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Differential roles of glucosinolates and camalexin at different stages of Agrobacterium-mediated transformation

PO-YUAN SHIH1,2,3, SHU-JEN CHOU1, CAROLINE MÜLLER4, BARBARA ANN HALKIER5, ROSALIA DEEKEN6 AND ERH-MIN LAI 1,2,7,*

1Institute of Plant and Microbial Biology, Academia Sinica, 115 Taipei, Taiwan
2Molecular and Biological Agricultural Sciences Program, Taiwan International Graduate Program, Academia Sinica, 115 Taipei, Taiwan
3Graduate Institute of Biotechnology, National Chung-Hsing University, 402 Taichung, Taiwan
4Chemical Ecology, Bielefeld University, 33615 Bielefeld, Germany
5Department of Plant and Environmental Sciences, DynaMo Center, University of Copenhagen, 1817 Frederiksberg C, Denmark
6Molecular Plant Physiology and Biophysics, Julius-von-Sachs-Institute for Biological Sciences, University of Wuerzburg, 97082 Wuerzburg, Germany
7Biotechnology Center, National Chung-Hsing University, 402 Taichung, Taiwan

SUMMARY

Agrobacterium tumefaciens is the causal agent of crown gall disease in a wide range of plants via a unique interkingdom DNA transfer from bacterial cells into the plant genome. Agrobacterium tumefaciens is capable of transferring its T-DNA into different plant parts at different developmental stages for transient and stable transformation. However, the plant genes and mechanisms involved in these transformation processes are not well understood. We used Arabidopsis thaliana Col-0 seedlings to reveal the gene expression profiles at early time points during Agrobacterium infection. Common and differentially expressed genes were found in shoots and roots. A gene ontology analysis showed that the glucosinolate (GS) biosynthesis pathway was an enriched common response. Strikingly, several genes involved in indole glucosinolate (iGS) modification and the camalexin biosynthesis pathway were up-regulated, whereas genes in aliphatic glucosinolate (aGS) biosynthesis were generally down-regulated, on Agrobacterium infection. Thus, we evaluated the impacts of GSs and camalexin during different stages of Agrobacterium-mediated transformation combining Arabidopsis mutant studies, metabolite profiling and exogenous applications of various GS hydrolysis products or camalexin. The results suggest that the iGS hydrolysis pathway plays an inhibitory role on transformation efficiency in Arabidopsis seedlings at the early infection stage. Later in the Agrobacterium infection process, the accumulation of camalexin is a key factor inhibiting tumour development on Arabidopsis inflorescence stalks. In conclusion, this study reveals the differential roles of GSs and camalexin at different stages of Agrobacterium-mediated transformation and provides new insights into crown gall disease control and improvement of plant transformation.

Keywords: Agrobacterium-mediated transformation, Agrobacterium tumefaciens, camalexin, crown gall, glucosinolates, plant defence, transcriptome.

INTRODUCTION

The plant pathogen Agrobacterium tumefaciens causes crown gall disease by a process initiated when A. tumefaciens senses phenolic compounds to induce the expression of virulence genes (Stachel et al., 1985). The transferred DNA (T-DNA) located on the tumour-inducing (Ti) plasmid is processed and transported from the bacterium into the plant cell. On T-DNA integration into the plant genome, the expression of T-DNA-encoded oncogenes causes the exogenous production of auxin and cytokinin to promote plant cell proliferation and lead to tumour formation. In addition to agrobacterial virulence genes that are required for T-DNA translocation, a successful transformation also involves several plant host factors (Gelvin, 2010; Gohlke and Deeken, 2014; Hwang et al., 2017; Pitzschke, 2013).

