vector pBIN1 (Narasimhulu et al., 1996) into *A. tumefaciens* EHA105, generating *A. tumefaciens* At804. pBIN1 contains a gusA-intron gene under the control of a strong ‘super-promoter’ that directs β-glucuronidase (GUS) expression in plants but not in bacteria (Ni et al., 1995). This strain can transfer both T-DNA and virulence (Vir) proteins into the plant cell. *Agrobacterium* strains that can transfer T-DNA and/or Vir proteins into the plant cell will be referred to as ‘transfer-competent’ strains. Another *Agrobacterium* strain (At793) contains pBIN1 but lacks a Ti-plasmid, and therefore cannot transfer T-DNA or Vir proteins to plant cells; we refer to this strain as ‘transfer-deficient’. We used *A. tumefaciens* At793 to distinguish the plant’s response to T-DNA and/or Vir protein transfer from a response merely to the presence of the bacterium.

To perform these experiments, we chose a system in which a high percentage of plant cells could be synchronously infected. Although *A. thaliana* suspension cells would appear preferable for use in transcriptional profiling experiments, we were unable, reproducibly, to achieve high levels of synchronous infection with the suspension cultures available. However, we had previously developed the tobacco BY-2 cell suspension culture system for these purposes (Mysore et al., 1998; Narasimhulu et al., 1996). Nearly 100% of the BY-2 cells showed GUS activity, as determined by staining with X-gluc, 48 h after infection with *A. tumefaciens* At804, whereas uninfected cells or cells infected with the transfer-deficient strain *A. tumefaciens* At793 did not express GUS activity (data not shown). To investigate early responses of plant cells to *Agrobacterium* infection, and to avoid long-term effects of T-DNA integration upon plant gene expression, we sampled the cells immediately after infection and at various time periods shortly thereafter (3, 6, 12, 18, 24, 30, and 36 h). Narasimhulu et al. (1996) previously detected expression of T-DNA-encoded genes with this system within 18 h, and further work in our laboratory indicated that we could detect mRNA directed by the gusA-intron gene of pBIN1 as little as 6 h (L.-Y. Lee and S.B. Gelvin, unpublished). T-DNA integration in this system could occur within 48 h (Mysore et al., 1998). For the experiments described below, we infected five flasks each containing approximately 10^7 plant cells with various *Agrobacterium* strains at a ratio of 1000 bacteria per plant cell (Narasimhulu et al., 1996), and then combined the contents of all flasks before sampling. Thus, infection of approximately 5 x 10^7 plant cells occurred synchronously.

**Identification of differentially expressed genes during Agrobacterium-mediated transformation**

We used a combinatorial approach of suppressive subtractive hybridization and macroarrays to identify plant genes that are differentially expressed during various stages of *Agrobacterium*-mediated transformation. Forward- and reverse-subtracted cDNA libraries were prepared using mRNA isolated from BY-2 cell suspension cultures either infected for 12 h with the transfer-competent strain *A. tumefaciens* At804, or uninfected suspension cultures. A total of 17, 280 cDNA clones were obtained from both libraries containing fragments approximately 0.2–1 kb in size. We performed initial screening of the subtracted libraries using colony arrays on a nylon membrane. Multiple rounds of differential screening of colony arrays, using radiolabeled reverse-transcribed RNA from uninfected BY-2 cell suspension cultures and cultures infected with either *A. tumefaciens* At804 or *A. tumefaciens* At793 for 12, 24, or 36 h, resulted in the identification of 421 potential differentially expressed clones.

We sequenced all the 421 potential differentially expressed clones and analyzed them for homology to gene and protein sequences in GenBank. Based upon sequence similarities (BLASTX E-value cut-off = 1 x 10^-9 and BLASTN E-value cut-off = 1 x 10^-10), we were able to assign putative functions to 339 cDNA clones. The remainder of the 82 clones were designated as ‘unknowns’ because of the low similarity of match to sequences in the database. We subsequently classified all genes into various categories such as cell division and growth, stress, chaperones, primary and secondary metabolism, and others (Table 2). The first category consists of 163 clones that mostly encode genes related to cell division and growth (including genes involved in DNA replication, recombination, and repair), and chaperones. The majority of these clones were similar to gene sequences that encode various types of ribosomal proteins, various members of core histone gene families including H2A, H2B, H3, and H4, and elongation factors. A few other clones encode RNA-binding protein RZ-1, translationally controlled tumor proteins (TCTP1), and a GTP-binding protein beta chain homolog that are known to be involved in DNA replication, recombination, and repair. Included in the same category were clones encoding proteins with similarity to various types of chaperonins such as DnaJ-like proteins, T-complex protein, chaperonin CPN60-1, heat shock cognate proteins 70 and 80, and a chaperone GrpE type 2. A second major group of clones consists mainly of cDNAs encoding proteins involved in hormonal regulation, detoxification, signaling, stress, and defense responses. The 101 clones in this category are
Table 2 Clones differentially expressed during Agrobacterium-mediated transformation

