Agroinfiltration by Cytokinin-Producing *Agrobacterium* sp. Strain GV3101 Primes Defense Responses in *Nicotiana tabacum*

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Transient infiltrations in tobacco are commonly used in plant studies, but the host response to different disarmed *Agrobacterium* strains is not fully understood. The present study shows that pretreatment with disarmed *Agrobacterium tumefaciens* GV3101 primes the defense response to subsequent infection by *Pseudomonas syringae* in *Nicotiana tabacum*. The presence of a *trans*-zeatin synthase (*tzs*) gene in strain GV3101 may be partly responsible for the priming response, as the *tzs*-deficient *Agrobacterium* sp. strain LBA4404 only weakly imparts such responses. Besides inducing the expression of defense-related genes like *PR-1* and *NHL10*, GV3101 pretreatment increased the expression of tobacco mitogen-activated protein kinase (MAPK) pathway genes like *MEK2*, *WIPK* (wound-induced protein kinase), and *SIPK* (salicylic acid-induced protein kinase). Furthermore, the GV3101 strain showed a stronger effect than the LBA4404 strain in activating phosphorylation of the tobacco MAPK, WIPK and SIPK, which presumably prime the plant immune machinery. Lower doses of exogenously applied cytokinins increased the activation of MAPK, while higher doses decreased the activation, suggesting a balanced level of cytokinins is required to generate defense response in planta. The current study serves as a cautionary warning for plant researchers over the choice of *Agrobacterium* strains and their possible consequences on subsequent pathogen-related studies.

Plants constantly encounter a plethora of both benign and harmful microorganisms. Some harmless microbes (e.g., certain *Bacillus* spp.) can benefit plants by inducing systemic resistance that primes the plants against disease-causing microbes. With the advent of modern biotechnology, some pathogenic microbes have been rendered harmless to the plant but their infection mechanisms are exploited for genetic engineering or other beneficial applications. The best example is *Agrobacterium tumefaciens*. Oncogenic *A. tumefaciens* strains like C58 transfer the T-DNA region of their Ti-plasmid into the plant cell, thereby causing crown gall disease in dicot plants

(McCullen and Binns 2006). The T-DNA carries genes for the biosynthesis of plant hormones (auxin and cytokinin) and alkaloid (opine) metabolism. The expression of these genes hijacks the plant metabolic machinery, eliciting a hyperplastic response and, finally, causing gall formation (Ditt et al. 2001). Disarmed *A. tumefaciens* (e.g., strain GV3101) lack the T-DNA locus but contain the *virulence* (*vir*) region necessary for plant transformation (Gelvin 2003).

Agrobacterium spp. can be broadly categorized into octopine- and nopaline-producing strains, which are known to differ in their virulence on plants like *Nicotiana glauca* (Melchers et al. 1990). This is mainly attributed to the presence of the *virF* locus in octopine-producing strains, which render them more virulent than nopaline-producing strains. On the other hand, octopine-producing wild-type strains like A6 are not able to efficiently transfer their T-DNA to maize cells, whereas nopaline-producing strains like C58 are (Jarchow et al. 1991). Thus, it was hypothesized that the octopine-producing strain-specific *virF* locus inhibits the T-DNA transfer into monocot maize cells. Additional *vir* loci also create a striking difference between octopine- and nopaline-producing strains. For example, the *trans*-zeatin synthase (*tzs*) locus is only present in nopaline-type Ti plasmids like pTiC58. It lies in the vicinity of the *vir* region and enables the bacteria to produce and secrete *trans*-zeatin (*t-Z*). Unlike plants, *Agrobacterium t-Z* is produced directly, without the requirement of cytochrome P450 monooxygenase-mediated hydroxylation (Sakakibara et al. 2005). The presence of the *tzs* gene in *Agrobacterium rhizogenes* 1855 is known to increase the induction of hairy roots in flax (*Linum usitatissimum* L.) (Zhan et al. 1990). *Nicotiana benthamiana* and tobacco leaf explants were recently found to exhibit increased shoot formation and regeneration when co-cultured with *tzs*-containing *Agrobacterium* sp. strain GV3101 (Han et al. 2013).

All natural cytokinins are chemical derivatives of adenine with different N⁶- side chains. Cytokinins are phytohormones that control plant growth and development, including the regulation of cell division, seed dormancy and germination, senescence, leaf expansion, bud development, and reversal of apical dominance (Spichal 2012). Plant-derived cytokinins have recently been shown to systematically induce plant defense responses, either through the salicylic acid (SA)-mediated pathway in *Arabidopsis thaliana* (Choi et al. 2010) or through the increase in phytoalexin production in tobacco (Grosskinsky et al. 2011). Gall-forming plant pathogenic bacteria like *A. tumefaciens* and *Rhodococcus fascians* as well as biotrophic fungi like *Puccinia striiformis* activate a complex interplay of auxin

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and cytokinin-regulated responses to possibly enhance their pathogenicity (Choi et al. 2011). Agonistic microbes that promote plant growth have evolved the ability to produce cytokinins to enhance the overall plant defense machinery or prime the host plants for a faster defense response. Accordingly, exogenously applied cytokinins have been shown to enhance disease resistance to powdery mildew infection (Liu and Bushnell 1986). It is, however, still unknown how cytokinins induce priming responses. A possible mechanism might be through the actions of mitogen-activated protein kinases (MAPK), which are required for proper priming responses in *A. thaliana* (Beckers et al. 2009).

