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Modulation of plant DNA damage response gene expression during *Agrobacterium* infection

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ABSTRACT

Agrobacterium T-DNA (transfer DNA) integration into the plant genome relies mostly on host proteins involved in the DNA damage repair pathways. However, conflicting results have been obtained using plants with mutated or down-regulated genes involved in these pathways. Here, we chose a different approach by following the expression of a series of genes, encoding proteins involved in the DNA damage response, during early stages of *Agrobacterium* infection in tobacco. First, we identified tobacco homologs of *Arabidopsis* genes induced upon DNA damage and demonstrated that their expression was activated by bleomycin, a DNA-break causing agent. Then, we showed that *Agrobacterium* infection induces the expression of several of these genes markers of the host DNA damage response, with different patterns of transcriptional response. This induction largely depends on *Agrobacterium* virulence factors, but not on the T-DNA, suggesting that the DNA damage response activation may rely on *Agrobacterium*-encoded virulence proteins. Our results suggest that *Agrobacterium* modulates the plant DNA damage response machinery, which might facilitate the integration of the bacterial T-DNA into the DNA breaks in the host genome.

1. Introduction

The process of plant genetic transformation by *Agrobacterium tumefaciens* culminates with the integration of the bacterial T-DNA into the genomic DNA of the host cell [1,2]. The T-DNA enters the host cell nucleus in the form of a single-stranded DNA segment, covalently linked to the *Agrobacterium* VirD2 protein at its 5′-end and likely associated with other bacterial and plant proteins. Within the nucleus, the T-DNA is integrated into the host chromosomal DNA mostly by the host cell machinery. Furthermore, that double-strand breaks (DSBs) in the genomic DNA have been suggested to represent target sites for T-DNA integration [3], and the preferential integration of T-DNA into DSBs [4,5], indicated a role for the host DNA repair mechanism in the T-DNA integration.

In plants, NHEJ (non-homologous end joining) is believed to be the major pathway for T-DNA integration, whereas integration mediated by the HR (homologous recombination) pathway occurs only at very low rates [6,7]. However, studies using plants with mutated or down-regulated genes involved in the main NHEJ pathways yielded conflicting results [1,2]. High levels of redundancy characterizing different DNA repair pathways may explain why it is difficult to assess their specific involvement in T-DNA integration. Thus, although a single pathway of DNA repair cannot be designated as a unique contributor to T-DNA integration, several concurrent DNA repair pathways most likely mediate T-DNA integration [1,2], similarly to their ability to complement each other during DNA damage repair [8].

The inconsistencies in identification of the components of the plant DNA repair machinery involved in T-DNA integration may derive from the experimental approaches that are based on reverse genetics (reviewed in Ref. [1]). We chose a different approach by following the expression of a series of genes encoding proteins involved in DNA damage repair pathways during early stages of *Agrobacterium* infection. Indeed, many genes involved in DNA damage response signaling or in DNA repair are induced in *Arabidopsis* and other plants in response to DNA damage [9–13], consistent with the notion that expression of these genes may be used as marker for activation of DNA damage repair [11–13]. Moreover, biotic stress, such as bacterial infection, can induce DNA

Abbreviations: T-DNA, transfer DNA; DSB, double-strand break; NHEJ, non-homologous end joining; HR, homologous recombination.

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Table 1
Tobacco genes selected for this study and their *Arabidopsis* homologs and known functions in the DNA damage response.

Gene name (*N. tabacum*)	Gene ID (*A. thaliana*)	%id (%qc)[a]	Function in *A. thaliana*
TAC9	At5g09810	90 (75)	Control, actin 9
PP2A	At3g25800	90 (100)	Control, protein phosphatase 2A
NAC82	At5g64060	47 (42)	Transcription factor (*NAC103* in *Arabidopsis*), DNA damage signaling
BRCA1	At4g21070	39 (99)	HR
RAD51	At5g20850	88 (99)	HR
Rad17	At5g66130	51 (100)	HR and NHEJ, DNA damage signaling
KU70	At1g16970	66 (100)	NHEJ, DSB detection
XRCC1	At1g80420	52 (93)	NHEJ, alt-NHEJ
XRCC4	At3g23100	61 (98)	NHEJ, ligase IV complex
PARP1	At2g31320	68 (100)	Alt-NHEJ
PARP2	At4g02390	70 (95)	Alt-NHEJ
WEE1	At1g02970	61 (89)	Cell cycle arrest (phosphorylation of cyclin dependent kinase CDKA:1)
AGO2	At1g31280	49 (88)	HR
FAM63	At4g22960	52 (99)	Cytosolic deubiquitinase

