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Is VIP1 important for *Agrobacterium*-mediated transformation?

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SUMMARY

Agrobacterium **genetically transforms plants by transferring and integrating T-(transferred) DNA into the host genome. This process requires both** ***Agrobacterium*** **and host proteins. VirE2 interacting protein 1 (VIP1), an Arabidopsis bZIP protein, has been suggested to mediate transformation through interaction with and targeting of VirE2 to nuclei. We examined the susceptibility of Arabidopsis** ***vip1*** **mutant and** ***VIP1*** **overexpressing plants to transformation by numerous** ***Agrobacterium*** **strains. In no instance could we detect altered transformation susceptibility. We also used confocal microscopy to examine the subcellular localization of Venus-tagged VirE2 or Venus-tagged VIP1, in the presence or absence of the other untagged protein, in different plant cell systems. We found that VIP1–Venus localized in both the cytoplasm and the nucleus of Arabidopsis roots, agroinfiltrated** ***Nicotiana benthamiana*** **leaves, Arabidopsis mesophyll protoplasts and tobacco BY-2 protoplasts, regardless of whether VirE2 was co-expressed. VirE2 localized exclusively to the cytoplasm of tobacco and Arabidopsis protoplasts, whether in the absence or presence of** ***VIP1*** **overexpression. In transgenic Arabidopsis plants and agroinfiltrated** ***N. benthamina*** **leaves we could occasionally detect small aggregates of the Venus signal in nuclei, but these were likely to be imagining artifacts. The vast majority of VirE2 remained in the cytoplasm. We conclude that VIP1 is not important for** ***Agrobacterium*****-mediated transformation or VirE2 subcellular localization.**

Keywords: ***Agrobacterium*****, plant transformation, VirE2 interacting protein 1, VirE2,** ***Arabidopsis thaliana*****,** ***Nicotiana benthamiana*****, protein subcellular localization.**

INTRODUCTION

Agrobacterium tumefaciens, a soil-borne pathogen, induces neoplastic growths on plants by transporting transferred DNA (T-DNA), a region of its Ti (tumor-inducing) plasmid, to the host cell and integrating it into the host genome. During the process of transformation, Ti-plasmid-encoded virulence (*vir*) genes are induced by phenolic molecules secreted from wounded plants. Some Vir proteins function in the bacterium, whereas others [virulence effector proteins VirD2, VirD5, VirE2, VirE3, VirF and GALLS (from *Agrobacterium rhizogenes*)], are secreted by the bacterium into the plant via a type IV secretion system (T4SS) made up of 11 VirB proteins and VirD4 (for reviews, see Gelvin, 2003, 2009, 2010a,b; McCullen and Binns, 2006; Pitzschke and Hirt, 2010; Lacroix and Citovsky, 2013b).

The VirD2 endonuclease, in conjunction with VirD1, nicks the Ti-plasmid at T-DNA border sequences and covalently attaches to the 5′ end of the resulting single-strand molecules (T-strands). VirD2 pilots T-strands into host cells through the T4SS. VirD2 contains a nuclear localization signal (NLS) sequence that interacts with importin α. Through this interaction, VirD2 directs T-strands to the nucleus (Herrera-Estrella *et al.*, 1990; Koukolikova-Nicola and Hohn, 1993; Koukolikova-Nicola *et al.*, 1993; Ballas and Citovsky, 1997; Mysore *et al.*, 1998; Bhattacharjee *et al.*, 2008).

A model that is currently popular posits that within the plant cell, VirE2, a single-stranded DNA-binding protein, binds T-strands, protects them from nucleolytic degradation and helps guide them to and/or through nuclear pores (Howard and Citovsky, 1990; Howard *et al.*, 1992; Ziemienowicz *et al.*, 2001; Gelvin, 2010a). T-strands, covalently linked to VirD2 at the 5′ end and coated with VirE2, have been termed T-complexes (Howard *et al.*, 1992), and T-complexes in association with host proteins have been termed super-T-complexes (Gelvin, 2010a,b). The model

suggests that these two complexes assemble in the plant cytoplasm and are subsequently disassembled in the nucleus prior to integration of T-DNA into the host genome (Tzfira *et al.*, 2004; Magori and Citovsky, 2012).

As with VirD2, VirE2 contains NLS sequences that presumably direct it to the nucleus. Some studies concluded that VirE2, tagged on its N-terminus with β-glucuronidase (GUS) or with an autofluorescent protein, localizes to nuclei of plant cells (Citovsky *et al.*, 1992, 1994; Tzfira and Citovsky, 2001) but not animal cell nuclei (Guralnick *et al.*, 1996; Tzfira and Citovsky, 2001). However, other studies indicated that VirE2 remains cytoplasmic, often in perinuclear rings (Bhattacharjee *et al.*, 2008; Lee *et al.*, 2008; Gelvin, 2010a) or as predominantly cytoplasmic strands (Sakalis *et al.*, 2013; Li *et al.*, 2014). Ballas and Citovsky (1997) demonstrated that, in yeast, VirE2 does not interact with the Arabidopsis importin α protein AtKAPα (now denoted as IMPa-1). They thus speculated that another protein could serve as a bridge between VirE2 and importin α to help direct VirE2 to the nucleus. Indeed, Tzfira *et al.* (2001) identified VirE2 interacting protein 1 (VIP1) as this possible bridge molecule. Citovsky *et al.* (2004) confirmed that VIP1 could form a ternary complex with VirE2 and importin α. Djamei *et al.* (2007) demonstrated that phosphorylation of VIP1 on serine-79 by mitogen-activated protein kinase 3 (MPK3) resulted in nuclear localization of VIP1, whereas alteration of this amino acid to alanine, mimicking the non-phosphorylated form of VIP1, resulted in cytoplasmic localization. These authors thus suggested that *Agrobacterium* infection of plants stimulated MPK3-mediated phosphorylation of VIP1, thus directing it and the associated T-complex to the nucleus (the 'Trojan horse' model).

The VIP1 protein is a transcription factor that responds to biotic and abiotic stress (Pitzschke *et al.*, 2009; Wu *et al.*, 2010; Tsugama *et al.*, 2012, 2013b). By binding the conserved VRE hexamer sequence ACNGCT (Pitzschke *et al.*, 2009; Lacroix and Citovsky, 2013a,b), VIP1 activates transcription of many defense-related genes (Pitzschke *et al.*, 2009). In addition VIP1 induces expression of *SYP707A1/3* (encoding enzymes inactivating the function of ABA and its biosynthesis) and thus regulates osmosensory signaling in Arabidopsis (Tsugama *et al.*, 2012, 2013a). Further, VIP1 can interact with the Arabidopsis GTP-binding protein Gβ (Tsugama *et al.*, 2013b) and has been implicated in growth of Arabidopsis on low-sulfur medium (Wu *et al.*, 2010).

Tzfira *et al.* (2001) examined the importance of VIP1 for *Agrobacterium*-mediated transformation. They showed that tobacco plants expressing an Arabidopsis *VIP1* antisense construction were resistant to both transient and stable transformation. Conversely, tobacco plants overexpressing Arabidopsis *VIP1* showed increased susceptibility to transformation (Tzfira *et al.*, 2002). The Arabidopsis T-DNA insertion mutant *vip1-1* still encodes the N-terminal portion (approximately 72%) of VIP1, and was reported to show wild-type levels of transient but decreased levels of stable transformation (Li *et al.*, 2005). Taken together, these results indicate that VIP1 is important for *Agrobacterium*-mediated transformation.

The importance of VirE2 in *Agrobacterium*-mediated transformation, and the role of VIP1 in binding VirE2, led us to investigate whether subcellular localization of VIP1 correlates with localization of VirE2 and transformation susceptibility. We used a quantitative Arabidopsis root assay to examine the transformation efficiency of wild-type and *vip1-1* mutant plants in the absence and presence of *VIP1* overexpression. Our assays used various *Agrobacterium* strains under a wide range of inoculum concentrations. Surprisingly, we found that manipulation of *VIP1* gene expression did not alter the susceptibility of Arabidopsis roots to *Agrobacterium*-mediated transformation. We also found that overexpression of several forms of *VIP1* did not alter the subcellular localization of VirE2. Similarly, overexpression of VirE2 did not alter the subcellular localization of VIP1. We conclude that VIP1 is not important for *Agrobacterium*-mediated transformation of Arabidopsis.