<table>
<thead>
<tr>
<th>Putative function</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell division and growth, and chaperones</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein</td>
<td>105</td>
</tr>
<tr>
<td>Histone</td>
<td>26</td>
</tr>
<tr>
<td>Elongation factor</td>
<td>10</td>
</tr>
<tr>
<td>Chaperone</td>
<td>5</td>
</tr>
<tr>
<td>Translationally controlled tumor protein</td>
<td>4</td>
</tr>
<tr>
<td>T-complex protein</td>
<td>4</td>
</tr>
<tr>
<td>DnaJ-like protein</td>
<td>3</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>1</td>
</tr>
<tr>
<td>RNA-binding protein RZ-1</td>
<td>1</td>
</tr>
<tr>
<td>RNA-binding glycin-rich protein</td>
<td>1</td>
</tr>
<tr>
<td>Polyubiquitin</td>
<td>1</td>
</tr>
<tr>
<td>GTP-binding protein beta chain homolog</td>
<td>1</td>
</tr>
<tr>
<td>Cyclophilin CYP5</td>
<td>1</td>
</tr>
<tr>
<td>Defense/stress, signaling, hormone regulation, and detoxification</td>
<td></td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>41</td>
</tr>
<tr>
<td>Extensin</td>
<td>25</td>
</tr>
<tr>
<td>Proline-rich protein</td>
<td>12</td>
</tr>
<tr>
<td>Sar8.2</td>
<td>7</td>
</tr>
<tr>
<td>Osmotin-like protein</td>
<td>5</td>
</tr>
<tr>
<td>Tumor-related protein</td>
<td>4</td>
</tr>
<tr>
<td>pap8</td>
<td>2</td>
</tr>
<tr>
<td>Tubulin</td>
<td>1</td>
</tr>
<tr>
<td>STR246</td>
<td>1</td>
</tr>
<tr>
<td>Inhibitor of microbial serine proteinases</td>
<td>1</td>
</tr>
<tr>
<td>Calcium-dependent protein kinase</td>
<td>1</td>
</tr>
<tr>
<td>Auxin-induced protein</td>
<td>1</td>
</tr>
<tr>
<td>Primary and secondary metabolism</td>
<td></td>
</tr>
<tr>
<td>Glucosyltransferase</td>
<td>5</td>
</tr>
<tr>
<td>F1-ATP synthase</td>
<td>5</td>
</tr>
<tr>
<td>Sucrose synthase</td>
<td>4</td>
</tr>
<tr>
<td>Glucan endo-1,3-β-D-glucosidase</td>
<td>4</td>
</tr>
<tr>
<td>Short-chain-type dehydrogenase</td>
<td>3</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>3</td>
</tr>
<tr>
<td>Caffeoyl CoA O-methyltransferase</td>
<td>3</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>3</td>
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<tr>
<td>Pyruvate decarboxylase</td>
<td>2</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>2</td>
</tr>
<tr>
<td>Non-specific monoxygenase</td>
<td>1</td>
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<tr>
<td>Translocase</td>
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</tr>
<tr>
<td>Superoxide dismutase</td>
<td>1</td>
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<tr>
<td>Pyruvate kinase</td>
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</tr>
<tr>
<td>Protein kinase</td>
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</tr>
<tr>
<td>Phosphoglycerate dehydrogenase</td>
<td>1</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>1</td>
</tr>
<tr>
<td>Phenylalanine ammonia-lyase</td>
<td>1</td>
</tr>
<tr>
<td>P-glycoprotein-like protein</td>
<td>1</td>
</tr>
<tr>
<td>OsTATC</td>
<td>1</td>
</tr>
<tr>
<td>Monodehydroascorbate reductase</td>
<td>1</td>
</tr>
<tr>
<td>Leucyl aminopeptidase</td>
<td>1</td>
</tr>
<tr>
<td>Ferredoxin-NADP reductase</td>
<td>1</td>
</tr>
<tr>
<td>β-xylodisaccharide</td>
<td>1</td>
</tr>
<tr>
<td>ATPase beta subunit</td>
<td>1</td>
</tr>
<tr>
<td>Arginine decarboxylase</td>
<td>1</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase</td>
<td>1</td>
</tr>
<tr>
<td>Acyl-CoA dehydrogenase</td>
<td>1</td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>Unknownb</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 2 continued

<table>
<thead>
<tr>
<th>Putative function</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-symbiotic hemoglobin</td>
<td>9</td>
</tr>
<tr>
<td>Early nodulin ENOD18</td>
<td>2</td>
</tr>
<tr>
<td>Senescence-associated protein</td>
<td>1</td>
</tr>
<tr>
<td>RNA expressed in roots</td>
<td>1</td>
</tr>
<tr>
<td>Retroviral-like transposon</td>
<td>1</td>
</tr>
<tr>
<td>G-beta repeat domains</td>
<td>1</td>
</tr>
</tbody>
</table>

*Table represents a short list of clones identified after differential screening of colony arrays. For additional data, see Table S1.

†Unknown proteins, function unknown, or no significant match to database.

dominated by stress-responsive genes, including glutathione S-transferases (parA, parB, parC, and PR-1), extensins, osmotin-like proteins, proline-rich proteins, SAR genes, and tumor-related proteins. A third category consists of 52 clones whose encoded proteins are mainly involved in energy production and metabolism. This group encodes proteins such as alcohol dehydrogenases, caffeoyl CoA O-methyltransferase, F1-ATP synthase, glucan endo-1,3-β-D-glucosidase, glucosyltransferase, glyceraldehyde-3-phosphate dehydrogenase, short-chain-type dehydrogenase, sucrose synthase, and pyruvate decarboxylase. Various other genes such as a β-xylodisaccharide, OsTATC, P-glycoprotein-like protein, phenylalanine ammonia-lyase, phosphoenolpyruvate carboxykinase, phosphoglycerate dehydrogenase, pyruvate kinase, superoxide dismutase, translocase, and a non-specific monooxygenase were also identified and grouped in this category. The last group consists of 105 clones mainly containing genes encoding unknown proteins or proteins whose biologic roles are not clear. Examples of these include non-symbiotic hemoglobin, early nodulin ENOD18, senescence-associated protein, protein containing G-beta repeat domains, retroviral-like transposons, and RNA expressed in roots. The majority of the clones (90 clones) grouped in this category were designated as unknowns; these clones had either substantial sequence similarity to genes with unknown functions, or only low-similarity matches to database sequences.

We PCR-amplified the cDNA inserts from all these clones and used them for preparation of cDNA macroarrays. Microarrays were used for expression profiling of tobacco BY-2 mRNA differentially expressed during various stages of Agrobacterium-mediated transformation. Figure 1 shows an example of one set of macroarrays. Total RNA isolated from BY-2 cultures infected with various Agrobacterium strains for various time points was used to generate hybridization probes for use with cDNA macroarrays. RNA for all hybridizations contained pooled samples from five different infection experiments. Figure S1 shows an example of differentially screened cDNA macroarrays. We quantified the data as described in Experimental procedures for the purpose of expression profiling. An analysis of variance

ANOVA) model indicated clones showing statistically significant differential expression (Table 3 and Table S1) during infection with *A. tumefaciens* At804 and At793 compared to the uninfected control (see Experimental procedures for details). In total, 46% of these clones were significantly differentially expressed in BY-2 cell suspension cultures at least once or more times following infection of BY-2 cell suspension cultures with various strains of *Agrobacterium*. Approximately 39% of the clones were differentially expressed in cultures infected with the transfer-deficient strain *A. tumefaciens* At793 compared to the uninfected control cells. Twenty-four per cent were differentially expressed when BY-2 cells were infected with the transfer-competent strain *A. tumefaciens* At804 compared to uninfected control cells. Approximately 23% were differentially expressed when infections with transfer-competent and transfer-deficient strains were compared to each other. During the period of 3, 6, 12, and 24 h of infection, 1.9, 11.4, 6.9, and 11% of the clones were differentially expressed, respectively. However, approximately 34% of clones showed differential expression during later stages (36 h) of infection (for details, see Table S1). Based on their differential expression profiles, and considering the significant similarity to other clones at the nucleotide level, in total, 90% of the classes of clones showed significant differential expression at some time after infection.

