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Letter to the Editor

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Arabidopsis NahG Plants as a Suitable and Efficient System for Transient Expression using *Agrobacterium tumefaciens*

Dear Editor,

Research in molecular plant biology has largely benefitted from technologies allowing exogenous gene expression. While the generation of stable transgenic lines is required in some cases, transient transformation methods offer several advantages over lengthier and labour-intensive stable transformation approaches.

A large variability in susceptibility to *Agrobacterium*-mediated genetic transient transformation exists among different plant species and tissues (Krenek et al., 2015). Transient transformation is highly efficient, simple, and consistent in leaves of *Nicotiana benthamiana*, making it to be considered as a model system for protein production. Unfortunately, this assay in the *bona fide* model plant *Arabidopsis thaliana* is inefficient and lacks robustness. Efforts to increase the efficacy of *Agrobacterium*-mediated transient transformation in *Arabidopsis* have been made in the past (reviewed in Krenek et al., 2015), although improvements in most cases are restricted to seedlings or suspension cells. One of the main reasons generally believed to underlie the recalcitrance of *Arabidopsis* to *Agrobacterium*-mediated transformation is the activation of plant immune responses upon perception of the bacteria. *Arabidopsis* cells are able to recognize the pathogen-associated molecular pattern (PAMP) Elongation Factor Thermo-unstable (EF-Tu) from this bacterial species (Zipfel et al., 2006). This recognition initiates a signaling cascade ultimately leading to the onset of the so-called PAMP-triggered immunity (PTI). Interestingly, perception of EF-Tu is able to restrict *Agrobacterium*-mediated transformation without apparent effect on bacterial growth; conversely, the *efr* mutant, lacking EFR, the receptor for EF-Tu, is more amenable to *Agrobacterium*-mediated transient transformation (Zipfel et al., 2006). In agreement with the idea that efficient *Agrobacterium*-mediated transformation requires suppression of PTI, inducible expression of the bacterial effector AvrPto, which suppresses plant immunity via interference with multiple PAMP recognition receptors, increases the efficiency of this procedure in *Arabidopsis* more than the lack of EFR alone (Tsuda et al., 2012). Agrobacterial infection also activates production of the defensive hormone salicylic acid (SA) (Lee et al., 2009), which has been shown to attenuate the formation of *Agrobacterium*-induced galls in *Arabidopsis* (Yuan et al., 2007). This observation may be explained by the finding that SA inhibits the expression of bacterial *vir* genes (Yuan et al., 2007; Anand et al., 2008). However, the fact that a *sid2* mutant, which is unable to synthesize SA through the isocorismate pathway, displays a lower efficiency of *Agrobacterium*-mediated transformation than a quadruple mutant affected in SA-, jasmonic acid (JA)-, and ethylene (ET)-mediated immune responses (*dde2/ein2/pad4/sid2*) (Tsuda et al., 2012), suggests that suppression of defense signaling pathways other than those dependent on SA is required to enhance *Agrobacterium*-mediated transformation in *Arabidopsis*.

In order to determine whether responses to SA or JA influence the efficiency of *Agrobacterium*-mediated transformation of *Arabidopsis* leaves, we inoculated rosette leaves of genotypes impaired in the accumulation or signaling pathways of SA and JA with the nontumorigenic strain LBA4404 harboring a binary plasmid to express β-glucuronidase (GUS)-intron (Zipfel et al., 2006). To evaluate the effect of SA, we used the *sid2* mutant and *NahG* transgenic plants. *Arabidopsis NahG* plants express an SA hydroxylase from the bacterium *Pseudomonas putida*, which metabolizes SA to catechol, leading to a dramatic decrease in the plant SA content. To test the effect of JA responses, we used the JA biosynthetic mutant *aos*, and the JA signaling mutants *coi1*, lacking the JA receptor, and *jin1*, lacking MYC2, a transcription factor acting downstream of JA perception. The *efr* mutant and wild-type plants were used as controls.

First, GUS accumulation was estimated as GUS staining (Figure 1A). In wild-type Col-0, GUS accumulation was limited and showed large leaf-to-leaf variation, as previously reported (Zipfel et al., 2006). Whereas *jin1* and *aos* mutants behaved similarly to wild-type plants, GUS accumulation in the *coi1* mutant was lower. GUS staining was more intense in the *efr* mutant than wild-type plants. A clear increase, similar to the one reported by Tsuda et al. (2012), was also observed in *sid2* plants. Interestingly, transgenic *NahG* plants displayed significantly higher GUS accumulation than the *sid2* mutant.