RESULTS

Mutation of *VIP1* does not affect *Agrobacterium*-mediated transient or stable transformation of Arabidopsis roots

The Arabidopsis *vip1-1* mutant (SALK_001014; Alonso *et al.*, 2003) contains a T-DNA insertion at the start of the third exon of the *VIP1* gene and allows expression of the first 244 amino acids of VIP1 (Li *et al.*, 2005; because of a misannotation of the correct VIP1 translation start site, Li *et al.* indicate that only the first 164 amino acids are made in this mutant). We generated a homozygous *vip1-1* mutant (Figure S1A in Supporting Information) from a heterozygous line; this mutant did not show any growth or developmental phenotypes under our standard growth conditions (Figure S1B). We used this homozygous line in all further experiments.

We tested root segments of wild-type Col-0 and *vip1-1* mutant plants for susceptibility to transient transformation (Nam *et al.*, 1997, 1999; Zhu *et al.*, 2003) using the non-tumorigenic nopaline-type strain *A. tumefaciens* At849. Within *A. tumefaciens* GV3101::pMP90 this strain harbors the T-DNA binary vector pBISN1, which contains a *uidA*-intron gene within its T-DNA (Narasimhulu *et al.*, 1996). We infected root segments for 2 days with bacterial inocula ranging from 10^5 to 5×10^8 colony forming units (cfu) ml^{-1}, after which we placed the segments on medium containing Timentin to kill the bacteria. Four days later, we stained the segments with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) and scored the percentage of root segments that stained blue. This assay requires transfer of T-DNA to the nucleus, conversion to a double-strand transcription-competent form and expression of GUS

activity, but does not require integration of T-DNA into the plant genome. Figure 1(a) shows that root segments of the *vip1-1* mutant had similar susceptibility to transient transformation as did root segments of wild-type Col-0 plants. This result agrees with that of Li *et al.* (2005), who inoculated root segments with *Agrobacterium* at a concentration of about 2×10^8 cfu ml^{-1}.

Stable transformation assays assess late steps of *Agrobacterium*-mediated transformation, including integration of T-DNA and stable transgene expression (Zhu *et al.*, 2003). When Li *et al.* (2005) used the nopaline-type strain *A. tumefaciens* A208 at about 2×10^8 cfu ml^{-1} to infect root segments of the *vip1-1* mutant, they observed substantially reduced stable transformation (around 35%) compared with root segments of wild-type plants. We tested the susceptibility of *vip1-1* and wild-type root segments to this same *Agrobacterium* strain following inoculation with bacteria at concentrations ranging from 10^5 to 5×10^8 cfu ml^{-1}. Surprisingly, we could not detect any differences in transformation susceptibility between these two lines (Figure 1b). We therefore tested the susceptibility of wild-type and *vip1-1* mutant root segments to stable transformation by other *Agrobacterium* strains. We could not detect any major differences (>1.5-fold) using *A. tumefaciens* A348 (an octopine-type strain; Figure S2A), *A. rhizogenes* R1000 (Figure S2B), *A. tumefaciens* At1811 (an *A. tumefaciens virE2*/*E3* mutant harboring a plasmid expressing a *GALLS* gene; Figure S2C) or *A. tumefaciens* At1982 (an *A. tumefaciens virE2*/*E3* mutant harboring a plasmid expressing *VirE1* and *VirE2* genes; Figure S2D).

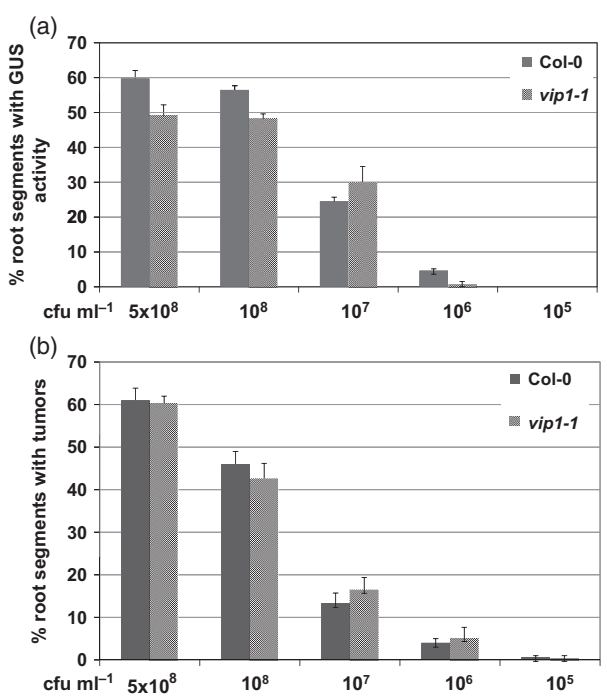

Figure 1. Transformation of Col-0 and *vip1-1* root segments.
Root segments were infected at various concentrations by (a) *Agrobacterium tumefaciens* GV3101(pBISN1) and stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) 6 days after infection to measure transient transformation, or (b) *A. tumefaciens* A208 and tumors were scored 1 month later for stable transformation. The figure shows the average values of three independent replicates with the indicated standard error. cfu, colony-forming units.

Root segments of the *vip1-1* mutant and wild-type Col-0 plants had equal ability to form calli on non-selective callus inducing medium (CIM) after inoculation with high concentrations (up to 10^9 cfu ml^{-1}) of *A. tumefaciens* A208 or the avirulent strain *A. tumefaciens* A136, which lacks a Ti-plasmid (Figure S3A,B). Thus, inoculation of *vip1-1* root segments with high *Agrobacterium* concentrations, as Li *et al.* (2005) did, does not interfere with essential cellular functions related to cell division.

Taken together, our results indicate that a full-length VIP1 protein is not essential for *Agrobacterium*-mediated transformation.

Overexpression of various forms of VIP1 does not increase *Agrobacterium*-mediated stable transformation of Arabidopsis roots

Although *VIP1* is not required for transient or stable transformation, it may still affect susceptibility to transformation. Previously, Tzfira *et al.* (2002) expressed an Arabidopsis *VIP1* cDNA in transgenic tobacco (*Nicotiana tabacum*) and showed that two derived transgenic plants had increased susceptibility to *Agrobacterium*-mediated transient and stable transformation. However, their cDNA did not encode amino acids 1–80, including the important serine-79 that is a phosphorylation site for MPK3 (Djamei *et al.*, 2007). We therefore overexpressed full-length cDNAs encoding the wild-type Arabidopsis VIP1 full open reading frame (ORF), or the VIP1^{S79A} or VIP1^{S79D} variants, in transgenic Arabidopsis and tested the roots of 59 resulting T_2 generation lines for susceptibility to stable transformation by *A. tumefaciens* A208. These overexpressing lines were in either the wild-type Col-0 or the *vip1-1* backgrounds. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of root RNA from selected transgenic plants confirmed various extents of overexpression of the corresponding *VIP1* mRNA in most tested lines (Figure S4).

We first assayed the transformation susceptibility of Col-0 plants overexpressing VIP1 to infection by *A. tumefaciens* A208 inoculated at 10^7 cfu ml^{-1}. Figure 2(a) shows that of the 10 independent lines assayed, only one line (#4) showed increased transformation susceptibility, and this was only about 1.5 times that of wild-type plants. We obtained similar results with the 10 *VIP1*^{S79A} and nine *VIP1*^{S79D} overexpressing lines that we tested (Figure 2b,c). There was no correlation between transformation efficiency and the extent of *VIP1* overexpression (Figure S4A).

