CRISPR/Cas-induced targeted mutagenesis with Agrobacterium mediated protein delivery

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Chapter 5

Transient expression of the isopentenyl transferase for (non)transgenic shoot induction in *Arabidopsis thaliana*

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Abstract

Plant transformation systems use a selectable marker gene which is co-delivered with the gene of interest for efficient selection of transformation events among the large numbers of non-transformed plants cells. Throughout the years several marker genes have been developed usually based on conditional dominant genes many of which are antibiotic resistance genes. An alternative non antibiotic marker gene is the isopentenyl transferase gene (*ipt*), found on the Ti-plasmid of *Agrobacterium tumefaciens*, which increases cytokinine levels stimulating organogenesis in many cultured plant tissues and which is widely used to regenerate transgenic plants from cultured cells after transformation. Constitutive expression of *ipt* however results in loss of apical dominance and an inability to form roots and therefore its removal after selection is essential to produce normal plants. Instead of integrating and subsequently removing the *ipt* gene we have tested whether the transient expression of IPT can be used for the selection of transformed plants. The first approach consisted of the delivery of a T-DNA encoding the *ipt* gene into the Pol-θ-deficient *Arabidopsis* integration mutant in which only transient expression of the T-DNA occurs but no integration. The second approach involved the direct delivery of the IPT protein through the *Agrobacterium* VirB/D4 T4SS into *Arabidopsis*. We show that the combined transfer of the IPT protein with a T-DNA encoding a CRISPR/Cas system can be used to obtain mutated shoots. Furthermore if the transfer of a T-DNA is combined with the transfer of the IPT protein, T-DNA transformants can be identified based on shoot induction without requiring selection for the T-DNA.

Introduction

For efficient selection of transformation events among the large numbers of non-transformed plants cells, plant transformation systems use a selectable marker gene which is co-delivered with the gene of interest. Throughout the years several marker genes have been developed usually based on conditional dominant genes. By selection for these genes transgenic plants can be obtained eventually. These selection systems do however have several shortcomings: (1) the presence of marker genes prevents usage of the same marker in a next round of transformation; (2) integration of the marker genes limits the usage of these plants due to regulatory concerns; (3) concerns have been raised specifically on the release of antibiotic resistance genes.

An alternative non antibiotic selection gene which been used successfully is the isopentenyl transferase gene (*ipt*) found on the Ti-plasmid of *Agrobacterium tumefaciens*. This isopentenyl transferase catalyzes the condensation of isopentenyl pyrophosphate, a precursor of several cytokinines [1,2]. Increased levels of these cytokinines have been shown to induce cell proliferation and shoot formation in several plants species [3–6]. The *ipt* gene derived from *Agrobacterium* has been used as a visible marker for identifying transgenic plants, which are bushy due to enhanced cytokinin levels. Unfortunately plants expressing *ipt* lose apical dominance and are unable to form roots and therefore removal of the *ipt* gene is required to obtain normal plants. For the removal of the *ipt* gene from transgenic cells two different approaches have been developed based on site specific recombination [7–13] or on transposition by the maize transposable element Ac [14]. Naturally the *ipt* gene is introduced into plant cells by *Agrobacterium* as part of the T-DNA. This bacterium has a type IV secretion system (T4SS), encoded by the *virB* genes and *virD4* gene on its Ti plasmid, through which translocation of the T-DNA occurs [15,16]. Several virulence protein are transported independently alongside the T-DNA into the host cell [17]. Recognition and translocation of proteins through the *Agrobacterium* VirB/D4 T4SS is dependent on a hydrophilic secretion signal with a net positive charge in the C-terminal part of the proteins [18]. Several heterologous proteins, fused to this secretion signal, have been translocated through the *Agrobacterium* VirB/D4 T4SS [17–20]. In this way the homing endonuclease I-SceI and the Cre recombinase were translocated into host cells to effect DNA recombination in the genome of target cells [17–19].

In this study we have developed two different methods for the transient expression of IPT that can be used for the selection of transformed plants. The first consists of delivery of a T-DNA encoding the *ipt* gene in the Pol-θ-deficient *Arabidopsis* mutant in which only transient expression of the T-DNA occurs but no integration [21]. The second involves the direct delivery of the IPT protein into *Arabidopsis* through the *Agrobacterium* VirB/D4 T4SS.