Although A. tumefaciens seems to be able to hijack host factors to promote T-DNA transfer and expression for crown gall development, plant defence and hormone response systems are also quickly activated to counteract Agrobacterium infection. Microbe-associated molecular patterns, such as EF-Tu of Agrobacterium, are recognized by the pattern recognition receptor EFR to activate rapid immune responses and compromise Agrobacterium infection (Wu et al., 2014; Zipfel et al., 2006). The activation of basal immune responses is highly regulated by a crosstalk between salicylic acid (SA) and jasmonic acid (JA) (Pieterse et al., 2009). Arabidopsis SA-deficient, but not JA-insensitive, mutants

*Correspondence: Email: emlai@gate.sinica.edu.tw
are more susceptible to Agrobacterium-mediated tumour formation, suggesting that SA, but not JA, is important for the control of Agrobacterium transformation (Lee et al., 2009; Yuan et al., 2007). In addition to SA, Agrobacterium-produced auxin (Kutacek and Rovenska, 1991; Lee et al., 2009) and cytokinin (Regier and Morris, 1982) also regulate immune responses and transformation efficiency (Hwang et al., 2010, 2013; Sardesai et al., 2013; Yu and Yang, 1979). These findings suggest that the regulation of plant hormone and immune systems is crucial in the control of Agrobacterium transformation efficiency.

Compared with several studies on the role of plant hormones in defence against Agrobacterium infection, little is known about plant secondary metabolites in the Agrobacterium transformation process. In the Brassicaceae family, to which Arabidopsis belongs, glucosinolates (GSs) and the phytoalexin camalexin are key secondary metabolites in defence against pathogen infection. They are synthesized from amino acids, such as tryptophan for indole glucosinolates (iGSs) or camalexin and methionine for aliphatic glucosinolates (aGSs). GSs and their hydrolysis enzymes, the myrosinases, provide a unique defence system which, when plant tissues are damaged, mixes and produces toxic hydrolysis products, typically isothiocyanates (ITCs) and nitriles (Halkier and Gershenzon, 2006). Moreover, the PEN2-mediated hydrolysis of 4-methoxyindol-3-ylmethyl GS (4MOI3M) serves as a major signal that activates innate immune responses to prevent fungal penetration (Bednarek et al., 2009; Clay et al., 2009). Camalexin can inhibit the growth of various plant pathogens by disrupting their cell membrane or inducing programmed cell death (Ahuja et al., 2012; Rogers et al., 1996; Shlezinger et al., 2011). Studies on GSs and camalexin deficiency mutants have suggested that both GSs and camalexin are crucial in protecting plants from microbial infection with a broad spectrum of antifungal activity (Bednarek et al., 2009; Stotz et al., 2011). Their role in bacterial pathogen defence has only been reported for Pseudomonas syringae pv. tomato DC3000 with a modest effect on bacterial growth in planta (Brader et al., 2006; Clay et al., 2009; Fan et al., 2011; Glazerbrook and Ausubel, 1994).

Although many studies have characterized the roles of GSs and camalexin in plant defence against herbivores and various pathogens (Bednarek, 2012), to our knowledge nothing is known about their roles in Agrobacterium–plant interaction. By transcriptome analysis of Arabidopsis seedlings on infection with the virulent A. tumefaciens wild-type strain C58, we noticed that several iGS modifying genes and camalexin biosynthetic genes were upregulated, whereas genes in aGS biosynthesis were generally downregulated. Similar expression patterns have also been observed in inflorescence stalks on Agrobacterium infection. Therefore, in this study, we aimed to elucidate the impact of GS (or rather its hydrolysis products) and camalexin on Agrobacterium-mediated transformation in both seedling and mature plant stages. We discovered that iGS hydrolysis products restrict Agrobacterium-mediated transient transformation at an early infection stage, and that camalexin plays a key role in the negative regulation of later stages of the tumorigenesis process to inhibit tumour development.

## RESULTS

### Time course analysis of Agrobacterium infection in Arabidopsis seedlings

In order to identify early responsive genes of Arabidopsis seedlings to Agrobacterium infection, the timing of gene expression of T-DNA-encoded genes was monitored in seedlings infected with the virulent Agrobacterium wild-type strain C58 carrying the β-glucuronidase (GUS) reporter gene (Narasimhu et al., 1996). GUS staining of 7-day-old seedlings infected at 2, 8, 24, 48, 72 and 96 h post-infection (hpi) showed that GUS signals were first detected as early as 48 hpi in both shoots and roots of seedlings and then increased with longer infection time (Fig. S1a–e, see Supporting Information). To determine whether T-DNA was translocated into plant cells, but not yet expressed prior to 48 hpi, infected Arabidopsis seedlings were further recovered in timentin-containing medium to abolish Agrobacterium cell growth, but allow GUS expression. With timentin treatment, GUS signals could be detected in both shoots and roots when seedlings at 24 hpi were recovered for an additional 3 days in timentin-containing medium (Fig. S1f–j). Our results suggest that T-DNA translocation into plant cells occurs within 24 hpi, but the accumulation of T-DNA-encoded gene products requires more than 1 day to reach detectable levels. Because this study aims to identify early responsive genes to Agrobacterium infection, i.e. prior to T-DNA gene expression, we chose 2 and 24 hpi for transcriptome analysis.