**Expression profiling**

A K-means clustering algorithm was used to obtain an overview of the gene expression profiles during the course of infection with various strains of *Agrobacterium*. We arbitrarily grouped the clones into 12 clusters that ranged in size between 11 and 114 genes per cluster (Figure 2). Additionally, these clusters could also be more broadly classified into two major groups based on their profiles. The first group consists of cluster numbers 2, 4, 5, 6, 8, and 10, all of which had a lower level of transcript expression during the initial stages of infection and showed a significant increase in expression during later stages of infection. Genes from uninfected BY-2 cells and cells infected with the transfer-competent *Agrobacterium* strain At804 had expression profiles with a similar magnitude. The magnitude of the expression profiles of genes infected with the

| Table 3 Number of statistically significant differentially expressed clones among all pair-wise treatments at each time point |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Infection comparison | Time of infection | Time of infection | Time of infection | Time of infection | Time of infection | Time of infection |
| At793 + BY-2/BY-2 | 3 h | 6 h | 12 h | 24 h | 36 h | Total number of clones |
| At804 + BY-2/BY-2 | 3 h | 6 h | 12 h | 24 h | 36 h | Total number of unique clones |
| At804 + BY-2/At793 + BY-2 | 3 h | 6 h | 12 h | 24 h | 36 h | Total number of unique clones |
| Total number of clones | 10 | 64 | 37 | 58 | 252 | 192 |
| Total number of unique clones | 8 | 48 | 29 | 46 | 145 | 192 |

*Sequences represented by more than one cDNA clone were considered duplicate.*

transfer-deficient strain was, however, relatively low. This group was dominated by genes that are involved in cell division and growth processes. These include various core histones (H2A, H2B, H3, and H4), ribosomal proteins, translation elongation factors, F1-ATP synthase, and several unknown proteins. The expression patterns in the second major group (cluster numbers 3, 7, 9, 11, and 12) showed a high level of expression at early stages of infection followed by a significant decline during later stages of infection with the transfer-competent Agrobacterium strain and uninfected BY-2 cell suspension cultures compared to BY-2 cells infected with the transfer-deficient Agrobacterium strain. The second major group consisted mainly of genes that are involved in defense responses such as various glutathione S-transferases (including parA, parB, and parC), SAR genes, extensins, osmotins, non-symbiotic hemoglobins, and several unknown proteins. Details of the expression profiles of these clones are presented in the Supplementary data.

Expression pattern of genes related to cell division and growth processes

We further analyzed the expression patterns of various genes by RNA blot and RNA array analyses. Figure 3 shows examples of RNA blots depicting expression patterns of several tobacco BY-2 genes that are known to be involved in cell division and growth processes following Agrobacterium infection (for RNA arrays, see Figure S2). The core histone gene families (H2A, H2B, H3, and H4) were expressed at relatively low levels during early times (3–12 h) after Agrobacterium infection, and these levels of
expression were similar to those of uninfected BY-2 cell suspension cultures. However, the levels of expression of these genes (especially histones H2B, H3, and H4) increased significantly during later times of infection (18–36 h) in tobacco cells infected with *A. tumefaciens* At804, a transfer-competent strain of *Agrobacterium* and in uninfected cell suspension cultures. In contrast, cells infected with *A. tumefaciens* At793, a transfer-deficient strain of *Agrobacterium*, showed a significantly lower level of transcript accumulation during these later stages of infection. Various ribosomal protein genes showed a similar pattern of expression.

Expression pattern of genes related to defense responses

Figure 4 shows the expression patterns of several stress-responsive genes following *Agrobacterium* transformation. Genes encoding β-1,3-glucanase, alcohol dehydrogenase, glutathione S-transferase *par*4, glutathione S-transferase T2, phenylalanine ammonia-lyase, and Sar8.2d showed significant induction, relative to uninfected tobacco cells, during the initial times (3–12 h) after *Agrobacterium* infection. This induction is similar in BY-2 cells infected with either *A. tumefaciens* At804 or *A. tumefaciens* At793.

However, during the later stages of infection (30 and 36 h), the relative accumulation of these transcripts is greater in cells infected with *A. tumefaciens* At793 compared to cells infected with *A. tumefaciens* At804. Genes encoding proteins showing significant similarity to tumor-related proteins and non-symbiotic hemoglobins showed a similar pattern of expression under these conditions. Interestingly, a gene encoding a proline-rich protein showed a similar initial induction in response to *Agrobacterium* infection. However, during the later stages of infection, it showed a higher level of expression only in cells infected with *A. tumefaciens* At804, a transfer-competent *Agrobacterium* strain. These observations, in conjunction with the RNA array results (see Figure S3), indicate significant differences in the plant response to *A. tumefaciens* At804 (transfer-competent) and *A. tumefaciens* At793 (transfer-deficient).

Expression pattern of differentially expressed plant genes in response to transfer of either T-DNA or Vir proteins from Agrobacterium

The differential response of tobacco cells to infection by transfer-competent and transfer-deficient *Agrobacterium* strains can result from a response to the transfer of T-DNA and/or virulence proteins to the plant cell. To discern the effect of T-DNA and/or Vir protein transfer on differential plant gene expression, we infected tobacco BY-2 cell suspension cultures with several additional *Agrobacterium* strains (Table 1). *A. tumefaciens* EHA105 lacks a T-DNA but contains genes encoding Vir proteins; this strain can therefore transfer only Vir proteins to the plant cells. *A. tumefaciens* At1221 contains, in addition to the disarmed Ti-plasmid pTiEHA105 and the T-DNA binary vector pBI511, a plasmid containing the oncogenic suppressive activity...
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Figure 5. Expression pattern of genes putatively involved in cell division and growth during transformation by various transfer-competent Agrobacterium strains. Total RNA was isolated from BY-2 cell suspension cultures infected with A. tumefaciens At542, At1221, or At1222 for various times (0, 3, 6, 12, 18, 24, 30, and 36 h) and subjected to RNA blot analysis. The blots were hybridized with radiolabeled cDNA fragments showing homology to genes involved in cell division and growth.

Figure 6. Expression pattern of genes putatively involved in stress responses during transformation by various transfer-competent Agrobacterium strains. Total RNA was isolated from BY-2 cell suspension cultures infected with A. tumefaciens At542, At1221, or At1222 for various times (0, 3, 6, 12, 18, 24, 30, and 36 h) and subjected to RNA blot analysis. The blots were hybridized with radiolabeled cDNA fragments showing homology to genes involved in stress responses, or an rDNA control.

Expression of the *osa* gene blocks transfer of *VirE2* protein but not T-DNA (Lee *et al.*, 1999). *A. tumefaciens* At1222 lacks the *osa* gene and served as a control for *A. tumefaciens* At1221. Figures 5 and 6 depict the expression profiles of various genes that showed differential expression following infection with *A. tumefaciens* At542, At1221, and At1222. As seen previously in Figure 3, various core histone and ribosomal protein genes showed higher levels of transcript accumulation during the later stages of infection with *Agrobacterium* strains that are capable of transferring Vir proteins and/or T-DNA into the plant cell. Interestingly, the level of induction of these transcripts was higher in cells infected with *A. tumefaciens* At1221 (can transfer T-DNA but not VirE2) than in cells infected with *A. tumefaciens* EHA105 (can transfer Vir proteins but not T-DNA) and At1222 (can transfer both Vir proteins and T-DNA) (Figure 5 and Figure S2). These data suggest that the transfer of VirE2 to tobacco cells may be somewhat inhibitory to the induction of these histone and ribosomal protein genes during later stages of *Agrobacterium* infection.

