Agrobacterium expressing a type III secretion system delivers Pseudomonas effectors into plant cells to enhance transformation

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Agrobacterium-mediated plant transformation (AMT) is the basis of modern-day plant biotechnology. One major drawback of this technology is the recalcitrance of many plant species/varieties to Agrobacterium infection, most likely caused by elicitation of plant defense responses. Here, we develop a strategy to increase AMT by engineering Agrobacterium tumefaciens to express a type III secretion system (T3SS) from Pseudomonas syringae and individually deliver the P. syringae effectors AvrPto, AvrPtoB, or HopAO1 to suppress host defense responses. Using the engineered Agrobacterium, we demonstrate increase in AMT of wheat, alfalfa and switchgrass by ~250%–400%. We also show that engineered A. tumefaciens expressing a T3SS can deliver a plant protein, histone H2A-1, to enhance AMT. This strategy is of great significance to both basic research and agricultural biotechnology for transient and stable transformation of recalcitrant plant species/varieties and to deliver proteins into plant cells in a non-transgenic manner.

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The soil-borne phytopathogen Agrobacterium tumefaciens causes crown gall tumors on various dicotyledonous plants by transferring a piece of its DNA (T-DNA; T-DNA) and virulence proteins into host cells through the type IV secretion system (T4SS). The ability to transfer T-DNA from Agrobacterium to plants has been widely adopted to generate transgenic plants expressing genes of interest for research purposes or for commercial applications. However, the generation of transgenic plants has several drawbacks, including the low transformation efficiency of some plant species/varieties. Plant defense responses against Agrobacterium significantly contribute to recalcitrance.

Active plant defense against microbial infection relies on innate immune responses triggered by several layers of microbial recognition. The first layer involves the perception of conserved microbial molecules called pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) leading to PAMP-triggered immunity (PTI) that often is sufficient to prevent pathogen growth. Perception of one of the most abundant A. tumefaciens proteins, the PAMP elicitation factor thermo unstable (EF-TU), by the Arabidopsis PRR EF-TU receptor (EFR) activates a set of signaling events and defense responses that reduce Agrobacterium-mediated plant transformation (AMT). Arabidopsis efr mutants are more susceptible to AMT. Therefore, reducing or dampening plant basal immunity is not only essential for a successful pathogen to cause disease, but also will aid in AMT.

In contrast to A. tumefaciens which has a T4SS, many Gram-negative plant pathogenic bacteria have a type III secretion system (T3SS) to deliver bacterial proteins directly into their eukaryotic hosts. Many such delivered proteins, known as type III effectors (T3Es), have virulence functions that interfere with host cellular processes to block PTI, thus allowing bacteria to thrive in their hosts and cause disease. T3SSs are macromolecular machines consisting of protein complexes that assemble a needle-like structure that spans the bacterial inner and outer membranes and traverses the plant cell wall and cell membrane. The synthesis of effector proteins is co-regulated with proteins encoding the type III secretion apparatus. Effector proteins contain an export signal at their N-termini with the characteristic composition of amphipathic and polar amino acids. Although the effector protein content varies among pathogens, the genes encoding the type III secretion apparatus are broadly conserved and functional when heterologously expressed. For example, the T3SS from Pseudomonas syringae pv. syringae 61 (Ps61) and Erwinia chrysanthemi expressed in P. fluorescens and Escherichia coli, respectively, allowed these non-pathogenic bacteria to deliver bacterial proteins into plant cells.

Many T3Es, including AvrPto from P. syringae pv. tomato, can suppress plant basal defense. The interaction between AvrPto and kinase domains of the PRRs Flagellin sensitive2 (FLS2) and EFR leads to the suppression of PTI. When AvrPto is expressed under the control of an inducible promoter, Arabidopsis becomes more susceptible to transient AMT. Transient expression of AvrPto by co-infiltration also improves transient transgene expression in Brassica sp. Earlier, we showed that Arabidopsis and Nicotiana benthamiana plants compromised for plant defense were more susceptible to AMT. Recently, the transient transformation was achieved in Arabidopsis NahG expressing plants in which the defense signaling hormone salicylic acid is reduced. Even though these results demonstrate that AMT can be increased by decreasing plant defense responses, practically this strategy cannot be used in the field because of the need for the generation of transgenic plants. An alternative approach to increase plant transformation is by altering the expression of host factors (other than genes involved in plant defense responses) that play a role in plant transformation and regeneration. Several plant proteins, including histones, have been identified to play a role in AMT. However, altering the expression of host factors needs a transgenic approach that is time-consuming and creates additional hurdles for deregulation.

Here, we report a strategy based on engineering A. tumefaciens with a T3SS to deliver proteins that suppress plant defense and/or increase transformation. P. syringae pv. tomato T3Es such as AvrPto, AvrPtoB, or HopA01, when co-delivered along with T-DNA through engineered A. tumefaciens during the transformation process, increase transformation efficiency in Arabidopsis, N. benthamiana, wheat, alfalfa, and switchgrass. Delivery of the plant protein histone H2A-1 also increases transformation efficiency.