Results

Shoot formation via transient expression of the ipt gene in Pol-θ-deficient Arabidoposis

To test if *Arabidopsis* shoots can be recovered after the transient expression of the *ipt* gene we created a T-DNA vector containing the *ipt* gene from the *Agrobacterium* octopine Ti plasmid (pSDM3679). This T-DNA was introduced into an *Agrobacterium* strain already containing a T-DNA vector that provides resistance to the herbicide phosphinothricin (PPT), but also encoded a CRISPR/Cas system targeting the protoporphyrinogen oxidase (*PPO*) locus (pSDM3905). Both of these T-DNA vectors could stably replicate together in *Agrobacterium* as their replication units were compatible: incP for pSDM3679 and pVS1 for pSDM3905. The resulting strain LBA3718 was used to transform the roots of wild type *Arabidopsis* and the roots of the T-DNA integration resistant Pol-θ-deficient mutant (*teb-5*). This second T-DNA, pSDM3905, was added to test if the transient expression of a CRISPR/Cas system was effective in inducing targeted mutations (discussed in the next paragraph). Although no T-DNA integration occurs in *teb-5* roots, genes on a transferred T-DNA are still expressed transiently [21].

After cocultivation for 3 days *Arabidopsis* wild type roots were transferred to hormone free medium containing PPT to select for the presence of the T-DNAs from pSDM3905. After three weeks such roots developed dark green callus tissue. Shoot formation was observed after six weeks with ~6.5% (47/720) of the calli (Fig. 1d). As this was not seen after cocultivation with strains lacking the *ipt* gene, this probably reflects the temporary or ongoing transient expression of the *ipt* gene or continuing activity of the encoded IPT protein. Cocultivated roots placed on shoot induction medium developed dark callus tissue with shoots on 14.6% of the calli (Fig. 1f). Similar cocultivations were done with the *teb-5* mutant. On hormone free medium containing PPT, dark green calli were formed from which shoots appeared after 6 weeks. Shoot formation was observed with ~1.2% (10/864) of the calli, a ~fivefold reduction compared to wild type roots. With *teb-5* roots dark green callus tissue always developed shoot tissue whereas with wild type roots dark green callus tissue did not always develop shoot tissue (Fig. 1a; Fig. 1d). Five of the ten shoots obtained after transformation of *teb-5* roots that were transferred to hormone free medium exhibited normal growth and root formation suggesting that they were not stably transformed with the *ipt* gene. This suggests that transient expression of the *ipt* gene from a T-DNA in *teb-5* roots is effective in inducing shoot formation. Neither non transformed *teb-5* roots nor wild type roots developed dark green calli and shoots (Fig. 1b; Fig 1e).