[a] Percentage of identity (%id) and percentage of query coverage (%qc) based on sequence comparisons between *Arabidopsis* proteins and their tobacco homologs. HR, homologous recombination; NHEJ, non-homologous end joining; alt-NHEJ, alternative NHEJ.

damage, which likely represents a general reaction to stress but may also plays a more specific role in the plant defense response [14,15]. Here, therefore, we explored the transcriptional activation of the DNA damage response machinery during early stages of *Agrobacterium* infection in tobacco leaves in three experimental steps. We identified tobacco homologs of *Arabidopsis* genes involved in DNA damage response, and demonstrated that their expression was indeed activated upon induction of DNA damage by a chemical agent, bleomycin. Then, we showed that these genes exhibit different patterns of transcriptional response to *Agrobacterium* inoculation.

2. Material and methods

2.1. A. tumefaciens strains

For testing the effects on expression of DNA damage response genes, we used three strains of *A. tumefaciens*: C58, a wild type virulent strain, harboring a nopaline-type pTiC58 Ti-plasmid; C58C1-pMP90 harboring a disarmed pTiC58 derivative pMP90 that lacks the T-DNA region but contains the *vir* region [16]; and C58C1 which is the C58 strain cured of its pTiC58 plasmid. We designated these strains A3, A10 and A66, respectively. Bacteria were grown at 28 °C, on LB medium without antibiotics, and the resulting bacterial cultures used for infiltration of leaf tissues.

2.2. Tobacco leaf infiltration

All infiltrations were performed in equivalent areas (see Fig. S3A) on both sides of the central vein and between secondary veins of fully developed, ca. 15-20-cm long, leaves of 4–6 week-old tobacco plants (*Nicotiana tabacum* cv. Turk) maintained in growth chamber at 23 °C with relative humidity of 50%–60% under a 16:8-h ratio of light to dark photoperiod with the light intensity of 100 μmol photons m^{-2} s^{-1}. For bleomycin infiltration, bleomycin (Bleocin, Millipore 203,408) stock solution was diluted in MES buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6) to final concentration of 20 ng l^{-1} and infiltrated into tobacco leaves using a needleless 1-ml syringe [17]. For negative, mock control, the infiltrations were performed using the MES buffer. The infiltrated tissue areas were harvested at 24 hpi and stored in liquid nitrogen. For *Agrobacterium* infiltration, bacterial strains were grown overnight at 28 °C, 250 rpm in LB, supplemented with the appropriate antibiotic. One milliliter of the bacterial suspension was diluted in 4 ml of LB and incubated for 1 h at 28 °C, 250 rpm, centrifuged, the bacterial pellet was resuspended in MES to $OD_{600nm} = 0.6$, and incubated for 2 h, 200 rpm at room temperature. The resulting bacterial suspension was syringe-infiltrated into the tobacco leaves as described above, and the infiltrated areas were harvested at 6, 24, 48 or 72 hpi and stored in liquid nitrogen.

2.3. Western blot analysis

Frozen tobacco leaf tissue (200 mg) was ground in liquid nitrogen, using mortar and pestle, and crude nuclear protein extract was prepared as described [18]. The supernatant, containing nuclear protein extract, was aliquoted and stored at −20 °C. Samples of the nuclear protein extract (20 μl/lane) were resolved on a 15% SDS polyacrylamide gel at 80 V for ca. 1.5 h, and proteins were either stained with Coomassie blue or transferred onto a nylon membrane by semi-dry transfer at 25V for 40 min. After blocking for 3 h in TBST supplemented with 5% BSA, membranes were incubated with anti-phospho-histone H2AX (pSer139) antibody (Sigma H5912) diluted 1/2000 in TBST with 1% BSA overnight at 4 °C. Following incubation with secondary antibody, anti-rabbit IgG coupled to horseradish peroxidase (HRP, Sigma H31440) diluted 1/2500 in TBST with 1% BSA for 6 h at 4 °C, the membrane was treated with a chemiluminescent HRP substrate (Millipore Immobilon, WBKLS0050), and analyzed by autoradiography (Thomas Scientific, 1141J52).