These results were further supported by a quantitative GUS activity assay using the same lines (Figure 1B). As reference, values from agroinfiltrated *Nicotiana benthamina* leaves are shown. Compared with Col-0, GUS activity was ~100 times lower in *coi1*, whereas it was almost 10–20 times higher in *efr* and *sid2* plants, respectively, and more than 300 times higher in *NahG* plants, which showed 1/10 of the activity detected in *N. benthamiana*. The data are consistent with an SA-induced restriction of *Agrobacterium*-mediated transformation in *Arabidopsis*; while transformation efficiency is increased in plants with lower SA content (*sid2* and *NahG*), it is reduced in plants with activated SA defenses such as *coi1*. Strikingly, decreased SA content, as previously reported for inactivation of EFR-mediated PTI (Zipfel et al., 2006), seems to have no effect on bacterial growth (Supplemental Figure 1). High efficiency of transient transformation in *NahG* plants was also achieved

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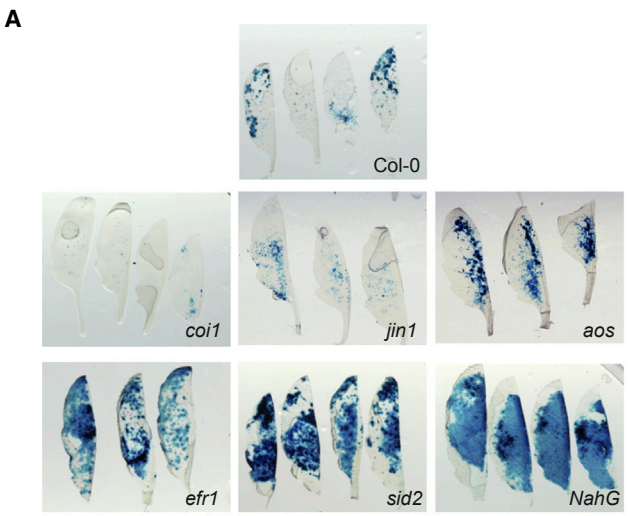

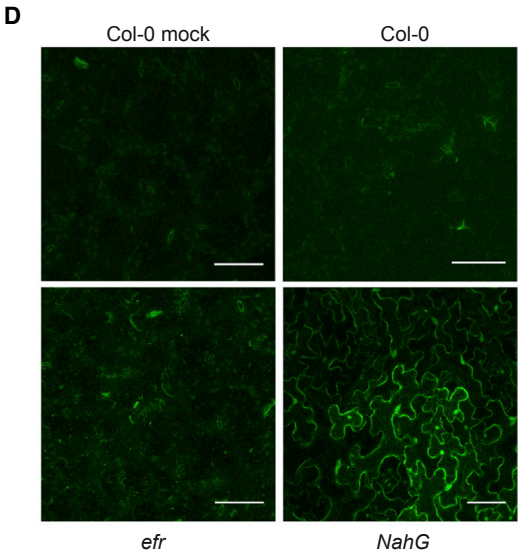

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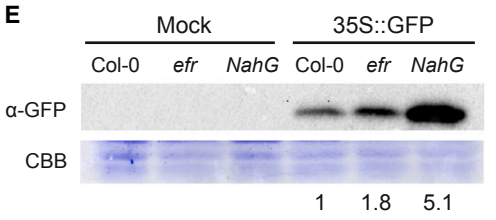
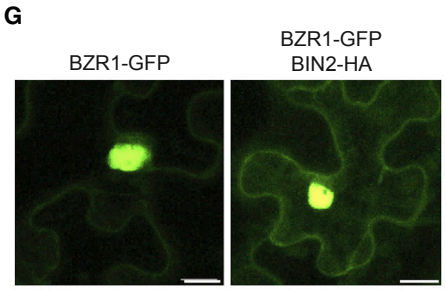
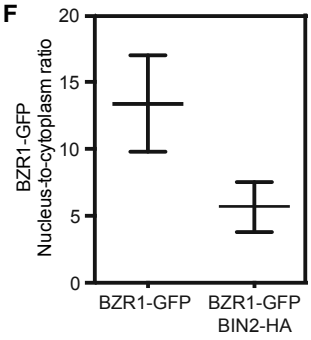
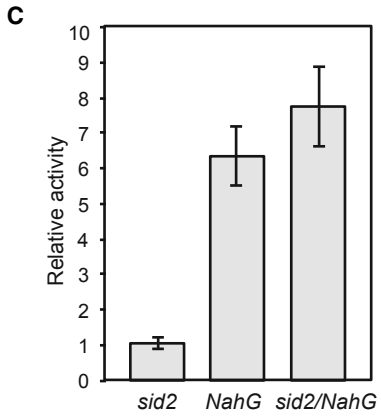
Genotypes	Relative activity
Col-0	1.00 +/- 0.23
coi1	0.01 +/- 0.01**
jin1	0.66 +/- 0.01
aos	1.63 +/- 1.30
efr	9.24 +/- 1.80*
sid2	19.25 +/- 8.38*
NahG	312.69 +/- 56.82**
N. benthamiana	3531.01 +/- 1701.07**