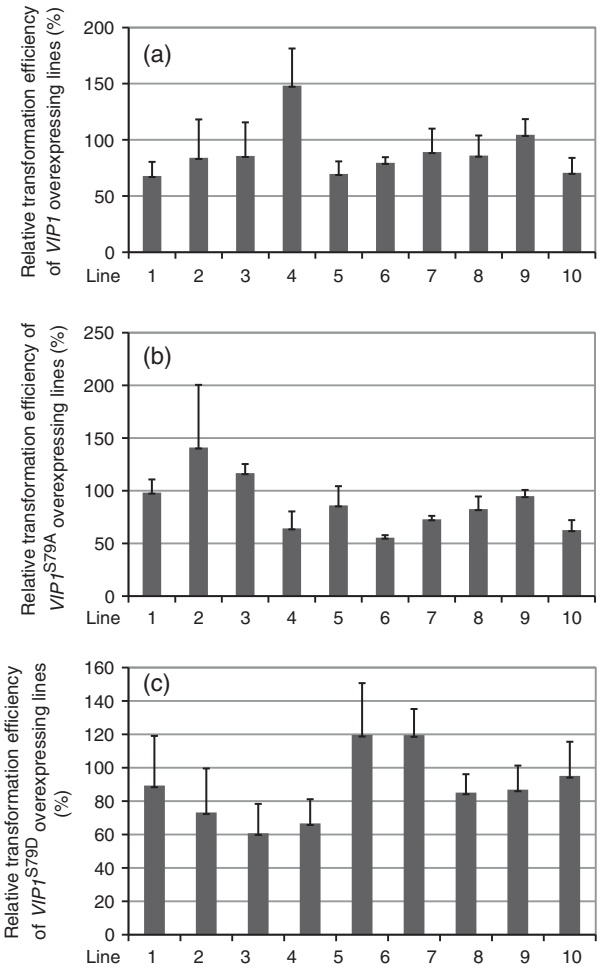

Figure 2. Stable transformation of root segments of *VIP1* overexpressing lines in the Col-0 background.
Root segments were infected by *Agrobacterium tumefaciens* A208 at 10^7 colony-forming units ml^{-1} and tumors were scored after 25–30 days. The figure shows the average values of three independent replicates with the indicated standard error. Relative transformation values are normalized to those of wild-type plants: (a) *VIP1*; (b) *VIP1*^{S79A}; (c) *VIP1*^{S79D}.

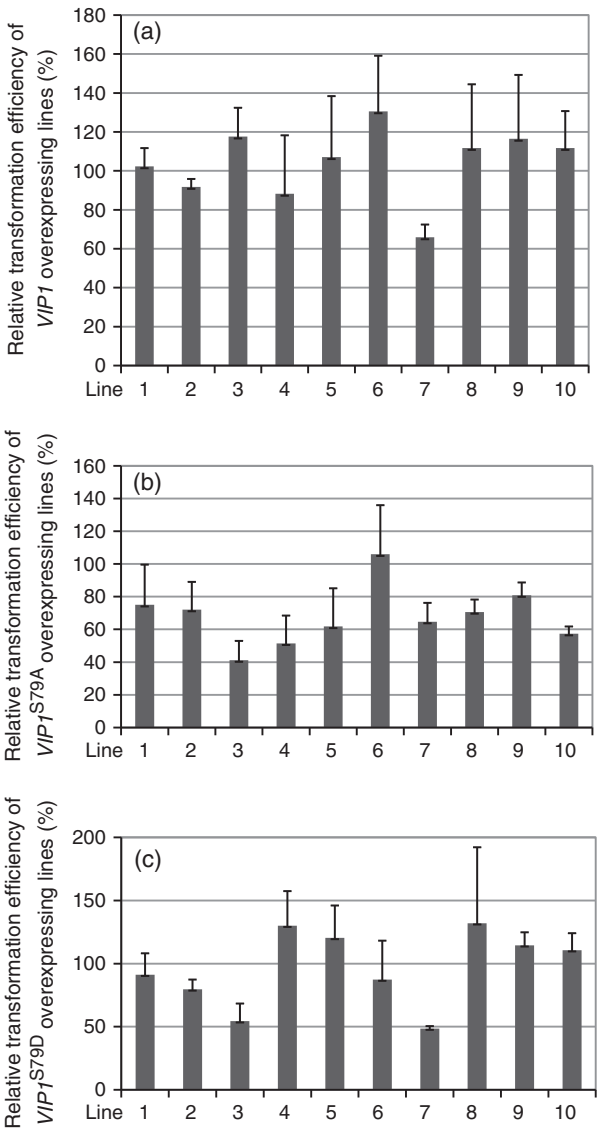

Figure 3. Stable transformation of root segments of *VIP1* overexpressing lines in the *vip1-1* background.
Root segments were infected by *Agrobacterium tumefaciens* A208 at 10^7 colony-forming units ml^{-1} and tumors were scored after 25–30 days. The figure shows the average values of three independent replicates with the indicated standard error. Relative transformation values are normalized to those of wild-type plants: (a) *VIP1*; (b) *VIP1*^{S79A}; (c) *VIP1*^{S79D}.

Because VIP1 is known to dimerize (Li *et al.*, 2005), we were concerned that dimerization of wild-type VIP1 protein with VIP1 variants may mask the effect of overexpressing these VIP1 variants. We therefore generated *VIP1* overexpressing lines in the *vip1-1* mutant background. Although this mutant produces the N-terminal portion of VIP1 protein, this protein cannot self-dimerize or form complexes with full-length VIP1 protein (Li *et al.*, 2005). For each overexpression construction (wild-type *VIP1*, *VIP1*^{S79A} and *VIP1*^{S79D}), we assayed the T_2 generation of 10 independent Arabidopsis lines. Figure 3(a–c) show that overexpression of these VIP1 variants did not increase transformation susceptibility (overexpression of *VIP1*^{S79A} may have slightly decreased transformation susceptibility). As with overexpression of *VIP1* in the wild-type Col-0 background, there was no correlation between transformation efficiency and the extent of *VIP1* overexpression in the *vip1-1* background (Figure S4B).

Our transformation data indicate that *VIP1* is not necessary for, and does not influence, the susceptibility of Arabidopsis roots to *Agrobacterium*-mediated transformation.

VirE2 localizes predominantly to the cytoplasm of plant cells

According to the T-complex model, VirE2 coats T-strands and helps localize them to plant nuclei (Howard and

Citovsky, 1990). Some studies have indicated that the VirE2 NLS sequences are responsible for VirE2 nuclear localization (Citovsky *et al.*, 1992). In these studies, VirE2 was tagged with a reporter protein at its N-terminus (Citovsky *et al.*, 1992, 1994; Tzfira and Citovsky, 2001). We have shown, however, that tagging VirE2 at its N-terminus renders it non-functional in assays in which plant-expressed VirE2 is tested for complementation of a *virE2* mutant *Agrobacterium* strain. Tagging VirE2 at its C-terminus maintains full VirE2 function in these assays (Bhattacharjee *et al.*, 2008). We have previously shown that when either N- or C-terminally tagged VirE2 is expressed in plants, it remains cytoplasmic, often in perinuclear rings (Bhattacharjee *et al.*, 2008; Lee *et al.*, 2008; Gelvin, 2010a). Only when importin α-4 (IMPa4) is overexpressed, or occasionally in leaf trichomes, could we find tagged VirE2 within the nucleus (Lee *et al.*, 2008; Gelvin, 2010a). Sakalis *et al.* (2013) recently showed that N-terminally tagged VirE2, when expressed in either yeast cells or in Arabidopsis protoplasts, remains cytoplasmic. In addition, both Sakalis *et al.* (2013) and Li *et al.* (2014) showed, using a split GFP approach, that tagged VirE2 transferred from *Agrobacterium* to yeast or plant cells remained predominantly cytoplasmic or perinuclear. Because of the discrepancies in the literature, we reevaluated the localization of VirE2 in several plant systems, in the absence or presence of *VIP1* overexpression.