Figure 6 indicates that, in accord with the data shown in Figure 4, various stress-responsive genes showed early induction after BY-2 cells were infected by *Agrobacterium* strains At542, At1221, and At1222. However, during the later stages of infection, these transcripts accumulated to only a low and similar level. These results contrast with those shown in Figure 4 and Figure S3, indicating that cocultivation with the transfer-deficient strain *A. tumefaciens* At793 resulted in induction of these stress genes at latter times of infection. However, transcripts of the proline-rich protein gene showed relatively higher accumulation at late times following infection with *A. tumefaciens* At1221 compared to strains EHA105 and At1222. Taken together, these data suggest that, in general, transfer of either T-DNA or Vir proteins can suppress the accumulation of these plant stress-defense-related gene transcripts late in infection.

**Discussion**

Despite the wide use of *Agrobacterium* for the genetic transformation of plants, we lack knowledge with regard to many details of the molecular events that occur during the interaction of *Agrobacterium* with plant cells. In particular, we know very little about the involvement of host factors during the process of *Agrobacterium*-mediated transformation. In an attempt to understand the transformation process more fully, we studied the differential expression of plant genes during the initial stages of transformation. We used various *Agrobacterium* strains to dissect general plant responses to the bacterium from specific responses to T-DNA and/or Vir protein transfer. These strains differed from each other in their ability to transfer T-DNA and/or Vir proteins to the plant cell. Using combinatorial approaches of suppressive subtractive hybridization, macroarray, and RNA blot analyses, we identified numerous tobacco cDNA clones that were differentially expressed during the initial stages of transformation. Further analyses permitted us to demonstrate the involvement of T-DNA and/or Vir proteins as factors that result in differential expression of these genes during *Agrobacterium* infection.

Genes identified in this study mainly include those involved in defense responses, cell division and growth, chaperones, and primary and secondary metabolism. We demonstrated that co-cultivation of plant cells with *Agrobacterium*, in general, results in the very early (3–6 h) induction of plant defense response genes. However, transfer of the T-complex (T-DNA and Vir proteins) to plant cells results in decreased expression of most of these genes 30–36 h after the initiation of infection. Hansen (2000) had previously demonstrated an apoptotic response of maize suspension cultures to *Agrobacterium* infection. Further experimentation would have to be performed to determine whether differences exist between infection of monocot and dicot plant species. In our studies, the transfer of the T-complex to plant cells additionally resulted in increased expression of genes involved in plant cell growth and division at these latter transformation times. Many of these genes encode proteins, such as histones, that have been implicated in the T-DNA integration process (Mysore *et al.*, 2000; Yi *et al.*, 2002). The correspondence of the timing of this differential gene expression with that at which T-DNA integration can first be detected (Mysore *et al.*, 1998) suggests that the T-complex may ‘hijack’ the plant metabolic machinery and direct it towards the successful integration of T-DNA into the plant genome. Figure 7 presents a model of how host gene expression is altered in response to infection by transfer-competent and transfer-deficient *Agrobacterium* strains.

A large number of differentially expressed cDNA clones identified in this study showed homology to genes that are regulated during abiotic and biotic plant defense responses. Various types of glutathione S-transferases showed differential expression during *Agrobacterium*-mediated transformation. In plants, glutathione S-transferases play a role in detoxification of xenobiotic compounds and are induced during cellular proliferation, hormonal stimuli, and various biotic and abiotic stresses (Delledonne *et al.*, 2001; Dixon *et al.*, 1998; Edwards *et al.*, 2000; Marrs, 1996). Included in this group are clones similar to *parA, parB*, and *parC*, which are also induced in response to auxins and may participate in the initiation of meristematic activity in non-dividing cells (Takahashi and Nagata, 1992; Takahashi *et al.*, 1989).

Genes involved in cell wall synthesis and degradation, such as those encoding extensins, pap8, osmotins, proline-rich proteins, caffeoyl-CoA O-methyltransferase, β-xilosidase, and β-1,3-glucanase also showed differential regulation during pathogen infection, wounding, salt, drought, and...
heat stresses (Okushima et al., 2000). These genes are stimulated during these stresses. Various osmotins, glucanases, proline-rich proteins, and expansins play roles in re-structuring the extracellular matrix during defense reactions and are considered indispensable for cell differentiation, signal transduction, and cell-to-cell recognition (Robertson et al., 1997; Showalter, 1993). Various genes encoding PR proteins and SARs that have defined roles in pathogenesis also showed differential expression in our analyses. We also identified differentially expressed genes encoding proteins involved in the biosynthesis of phenylpropanoids, including phenylalanine ammonia-lyase, glucosyl transferase, and a monooxygenase. Phenylpropanoids are secondary metabolites that can have antimicrobial activity and play a role in signaling and chemotaxis with both pathogenic and symbiotic microorganisms (Paiva, 2000).

Our studies additionally identified genes encoding various enzymes involved in carbon metabolism including alcohol dehydrogenases, monodehydroascorbate reductase, a gluconeogenic pathway enzyme phosphoenolpyruvate carboxykinase, phosphoglycerate dehydrogenase, and sucrose synthase. Most of these genes are induced during abiotic and biotic stresses (Peng et al., 2001; Potenza et al., 2001; Wang et al., 2000). Genes similar to a glucosyltransferase, an alcohol dehydrogenase, and a monooxygenase were also differentially regulated in Agrobacterium plant cell suspension cultures following infection by Agrobacterium (Ditt et al., 2001). Differential expression of these genes suggests an alteration in carbohydrate metabolism following Agrobacterium infection. However, the significance of this alteration is not clear.

The majority of genes grouped in the above-mentioned categories showed induced expression during early stages of infection with various strains of Agrobacterium irrespective of the bacterium’s ability to transfer T-DNA and/or Vir proteins into the plant cell. Differential expression of plant genes in response to Agrobacterium infection was also studied by Ditt et al. (2001). Using AFLP RNA differential display, they observed that Agrobacterium infection of Ageratum suspension culture cells could induce plant defense machinery, and that these responses were similar to those obtained during infection of plant cells with Escherichia coli. In their study, only expression of a nodulin-like gene was uniquely regulated by Agrobacterium. Ditt et al. (2001) utilized only a transfer-competent Agrobacterium strain, and their study investigated only late (24 and 48 h) times of infection. In contrast, our studies showed that expression of defense response genes was significantly reduced during the later stages of infection in plant cells infected with transfer-competent strains of Agrobacterium. However, cells infected with a transfer-deficient Agrobacterium strain showed significant re-induction of these genes during the later stages of transformation. These observations suggest that suppression of host defense responses is prerequisite to successful plant transformation. Genes encoding proline-rich proteins showed an early induction in response to Agrobacterium infection, similar
to that of other defense-related genes. However, unlike the expression patterns of these other defense-related genes, during later stages of infection, transcripts encoding proline-rich proteins had induced expression during infection with a transfer-competent strain compared to a transfer-deficient strain of *Agrobacterium*, thereby suggesting the importance of these proteins in later stages of *Agrobacterium*-mediated transformation. We additionally observed significant induction of the proline-rich protein genes in response to infection by an *Agrobacterium* strain lacking virE2, but less so using a strain containing virE2. However, the significance of this induced expression needs to be explored in greater detail.