Disarmed *Agrobacterium* strains are widely used in transient gene-expression assays in plants, especially in *Nicotiana* spp. Though the plants do not show any obvious macroscopic response to it, agroinfiltration has an impact on the plant immune system and priming responses (Conrath et al. 2002; Ditt et al. 2001). Pretreatment with a nonpathogenic bacterium induces PTI (pathogen-associated molecular pattern [PAMP]-triggered immunity) and suppresses the hypersensitive response (HR) or disease symptoms arising from subsequent infection by a pathogenic bacterium (De Vleeschauwer et al. 2008). Infection of *Nicotiana tabacum* leaves with disarmed *A. tumefaciens* induced *PR-1* expression in the infiltrated zone and protected the leaves from subsequent *Tobacco mosaic virus* infection (Pruss et al. 2008). The preinfiltration of disarmed *A. tumefaciens* nopaline-producing strain GV3101 into *N. taba-*

cum leaves reduced the macroscopic symptoms of disease or HR associated with subsequent *Pseudomonas syringae* infection in the infiltrated zone (Rico et al. 2010). The agroinfiltration elicited mild callose deposition and reduced abscisic acid (ABA) levels in tobacco leaves. After *P. syringae* infection, the preagroinfiltrated leaves displayed reduced SA biosynthesis and increased callose deposition, thereby reducing the pathogen growth. The one or more factors responsible for the effect remain largely unclear.

In this study, we compared the representative nopaline and octopine disarmed strains, GV3101 and LBA4404, for macroscopic symptom reduction following *P. syringae* infection in *N. tabacum*. It was observed that only the GV3101 strain was able to reduce disease symptoms, while the LBA4404 strain failed to do so. Despite the possibility of a complex crosstalk, we hypothesize that *Agrobacterium* sp. strain GV3101 may prime the plant defense machinery, possibly through the production of cytokinins. The priming effect may occur by inducing the levels of MAPK at transcription level or increasing the activity of MAPK by phosphorylation at post-translational level. Additionally, the exogenous application of low doses of cytokinins was shown to activate the tobacco MAPK. Our data indicates that agroinfiltration can have significant effects on plant defense-signal transduction pathways and supports the hypothesis that cytokinins prime the plant defense machinery in addition to being a vital hormone for normal plant growth and development.

Fig. 1. AgroGV3101 but not AgroLBA4404 infiltration of tobacco leaves prior to *Pseudomonas syringae* infection reduces macroscopic symptoms and *P. syringae* growth. **A**, *Nicotiana tabacum* leaves were infiltrated with IM (infiltration media) or approximately 10^8 CFU of AgroGV3101 or AgroLBA4404 per milliliter. After 24 h, leaves were infected with approximately 2.5×10^7 CFU/ml of *P. syringae* pv. *tabaci* (Pta) or *P. syringae* pv. *tomato* DC3000 (Pto). Images shown are the infiltrated leaf area after 7 days of infection (for better visualization of the disease symptoms). **B**, AgroLBA4404 alone and 4xJERE:ipt (in pCambia1380 vector) containing AgroLBA4404 were infiltrated in the tobacco leaves. After 24 h, the infiltrated regions were infected with either *P. syringae* pv. *tabaci* or *P. syringae* pv. *tomato* DC3000, and macroscopic symptoms were photographed after 14 days of infection. **C**, Quantitative reverse transcription-polymerase chain reaction expression analysis showing the expression of *ipt* (*Agrobacterium tmr* gene) in 4xJERE:ipt-transformed AgroLBA4404. **D**, Bacterial growth quantification of *P. syringae* pv. *tabaci* in agroinfiltrated leaves. Tobacco leaves were infiltrated with either IM, AgroGV3101, or AgroLBA4404 as described in A, followed by *P. syringae* pv. *tabaci* infection after 24 h. CFU per 10 mm² leaf disc were calculated at indicated timepoints postinfection. Leaf extracts (10^3 times diluted) were selected for *P. syringae* pv. *tabaci* colonies on LB+tetracycline plates. Data shown is the average of three triplicates with error bars indicating standard deviation. T-test performed within the same-day CFU counts shows significant results as * ($P \leq 0.05$) and highly significant as ** ($P \leq 0.001$; agrotreated in comparison with IM treated).

RESULTS

Preinfiltration of *Agrobacterium* sp. strain GV3101 but not strain LBA4404 reduces the macroscopic symptoms associated with *P. syringae* infection.

Using different infiltration and infection combinations, we observed that leaves preinfiltrated with *A. tumefaciens* GV3101 (hereafter called AgroGV3101) exhibited a loss of macroscopic cell-death symptoms that typically developed after infection with virulent *P. syringae* pv. *tabaci* or with the nonadapted *P. syringae* pv. *tomato* DC3000. Surprisingly, leaves preinfiltrated with *Agrobacterium* sp. strain LBA4404 (hereafter AgroLBA4404) did not prevent the formation of macroscopic symptoms, albeit only slightly, during the early phase of infection (Fig. 1A). Since AgroGV3101 has the ability to produce *t-Z* because it contains the *tzs* gene, which is absent in octopine-producing AgroLBA4404, we next asked whether the cytokinin-producing ability of AgroGV3101 is responsible for this phenomenon. To prove this, we transformed AgroLBA4404 with the pCambia1380 vector expressing the *ipt* gene (the *Agrobacterium* *tmr* gene, which is

highly similar to *tzs*) under the control of a synthetic promoter with four copies of the jasmonate- and elicitor-responsive elements (*JERE*). Symptoms of plants preinfiltrated with AgroLBA4404 containing this *4xJERE:ipt* construct now resembled the AgroGV3101 preinfiltrated leaves (Fig. 1B). The expression of *ipt* in the AgroLBA4404(*4xJERE:ipt*)-infiltrated leaves was confirmed by quantitative real time-polymerase chain reaction (qRT-PCR) (Fig. 1C).