VirE2 oligomerizes (Sen *et al.*, 1989; Abu-Arish *et al.*, 2004; Citovsky *et al.*, 2004; Frenkiel-Krispin *et al.*, 2007) and forms cytoplasmic aggregates *in planta* (Bhattacharjee *et al.*, 2008; Grange *et al.*, 2008; Sakalis *et al.*, 2013; Li *et al.*, 2014). In order to minimize self-aggregation, we expressed VirE2 from the relatively weak nopaline synthase promoter (P_{nos}) and affixed to its C-terminus the bright autofluorescent protein Venus, a derivative of yellow fluorescent protein (YFP; Nagai *et al.*, 2002). When co-expressed, transcription of untagged *VIP* cDNAs was directed by the strong cauliflower mosaic virus (CaMV) 35S promoter.

Figure 4(a–c) show that VirE2 localizes almost exclusively to the cytoplasm of transgenic Arabidopsis roots (the target tissue for our transformation assays), Arabidopsis leaf mesophyll protoplasts and tobacco BY-2 protoplasts, respectively. Occasionally, small aggregates of yellow fluorescence could be seen in nuclei of transgenic Arabidopsis roots (white arrow in Figure 4a). However, it is unlikely that this signal represents VirE2–Venus, because nuclei of transgenic Arabidopsis expressing only a mRFP–NLS protein, and not VirE2–Venus, also occasionally showed small aggregates of yellow fluorescence (Figure

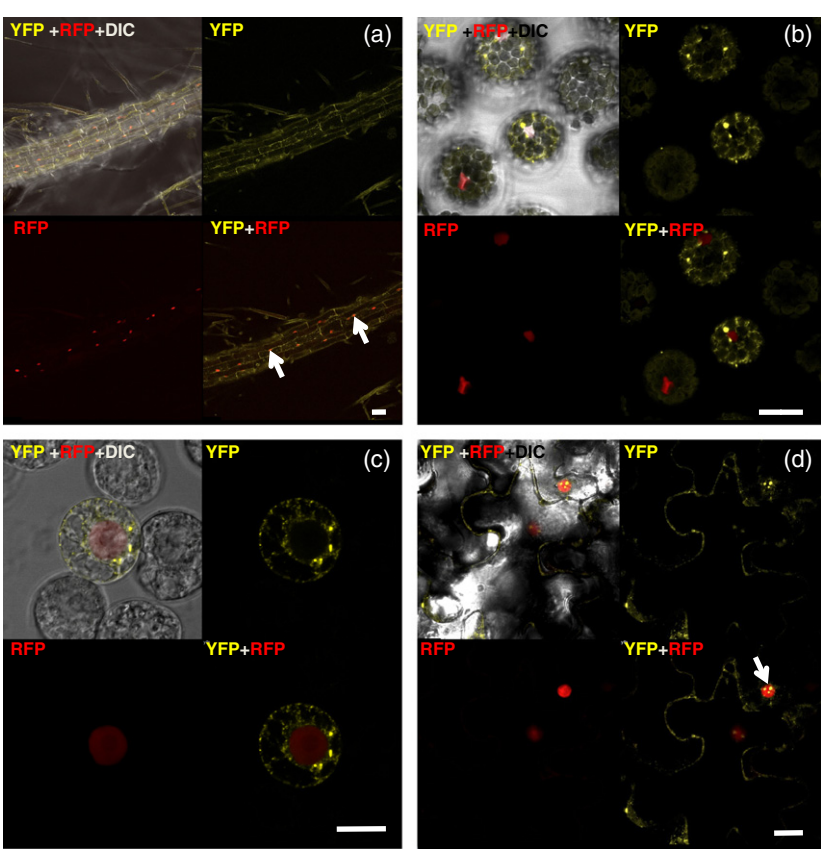

Figure 4. Subcellular localization of VirE2–Venus.
A T-DNA encoding VirE2–Venus was used to generate transgenic Arabidopsis (a) or to agroinfiltrate *Nicotiana benthamiana* leaves (d). A similar construction was used to transfect Arabidopsis leaf mesophyll protoplasts (b) or tobacco BY-2 protoplasts (c). A construction encoding mRFP–NLS was co-introduced to mark nuclei. Plant tissues or protoplasts were imaged by confocal microscopy. Arrows indicate small aggregates of VirE2–Venus in nuclei. Scale bars = 20 μm. YFP, yellow fluorescent protein; RFP, red fluorescent protein; DIC, differential interference contrast imaging.

S5). This signal is therefore likely to be an imaging artifact. The cytoplasmic localization of VirE2 confirms our previous observations (Bhattacharjee *et al.*, 2008; Lee *et al.*, 2008; Gelvin, 2010a) and those of others (Grange *et al.*, 2008; Sakalis *et al.*, 2013; Li *et al.*, 2014).

Djamei *et al.* (2007) previously noted that transgenic Arabidopsis leaves expressing functional YFP-tagged VIP1 relocalize VIP1 to nuclei through activation of MAP kinases after a pathogen-associated molecular pattern (PAMP) response is initiated by either flagellin treatment or *Agrobacterium* infection. Because VIP1 binds VirE2, we considered that VirE2 may translocate to nuclei after *Agrobacterium* contacts a plant cell. We therefore infiltrated *Nicotiana benthamiana* leaves with an *Agrobacterium* strain encoding VirE2–Venus on the T-DNA. Figure 4(d) shows that even in the presence of *Agrobacterium*, VirE2 localizes predominantly to the cytoplasm. We occasionally noted small aggregates of yellow fluorescence in the nucleus (white arrow), but this occurred in a small minority of the nuclei examined.

VIP1 is expressed at very low levels in Arabidopsis and tobacco (Figure S4; Tzfira *et al.*, 2002). We therefore overexpressed an untagged full-length *VIP1* cDNA, or cDNAs encoding the variants *VIP1*^{S79A} and *VIP1*^{S79D}, from a strong CaMV 35S promoter while expressing VirE2–Venus from the weaker *nos* promoter. Figure 5 shows that overexpression of wild-type *VIP1* or the *VIP1* variants did not alter the subcellular localization of VirE2–Venus in Arabidopsis leaf mesophyll (Figure 5a) or tobacco BY-2 (Figure 5b) protoplasts: VirE2–Venus remained predominantly cytoplasmic. We obtained similar results when VIP1 was overexpressed, in the presence of VirE2–Venus, in transgenic Arabidopsis roots (Figure 5c) or in agroinfiltrated *N. benthamiana* leaves (Figure 5d). Thus, contrary to expectations, *VIP1* did not mobilize VirE2 into the nucleus in any of the cases of overexpression.

The VIP1 protein and VIP1 variants fractionate between the nucleus and cytoplasm of plant cells

Tzfira *et al.* (2001) showed that a GUS–VIP1 fusion protein localized exclusively to the nucleus. However, this VIP1 construction lacked the first 80 amino acids. We confirmed the exclusive nuclear localization of a similarly truncated VIP1–YFP fusion protein in tobacco BY-2 protoplasts (Figure S6). However, Djamei *et al.* (2007) showed that induced full-length VIP1–YFP fractionated between the nucleus and cytoplasm in transgenic Arabidopsis leaves. They additionally showed that a YFP fusion to the induced VIP1 variant VIP1^{S79A} fractionated between the nucleus and cytoplasm, whereas induced VIP1^{S79D} localized predominantly to the nucleus. We confirmed the nuclear/cytoplasmic fractionation of wild-type VIP1–Venus and the variant VIP1^{S79A} in Arabidopsis leaf mesophyll and tobacco BY-2 protoplasts. However, in these plant systems, VIP1^{S79D} also fractionated between the cytoplasm and nucleus in a pattern indistinguishable from that of wild-type VIP1 (Figure 6a,b). We obtained similar results in transgenic Arabidopsis roots and agroinfiltrated *N. benthamiana* leaves (Figure 6c,d). We additionally tested whether subcellular localization of VIP1–Venus was altered when untagged VirE2 was co-expressed. We could not detect any major changes in localization of VIP1–Venus (Figure S7).