### Results

**T3SS from Pseudomonas syringae pv. syringae 61 is functional in A. tumefaciens.** T3SS encoding genes cloned from Ps6126, contained in the plasmid pLN18, are functional in P. fluorescens27 and E. coli22. Here, we tested whether the expression of the Ps61 T3SS in A. tumefaciens is functional to secrete and translocate T3Es. We introduced pLN18, containing the T3SS genes, and a plasmid that can express the effector protein AvrPto tagged with the fluorescent reporter PhiLOV into A. tumefaciens (Fig. 1a). To monitor hrp-dependent effector secretion into the medium, the A. tumefaciens strain expressing T3SS and AvrPto-PhiLOV along with appropriate control strains were cultured in hrp-derepressing medium. Both cell pellet and supernatant fractions were used for immunoblot analysis. AvrPto-PhiLOV could be found in both the cell pellet and the supernatant fractions for A. tumefaciens containing pLN18 and expressing AvrPto-PhiLOV (Fig. 1b). An A. tumefaciens strain expressing AvrPto-PhiLOV without pLN18 showed the presence of AvrPto-PhiLOV only in the cell pellet and not in the supernatant fraction (Fig. 1b). These results demonstrate that an A. tumefaciens strain expressing a T3SS is able to express a T3E and secrete it from A. tumefaciens into the culture medium.

To demonstrate that the T3E secreted from the engineered A. tumefaciens strain can be delivered into plant cells, we used a previously established split GFP system by infiltrating N. benthamiana leaves with A. tumefaciens that contain a GFP10 gene within the T-DNA of a binary vector (Fig. 1c), followed by infiltration of the same leaves with A. tumefaciens containing pLN18 and expressing AvrPto-GFP11 (Fig. 1c). Live-cell imaging showed green fluorescence signals inside the plant epidermal cells resulting from the assembly of full-length GFP from the interaction of GFP11 and GFP10, indicating delivery of AvrPto-GFP11 into plant cells (Fig. 1d). As expected, green fluorescence was not observed in leaves infiltrated with Agrobacterium strains lacking either the T3SS or the tagged effector protein gene (Fig. 1d). FM4-64 staining of the leaves showed plasma membrane localization of AvrPto-GFP, similar to previous reports.

**T3Es delivered by a T3SS in A. tumefaciens improves transformation.** The T3E AvrPto suppresses plant innate immunity that hinders AMT. Inducible expression of AvrPto in transgenic...
Arabidopsis increases transient transformation efficiency. To determine if AvrPto delivered through a T3SS can increase transient transformation, we transferred plN18 (containing T3SS genes) and a plasmid that expresses AvrPto under its native promoter into the disarmed A. tumefaciens strain EHA105 containing a binary vector with a β-glucuronidase (GUS)-intron gene within the T-DNA (Supplementary Fig. 2a). This engineered A. tumefaciens strain, along with appropriate controls, was infiltrated into the leaves of Arabidopsis plants. GUS expression significantly increased when A. tumefaciens expressed a T3SS and AvrPto (Fig. 2a and b). To determine if T3SS delivery of AvrPto can also increase stable transformation, we introduced plN18 and a plasmid expressing AvrPto into the tumorigenic strain A. tumefaciens A208 (Supplementary Fig. 2b). This engineered A. tumefaciens strain A208 was used for Arabidopsis root transformation assay.

**Fig. 1** *Pseudomonas* type III secretion system when expressed in *A. tumefaciens* can deliver T3Es to plant cells. **a** Schematics of engineering *A. tumefaciens* to deliver proteins through a T3SS assay. *A. tumefaciens* strain GV2260 was mobilized with plasmids containing the *P. syringae* T3SS (pLN18) and T3E AvrPto tagged with PhiLOV (pBR1MCS5-AvrPto-PhiLOV) to express the T3SS and AvrPto-PhiLOV. Promoter (P_AvrPto) and coding sequences (CDS) of AvrPto without a stop codon were fused to codon-optimized sequences of PhiLOV. CDS of AvrPto includes sequences encoding a type III secretion signal peptide (T3SS). **b** AvrPto-PhiLOV secreted in the culture medium by the engineered *A. tumefaciens* were detected by immunoblotting. GV2260 derived *A. tumefaciens* strains grown in hrp-derepressing medium were separated into cell pellet and supernatant fractions and probed with PhiLOV-specific antibody. **c** Schematics of engineering *A. tumefaciens* to deliver proteins through a T3SS for *in planta* visualization. *A. tumefaciens* strain GV2260 was mobilized with plN18 and a plasmid containing AvrPto-GFP11 to express a T3SS and AvrPto-GFP11. **d** AvrPto-GFP11 delivered through a T3SS of an engineered *A. tumefaciens* can complement GFP1-10 expressed in plants. Representative confocal images of *N. benthamiana* leaves transiently expressing GFP1-10 individually infiltrated with *A. tumefaciens* strains GV2260, GV2260 (pLN18), GV2260 (AvrPto-GFP11) and GV2260 (AvrPto-GFP11, pLN18) are shown. Confocal microscopy was used to visualize GFP fluorescence 48 h post-infiltration. GFP signals were pseudo-colored to green and chlorophyll autofluorescence is shown in red. AvrPto-GFP11 translocated to plant cells through a T3SS of engineered *A. tumefaciens* complemented GFP1-10 produced *in planta* to form functional GFP. Scale bars, 10 μm. Experiments were repeated three times with similar results. Source data are provided as a Source Data file.
4 weeks after two-way ANOVA analysis (Fig. 2c and d). T-DNA encoded AvrPto increased the transient and stable transformation efficiency of Arabidopsis. The percentage of root segments forming tumors was calculated. Data presented in b and d are mean ± standard error of three replicates. Bars with different letters are significantly different based on Tukey’s post-hoc one-way ANOVA analysis (p < 0.05). Brown-Forsythe test was done to test for variance (p = 0.6830 for b, 0.1433 for d). Data presented in e are mean of two replicates. Bars with different letters are significantly different based on Tukey’s post-hoc two-way ANOVA analysis (p = 0.0102). Experiments were repeated three times with similar results. Source data are provided as a Source Data file.