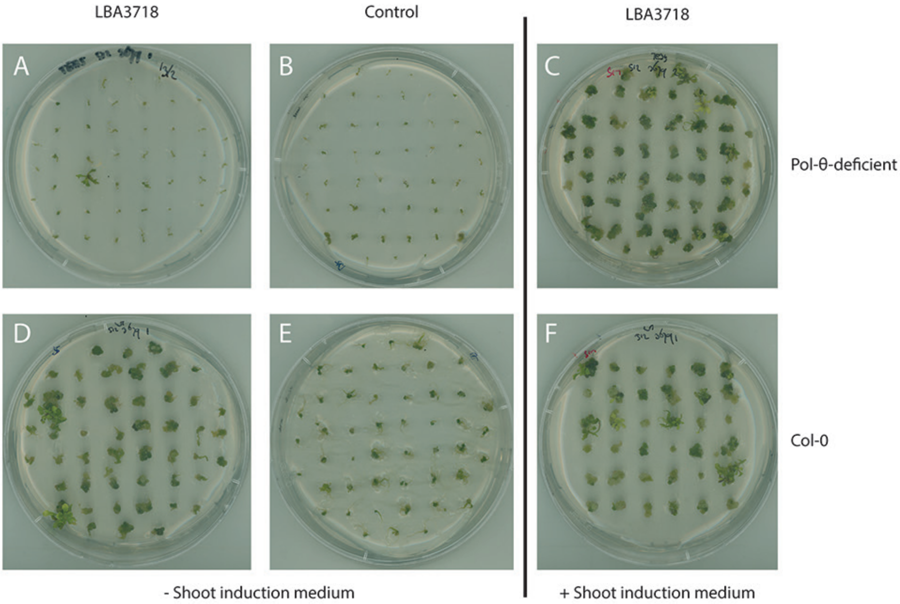


Figure 1. Shoot formation after cocultivation of wild-type (**D**, **F**) and *teb-5* roots (**A**,**C**) with *Agrobacterium* strain LBA3718 that transfers a T-DNA encoding the *ipt* gene (pSDM3679) and a T-DNA encoding a CRISPR/Cas system targeting the *PPO* locus (pSDM3905). (**A**,**D**) Roots cocultivated with LBA3718 placed on hormone free medium containing PPT. (**B**,**E**) Non cocultivated roots on hormone free medium containing PPT. (**C**,**F**) Roots cocultivated with LBA3718 placed on shoot induction medium containing PPT.

Targeted mutagenesis in shoots recovered after transient expression of the ipt gene and CRISPR/Cas

In the previous experiments we were able to recover several shoots on selection medium after the cocultivation of *teb-5* roots with LBA3718. To test if (transient) presence of the T-DNA expressing CRISPR/Cas9 had resulted in targeted mutations the recovered shoots were analyzed for the presence of footprints at the *PPO* locus. A 950 basepair (bp) fragment was amplified by PCR from the *PPO* locus with primers flanking the target sequence from genomic DNA isolated from a single leaf. This PCR product was digested with FauI, as the target site of the sgRNA overlaps with a FauI site, and restriction digestion resistant bands were cloned and analyzed by sequencing. Three shoots (3/10) that were analyzed showed FauI resistant PCR products (Fig. 2a). Sequencing of the PCR products showed that mutations were present consisting of small deletions several bp upstream of the PAM (Fig. 2c). Several of the targeted mutations we detected after the co-transfer of the T-DNA expressing *ipt* and the T-DNA expressing the CRISPR/Cas system using the restriction enzyme site loss method [22] did not contain a mutated FauI site and therefore are probably the result of incomplete digestion of the PCR product. The three shoots with targeted mutations (2, 6, and 10) showed a bushy phenotype consistent with constitutive expression of the *ipt* gene suggesting that the T-DNA encoding *ipt* might still be present (Fig. 2d). Therefore all shoots were analyzed for the presence of both T-DNA's using PCR. The pSDM3905 T-DNA was still detected in shoot number 2, 5, 6 and 10 and the T-DNA encoding the *ipt* gene (pSDM3679) was detected in shoot number 2, 6 and 10 (Fig. 2b).

These results indicated that non-integrated T-DNA can remain present for a long period of time or that T-DNA's can integrate by a process independent of Pol θ in a low percentage of the cells.

