Characterization of VIP1 activity as a transcriptional regulator in vitro and in planta

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VIP1 (VirE2 interacting protein 1), initially discovered as a host protein involved in Agrobacterium-plant cell DNA transfer, is a transcription factor of the basic leucine-zipper (bZIP) domain family that regulates several defence-related genes in Arabidopsis. We have developed assays to assess VIP1 binding to its DNA target in vitro and transcriptional activation efficiency in planta. Several point mutations in the VIP1 response element VRE affected the VIP1 activity, and a strong correlation between VIP1-VRE binding and transcriptional activation levels was observed. Promoter activation by VIP1 was influenced by bacterial and plant proteins known to interact with VIP1 during Agrobacterium infection, i.e., VirE2, VirF and VIP2. VirF, an F-box protein, strongly decreased VIP1 transcriptional activation ability, but not its binding to VRE in vitro, most likely by triggering proteasomal degradation of VIP1. Finally, activation of a VRE-containing promoter was observed in dividing cells, probably resulting from activation of endogenous VIP1.

VIP1 (VirE2 interacting protein 1) is a plant basic leucine zipper (bZIP) domain transcription factor initially identified as an interactor of the virulence protein E2 (VirE2) of Agrobacterium. VirE2 plays an important role during Agrobacterium-mediated genetic transformation of host cells by packaging the bacterial transferred DNA (T-DNA) into a nucleoprotein transfer complex (T-complex) and interacting with VIP1 in the course of several critical events of the infection process. Specifically, VIP1 is thought (i) to enhance the entry of the T-complex into the host cell nucleus via VIP1 interactions with the importin alpha-dependent nuclear import machinery, (ii) to mediate the T-complex targeting to the host chromatin via VIP1 interactions with core histones, and (iii) to present the T-complex to the proteasomal degradation machinery for uncoating via VIP1 interactions with the bacterial and/or host F-box proteins, VirF and VIPB, respectively.

Whereas it appears that Agrobacterium has evolved to exploit VIP1 for infection purposes, the natural function of VIP1 is informed from several recent studies showing its involvement in responses to different types of biotic and abiotic stresses. For example, VIP1 has been shown to participate in defence signalling, and, particularly, to act as substrate for the mitogen-activated protein kinase (MAPK) MPK3. When phosphorylated by MPK3, VIP1—which normally partitions between the cell cytoplasm and the nucleus—becomes largely nuclear, presumably allowing it to activate its target genes. Interestingly, enhancement of VIP1 nuclear uptake is also involved in transcriptional regulation of plant osmosensory signalling genes CYP707A1 and CYP707A3. In the MPK3 pathway, the VIP1 target genes include Trxh8 and MYB44. The promoters of these latter genes, as well as others that respond to activation by the MPK3 pathway, were shown to contain a DNA hexamer motif that acts as the VIP1 response element (VRE). VIP1 specifically binds to VRE, and strongly enhances expression of a synthetic promoter harbouring multiple VRE copies.

Here, we analysed in further detail the VIP1-VRE interaction and identified VRE nucleotides important for VIP1 binding and promoter activation. We then showed that major Agrobacterium effector proteins known to interact with VIP1 can modulate its VRE transcriptional activation ability.

Results

Activation of a VRE-containing promoter by VIP1 in planta. Functional studies of the VIP1-VRE interaction require a simple and reliable system for detection of VIP1-mediated transcriptional activation directly in living plant tissues. We developed such a system by constructing an artificial promoter that contained a direct tandem repeat of the VRE1 sequence, followed by the CaMV 35S minimal promoter, i.e., a 46-bp fragment containing the TATA box and located immediately upstream of translation initiation codon. This promoter was then used to drive
expression of two different reporters, GFP and an intron-containing beta-glucuronidase (GUSintron) (Fig. 1A). Unlike previous studies focused on the VRE function in plant protoplasts, we introduced these expression constructs into plant tissues, either transiently by agroinoculation into tobacco leaves, or stably, in transgenic tobacco plants.