A. tumefaciens strain carrying pLN18 and expressing AvrPto developed significantly more tumors compared to controls (Fig. 2c and d). T-DNA encoded iaaM, iaaH, and ipt genes of tumorigenic strains cause overproduction of phytohormones such as auxin and cytokinin in plants that lead to tumor formation and may affect plant defense responses. Therefore, we tested stable transformation efficiency in Arabidopsis roots using non-tumorigenic strain EHA105 carrying binary vector pCAS120 that gives phosphinothricin (PPT) resistant calli because of a chimeric nos-bar gene expression in plants. Consistent with the tumor results, engineered A. tumefaciens strain carrying pLN18 and expressing AvrPto developed significantly more PPT-resistant calli compared to controls (Fig. 2c and Supplementary Fig. 3a). In addition, we also tested if our engineered A. tumefaciens strain can also enhance the floral dip transformation that is commonly used in Arabidopsis. We used a low concentration of A. tumefaciens (A600 = 0.1) to see subtle differences between the A. tumefaciens strains used. Surprisingly, delivery of AvrPto through T3SS increased the floral dip transformation efficiency by two-fold (Supplementary Fig. 3b).

Similar experiments were performed in a different plant species, N. benthamiana, using the disarmed strain A. tumefaciens GV2260 for transient expression and the tumorigenic strain A. tumefaciens A348 for stable leaf disk transformation. These results were similar to those using Arabidopsis wherein expression of a T3SS and AvrPto in A. tumefaciens significantly increased both transient and stable transformation (Fig. 3a–d).

Like AvrPto, several other T3Es have the ability to suppress plant basal defense to establish/aid the growth of pathogens and
cause disease\textsuperscript{14}. To further examine the effect of other T3Es on AMT, we selected two T3Es from \textit{P. syringae} \textit{pv. tomato}: AvrPtoB and HopAO1. Similar to AvrPto, both AvrPtoB and HopAO1 significantly increased the percentage of root segments forming tumors and the weight of leaf disk tumors (Fig. 4 and Supplementary Fig. 4). As a negative control, another set of Arabidopsis root tumor assays was carried out using a HopAI1 construct. Since HopAI1 is targeting the PTI pathway by inhibiting MAPKs downstream of PAMP receptors\textsuperscript{36}, we hypothesized that expression HopAI1 would not increase the susceptibility of the host to \textit{A. tumefaciens} infection. As expected, we did not see any increase in transformation efficiency in the negative control (Supplementary Fig. 5a). These results suggest that T3Es when delivered through T3SS of engineered \textit{A. tumefaciens} can enhance both transient and stable transformation in \textit{N. benthamiana} and Arabidopsis.

Delivery of plant defense suppressing T3Es improves the transformation of crop plants. Both Arabidopsis and \textit{N. benthamiana} are highly susceptible to stable AMT, and therefore the increase in transformation efficiency we observed by co-delivery of T3Es was only incremental for these species. Despite continuous efforts by many groups, efficient and reproducible \textit{Agrobacterium}-mediated wheat transformation remains challenging\textsuperscript{37,38}. Most reports of AMT of wheat have
focused on the model spring wheat genotypes Fielder and Bobwhite. We chose to determine if AMT of the wheat cultivar Fielder could be improved by co-delivery of T3Es. Immature embryos of wheat were infected with engineered A. tumefaciens strain AGL1 (pANIC6B) harboring pLN18 and a plasmid expressing T3E. The number of immature embryos that produced transgenic calli and subsequently regenerated shoots were counted. Transgenic plants derived from these regenerated shoots were tested for the activity and presence of reporter genes by GUS histochemical staining and PCR analysis of the GUSPlus and hph genes (Supplementary Fig. 6). A. tumefaciens strains individually delivering AvrPto, AvrPtoB, or HopAO1 through engineered T3SS greatly increased the percentage of individual transgenic plants obtained (Fig. 5a and Supplementary Fig. 7). The A. tumefaciens strain expressing AvrPto produced the best results, with a transformation efficiency ~400% that of the control strain lacking the T3SS components. These results indicate that A. tumefaciens with an engineered T3SS that delivers T3Es can increase the transformation efficiency of a recalcitrant crop species. Wheat transformation assay was also carried out using A. tumefaciens strains expressing HopAI1. Delivery of HopAI1 through T3SS did not have any effect on wheat transformation similar to the results obtained for Arabidopsis root assay (Supplementary Fig. 5b).