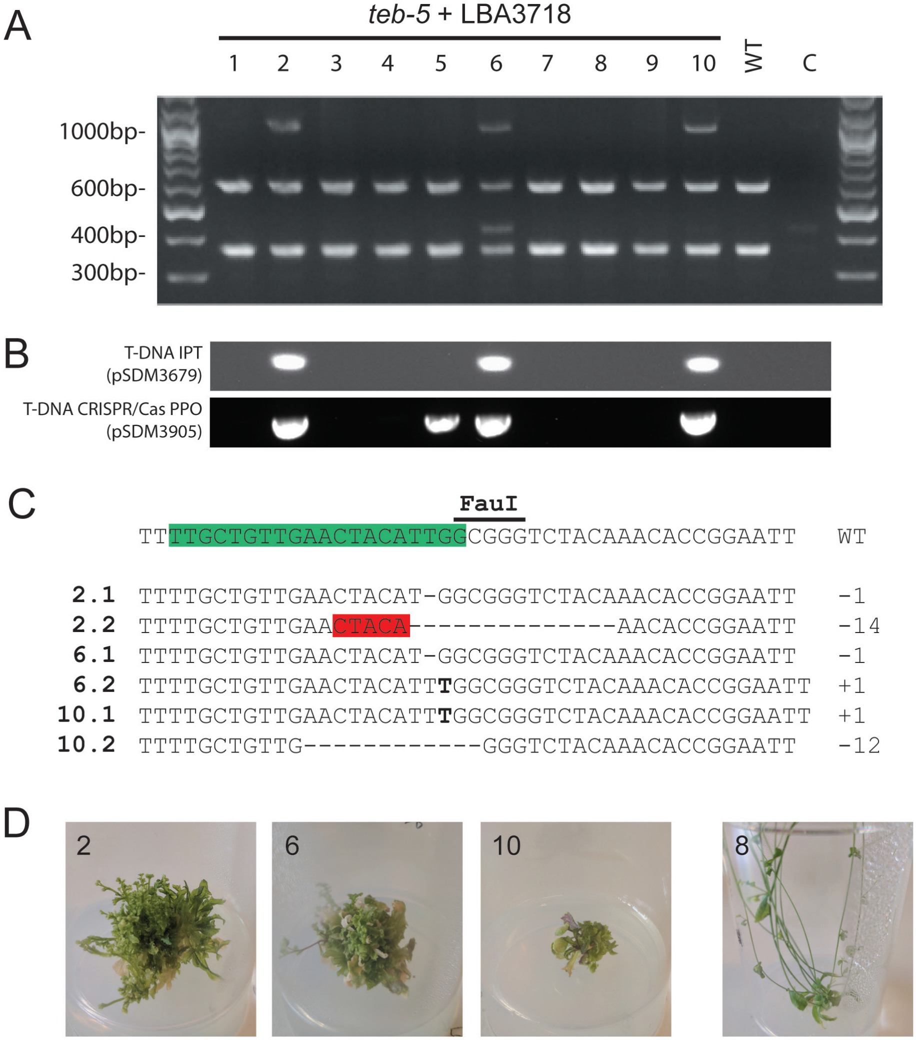


Figure 2. CRISPR/Cas induced mutagenesis in *teb-5* roots. (**A**) The *PPO* target site was amplified using genomic DNA of 10 *teb-5* shoots transformed by LBA3718 and a wild type leaf and the resulting PCR products were digested with FauI and separated on agarose gel. A control PCR sample without template (**C**) was included. (**B**) Detection of the presence of the T-DNA from pSDM3679 and pSDM3905 via PCR. (**C**) Sequence analysis of mutations in shoot number 2, 6 and 10. The sgRNA is in green, the restriction site is underlined, deletions are shown by dashes, insertions in bold and microhomology in red. Numbers on the right are length of deletions (-) and insertions (+). (**D**) Shoot number 2, 6, and 10 showing a bushy phenotype. Shoot number 8 is an example of a shoot showing a normal phenotype.

Shoot induction after translocation of the IPT protein

The previous experiments suggested that transient expression of *ipt* from a non-integrated T-DNA is effective in inducing shoot formation in the roots of *teb-5*. To eliminate the requirement for this mutant line we tested if the IPT protein can be translocated through the *Agrobacterium* VirB/D4 type IV secretion system and is capable of inducing shoot formation after translocation.

For the translocation of the IPT protein an expression plasmid was created encoding the isopentenyl transferase fused to the C-terminal 37 amino acid translocation signal of the *Agrobacterium* virulence protein VirF. This translocation signal has previously been used for the translocation of several heterologous proteins [17–19]. The production of the fusion protein (IPTF) was under the control of the acetosyringone inducible *virF* promoter to ensure that production would occur concomitantly with formation of a functional VirB/D4 type IV channel.

To assay for the translocation of the IPTF protein roots had been co-cultivated with *Agrobacterium* expressing the IPTF protein (LBA3720) after which the root segments were placed on hormone free medium. After six weeks shoot formation was observed on the plates with root fragments that had been co-cultivated with *Agrobacterium* strain expressing the IPTF protein (Fig. 3a; Fig. 3b). On plates with root fragments that were co-cultivated with an *Agrobacterium* strain not expressing IPTF no shoot induction was observed (Fig. 3c).

Because translocated IPTF successfully initiated shoot formation we tested if shoot formation could be used to visually identify T-DNA transformants if the translocation of a T-DNA is combined with the transfer of IPTF. A binary vector (pBIN19) was introduced into the *Agrobacterium* expressing the IPTF protein. The resulting strain (LBA3721) was used to transform the roots of wild type *Arabidopsis*. After co-cultivation for three days roots were transferred to hormone free medium. After five weeks shoot formation was observed on several root fragments. These shoots were analyzed for the presence of the *nptII* gene present on the T-DNA by PCR. Shoot number 1, 3 and 5 contained the T-DNA showing that T-DNA transformants can be selected for using shoot formation induced by transferred IPTF as a visual selection marker (Fig. 3d).