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted using the GeneJEt plant RNA purification mini Kit (Thermo Fisher, K0801) according to the manufacturer's instructions. Concentration and purity of total RNA were verified by spectrophotometry. DNase treatment was done by incubating 2 μg of total RNA in a total 20 μl volume, containing 2 μl DNase I buffer and 0.4 μl DNase I (NEB, M0303) for 30 min at 37 °C, after which DNase was inactivated by adding 2 μl of 50 mM EDTA followed by a 10 min incubation at 75 °C. RNA preparation was aliquoted and stored at −80 °C.

Reverse transcription was performed using the RevertAid RT Kit (Thermo Scientific, K1691) according to the manufacturer's instructions.

2.5. Quantitative RT-PCR (RT-qPCR)

For RT-qPCR analyses, the reaction mixture was composed of 2 μl of 3-times diluted cDNA, 10 μl of 2x Power SYBR Green PCR Master Mix (Thermo Scientific, 4368706), 0.4 μl each of forward and reverse primers (Table S1), and 7.2 μl H_2O. qPCR was performed

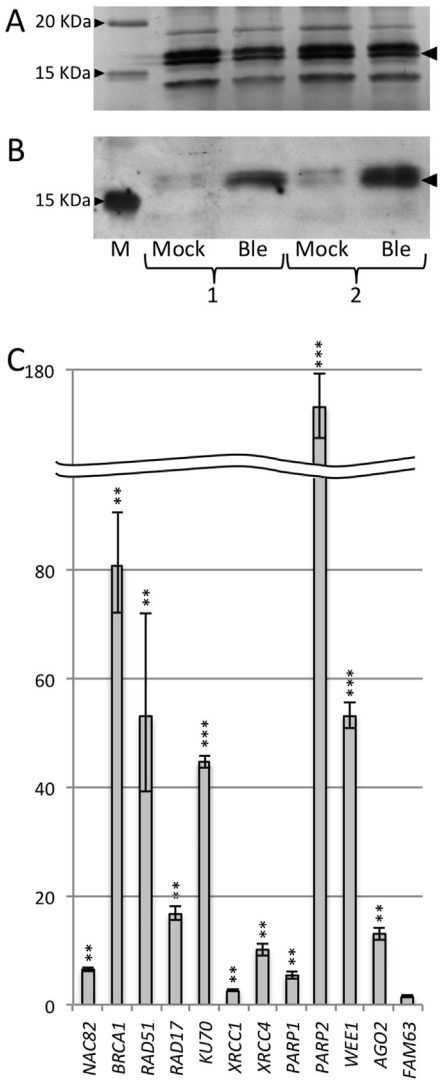

Fig. 1. Effects of bleomycin on formation of DSBs and expression of DNA damage response genes in tobacco leaves. (A) H2AX phosphorylation. Total protein load detected by Coomassie blue staining. (B) H2AX phosphorylation. Western blot with anti-gamma-H2AX. M, molecular size markers. Lanes 1 and 2, two independent biological repeats; mock or bleomycin (Ble) indicate infiltration with buffer or bleomycin, respectively. Arrowheads on right indicate the expected position of tobacco gamma-H2AX. (C) Transcriptional activation of the indicated DNA damage response genes in response to bleomycin treatment. The expression levels were measured 24 h after bleomycin treatment and are expressed as fold change after bleomycin treatment relatively to mock infiltration. Error bars represent the SD from two independent biological replicates. Statistical significance is indicated by asterisks (** = $p < 0.05$; *** = $p < 0.01$).

with a QuantStudio 3 thermocycler (Thermo Scientific), with one cycle of 10 min at 95 °C, 40 cycles of 30 s at 95 °C and 1 min at 60 °C. For each set of primers, the specificity of amplification was verified by agarose gel electrophoresis and melting curves (Fig. S1). Each sample was analyzed in two biological replicates, each consisting of three technical replicates, using validated constitutive reference gene *TAC9* to normalize RT-qPCR data by the comparative C_t method; the relative transcript levels were calculated by the cycle threshold (CT) $2^{-\Delta\Delta C}{}_t$ method [19].

2.6. Statistical analysis

Quantitative data are presented as averages with lower and upper standard deviation values. Statistical significance was calculated using the paired *t*-test method using GraphPad Prism 7, with *p*-values <0.1, 0.05, or 0.01 corresponding to the statistical probability of >90%, 95% or 99%, respectively, considered statistically significant.