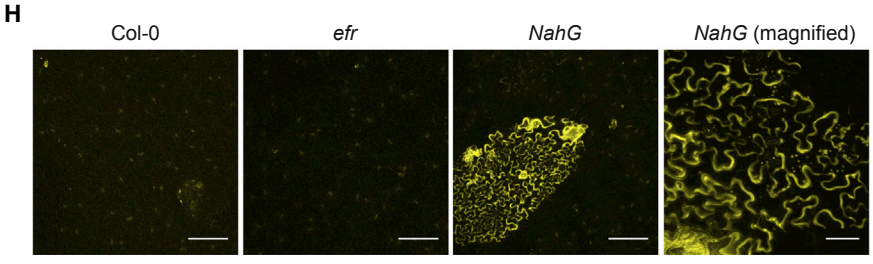


Figure 1. Expression of *NahG* Increases the Efficiency of *Agrobacterium*-Mediated Transient Transformation in *Arabidopsis* Leaves.
(A and B) GUS staining and activity in agroinfiltrated leaves of wild-type *Arabidopsis thaliana* and mutants lacking the receptor for bacterial EF-Tu (*efr*), or impaired in SA (*sid2* and *NahG*) or JA (*coi1*, *jai1* and *aos*) pathways. **(A)** Leaves were stained with X-Gluc at 4 days post infiltration (dpi) for detection of GUS activity. Each picture shows three to four leaves per mutant detached from a single plant. **(B)** GUS activity was measured using protein extracts obtained from four agroinfiltrated leaves of the same plant. The activity values have been normalized to the activity detected in wild-type (Col-0) plants. Values represent means and SE of n = 9 (wild-type Col-0 indicated as Col-0), n = 6 (*coi1*), n = 6 (*jin1*), n = 6 (*aos*), n = 9 (*efr*), n = 6 (*sid2*), n = 9 (*NahG*), and n = 6

(legend continued on next page)

employing other commonly used *Agrobacterium* strains (Supplemental Figure 2).

To determine whether the enhanced efficiency of *Agrobacterium*-mediated transient transformation of *NahG* plants depends on the accumulation of the SA-degradation product catechol rather than depletion of the hormone, we used *sid2*/*NahG* plants, which accumulate very low levels of SA and hence also of catechol (Nawrath and Metraux, 1999; Heck et al., 2003). As shown in Figure 1C, β-glucuronidase activity was similar in *NahG* and *sid2*/*NahG* plants, supporting that the enhanced transformability of *NahG* plants is not linked to catechol accumulation. However, differences between *sid2* mutants and *NahG* plants in susceptibility to *Pseudomonas syringae* and *Pernospora parasitica*, as well as in ethylene and camalexin accumulation in response to *Pseudomonas syringae*, have been described (Nawrath and Metraux, 1999; Heck et al., 2003), suggesting the induction of pleiotropic changes in defense signaling in *NahG* plants that are unlikely to be a result of their low SA content alone. Whether those SA-independent compensation pathways, which do not operate in *NahG* plants but are active in the *sid2* mutant, are responsible for the observed differences in transformation efficiency remains to be determined.

We next explored whether assays to study protein subcellular localization or protein–protein interactions could be successfully carried out in *Arabidopsis NahG* plants. As shown in Figure 1D, while GFP accumulation upon *Agrobacterium*-mediated transformation is weak in wild-type Col-0 and only slightly improved in the *efr* mutant, it is largely increased in *NahG* plants, and the nuclear/cytoplasmic subcellular localization of this protein can be clearly observed using confocal microscopy. To further evaluate the potential of transient expression in *NahG* plants to detect changes in subcellular localization of a protein of interest, we used the *Arabidopsis* protein BZR1 (BRASSINAZOLE RESISTANT 1), which is in the nucleus in its unphosphorylated form, but whose phosphorylation by BIN2 (BRASSINOSTEROID INSENSITIVE 2) favors its cytoplasmic retention (reviewed in Belkhadir and Jaillais, 2015). We analyzed the subcellular localization of transiently overexpressed BZR1-GFP in the presence or absence of transiently overexpressed BIN2-HA. The results obtained confirm that, in the absence of overexpressed BIN2-HA, BZR1-GFP accumulates mainly in the nucleus, while overexpression of BIN2-HA increases its cytoplasmic retention (Figure 1F). Quantification of BZR1-GFP fluorescent intensity ratified that the ratio of nuclear to cytoplasmic BZR1-GFP signal is reduced when co-overexpressed with BIN2-HA (Figure 1G).

