PLANT BIOLOGY

Cytokinins Secreted by *Agrobacterium* Promote Transformation by Repressing a Plant Myb Transcription Factor

Nagesh Sardesai,[1]* Lan-Ying Lee,[1] Huabang Chen,[1]† HoChul Yi,[1]‡ Gayla R. Olbricht,[2]§ Alexandra Stirnberg,[1] Jacob Jeffries,[1] Kia Xiong,[1] R.W. Doerge,[2] Stanton B. Gelvin[1]¶

***Agrobacterium*-mediated transformation is the most widely used technique for generating transgenic plants. However, many crops remain recalcitrant. We found that an *Arabidopsis* myb family transcription factor (MTF1) inhibited plant transformation susceptibility. Mutating *MTF1* increased attachment of several *Agrobacterium* strains to roots and increased both stable and transient transformation in both susceptible and transformation-resistant *Arabidopsis* ecotypes. Cytokinins from *Agrobacterium tumefaciens* decreased the expression of *MTF1* through activation of the cytokinin response regulator ARR3. Mutating *AHK3* and *AHK4*, genes that encode cytokinin-responsive kinases, increased the expression of *MTF1* and impaired plant transformation. Mutant *mtf1* plants also had increased expression of *AT14A*, which encodes a putative transmembrane receptor for cell adhesion molecules. Plants overexpressing *AT14A* exhibited increased susceptibility to transformation, whereas *at14a* mutant plants exhibited decreased attachment of bacteria to roots and decreased transformation, suggesting that AT14A may serve as an anchor point for Agrobacteria. Thus, by promoting bacterial attachment and transformation of resistant plants and increasing such processes in susceptible plants, treating roots with cytokinins may help engineer crops with improved features or yield.**

INTRODUCTION

Agrobacterium tumefaciens causes crown gall disease in plants and genetically transforms numerous plant (*1*, *2*), fungal (*3*), and animal (*4*) species. Virulent Agrobacteria harbor a tumor-inducing (Ti) plasmid containing virulence (*vir*) genes required by the pathogen to transport transferred DNA (T-DNA) and virulence effector proteins to host cells. Induction of *vir* genes, processing of T-DNA from the Ti plasmid, attachment of the bacteria to plants, and subsequent transfer of T-DNA and Vir proteins to host cells are complex processes. Numerous studies have elucidated the events governing these processes in the bacterium, but relatively little is known about the plant contribution to transformation (*5*–*9*).

Agrobacterium-mediated plant transformation forms the basis for the modern agricultural biotechnology industry, and although *Agrobacterium* has a broad host range, many economically important plants remain recalcitrant to transformation. Scientists have used a variety of techniques to identify plant genes that are involved in *Agrobacterium*-mediated transformation. Among these, forward and reverse genetic screens revealed more than 125 such *Arabidopsis* (*6*, *10*, *11*) and tobacco (*12*) genes. Collectively, the plant lines used in these screens are designated *rat* (resistant to *Agrobacterium* transformation), reflecting their attenuated transformation response. The genes identified encode proteins that function in specific steps necessary for successful transformation (*6*). However, none of these mutants identify genes regulating the extent of transformation susceptibility.

Therefore, we conducted a genetic screen to search for *Arabidopsis* mutants that are "hyper-susceptible to *Agrobacterium* transformation (*hat*)" from a T-DNA activation-tagged library (*13*). This screen identified the mutant *mtf1-1*, in which the gene *At2g40970*, encoding a putative myb family transcription factor (MTF1), is disrupted. *MTF1* is a member of a five-gene family whose encoded proteins have a single SHAQKYF-type Myb DNA binding domain that is unique to plants (*14*) and is similar to those found in proteins associated with two-component signal transduction systems (*15*), including GOLDEN2-LIKE (GLK), the B-type *Arabidopsis* response regulators (ARRs), and pseudoresponse regulator 2 (PRR2) (*14*). MTF1 negatively regulates cold and osmotic stress tolerance (*16*). Here, MTF1 inhibited the susceptibility of *Arabidopsis* to *Agrobacterium*-mediated transformation, suggesting that targeting MTF1 or its gene *At2g40970* could increase transformation susceptibility in crop plants.

[1]Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA. [2]Department of Statistics, Purdue University, West Lafayette, IN 47907, USA.
*Present address: Dow AgroSciences, 9330 Zionsville Road, Indianapolis, IN 46268, USA.
†Present address: State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China.
‡Present address: Syngenta Biotechnology, Research Triangle Park, NC 27709, USA.
§Present address: Department of Mathematics and Statistics, Missouri University of Science and Technology, Rolla, MO 65409, USA.
¶Corresponding author. E-mail: gelvin@purdue.edu

RESULTS

mtf1 mutants show increased transformation

To verify the importance of MTF1 in transformation, we analyzed transformation susceptibility of independent *mtf1* mutants. The heterozygous mutant *mtf1-1* contains a T-DNA insertion in the 5′ untranslated region of *MTF1*, 36 bp (base pair) upstream of the start codon (Fig. 1A), and displayed about 10-fold increase in transformation susceptibility compared with that of the wild-type Columbia ecotype, Col-7 (Fig. 1B). Likewise, heterozygous *mtf1-2* and *mtf1-3* mutants displayed a four- to

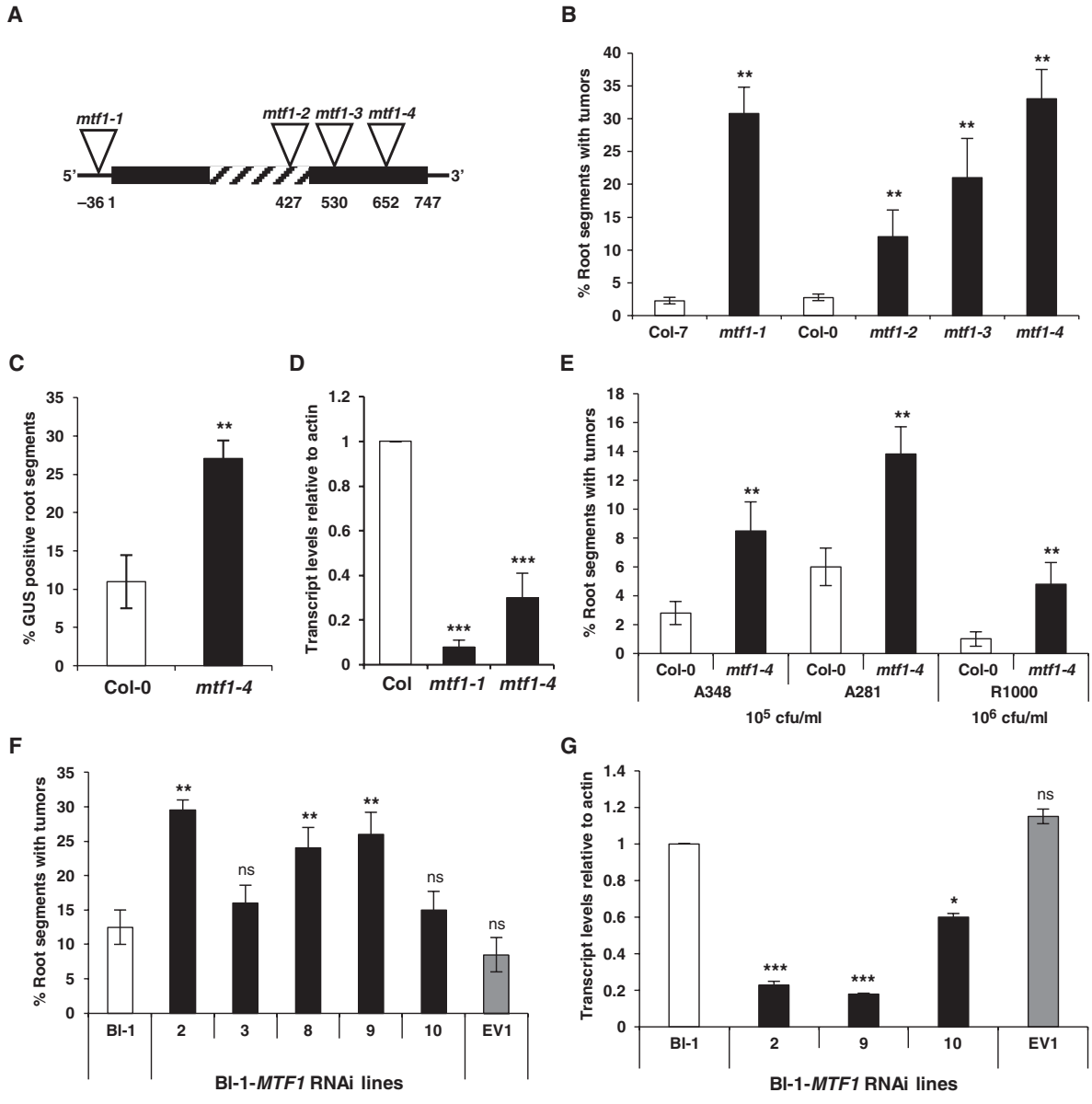

Fig. 1. Expression of *MTF1* influences plant susceptibility to *Agrobacterium*-mediated transformation. (**A**) Map of T-DNA insertion positions in *MTF1* mutants. Numbers indicate nucleotide positions; +1 indicates translation start site. Striped box denotes Myb DNA binding domain. (**B**) Percentage of root segments developing tumors after inoculation with *A. tumefaciens* A208 [10^5 colony-forming units (CFU)/ml]. $^{**}P < 0.02$, *mtf* lines to Col lines. (**C**) Percentage of GUS-positive root segments after inoculation with *A. tumefaciens* At849 (10^5 CFU/ml). $^{**}P < 0.02$, *mtf1-4* to Col-0. (**D**) Relative *MTF1* transcript abundance in wild-type (Col-7 or Col-0), *mtf1-1*, and *mtf1-4* roots. $^{***}P < 0.001$, *mtf1-1* and *mtf 1-4* to Col. (**E**) Percentage of root segments developing tumors after inoculation with *A. tumefaciens* A348, A281, and *A. rhizogenes* R1000. $^{**}P < 0.02$, *mtf1-4* to Col-0. (**F**) Percentage of root segments developing tumors on T2 generation Bl-1–*MTF1*-RNAi lines inoculated with *A. tumefaciens* A208 (10^8 CFU/ml). $^{**}P < 0.02$, lines 2, 8, and 9 to Bl-1. (**G**) Relative *MTF1* transcript abundance in roots of Bl-1 and *MTF1*-RNAi lines 2, 9, and 10, and empty vector (EV). $^{***}P < 0.001$, $^{*}P < 0.05$, MTF1-RNAi lines to Bl-1. Data are means ± SEM from $n = 3$ (for relative transcript abundance) or $n = 5$ (for percentage of roots developing tumors or GUS activity) biological replicates. ns, not significant.

sevenfold increase in stable transformation susceptibility (Fig. 1B). We could not recover homozygous mutant plants from self-fertilized progeny of *mtf1-1*, *mtf1-2* (SALK_072082), or *mtf1-3* (SALK_102624), suggesting that loss of all *MTF1* functions may be lethal. Self-fertilization of only *mtf1-4* (SALK_072083) resulted in a homozygous mutant. The insertion in *mtf1-4* permits expression of about 85% of the *MTF1* open reading frame, indicating that the majority of MTF1 protein is essential for *Arabidopsis* viability. Homozygous *mtf1-4* plants showed an about 11-fold increase in stable transformation susceptibility (Fig. 1B). Other than the inviability of homozygous *mtf1-1*, *-2*, and *-3* mutants, neither

any of the heterozygous *mtf* mutants nor the homozygous *mtf1-4* displayed any additional visible phenotype.