3. Results

3.1. Choice of plant host and DNA damage response genes

We elected to utilize tobacco (*Nicotiana tabacum*) as a model plant because, unlike *Arabidopsis*, it allows harvesting sufficient amounts of tissue uniformly infected with *Agrobacterium* for reliable and sensitive analyses. Next, we selected a set of 12 genes known to be induced in a dose-dependent manner by DNA damaging treatment in *Arabidopsis*, which therefore can serve as reliable genetic markers for the DNA damage response [11–13] (Table 1). These genes encode proteins that function in different pathways of DNA damage response and repair (reviewed in Refs. [20,21]). Specifically, *NAC103* (termed *NAC82* in tobacco) is involved in signaling of DNA damage response, acting as a transcription factor that induces expression of several DNA repair genes [13]. Other selected genes are involved in the actual DNA repair, via HR (*BRCA1, RAD51, RAD17*), NHEJ (*RAD17, KU70, XRCC1, XRCC4*), or alt-NHEJ (*PARP1, PARP2*). Finally, *WEE1* is a protein kinase involved in cell cycle arrest in response to genotoxic stress [22], and *FAM63* is a cytoplasmic deubiquitinase, strongly induced by DNA damage in *Arabidopsis*, although its exact role in DNA repair remains unknown [11]. We then identified the closest homologs of these *Arabidopsis* genes in the *N. tabacum* genome; these genes are annotated in both tobacco and in *Arabidopsis* with the same name, except for *NAC103* that is termed *NAC82* in tobacco (Table 1). As reference gene, we chose *ACTIN9* (Tac9, X69885), previously used as control in tobacco [23–25], which exhibited minimal variation in expression following inoculation with *Agrobacterium*, as compared to four other reference genes (Fig. S2).

3.2. Transcriptional outcomes of genotoxic stress

We investigated the transcriptional response of the selected genes to genotoxic stress elicited by bleomycin, a DSB inducer [26] and activator of both HR and NHEJ pathways [12]. Formation of DSBs following bleomycin infiltration was detected using Western blot analysis with antibody against the phosphorylated histone 2X (gamma-H2AX), a diagnostic marker of DSBs in all eukaryotes, including plants [27]. We observed a strong increase in protein species with electrophoretic mobility of 15–17 kDa, corresponding to the tobacco gamma-H2AX protein, 24 h after the bleomycin treatment and similar in two biological replicates (Fig. 1A and B).

Next, we measured the transcriptional response of our DNA damage response genes 24 h after application of bleomycin. Fig. 1C shows that these genes were transcriptionally activated to varying degrees, ranging from 1.4 to 170-fold change compared to the mock infiltration treatment. The expression of most of these genes significantly and strongly increased upon bleomycin treatment, with levels over 40 times the mock inoculation control for five of them (*BRCA1*, *RAD51*, *PARP2*, *WEE1*). The response of one gene, *FAM63*, was not statistically significant (Fig. 1C). Collectively, these data demonstrate that the selected tobacco genes respond to DNA damage by a strong activation of their transcription.

3.3. Transcriptional outcomes of Agrobacterium inoculation

The genes that showed statistically significant response to bleomycin were examined for their ability to respond to