To determine if the engineered A. tumefaciens strains can also be used to improve the transformation efficiency of other commercial crop plants, we used our engineered strain that can deliver AvrPto through T3SS on alfalfa line R2336 and switchgrass line NFCX01. We observed 260% increase in transformation efficiency in alfalfa and 400% increase in transformation efficiency in switchgrass (Fig. 5b and c). These results indicate that engineered A. tumefaciens delivering AvrPto can be used to enhance AMT in many commercially important crop plants.

Virulence gene expression is not altered in the engineered A. tumefaciens strains expressing T3SS. As shown above, A. tumefaciens strains expressing T3SS and T3Es effectively increased AMT efficiency. The expression of T3Es in A. tumefaciens may increase virulence gene (vir) expression and thus increase transformation efficiency. To test this, we measured the expression of several vir genes in engineered A. tumefaciens A208 strains using reverse transcription-quantitative PCR (RT-qPCR). No major differences were observed in virA, virB2, virD2, and virE3 gene induction, after acetosyringone treatment, among A. tumefaciens strains with or without the T3SS + T3E (Supplementary Fig. 8). These results, along with those reported in Fig. 2, indicate that the increase in transformation by A. tumefaciens strains expressing T3SS and T3Es is not due to increased expression of vir genes and is most likely due to the delivery of T3Es into plant cells.

AvrPto delivered through engineered A. tumefaciens T3SS reduces the expression of plant defense genes. Based on the role of AvrPto in suppressing plant defense, and our results showing the delivery of AvrPto along with T-DNA into plants increases AMT (Figs. 2 and 3), we speculated that the increase in AMT efficiency was due to the suppression of plant defense responses. To show that AvrPto delivered through engineered A. tumefaciens T3SS can suppress plant defense responses, we infected Arabidopsis roots with tumorigenic A. tumefaciens A208 expressing a T3SS and AvrPto, or with negative controls, and measured the expression of well-known PTI marker genes including FLG22-induced receptor-like kinase 1 (FRK1), and...
Irrespective of the A. tumefaciens strain used, both the tested defense-related genes were induced in response to A. tumefaciens infection at 2 h after infection when compared to a mock-infected control (Fig. 6). However, 16 h after A. tumefaciens infection, transcripts of defense-related genes were significantly reduced in root samples infected with A. tumefaciens expressing the T3SS and AvrPto compared to control strains (Fig. 6). These results indicate that AvrPto, when delivered through an engineered T3SS of A. tumefaciens, can suppress the plant defense response, thus contributing to increased AMT efficiency.

Delivery of a plant protein from an engineered A. tumefaciens strain expressing a T3SS enhances stable transformation. AMT is a complex process involving functions of both bacterial virulence proteins and plant proteins. Histone H2A-1 (encoded by the gene HTA1) is involved in T-DNA integration, and overexpression of HTA1 and truncated HTA1 (HTA1; coding only the first 39 amino acids) in plants increases transformation efficiency. To verify if plant proteins that enhance AMT can also be delivered through an engineered T3SS of A. tumefaciens, we chose HTA1 and tHTA1. Two different promoters, along with N-terminal sequences containing a type III signal from the T3Es AvrRpm1 and AvrRps4, designated as AvrRpm1N and AvrRps4N, respectively, were selected to drive the expression of HTA1 and tHTA1 and export from A. tumefaciens. Using the engineered A. tumefaciens strains, we conducted Arabidopsis root and N. benthamiana leaf disk tumor assays. Both HTA1 and tHTA1 expressing A. tumefaciens strains enhanced the stable transformation efficiency of Arabidopsis and N. benthamiana (Fig. 7a–c and Supplementary Fig. 9a). Transformation assays in crop plants using engineered strains expressing HTA1 also showed increased transformation efficiency in wheat, alfalfa, and switchgrass (Fig. 7d–f, Supplementary Fig. 9b and c). These results indicate that AvrPto, when delivered through an engineered T3SS of A. tumefaciens, can suppress the plant defense response, thus contributing to increased AMT efficiency.
results indicate that engineered *A. tumefaciens* expressing a T3SS can also be used to deliver plant proteins to enhance AMT.

**Discussion**

*A. tumefaciens* is a plant pathogen that causes crown gall disease in many plant species and has been widely used in the field of plant biotechnology and functional genomic studies, including the recently developed genome-editing technologies. The T3SS has some limitations in the size and structure of proteins that it can deliver to the host cell. For example, the fusion of T3Es with a large fluorescent protein-like GFP interferes with effector secretion and translocation because of the inability of the type III ATPase to unfold the tightly packed GFP domain. Therefore, we explored the possibility of using a small 13 KDa fluorescent reporter PhiLOV and successfully demonstrated its use in visualizing effector expression, secretion, and translocation to plant cells (Fig. 1 and Supplementary Fig. 1). Previously, PhiLOV has been used to tag T3Es of animal pathogens to monitor effector secretion and translocation and to visualize translocation and localization of *A. tumefaciens* virulence proteins. In another study, a split GFP system was used to monitor the secretion of T3Es of *P. syringae* and *R. solanacearum*. Recently, a T3SS gene cluster from *Xanthomonas euvesicatoria*, which is known to secrete large effectors, was shown to secrete effectors into the plants. However, heterologous expression of a *Xanthomonas* T3SS has not yet been reported.