To evaluate the feasibility of performing bimolecular fluorescent complementation (BiFC) assays upon *Agrobacterium*-mediated transient transformation of *NahG* plants, we used the capsid protein (CP) of *Prunus necrotic ringspot virus*, which is known to self-interact (Aparicio et al., 2006). Restoration of YFP fluorescence was visible in *NahG* plants transiently transformed with constructs to express nYFP-CP and cYFP-CP, whereas it was not in wild-type or *efr* mutant plants (Figure 1H). Accumulation of YFP was also dramatically increased in *NahG* plants when compared with the *efr* mutant or wild-type plants (Supplemental Figure 3).

Taken together, our results show that expression of the *NahG* transgene in *Arabidopsis* dramatically enhances the efficiency of *Agrobacterium*-mediated transformation in rosette leaves, enabling the routine use of this technique in transient assays. This method can be successfully applied in SA-depleted *Arabidopsis* plants for transient expression-based functional assays routinely done in *N. benthamiana*, which would facilitate the use of the plethora of tools and knowledge generated in *Arabidopsis*. The use of this assay does not require complex inoculation media, supplements, or specific growth conditions, and can be used with different *Agrobacterium* strains. A high level of expression has also been previously achieved in *Arabidopsis* transgenic plants containing an inducible cassette to express the bacterial effector AvrPto from a DEX-inducible promoter (Tsuda et al., 2012). However, the assay reported here overcomes the use of DEX treatment and the putative multi-side effects potentially derived from the expression of a bacterial effector that interferes with multiple receptor-like kinases and whose constitutive expression is most likely detrimental for the plant.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

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(*N. benthamiana*) plants. Asterisks indicate samples that are statistically different from the Col-0 value (**$P < 0.01$; *$P < 0.05$), as determined by Student's t test.

(C) GUS activity in *sid2*, *NahG*, and *sid2*/*NahG* agroinfiltrated leaves. Activity values have been normalized to the activity detected in *sid2* plants. Values represent means of n = 10 plants from two independent experiments. Bars represent SE.

(D and E) Expression of GFP in agroinfiltrated leaves of wild-type (Col-0), *efr*, or *NahG Arabidopsis* plants. **(D)** Confocal images show GFP accumulation in rosette leaves at 4 dpi of agroinfiltration solution (Col-0 mock) or 35S::GFP (Col-0, efr, and NahG). At least three plants/genotype and four leaves/plant were used. Scale bars represent 50 μm. **(E)** Western blot of leaves shown in **(D)** with anti-GFP. Coomassie blue staining of the blot is shown as loading control. Numbers represent the amount of GFP protein relative to the protein accumulated in Col-0 plants. Quantification of GFP was determined from western blot images using ImageJ. Similar results were obtained in three independent experiments.

(F and G) BIN2-HA induces cytosolic accumulation of BZR-GFP in *NahG Arabidopsis* plants upon *Agrobacterium*-mediated transient transformation. **(F)** Subcellular localization of BZR1-GFP alone or co-expressed with BIN2-HA in *NahG Arabidopsis* leaves. **(G)** Average ratios between nuclear and cytoplasmic signal intensities and standard errors (SEM) calculated based on three independent measures from three cells per plant. This experiment was repeated twice with similar results. Scale bars represent 10 μm.

(H) Bimolecular fluorescent complementation (BiFC) assay using Col-0, *efr*, and *NahG Arabidopsis* leaves agroinfiltrated with a construct to express the capsid protein of PNRSV fused to the C terminus or the N terminus of YFP (CYFP-CP and NYFP-CP, respectively) and observed under the confocal microscope at 3 dpi. Scale bars represent 150 μm. Detail of *NahG* leaves co-infiltrated with NYFP-CP and CYFP-CP is shown. Scale bar represents 50 μm. A minimum of seven plants per genotype and four leaves per plant were used in this experiment.