VirE2 forms complexes with VIP1 in the cytoplasm

We used bimolecular fluorescence complementation (BiFC; Citovsky *et al.*, 2006; Lee *et al.*, 2008) and confocal microscopy to investigate the subcellular location of VirE2–VIP1 protein complexes. In both Arabidopsis leaf protoplasts (Figure 7a) and tobacco BY-2 protoplasts (Figure 7b) nVenus-VIP1 interacted with VirE2–cCFP in the cytoplasm. Multicolor BiFC, in which VirE2–cCFP interacted with either nVenus–VIP1 or VirE2–nCerulean, indicated exclusive cytoplasmic localization of both VirE2–VIP1 and VirE2–VirE2 complexes (Figure 7b).

The exclusive cytoplasmic localization of VirE2–VIP1 complexes (Figure 7) may seem at odds with our findings that overexpression of untagged VirE2 did not alter the subcellular localization of VIP1–Venus (Figure S7). However, this latter experiment revealed the localization of VIP1, not VIP1 in a complex with VirE2. We speculate that the large amount of VIP1–Venus produced in transgenic cells (Figure S7) exceeds the amount of VirE2 required to form complexes with all VIP1 proteins. Plants normally produce only small amounts of VIP1 mRNA (Tzfira *et al.*, 2002; Figure S4), and therefore the relatively large amounts of VirE2 protein entering the plant cell (Sakalis *et al.*, 2013; Li *et al.*, 2014) may be sufficient to relocalize VIP1 to the cytoplasm. Thus, VirE2 can interact with at least part of the VIP1 protein pool and exclude this portion of the pool from the nucleus.

DISCUSSION

VirE2 plays an important role in plant transformation by *A. tumefaciens*. *VirE2* mutants are almost avirulent (Stachel and Nester, 1986; Gelvin, 1998) and the few integrated T-DNA molecules that are delivered from a *virE2* mutant *Agrobacterium* strain are severely truncated from the 3′ end (Rossi *et al.*, 1996). It has been proposed that VirE2 interacts with T-strands in the plant cell and protects them from nucleolytic degradation (Yusibov *et al.*, 1994; Rossi *et al.*, 1996). A role for VirE2 in nuclear targeting of T-strands has also been proposed (Citovsky *et al.*, 1992; Gelvin, 1998; Ziemienowicz *et al.*, 2001).

Several studies indicated that VirE2, tagged at its N-terminus, localizes to the nucleus (Citovsky *et al.*, 1992, 1994; Tzfira and Citovsky, 2001). However, we have shown that N-terminal, but not C-terminal, tagging of VirE2 destroys its function in mediating transformation (Bhattacharjee

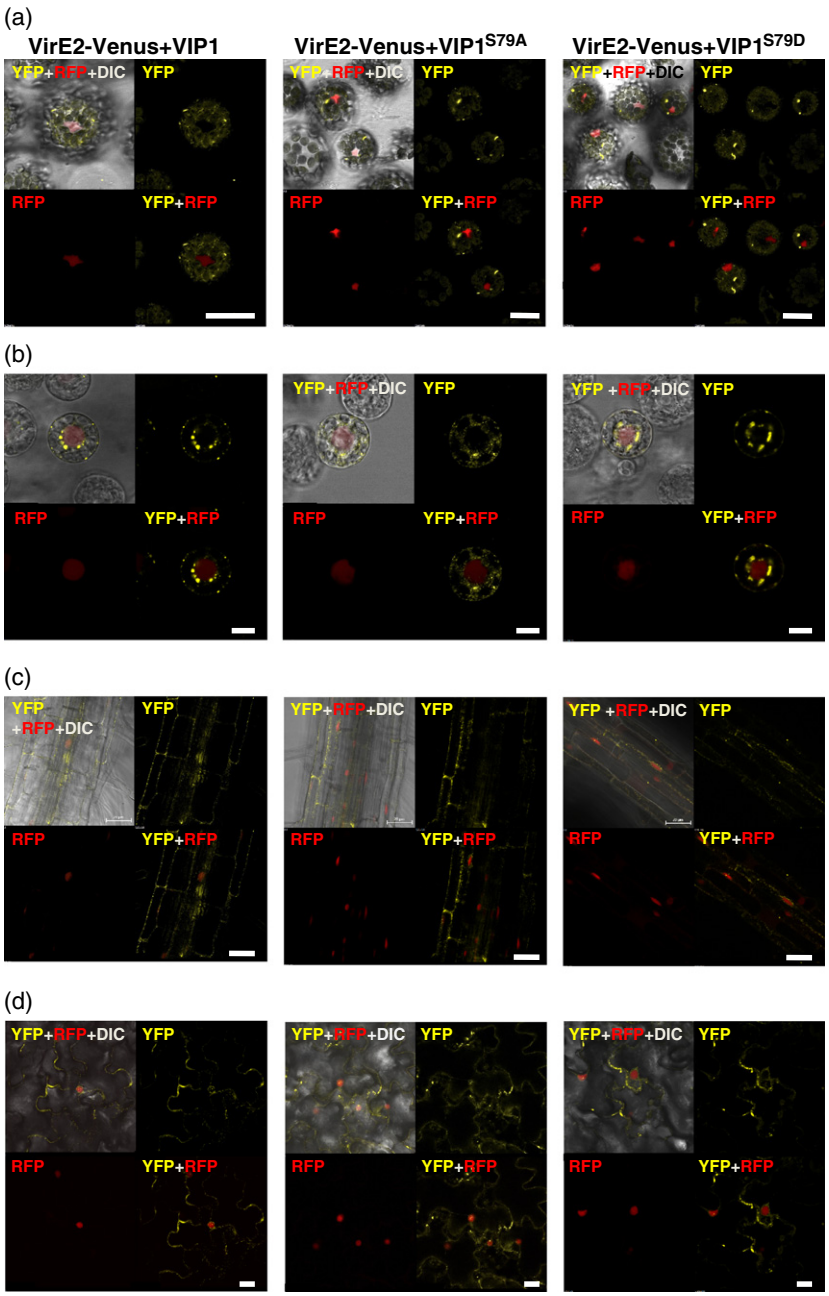

Figure 5. Subcellular localization of VirE2–Venus in the presence of overexpressed VIP1.
A construct encoding VirE2–Venus and various forms of VIP1 was used to transfect Arabidopsis (a) or tobacco BY-2 (b) protoplasts. A T-DNA with a similar construction was used to generate transgenic Arabidopsis (c) or agroinfiltrate *Nicotiana benthamiana* leaves (d). A construction encoding mRFP–NLS was co-introduced to mark the nuclei. Plant tissues or protoplasts were imaged by confocal microscopy. Scale bars = 20 μm. YFP, yellow fluorescent protein; RFP, red fluorescent protein; DIC, differential interference contrast imaging.

et al., 2008). Ballas and Citovsky (1997) showed that VirE2 does not interact with the Arabidopsis importin α protein AtKAPα (IMPa-1) in yeast. Because their experiments indicated that VirE2 contained NLS sequences and that it localized to the nucleus, it was somewhat surprising that VirE2 did not interact with importin α. They thus postulated an adapter protein VIP1 that interacts with VirE2 and importin α and serves as the mediator of nuclear transport of VirE2. Our studies, however, indicated that VirE2 does not normally enter the nucleus (Bhattacharjee *et al.*, 2008; Lee *et al.*, 2008; Gelvin, 2010a; this study), and that VirE2 does interact with all tested Arabidopsis importin α isoforms (including IMPa-1) in yeast, *in vitro* and *in planta* (Bhattacharjee *et al.*, 2008). In addition, VirE2 can enter the nucleus, but only when IMPa-4 is overexpressed (Bhattacharjee *et al.*, 2008; Lee *et al.*, 2008). Grange *et al.* (2008) showed that VirE2 expressed in tobacco BY-2 cells remains perinuclear with additional cytoplasmic strands. Both Sakalis *et al.* (2013) and Li *et al.* (2014) showed that VirE2 transferred from *Agrobacterium* forms predominantly cytoplasmic strands in yeast and plant cells. Formation of these filaments occurred both in the absence and the presence