To check whether the macroscopic symptom reduction correlates with pathogen progress, bacterial growth was evaluated. The AgroGV3101 preinfiltrated leaves showed a statistically significant decrease in *P. syringae* pv. *tabaci* bacterial numbers after 2 and 10 days compared with leaves pretreated with infiltration medium (IM), which is stronger than the reduction seen with AgroLBA4404 pretreatment (Fig. 1D). The *P. syringae* pv. *tabaci* growth in the leaves preinfiltrated with AgroLBA4404(*4xJERE:ipt*) is (at least at the 2-days postinfection [dpi] timepoints) intermediate between that of the AgroGV3101- and AgroLBA4404-treated leaves. At 10 dpi, the differences between AgroLBA4404(*4xJERE:ipt*) and AgroLBA4404 are no longer apparent, thus suggesting only a

Fig. 2. AgroGV3101 infiltration primes callose deposition and enhances pathogen-triggered reactive oxygen species (ROS) production in tobacco. **A**, Callose deposition in agroinfiltrated leaves before and after the *Pseudomonas syringae* infection. Tobacco leaves were infiltrated with infiltration medium (IM), AgroGV3101, and AgroLBA4404 and, after 48 h, were challenged with mock (10 mM MgCl₂), *P. syringae* pv. *tabaci* (Pta) or *P. syringae* pv. *tomato* DC3000 (Pto). After 24 h, leaf sections were stained with aqueous aniline blue and were imaged under UV light (420 nm). Photos are representative of three leaf areas of 2 mm² each (scale bar = 100 µm). The experiment was repeated twice with similar results. **B**, Quantification of callose deposition after agroinfiltrations in tobacco leaves. Prominent callose spots were counted per square millimeter of leaf area (error bars depict standard deviations). Asterisks mark significant differences compared with the mock inoculation on the basis of a Student's *t* test (* indicates *P* ≤ 0.001). **C** and **D**, ROS production in agroinfiltrated leaves after pathogen-associated molecular pattern treatments. H₂O₂ production in agroinfiltrated tobacco leaf discs was measured using a luminol-based ROS assay after elicitation with 100 nM flg22 (C) or 200 µg of crab shell chitin per milliliter (D).

partial “complementation” through cytokinin production. Hence, while there are clearly other bacterial factors that contribute to *A. tumefaciens*–mediated resistance, cytokinin appears to contribute to this process.

AgroGV3101 preinfiltrated leaves show higher callose deposition and an enhanced reactive oxygen species (ROS) burst compared with AgroLBA4404.

As the AgroGV3101 pretreated leaves showed reduced *P. syringae* pv. *tabaci* growth, there might be an increase in plant-defense responses in the pretreated leaves. We therefore determined callose deposition, a defense response against pathogens (Nguyen et al. 2010), 24 h after infection. The AgroGV3101 pretreated leaves showed higher callose staining than the IM- or AgroLBA4404-treated leaves (Fig. 2A). Quantification of the number of callose deposits confirmed increased callose deposition after secondary infection with *P. syringae* pv. *tabaci* or *P. syringae* pv. *tomato* DC3000 in the AgroGV3101 pretreated leaves (Fig. 2B). Based on the callose deposition readout, our data show that the priming effect of *N. tabacum* is stronger with AgroGV3101 pretreatment than with AgroLBA4404.

Since ROS regulate priming responses (Pastor et al. 2013), we also measured H₂O₂ production in *N. tabacum* leaf discs, using a luminol-based ROS assay (Ranf et al. 2011). Leaf discs preinfiltrated with either AgroGV3101 or AgroLBA4404 (for 24 h) were subsequently challenged with 100 nM flg22 and the H₂O₂ accumulation was monitored over an 8-h time period. Leaf discs pretreated with AgroGV3101 showed a very high ROS burst after flg22 perception in comparison with AgroLBA4404-pretreated leaf discs (Fig. 2C). A similar priming response was observed when AgroGV3101-pretreated leaf discs were challenged with the fungal elicitor chitin (Fig. 2D).

AgroGV3101 primes plant defense by inducing expression of MAPK signaling pathway genes.

One of the key PTI and priming responses is the activation of the MAPK pathway in plants (Beckers et al. 2009; Rodriguez et al. 2010). In tobacco, two MAPK, SA-induced and wound-induced protein kinases (SIPK and WIPK), play central roles in modulating the plant defense response. Therefore, we wanted to evaluate the possible induction of MAPK expression in leaves pretreated with *Agrobacterium* strains. By performing a real-time qPCR analysis after 6 h of agroinfiltration, it was observed that AgroGV3101-pretreated leaves but not the AgroLBA4404-pretreated leaves accumulated higher levels of *MEK2* (MAP2K) and *WIPK* transcripts than the IM-treated leaves (Fig. 3A). By contrast, the expression of *SIPKK* (encoding the upstream MAP2K for NtMPK4) was only slightly changed after agroinfiltrations in either case.

In addition, the AgroGV3101-infiltrated leaves also had higher *pathogenesis-related 1a* (*PR-1a*) expression in comparison with AgroLBA4404-infiltrated leaves (Fig. 3B). We quantified the expression of *PAL-4*, encoding phenylalanine ammonia-lyase, which catalyzes the first step of the phenylpropanoid biosynthesis pathway to produce precursors for a variety of important secondary metabolites. However, no significant changes could be observed in *PAL-4* expression in agroinfiltrated leaves in comparison with IM-treated leaves (Fig. 3C).

Enhanced activation of WIPK, SIPK, and defense-gene promoter activity in AgroGV3101 pretreated leaves.