Additionally, we investigated whether disruption of *MTF1* also alters transient transformation. β-Glucuronidase (GUS) activity, resulting from the transfer of a *gusA*-intron gene from *Agrobacterium* to plants, is a standard assay to assess transient transformation that does not rely on hormone responses, unlike tumorigenesis assays (*11*, *17*). Compared with Columbia ecotype Col-0 plants, *mtf1-4* plants showed about 2.5-fold increase in transient transformation (Fig. 1C). Thus, all four *mtf1* mutant lines displayed a *hat* phenotype, highlighting the importance of *MTF* in transformation.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) assays using primers directed to the 5′ end of the *MTF1* gene revealed that *MTF1* transcript abundance decreased 3-fold in homozygous *mtf1-4* mutants and more than 12-fold in heterozygous *mtf1-1* mutants compared with that in wild-type plants (Fig. 1D), demonstrating that transformation susceptibility is inversely correlated with *MTF1* transcript abundance. Our primers amplified a region of the *mtf1* complementary DNA (cDNA) upstream of the T-DNA insertion site in *mtf1-4*, and because *mtf1-4* is homozygous, our assay detected only the truncated transcript. The threefold decrease in the amount of this 5′ region of the *MTF1* transcript in the *mtf1-4* plants likely reflects instability of this truncated transcript. Because *mtf1-1* is heterozygous for the *MTF1* locus, one may expect a 50% reduction in *MTF1* transcript abundance. However, because we detected a more than 12-fold decrease in *MTF1* transcript abundance, our results suggest a complex unknown mechanism that may involve partial silencing of the remaining *MTF1* allele. The observed haploinsufficiency of the *mtf1-1*, *-2*, and *-3* mutants suggests that dosage of this gene is critical for transformation susceptibility. Further experiments in this study therefore used homozygous *mtf1-4* plants.

The transformation experiments described above were carried out using *A. tumefaciens* A208 that contains a nopaline-type Ti plasmid. To assess whether *mtf1-4* shows increased susceptibility to other *Agrobacterium* strains, we conducted root transformation assays using the octopine-type strain A348, the succinamopine-type strain A281, and *Agrobacterium rhizogenes* R1000 containing the Ri plasmid pRiA4b. The *mtf1-4* mutant displayed two- to fivefold increased transformation susceptibility to these strains (Fig. 1E). Thus, *MTF1* plays an important role in plant susceptibility to various *Agrobacterium* strains.

Ectopic expression of wild-type *MTF1* cDNA in *mtf1-4* plants resulted in several transgenic lines that exhibited transformation susceptibility similar to that of wild-type plants (fig. S1A). These transgenic lines individually expressed increased abundance of *MTF1* mRNA (fig. S1B). These complementation experiments confirmed that disruption of the *MTF1* gene is responsible for increased transformation susceptibility.

Targeting *MTF1* increases transformation of a recalcitrant *Arabidopsis* ecotype

The *mtf1-1*, *-2*, *-3*, and *-4* mutants were of the Columbia background, an ecotype relatively amenable to root transformation. *Arabidopsis* ecotype Bl-1 is highly recalcitrant to root transformation (*18*), even when inoculated with 10^8 bacteria/ml, but can be transformed using a floral dip method (*19*). *MTF1* genes of ecotypes Columbia and Bl-1 have identical sequences. We introduced into ecotype Bl-1 an RNA interference (RNAi) expression construction targeting *MTF1* transcripts and screened the derived transgenic lines for root transformation susceptibility. Nine of the 10 tested T1 generation transgenic plants exhibited increased susceptibility (fig. S2). We tested 25 T2 generation plants from each of five *MTF1*-RNAi lines, along with an RNAi empty vector line. Three of these transgenic lines continued to show greater transformation susceptibility (Fig. 1F). RNAi lines 2 and 9, which had increased transformation susceptibility, showed a 4.6- and 7-fold decrease in *MTF1* transcript abundance, respectively, whereas line 10, which did not have altered susceptibility, showed a more than 2-fold decrease in *MTF1* transcript abundance (Fig. 1G). A transgenic line containing an empty RNAi vector did not display altered transformation susceptibility or altered *MTF1* transcript abundance. These results indicate that transformation susceptibility of Bl-1 plants inversely correlated with the abundance of *MTF1* transcripts.

Cytokinins secreted by *Agrobacterium* repress *MTF1*

The expression of *MTF1* is decreased by cytokinin treatment (*20*), and thus, we sought to examine whether the transformation process was influenced by cytokinins. Ti plasmids of nopaline-type *Agrobacterium* strains carry a *Tzs* gene that directs synthesis and secretion of cytokinins (*21*–*23*). *Tzs* promotes transformation by both nopaline-type *A. tumefaciens* strains (*24*) and, when transferred to strain 1855, *A. rhizogenes* strains (*25*). *A. tumefaciens* strains harboring nopaline-type Ti plasmids secrete *trans*-zeatin or *trans*-zeatin ribosides into the medium in amounts greater than 1 μg/liter (*24*, *26*, *27*). In addition, all *Agrobacterium* strains produce a second smaller but substantial source of cytokinins from derivatives of isopentenylated transfer RNA (tRNA), which is catalyzed by the enzyme tRNA:isopentenyltransferase encoded by the chromosomal *MiaA* gene (*28*).

Therefore, to determine the effect of *Agrobacterium*-secreted cytokinins on transformation and *MTF1* expression, we conducted stable transformation assays on *Arabidopsis* roots infected with (i) the wild-type *Tzs* strain *A. tumefaciens* NT1RE(pJK270) or the *tzs* frameshift mutant NT1RE(pJK270*tzs*-fs) (*24*), and (ii) the wild-type *A. tumefaciens* A348 (pBISN2) or the corresponding *miaA* mutant containing the octopine-type plasmid pTiA6 and pBISN2 (*28*). *Arabidopsis* root segments infected with the *tzs* mutant developed about threefold fewer tumors than did roots infected with the corresponding wild-type strain (Fig. 2A). Antibiotic resistance callus assays showed that root segments infected with the *miaA* mutant developed about twofold fewer calli than did those infected with the wild-type strain (Fig. 2B). In addition, transient transformation assays carried out with the *miaA* mutant also revealed about twofold lower percentage of GUS-positive root segments than did infection by wild-type bacteria (fig. S3). The lowest abundance of *MTF1* transcripts was observed in root segments infected with the wild-type strain NT1RE(pJK270) that produced cytokinins from both sources, followed by roots infected with the strain A348 that secretes tRNA-derived cytokinins. *tzs* mutant–infected root segments had about 20% decrease in *MTF1* transcript abundance than did uninfected roots, whereas the *miaA* mutant–infected roots did not show a change in *MTF1* transcript abundance (Fig. 2C). These results confirm that the expression of *MTF1* is decreased by cytokinins produced by *A. tumefaciens* and indicate that cytokinin-mediated repression of *MTF1* expression leads to altered transformation susceptibility. Further, the data suggest that *Tzs* has a greater effect than does *MiaA* on decreasing *MTF1* expression.

To determine in which root tissues this decrease in *MTF1* expression was most pronounced, we generated transgenic *Arabidopsis* lines expressing enhanced yellow fluorescent protein (EYFP) under the control of the *MTF1* promoter. *MTF1* promoter activity was constitutive in all examined plant seedlings (Fig. 2D). We used the high EYFP-expressing line Col-7–P_{MTF}–EYFP4 to assess whether root tissues exhibited altered *MTF1* expression when infected with a *Tzs A. tumefaciens* strain. Fluorescence decreased in roots by 48 hours of cocultivation, most noticeably in the epidermal and cortical cells of the elongation zone, the region most susceptible to transformation (*29*) (Fig. 2D). This decrease in fluorescence was not observed in roots incubated with the *tzs* mutant. These results are consistent with the decreased *MTF1* transcript abundance we observed in roots cocultivated with *Tzs* bacteria.