### Common and distinct sets of genes are differentially expressed in shoots and roots of Arabidopsis seedlings in response to Agrobacterium infection

As transient GUS expression signals were stronger and more detectable in shoots (100%) than in roots (no more than 25%) of all infected seedlings (Fig. S1), we separated shoots and roots of infected and non-infected (mock control) seedlings for gene expression analysis with Affymetrix ATH1 chips. The genes with an intensity higher than the background (value > 75), and with at least two-fold changes determined by signals of infected plant tissues versus mock controls in all three biological replicates, were classified as differentially expressed genes (DEGs). This analysis resulted in 25 DEGs in shoots (17 up, 8 down) and 107 DEGs in roots (85 up, 22 down) at 2 hpi [false discovery rate (FDR), P< 0.05; Fig. 1a and Datasheet S1, see Supporting Information]. At 24 hpi, 353 genes were differentially expressed in shoots (296 up, 57 down) and 1178 in roots (677 up, 501 down) (Fig. 1a and Datasheet S1). Thus, more DEGs were found at 24 hpi than at 2 hpi in both shoots and roots. More than 50% of DEGs in shoots
and roots at 2 hpi were not identified at 24 hpi, suggesting that these were only transiently regulated at an early infection stage of Agrobacterium infection (Fig. 1b).

The number of DEGs in roots was higher than in shoots (Fig. 1a), although transiently expressed GUS signals were not detected in most roots (Fig. S1e,j). In shoots, more genes were up-regulated, rather than down-regulated, whereas, in roots, the number of down-regulated genes was increased and close to the number of up-regulated genes (677 up and 501 down at 24 hpi). Strikingly, only one of 25 DEGs (4%) in shoots was shared with DEGs in roots at 2 hpi, whereas 92 of 353 DEGs (26%) in shoots were shared with DEGs in roots at 24 hpi (Fig. 1c). The data suggest a highly tissue-specific response to Agrobacterium infection. In agreement with this notion, scatter plots illustrating whole genome expression patterns showed low correlations between shoots and roots (Fig. 1d).

We identified the majority of DEGs as distinct for shoots and roots. Despite this, a gene ontology (GO) analysis using the 24-hpi dataset revealed that most enriched GO categories were shared by shoots and roots, except for the development GO, which was only found in roots (Fig. 1e and Tables S1–S4, see Supporting Information). However, the percentage and/or number of gene counts in certain GO categories were significantly different between roots and shoots. For example, both the percentage and number of gene counts in the defence response GO were higher in shoots (30.1%, 63) than in roots (8.7%, 53) at 24 hpi (Fig. 1e and Tables S3 and S4). In contrast, a higher percentage and number of gene counts in the hormone response GO were found in roots (17.8%, 109) than in shoots (11%, 23) (Fig. 1e). In roots, many of the hormone response GO terms related to cytokinin, abscisic acid (ABA), JA and auxin were enriched (Tables S1–S4), with the most significant and abundant GO terms being related to cytokinin. Taken together, these results show that defence responses are regulated more significantly in shoots than in roots, but roots display stronger responses to various hormones than shoots, probably responsible for root growth and development.