As the transcript levels of tobacco MAPK are higher in AgroGV3101-infiltrated leaves, we wanted to evaluate any change in the proteins at the post-translational level. IM and

agroinfiltrated leaves were secondarily challenged with *Pseudomonas* spp. to check the status of phosphorylated WIPK and SIPK (the homologs of *Arabidopsis* AtMPK3 and AtMPK6, respectively). After 24 h of agroinfiltration, leaves were treated with mock (10 mM MgCl₂), *P. syringae* pv. *tabaci*, or *P. syringae* pv. *tomato* DC3000 (at approximately 2.5×10^7 CFU/ml). Six hours after the second infection, leaves were harvested and the extracted proteins were analyzed by Western blotting to detect phosphorylated forms of the MAPK. No differences could be seen between the additional *Pseudomonas* infection compared with the MgCl₂ mock infiltration, suggesting the activated MAPK activities observed here are predominantly due to wounding effects from the infiltration. However, the AgroGV3101 pretreated leaves, indeed, have higher amounts of phosphorylated MAPK (Fig. 4A). Taken together, enhanced expression of MAPK pathway genes (Fig. 3A) is also reflected by elevated SIPK or WIPK phosphorylation (Fig. 4A) in the AgroGV3101 pretreated tobacco leaves, which may boost defense responses.

To further investigate whether the priming effect proposed above is reflected in transcriptional regulation, we adapted a routine defense promoter–luciferase (LUC) reporter assay (Ranf et al. 2011) for the current tobacco system. Tobacco protoplasts isolated from the *Agrobacterium*-treated plants were transfected with plasmids of LUC gene driven by the PAMP-inducible *NHL10* promoter and, subsequently, elicited. Protoplasts from *Agrobacterium*-treated plants showed an enhancement of the basal (i.e., water-treated control) as well as the chitin-elicited *NHL10* promoter activities. However, those pretreated with AgroGV3101 showed a stronger boost in *NHL10* promoter activity than from AgroLBA4404-treated plants (Fig. 4B). Taken together, these observations suggest that AgroGV3101 treatment activates the plant defense machinery and helps in the priming response.

Dose-dependent regulation of plant defense response by exogenous cytokinin application.

One of the features differentiating AgroGV3101 from AgroLBA4404 is the ability to produce cytokinins. The stronger priming effects observed by pretreatment of tobacco leaves with AgroGV3101 as compared with AgroLBA4404 suggest cytokinins have one or more roles in immune priming. To validate the role of cytokinins in plant defense priming, tobacco leaves were infiltrated with different concentrations of *t*-Z. After 24 h of 0-, 1-, 10-, and 20-μM cytokinin administration, leaves were treated with either mock (10 mM MgCl₂), *P. syringae* pv. *tabaci*, or *P. syringae* pv. *tomato* DC3000 (at approximately 2.5×10^7 CFU/ml). Leaf tissue was harvested 6 h later, and the phosphorylation status of MAPK was analyzed as described above. Lower doses of *t*-Z (1 μM) increased the activation of WIPK and SIPK, while MAPK activation appears to be suppressed with higher (20 μM) *t*-Z concentration (Fig. 5A). This is also reflected in the lower growth of *P. syringae* pv. *tabaci* in 1 μM kinetin-treated tobacco leaves, whereas 20 μM kinetin-pretreated plants were as susceptible to *P. syringae* pv. *tabaci* infection as the mock controls (Fig. 5B). Next, the ROS burst assay with 1 and 20 μM cytokinin-pretreated *Nicotiana* leaves was performed. The lower dose of cytokinin enhanced the first peak of the biphasic flg22-mediated H₂O₂ production. By contrast, the higher 20-μM kinetin treatment suppressed this initial ROS burst (Fig. 5C).

Next, we wanted to validate the cytokinin-mediated defense responses in the model plant *Arabidopsis thaliana*. Similarly, *Arabidopsis* protoplasts showed enhanced MAPK phosphorylation after overnight treatment with low concentrations of *t*-Z but an apparent inhibition with higher cytokinin concentrations (Fig. 5D). To address whether this effect was SA dependent,

we administered either mock (0 μ M) or 10 μ M *t*-Z into *Arabidopsis* Col-0 or *sid2-1* plants. However, there was no difference between the two genotypes in decreasing the activation of WIPK and SIPK upon treatment with a higher dose (10 μ M) of *t*-Z (Supplementary Fig. 1), thus suggesting a SA-independent mode of action.

Since the *NHL10* gene is, in part, regulated by MAPK pathway, we next tested if the altered MAPK activities in the cytokinin-pretreated plants affected *NHL10* expression. Transfected *Arabidopsis* protoplasts were treated overnight with different concentrations of kinetin and were elicited with 100 nM flg22, and the luminescence from the *pNHL10-LUC* construct was monitored. However, we observed a dose-dependent reduction in flg22-induced *pNHL10* promoter activity (Fig. 5E). This reduction could be influenced by the fact that, besides being a PAMP-inducible gene, *NHL10* is also regulated by senescence in *Arabidopsis* (Zhang et al. 2004).

***Agrobacterium* preinfiltration maintains the photosynthetic machinery of the plant after *P. syringae* infection.**

Pathogenic infections in plants also modulate primary metabolic processes by either suppressing the photosynthesis of the

plant or creating a nutrient sink at the infection site. The dynamics of chlorophyll fluorescence values are indicators of plant stress (Berger et al. 2007). Tobacco leaves were preinfiltrated with AgroGV3101, AgroLBA4404, or cytokinin-producing 4xJERE:ipt AgroLBA4404 strains and, after 24 h, were treated with either 10 mM MgCl₂ (mock), *P. syringae* pv. *tabaci*, or *P. syringae* pv. *tomato* DC3000. The maximum quantum yield (Fv/Fm) and nonphotochemical quenching (NPQ) parameters were quantified after 72 h of infection in the dark-adapted leaves. It was observed that the agroinfiltrated leaves could maintain the photosynthetic parameters, while untreated leaves showed reduced Fv/Fm values after infection. No significant changes in the Fv/Fm values were observed in AgroGV3101- and AgroLBA4404-treated plants, at least during the first 3 days of infection (Fig. 6A). However, NPQ values were drastically reduced in AgroLBA4404-preinfiltrated leaves after *P. syringae* pv. *tomato* DC3000 infection (Fig. 6B). The leaf damage due to *P. syringae* pv. *tomato* DC3000 infection in AgroLBA4404-pretreated leaves may lead to the altered NPQ values as well. Cytokinin-producing AgroLBA4404 was able to maintain the NPQ values in leaves after the *P. syringae* pv. *tabaci* or *P. syringae* pv. *tomato* DC3000 infections. However, preinfiltration with 1 or 20 μ M kinetin