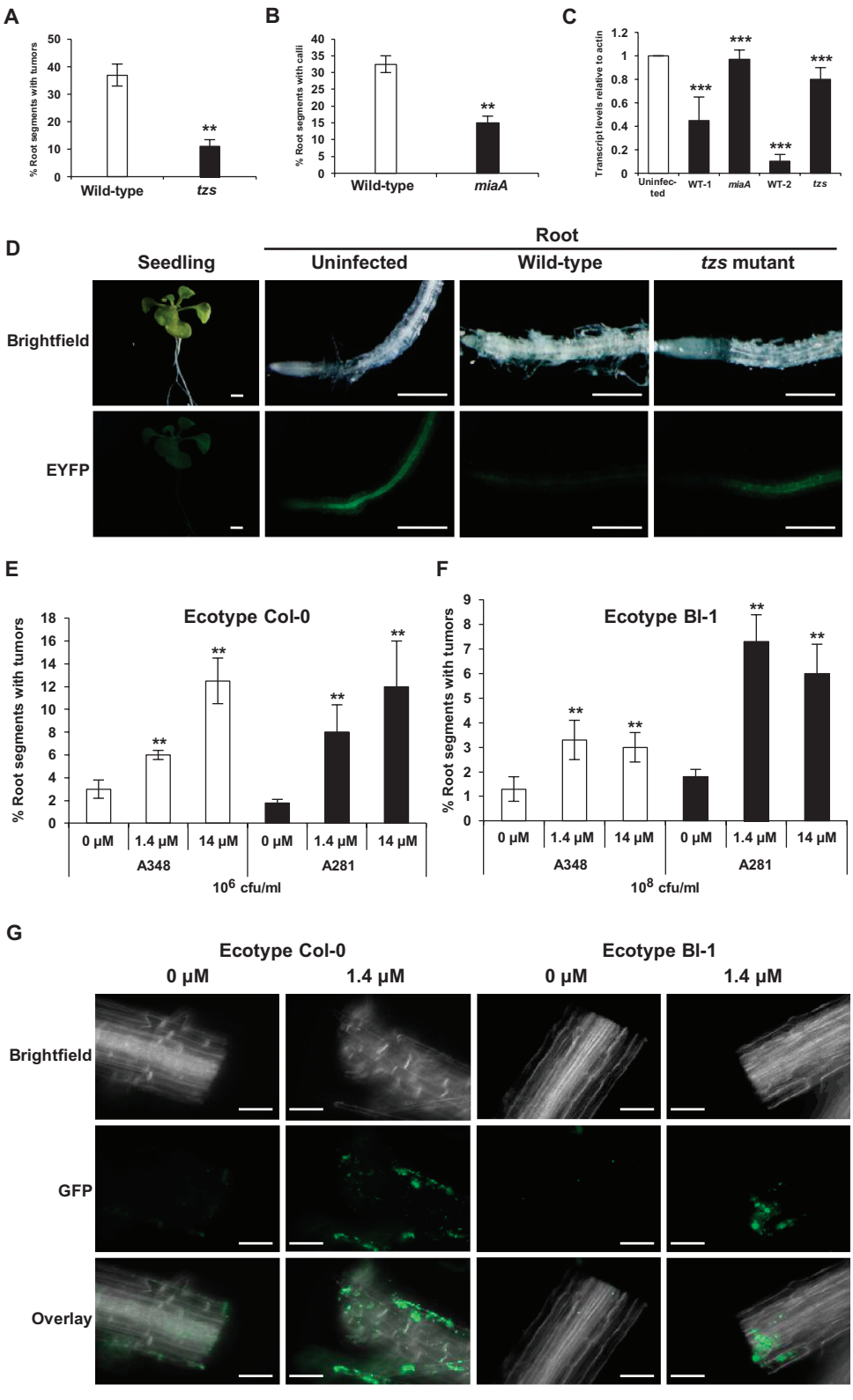

Fig. 2. Cytokinins increase susceptibility to *Agrobacterium*-mediated transformation. (**A**) Percentage of root segments developing tumors after inoculation with 10^7 CFU/ml wild-type NT1RE(pJK270) and *tzs* mutant *A. tumefaciens*. $^{**}P < 0.02$, *tzs* to wild-type. (**B**) Percentage of root segments developing antibiotic-resistant calli after inoculation with wild-type A348(pBISN2) (10^7 CFU/ml) and *miaA* mutant *A. tumefaciens*. $^{**}P < 0.02$, *miaA* to wild-type. (**C**) Relative *MTF1* transcript abundance in roots infected with *miaA* and *tzs* mutants and their respective wild-type *A. tumefaciens* strains, WT-1 [A348 (pBISN2)] and WT-2 [NT1RE(pJK270)]. $^{***}P < 0.001$, WT-1 and WT-2 to uninfected, *miaA* to WT-1, and *tzs* to WT-2. (**D**) The *MTF1* promoter is repressed in response to inoculation with wild-type *A. tumefaciens*. EYFP expression in an *MTF1* promoter–EYFP transgenic seedling (scale bars, 1 mm), and in roots either uninfected or inoculated with wild-type NT1RE(pJK270) (10^8 CFU/ml) or *tzs* mutant *A. tumefaciens* (scale bars, 250 µm). Analyses were conducted 48 hours after inoculation. (**E** and **F**) Percentage of root segments developing tumors on (E) Col-0 ($^{**}P < 0.02$, 1.4 and 14 µM to 0 µM) and (F) Bl-1 ($^{**}P < 0.02$, 1.4 and 14 µM to 0 µM) roots after inoculation with *A. tumefaciens* A348 and A281 in the absence or presence of *trans*-zeatin. (**G**) Attachment of GFP-tagged *A. tumefaciens* A281 to root segments of Col-0 and Bl-1 pretreated for 24 hours without or with 1.4 µM *trans*-zeatin. Scale bars, 50 µm. Data are means ± SEM from $n = 3$ (C) or $n = 5$ (A, B, E, and F) biological replicates. Images in (D) and (G) are representative of $n = 6$ plants.

Cytokinins enhance transformation

Chateau *et al*. (*30*) reported that preincubation of *Arabidopsis* petioles with phytohormones increases transformation, and hormone pretreatment is part of the protocol to generate transgenic *Arabidopsis* plants from roots (*31*). Because cytokinins from *A. tumefaciens* influence transformation, we speculated that phytohormone pretreatment of *Arabidopsis* root segments may enhance transformation susceptibility of transformation-recalcitrant ecotypes. We incubated root segments from five transformation-recalcitrant *Arabidopsis* ecotypes [Bl-1, Bla-2, Cal-0, Dijon-G, and Petergof (*18*)] and a transformation-susceptible ecotype (Ws-2) on callus-inducing medium containing kinetin, indole acetic acid, and 2,4-dichlorophenoxyacetic acid before infection by *Agrobacterium* and scored transformation susceptibility. All ecotypes displayed increased transformation susceptibility after 1 day of phytohormone pretreatment (fig. S4, A and B). Pretreatment of roots also increased transient transformation, a process that does not require T-DNA integration into the plant

genome (fig. S4C). Thus, phytohormone treatment sensitized roots to *Agrobacterium*-mediated transformation at an early step (before T-DNA integration) in the process.

To test whether only exogenous application of the cytokinin *trans*-zeatin to roots could influence transformation susceptibility, we incubated *Arabidopsis* roots on medium containing *trans*-zeatin and infected them with *A. tumefaciens* A348 or A281. Neither of these strains harbors *Tzs*. We used *trans*-zeatin concentrations representing the range secreted by nopaline-type *A. tumefaciens* strains (*24*, *26*, *27*). *Trans*-zeatin treatment of Col-0 roots resulted in a four- to eightfold increase in transformation efficiency by these *A. tumefaciens* strains. Ecotype Bl-1 roots infected with these strains showed a two- to threefold increase in susceptibility (Fig. 2, E and F). Incubation of roots on *trans*-zeatin decreased *MTF1* transcript abundance by 30 to 60% (fig. S5, A and B) and increased attachment of green fluorescent protein (GFP)–expressing *A. tumefaciens* A281 (Fig. 2G), indicating that exogenous cytokinins increase transformation susceptibility.

Cytokinins influence *MTF1* via *ARR3*

To determine which components of the plant cytokinin signaling pathway are involved in influencing *MTF1* by *Agrobacterium*-secreted cytokinins, we analyzed *Arabidopsis* lines with mutant genes in this pathway. In *Arabidopsis*, four major steps in cytokinin signaling are as follows: (i) perception of cytokinins by hybrid histidine protein kinases (AHKs), (ii) transfer of the phosphosignal by histidine phosphotransfer proteins (AHPs) to the nucleus, (iii) phosphorylation of type B ARRs that activate transcription, and (iv) a negative feedback loop through cytokinin-inducible type A *ARR* gene products (*32*, *33*). We screened mutants of various genes, encoding proteins involved in the cytokinin signaling pathway, for transformation susceptibility, using a phytohormone-independent stable root transformation assay. Root segments were infected with *A. tumefaciens* carrying a *bar* gene in the T-DNA, and selected for formation of phosphinothricin (PPT)–resistant calli. Mutants of *ahk3* and *ahk4*, which encode the cytokinin receptors Arabidopsis histidine kinase 3 and 4, respectively, displayed decreased transformation susceptibility, whereas an *ahk2* mutant showed susceptibility similar to that of wild-type plants (Fig. 3A). *MTF1* transcripts increased in the roots of *ahk3* and *ahk4* mutants, but not in those of *ahk2* (fig. S6A). Likewise, a quintuple mutant of *ahp1,2,3,4,5*, genes that encode histidine-containing phosphotransmitters, showed decreased transformation susceptibility (Fig. 3A) and increased *MTF1* transcript accumulation (fig. S6A), together indicating the promotional roles of histidine phosphotransfer proteins and select cytokinin receptors in transformation.