A previous attempt by Tsuda et al. to express T3Es other than AvrPto in planta to increase the transient transformation of Arabidopsis was not successful, likely because these effectors are not targeting the PTI pathway at the PAMP receptor level. We therefore selected the *P. syringae* T3Es AvrPtoB and HopAO1, which are involved in the early immune signaling of PTI. These proteins target PRRs and disrupt their functions in *Arabidopsis* by T3E AvrPto is associated with an attenuated plant defense response. Many plant pathogenic bacteria contain T3SSs that act as an essential virulence determinant to deliver T3Es that remodel normal plant cellular functions, compromising the plants’ immune responses and promoting bacterial multiplication.

In our study, both AvrPtoB and HopAO1, when co-delivered with T-DNA, increased the stable transformation of Arabidopsis, *N. benthamiana*, and wheat (Figs. 4 and 5a).

Research in several laboratories had previously suggested that AMT of wheat was not reproducible across laboratories. Recently, up to 25% AMT frequency of *N. benthamiana* using T3SS had been achieved by *P. syringae* T3Es AvrPtoB and HopAO1, which are involved in the early immune signaling of PTI. These proteins target PRRs and disrupt their functions in *Arabidopsis* by T3E AvrPto is associated with an attenuated plant defense response. Many plant pathogenic bacteria contain T3SSs that act as an essential virulence determinant to deliver T3Es that remodel normal plant cellular functions, compromising the plants’ immune responses and promoting bacterial multiplication. We used the T3SS of *P. syringae* species because it is well characterized.

The T3SS has some limitations in the size and structure of proteins that it can deliver to the host cell. For example, the fusion of T3Es with a large fluorescent protein-like GFP interferes with effector secretion and translocation because of the inability of the type III ATPase to unfold the tightly packed GFP domain. Therefore, we explored the possibility of using a small 13 KDa fluorescent reporter PhiLOV and successfully demonstrated its use in visualizing effector expression, secretion, and translocation to plant cells (Fig. 1 and Supplementary Fig. 1). Previously, PhiLOV has been used to tag T3Es of animal pathogens to monitor effector secretion and translocation and to visualize translocation and localization of *A. tumefaciens* virulence proteins. In another study, a split GFP system was used to monitor the secretion of T3Es of *P. syringae* and *R. solanacearum*. Recently, a T3SS gene cluster from *Xanthomonas euvesicatoria*, which is known to secrete large effectors, was shown to secrete effectors into the plants. However, heterologous expression of a *Xanthomonas* T3SS has not yet been reported.

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In the present study, the expression of vir genes in the *Agrobacterium*
strain expressing T3SS and AvrPto did not show major differences from control strains. *Agrobacterium* infection induces the expression of various plant defense genes, including PR genes and those encoding chitinases and defensins.\textsuperscript{7,32,60} *FRK1*, a commonly used molecular marker gene to study T3E-mediated suppression of plant defense,\textsuperscript{61,62} is activated by PAMPs and not by other stress-related signals.\textsuperscript{62} Many studies designed to optimize media composition for AMT rely on the expression of *FRK1* and *NHL10* to monitor plant defense responses.\textsuperscript{63–65} We showed that the expression of *FRK1*, and *NHL10* is reduced by an engineered
Agrobacterium strain that delivers AvrPto through a T3SS, thus increasing AMT (Fig. 6).

One way to increase the efficiency of AMT is by suppressing the plant defense response as described above or by altering the expression of plant genes that play a role in AMT25. To determine if the engineered Agrobacterium with a T3SS can deliver plant proteins into plants cells, we used A. tumefaciens codon-optimized sequences of HTA1 and hHTA1. The expression of functional proteins in heterologous hosts is often enhanced by codon optimization66,67. Delivery of either HTA1 or HHTA1 through the T3SS increased AMT (Fig. 7).

Transformation and regeneration are major bottlenecks in the generation of transgenic plants or genome-edited plants. Our study aimed to improve transformation in the delivery and integration of T-DNA steps. The chimeric protein of plant developmental gene encoding GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) (GRF4-GIF1) and GRF5 were shown to enhance regeneration and transformation of both monocot and dicot species68,69. Specific morphogenic genes that are known to induce somatic embryogenesis and regeneration are also used to improve transformation efficiency in monocots70,71. Since constitutive expression of these genes has negative phenotypic and reproductive effects, altruistic transformation is used in maize and sorghum which uses the transient expression of Baby boom and Wuschel2 to promote somatic embryogenesis and regeneration in nearby transformed cells72,73. In the future, these proteins can be potentially delivered through Agrobacterium strain expressing T3SS to improve somatic embryogenesis and plant regeneration.