Fig. 1A shows very large areas of GUS histochemical staining on a leaf disk derived from the plant that was transiently co-transformed with the VRE1-35Smin-GUSintron reporter construct and a VIP1-expressing construct. No GUS expression at all was observed when the reporter construct was co-agroinoculated with the same expression vector, but lacking the VIP1 sequence (empty vector); furthermore, that no GUS staining was observed at the cut edges of the leaf disk indicated that the VRE element was not induced by tissue wounding. Similarly, transient expression of VIP1 in the VRE1-35Smin-GFP transgenic tobacco plants activated expression of the GFP reporter, whereas agroinoculating the empty vector into these transgenic tissues or mock-inoculating them elicited no GFP expression (Fig. 1C). That the endogenous VIP1 did not detectably activate the reporter is consistent with the known naturally low levels of this protein in plant cells.

Collectively, the data in Fig. 1B, C, therefore, indicate that the VRE element indeed activates gene expression in the presence of VIP1, and that this control is stringent, i.e., no expression occurs in the absence of VIP1, and specific, i.e., virtually no activation is achieved by wounding or bacterial challenge. These observations also demonstrate that our reporter constructs can be used for simple and specific detection of VIP1-mediated transcriptional activation of the VRE element in plant tissues.

**Binding of VIP1 to VRE in vitro.** Next, we developed a simple quantitative assay for the VIP1-VRE binding. To this end, we adapted the DNA-Protein-Interaction (DPI)-ELISA technology. In this method, biotin-labelled DNA probes are bound onto streptavidin-coated 96-well plates; then, binding of VIP1 to DNA probes is detected by anti-VIP1 primary antibody and followed by alkaline phosphatase-conjugated secondary antibody. Based on this rationale, we produced a DNA probe that essentially replicated the VRE1-35Smin-GFP transgene used to detect the VRE response to VIP1 in planta (see Fig. 1A); specifically, it contained the VRE1-35Smin promoter and the full-length GFP coding sequence with a covalently-attached biotin molecule (Fig. 2A). When this probe was incubated with purified recombinant histidine-tagged VIP1 (Fig. 2B), protein-DNA binding was observed (Fig. 2C). Note that our previous studies utilizing histidine-tagged VIP1 demonstrated that this the tagged protein retains its known biological activities, such as specific binding to VirE2 and VirF. This assay produced only low levels of signal with either no probe or with a non-specific GFP probe, lacking the VRE sequence (Fig. 2C); thus, in all subsequent experiments, the absorbance values obtained with the non-specific GFP probe were subtracted from the total experimental measurements.

The specificity of VIP1-VRE binding was directly demonstrated by competition experiments, using unlabelled competitor DNA. The results in Fig. 2D show that significant, 80–95%, inhibition of the interaction was achieved in the presence of increasing amounts of the specific VRE1-35Smin-GFP competitor, i.e., 1.0 and 2.5 molar excess, respectively, whereas no such inhibitory effect was observed with 2.5 molar excess of a non-specific GFP competitor.

For some bZIP proteins, such as CAMP responsive element-binding protein (CREB), magnesium ions played a major role in its interaction with the target DNA, enhancing the binding up to 25 fold. Thus, we tested whether the VIP1-VRE binding also was enhanced by magnesium ions. Fig. 2C shows that this was not the case. In fact, increasing the concentration of the magnesium ions even inhibited binding, potentially via ionic screening. Thus, unlike CREB, VIP1 most likely does not trap magnesium ions in the binding interface with DNA.

**Effects of mutations in VRE on VIP1 binding and promoter activation.** Having established binding and transcriptional activation assays for the VIP1-VRE interaction, we set out to use this methodology to examine the role of individual VRE nucleotides in VIP1 binding and subsequent function. First, we aimed to assess the importance of nucleotides flanking the core consensus hexamer. Indeed, whereas they are not conserved and not absolutely required for the binding of bZIP proteins to their target sequence, these nucleotides have been shown to affect the specificity of DNA binding of several other bZIP proteins. Using the VRE1 sequence as reference, we designed a similar construct harbouring modified VRE versions. VRE2, corresponding to the VIP1 response element found in the MYB44 gene promoter region, differs from VRE1 in the T1G and C10A substitutions, while VRE3 harbours a T1G substitution. Fig. 3B shows that these mutated VRE sequences differed in their ability to be recognized by and bind VIP1. VIP1 binding to VRE2 was reduced by about 25% relative to VRE1 whereas binding to VRE3 was essentially unaffected. That VRE1 and VRE3 displayed similar levels of VIP1 binding suggests that the cytosine nucleotide in position 10...
enhances VIP1 binding efficiency (relative to VRE2), whereas the presence of a thymidine or guanosine nucleotides in position 1 produces similar binding levels. Further, we modified the VRE3 sequence by inserting two additional substitutions in the core consensus VRE sequence, A4T and T7A, resulting in VRE4. VRE4 almost completely lost its ability to interact with VIP1. Previous studies indicated that multiple mutations in the VRE core consensus sequence disrupt VIP1 binding and transcriptional activity. Importantly, however, these mutations also affected the palindromic structure of the VRE sequence, while, in VRE4, the A4T substitution restored the palindromic structure.