Type A ARRs encode transcription repressors that act as negative regulators of cytokinin signaling (*34*), and one model for the mechanism of regulation suggests that unphosphorylated type A ARRs inhibit type B–mediated transcription, whereas phosphorylation of type A ARRs may relieve their negative action (*35*). MTF1 contains a Myb DNA binding domain similar to that of type B ARRs. We therefore assessed whether type A ARRs negatively regulate *MTF1* expression and transformation susceptibility. Because of functional redundancy among the type A ARRs, we screened several type A ARR multiple mutants to identify genes influencing transformation. The mutants *arr3,4,5,6,8,9*, *arr3,4,5,6* (fig. S6, B and C), and *arr3,4* (fig. S6, D and E) consistently showed decreased transformation susceptibility, whereas *arr5,6,8,9* mutants did not show a significant difference in transformation (fig. S6, B and C). Among single mutants, an *arr3* mutant showed decreased transformation susceptibility, whereas *arr4* did not (Fig. 3, B and C). *arr3* roots infected with *A. tumefaciens* also showed a 3.5-fold greater abundance of *MTF1* transcripts than did wild-type roots (fig. S6A). *mtf1-4* did not display differences in *ARR3*

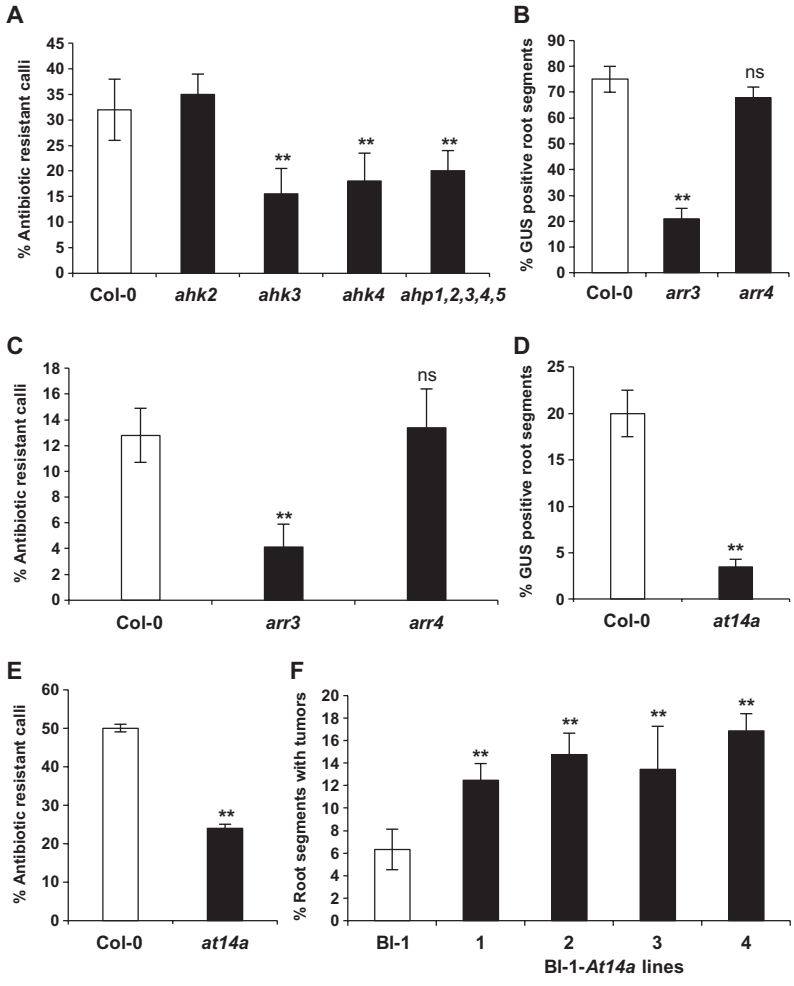

Fig. 3. *Agrobacterium*-mediated transformation is mediated by the cytokinin signaling pathway and *At14a*. (**A**) Percentage of wild-type (Col-0) and mutant *ahk* or *ahp* root segments developing antibiotic-resistant calli after inoculation with *A. tumefaciens* At872 (10^6 CFU/ml). $^{**}P < 0.02$, *ahk3*, *ahk4*, and *ahp1,2,3,4,5* to Col-0. (**B**) Percentage of GUS-positive root segments of wild-type and mutant *arr3* or *arr4* plants inoculated with *A. tumefaciens* At849 (10^6 CFU/ml). $^{**}P < 0.01$, *arr3* to Col-0. (**C**) Percentage of wild-type and mutant *arr3* or *arr4* root segments developing antibiotic-resistant calli after inoculation with *A. tumefaciens* At872 (10^6 CFU/ml). $^{**}P < 0.02$, *arr3* to Col-0. (**D**) Percentage of GUS-positive root segments of wild-type and *at14a* plants inoculated with *A. tumefaciens* At849 (10^7 CFU/ml). $^{**}P < 0.02$, *at14a* to Col-0. (**E**) Percentage of wild-type and *at14a* mutant root segments developing antibiotic-resistant calli after inoculation with *A. tumefaciens* At872 (10^7 CFU/ml). $^{**}P < 0.02$, *at14a* to Col-0. (**F**) Percentage of wild-type and Bl-1 *At14a* transgenic root segments developing tumors after inoculation with *A. tumefaciens* A208 (10^7 CFU/ml). $^{**}P < 0.02$, Bl-1-*At14a* lines to Bl-1. Data are means ± SEM from $n = 5$ biological replicates. ns, not significant.

transcript abundance (fig. S6F). Together, these data indicate that *ARR3* promotes transformation and functions upstream of *MTF1*.

MTF1 regulates *Agrobacterium* attachment

We used *Arabidopsis* ATH1 Genome Arrays to identify genes whose expression is altered in wild-type, heterozygous *mtf1-1*, and homozygous *mtf1-4 Arabidopsis* roots. Of the 40 genes that exhibited statistically significant differential expression between both *mtf1* mutants and wild-type roots and had a difference greater than 1.5-fold in the microarray (table S1), 13 were independently validated to be differentially expressed. Of these, the expression of seven genes was commonly increased and that of six genes was commonly decreased in both *mtf* mutants compared to wild-type roots, including *At2g40970*. To assess the importance of these genes in transformation, we tested the transformation susceptibility of plants that were mutant for one of the other five genes with decreased transcript abundance: *At3g56710*, *At5g39670*, *At2g43290*, *At4g25470*, and *At5g49520*. Only the mutant for *At5g49520*, encoding the transcriptional activator WRKY48, displayed a mild *hat* phenotype (fig. S7A). We also individually ectopically expressed each of the four genes that showed increased transcript abundance (*At2g40960*, *At1g50060*, *At5g46295*, and *At5g15725*) in *Arabidopsis* Col-0 plants and tested transformation susceptibility of the transgenic lines. Transformation susceptibility increased in *At1g50060* and *At5g15725* transgenic lines (fig. S7B), but not in the other lines (fig. S7C). *At1g50060* encodes a basic PR1 (pathogenesis-related 1)–like protein, whereas *At5g15725* encodes a putative glycosyltransferase. These data indicate that MTF1 regulates multiple genes that promote transformation.

Another gene whose expression was increased in both *mtf1* mutants, *At3g28290* (table S1), encodes AT14A, a putative transmembrane receptor for cell adhesion molecules. AT14A has a small domain similar to that of the mammalian β_1 integrins (*36*) and may mediate the cell wall–plasma membrane–cytoskeleton continuum (*37*). Human β_1 integrins bind to CagL proteins of the *Helicobacter pylori* type IV secretion system (T4SS) pilus that are homologous to VirB5 proteins of the *Agrobacterium* T4SS T-pilus (*38*). To assess the importance of *At14a* in transformation, we assayed a T-DNA insertion mutant (SALK_005316C) for susceptibility to *A. tumefaciens*. Mutant *at14a* plants showed decreased susceptibility to both transient and stable transformation (Fig. 3, D and E). In contrast, transgenic *Arabidopsis* ecotype Bl-1 ectopically expressing *At14a* displayed increased transformation susceptibility (Fig. 3F). Together, these results indicate that *At14a* promotes MTF1-mediated transformation, perhaps by increasing anchor points for *A. tumefaciens* attachment.

Because increased expression of *At14a* in *mtf1-4* could, in theory, result in increasing the number of binding sites available to *Agrobacterium* cells, leading to increased transformation, we determined if MTF played a role in promoting bacterial attachment. *A. tumefaciens* constitutively expressing GFP showed increased aggregation around *mtf1-4* roots compared to Col-0 roots (Fig. 4A). On the contrary, lower bacterial aggregation was seen around *at14a* roots (Fig. 4B). Scanning electron microscopy (SEM) quantitatively confirmed that higher numbers of bacteria directly bind to *mtf1-4* roots and lower numbers to *at14a* roots than to wild-type roots (Fig. 4C), indicating that decreased expression of *MTF1* results in increased susceptibility during the early attachment stage of the transformation process.

Earlier studies indicated that roots of ecotype Bl-1 do not bind Agrobacteria well (*18*). We therefore examined bacterial attachment to roots of Bl-1–*MTF1*-RNAi lines and Bl-1 lines ectopically expressing *At14a*. *A. tumefaciens* expressing GFP showed increased bacterial aggregation in the high-transforming transgenic Bl-1 RNAi lines 2, 8, and 9 compared to that of the low-transforming line 10, the empty RNAi vector line, and wild-type Bl-1 (Fig. 4D). Furthermore, SEM studies showed a greater number of bacteria attached to roots of the RNAi lines 2, 8, and 9 than to roots of wild-type and control lines (Fig. 4E). Bl-1 lines ectopically expressing *At14a* had increased bacterial aggregation around cut root segments (Fig. 4F). These results strongly support the role of *At14a* in increasing transformation susceptibility by promoting bacterial attachment.

DISCUSSION

The *mtf1-4* mutant was hypersusceptible to transformation by several *Agrobacterium* strains, indicating that *MTF1* is a global negative regulator of transformation susceptibility. Decreasing *MTF1* expression in the transformation-recalcitrant *Arabidopsis* ecotype Bl-1 increased its transformation susceptibility. *MTF1* expression was down-regulated by cytokinins, and other studies have reported increased transformation after phytohormone treatment of plant explants (*24*, *30*, *31*). However, the mechanism of this increased susceptibility was unknown. Our study provides a molecular explanation for the role of cytokinins in increasing transformation by decreasing *MTF1* expression.

All Agrobacteria produce cytokinins (*24*, *26*–*28*), and decreasing cytokinin production by the bacteria attenuates virulence (*24*). *Agrobacterium* strains containing nopaline-type Ti plasmids synthesize and secrete *trans*-zeatin, mediated by the *vir* region-localized gene *Tzs*. *A. tumefaciens tzs* mutants are less virulent than are *Tzs* strains (*24*). TZS is present on the bacterial surface (*39*), suggesting that metabolites from wounded plant cells may be converted into *trans*-zeatin at infection sites. The decreased virulence of *Agrobacterium tzs* mutants can be restored by exogenous application of cytokinins (*24*). Bioactive cytokinins are produced and secreted by *Rhizobium* (*40*) and play a key role in nodule development. *Bradyrhizobium miaA* mutants complemented with *Tzs* restore normal nodulation of *Aeschynomene* legumes (*41*).