Heterologous protein expression in plants is achieved by stable or transient transformation by delivery of genes of interest harbored in Agrobacterium T-DNA74. AMT has emerged as a vehicle for the application of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated genome editing of plants75. Genome-editing reagents are delivered to plants mostly through a stable transformation that requires segregation of Cas9 by Mendelian segregation to achieve transgene-free genome-edited plants (null segregants)13. In addition, off-target mutations may be increased by constitutive Cas9 expression76, which can be significantly reduced by conditional and transient expression of Cas977. Our approach of delivering proteins from Agrobacterium through a T3SS can not only increase AMT but also has the potential to alleviate the above-mentioned problems of CRISPR-mediated genome editing by delivering bacterially expressed Cas9 to plants through a T3SS instead of generating Cas9 expressing transgenic plants. In addition, our technology side-steps the disadvantages of making transgenic plants that overexpress genes that enhance transformation. Recently, direct delivery of proteins using Agrobacterium’s T4SS is getting attention, particularly in the field of DNA-free genome editing. For example, Cas9 transformation to plants through the T4SS by fusion with VirF translocation signal has been shown78. Previously, DNA modifying proteins such as site-specific recombinase Cre79 and homing endonuclease I-Scei79 have been translocated to plants through the T4SS. Our technology would be a good alternative to deliver genome-editing proteins because the T3SS is evolved for fast and efficient translocation of multiple effectors80.

Methods

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are shown in Supplementary Data 1. E. coli DE5a was used for molecular cloning and was grown at 37°C in Luria-Bertani (LB) medium. HB101 was used for maintaining the helper plasmid pRK2013. A. tumefaciens strains were grown at 28°C on YEP agar plates or in YEP liquid medium, Agrobacterium minimal medium containing sucrose (AB-sucrose)81, hrp-depressing liquid medium (HDM)28, or mannitol glutamate/lysozyme (MGIL) medium. Acetosyringone (200 µM) added to AB-MES29 (17.2 mM KH2PO4, 8.3 mM NaH2PO4, 18.7 mM NH4Cl, 2 mM KCl, 1.25 mM MgSO4, 100 mM CaCl2, 10 mM FeSO4, 50 mM MES, 2% glucose (w/v), pH 5.5) and ABM-MS65 (½ AB-MES, ¼ MS, 0.25% sucrose (w/v), pH 5.5) medium was also used. Antibiotics used were spectinomycin (25 µg mL−1), carbencillin (10 µg mL−1), rifampicin (10 µg mL−1), kanamycin (50 µg mL−1), gentamycin (25 µg mL−1), and tetracycline (5 µg mL−1).

Bacterial genetic manipulations and plasmid construction. Promoter sequences of AvrPto (116 bp upstream of ATG) followed by coding sequences without a stop codon, from P. syringae pv. tomato strain DC3000, as well as codon-optimized PluOv21 sequences, were synthesized and cloned into the broad host range vector pBRR1MCS5 at Eco53kI and KpnI sites to generate pBRR1MCS5-AvrPto-PluOv. AvrPto containing its native promoter was also synthesized and cloned into the Eco53kI and KpnI site of pBRR1MCS5 to generate pBRR1MCS5-AvrPto. The coding sequences of AvrPtoB and HopAOI, along with their native promoters (from P. syringae pv. tomato strain DC3000), were synthesized and cloned into the Eco53kI and KpnI site of pBRR1MCS5. Promoter sequences from AvrPtoB and HopAOI are 93 bp and 86 bp, respectively, upstream of ATG. AvrRpmlN1 consists of 199 bp upstream of ATG and the first 267 bp of the CDS from P. syringae pv. pisi82. Full-length and truncated HHTA1 (coding first 39 amino acids)1 sequences codon-optimized for A. tumefaciens driven by promoters and N-terminal sequences of AvrPmp1 as well as AvrPs4 were synthesized and cloned into Eco53kI and KpnI site of pBRR1MCS5. GenScript (Piscataway, NJ) carried out all DNA syntheses reported here. E. coli DH5α competent cells were transformed by a standard heat-shock procedure. Electroporation was used to introduce all the plasmids into A. tumefaciens strains, with the exception of pLN18. Triparental mating83 was used to mobilize pLN18 into A. tumefaciens strains.

Plant materials and growth conditions. For root transformation assays, Arabidopsis thaliana Columbia-0 (Col-0) plants were grown in B5 medium in an environment-controlled growth chamber at 24°C, 70% humidity, and a 16/8 h light/dark photoperiod with the light intensity of 50–80 µmol m−2 s−1. For leaf disk experiments, Arabidopsis (Col-0) plants were grown in metromix soil in a controlled growth chamber at 22°C, 75% humidity, and a 16/8 h light/dark photoperiod.
photoperiod with the light intensity of 140 μE m⁻² s⁻¹ using fluorescent tubes. *N. benthamiana* plants were grown in soil in a controlled growth room at 24 °C, 75% humidity, and a 16/8 h light/dark photoperiod with the light intensity of 140 μE m⁻² s⁻¹ using fluorescent tubes.