These binding data paralleled the biological functionality of the VRE sequence in the promoter activation assay (Fig. 3C, D). Based on the levels of the GFP reporter expression, VRE2 activation was about 30% lower than the VRE1 control, but VRE3 was activated more efficiently, by about 20%. VRE4 activation capacity was very low, about 10% of the VRE1 control.

**Effect of coexpression of VIP1 interactors.** Besides acting as transcriptional activator of plant defence and stress response genes, VIP1 represents one of the major host factors involved in several key steps of plant infection and genetic transformation by Agrobacterium. During these activities, VIP1 interacts with several other bacterial and plant proteins, such as VirE2, VirF, and VIP2. These interactions are presumed to occur when VIP1 is associated with the chromatin or even bound to DNA. Thus, we set out to assess whether VIP1 retains its transcriptional activation and VRE binding activities in the presence of VirE2, VirF, or VIP2.

In our transcriptional activation assay, we transiently expressed the VRE1-35Smin-GFP reporter construct together with VIP1 alone or VIP1/VirE2, VIP1/VirF, or VIP1/VIP2 pairs; to standardize expression, the tested proteins were coexpressed from the same vector. Fig. 4A shows that the presence of VirE2 did not significantly alter expression of the reporter, whereas coexpression of VirF with VIP1 had a pronounced inhibitory effect. Similarly to VirE2, VIP2 did not affect the reporter expression in a major way when coexpressed with VIP1. Quantification of these data (Fig. 4B) demonstrated that the presence of VirE2 or VIP2 only slightly, i.e., by 10–15%, decreased the activation efficiency of VIP1, but coexpression of VirF reduced this VIP1 activity by more than 50%.

Does VirF interfere with VIP1 binding to VRE to produce this inhibitory effect on transcriptional activation ability of VIP1? To address this question we assayed the VIP1-VRE1 binding in vitro. Comparison between the binding activity of purified recombinant histidine-tagged VIP1 incubated with the VRE1-35Smin-GFP probe alone or in the presence of equimolar amount of purified recombinant histidine-tagged VirF detected only minor, less than 15%, decrease in VRE binding (Fig. 4C). As mentioned above, our earlier studies using similarly tagged and purified VIP1 and VirF demonstrated their protein-protein interaction functionality. Thus VirF, similarly to VirE2 and VIP2, has no significant effect on VIP1 recognition of VRE. Instead, VirF, which is known to destabilize VIP1 via proteasomal degradation, most likely depletes the amounts of VIP1 in the expressing cells.

**Induction of VRE activity by cell division.** Besides its functions in defence and stress responses, VIP1 is induced during cell dedifferentiation, which occurs in the course of cell division elicited by growth regulators. Thus, we examined whether induction of cell division by cytokinin and auxin treatments activates the VRE1-35Smin-GUS intron reporter. To this end, leaf explants from the VRE1-35Smin-GUS intron transgenic tobacco plants were grown on a solid medium supplemented with BAP (6-benzylaminopurine) and NAA (naphthalene-1-acetic acid). Fig. 5 shows that, after two weeks of cultivation, the GUS reporter activity was observed in the areas of active callus formation (panels B, C, arrowheads), which indicate the zones where cell division is induced; in control experiments, in which the leaf disks were cultivated in the absence of hormones, no reporter expression or callus formation were observed (panel A). Because these experiments did not involve coexpression of VIP1, the reporter expression, and thus activation

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**Figure 2** | VIP1 binding to VRE1 in vitro. (A) Schematic representation of DNA probe, containing the VRE1-35Smin synthetic promoter (indicated in black), GFP coding sequence (indicated in green), and a covalently attached biotin molecule (indicated in yellow). (B) SDS PAGE analysis of purified VIP1. The gel was stained with Coomassie blue. The position of the VIP1 protein band (right lane) is indicated by arrowhead; protein molecular mass standards (left lane) are indicated in kDa. (C) VIP1 binding to VRE1-35Smin-GFP is slightly decreased in the presence of magnesium ions. (D) Binding competition by unlabeled VRE1-35Smin-GFP or GFP. Standard deviations are indicated.