Perception of the *Agrobacterium*-secreted cytokinins was achieved through the AHK3 and AHK4 receptors, and the signal was transduced through a phosphotransfer cascade mediated by the AHP group of proteins (*42*, *34*). In our study, mutations in these genes resulted in decreased transformation susceptibility and the accumulation of *MTF1* transcripts. Type A ARRs act as negative regulators of cytokinin signaling (*43*), and *ARR3* negatively regulated *MTF1* expression. Cytokinins play an important role in determining the outcome of plant-pathogen interactions, in which high concentrations (10 to 100 μM) prime salicylic acid–dependent defense responses, and low concentrations (up to 10 μM) lead to increased susceptibility of *Arabidopsis* to the virulent oomycete *Hyaloperenospora arabidopsidis* Noco2 (*44*). Whereas cytokinins can increase plant defense responses and therefore resistance to bacterial pathogens (*45*), *A. tumefaciens* seems to have devised a mechanism to circumvent or minimize the triggering of a plant defense response by secreting low concentrations of cytokinins that influence the expression of *MTF1* and type A ARR genes (*46*).

Regulation of gene expression by MTF1 was highly specific. Only about 40 genes had a significantly increased or decreased (by greater than or equal to 1.5-fold) expression in roots of *mtf1* mutant plants (table S1). One gene involved in transformation, *At14a*, was up-regulated in *mtf1-4*. AT14A may mediate the cell wall–plasma membrane–cytoskeleton continuum that plays important roles in the regulation of plant responses to environmental cues (*37*). NDR1 (non–race-specific disease resistance 1), a plant integrin-like protein, mediates primary cellular functions in *Arabidopsis* through maintaining the integrity of the cell wall–plasma membrane connection during *Pseudomonas syringae* pv *tomato* DC3000 infection (*47*). Cowpea plants, which are resistant to the powdery mildew fungus and susceptible to the cowpea rust fungus, show increased plasma membrane–cell wall adhesion when inoculated first with conidia of powdery mildew fungus for 24 hours before inoculation with the virulent rust fungal basidiospores

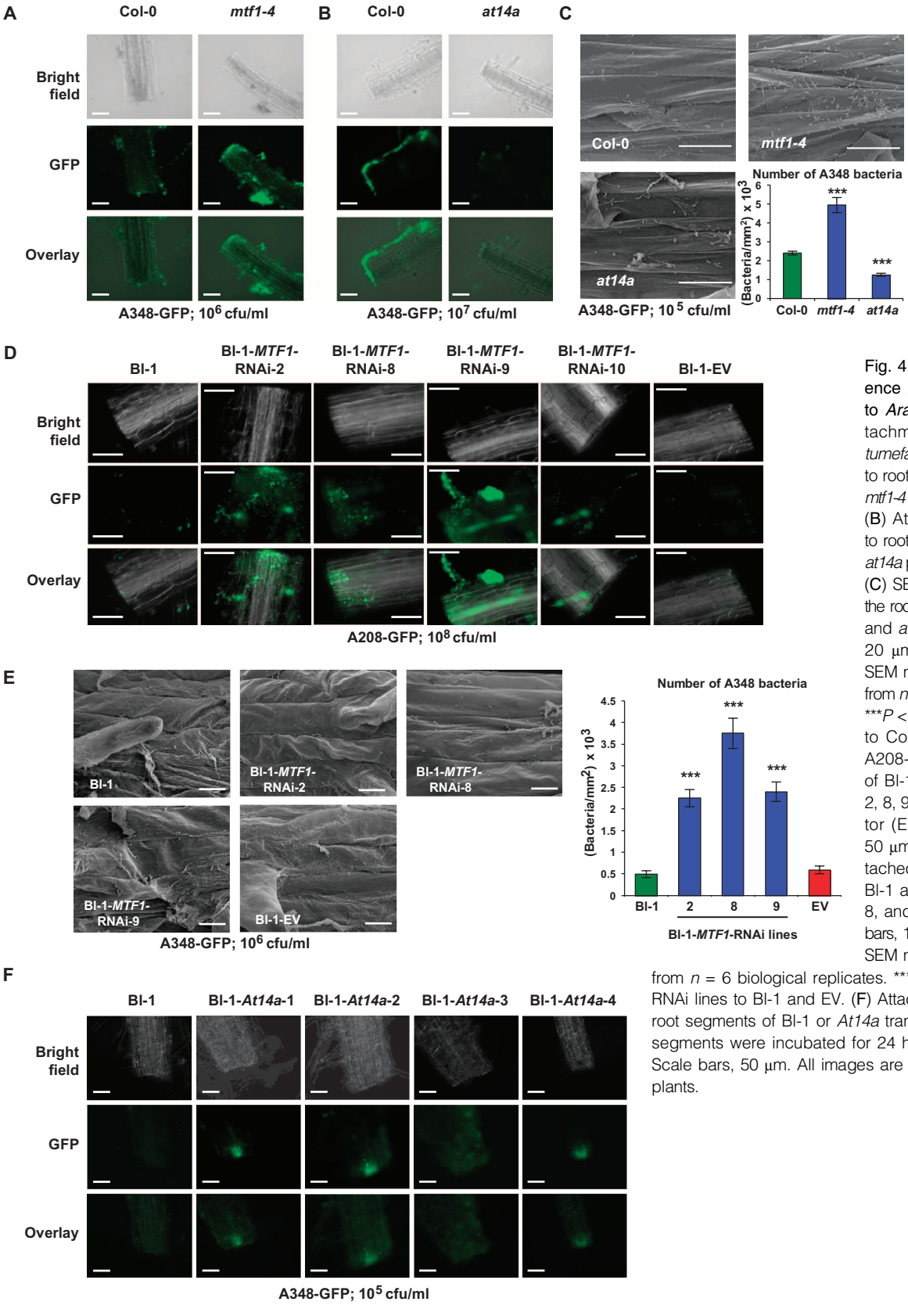

Fig. 4. MTF1 and At14a influence *Agrobacterium* binding to *Arabidopsis* roots. (**A**) Attachment of GFP-tagged *A. tumefaciens* A348 (A348-GFP) to root segments of Col-0 and *mtf1-4* plants. Scale bars, 50 μm. (**B**) Attachment of A348-GFP to root segments of Col-0 and *at14a* plants. Scale bars, 50 μm. (**C**) SEM of A348 attached to the root surface of Col-0, *mtf1-4*, and *at14a* plants. Scale bars, 20 μm. Data are means ± SEM number of bacteria/mm^2 from $n = 6$ biological replicates. ***$P < 0.001$, *mtf1-4* and *at14a* to Col-0. (**D**) Attachment of A208-GFP to root segments of Bl-1 and *MTF1*-RNAi lines 2, 8, 9, and 10, or empty vector (EV) plants. Scale bars, 50 μm. (**E**) SEM of A348 attached to the root surface of Bl-1 and *MTF1*-RNAi lines 2, 8, and 9, or EV plants. Scale bars, 10 μm. Data are means ± SEM number of bacteria/mm^2 from $n = 6$ biological replicates. ***$P < 0.001$, Bl-1–*MTF1*-RNAi lines to Bl-1 and EV. (**F**) Attachment of A348-GFP to root segments of Bl-1 or *At14a* transgenic Bl-1 lines. Root segments were incubated for 24 hours with the bacteria. Scale bars, 50 μm. All images are representative of $n = 6$ plants.

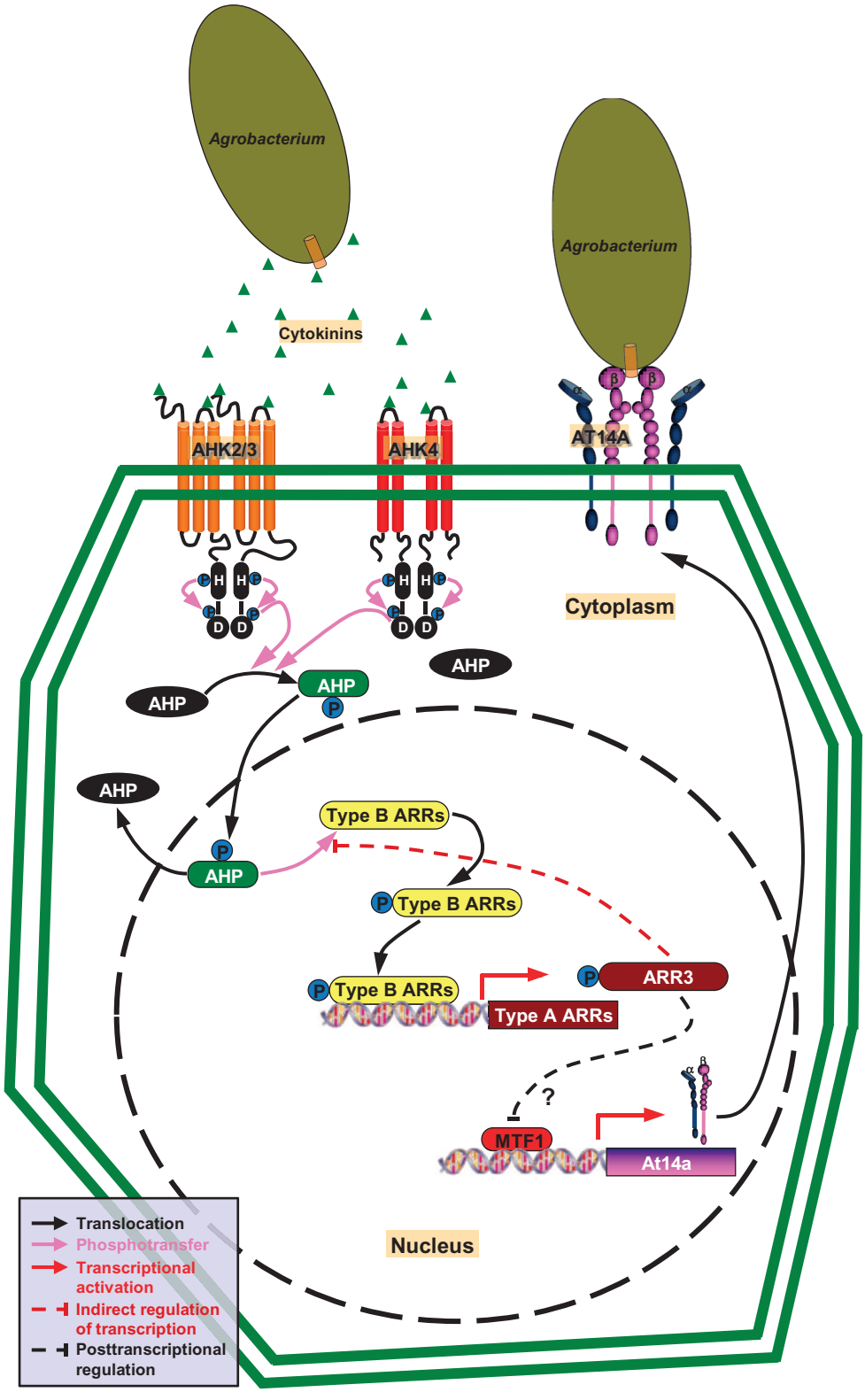

Fig. 5. Model for *Agrobacterium*-mediated transformation by inhibiting MTF1 through the cytokinin signaling pathway. *Agrobacterium*-secreted cytokinins are perceived in *Arabidopsis* by the AHK3 and AHK4 receptors. Histidine (H) is autophosphorylated, and the phosphate group (P) is transferred to aspartate (D). The phosphate group is then transferred to the AHPs, activating them. Activated AHPs are translocated to the nucleus, where the phosphate group is transferred to the receiver domain of B type ARRs. Dephosphorylated AHPs shuttle back to the cytosol, and activated ARRs bind to multiple *cis* elements in the promoters of target genes that include the type A ARR, *ARR3*. ARR3 inhibits *MTF1* expression, resulting in increased expression of *At14a*. AT14A localizes to the cell wall–plasma membrane continuum and serves as anchor points for *A. tumefaciens* attachment.