Fig. 3. AgroGV3101 primes the plant immune response by inducing the expression of several defense-related genes in tobacco. **A**, Quantification of the mitogen-activated protein kinase (MAPK) signaling cascade gene expression upon agroinfiltration. RNA was isolated after 24 h from agroinfiltrated (AgroGV3101 or AgroLBA4404) tobacco leaves and relative gene expression (normalized against housekeeping gene *actin* expression) was quantified by quantitative real time-polymerase chain reaction (qRT-PCR). The expression of tobacco MEK2 (MAPK/ERK kinase2, a MAPKK), salicylic acid-induced protein kinase (SIPK, tobacco homolog of *Arabidopsis* MPK6), wound-induced protein kinase (WIPK, tobacco homolog of *Arabidopsis* MPK3), and SIPKK (upstream MAPKK of NtMPK4) was analyzed. Similar ratios of gene expression were observed in at least two separate experiments. **B**, *Pathogenesis-related 1a* (*PR-1a*) gene expression relative to the *actin* expression measured by qRT-PCR (24 h after infiltration). The illustration is representative of at least two other similar experiments. **C**, qRT-PCR measurement of the gene expression of *PAL4* (*phenylalanine ammonia-lyase 4*) gene relative to tobacco *actin* expression. *T*-test performed for all the experiments with * indicating highly significant results ($P \leq 0.001$; agrotreated compared with infiltration medium [IM]).

could only partially maintain the Fv/Fm and NPQ values after *P. syringae* pv. *tabaci* and *P. syringae* pv. *tomato* DC3000 infections, as compared with mock infiltrations.

DISCUSSION

Agroinfiltration is often employed for transient transformation of plants to study the effect of a particular gene or subset of genes. The *Agrobacterium* strains commonly used are cured or disarmed of their T-DNA and are generally cotransformed with a helper plasmid carrying *vir* genes to transfer the gene of interest into plant cells (Gelvin 2003). There are different *Agrobacterium* strains used in transient infiltration assays, depending on their efficiency to transform different plant species. We used two *Agrobacterium* strains, namely GV3101 and LBA4404, in our transient infiltration studies in *N. tabacum* leaves. AgroGV3101, having the ability to produce *t-Z*, could suppress the macroscopic symptoms associated with *Pseudomonas* spp. infection, while AgroLBA4404 pretreatment failed to do so. It is well-known that the oncogenic *Agrobacterium* infection induces the plant's defense response at the earlier stages of infection but later suppresses it to enable gall disease development (Veena et al. 2003). Pruss and associates (2008) showed that agroinfiltration of oncogenic wild-type *A. tumefaciens* elicited a T-DNA-dependent increase in miR393, which has been linked to PAMP-dependent repression of auxin signaling. It is proposed that the disarmed *Agrobacterium*-pretreated plants can respond to subsequent pathogenic infections

in three different possible ways. i) The *Agrobacterium* spp. can directly inhibit the proliferation or virulence gene expression in *P. syringae*. ii) *Agrobacterium*-mediated priming of the basal immune response can inhibit proliferation or virulence gene expression in *P. syringae*. iii) *Agrobacterium*-mediated priming of the basal immune response can suppress SA and ABA synthesis (Rico et al. 2010). We observed the increase in basal plant defense responses, like callose deposition, after AgroGV3101 pretreatment, which is in agreement with earlier observations made by Rico and associates (2010). However, we observed an increase in *PR-1a* gene expression that contradicts the observations of Rico and associates (2010) but is in agreement with Pruss and associates (2008). These discrepancies could arise due to different inoculation concentrations used in the individual experiments and also the different timepoints of *PR-1a* expression measurements.

A hallmark of PTI induction is the rapid production of ROS after elicitation. It was observed that the leaves infiltrated with AgroGV3101 showed a sharp ROS peak at about 5 to 20 min after elicitation with either flg22 or chitin. A second peak of lower amplitude was observed at around 2 to 3 h after elicitation. The ROS peaks for AgroLBA4404 infiltrated leaf discs were very weak in amplitude and were delayed in comparison with AgroGV3101-infiltrated leaves. These findings are in agreement with the biphasic ROS bursts observed in *N. benthamiana* leaves after flg22 elicitation (Segonzac et al. 2011). The leaves infiltrated with AgroGV3101 were observed to have higher basal ROS levels prior to elicitation, reflecting that

Fig. 4. AgroGV3101 preinfiltration activates the mitogen-activated protein kinase (MAPK) pathway in tobacco. **A**, Western blot showing the activated/phosphorylated salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) in tobacco after agroinfiltrations. Tobacco leaves were infiltrated with IM (infiltration media), AgroGV3101, or AgroLBA4404, and after 24 h, were challenged with 10 mM MgCl₂ (mock), *P. syringae* pv. *tabaci* (*Pta*), or *P. syringae* pv. *tomato* DC3000 (*Pto*) as shown in the upper panel. At 6 h after challenge, leaf tissue was harvested and isolated proteins were immunoblotted against anti-phospho p44/42 MAPK antibody (α-pTEpY). The band intensities of phosphorylated SIPK and WIPK are shown, with the lower panel showing the Ponceau staining of the same blot. **B**, Relative *pNHL10* promoter activity as indicated by the relative luciferase (LUC) signal in either IM-, AgroLBA4404-, or AgroGV3101-treated tobacco protoplasts. Tobacco leaves were preinfiltrated with indicated *Agrobacterium* strains, and protoplasts were isolated after 24 h. Protoplasts were transformed with *pNHL10-LUC* and *pUBQ10-GUS* (β-glucuronidase) constructs and, later, were elicited with either water or chitin. The LUC signal was measured and expressed as LUC/GUS ratios and was normalized to the untreated controls.