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AUTHOR CONTRIBUTIONS

E.R.B. and R.L.-D. conceived the project. All authors designed the experiments, which were performed by T.R.-D., P.C.-Q., and V.A.-S., and supervised by E.R.B. and M.A.B. T.R.-D., P.C.-Q., and E.R.B. analyzed data. All authors contributed to the writing of the manuscript.

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REFERENCES

Anand, A., Uppalapati, S.R., Ryu, C.M., Allen, S.N., Kang, L., Tang, Y., and Mysore, K.S. (2008). Salicylic acid and systemic acquired resistance play a role in attenuating crown gall disease caused by *Agrobacterium tumefaciens*. Plant Physiol. **146**:703–715.

Aparicio, F., Sanchez-Navarro, J.A., and Pallas, V. (2006). In vitro and in vivo mapping of the *Prunus* necrotic ringspot virus coat protein C-terminal dimerization domain by bimolecular fluorescence complementation. J. Gen. Virol. **87**:1745–1750.

Belkhadir, Y., and Jaillais, Y. (2015). The molecular circuitry of brassinosteroid signaling. New Phytol. **206**:522–540.

Heck, S., Grau, T., Buchala, A., Metraux, J.-P., and Nawrath, C. (2003). Genetic evidence that expression of NahG modifies defence pathways independent of salicylic acid biosynthesis in the *Arabidopsis-Pseudomonas syringae* pv. tomato interaction. Plant J. **36**:342–352.

Krenek, P., Samajova, O., Luptovciak, I., Doskocilova, A., Komis, G., and Samaj, J. (2015). Transient plant transformation mediated by *Agrobacterium tumefaciens*: principles, methods and applications. Biotechnol. Adv. **33**:1024–1042.

Lee, C.W., Efetova, M., Engelmann, J.C., Kramell, R., Wasternack, C., Ludwig-Muller, J., Hedrich, R., and Deeken, R. (2009). *Agrobacterium tumefaciens* promotes tumor induction by modulating pathogen defense in *Arabidopsis thaliana*. Plant Cell **21**:2948–2962.

Nawrath, C., and Metraux, J.P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11**:1393–1404.

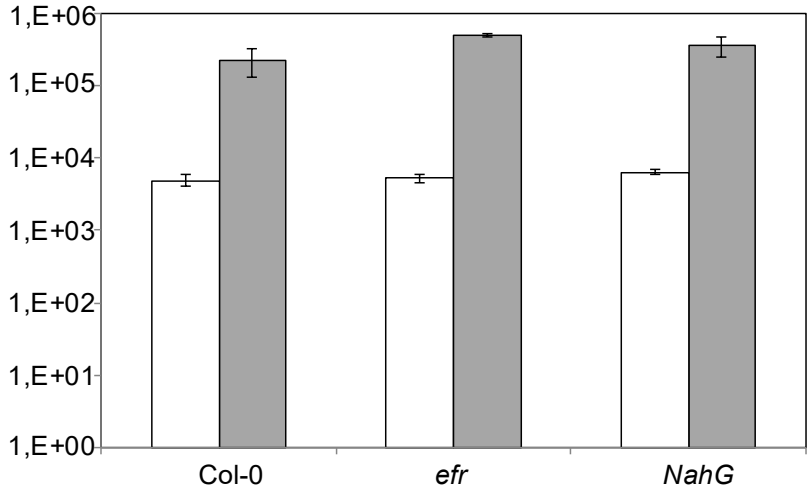
Tsuda, K., Qi, Y., Nguyen le, V., Bethke, G., Tsuda, Y., Glazebrook, J., and Katagiri, F. (2012). An efficient *Agrobacterium*-mediated transient transformation of *Arabidopsis*. Plant J. **69**:713–719.

Yuan, Z.C., Edlind, M.P., Liu, P., Saenkham, P., Banta, L.M., Wise, A.A., Ronzone, E., Binns, A.N., Kerr, K., and Nester, E.W. (2007). The plant signal salicylic acid shuts down expression of the vir regulon and activates quormone-quenching genes in *Agrobacterium*. Proc. Natl. Acad. Sci. USA **104**:11790–11795.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell **125**:749–760.