and resist penetration by the rust fungus (*48*). Thus, although plant integrin-like proteins play a role in cell wall–associated defenses, Agrobacteria may have discovered a way to use this defense mechanism to their advantage. As with other integrin-like plant proteins, the AT14A protein family contains a membrane-localized integrin-like β_1 domain. In animal systems, this domain interacts with *H. pylori* CagL protein, an ortholog of the *Agrobacterium* T-pilus protein VirB5, and acts as a host membrane anchor for the bacterial protein delivery machinery (*38*). Because transformation recalcitrance of several *Arabidopsis* ecotypes results from decreased binding of *Agrobacterium* to roots (*18*) and the expression of *At14a* in the *mtf1* mutants resulted in increased attachment of *Agrobacterium*, we speculated that decreased transformation susceptibility of *at14a* mutants may result from fewer *Agrobacterium* binding sites on the plant cells. Indeed, high-resolution SEM confirmed that roots of *at14a* plants bound fewer Agrobacteria than did either wild-type or *mtf1-4* mutant plants. Our hypothesis was further supported by observations that decreased *MTF1* expression or ectopic overexpression of *At14a* in Bl-1, a highly recalcitrant ecotype, increased both transformation and bacterial attachment.

In conclusion, we identified *MTF1* as a negative regulator of plant susceptibility to *Agrobacterium*-mediated transformation. Enhanced plant transformation is dependent on integration of *Agrobacterium*-secreted cytokinin perception and signal transduction by the cytokinin signaling pathway with negative regulation of *MTF1* via ARR3. We propose that mutation of *MTF1* increases transformation by increasing the expression of *At14a*, thereby increasing the number of *Agrobacterium* attachment sites on the plant cells (Fig. 5). *MTF1* orthologs exist in transformation-

recalcitrant crop species. Thus, we are currently manipulating these genes to increase transformation susceptibility of economically important crops to improve product quality and tolerance to biotic and abiotic stress factors and increase crop yield.

MATERIALS AND METHODS

Agrobacterium culture, plant growth conditions, and transformation assays

A. tumefaciens was cultured in yeast extract–peptone medium (*49*) containing the appropriate antibiotics. Root transformation assays were carried out as previously described (*18*) with minor modifications (*50*). MS (Murashige and Skoog) basal medium lacking phytohormones was used to select for tumors, and callus-inducing medium (*18*) containing PPT (10 μg/ml) was used to select for antibiotic-resistant calli. GUS activity assays were carried out after infection of root segments with *A. tumefaciens* At849 (*17*) for 4 to 6 days, using 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-Gluc) (*51*).

Arabidopsis mutants

We screened about 4000 mutagenized plants from a T-DNA activation-tagged library (*13*) at low *Agrobacterium* inoculation densities (10^5 and 10^6 CFU/ml) for increased root transformation. We used TAIL (thermal asymmetric interlaced)–PCR (*52*) to identify the T-DNA/plant junction from *mtf1-1*. Primers for TAIL-PCR are listed in table S2.

Seeds of the T-DNA insertion *MTF1* mutants SALK_072082 (*mtf1-2*), SALK_102624 (*mtf1-3*), and SALK_072083 (*mtf1-4*), and a T-DNA insertion *At14a* mutant SALK_005316C (*53*) were obtained from the Arabidopsis Biological Resource Center (ABRC). The mutants were genotyped with primers listed in table S2.

Generation of transgenic *MTF1*-complemented plants

MTF1 cDNA was synthesized from 1 to 2 μg of RNA using oligo(dT) and the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's protocol. Primer sequences are listed in table S2. The PCR was conducted with PfuTurbo DNA polymerase (Stratagene) and 200 ng of *Arabidopsis* Columbia root cDNA. PCR products were cloned into the Sma I site of pBluescript II SK+ (Stratagene). *MTF1* cDNA was excised with Xho I and Spe I and cloned into the binary vector pE1775 (*54*). The resulting construction, pE3263, was introduced into *A. tumefaciens* GV3101 by electroporation and used for floral dip transformation (*55*) of the mutant *mtf1-4*. Transgenic plants were selected on B5 medium containing hygromycin (20 μg/ml).

Quantitative real-time RT-PCR analysis

Real-time RT-PCR was carried out with total RNA isolated in triplicate from roots of plants grown in liquid B5 medium. PCR was performed in triplicate on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Expression was calculated by the relative standard curve method (Applied Biosystems) and normalized to *Actin2* transcript abundance. Transcript abundance of genes identified in our microarray experiments was validated by RT-PCR. The list of primers is given in table S2.

Generation of *MTF1*-RNAi lines

We generated *MTF1*-RNAi lines with the T-DNA binary vector pFGC1008 (GenBank accession AY310333). The RNAi construction (pE3387) contained about 400-bp cDNA fragment of *MTF1* amplified with primers listed in table S2. The *MTF1* fragment was oriented as an inverted repeat, with each repeat separated by a fragment from the *gusA* gene. RNAi lines, in ecotype Bl-1, were produced by floral dip transformation using *A. tumefaciens* GV3101. Transgenic plants were selected on B5 medium containing hygromycin.

Bacterial attachment assays

Root segments of *Arabidopsis* Col-0, *mtf1-4, at14a*, and *At14a*-overexpressing plants were incubated with *A. tumefaciens* A348 containing pJZ383 (P_{tac}::GFP), and Bl-1 and *MTF1*-RNAi lines were incubated with *A. tumefaciens* A208 containing pJZ383. Root segments were cocultivated for 24 hours in B5 minimal medium. Root segments were rinsed, placed on glass slides, and visualized by epifluorescence microscopy.

Scanning electron microscopy

Root segments of *Arabidopsis* Col-0, *mtf1-4*, *at14a*, Bl-1, and *MTF1*-RNAi lines incubated with *A. tumefaciens* A348-pJZ383 were processed for SEM by fixation of the tissues in 1% (w/v) OsO_4 in 0.1 M cacodylate buffer and dehydration through an ethanol series, followed by critical point drying. Samples were sputter-coated with platinum and imaged with a Nova NanoSEM 200 scanning electron microscope (FEI) using a high-vacuum Everhart-Thornley detector operating at a 5-kV accelerating voltage and a 4.5- to 5-mm working distance. Attachment was quantified by recording the number of bacteria bound to root segments per mm^2 over a length of 3 mm for six independent root samples per plant line. Means were compared by the Tukey-Kramer method that corrects for type I errors using JMP Pro 9 (SAS Institute).

Generation of *MTF1* promoter–EYFP transgenic plants

About 1.2 kb of the *MTF1* promoter region was amplified with a forward primer incorporating an Age I restriction site at the 5′ end and a reverse primer incorporating the sequence encoding the first 10 amino acids of MTF1 and a Bam HI restriction site. Primers are listed in table S2. The amplification product was cloned into the Sma I site of pBluescript II SK+. The *MTF1* promoter was excised with Age I and Bam HI and cloned into these sites of pSAT6-EYFP-N1 (*56*) as a translational fusion with EYFP. The expression cassette was cloned as a PI-*Psp*I fragment into pPZP-RCS2-hptII (*56*). The resulting plasmid was transformed into *A. tumefaciens* GV3101 and used for floral dip transformation of *Arabidopsis* Col-0. Transgenic plants were selected on B5 medium supplemented with hygromycin (20 μg/ml).

Generation of Bl-1 *At14a* overexpression lines

At14a (*At3g28290*) was PCR-amplified from 200 ng of *Arabidopsis* Columbia root cDNA with PfuTurbo DNA polymerase. Primer sequences are listed in table S2. PCR products were cloned into the Sma I site of pBluescript II SK+ (Stratagene). *At14a* cDNA was excised with Kpn I and Sac I and cloned into the binary vector pE1775 (*54*). The resulting construction was introduced into *A. tumefaciens* GV3101 by electroporation and used for floral dip transformation (*55*) of *Arabidopsis* Bl-1. Transgenic plants were selected on B5 medium containing hygromycin (20 μg/ml).

Phytohormone treatment of plant roots

Plants of *Arabidopsis* ecotypes Ws-2, Bl-1, Bla-2, Cal-0, Dijon-G, and Petergof were grown as described (*18*), and roots were excised and incubated on callus-inducing medium for 0, 1, or 3 days before cutting into segments and infection with *A. tumefaciens* A208 for tumorigenesis assays or strain At849 for transient GUS expression assays (*17*).

For assessing the effect of cytokinins on *MTF* transcript abundance and transformation, root segments from *Arabidopsis* Col-0 or Bl-1 were incubated on MS medium supplemented with 0, 1.4, or 14 μM *trans*-zeatin and cocultivated with either *A. tumefaciens* A348 or A281 for 48 hours. Roots were infected with bacteria at 10^6 CFU/ml (Col-0) or 10^8 CFU/ml

(Bl-1). After infection, root segments were either transferred to MS basal medium containing Timentin (100 μg/ml) and incubated for 4 to 5 weeks before recording the percentage of root segments developing tumors, or used for RNA isolation 48 hours later.