Defense-related genes were significantly induced during later stages of infection with a transfer-deficient strain. The late differential induction of defense-related genes in cells infected with this strain compared to a transfer-competent strain suggests that transfer of T-DNA and Vir proteins was somehow able to mask the induction of defense responses during later stages of transformation. A suppression of defense genes was observed during infection with various transfer-competent *Agrobacterium* strains, suggesting that general plant defense responses are suppressed during a successful transformation event. It is significant to note that an *A. thaliana* cep1 mutant identified by Silva *et al.* (1999), which shows constitutive expression of PR-1, PR-2, and PR-5 defense-related genes, has reduced susceptibility to *Agrobacterium* infection (Veena and S.B. Gelvin, unpublished). Induction of defense response genes during the early stages of infection may result from attachment of the bacterium to the plant cell or merely the presence of *Agrobacterium* in the vicinity of plant cells. Induction of defense responses during infection of *Ageratum* cell suspension cultures with *Agrobacterium* and *E. coli* has been suggested by Ditt *et al.* (2001). These authors speculated that induction of plant defense genes may result from the transfer of certain soluble factors from the bacterium to plant cells. A detailed investigation of plant responses to attachment-deficient and attachment-proficient *Agrobacterium* strains will provide additional clues to these responses.

Genes similar to the early nodulin ENOD18 of *Vicia faba* showed differential expression after *Agrobacterium* infection. During *Rhizobium* infection, ENOD18 is differentially regulated during various stages of nodule development (Becker *et al.*, 2001; Hohnjec *et al.*, 2000). Ditt *et al.* (2001) identified an *Ageratum* putative nodulin gene that was differentially regulated following infection by *Agrobacterium*. Several clones encoding Class 1 non-symbiotic hemoglobins identified in this study showed expression patterns similar to that of various defense-related genes. Non-symbiotic hemoglobins have a high affinity for oxygen; however, the exact biological function of these proteins in plants is still unknown (Hunt *et al.*, 2001; Seregelyes *et al.*, 2000).

Tobacco genes generally involved in cell division and growth processes showed differential expression during *Agrobacterium*-mediated transformation. These genes were induced during later stages of infection, and the level of their transcripts remained significantly higher when plant cells were infected with a transfer-competent *Agrobacterium* strain compared to a transfer-deficient strain. This result suggests the importance of these genes during *Agrobacterium*-mediated transformation, and also indicates a possible role of T-DNA and Vir proteins in modulating expression of these genes. A number of genes encoding ribosomal proteins fall into this category. These genes are highly induced in cells that are proliferating and undergoing cell division. High expression of various ribosomal proteins was also reported during nodule formation in legumes (Gyorgyey *et al.*, 2000). In eukaryotes, four ribosomal RNAs and approximately 80 ribosomal proteins are known to contribute to the structure of ribosomes. Coordinated expression of all ribosomal constituents is essential for correct ribosome assembly (Mager, 1988).

Various members of core histone gene families (including histones H2A, H2B, H3, and H4) also showed differential expression during various stages of *Agrobacterium*-mediated transformation of tobacco BY-2 cells. Yi *et al.* (2002) recently showed that the *A. thaliana* histone H2A-1 gene is similarly induced upon *Agrobacterium* infection of roots. Of special significance is the induced expression of these genes observed during the later stages of transformation by transformation-proficient *Agrobacterium* strains, but not by a transformation-deficient strain. This latter induction occurs at a time at which T-DNA integration takes place (Mysore *et al.*, 1998). Mysore *et al.* (2000) demonstrated that disruption of the *A. thaliana* histone H2A-1 gene in the rats5 mutant results in decreased T-DNA integration into the plant genome. Mutant *Arabidopsis* T-DNA insertion lines with disruptions of other histone genes, including histones H2B, H3, and H4, are also recalcitrant, to various extents, to *Agrobacterium* transformation (H. Cao, J. Li, A. Kaiser, A. Kopecki, Y. Zhu, and S.B. Gelvin, unpublished). In addition, over-expression of the *Arabidopsis* histone H2A-1 gene or cDNA can result in increased transformation efficiency of *Arabidopsis* (Mysore *et al.*, 2000; H. Yi, T. Fujinuma, and S.B. Gelvin, unpublished) and *Brassica napus* (S. Davis, L.-Y. Lee, and S.B. Gelvin, unpublished), and over-expression of this same gene can sensitize recalcitrant *Arabidopsis* ecotypes and rats mutants to *Agrobacterium*-mediated transformation (L.-Y. Lee, X. Sui, S. Davis, and S.B. Gelvin, unpublished).

Several clones encoding elongation factor-1 (EF-1) were also identified in this study. EF-1 is known to be involved in the coordinated regulation of multiple cellular processes including growth, division, and transformation (Negrutskii and El’skaya, 1998). EF-1 has significant similarities with
vitronectin-like proteins that have previously been proposed to play a role in attachment of Agrobacterium to plant cells (Wagner and Mattheyse, 1992). Clones similar to RNA-binding protein RZ-1, a transcriptionally controlled tumor protein (TCTP), and a GTP-binding protein beta chain homolog that are known to be involved in DNA replication, recombination, and repair (Hanano et al., 1996; Ishida et al., 1993; Sanchez et al., 1997) were also identified in this study. Transcriptionally controlled tumor-related proteins also have tubulin-binding properties and associate with microtubules in a cell-cycle-dependent manner (Gachet et al., 1999).

Certain chaperonins, such as T-complex protein, chaperonin CNP60-1, heat shock cognate protein 80, heat shock protein cognate 70, and Nicotiana tabacum chaperone GrpE type 2, were also identified as differentially expressed following Agrobacterium infection. Similarly, clones encoding proteins related to a sec-independent translocase TATC protein from Oryza sativa (Agrawal et al., 2001) and a P-glycoprotein-like protein (an ABC transporter) were also identified. One of the hypothetical proteins (BQB42824) identified in this study showed significant similarity to the common tobacco retrotransposon Tto 1. Tto 1 is known to carry all functions required for autonomous transposition through reverse transcription. Hirochika et al. (1996) suggested that Tto 1 can transpose autonomously through reverse transcription, and that host factors required for transposition are conserved among monocots (class Magnoliopsida; rice) and dicots (class Liliopsida; tobacco).