AgroGV3101 could induce the plant defense to a higher extent than AgroLBA4404. As the accumulation of the dormant MAPK is considered to be a feature for the priming response, we sought to check the expression of some selected MAPK in agroinfiltrated leaves. The increased transcript accumulation of *MEK2* and *WIPK* may reflect their subsequent protein accumulation in response to AgroGV3101 infiltration. The accumulated MAPK, particularly WIPK, may be partly responsible for priming of the defense response, which is in agreement with the finding that AtMPK3 (*Arabidopsis* ortholog of WIPK) was observed as the main player for priming in *Arabidopsis* (Beckers et al. 2009). The slightly stronger activation of MAPK after 6 h of infection in AgroGV3101-infiltrated leaves

may be because of the already increased accumulation of the dormant MAPK in those leaves.

AgroGV3101 differs from AgroLBA4404 in having the ability to produce *t-Z*. It is highly possible that the suppression of macroscopic disease symptoms by AgroGV3101 could be attributed to the *t-Z* produced by AgroGV3101. Recently, cytokinins were investigated for their role in plant defense responses. Choi and associates (2010) observed that cytokinin signaling activated the SA-dependent plant defense pathways in *Arabidopsis*, while Grosskinsky and associates (2011) could show an increase in phytoalexins in cytokinin-treated tobacco leaves. It was reported that BAP (6-benzylaminopurine, a cytokinin) treatment affects the tissue-specific endogenous hormo-

Fig. 5. Mitogen-activated protein kinase (MAPK) activation by different concentrations of exogenously applied cytokinins. **A**, Western blot showing the phospho-SIPK (salicylic acid-induced protein kinase) and phospho-WIPK (wound-induced protein kinase) in tobacco leaves after treatment with the indicated concentrations of *trans*-zeatin (*t-Z*, a cytokinin). Tobacco leaves were syringe-infiltrated with H₂O or the indicated concentrations of *t-Z* and, after 24 h, were challenged with 10 mM MgCl₂ or *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*). Leaf tissue was harvested 6 h after the challenge and isolated proteins were subjected to immunoblotting against anti-pTEpY antibody. Upper panel, Western blot; lower panel, Ponceau stain of the same blot. **B**, Bacterial growth quantification of *P. syringae* pv. *tabaci* in *t-Z*-treated leaves. Tobacco leaves were infiltrated with water or with 1 or 20 μM *t-Z*, followed by *P. syringae* pv. *tabaci* infection after 24 h. CFU per 10-mm² leaf disc were calculated at 0 and 2 days postinfection. Data shown is the average of three replicates with error bars indicating standard deviation. Significant results in the *T*-test performed within the same-day CFU counts are indicated with an asterisk (*) (*P* ≤ 0.05; kinetin treatment compared with mock). **C**, Reactive oxygen species (ROS) production in tobacco leaves after kinetin administration. H₂O₂ production in mock- or 1 or 20 μM kinetin-treated leaf discs was measured using luminol-based ROS assay after 100 nM flg22 elicitation. **D**, Western blot showing phosphorylated MPK3 and MPK6 in *Arabidopsis* protoplasts after kinetin treatment. *Arabidopsis* mesophyll protoplasts were isolated and treated overnight with the indicated concentrations of kinetin. Protoplasts were harvested and isolated proteins were analyzed by Western blotting with the anti-pTEpY antibody. The lower panel shows the amido black staining of the same blot. **E**, Relative *pNHL10* promoter activity, as indicated by the relative luciferase (LUC) signal in kinetin-treated *Arabidopsis* protoplasts. *Arabidopsis* protoplasts were transformed with *pNHL10-LUC* and *pUBQ10-GUS* (β-glucuronidase) constructs and were treated overnight with the indicated concentrations of kinetin. The next day, protoplasts were elicited with 100 nM flg22 and the LUC signal was measured. Results are expressed as LUC/GUS ratios normalized to the untreated controls.

nal homeostasis in *Arabidopsis*. BAP upregulated the abundance of proteins involved in the ABA response and the ethylene biosynthetic pathway, thereby making it a strong candidate to trigger plant stress responses (Zd`arska et al. 2013).

On the other hand, it was also reported that the increase in cytokinin content is associated with the formation of 'green-islands' to foster bacterial growth and also, in some cases, fungal growth (Babosha 2009; Walters and McRoberts 2006). From these examples, it is evident that the levels of host- or pathogen-derived cytokinins can alter the outcome of a plant-pathogen interaction. From our experimental data, it was observed that lower levels of exogenous cytokinins (1 μ M) increased the activity of tobacco MAPK, while higher levels (10 or 20 μ M) decreased it. The levels of active MAPK can be directly linked to the primed state of the plant for possible upcoming infection. However, the recent work of Argueso and associates (2012) reported that the lower levels of benzyl adenine (BA) treatment made *Arabidopsis* more susceptible to the biotrophic oomycete *Hyaloperonospora arabidopsidis* Noco 2. The contrasting results might reflect the different levels of signaling elicited by different cytokinins (BA vs. *t*-Z) during plant-pathogen interactions and their different effect on pathogen growth in different plant species (*Arabidopsis* vs. tobacco) or pathosystems. Additionally, the effect of higher concentrations of

cytokinins is largely dependent on SA biosynthesis. A recent publication by Hann and associates (2014) reported that lower doses of exogenous *t*-Z can suppress the plant defense in *N. benthamiana* leaves, while higher doses can increase the plant defense response. The contradictory results may arise due to varied concentrations of cytokinins used and the time span of exogenous cytokinin treatment (5 vs. 24 h, in our case). The altered plant growth conditions and the age of the plants used may also contribute to the contradiction. However, we observed the SA-independent induction of plant defenses upon *t*-Z administration, which is in agreement with the insignificant changes in the SA levels upon modulation of cytokinin levels with the HopQ1 effector (Hann et al. 2014). The decrease in plant defense responses using higher doses of cytokinin may also occur due to a decrease in the NO (nitric oxide) content in the plant (Liu et al. 2013), as NO is considered as a positive regulator of plant defense responses. From our work, however, it can be presumed that lower levels of *t*-Z in tobacco plants induce the defense response by activating the MAPK signaling pathway.