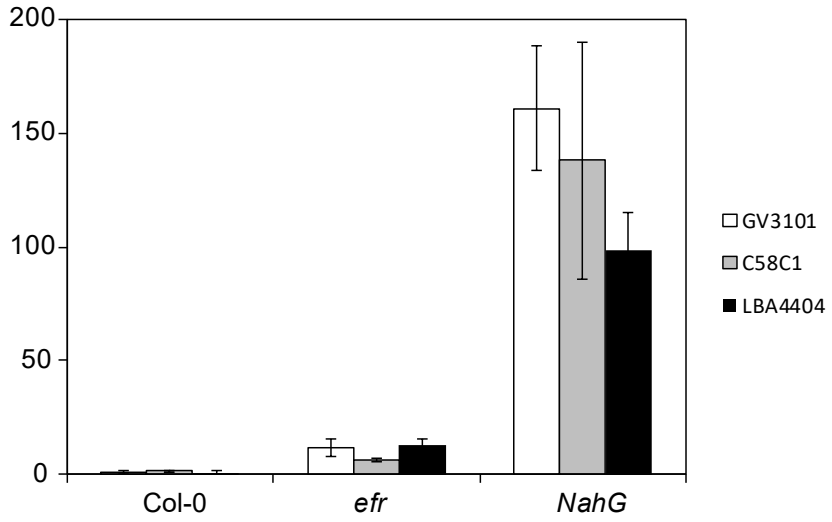
SUPPLEMENTAL INFORMATION

Figure S1



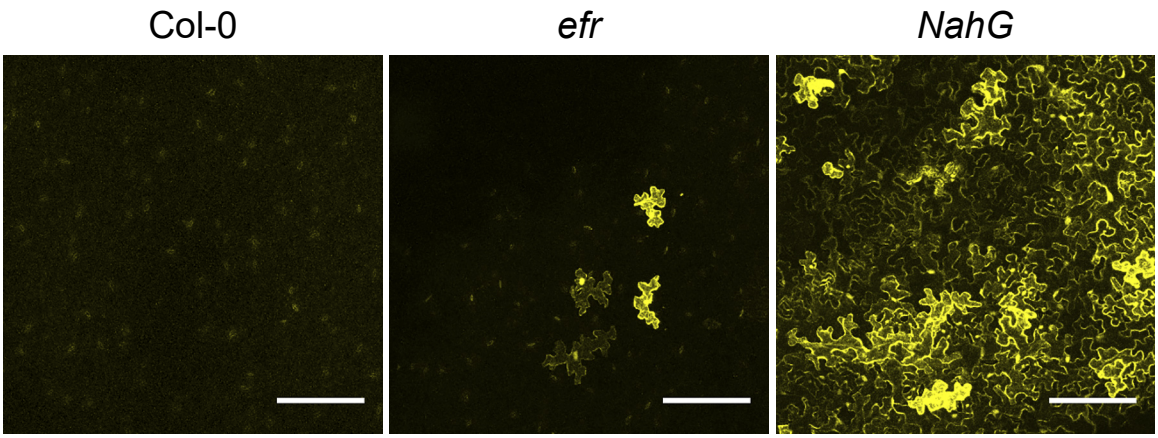
Supplemental figure 1. Bacterial growth in wild type (Col-0), *efr* and *NahG* plants. Bacterial counts were obtained at 0 and 4 days post infiltration (dpi). The values are the mean of 3 independent plants and 3 leaf discs per plant. Bars represent standard error. The experiment was repeated twice with similar results.

Figure S2



Supplemental figure 2. GUS activity from leaves of wild type (Col-0), *efr* and *NahG* plants agroinfiltrated with three Agrobacterium strains (GV3101, C58C1 or LBA4404). The activity values have been normalized to the activity detected in wild type plants agroinfiltrated with GV3101. Values represent means of n=15 plants from three independent experiments. In all cases, GUS activity was measured using protein extracts obtained from four leaves from the same plant four days post-infection. Bars represent standard error.

Figure S3



Supplemental figure 3. YFP accumulation in infiltrated leaves from wild-type (Col-0) and *efr* and *NahG* plants with a construct to express YFP. Scale bars represent 150 µm. A minimum of 7 plants per genotype and 4 leaves per plant were used in this experiment

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana (Arabidopsis) plants accession Columbia (Col-0) wild type (wt) and mutant derivatives were grown in growth chambers with 8 h light:16 h dark cycles at 21°C. The following mutants or transgenics have been described elsewhere: *efr-1* (Zipfel et al., 2006), *NahG* (Lawton et al., 1995), *sid2-1* (Wildermuth et al., 2001), *coi1-1* (Xie et al., 1998), *jin1-1* (Berger et al., 1996), *aos*/*dde2-2* (von Malek et al., 2002), *sid2/NahG* (Zipfel et al., 2006). *Nicotiana benthamiana* plants were grown in soil at 22ºC in long day conditions (16 h light:8 h dark photoperiod).