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of the VRE-containing promoter, was most likely induced by the endogenous VIP1 activated by cell division.

**Discussion**

VIP1 is a multifunctional protein involved in several critical aspects of plant interactions with biotic and abiotic environment, such as defence response and osmosensory signalling\(^{10-12}\). Evolutionarily, this central role of VIP1 in plant defence makes it a likely target for at least some of the invading pathogens. Indeed, Agrobacterium is thought to subvert some of the VIP1 activities, i.e., import into the nucleus, targeting to the chromatin, and serving as a substrate for the ubiquitin/26S proteasome system (UPS)\(^{1,3,4,7}\), to facilitate its own infection. Thus, it would be useful to understand better the natural VIP1 function, i.e. its binding to the target gene response element (VRE) and subsequent transcriptional activation, and then examine how they might be affected by interactions with bacterial and cellular factors known to associate with VIP1 during genetic transformation by Agrobacterium. To this end, we have developed methods to assay the VIP1-VRE binding *in vitro* and VIP1-induced activation of expression of VRE-containing reporter genes *in planta*. We then used different VRE mutants to demonstrate direct correlation between the protein-DNA binding levels and the resulting transcriptional activity observed in plant tissues. These experiments also revealed the functional significance of the DNA sequences immediately flanking the VRE core consensus. Specifically, the conserved VRE hexamer, ACNGCT, is sufficient for VIP1 binding\(^{12}\), and mutations within this element almost completely disrupt VIP1 binding and transcriptional activation. The nucleotides located outside of and adjacent to the VRE core, on the other hand, had a much milder effect both on the VIP1-VRE binding and on the induction of transcriptional activity. Thus, different target genes of VIP1 may be activated at different levels, depending on the nature of the VRE flanking nucleotides, which therefore might function to fine-tune the VIP1-mediated transcriptional activation. These data are consistent with previous observations that nucleotides at the periphery of the conserved target sequences may modify binding affinity of several other bZIP proteins\(^{17}\).

How are these natural activities of VIP1 influenced by its recruitment by Agrobacterium for molecular reactions of the infection? To address this question, we investigated whether VIP1 is still able to act as a transcriptional activator in presence of its protein interactors involved in Agrobacterium-mediated genetic transformation. These experiments demonstrated that VirE2 and VIP2 interfered with the VIP1 activities only slightly. Whereas the effect of free VirE2 on VIP1 may differ from that of the ssDNA-bound VirE2, as in the bacterial T-complex, our previous study indicates that the VIP1-VirE2 interaction occurs with the free as well as with the ssDNA-bound VirE2 to a comparable degree\(^{1}\). That neither VirE2 nor VIP2 markedly impaired the transcriptional activation activity of VIP1 may be because binding of these proteins to the VIP1 molecule does not significantly affect its domains involved in recognition of VRE and/or activation of transcription. Alternatively, the affinity of VIP1 to VRE might be higher than that toward VirE2 or VIP2, shifting the binding equilibrium toward the formation of VIP1...

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**Figure 3** | Effects of mutations in VRE on VIP1 binding and promoter activation. (A) Sequences of VRE1 and its VRE2-4 mutants. The mutated nucleotides are indicated in red. (B) VIP1 binding to the VRE-35Smin-GFP probe containing VRE1-4 sequences. GFP is in green, plastid autofluorescence is in red. All images are single confocal sections. (D) Quantification of VIP1-induced expression of VRE-35Smin-GFP containing VRE1-4 sequences. GFP signal was calculated as percent of the signal measured with the VRE1-35Smin-GFP reporter, which was defined as 100% signal. All quantified data are shown as mean of three experiments with indicated standard deviations; standard deviation for measurements of the VRE1-35Smin-GFP reporter itself was 9.6%.
complexes with DNA rather than its protein interactors. In contrast, the presence of VirF substantially reduced the ability of VIP1 to induce target gene transcription, yet had no major effect on the VIP1-VRE binding. Thus, VirF most likely reduced the overall cellular levels of VIP1 by targeting it to degradation via the SCFVirF pathway, which represents a well-known function of VirF during Agrobacterium infection. In this scenario, VirF would not only act to uncoat the T-complex, but also mitigate the induction of the host defence genes mediated by VIP1.