During pathogen defense, the plant needs to efficiently counteract the infection process but, at the same time, limit the negative impacts of immune responses on tissue integrity, photosynthetic capacity, and plant primary metabolism (Sato

Fig. 6. Agroinfiltration maintains the photosynthetic parameters of the plant upon *Pseudomonas syringae* infection. **A**, Effective fluorescence yield (Fv/Fm) quantification in agroinfiltrated leaves preceded by *P. syringae* pv. *tabaci* (Pta) or *P. syringae* pv. *tomato* DC3000 (Pto) infection. Tobacco leaves were infiltrated with IM (infiltration media), AgroGV3101, AgroLBA4404, or 4xJERE:ipt AgroLBA4404 and, after 24 h, were treated with 10 mM MgCl₂, *P. syringae* pv. *tabaci* or *P. syringae* pv. *tomato* DC3000 (2.5×10^7 CFU/ml). After 3 days, Fv/Fm values of the dark-adapted leaves were measured with a GFS3000 chlorophyll fluorometer. All the measurements were taken at predawn time. **B**, Nonphotosynthetic quenching (NPQ) values of the dark-adapted leaves preinfiltrated with IM, AgroGV3101, AgroLBA4404, or 4xJERE:ipt AgroLBA4404 and, after 24 h, treated with 10 mM MgCl₂, *P. syringae* pv. *tabaci* or *P. syringae* pv. *tomato* DC3000 ($n = 3$).

et al. 2010). As cytokinins are known to have a role in carbon partitioning and energy allocation, it is possible that cytokinin signaling might play a role in regulating the levels of energy that can be allocated into defense responses (Roitsch and Ehness 2000). Our results suggest that *Agrobacterium* pretreatment could maintain the photosynthetic machinery of the plant during the *P. syringae* pv. *tabaci* and *P. syringae* pv. *tomato* DC3000 infections. The maintenance of the photosystem II (PSII), as reflected by Fv/Fm values, seems to be independent of cytokinin-producing activity in *Agrobacterium* spp. However, the NPQ values were maintained to normal values in AgroGV3101 and cytokinin-producing AgroLBA4404 strain pretreated leaves only. It is known that higher cytokinin doses can increase the NPQ values in tobacco leaves (Novák et al. 2013) and the pathogen will try to decrease the same. Also, the complete damage of the leaf integrity in AgroLBA4404 preinfiltrated leaves after *P. syringae* pv. *tomato* DC3000 infection could lead to the observed decrease in NPQ values. This alludes to the fact that the pretreatment of disarmed *Agrobacterium* spp. (particularly AgroGV3101) during the early phase of infection could maintain the photosynthetic machinery, thereby helping the plant to fight the infection in a better way.

In conclusion, this study has shed light on the different aspects of plant priming by cytokinin-producing disarmed *Agrobacterium* strains frequently used in transient agroinfiltration assays. Agroinfection with AgroGV3101 was found to induce the expression of and also activate the MAPK in tobacco leaves. This study has helped us to partly understand the unknown priming mechanism in plants and the evolution of plant-pathogen interactions. At the same time, this work is a cautionary warning for the plant research community when interpreting results involving agroinfiltrations, particularly when pathogen studies are involved. Based on the results of this work, AgroLBA4404 appears to be a better choice for *N. tabacum* infiltration assays, and then, if AgroGV3101 is used, the results should be always compared with AgroGV3101 strain control infiltrations.

MATERIALS AND METHODS

Plant material and bacterial strains.

Tobacco (*N. tabacum* L. cv. *tabaci*) seeds were sown in a 3:1 soil/vermiculite mix and were grown in a growth cabinet (National Institute of Plant Genome Research) under a 16-h photoperiod and 23°C temperature. Seedlings (10 to 15 days old) were transferred to bigger pots and were grown under the same conditions for 5 to 8 weeks in the greenhouse.

For agroinfiltrations, disarmed *Agrobacterium* sp. strains GV3101 and LBA4404 were used. Strain GV3101 was streaked out on YEB plates (per liter: 5 g of beef extract, 1 g of yeast extract, 5 g of peptone, 5 g of sucrose, 0.5 g of MgCl₂; 1.5% agar) supplemented with 50 µg of rifampicin per milliliter and incubated overnight at 28°C. The colonies were used to inoculate liquid YEB media to grow until the optical density at 600 nm (OD₆₀₀) reached 1. *Agrobacterium* sp. strain LBA4404 was grown in Luria Bertani (LB) medium till the growth reached OD₆₀₀ = 1. For infection purposes, both host and nonhost *P. syringae* strains were used. Nonhost *Pseudomonas syringae* pv. *tomato* DC3000 was streaked out on Kings B media plates (per liter: 20 g of peptone, 1.5 g of K₂HPO₄, 1.5 g of MgSO₄·7H₂O, 15 g of agar, final pH 7.2) supplemented with 50 µg of rifampicin per milliliter and were incubated overnight at 28°C. Likewise, host *Pseudomonas syringae* pv. *tabaci* was streaked out on LB plates supplemented with 20 µg of tetracycline per milliliter and incubated overnight at 28°C.

Plant inoculation and CFU assay.