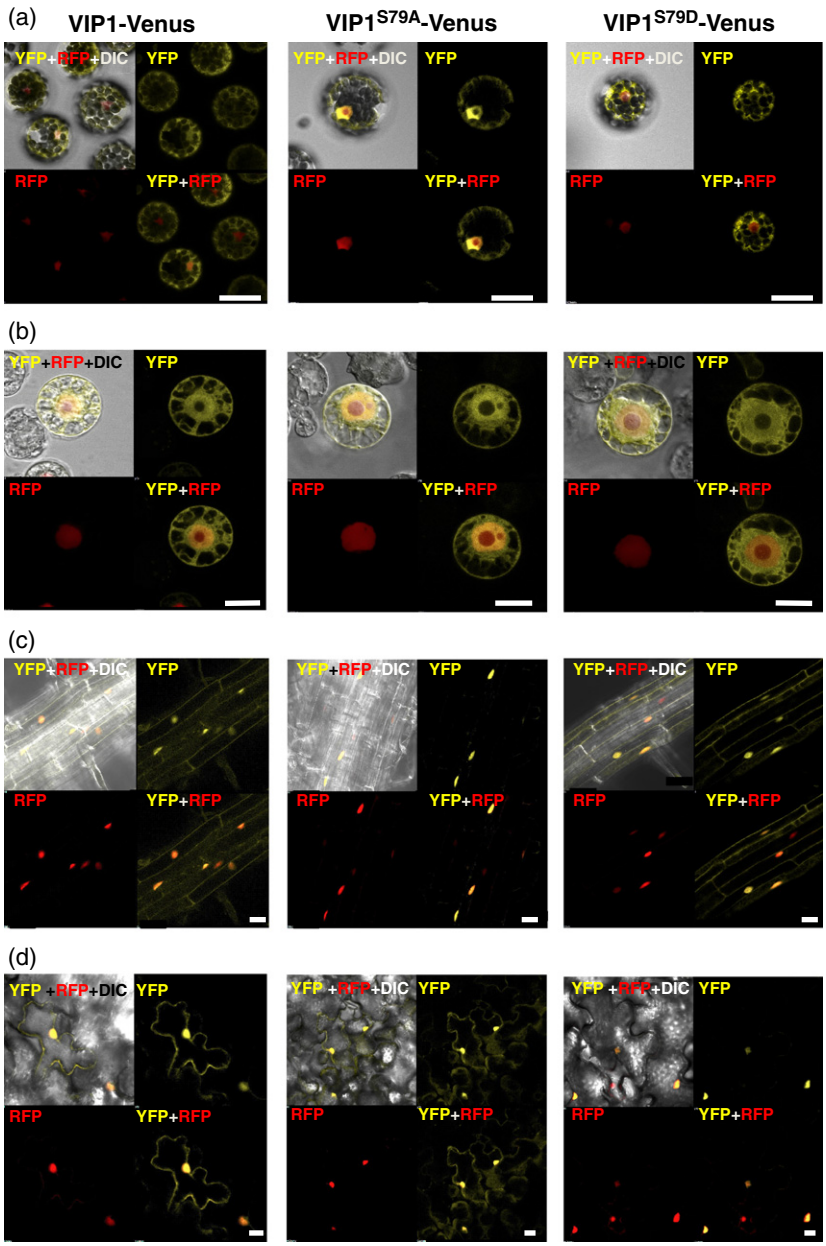

Figure 6. Subcellular localization of VIP1–Venus and VIP1 variants in various plant cell systems.
A construct encoding VIP1–Venus, VIP1^{S79A}–Venus, or VIP1^{S79D}–Venus was used to transfect Arabidopsis (a) or tobacco BY-2 (b) protoplasts. A T-DNA with a similar construction was used to generate transgenic Arabidopsis (c) or agro-infiltrate *Nicotiana benthamiana* leaves (d). A construction encoding mRFP–NLS was co-introduced to mark nuclei. Plant tissues or protoplasts were imaged by confocal microscopy. Scale bars = 20 μm. YFP, yellow fluorescent protein; RFP, red fluorescent protein; DIC, differential interference contrast imaging.

of T-DNA transfer. Cytoplasmic localization of VirE2 occurred even in the presence of excess VIP1 (Figure 5), and VirE2–VIP1 complexes remained cytoplasmic (Figure 7). Thus, the importance of VIP1 as an adaptor for translocating VirE2 into the nucleus is equivocal.

The importance of VIP1 in transformation was previously investigated by overexpressing an Arabidopsis *VIP1* cDNA in tobacco (Tzfira *et al.*, 2002), by using an antisense construction targeted against the Arabidopsis *VIP1* mRNA in tobacco (Tzfira *et al.*, 2001), and by investigating the transformation susceptibility of the Arabidopsis *vip1-1* mutant (Li *et al.*, 2005). Tzfira *et al.* (2002) investigated only two tobacco lines overexpressing an Arabidopsis *VIP1* cDNA; these lines showed increased susceptibility to transient and stable *Agrobacterium*-mediated transformation. However, the cDNA they used did not encode full-length VIP1 protein, but rather a protein that localizes exclusively to the nucleus (Tzfira *et al.*, 2001). We analyzed 59 T_2 generation Arabidopsis lines that overexpressed full-length wild-type *VIP1* cDNA, or the VIP1 variants VIP1^{S79A} and VIP1^{S79D}. Other than a very few lines that showed slightly increased (or occasionally reduced) transformation susceptibility, we saw no overall increase in transformation of these lines.

Tzfira *et al.* (2001) investigated two tobacco lines expressing an antisense construction directed against the

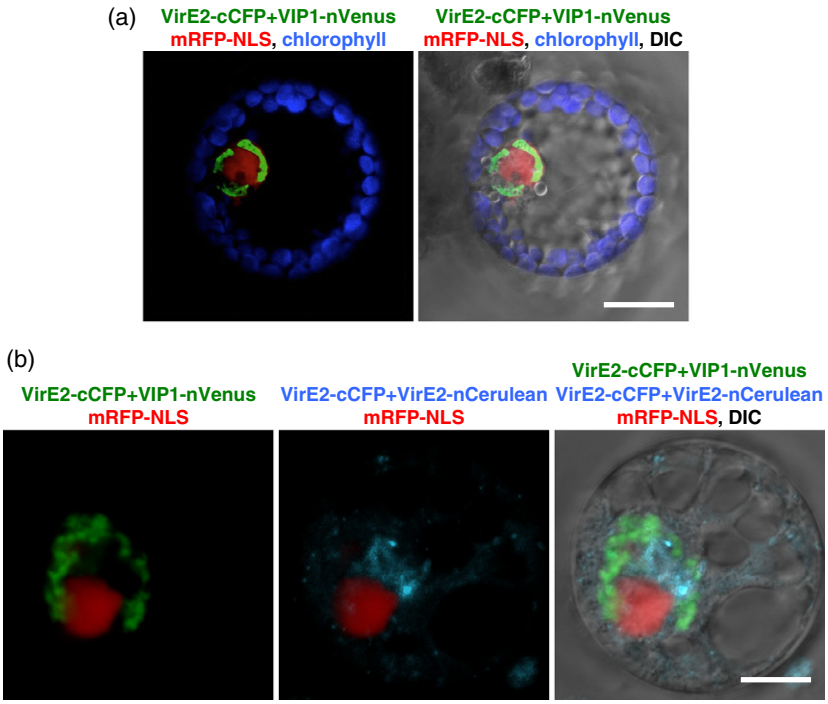

Figure 7. Subcellular localization of VirE2–VIP1 bimolecular fluorescence complementation complexes in plant protoplasts.
(a) Arabidopsis protoplasts showing interaction of VirE2–cCFP with VIP1–nVenus. Blue indicates chlorophyll autofluorescence. Scale bar = 10 μm.
(b) Tobacco BY-2 protoplasts showing interaction of VirE2–cCFP with VIP1–nVenus (left panel), or VirE2–cCFP with VirE2–nVenus (central panel).
Scale bar = 20 μm. mRFP-NLS is a nuclear marker. DIC, differential interference contrast imaging. Ten micrograms of each plasmid was transfected into protoplasts and imaged by confocal microscopy after 24 h.