It would be interesting to determine if any of the genes mentioned above play a role in Agrobacterium-mediated transformation, especially in relation to transport of the T-complex from the cytoplasm to the nucleus and/or integration of the T-DNA into the plant genome.

One of the Agrobacterium strains used in our study expresses osa, a gene that has been previously shown to block VirE2 export from Agrobacterium to the plant cell (Lee et al., 1999). VirE2 is a single-stranded DNA-binding protein that presumably coats the T-strand and prevents nucleolytic degradation during the transfer of the T-DNA complex from the plant cytoplasm to the nucleus (Citozky et al., 1997; Rossi et al., 1996; Yusibov et al., 1994). T-DNAs delivered from a virE2 mutant bacterium are often severely truncated after integration into the plant genome (Rossi et al., 1996). Infection of plants by an Agrobacterium strain that cannot transfer VirE2 protein rarely results in transformation (Lee et al., 1999). Infection of BY-2 cell suspension cultures with an osa-containing Agrobacterium strain similarly resulted in negligible transient transformation. Interestingly, the expression of various histones and ribosomal proteins after infection by this strain appeared to be greater relative to expression of these plant genes following infection with an Agrobacterium strain that is capable of transferring only Vir proteins or T-DNA and Vir proteins to plant cells. These results suggest that naked T-DNA can induce the genes to a higher level than can the transfer of Vir proteins alone or T-DNA coated with Vir proteins.

Agrobacterium-mediated transformation is a highly complex process involving genetic determinants of both the bacterium and the host plant cell. Differential expression of various plant genes during Agrobacterium-mediated transformation indicates successful utilization of existing host cellular machinery by Agrobacterium for genetic transformation purposes. Our observations suggest that Agrobacterium infection induces plant genes necessary for the transformation process while simultaneously repressing host defense response genes. In-depth analysis of these genes will contribute to a better understanding of basic biological processes such as cell communication, intracellular molecular transport, and DNA repair and recombination that occur during the process of Agrobacterium-mediated transformation. This fundamental knowledge may help in designing new strategies to improve the transformation efficiency of various recalcitrant plant species.

**Experimental procedures**

Agrobacterium strains and growth conditions

All Agrobacterium strains were cultured at 30°C in Yeast extract-Peptone (YPE) medium or AB-sucrose medium (Lichtenstein and Draper, 1986) supplemented with the appropriate antibiotics (tetracycline, 10 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; carbenicillin, 50 µg ml⁻¹). Characteristics of the various Agrobacterium strains used in this study are listed in Table 1.

Agrobacterium-mediated transformation of BY-2 cell suspension cultures

Nicotiana tabacum BY-2 cell suspension cultures were maintained in Murashige and Skoog medium (Gibco-BRL, Grand Island, NY, USA) containing 3% sucrose, 1 µg ml⁻¹ thiamine, 0.2 µg ml⁻¹ 2,4-dichlorophenoxyacetic acid, and 370 µg ml⁻¹ K2HPO4 with continuous shaking at 140 r.p.m. at 23°C in the presence of light. Agrobacterium cells induced with acetosyringone were used for the infection of plant cells. Agrobacterium cells were grown to a density of 2 × 10⁸ cells ml⁻¹ (A = 100, using Klett–Summerson spectrophotometer, red filter) in AB-sucrose medium. Cells were harvested by centrifugation at 10,000 g and re-suspended in two volumes of induction medium (AB salts, 0.5% glucose, 2 mM sodium phosphate, 50 mM MES, pH 5.6, 100 µM acetosyringone), and incubated at 23°C for 14–18 h with gentle shaking. Induced Agrobacterium cells were washed with plant cell culture medium and used to infect BY-2 cell suspension cultures (approximately 1000 bacterial cells per plant cell). For infection, 5-6-day-old BY-2 cell suspension cultures were diluted with three volumes of plant cell suspension medium and mixed with induced Agrobacterium cells. Co-cultivation was carried out at 23°C with gentle shaking at 80 r.p.m. for various time periods (0, 3, 6, 12, 24, 30, and 36 h). After infection, plant cells were pelleted by centrifugation at 50 g (model GLC-2 clinical centrifuge; Beckman Sorvall, Newton, CT, USA) and washed three times in plant cell suspension medium. Five independent infection experiments (50 ml volume of BY-2 cells) were
performed for each treatment (uninfected BY-2 cell suspension cultures and cultures infected with various strains of Agrobacterium), followed by pooling of samples for RNA isolation at each time point. For RNA isolation, plant cell pellets were frozen in liquid nitrogen and stored at −80 °C.

To determine the efficiency of Agrobacterium-mediated transformation, the plant cell suspension cultures were collected after 48 h of Agrobacterium infection, washed as described above, and stained with GUS staining solution (50 mM sodium phosphate buffer, pH 7.0, 0.1% Tween 20, 3% sucrose, and 1–2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc)) overnight at 37 °C. Stained cells were visualized using a light microscope and scored to determine the percentage of cells transformed.

**Preparation of subtracted cDNA libraries**

Subtracted libraries were prepared by ‘suppressive subtractive hybridization’ (SSH) using the Clontech PCR-select™ cDNA subtraction kit (Clontech Laboratories Inc., Palo Alto, CA, USA). Fifty micrograms of total RNA was used to isolate mRNA using the PolyATtract™ mRNA Isolation System (Promega, Madison, WI, USA) following the protocols provided by the manufacturer. SSH was performed using RNA isolated from uninfected BY-2 cell suspension cultures and cultures infected for 12 h with the transfer-competent Agrobacterium strain A8904. For ‘forward’ subtraction, RNA isolated from BY-2 cell suspension cultures infected with A. tumefaciens A804 was used as the ‘tester’; for ‘reverse’ subtraction, it was used as the ‘driver’.

After completion of SSH, forward- and reverse-subtracted PCR amplified products were cloned into the TA cloning vector pCRTM-2-1-TOPO (Invitrogen Corporation, Carlsbad, CA, USA) and used to transform electro-competent E. coli DH10B ( Gibco-BRL) under standard conditions. The electropercolated cells were plated on LB agar plates containing 50 μg ml⁻¹ of ampicillin with blue/white selection. A total of 17, 280 white colonies (8,064 from the forward-subtracted library, 9,216 from the reverse-subtracted library) were picked robotically (OPix, Genetix) and transferred to 384 well plates containing 30 μl of liquid LB supplemented with 50 μg ml⁻¹ of ampicillin and 10% glycerol. The plates were incubated at 37 °C to prepare master plates. Overnight-grown cultures were either stored at −80 °C or instantly used for gridding.