These results show that the IPTF protein is effectively translocated to *Arabidopsis* roots at sufficient levels to induce shoot formation in *Arabidopsis* root fragments and this induction of shoot formation can be used to identify T-DNA transformants.

Combined transfer of the IPT protein and a T-DNA encoding the CRISPR/Cas system

As the previous experiments showed that translocated IPTF is effective in inducing shoot formation we added the binary vector encoding the CRISPR/Cas system targeting the *PPO* locus (pSDM3905) to the *Agrobacterium* strain expressing the IPTF protein resulting in *Agrobacterium* strain LBA3719. Roots were co-cultivated with LBA3719 for three days after which roots were placed on hormone free medium containing PPT to select for the T-DNA encoding the CRISPR/Cas system targeting the *PPO* locus. Shoot formation was observed on one root fragment (1/1392). DNA was isolated from three individual leaves of this shoot. To easily detect sgRNA-guided mutations induced by Cas9 we used the same restriction enzyme loss method described above. The target locus was amplified via PCR with primers flanking the target sequence using three different parts of the shoot and the resulting PCR products were digested with FauI. Resistant bands were observed in all three samples (Fig. 4a).

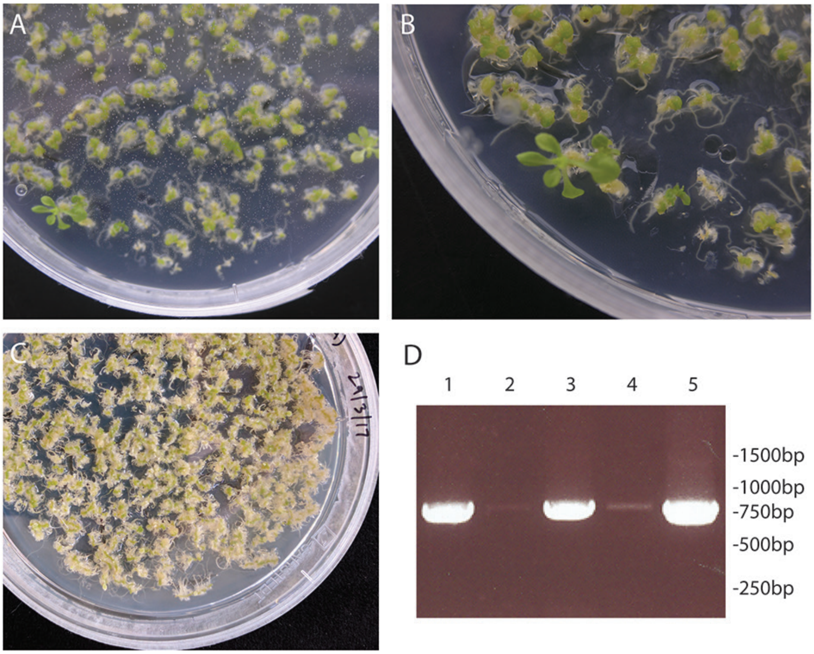
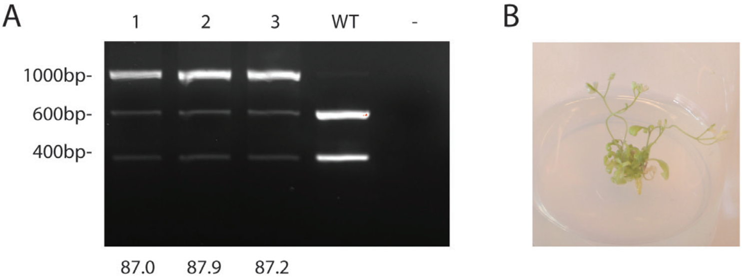


Figure 3. Shoot regeneration after translocation of the IPTF protein. (**A**) Roots after co-cultivation with *Agrobacterium* expressing IPTF. (**B**) Close up of shoot after co-cultivation of roots with *Agrobacterium* expressing IPTF (**C**). Non co-cultivated roots on hormone free medium (**D**). PCR on the *nptII* locus in shoots that were recovered after co-cultivation with *Agrobacterium* containing pBIN19 and expressing IPTF.