Arabidopsis *VIP1* coding region. These lines showed decreased transformation susceptibility. *Nicotiana tabacum* encodes a likely ortholog of Arabidopsis VIP1 protein (annotated as the RSG activator protein), but the nucleotide sequence does not contain sufficient identity to be a homology-dependent gene silencing target of Arabidopsis *VIP1*. However, Arabidopsis *VIP1* may contain sufficient nucleotide identity (20/22 nucleotides) to target the tobacco myosin XI-F gene. Mutation of some Arabidopsis myosin XI genes can decrease transformation susceptibility. It is therefore possible that the transformation susceptibility decrease seen by Tzfira *et al.* (2001) resulted from off-target effects of the Arabidopsis *VIP1* antisense construction that they used.

Li *et al.* (2005) showed that the Arabidopsis *vip1-1* mutant, that makes only the N-terminal portion of the VIP1 protein, showed wild-type levels of transient transformation but decreased stable transformation. We could not detect a difference in the susceptibility to transient or stable transformation of this mutant. We note that Li *et al.* used only one *Agrobacterium* strain (A208) and one concentration of bacterial inoculum (about 2×10^8 cfu ml^{-1}). Furthermore, they tested a total of only 90–150 root segments from three plants. We used five different *Agrobacterium* strains (including *A. tumefaciens* A208 and one *A. rhizogenes* strain) and tested transformation susceptibility over a broad range (10^5 to 5×10^8 cfu ml^{-1}) of inoculum concentrations. We pooled roots from 15 to 30 plants, and for each experimental point assayed about 300 root segments (i.e. three independent replicates for each experimental point).

Full-length VIP1 fractionates between the nucleus and the cytoplasm (Djamei *et al.*, 2007; Tsugama *et al.*, 2012; Figure 6). The serine-79 of VIP1 is a phosphorylation target for the MAP kinase MPK3; phosphorylation of serine-79 may direct VIP1 to the nucleus. Djamei *et al.* (2007) showed that the phosphomimic $VIP1^{S79D}$ localizes to the nucleus, whereas the non-phophorylatable mutant $VIP1^{S79A}$ fractionates between the nucleus and cytoplasm. We were unable to reproduce the $VIP1^{S79D}$ results. We note, however that Djamei *et al.* (2007) observed transiently expressed VIP1 under an inducible system, whereas we investigated constitutively expressed VIP1. In transgenic Arabidopsis roots, agroinfiltrated *N. benthamiana* leaves and Arabidopsis and BY-2 protoplasts, we observed that all variants of VIP1 fractionated between the nucleus and the cytoplasm (Figure 6). Localization of VIP1 was not affected by overexpression of untagged VirE2 protein (Figure S7). We note that as well as having a NLS sequence, VIP1 contains a NES sequence. The subcellular localization of VIP1 responds to the turgor pressure of the cells: high turgor pressure drives VIP1 to the nucleus, after which it returns to the cytoplasm when plant cells osmotically adjust (Tsugama *et al.*, 2012). The conflicting results between Djamei *et al.* (2007) and this study may reflect osmotic differences in plant leaf material.

A model for the role of VirE2–VIP1 interactions in *Agrobacterium*-mediated plant transformation

Considering the importance of VirE2 in *Agrobacterium*-mediated transformation (Stachel and Nester, 1986; Rossi *et al.*, 1996), the predominant cytoplasmic localization of

VirE2 (Bhattacharjee *et al.*, 2008) (Figures 4 and 5), the cytoplasmic localization of VirE2–VIP1 complexes (Figure 7) and the role of VIP1 as a positive regulator of plant defense responses (Djamei *et al.*, 2007; Pitzschke *et al.*, 2009; Pitzschke, 2013), we propose a model for the role of VirE2–VIP1 interactions in *Agrobacterium*-mediated transformation.

In this model, nuclear targeting of T-strands is predominantly effected by VirD2, although VirE2 may play a 'structural' role in 'unwinding' structures in T-strands, thus facilitating nuclear entry of T-strands (Ziemienowicz *et al.*, 2001). VirE2 enters the plant cell where, in addition to its structural role in T-complex formation, it serves as a transcriptional modulator by interacting with VIP1 in the cytoplasm. Cytoplasmic sequestration of at least part of the small endogenous VIP1 pool prevents VIP1 from activating plant defense genes, thus facilitating transformation. In support of this model, we previously showed that when tobacco cells were co-cultivated with an avirulent *Agrobacterium* strain lacking a Ti-plasmid (and thus lacking T-DNA and *vir* genes), host defense genes were activated within a few hours (probably a PAMP response), and were again induced 30–36 h after infection. However, when we infected tobacco cells with an *Agrobacterium* strain harboring a Ti-plasmid we detected only the initial induction of defense response genes (Veena *et al.*, 2003). These data indicate that incoming virulence proteins can suppress 'late' host defense responses. Transcriptional modulation by interactions between effector protein and host transcription activator protein occurs after infection of plant cells by several plant pathogens (Cannone *et al.*, 2011; Deslandes and Rivas, 2011; Canonne and Rivas, 2012). We are currently testing the model that VirE2 can modulate expression of VIP1-responsive genes.

EXPERIMENTAL PROCEDURES

Bacterial and plant growth

Escherichia coli strains were grown at 37°C on Luria–Bertani medium (Sambrook and Russell, 2001) containing the appropriate antibiotics (ampicillin, 100 mg L^{-1}; kanamycin, 25 mg L^{-1}; spectinomycin, 100 mg L^{-1}). *Agrobacterium* strains were grown at 30°C in either YEP rich or AB minimal medium (Lichtenstein and Draper, 1986) with 0.5% sucrose and the appropriate antibiotics (kanamycin, 25 mg L^{-1} in liquid medium and 50 mg L^{-1} in solidified medium; rifampicin, 10 mg L^{-1}; spectinomycin, 50 mg L^{-1} in liquid medium and 100 mg L^{-1} in solidified medium).

Arabidopsis plants (ecotype Col-0) were grown either in soil or on Gamborg's B5 medium (Gibco-BRL; http://www.lifetechnologies.com/us/en/home/brands/gibco.html) at 25°C under a 16/8 h light/dark regime. When grown *in vitro*, Arabidopsis seeds were first surface sterilized for 15 min using 50% commercial bleach, 0.1% sodium dodecylsulfate (SDS), washed five times in sterile water, then plated in Petri dishes containing 100 mg L^{-1} Timentin and (when appropriate) 20 mg L^{-1} hygromycin or 50 mg L^{-1} kanamycin. Before assaying for transformation susceptibility, 10-day-old seedlings were transferred to baby-food jars containing B5 medium. Arabidopsis plants used for imaging were grown on B5 medium on vertical plates. *Nicotiana benthamiana* plants were grown in soil at 25°C under a 16/8 h light/dark regime.

Plasmid and bacterial strain constructions

Table S1 describes the plasmids and strains used for this study. For overexpression of *VIP1*, *VIP1*^{S79A}, and *VIP1*^{S79D}, PCR products (with *Spe*I/*Kpn*I flanking sites) of corresponding *VIP1* ORF variants were cloned into the same sites of the T-DNA binary vector pE1774 (Lee *et al.*, 2007) to make pE4130, pE4126 and pE4127, respectively. These three plasmids were individually introduced into *A. tumefaciens* GV3101 to generate *A. tumefaciens* At1983, 1984 and 1985, respectively.