Our observations also suggest that the activity of VIP1 itself is regulated in a developmentally controlled manner. This is inferred from activation of the VRE element by induction of cell division; potentially, when cells divide, their pool of endogenous VIP1 is increased and/or activated, which in turn activates the VRE-regulated target genes. Potentially, this could be achieved in two ways, both of which have been reported for VIP1: enhancement of nuclear import, and, by implication, transcriptional activity, by phosphorylation, or complexes with DNA rather than its protein interactors. In contrast, the presence of VirF substantially reduced the ability of VIP1 to induce target gene transcription, yet had no major effect on the VIP1-VRE binding. Thus, VirF most likely reduced the overall cellular levels of VIP1 by targeting it to degradation via the SCFVirF pathway, which represents a well-known function of VirF during Agrobacterium infection. In this scenario, VirF would not only act to uncoat the T-complex, but also mitgate the induction of the host defence genes mediated by VIP1.
simply upregulation of the VIP1 gene transcription2. The latter scenario is consistent with the known increase in VIP1 transcription levels upon cell dedifferentiation22. Collectively, our data shed new light on the function of VIP1 as a transcriptional activator and emphasize how this function fits into participation of VIP1 in the reactions of Agrobacterium-mediated genetic transformation of plant cells.

Methods

Plasmids. For protein expression in E. coli, the full-length VIP1 coding sequence from Arabidopsis thaliana ecotype Col-0 (At1G3700) was PCR-amplified from a cDNA library, using the primer pair 5’GGCGAGTTCTTACACAGTTCAATGACAGGAGGGAAAGAGATGAGATGGCACG/5’CCGCTCTAGAATTCGCTTCTGTAAGATATCCATGGAAGCTGCACTG and cloned into the EcoRI-XhoI sites of pET-28a(+) (Clontech), resulting in pET28-VIP1. The vipf gene from the Agrobacterium strain 15955 was PCR-amplified, using the primer pair 5’GGCGGATCCATTATCTGACATGAGCTACAGGAGGGAGAAGAGATGAGATGGCACG/5’CCGCTCTAGAATTCGCTTCTGTAAGATATCCATGGAAGCTGCACTG and cloned into the BamHI-HindIII sites of pET28c(+) (Novagen), resulting in pET28-vipF.

For VRE-controlled transient expression in plants, a new mini-binary vector was constructed in the following sequential steps. First the pCB302 plasmid23 was PCR-amplified using the primer pair 5’GGCGGATCCATTATCTGACATGAGCTACAGGAGGGAGAAGAGATGAGATGGCACG/5’CCGCTCTAGAATTCGCTTCTGTAAGATATCCATGGAAGCTGCACTG and self-ligated, resulting in a plasmid, designated pCB302T, with the pCB302 backbone that includes the T-DNA borders, the restriction sites AgeI and PspOMI between these borders, but lacks the original pCB302 expression cassette. The expression cassette, i.e., the 35S promoter-MCS-35S terminator sequence, from the pSAT3A plasmid24 was then inserted into the EcoRI-BamHI fragment into the EcoRI-SalI sites of pSAT1A-MCS, resulting in pSAT1A-virE2. The octopine-type VirF was then eluted with the wash buffer containing 1 M urea and 250 mM imidazole, dialyzed overnight at 4°C against 2,000 volumes of 4 mM HEPES, pH 7.5, 100 mM KCl, 8% (v/v) glycerol, 0.2% bovine serum albumin (BSA), 5 mM dithiothreitol (DTT), and 1 mM PMSF, and aliquoted and stored at ~80°C until use. The absence of significant contamination was confirmed by SDS polyacrylamide gel electrophoresis (PAGE) on Coomassie blue-stained 12.5% SDS-polyacrylamide gels as described24. All experiments used the same protein preparation batch, making data comparison more meaningful.

Preparation of biotinylated DNA probes. The EGFP coding sequence was PCR-amplified, using Pfu polymerase (Agilent), with a 5’-biotinylated reverse primer 5’-5Biosg/TATGTCTGAGCTGTCATGGC3’ (Integrated DNA Technologies) and forward primers with or without the VRE-1 sequences, thereby producing probes that corresponded to the full-length GFP with a biotin molecule downstream of GFP and with or without VRES upstream of GFP. Similar probes with a non-biotinylated reverse primer were produced for binding competition experiments. All probes were purified using Zymoclean gel DNA purification kit (Zymoresearch) according to manufacturer’s instructions; this method eliminates free biotinylated primer.