C

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                                FauI
      WT TTTTGCTGTTGAACTACATTGGCGGGTCTACAAACACCGGAATT

         TTTTGCTGTTGAACTACATTGGCGGGTCTACAAACACCGGAATT    2x
-19/+21  TTTTGCTGTTGAATTCTGTCCAAGGTAAATGTCCAACACCGGAATT  6x
     -14 TTTTGCTGTTGAACTACGC--------------ACACCGGAATT    2x
     -36 TTTGCTGTTGAAC...........-36...........GTAAA     2x
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Figure 4. CRISPR/Cas induced mutagenesis in a wild type shoot after co-cultivation with LBA3719. (**A**) The *PPO* target site was amplified using genomic DNA from three individual leaves from a shoot recovered after co-transfer of IPTF and the T-DNA from pSDM3905 (1-3) and a wild type leaf (WT). A control sample without template (-) was included. The resulting PCR products were digested with FauI and separated on an agarose gel. (**B**) Shoot regenerated after co-transfer of IPTF and the T-DNA from pSDM3905. (**C**) Sequence analysis of mutations detected. The sgRNA is in green, restriction site is underlined, deletions are shown by dashes, insertions in red and templated insertion is in bold. Numbers on the left are length of deletions (-) and insertions (+). Numbers of multiple clones with the same sequence are shown on the right.

Using the relative band intensities the number of mutations was estimated at around ~87% in each of the three samples (Fig. 4a). The resistant PCR products were cloned into a high-copy vector, transformed to *E. coli* and individual clones were sequenced. Analysis of these sequences showed that the plant contains three different kinds of mutations; a 16 bp deletion, a 36 bp deletion and a templated insertion (Fig. 4c). Because of the high mutation frequency in the essential *PPO* gene stunted growth was observed in the recovered plant (Fig. 4a).

These results combined show that the translocation of the IPTF protein combined with the transfer of a T-DNA encoding a CRISPR/Cas system resulted in a shoot with targeted mutations.

Discussion

In this study we have shown that the *ipt* gene can be transiently expressed from a T-DNA in the Pol- θ-deficient *teb-5* mutant to induce shoot formation. Furthermore we showed that the IPT protein can be translocated through the *Agrobacterium* VirB/D4 T4SS and is effective in inducing shoot formation. If the translocation of the IPTF protein is combined with the transfer of a T-DNA encoding a CRISPR/Cas system it is possible to recover plants with mutations. We also showed that T-DNA transformants could be identified using shoot formation induced by translocated IPTF as a visual identification method.

The T-DNA encoding the *ipt* gene and the T-DNA encoding the CRISPR/Cas system which were used to transform *teb-5* roots could still be detected via PCR in 40% (4/10) and 30% (3/10) of the obtained shoots, respectively. This suggests that non-integrated T-DNA persists in the plant cells for a prolonged period up to five weeks. We can however not exclude that the T-DNA was still detected due to incomplete removal of all *Agrobacterium* or that T-DNA integration still occurs in *teb-5* plants via an alternative integration pathway.

When selection of shoot formation by IPTF protein transfer was done after cocultivation with an *Agrobacterium* strain containing a T-DNA encoding a CRISPR/Cas system, a shoot was obtained with a high frequency of targeted mutations that were evenly distributed throughout the plant. This suggests that these mutations occurred early in the development of the shoot. If the frequency of shoot formation after co-transfer can be improved, it will allow for the easy recovery of plants with a high frequency of targeted mutations.

In summary we developed two systems for which we employ shoot regeneration by IPT activity to identify transformants, which grow with a normal phenotype, because they do not contain an integrated *ipt* gene as in previous methods.

Material & Methods

Agrobacterium strains and media

Agrobacterium strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. All *Agrobacterium* strain were grown in LB (5 g/l NaCl) with the appropriate antibiotics: gentamicin (40 µg/ml); carbenicillin (75 µg/ml); kanamycin (100 µg/ml); spectinomycin (250 µg/ml). Plasmids were electroporated into AGL1 as described in den Dulk-Ras and Hooykaas (1995).