To generate a T-DNA binary vector containing VirE2–Venus, an *Asc*I fragment containing P_{nos}-VirE2–Venus was cloned into the *Asc*I site of the binary vector pE4145, generating pE4147. To add a mRFP–NLS expression cassette into this plasmid, a 2522-bp *Sph*I–I–*Ceu*I fragment from pE4234 was used to replace the *Sph*I–I-*Ceu*I fragment of pE4147 to make pE4260. This plasmid was introduced into *A. tumefaciens* GV3101 to generate *A. tumefaciens* At2065.

To overexpress untagged VIP1 in the presence of VirE2–Venus, the I–*Sce*I fragments encoding VIP1, VIP1^{S79A}, or VIP1^{S79D}, under the control of the CaMV 35S promoter, were taken from their corresponding pSAT4 vectors and cloned into the same sites of pE4260, generating pE4262, pE4261 and pE4263, respectively. These three plasmids were individually introduced into *A. tumefaciens* GV3101 to generate *A. tumefaciens* At2067, At2066 and At2068, respectively.

To generate T-DNA binary vectors containing *VIP1*–Venus, *VIP1*^{S79A}-Venus, and *VIP1*^{S79D}–Venus, we first generated binary vectors expressing untagged VirE2 in the presence of these tagged *VIP1* cDNAs, then deleted VirE2 from them. An I–*Ceu*I fragment containing P_{35S}–VirE2 was isolated from pE4229 and cloned into the same site of the T-DNA binary vector pE4234, generating pE4220. The *npt*II expression cassette was replaced with a *hpt*II expression cassette through an *Asc*I/I–*Spe*I fragment (from pE4145) replacement to make pE4248. The *Asc*I fragments containing *VIP1*–Venus, *VIP1*^{S79A}–Venus, and *VIP1*^{S79D}–Venus under control of P*ocs* (Octopine synthase gene promoter) from their corresponding pSAT1 vectors pE4231, pE4232 and pE4233 were cloned into the *Asc*I site of pE4248 to make pE4249, pE4250 and pE4251. The I–*Ceu*I fragments (containing P_{35S}–VirE2) were deleted from pE4249, pE4250, and pE4251 to make pE4257, pE4258, and pE4259. These six plasmids (pE4249, pE4250, pE4251, pE4257, pE4258 and pE4259) were individually introduced into *A. tumefaciens* GV3101 to generate *A. tumefaciens* At2055, At2056, At2057, At2058, At2059 and At2060, respectively.

Confirmed constructions in T-DNA binary vectors were introduced into *A. tumefaciens* GV3101 (Koncz and Schell, 1986) by electroporation.

Generation of transgenic Arabidopsis plants

Transgenic Arabidopsis plants were generated using a flower dip protocol (Clough and Bent, 1998).

Agrobacterium-mediated transient and stable transformation assays

Roots of 18-day-old Arabidopsis plantlets were cut into segments of 3–5 mm and assayed as previously described (Tenea *et al.*, 2009). *Agrobacterium tumefaciens* GV3101(pBISN1) was used for transient transformation assays and *A. tumefaciens* A208, A348,

At1811, At1982 or *A. rhizogenes* R1000 were used for stable tumorigenesis assays. For each replicate, root segments were pooled and randomized from five to ten plants; 80 or more root segments were scored for each experimental point. Three replicates were conducted for each experiment.

Agroinfiltration of *Nicotiana benthamiana*

Agroinfiltration was performed on leaves of 3–4-week-old *N. benthamiana* plants. Two milliliters of a bacterial overnight culture (in YEP medium) was centrifuged($\sim$10,000 ***g*** for 1 min in a microcentrifuge) and suspended in 6 ml of induction medium [1$\times$ AB salts, 1% glucose, 30 mM 2-(*N*-morpholine)-ethanesulfonic acid (MES), 2 mM NaH_2PO_4, 2 mM Na_2HPO_4, 200 μM acetosyringone, pH 5.6] and grown for another 6 h. Cells were centrifuged as above and suspended in agroinfiltration medium (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone) to a final density of 5×10^8 cfu ml^{-1}. The bacteria were infiltrated into leaves using a 1-ml syringe. Fluorescence was imaged 48 h later by confocal microscopy.

Arabidopsis and tobacco BY-2 protoplast isolation and transfection

Protoplasts were isolated from leaves of Arabidopsis plants (ecotype Col-0) and tobacco BY-2 cells as previously described (Lee *et al.*, 2012). Protoplasts were transfected with 10 μg of DNA of the relevant clones, and imaged 12–48 h later by confocal microscopy.

Confocal laser scanning microscopy

Plant cells and tissues were imaged using a Nikon A1R confocal microscope with a Plan Fluor 20 $\times$ MImm DIC N2 objective (http://www.nikon.com/). The wavelengths of excitation and emission were 488 and 525 nm for both YFP and Venus, 561 and 595 nm for mRFP, and 433 nm and 475 nm for Cerulean.

PCR and RT-PCR

We genotyped *vip1-1* mutant plants using *VIP1*-specific primers. In the homozygous mutant, the N-terminal-specific forward 5′-CGAGATCTATGGAAGGAGGAGGAAG-3′ and reverse 5′-CTGT AACATAGTGACTTGAGCAG-3′ primers of *VIP1* would generate an 835-bp product from both Col-0 and *vip1-1* mutant genomic DNA, and forward 5′-AATAGACAATCTGCGGC-3′ and reverse 5′-AAGG ATCCCGCCTCTCTTGGTGAAAT-3′ primers specific to the *VIP1* sequence spanning the T-DNA insertion site would generate a 968-bp product from Col-0 genomic DNA but no product from homozygous *vip1-1* genomic DNA samples.

To conduct RT-PCR, total RNA was extracted from 100 mg of roots. The RNA samples (2 μg) were treated with DNaseI (Ambion Turbo DNA free Kit; http://www.lifetechnologies.com/us/en/home/brands/ambion.html) and reverse transcribed with SuperScriptIII reverse transcriptase (Invitrogen RT-PCR Kit, http://www.invitrogen.com/). The cDNA products were amplified through PCR. The forward 5′-CGAGATCTATGGAAGGAGGAGGAAG-3′ and reverse 5′-CTGTAACATAGTGACTTGAGCAG-3′ primers would generate a 740-bp product from both Col-0 and *vip1-1* mutant genomic DNA, and the forward 5′-AATAGACAATCTGCGGC-3′ and reverse 5′-AAG GATCCCGCCTCTCTTGGTGAAAT-3′ primers would generate a 423-bp product from Col-0 genomic DNA but no product from the *vip1-1* genomic DNA samples. The 50-μl PCR reaction contained 0.3 μg of cDNA as a template and was conducted for 40 cycles. Starting at cycle 25 we took out 7-μl samples every five cycles. All samples were analyzed by electrophoresis through a 1% agarose gel. Amplification of *ACT2* cDNA was used as an internal control using the actin primers (5′-GAAGTACAGTGTCTGGATCGGTGGTT-3′ and 5′-ATTCCTGGACCTGCCTCATCATACTC-3′) for RT-PCR. The gel images were scanned and analyzed using IMAGE J software (Image Metrology A/S; info@imagemet.com).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Characterization of a homozygous *vip1-1* mutant.

Figure S2. Stable transformation of *vip1-1* and Col-0 root segments by various *Agrobacterium* strains.

Figure S3. Callus induction efficiency of Col-0 and *vip1-1* root segments.

Figure S4. Semi-quantitative RT-PCR analysis of *VIP1*, $VIP1^{S79A}$, and $VIP1^{S79D}$ overexpressing Col-0 (a) and *vip1-1* (b) plants.

Figure S5. Confocal imaging of a transgenic Arabidopsis root expressing mRFP–NLS.

Figure S6. Subcellular localization of VIP1 (lacking the first 80 amino acids)–YFP in tobacco BY-2 protoplasts.

Figure S7. Overexpression of VirE2 does not alter the subcellular localization of VIP1–Venus.

Table S1. Bacterial strains used in this study.

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