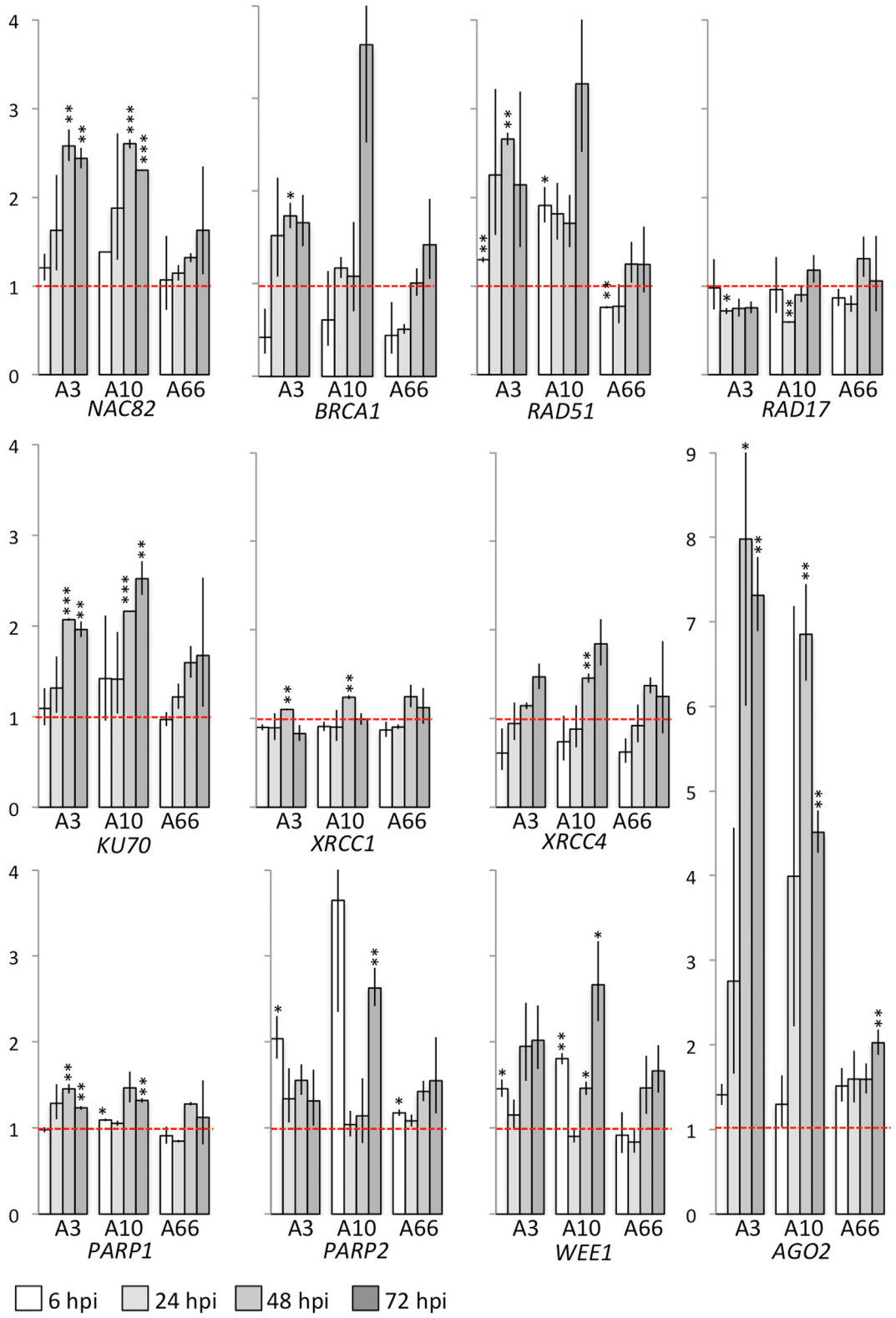

Fig. 2. *Agrobacterium* inoculation alters transcriptional outcomes of DNA damage response gene expression. Bacterial strains: A3, C58; A10, C58C1-pMP90; and A66, C58C1. The expression levels were measured at 6, 24, 48, and 72 hpi indicated by white, light gray, medium gray, and dark gray bars, respectively, and are expressed as fold change after bacterial inoculation relatively to mock inoculation. The dotted line represents the expression levels after mock inoculation, which were set at 1. Error bars represent the SD from two independent biological replicates. Statistical significance is indicated by asterisks (* = $p < 0.1$; ** = $p < 0.05$; *** = $p < 0.01$).

Agrobacterium inoculation. Three bacterial strains were used: A3, which is the wild-type, fully virulent C58 nopaline strain, harboring the native pTiC58-plasmid that contains both its *vir* region and T-DNA; A10, which is the avirulent C58C1-pMP90 strain, carrying the pMP90 variant of pTiC58 that contains the *vir* region but no T-DNA [16]; and A66, which is the avirulent C58C1 strain, harboring no Ti plasmid at all. Inoculations were performed into four areas of a tobacco leaf (Fig. S3A) and each of these areas was confirmed to exhibit comparable, with the variation of less than 20%, levels of expression of all of the tested DNA damage response genes as well as of two house-keeping genes, *TAC9* and *PP2A*, 48 h after mock inoculation; this variability was considered statistically insignificant in our analyses (Fig. S3B).

Quantification of changes in transcription 6, 24, 48 and 72 h post inoculation (hpi) revealed that most of the tested genes, with the exception of *RAD17* and *XRCC1,* exhibited varying degrees of statistically significant increase in expression induced by *Agrobacterium* infection at least at some time points as compared to mock inoculation performed at the same time point on the equivalent area of the leaf (Fig. 2).