In vitro protein-DNA binding assay. The binding of VIP1 to VRE was assayed as described in25, with modifications. A 96-well plate (Nunc, Maxi-Sorp, 420402) was coated with streptavidin (Sigma S4762) by placing 60 μL of a 10-μg/ml1 streptavidin solution in each well and incubating the plate uncovered at 37°C until the water was completely evaporated, typically, for 6 to 8 h. The wells were then blocked overnight at 4°C with 300 μL/well of biotin-free 5% BSA (Sigma A7906) in TBST (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.05% (v/v) Tween-20). Next, 2 pmoles of biotinylated DNA probe in 60 μL TBST were added to each well and incubated for 1 h at 37°C. After washing the wells with 300 μL TBST pH 7.5, a second blocking step was performed with 300 μL/well of 5% non-fat dry milk (BioRad, 170-6404) in TBST, followed by additional washes with TBST. Purified VIP1, 1 μg protein per well in 60 μL of 4 mM HEPES, pH 7.5, 100 mM KCl, 8% (v/v) glycerol, 0.2% BSA, 5 mM DTT, was added for 1 h at room temperature, followed by three washes with 300 μL TBST per well. When necessary, MgCl2 or unlabeled DNA probe at the concentrations indicated for each specific experiment were preincubated for 20 min at 4°C with the VIP1 solution prior to the binding step. For the VIP1 binding in presence of Vifr, approximately equal amounts of VIP1 and Vifr, i.e., 1.0 μg of each, were added per well, for which per well were preincubated for 20 min at 4°C. The precipitation buffer performed before the DNA binding assay because our objective was to examine whether this protein-protein interaction affects the binding between VIP1 and VRE.

DNA-bound VIP1 was detected using rabbit anti-VIP1 antibody (1: 1,000 dilution in TBST) followed by goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich, 1: 500 dilution in TBST). Photometric detection of alkaline phosphatase was performed using p-nitrophenyl phosphate (Sigma, N2770) as substrate according to manufacturer’s instructions and measuring the absorbance at 405 nm with a FluorOpta Optima plate reader (BMG). For all experiments, three measurements were done for each condition, and each experiment was repeated two or three times. Results are presented as average values with standard deviations.

Plants. Tobacco plants (Nicotiana tabacum, var. Turk) were grown either in soil on or on 2% (v/v) 1/2 BAP, 1.0 g/L 1 NAA for 2 to 3 weeks. The experiment was repeated three times. As controls, tobacco plants were grown on high sucrose MS medium (30 g/L sucrose, 8 g/L agar) after seed germination and 1.7 g/L 1 BAP, 1.0 g/L 1 NAA, containing 50 mg/L 1’-timentin and 50 mg/L 1’-kanamycin, and then transferred to MS rooting medium (30 g/L sucrose, 8 g/L agar). For induction of callus, hence cell division, leaf discs from the VRE1-GUSintron tobacco plants were cultured on MS medium supplemented with 10 g/L 1 BAP and 1.0 g/L 1 NAA for 2 to 3 weeks. The experiment was repeated three times using two leaf discs per experiment.

In planta promoter activation assay. Agrobacterium strain EHA105 was transformed with the binary construct pCB302T-VRE1-GFP or pCB302T-VRE1-GUSintron and one of the constructs pRCS2-VIP1, pRCS2-VIP1-virE2, pRCS2-VIP1-virF or pRCS2-VIP1-VIP2, grown overnight at 25°C, and agroinfiltrated into N. tabacum leaves as described26. The agroinfiltrated tissues were viewed under a Zeiss LSM 5 Pascal confocal laser scanning microscope for detection of GFP expression, or subjected to GUS histochemical assay, as described27. GFP signal was quantified using the LSM Pascal software (Zeiss) by measuring the total GFP fluorescence in one field in the infiltration area with a low magnification objective (10×); all images used for fluorescence measurement were taken with the same settings. Basal signal measured in area infiltrated with VRE1-35S-MNN-GFP alone was subtracted from the values measured for each experimental condition. For each vector combination, three agroinfiltrations were performed on three different leaves.
and the GFP fluorescence of three microscope fields was measured in each infiltration area; each experiment was repeated twice.

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**Author contributions**

B.L. designed experiments, performed experiments and wrote manuscript. V.C. designed experiments and wrote manuscript.

**Additional information**

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