**Preparation of colony, DNA, and RNA macroarrays**

Colonies were prepared robotically (BioGrid, BioRobotics Limited, UK) by spotting 17,280 bacterial clones from subtracted cDNA libraries onto positively charged nylon membranes (Schleicher and Schuell, Keene, NH, USA) placed on LB plates containing 50 μg ml⁻¹ of ampicillin. The colonies were grown at 37 °C to the optimum size. Lysis of colonies and DNA binding to the membrane were performed as described by Sambrook et al. (1989). On each DNA macroarray, PCR-amplified products from 421 potential differentially expressed clones and 59 null (control) clones were spotted. PCR was performed in 100-μl reaction volumes using 1 μl of overnight grown E. coli cultures and M13 forward and M13 reverse primers for 30 cycles (94 °C, 1 min; 56 °C, 1 min; 72 °C, 1 min). The PCR products were analyzed by electrophoresis through 2% agarose gels before use. PCR products were denatured by addition of NaOH and Na₂EDTA (pH 8.0) to final concentrations of 0.4 M and 10 mM, respectively, followed by incubation at 80 °C for 10 min. RNA arrays were prepared by spotting 43 different samples of RNA on nylon membranes. Before spotting, total RNA was treated with 1× MOPS buffer (20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 8 mM sodium acetate, 1 mM Na₂EDTA, pH 7.0), 7% (w/v) formaldehyde, 5% (v/v) glycerol, 50% (v/v) formamide, and 0.025% (v/v) of saturated aqueous bromophenol blue solution followed by incubation at 65 °C for 15 min. Bacterial colonies and DNA samples were spotted in duplicates with print pins size 0.4 mm (BioGrid), and RNA samples were spotted in quadruplicate with print pin size 2 mm (BioGrid).

**RNA extraction and RNA blot hybridization**

Total RNA was isolated from various samples according to Chomczynski and Sacchi (1987). Approximately 20 μg of total RNA was subjected to electrophoresis through 1.2% formaldehyde-agarose gels and transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). RNA arrays and RNA blots were probed with selected gene fragments. Labeling was performed in the presence of 32P-dCTP (Amersham Pharmacia Biotech) using a random primer Ready-to-Go labeling kit (Amersham Pharmacia Biotech) for at least 3 h. Unincorporated radiolabeled nucleotides were removed by passing the reaction mix through Sephadex G-50 columns (Sambrook et al., 1989). Membranes were pre-hybridized for 3–4 h at 65 °C in buffer containing 0.5 M sodium phosphate buffer, pH 7.0, 7% SDS, 10 mM EDTA, pH 8.0, and 100 μg ml⁻¹ of denatured salmon sperm DNA. Hybridization was carried out under the same conditions in the presence of the radiolabeled probe for 16–18 h. Membranes were washed twice in 0.1 SSC, 0.1% SDS at 65 °C and 0.1× SSC, 0.1% SDS for 15 min each at 65°C and exposed for autoradiography.

**Colony and DNA macroarray hybridization**

Macroarrays were pre-hybridized in 10 ml of hybridization solution (0.5 M sodium phosphate buffer, pH 7.0, 7% SDS, 10 mM EDTA, pH 8.0, and 100 μg ml⁻¹ of denatured salmon sperm DNA) for 3 h at 65 °C. Each RNA sample was reverse-transcribed in the presence of 32P-dCTP. Reverse-transcribed single-stranded radiolabeled cDNA probe (20 μl) was mixed with 2 μl of 50 mM EDTA, pH 8.0, and 1 μl of 10 N NaOH and incubated at 65 °C for 20 min. Samples were neutralized by addition of 2 μl of 5 M glacial acetic acid and purified through Sephadex G-50 columns. Purified probes were denatured and added to 10 ml of hybridization solution. Hybridization conditions for colony array and DNA arrays were same as described above for RNA blot hybridization.

**Sequencing and analysis of cDNA clones**

Clones showing significant differential expression were selected and used for preparation of cDNA macroarrays. All clones were sequenced using an automated sequencer using T7 and M13 R primers at the Purdue University DNA Sequencing Facility, and sequence homologies were determined by comparison with databases using BLAST at the National Center for Biotechnology Information (BLASTX: E-value cut-off = 1e⁻05; BLASTN: E-value cut-off = 1e⁻10).

**Data acquisition, processing, and analysis**

The acquisition and analysis of the data presented in this paper conform fully with the MIAME guidelines (http://www.mged.org/Workgroups/MIAME/miame.html).

Hybridized macroarrays were subjected to autoradiography and scanned using a personal densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Spot intensities from scanned images were quantified using QuantArray™ analysis software (GSI Lumonics, Billerica, MA, USA). Grids were pre-defined and manually adjusted to insure optimal spot recognition. Spots were individually quantified using QuantArray’s fixed circle method; spot intensity...
was measured as the mean of pixels within a circle encompassing the spot. Clones that did not show a single strong PCR band on agarose gels before macroarray printing were not considered for statistical analysis. For statistical analysis, we used a split plot experimental design with the experimental unit for whole plot being the array, and the experimental unit for sub plot being each clone. An analysis of variance (ANOVA) model was employed for the purpose of testing clone expression changes among different treatments. The actual clone expression measurement is the response variable because our model takes background noise into account within its conceptual framework. Every clone intensity measurement is associated with a particular combination of array, treatment, time point, and gene. Let \( y_{ijk} \) be the clone intensity on a logarithmic scale from the \( P \)th spot for clone \( k \), and treatment \( i \) at time point \( j \). The ANOVA model is:

\[
Y_{ijk} = \mu + \alpha_i + \beta_j + \chi_{ijk} + \epsilon_{ijk}
\]

where \( i = 1, 2, 3; j = 1, \ldots, 5; k = 1, \ldots, 480; \) and \( \mu \) is the mean intensity of clone \( k \) under treatment \( i \) at time point \( j \) and \( \mu \) represents the overall mean clone expression. The main effects \( \alpha, \beta, \chi \) and \( \epsilon \) account for the variation in treatments and time points. The random error \( \epsilon_{ijk} \) is associated with variation between arrays with mean 0 and variance \( \sigma^2 \). As one array was used for each combination of treatment and time point, the treatment by time interaction is confounded with the array effect, \( \epsilon_{ijk} \). In other words, if the treatment and the time point were known, one knows which array is associated with that observation. Therefore, it is required that we use the treatment by time interaction as the whole-plot error. The clone effect \( \epsilon_{.ij} \) accounts for the overall average intensity for clone \( k \) across treatments and time points. Additionally, \( (\overline{y}_{.ij} - \mu) \) captures the expression of clone \( k \) under treatment \( i \) at time_point \( j \). \( (\overline{y}_{.ij} - \mu) \) captures the expression of clone \( k \) at time point \( j \), and \( (\overline{y}_{.ij} - \mu) \) is the interaction between treatment \( i \), time \( j \) and clone \( k \) (i.e. the ‘spot’ effect). The term \( \overline{y}_{.ij} \) represents independent random error associated with variation within an array with mean 0 and variance \( \sigma^2 \). The least square estimates of the parameters were computed subject to the usual constraints for the linear models.