Plasmid construction

To create the expression plasmid for expression of the IPT protein fused to the 37 last amino acids of the *Agrobacterium* virulence protein VirF (IPTF) in *Agrobacterium*, the *ipt* gene was amplified by PCR from LBA1 with primers IPT1/IPT2 and was inserted into the EcoRV and SalI sites of pSDM3190. From this modified pSDM3190 vector a 1900bp HindIII/XbaI fragment was cut and inserted into the HindIII and XbaI sites of pBBR6, creating pSDM3678. For construction of the binary vector for the expression of *ipt* in plant cells, the *ipt* gene was amplified by PCR from LBA1 with primers IPT3 and IPT4 and inserted into the XbaI and XhoI sites of pART7-YFP-HAII. The NotI fragment (p35S::ipt::t35S) from this vector was cut and inserted into the NotI site of pBluescript creating pBSK-p35S-IPT-t35S. From pBSK-p35S-IPT-t35S a HindIII and SacI fragment (p35S::ipt::t35S) was cut and inserted into the HindIII and SacI sites of pBIN19 creating pSDM3679.

Protein translocation and plant transformation experiments

Root transformations were performed as described previously [17,24,25], using *Agrobacterium* strain AGL1. Briefly, seedlings from wild type *Arabidopsis* (ecotype Col-0) and the *teb-5* mutant (Pol-θ-deficient line, ecotype Col-0, [26]) were grown for 10 days. Roots were removed from seedlings and precultured on callus induction medium [25], followed by a three day co-cultivation period with *Agrobacterium*. After co-cultivation roots were transferred to B5 medium [27] containing vancomycin (100 µg/ml) and timentin (100 µg/ml) to kill remaining *Agrobacterium*. The selection for the T-DNA of pSDM3905 was done by adding PPT (30 µg/ml) to the medium. After three weeks calli were transferred to medium without PPT.

Detection of the T-DNAs

The presence of pSDM3905 and pSDM3679 T-DNAs was performed with PCR using primers pair SP558/SP559 (detecting Cas9) and DS585/DS589 (detecting *ipt*), respectively. The presence of the pBIN19 T-DNA was detected using primers nosNPTII1/nosNPTII2 (detecting *nptII*).

Detection of mutations

DNA was isolated from a single leaf using CTAB DNA extraction [28]. The target sequence was amplified using primers SP392 and SP538. Amplified products were digested with FauI (New England Biolabs) after which resistant bands were cloned into pJET1.2 (Thermo Scientific Inc.). Individual clones were sent for Sanger sequencing (Macrogen Inc.).

Table 1. Overview of plasmids used in this study

Plasmid	Marker	Origin of replication	Properties	Source
pART7-YFP-HAII	Sp	ColE1, incP	Modified pART7 backbone	Galvan Ampudia (unpublished)
pBBR6	Gm	pBBR	Derivative of the broad host-range plasmid pRL662	[17]
pBIN19	Km	incP	Plant binary vector	[29]
pBSK-p35S-IPT-t35S	Cb	ColE1	pBluescript containing 35S promoter, ipt and 35S terminator	This study
pSDM3190	Cb	ColE1	pUC21 pvirFpromoter:NLS::Cre::VirFΔ42N	den Dulk (unpublished)
pSDM3678	Gm	pBBR	IPTF expression vector0	This study
pSDM3679	Km	incP	Binary vector for the expression of the ipt gene	This study
pSDM3905	Sp	ColE1, pVS1	T-DNA vector with CRISPR/Cas system targeting the PPO locus	[30]

Sp = spectinomycin, Gm = gentamicin, Cb = carbenicillin, Km = kanamycin

Table 2. Overview of strains used in this study

Strain	Genomic background	Plasmids	Source
AGL1	AGL1	-	[31]
LBA3718	AGL1	pSDM3679 + pSDM3905	This study
LBA3719	AGL1	pSDM3578 + pSDM3905	This study
LBA3720	AGL1	pSDM3679 + pBIN19	This study
LBA3721	AGL1	pSDM3679	This study

Table 3. Overview of primers used in this study

Primer name	Sequence
IPT1	agcgatatcATGGACCTGCATCTAATTTTCG
IPT2	acggtcgactATACATTCCGAACGGATGAC
IPT3	ccgctcgagCAGTTTGTATTCAATATACTGC
IPT4	gctctagaATACATTCCGAACGGATGAC
SP392	CACTTTGACAGATTAGGTAG
SP538	CTAAGGCTACACCAGCGACG
DS558	GGAACTAACTCTGTGGGATG
DS559	CACACCTGAAGCGTTGATAG
NOSNPTII1	AAGCCTGAACTCACCGCGAC
NOSNPTII2	CCGGCACCAACCGAACATAC

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