For the majority of the genes, the induction of expression was stronger after inoculation with bacterial strains A3 and A10, compared with the levels observed with A66 (Fig. 2). Moreover, there was an evolution of expression at different time points after inoculation. For the *NAC82, KU70, PARP1,* and *AGO2* genes, strain A3 had no significant effect at 6 hpi, but caused an increase in expression at 24 hpi, which plateaued, or decreased for *PARP1*, at 48–72 hpi. These genes responded to strain A10 in a very similar pattern, whereas their expression in response to strain A66 was much weaker or statistically insignificant (Fig. 2). In contrast, the expression of *BRCA1* and *XRCC4* decreased at 6 hpi with strain A3, followed by slight, and not always statistically significant, increase at later time points. With strains A10 and A66, both genes exhibited a similar decrease in expression at 6 hpi as well as a weak increase at 24, 48 and 72 hpi, which was more pronounced with A3 and A10 than with A66 (Fig. 2). After inoculation with strain A3, *RAD51, PARP2*, and *WEE1* showed higher levels of expression at 6 hpi, and the expression of *RAD51* and *WEE1* mostly remained elevated, relatively to mock inoculation controls, at later time points whereas the expression of *PARP2* slightly decreased. For these genes as well, the increase in expression at 6 hpi was more significant with strains A3 and A10 than with A66, while the differences between strains were less consistent at later time points.

4. Discussion

The *Agrobacterium* T-DNA and effector Vir proteins do not provide any known components of the DNA repair machinery; thus, the T-DNA integration most likely relies on these functions provided by the host plant cell. It would make biological sense that *Agrobacterium* has evolved to modulate this host machinery—potentially using the bacterial virulence capabilities—to optimize efficiency of its own infection. Here, we began to examine this idea by defining transcriptional outcomes of *Agrobacterium* infection on expression of a group of genes that represent genetic hallmarks of DNA damage response in plants, including *Arabidopsis* [13] and tobacco (Fig. 1B) exposed to a DNA break-inducing drug bleomycin. Transcription of the majority of these genes, i.e., 9 out of 11 that showed a strong response to bleomycin, was also increased to varying extent after *Agrobacterium* infiltration, albeit at substantially lower levels, most likely due to different capabilities of bacterial pathogens and chemical drugs to induce DNA damage.

Our observations of transcriptional activation of *NAC82*—a homolog of *Arabidopsis NAC103*, a transcription factor involved in DNA damage response signaling [13]—show that *Agrobacterium* can activate the DNA damage response of the host cell. The expression levels of other DNA damage response genes, in particular *KU70, RAD51*, *WEE1* and *AGO2*, were also elevated by *Agrobacterium*. This transcriptional response also displayed different temporal patterns; specifically, some genes, such as *RAD51, PARP2, WEE1*, became induced as early as 6 hpi whereas others, such as *NAC82, KU70, AGO2*, became activated only from 48 hpi.

Besides activating the host plant DNA damage response, *Agrobacterium* induces more complex reactions involving host defense and developmental and phytohormonal mechanisms [28,29]. Moreover, the existence of a crosstalk between activation of the DNA damage response and immune defense response upon plant infection by bacterial pathogens has been demonstrated [14,15]. In this regard, noteworthy are our observations that the *AGO2* gene is induced by *Agrobacterium* to higher levels, comparable to those elicited by bleomycin. This is likely because whereas the *AGO2* expression is induced upon DNA damage [11] and plays a role in DNA repair by recruiting RAD51 [30,31], AGO2 also participates in defensive RNA silencing [32]. For example, AGO2 is involved in defense against viruses [33], induced by bacterial pathogens and regulates anti-bacterial immunity [34] and, more specifically, down-regulation of *AGO2* expression results in increased susceptibility to *Agrobacterium* [35]. Thus, during infection, *AGO2* may become induced to play a dual role: participating in host defenses, acting against *Agrobacterium* and mitigating infection, and participating in DNA repair, likely facilitating the T-DNA integration and the infection. Different response to the *Agrobacterium* strains, i.e., A3 and A10 on the one hand and A66 on the other hand, suggests that the presence of the *vir* region results in stronger induction of the DNA damage response genes—particularly, *NAC82, RAD51, PARP2,* and *AGO2*—whereas the presence of the T-DNA region is not required for the induction.

Overall, our results show that *Agrobacterium* infection induces the expression of several gene markers of the host DNA damage response, and that this induction largely depends on *Agrobacterium* virulence factors, but not on the T-DNA. These observations, in turn, strongly suggest that the DNA damage response is activated in the host plant upon *Agrobacterium* infection, and that the Vir effectors exported by the bacterium into the plant cell might contribute to this transcriptional activation. It is tempting to speculate that the modulation of the plant DNA damage response machinery by *Agrobacterium* facilitates the ultimate step of the infection process, i.e. the integration of the bacterial T-DNA into the DNA breaks in the host genome.

Declaration of competing interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.03.044.

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