Agrobacterium attachment assays were conducted as described above. Col-0 and Bl-1 root segments were cocultivated with A281 at 10^6 or 10^8 CFU/ml, respectively, for 24 hours in the presence or absence of 1.4 μM *trans*-zeatin.

Microarray analysis

Surface-sterilized seeds of wild type, *mtf1-1*, and *mtf1-4* were germinated in B5 medium, and seedlings were grown for 2 weeks at 23°C under a 16-hour light/8-hour dark photoperiod. Three biological replicates, each consisting of 20 seedlings of each line transferred to liquid B5 medium, were grown for 12 days. Roots were frozen in liquid N_2. RNA was isolated with TRIzol reagent (Invitrogen). Microarray experiments were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com) using Arabidopsis ATH1 Genome Arrays (Affymetrix) at the Purdue University Genomics Center. GeneChip operating software was used to produce CEL files containing raw probe intensities for the arrays. Data from these files were read with "Biobase" and "affy" packages in R/Bioconductor (*57*) for analysis of genomic data. A background correction was performed on the perfect match intensities to make signals from different chips comparable. A robust local regression was used to normalize background-corrected data. Using the natural log of the background-corrected, normalized data as the gene expression information, we used an analysis of variance (ANOVA) as previously described (*58*) to identify statistically significant probe sets that are differentially expressed between two lines. To determine whether there was a statistically significant difference between two lines, it was sufficient to test whether the line effect was different from zero. This ANOVA model was performed for Col versus *mtf1-1*, Col versus *mtf1-4*, and *mtf1-4* versus *mtf1-1*. Both the false discovery rate approach (*59*) and Holm's sequential Bonferroni correction procedure (*60*) were used to adjust for multiple testing, with a significance α of 0.05.

Statistical analysis

Data for root transformation assays and transcript abundance were analyzed by nonparametric pairwise comparisons using the Wilcoxon method with JMP Pro 9 software (SAS Institute).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/6/302/ra100/DC1

Fig. S1. Expression of *MTF1* influences plant susceptibility to *Agrobacterium*-mediated transformation.

Fig. S2. Silencing *MTF1* by RNAi in roots of *Arabidopsis* ecotype Bl-1 increases transformation susceptibility.

Fig. S3. Decreased transient transformation of Col-0 roots inoculated with *miaA* mutant *A. tumefaciens*.

Fig. S4. Phytohormone pretreatment of *Arabidopsis* roots increases susceptibility to *Agrobacterium*-mediated transformation.

Fig. S5. *MTF1* expression is negatively regulated by cytokinin treatment.

Fig. S6. Genes encoding cytokinin signaling pathway proteins are involved in *Agrobacterium*-mediated transformation.

Fig. S7. Manipulation of *Arabidopsis* genes that are regulated by MTF1 increases susceptibility to *Agrobacterium*-mediated transformation.

Table S1. Microarray data.

Table S2. Sequences of primers used.

REFERENCES AND NOTES

1. M. DeCleene, J. DeLey, The host range of crown gall. *Bot. Rev.* **42**, 389–466 (1976).
2. M. DeCleene, The susceptibility of monocotyledons to *Agrobacterium tumefaciens*. *J. Phytopathol.* **113**, 81–89 (1985).
3. B. Lacroix, T. Tzfira, A. Vainstein, V. Citovsky, A case of promiscuity: *Agrobacterium's* endless hunt for new partners. *Trends Genet.* **22**, 29–37 (2006).
4. T. Kunik, T. Tzfira, Y. Kapulnik, Y. Gafni, C. Dingwall, V. Citovsky, Genetic transformation of HeLa cells by *Agrobacterium*. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 1871–1876 (2001).
5. S. B. Gelvin, *Agrobacterium* in the genomics age. *Plant Physiol.* **150**, 1665–1676 (2009).
6. S. B. Gelvin, Plant proteins involved in *Agrobacterium*-mediated genetic transformation. *Annu. Rev. Phytopathol.* **48**, 45–68 (2010).
7. T. Tzfira, V. Citovsky, *Agrobacterium: From Biology to Biotechnology* (Springer, New York, 2008).
8. C. A. McCullen, A. N. Binns, *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. *Annu. Rev. Cell Dev. Biol.* **22**, 101–127 (2006).
9. A. Pitzschke, H. Hirt, New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation. *EMBO J.* **29**, 1021–1032 (2010).
10. J. Nam, K. S. Mysore, C. Zheng, M. K. Knue, A. G. Matthysse, S. B. Gelvin, Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. *Mol. Gen. Genet.* **261**, 429–438 (1999).
11. Y. Zhu, J. Nam, J. M. Humara, K. S. Mysore, L. Y. Lee, H. Cao, L. Valentine, J. Li, A. D. Kaiser, A. L. Kopecky, H. H. Hwang, S. Bhattacharjee, P. K. Rao, T. Tzfira, J. Rajagopal, H. Yi, Veena, B. S. Yadav, Y. M. Crane, K. Lin, Y. Larcher, M. J. K. Gelvin, M. Knue, C. Ramos, X. Zhao, S. J. Davis, S. I. Kim, C. T. Ranjith-Kumar, Y. J. Choi, V. K. Hallan, S. Chattopadhyay, X. Sui, A. Ziemienowicz, A. G. Matthysse, V. Citovsky, B. Hohn, S. B. Gelvin, Identification of Arabidopsis *rat* mutants. *Plant Physiol.* **132**, 494–505 (2003).
12. A. Anand, Z. Vaghchhipawala, C. M. Ryu, L. Kang, K. Wang, O. del-Pozo, G. B. Martin, K. S. Mysore, Identification and characterization of plant genes involved in *Agrobacterium*-mediated plant transformation by virus-induced gene silencing. *Mol. Plant Microbe Interact.* **20**, 41–52 (2007).
13. D. Weigel, J. H. Ahn, M. A. Blázquez, J. O. Borevitz, S. K. Christensen, C. Fankhauser, C. Ferrándiz, I. Kardailsky, E. J. Malancharuvil, M. M. Neff, J. T. Nguyen, S. Sato, Z. Y. Wang, Y. Xia, R. A. Dixon, M. J. Harrison, C. J. Lamb, M. F. Yanofsky, J. Chory, Activation tagging in Arabidopsis. *Plant Physiol.* **122**, 1003–1013 (2000).
14. S. P. Hazen, T. F. Schultz, J. L. Pruneda-Paz, J. O. Borevitz, J. R. Ecker, S. A. Kay, *LUX ARRHYTHMO* encodes a Myb domain protein essential for circadian rhythms. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 10387–10392 (2005).
15. I. Hwang, H. C. Chen, J. Sheen, Two-component signal transduction pathways in Arabidopsis. *Plant Physiol.* **129**, 500–515 (2002).
16. H. Zhai, X. Bai, Y. Zhu, Y. Li, H. Cai, W. Ji, Z. Ji, X. Liu, X. Liu, J. Li, A single-repeat R3-MYB transcription factor *MYBC1* negatively regulates freezing tolerance in *Arabidopsis*. *Biochem. Biophys. Res. Commun.* **394**, 1018–1023 (2010).
17. S. B. Narasimhulu, X. B. Deng, R. Sarria, S. B. Gelvin, Early transcription of Agrobacterium T-DNA genes in tobacco and maize. *Plant Cell* **8**, 873–886 (1996).
18. J. Nam, A. G. Matthysse, S. B. Gelvin, Differences in susceptibility of Arabidopsis ecotypes to crown gall disease may result from a deficiency in T-DNA integration. *Plant Cell* **9**, 317–333 (1997).
19. K. S. Mysore, C. T. R. Kumar, S. B. Gelvin, *Arabidopsis* ecotypes and mutants that are recalcitrant to *Agrobacterium* root transformation are susceptible to germ-line transformation. *Plant J.* **21**, 9–16 (2000).
20. D. J. Lee, J. Y. Park, S. J. Ku, Y. M. Ha, S. Kim, M. D. Kim, M. H. Oh, J. Kim, Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR (ARR7) overexpression in cytokinin response. *Mol. Genet. Genomics* **277**, 115–137 (2007).
21. D. A. Regier, R. O. Morris, Secretion of *trans*-zeatin by *Agrobacterium tumefaciens*: A function determined by the nopaline Ti plasmid. *Biochem. Biophys. Res. Commun.* **104**, 1560–1566 (1982).
22. J. S. Beaty, G. K. Powell, L. Lica, D. A. Regier, E. M. S. MacDonald, N. G. Hommes, R. O. Morris, *Tzs*, a nopaline Ti plasmid gene from *Agrobacterium tumefaciens* associated with *trans*-zeatin biosynthesis. *Mol. Gen. Genet.* **203**, 274–280 (1986).
23. G. K. Powell, N. G. Hommes, J. Kuo, L. A. Castle, R. O. Morris, Inducible expression of cytokinin biosynthesis in *Agrobacterium tumefaciens* by plant phenolics. *Mol. Plant Microbe Interact.* **1**, 235–242 (1988).
24. H. H. Hwang, M. H. Wang, Y. L. Lee, Y. L. Tsai, Y. H. Li, F. J. Yang, Y. C. Liao, S. K. Lin, E. M. Lai, *Agrobacterium*-produced and exogenous cytokinin-modulated *Agrobacterium*-mediated plant transformation. *Mol. Plant Pathol.* **11**, 677–690 (2010).
25. X. Zhan, D. A. Jones, A. Kerr, The pTiC58 *tzs* gene promotes high-efficiency root induction by agropine strain 1855 of *Agrobacterium rhizogenes*. *Plant Mol. Biol.* **14**, 785–792 (1990).
26. M. Claeys, E. Messens, M. Van Montagu, J. Schell, GC/MS determination of cytokinins in *Agrobacterium tumefaciens* cultures. *Fresenius J. Anal. Chem.* **290**, 125–126 (1978).
27. J. A. McCloskey, T. Hashizume, T. B. Basile, Y. Ohno, S. Sonoki, Occurrence and levels of *cis*-and *trans*-zeatin ribosides in the culture medium of a virulent strain of *Agrobacterium tumefaciens*. *FEBS Lett.* **111**, 181–183 (1980).