**Secretion assays and immunoblotting.** To monitor the hrp-dependent effector secretion into the medium, *A. tumefaciens* strain GV2260 expressing different combinations of *AvrPto-PhiLOV* and T3SS were grown on YEP agar plates at 28 °C for 2 days. Two colonies were transferred to HDM medium and cultured for 16 h at 28 °C with shaking at 220 rpm. Pellets from bacterial cultures at A600 = 0.25 were taken for protein analysis. After centrifugation (normalized based on the A600 of the bacterial cultures to get an equal protein amount) were separated into pellet and supernatant fractions by centrifuging the culture at 3220 × g for 15 min at 21 °C. The top 15 mL of the supernatant solution was carefully removed without disturbing the pellet. The supernatant solution was passed through a 0.45 μm Durapore PVDF Membrane Millipore filter (Cat. No. SEIM00300M, EMD Millipore Corporation, Burlington, MA), and further centrifugation steps were carried out at 4 °C. The supernatant fractions were concentrated by ultrafiltration using Amicon Ultra-15 Centrifugal Filters (Catalog No. UFC901024, Merck Millipore Ltd, Tullgreen, Ireland), and further concentrated to ~30 μL using Amicon Ultra-0.5 Centrifugal Filters (Catalog No. UFC0244, Merck Millipore Ltd, Tullgreen, Ireland) according to the manufacturer’s instructions. Proteins from the pellet as well as supernatant fractions were subjected to electrophoresis through a SDS-PAGE gel, and immunoblot analysis was carried out using PHILoV-specific antibody (dilution 1:5000).

**Confocal microscopy.** *A. tumefaciens* strain GV2260 carrying GFP1, 11, 21 or in a binary vector and the engineered *A. tumefaciens* strain GV2260 expressing a T3SS and various combinations of GUS reporter and T-DNA effector were used. All appropriate antibiotics for two days at 28 °C. Single colonies were inoculated into YEP liquid medium and incubated for 16 h, cells were resuspended in an induction medium containing 10 mM MES and 200 μM acetoxyrroicin, and incubated at room temperature for 3 h with slow shaking. The A600 of the culture was adjusted to 0.4. Bacterial strains were grown in nutrient broth containing 25 μg/mL acetoxyrroicin and 100 μg/mL hygromycin. *A. tumefaciens* strains were grown on YEP plates containing timentin, ticarcillin, and phosphinothricin (PPT) (10 mg L⁻¹). Four weeks after infection, the number of root segments forming PPT-resistant calli was recorded. All GUS assay performed as previously described22. Experiments were carried out in two biological replicates. In each experiment, ten plants were inoculated with strain EHA105 harboring different constructs (A600 = 0.1). TD seedlings were germinated on half-strength MS media containing hygromycin (20 mg L⁻¹). Hygromycin-resistant plants were selected and stained for GUS activity as described above.

For *N. benthamiana* transient transformation assays, engineered *A. tumefaciens* strain GV2260 carrying different plasmids were prepared and infiltrated as described above for microscopy. Leaf disks were collected after 4 days of infection and frozen in liquid nitrogen. Frozen leaf disks were ground in liquid nitrogen using a mortar and pestle. The crude leaf disk tumor assays were carried out as previously described22. In brief, leaves harvested from greenhouse-grown plants were sterilized using 8% bleach for five minutes, then washed four times with sterile distilled water. Leaf disks made using a cork borer (0.9 cm) were infected with *A. tumefaciens* strain A348 or its derivatives for 15 min followed by co-cultivation on MS-basal medium for 2 days in the dark at room temperature. Leaf disks were transferred onto MS-basal medium supplemented with cefotaxime (200 mg L⁻¹) and ticarcillin (100 mg L⁻¹). Fifteen days after transfer, the fresh weights of leaf disks were measured for the leaf disk tumorigenesis assay.

**Wheat transformation.** Wheat plants (cv. Fielder) were grown in a greenhouse with a 16/8 h light/dark photoperiod and 20–22 °C day/19–21 °C night. Immature ears were collected the 14 days after anthesis. The ears were strewn with 1% ethanol. After removing the glume, lemma, and palea, the immature embryos (IEs) were isolated from these immature seeds under a dissecting microscope in a laminar flow hood. The IEs were centrifuged in 2 mL embryo-collection medium using a fixed-angle rotor at 17,000 × g for 4 °C for 10 min. The IEs were resuspended in an induction medium containing 15 mg L⁻¹ hygromycin for 2 weeks followed by 30 mg L⁻¹ A. tumefaciens strain AGL1 carrying different plasmids grown in M7G/L medium with 100 μM acetoxyrroicin. The mixture was shaken at 90 rpm at room temperature for 15 min. The infected IEs were transferred onto co-cultivation medium with the scutellum side up. Plates were sealed with microfilm tape and then incubated at 28 °C for 3 weeks. The proliferating explants were then transferred onto a shoot regeneration medium with 30 mg L⁻¹ hygromycin and cultured at 24 °C under illumination (16/8 h light/dark) until shoots were produced. The regenerated shoots were transferred to a root regeneration medium containing 15 mg L⁻¹ hygromycin under the same growth conditions. Regenerated plants were transferred to soil and sampled for GUS activity staining (as described above) and genomic DNA extraction to test for the presence of transgenes by PCR for hph and GUSPlus genes. All primers used are listed in Supplementary Data 2.