Agrobacterium sp. strains GV3101 and LBA4404 were grown to OD₆₀₀ = 0.6 in YEB and LB media, respectively. Bacteria were pelleted at 2,000 × g for 5 min and were resuspended in IM (10 mM morpholineethanesulfonic acid (pH 5.6), 10 mM MgCl₂, and 150 µM acetosyringone) to give a final bacterial concentration of approximately 10⁸ CFU/ml. *4xJERE:ipt* in pCambia1380 in LBA4404 was grown like AgroLBA4404. Bacteria were infiltrated on the abaxial leaf surface of the plants. IM only was used as a vehicle control. Plants were grown in a greenhouse and, after 24 or 48 h, were infected with *P. syringae* pv. *tabaci* or *P. syringae* pv. *tomato* DC3000. *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tabaci* were grown in Kings B and LB media, respectively, until the OD₆₀₀ reached 0.6. Bacteria were pelleted and resuspended in 10 mM MgCl₂ at 2.5 × 10⁷ CFU/ml.

For bacterial colony-counting assays, three 10-mm² leaf discs were collected from leaves after 0, 3, and 10 days of *P. syringae* pv. *tabaci* infection and were homogenized in 10 mM MgCl₂ and diluted 10³ times, and 100 µl was plated on LB + tetracycline plates.

Callose deposition and ROS assays.

Tobacco leaves were agroinfiltrated with AgroGV3101 and AgroLBA4404 and, after 48 h, were infected with *P. syringae* pv. *tabaci* or *P. syringae* pv. *tomato* DC3000. After 24 h, 10-mm² leaf discs were collected in 24-well microtiter plates containing 3 ml of 95% ethanol. Leaf discs were incubated at 37°C for 6 h until all the chlorophyll was removed. Cleared leaf discs were washed twice with 70% ethanol and, then, three times with water. Leaf discs were incubated for 3 h in the dark in 0.1% aniline blue (Methyl Blue from Sigma) dissolved in 150 mM K₂HPO₄ (pH 9.5, set with KOH). Finally, leaf discs were mounted on 60% glycerol and were observed under a fluorescent microscope with UV filter (path length 420 nm).

ROS production was measured with the help of a luminol-based assay as described earlier by Ranf and associates (2011).

Analysis of defense gene expression by qRT-PCR.

To study the effect of agroinfiltration on defense gene expression, plants were inoculated with AgroGV3101 (10⁸ CFU/ml) and AgroLBA4404 (10⁸ CFU/ml) and, after 24 h, were treated with either 10 mM MgCl₂, *P. syringae* pv. *tabaci*, or *P. syringae* pv. *tomato* DC3000 (10⁷ CFU/ml), as described above. The relative mRNA levels were analyzed 6 h after inoculation of *P. syringae* by real-time qRT-PCR, using actin as internal control. Leaf samples were homogenized in liquid nitrogen and RNA was isolated using Trizol reagent. RNA was treated with RNase-free DNase, and first-strand cDNA was generated using the RevertAid first-strand cDNA synthesis kit (Thermo Scientific). The primers used to detect the relative abundance of different genes are given in Supplementary Table S1. qRT-PCR was performed with the SYBR Green PCR master mix kit (Applied Biosystems). Each reaction was performed in triplicate with each experiment repeated at least twice.

MAPK activation assay.

After agroinfiltrations, plants were challenged with either mock (10 mM MgCl₂), *P. syringae* pv. *tabaci*, or *P. syringae* pv. *tomato* DC3000. Leaf tissue was harvested 6 h postinfection and was ground in liquid nitrogen. Proteins were isolated using kinase extraction buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM dithiothreitol, 10 mM Na₃VO₄, 10 mM NaF, 50 mM beta-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail, 10% glycerol) and were quantified with the Bradford assay. total protein (30 to 40 µg) was separated by 10% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE), and later, immunoblotting analysis was performed using anti-p42/44-phospho-ERK antibody (Cell Signaling).

Exogenous cytokinin treatment in leaves and protoplasts.

Tobacco leaves were infiltrated with water, 1, 10, or 20 μ M kinetin, or *t*-Z (Sigma). After 24 h, leaves were challenged with *P. syringae* pv. *tabaci* or *P. syringae* pv. *tomato* DC3000. Leaf tissues were harvested at different timepoints. The proteins were isolated and subjected to immunoblotting as described above.

Arabidopsis and *Nicotiana* protoplasts were isolated as per Yoo and associates (2007), with slight modifications. Isolated protoplasts were treated overnight with different concentrations of kinetin and, the next day, were treated with water or 100 nM flg22. Protoplasts were harvested and directly boiled in SDS-PAGE loading buffer. Proteins were separated by 10% SDS-PAGE and were analyzed by immunoblotting using anti-p42/44-phospho-ERK antibody (Cell Signaling).

For the LUC assay, *Arabidopsis* protoplasts were isolated and transformed according to Yoo and associates (2007). The *pNHL10*-promoter-LUC construct was used as a reporter and *pUBQ10*-GUS was cotransfected for normalization (Ranf et al. 2011). Luminescence of protoplast suspensions containing D-luciferin (200 μ M; Invitrogen), treated with water, 100 nM flg22, or 200 μ g of crab shell chitin per milliliter (Sigma), was recorded in 96-well plates at indicated intervals (Thermo Luminoskan Ascent 2.1). Results are expressed as LUC/GUS ratios and were normalized to the LUC/GUS values in the untreated controls.

Chlorophyll fluorescence measurements.

Both photosynthetic and nonphotosynthetic chlorophyll fluorescence parameters of the agroinfiltrated tobacco leaves were measured using a GFS 3000 chlorophyll fluorometer (Heinz Walz, Germany). The maximum PSII quantum yield of the dark-adapted sample (Fv/Fm) and NPQ were measured as described by Sinha and associates (2002). All the measurements were taken at predawn times to ensure dark-adaptation of the leaves.

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