28. J. Gray, S. B. Gelvin, R. Meilan, R. O. Morris, Transfer RNA is the source of extracellular isopentenyladenine in a Ti-plasmidless strain of *Agrobacterium tumefaciens*. *Plant Physiol.* **110**, 431–438 (1996).
29. H. Yi, K. S. Mysore, S. B. Gelvin, Expression of the *Arabidopsis* histone *H2A-1* gene correlates with susceptibility to *Agrobacterium* transformation. *Plant J.* **32**, 285–298 (2002).
30. S. Chateau, R. S. Sangwan, B. S. Sangwan-Norreel, Competence of *Arabidopsis thaliana* genotypes and mutants for *Agrobacterium tumefaciens*-mediated gene transfer: Role of phytohormones. *J. Exp. Bot.* **51**, 1961–1968 (2000).
31. D. Valvekens, M. V. Montagu, M. V. Lijsebettens, *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5536–5540 (1988).
32. J. P. C. To, J. J. Kieber, Cytokinin signaling: Two-components and more. *Trends Plant Sci.* **13**, 85–92 (2008).
33. I. Hwang, J. Sheen, B. Müller, Cytokinin signaling networks. *Annu. Rev. Plant Biol.* **63**, 353–380 (2012).
34. I. Hwang, J. Sheen, Two-component circuitry in *Arabidopsis* cytokinin signal transduction. *Nature* **413**, 383–389 (2001).
35. T. Kakimoto, Perception and signal transduction of cytokinins. *Annu. Rev. Plant Biol.* **54**, 605–627 (2003).
36. P. Nagpal, R. S. Quatrano, Isolation and characterization of a cDNA clone from *Arabidopsis thaliana* with partial sequence similarity to integrins. *Gene* **230**, 33–40 (1999).
37. B. Lü, J. Wang, Y. Zhang, H. Wang, J. Liang, J. Zhang, AT14A mediates the cell wall–plasma membrane–cytoskeleton continuum in *Arabidopsis thaliana* cells. *J. Exp. Bot.* **63**, 4061–4069 (2012).
38. T. Kwok, D. Zabler, S. Urman, M. Rohde, R. Hartig, S. Wessler, R. Misselwitz, J. Berger, N. Sewald, W. König, S. Backert, *Helicobacter* exploits integrin for type IV secretion and kinase activation. *Nature* **449**, 862–866 (2007).
39. K. A. Aly, L. Krall, F. Lottspeich, C. Baron, The type IV secretion system component VirB5 binds to the *trans*-zeatin biosynthetic enzyme Tzs and enables its translocation to the cell surface of *Agrobacterium tumefaciens*. *J. Bacteriol.* **190**, 1595–1604 (2008).
40. A. Kisiala, C. Laffont, R. J. N. Emery, F. Frugier, Bioactive cytokinins are selectively secreted by *Sinorhizobium meliloti* nodulating and nonnodulating strains. *Mol. Plant Microbe Interact.* **26**, 1225–1231 (2013).
41. K. Podlešáková, J. Fardoux, D. Patrel, K. Bonaldi, O. Novák, M. Strnad, E. Giraud, L. Spíchal, N. Nouwen, Rhizobial synthesized cytokinins contribute to but are not essential for the symbiotic interaction between photosynthetic Bradyrhizobia and *Aeschynomene* legumes. *Mol. Plant Microbe Interact.* **26**, 1232–1238 (2013).
42. A. Imamura, N. Hanaki, A. Nakamura, T. Suzuki, M. Taniguchi, T. Kiba, C. Ueguchi, T. Sugiyama, T. Mizuno, Compilation and characterization of *Arabidopsis thaliana* response regulators implicated in His-Asp phosphorelay signal transduction. *Plant Cell Physiol.* **40**, 733–742 (1999).
43. J. P. C. To, G. Haberer, F. J. Ferreira, J. Deruère, M. G. Mason, G. E. Schaller, J. M. Alonso, J. R. Ecker, J. J. Kieber, Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* **16**, 658–671 (2004).
44. C. T. Argueso, F. J. Ferreira, P. Epple, J. P. C. To, C. E. Hutchinson, G. E. Schaller, J. L. Dangl, J. J. Kieber, Two-component elements mediate interactions between cytokinin and salicylic acid in plant immunity. *PLoS Genet.* **8**, e1002448 (2012).
45. J. Choi, S. U. Huh, M. Kojima, H. Sakakibara, K. H. Paek, I. Hwang, The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in *Arabidopsis*. *Dev. Cell* **19**, 284–295 (2010).
46. J. P. C. To, J. Deruère, B. B. Maxwell, V. F. Morris, C. E. Hutchinson, F. J. Ferreira, G. E. Schaller, J. J. Kieber, Cytokinin regulates type-A *Arabidopsis* response regulator activity and protein stability via two-component phosphorelay. *Plant Cell* **19**, 3901–3914 (2007).
47. C. Knepper, E. A. Savory, B. Day, Arabidopsis NDR1 is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. *Plant Physiol.* **156**, 286–300 (2011).
48. D. G. Mellersh, M. C. Heath, Plasma membrane–cell wall adhesion is required for expression of plant defense responses during fungal penetration. *Plant Cell* **13**, 413–424 (2001).
49. C. Lichtenstein, J. Draper, Genetic engineering of plants, in *DNA Cloning. A Practical Approach*, D. M. Glover, Ed. (IRL Press, Washington, DC, 1986), **vol. 2**, pp. 67–119.
50. G. N. Tenea, J. Spantzel, L. Y. Lee, Y. Zhu, K. Lin, S. J. Johnson, S. B. Gelvin, Overexpression of several *Arabidopsis* histone genes increases *Agrobacterium*-mediated transformation and transgene expression in plants. *Plant Cell* **21**, 3350–3367 (2009).
51. R. A. Jefferson, T. A. Kavanagh, M. W. Bevan, GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907 (1987).
52. Y.-G. Liu, N. Mitsukawa, T. Oosumi, R. F. Whittier, Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* **8**, 457–463 (1995).
53. J. M. Alonso, A. N. Stepanova, T. J. Leisse, C. J. Kim, H. Chen, P. Shinn, D. K. Stevenson, J. Zimmerman, P. Barajas, R. Cheuk, C. Gadrinab, C. Heller, A. Jeske, E. Koesema, C. C. Meyers, H. Parker, L. Prednis, Y. Ansari, N. Choy, H. Deen, M. Geralt, N. Hazari, E. Hom, M. Karnes, C. Mulholland, R. Ndubaku, I. Schmidt, P. Guzman, L. Aguilar-Henonin, M. Schmid, D. Weigel, D. E. Carter, T. Marchand, E. Risseeuw, D. Brogden, A. Zeko, W. L. Crosby, C. C. Berry, J. R. Ecker, Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653–657 (2003).
54. L. Y. Lee, M. E. Kononov, B. Bassuner, B. R. Frame, K. Wang, S. B. Gelvin, Novel plant transformation vectors containing the superpromoter. *Plant Physiol.* **145**, 1294–1300 (2007).
55. S. J. Clough, A. F. Bent, Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743 (1998).
56. T. Tzfira, G. W. Tian, B. Lacroix, S. Vyas, J. Li, Y. Leitner-Dagan, A. Krichevsky, T. Taylor, A. Vainstein, V. Citovsky, pSAT vectors: A modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. *Plant Mol. Biol.* **57**, 503–516 (2005).
57. R. C. Gentleman, V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. Yang, J. Zhang, Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80 (2004).
58. T. M. Chu, B. Weir, R. Wolfinger, A systematic statistical linear modeling approach to oligonucleotide array experiments. *Math. Biosci.* **176**, 35–51 (2002).
59. Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methodol.* **57**, 289–300 (1995).
60. S. Holm, A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* **6**, 65–70 (1979).

Acknowledgments: We thank the Purdue Genomics Center for conducting microarray hybridizations and DNA sequencing, and C.-P. Hwang of the Purdue Life Sciences Microscopy Facility for conducting SEM. We thank S. Subramanyam and C. E. Williams for help with real-time RT-PCR, E.-M. Lai and H.-H. Hwang for providing *A. tumefaciens* NT1RE(pJK270) and the *tzs*-fs mutant, E. Parsons for help with the transformation assays, and P. Pijut for providing *trans*-zeatin. **Funding:** This work was supported by grants from the National Science Foundation, the Consortium for Plant Biotechnology Research, the Biotechnology Research and Development Corporation, and a P30 grant to the Purdue University Cancer Center. **Author contributions:** N.S. designed the project and carried out most of the experimental work, H.C. and H.Y. identified the *mtf1-1* mutant, A.S. carried out root transformation assays of Bl-1–At14a lines, K.X. carried out root transformation assays of some mutants, L.-Y.L. and J.J. conducted fluorescence studies of *A. tumefaciens* attachment to Bl-1–At14a transgenic roots, G.R.O. and R.W.D. performed statistical analyses of microarray data, S.B.G. directed the project, and N.S. and S.B.G. interpreted the data and wrote the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** A patent has been filed on the use of MTF1 to increase transformation susceptibility. Microarray data have been deposited to the NCBI (National Center for Biotechnology Information) Gene Expression Omnibus under accession number GSE51236 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51236).

Submitted 16 July 2013
Accepted 4 October 2013
Final Publication 19 November 2013
10.1126/scisignal.2004518
Citation: N. Sardesai, L.-Y. Lee, H. Chen, H. Yi, G. R. Olbricht, A. Stirnberg, J. Jeffries, K. Xiong, R. W. Doerge, S. B. Gelvin, Cytokinins secreted by *Agrobacterium* promote transformation by repressing a plant Myb transcription factor. *Sci. Signal.* **6**, ra100 (2013).

Cytokinins Secreted by *Agrobacterium* Promote Transformation by Repressing a Plant Myb Transcription Factor

Nagesh Sardesai, Lan-Ying Lee, Huabang Chen, HoChul Yi, Gayla R. Olbricht, Alexandra Stirnberg, Jacob Jeffries, Kia Xiong, R. W. Doerge and Stanton B. Gelvin (November 19, 2013)

Science Signaling **6** (302), ra100. [doi: 10.1126/scisignal.2004518]