Transient expression assays

Agrobacterium tumefaciens (Agrobacterium)-mediated expression in Arabidopsis was performed as described in Zipfel et al., (2006) with some modifications. Agrobacterium LBA4404, GV3101 and C58C1 carrying a GUS-intron or a GFP transgene cloned into pBIN19g vector bacteria were resuspended in infiltration solution (10 mM morpholineethanesulfonic acid pH 5.6, 10 mM $MgCl_2$, and 100 µM acetosyringone) at OD_{600}=0.05 for injection into leaves of 4- to 5-week-old plants. At least 6 plants/genotype and 4 leaves/plant were used per experiment.

Agrobacterium replication assay

Agrobacterium strain LBA4404 carrying a GUS-intron transgene cloned into pBIN19g vector (Zipfel et al., 2006) was grown at 28ºC in LB medium supplemented with rifampicin (50 µg/ml) and kanamycin (50 µg/ml). Bacteria were suspended in 10 mM $MgCl_2$ before inoculations. Four- to five-week-old Arabidopsis plants were inoculated by infiltrating with a $5x10^4$ cfu/ml bacterial suspension using a blunt syringe. Samples were taken from inoculated leaves at 4 dpi using a 10 mm-diameter cork borer. Three disks were taken per plant, placed into 1ml of 10 mM $MgCl_2$, and homogenized by mechanical disruption. Serial dilutions of the resulting bacterial suspensions were plated onto LB plates supplemental with cycloheximide (2 µg/ml), rifampicin (50 µg/ml) and kanamycin (50 µg/ml). At least 6 plants/genotype and 4 leaves/plant were used per experiment.

GUS assays

GUS staining was performed according to the protocol described by (Ranjan et al., 2012) with minor modifications. Plant tissues were immersed in histochemical GUS staining buffer (100 mM $NaPO_4$ pH7, 0.5 mM $K_3[Fe(CN)_6$, 0.5 mM $K_4[Fe(CN)_6]$, 20% Methanol, 0.3% Triton X-100 and 2 mM 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium (X-gluc)

(Gold Biotechnology, USA) on multi-well plates, vacuum-infiltrated (60 cm Hg) for 10 min three times, and then wrapped in aluminium foil and incubated at 37°C for 12 h. Samples were then washed several times with 95% ethanol until complete tissue clarification, stored in 50% glycerol and photographed.

Quantitative measurements of GUS activity were performed adapting the protocol described by (Francis and Spiker, 2005). Plant tissues were collected in 2ml Eppendorf tubes and frozen in liquid nitrogen. Samples were grinded using TissueLyser II (Qiagen), given 2 pulses of 10 s at 30 s^{-1}. Fluorescence was measured using a fluorometer BIO-TEK, FL600. Every plate included a standard curve corresponding to 1000, 500, 100, 50, 25, 5, 2.5, 0.5, 0.25, and 0 µM 4-Methylumbelliferone (MU) used to calculate the amount of liberated MU per sample. Activity values were then relativized to protein concentration. Protein concentration was determined using the method described by (Bradford, 1976) and analysed on a microplate reader 2001 (WITTAKER) set to measure absorbance at 595 nm. Two to three independent experiments were performed; the values obtained in every experiment have been considered to calculate the means.

Subcellular localization and Bimolecular fluorescence complementation (BiFC) assays

To express GFP, leaves were infiltrated with Agrobacterium strain GV3101 harbouring the binary plasmid 35S::GFP clone (Morilla et al., 2006). Assay for BZR1 localization was performed as described in Kim et al., 2009. The binary plasmid for BIN2-HA is described elsewhere (Bernardo-Garcia et al., 2014). For expression of BZR1-GFP, BZR1 full length coding region was Gateway-cloned from pDONR-BZR1 (Lozano-Duran et al., 2014) to the Gateway-destination vector pGWB5 (Nakagawa et al., 2007).

BiFC assays were carried out co-agroinfiltrating using the plasmids described in Aparicio et al. (2006) to express YFP (pMOG YFP), nYFP (pMOG nYFP), cYFP (pMOG nYFP), or CP fused to nYFP (pMOG nYFP-CP) or to cYFP (pMOG cYFP-CP) in the Agrobacterium strain GV3101.

Confocal imaging

BiFC and GFP, BZR1-GFP overexpression images were obtained using a Leica TCS SP5 II equipped with a 488-nm argon laser for GFP and YFP. Leica LAS AF Lite platform or the Java-based image-processing program ImageJ (National Institutes of Health) were used in the processing of all microscopy images. Images for BiFC, GFP and BZR1-GFP are, respectively, a maximum projection of 3, 7 or 1µm-spaced stacks in the z-axis.