As our main interest was to identify differentially expressed genes in response to different treatments at each of the five time points, at each time point \( j \), for each clone \( k \), and two different treatments \( (i \) and \( t \) ), we tested the difference in the mean clone expression intensities. The alternative conclusions we wish to consider are \( H_0^i: \overline{y}_{.ij} = \mu \) against \( H_1^i: \overline{y}_{.ij} \neq \mu \). The classical \( t \)-statistic is:

\[
T = \frac{\overline{y}_{.ij} - \overline{y}_{.ik}}{\sqrt{2\sigma^2 + \sigma^2}}
\]

where \( \overline{y}_{.ij} \) is the average intensity reading over index \( r \), \( \sigma^2 \) and \( \sigma^2 \) are the estimated variances for the whole-plot errors and subplot errors, respectively.

It is well known that the distribution of the error terms is often affected in macroarray data. In anticipation of these issues, randomization techniques such as bootstrapping (over the classical \( t \)-tests and \( F \)-tests) have been suggested by Kerr and Churchill (2001). For the split-plot design used here, a bootstrap procedure (Davison and Hinkley, 1997) was employed for the purpose of obtaining an accurate assessment of both the whole-plot and subplot error distributions. The \( P \)-values of each \( t \)-statistic were computed via bootstrapping the residuals. As there are three treatments and five time points, the total number of pair-wise comparisons made is \( 3 \times 5 \times 480 \times 7200 \). An approach to control the false discovery rate (Benjamini and Hochberg, 1995) of the multiple tests was employed to produce a list of statistically differentially expressed clones. In order to summarize the expression profiles of all treatments with respect to time, K-mean clustering was performed using Spotfire DecisionSite 6.3 software. Euclidean distance- and data-centroid-based searches were used as the criteria for similarity measurement.

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Supplementary Material

The following material is available from http://www.blackwell-publishing.com/products/journals/suppmat/TPJ/TPJ796/ TPJ1796sm.htm

Table S1 Detailed list of clones differentially expressed during early stages of Agrobacterium-mediated transformation

Figure S1. cDNA microarray analysis during early stages of Agrobacterium-mediated transformation.

(a) Representative image of macroarrays showing pattern of DNA spotting. Macaroarrays were prepared robotically by spotting PCR-amplified cDNA clones on a nylon membrane. Each sample was spotted twice in diagonal orientation (inset).

(b) Images of differentially screened cDNA macroarrays during Agrobacterium-mediated transformation. Macaroarrays were hybridized with equal amounts of reverse-transcribed radiolabeled probe of RNA obtained from uninjected BY-2 cell suspension cultures and cultures infected for 0, 3, 6, 12, 24, and 36 h with transfer-competent A. tumefaciens At804 or transfer-deficient A. tumefaciens At793.

Figure S2. Expression pattern of genes putatively involved in the process of cell division and growth during Agrobacterium-mediated transformation.

RNA arrays were prepared robotically by spotting 2 \( \mu \)g of total RNA isolated from uninfected BY-2 cell suspension cultures (control) and cultures infected with A. tumefaciens At793, At804, At542, At1221, or At1222 for various periods of time (0, 3, 6, 12, 18, 24, 30, and 36 h). RNA arrays were hybridized with various radiolabeled gene fragments showing homology with genes involved in cell division and growth processes. RNA array hybridized with rRNA was used as a loading control.

Figure S3. Expression pattern of genes putatively involved in the process of cell division and growth during Agrobacterium-mediated transformation.

RNA arrays were prepared robotically by spotting 2 \( \mu \)g of total RNA isolated from uninfected BY-2 cell suspension cultures (control) and cultures infected with A. tumefaciens At793, At804, At542, At1221, or At1222 for various time points (0, 3, 6, 12, 18, 24, 30, and 36 h). RNA arrays were hybridized with various radiolabeled gene fragments showing homology with genes involved in stress responses. RNA array hybridized with rRNA was used as a loading control.

References


primary cell wall proteins from plants. J. Biol. Chem. 272, 15841–15848.
Rossi, L., Hohn, B. and Tinland, B. (1996) Integration of complete
transferred DNA units is dependent on the activity of virulence
USA 93, 126–130.
Harbor Laboratory Press.
Sanchez, J.C., Schaller, D., Ravier, F., Golaz, O., Jaccoud, S., Belet,
M., Wilkins, M.R., James, R., Deshusjes, J. and Hochstrasser, D.
(1997) Translational controlled tumor protein: a protein iden-
tified in several nontumoral cells including erythrocytes.
Electrophoresis, 18, 150–155.
Schrammeijer, B., Risseeuw, E., Pansegrau, W., Regensburg-Tuin,
T.J., Crosby, W.L. and Hooykaas, P.J. (2001) Interaction of
the virulence protein VirF of Agrobacterium tumefaciens
with plant homologs of the yeast Skp1 protein. Curr. Biol. 11,
258–262.
localization of a hypoxia-inducible novel non-symbiotic hemo-
transport: have virulence proteins, will travel. Plant Cell, 8,
1699–1710.
Showalter, A.M. (1993) Structure and function of plant cell wall
Characterization of a new Arabidopsis mutant exhibiting
enhanced disease resistance. Mol. Plant Microbe Interact. 12,
1053–1063.
Smarrelli, J., Watters, M.T. and Diba, L.H. (1986) Response of
various cucurbits to infection by plasmid-harboring strains of
encoding glutathione S-transferase. Proc. Natl. Acad. Sci. USA,
89, 56–59.
Takahashi, Y., Kuroda, H., Tanaka, T., Machida, Y., Takebe, I.
eDNA expressed during the transition from G0 to S phase in
tobacco mesophyll protoplasts. Proc. Natl. Acad. Sci. USA, 86,
9279–9283.
Tzfira, T. and Citovsky, V. (2002) Partners-in-infection: host pro-
tein involved in the transformation of plant cells by Agrobac-
Nucleic acid transport in plant–microbe interaction: the mole-
cules that walk through the walls. Annu. Rev. Microbiol. 54,
protein that interacts with Agrobacterium VirE2, is involved in
VirE2 nuclear import and Agrobacterium infectivity. EMBO J. 20,
3596–3607.
Wagner, V.T. and Matthissyse, A.G. (1992) Involvement of a vito-
nectin-like protein in attachment of Agrobacterium tumefaciens
Osmotic stress-induced changes of sucrose metabolism in cul-
Arabidopsis histone H2A-1 gene correlates with susceptibility to