**RT-qPCR.** To study the expression levels of *A. tumefaciens* vir genes, engineered *A. tumefaciens* strain A208 grown in YEP medium overnight at 28 °C was harvested and resuspended in AB-MS® medium containing 200 μM acetoxyrroicin. After incubation at room temperature with minimal shaking, bacterial cells were harvested by centrifugation, and RNA was extracted using a NucleoSpin RNA mini kit (Catalog No. 740955, Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions, including in-column genomic DNA digestion. Superscript III Reverse Transcriptase (Catalog No. 18080085, Invitrogen) was used for the synthesis of cDNAs using 1.5 μg of RNA and random hexamers (Catalog No. N8080127, Invitrogen). Quantitative PCR (qPCR) reactions were performed using a CFX Real-time PCR system (Applied Biosystems, Foster City, CA) and a KicqStart SYBR Green qPCR ReadyMix (MilliporeSigma, St Louis, MO). Quantitative PCR data were collected using Bio-Rad CFX Manager Version 2.0.1.202.0523 software. A minimum of three technical replicates and three biological replicates were used, and all primers used are listed in Supplementary Data 2.

For measuring the expression of plant defense genes, Arabidopsis plants were grown vertically on B5 medium for 12 days. Overnight cultures of engineered *A. tumefaciens* strain A208 were diluted to ~70° C for fermentation. 28 °C RNA extraction was harvested using a NucleoBond RNA mini kit (Catalog No. 740940, Qiagen, Valencia, CA). Quantitative RT-qPCR measurements were performed with TURBO DNase (Catalog No. AM1907, Invitrogen) to remove genomic DNA. Reverse transcription reactions were performed with 1 μg of RNA in 4 μL RT buffer using M-MLV reverse transcriptase (Catalog No. M1804A, Promega) and SuperScript III Reverse Transcriptase (Invitrogen), and qPCR reactions were performed using a CFX Real-time PCR system (Applied Biosystems) using KicqStart SYBR Green fluorescence assay mix.

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Alphafector transformation. Alphafector transformation was carried out following the previously described method. Briefly, A. tumefaciens strain EHA105 harboring different constructs were streaked and cultured on AB agar plates containing different antibiotics at 28 °C for 2–3 days. A single colony was then cultured in AB liquid media, containing the same antibiotics, overnight till the A_{600} was 0.6–0.8. The young leaves from 4–6-week-old alfalfa line R2336 plants were sterilized with 20% commercial bleach containing a drop of Tween-20 for 10 min and then washed three times with sterilized water. These leaves were then infected with the A. tumefaciens suspension by resuspending the pellets in a liquid infection medium to an A_{600} of 0.05 or 0.12 after centrifuging the A. tumefaciens liquid culture at 3500 rpm for 20 min. The infected leaves were blotted dried and plated on cultures medium and cultured under 24 °C in the dark for 24–30 h. These triplicate plates were transferred onto selection medium containing 10 mg mL⁻¹ hygromycin and continued growth for a total of 6–8 weeks under the same conditions. During this period, the plates were subcultured every 2 weeks until enough resistant calli produced. The resistant calli were then transferred onto a shoot regeneration medium with 5 mg mL⁻¹ hygromycin and cultured at 24 °C day/20 °C night and 16/8 h light/dark photoperiod with 150 μmol m⁻² s⁻¹ light. Shoots regenerated after 2–3 months of transfer were counted and checked by PCR and GUS activity staining.

Switchgrass transformation. Switchgrass transformation was carried out according to previously reported protocol. Briefly, switchgrass NFCX01 calli induced from inflorescence were infected with A. tumefaciens strain AG1.1 carrying different plasmids. After co-cultivation in the dark chamber at 24 °C for 3 days, infected calli were transferred onto a selection medium with 50 mg L⁻¹ hygromycin and cultured in the same conditions for 6–8 weeks. Resistant calli were then transferred onto a regeneration medium with 30 mg L⁻¹ hygromycin and cultured in the light chamber 16/8 h light/dark photoperiod, 24 °C day/20 °C night, and 150 μmol m⁻² s⁻¹ light. The regenerated shoots were transferred onto a rooting medium with 10 mg L⁻¹ hygromycin till plants grew big enough for screening by methods described for wheat transgenic plants.

Statistical analysis. GraphPad Prism version 8.0.1 was used for making graphs and ANOVA tests. R version 3.5.2 was used for all Tukey’s post-hoc tests.

Data availability
All relevant data supporting the key findings of this study are available within the article and its Supplementary Information files. Source data are provided with this paper.

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Author contributions
K.S.M. conceptualized the hypothesis. V.R., C.M.R., B.V., Q.J., and K.S.M. conceived and designed the experiments, and did data analysis and wrote the manuscript. V.R., C.M.R., B.V., K.D., J.K., S.O., J.Y., L.Y., G.L., and B.D.P conducted the experiments.

Competing interests
The authors declare no competing interests.

Additional information
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