Western blotting

Total proteins were extracted from three plants (four agroinfiltrated leaves per plant) using Laemmli buffer, resolved by SDS polyacrylamide gel electrophoresis, and transferred by electroblotting onto a polyvinylidene diflouride membrane (Immobilon-P; Millipore). Proteins were stained with Coomassie blue and analysed for antibody reaction: GFP antibody (Santa Cruz Biotechnology; sc 9996) was used at a dilution 1:200 and developed by a second peroxidase-conjugated antibody (Sigma-Aldrich; A9044) through enhanced chemiluminescence (SuperSignal™ West Femto Maximum Sensitivity Substrate, ThermoFisher Scientific) using a Chemidoc XRS+System (Biorad).

Quantification of Fluorescent Protein Signal

Images were analysed using FIJI (Schindelin et al., 2012). To measure the ratio between nuclear and cytoplasmic signals of BZR1-GFP for each cell, a small area of fixed size (20 pixels) was drawn, and measurements of integrated densities were taken from representative areas within the nucleus and cytoplasm of each cell.

REFERENCES

Aparicio, F., Sanchez-Navarro, J.A. and Pallas, V. (2006) In vitro and in vivo mapping of the Prunus necrotic ringspot virus coat protein C-terminal dimerization domain by bimolecular fluorescence complementation. J Gen Virol **87:** 1745-1750

Berger, S., Bell, E. and Mullet, J.E. (1996) Two Methyl Jasmonate-Insensitive Mutants Show Altered Expression of AtVsp in Response to Methyl Jasmonate and Wounding. Plant Physiol **111:** 525-531

Bernardo-Garcia S., de Lucas M., Martinez C., Espinosa-Ruiz A., Daviere J.M. and Prat S. (2014) BR-dependent phosphorylation modulates PIF4 transcriptional activity and shapes diurnal hypocotyl growth. Genes Dev **28:** 1681-1694

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248-254

Francis, K.E. and Spiker, S. (2005) Identification of Arabidopsis thaliana transformants without selection reveals a high occurrence of silenced T-DNA integrations. Plant J **41:** 464-477

Kim, T.W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J.X., Sun, Y., Burlingame, A.L. and Wang, Z.Y. (2009) Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. Nat Cell Biol **11:** 1254-1260

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S. and Ryals, J. (1995) Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. Mol Plant Microbe Interact **8:** 863-870

Lozano-Duran, R., Bourdais, G., He, S.Y. and Robatzek, S. (2014) The bacterial effector HopM1 suppresses PAMP-triggered oxidative burst and stomatal immunity. New Phytol **202:** 259-269

Morilla, G., Castillo, A.G., Preiss, W., Jeske, H. and Bejarano, E.R. (2006) A versatile transreplication-based system to identify cellular proteins involved in geminivirus replication. J Virol **80:** 3624-3633

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T. and Kimura, T. (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng **104:** 34-41

Ranjan, R., Patro, S., Pradhan, B., Kumar, A., Maiti, I.B. and Dey, N. (2012) Development and functional analysis of novel genetic promoters using DNA shuffling, hybridization and a combination thereof. PLoS One **7:** e31931

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V, Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods **9:** 676-682

von Malek, B., van der Graaff, E., Schneitz, K. and Keller, B. (2002) The Arabidopsis male-sterile mutant dde2-2 is defective in the ALLENE OXIDE SYNTHASE gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta **216:** 187-192

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562-565

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280:** 1091-1094

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, JD., Boller, T. and Felix, G. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell **125:** 749-760

FIGURE LEGENDS

Supplemental figure 1. Bacterial growth in wild type (Col-0), *efr* and *NahG* plants. Bacterial counts were obtained at 0 and 4 days post infiltration (dpi). The values are the mean of 3 independent plants and 3 leaf discs per plant. Bars represent standard error. This experiment was repeated twice with similar results.

Supplemental figure 2. GUS activity from leaves of wild type (Col-0), *efr* and *NahG* plants agroinfiltrated with three Agrobacterium strains (GV3101, C58C1 or LBA4404). The activity values have been normalized to the activity detected in wild type plants agroinfiltrated with GV3101. Values represent means of n=15 plants from three independent experiments. In all cases, GUS activity was measured using protein extracts obtained from four leaves from the same plant four days post-infection. Bars represent standard error.

Supplemental figure 3. YFP accumulation in leaves from wild type (Col-0) and *efr* and *NahG* plants agroinfiltrated with a construct to express YFP. Scale bars represent 150 μm. A minimum of 7 plants per genotype and 4 leaves per plant were used in this experiment.