**Genes in GS and camalexin biosynthesis are regulated in both Arabidopsis seedlings and inflorescence stalks on Agrobacterium infection**

Among the enriched GO terms in the secondary metabolism category, we noticed that GS biosynthesis-related GO terms were enriched in both shoots and roots (Tables S3 and S4), suggesting...
a common role of GSs in Arabidopsis seedlings in response to Agrobacterium infection. Therefore, we analysed the expression profiles of the genes involved in GS biosynthesis in Agrobacterium-infected Arabidopsis seedlings, and found that most key genes in iGS modification and camalexin biosynthesis were highly up-regulated, especially at 24 hpi (Fig. 2a and Datasheet S2). The gene encoding CYP81F2, which converts indol-3-ylmethyl GS (I3M) to 4-hydroxyindol-3-ylmethyl GS (4OHI3M), was induced at 2 hpi, and further enhanced at 24 hpi, in roots (Fig. 2a and Datasheet S2). The other genes encoding indole glucosinolate methyltransferase 1 and 2 (IGMT1 and IGMT2), converting 4OHI3M or 1OHI3M to 4MOI3M or 1MOI3M, respectively, were also up-regulated at 24 hpi in roots (IGMT1 and IGMT2) and shoots (IGMT2) (Fig. 2a and Datasheet S2). The camalexin biosynthesis genes, including CYP71A12, CYP71A13 and PAD3, were highly up-regulated in both plant parts at 24 hpi, although they were only identified to be statistically significant in roots (CYP71A12 also in shoots) (Fig. 2a and Datasheet S2). In contrast, most genes involved in aGS biosynthesis were highly suppressed at 24 hpi in roots and shoots (Fig. 2b and Datasheet S2).

Subsequently, we tested whether the observed differential gene expression of GS and camalexin biosynthesis could also be seen in mature plants and at a later stage of infection. According to microarray data (Lee et al., 2009), the transcription of genes in camalexin biosynthesis from tryptophan via indole-3-aldoxime (IAOx) and indole-3-acetonitrile (IAN) was induced at 6 days post-infection (dpi) and in crown gall tumours, whereas the transcription of genes in iGS biosynthesis from IAOx to I3M was inhibited mainly in tumours (Fig. S2a, see Supporting Information). All genes in aGS biosynthesis were strongly down-regulated at the later time points, similar to their expression in infected seedlings (Figs 2b and S2b). These findings imply that GS and camalexin may play important roles during Agrobacterium infection and may modulate transformation efficiency. Therefore, we aimed to study the impact of GS, camalexin and the derived metabolites on: (i) Agrobacterium transient transformation; and (ii) crown gall development.

iGS hydrolysis plays a negative role in Agrobacterium transient transformation efficiency of Arabidopsis seedlings

We first utilized a variety of Arabidopsis mutants impaired in iGS, camalexin and aGS biosynthesis to determine their transient transformation efficiencies at the seedling stage. On Agrobacterium infection, no statistical difference could be identified in transient GUS activity between Col-0 and the aGS biosynthesis mutant myb28/29 (Fig. 3a,b). In contrast, the mutant cyp79B2/B3, deficient in iGS and camalexin biosynthesis, showed significantly
higher transient GUS activity than Col-0. Interestingly, the two iGS hydrolysis mutants (pen2-1 and pen2-2) were also more susceptible to Agrobacterium transformation relative to Col-0, but there was no detectable difference in transient GUS activity in the camalexin mutants (cyp71A12, cyp71A13-1, cyp71A13-3 and pad3-1). These results together suggest that the susceptible phenotype detected in the cyp79B2/B3 mutant should be a result of the depletion of iGS hydrolysis products, but not the depletion of camalexin.

**Arabidopsis seedlings reduce the content of specific GSs after Agrobacterium infection**

To determine the impact of differential gene expression of GS biosynthesis pathways in Arabidopsis seedlings on Agrobacterium infection, we next analysed the GS profiles of Arabidopsis Col-0 seedlings and selected GS mutants (myb28/29, cyp79B2/B3 and pen2-1) with or without infection at 3 dpi (Figs 4 and S3, and Table S5, see Supporting Information). The concentrations of I3M, 4OH13M, 4-methylsulfinylbutyl GS (4MSOB), 4-methylthiobutyl GS (4MTB) and 8-methylthiooctyl GS (8MTO), but no other iGSs and aGSs, were significantly reduced in infected Col-0 seedlings (Figs S3c,d and 4a,b). The most basic iGS, I3M, was significantly reduced from 72 to 32 nmol/g fresh weight (FW) after infection (Fig. 4a). In the secondarily modified iGSs, only 4OH13M showed a significant reduction in Col-0 seedlings after infection, but the concentration was too low for it to be considered as a main metabolite (Fig. 4a). No aGSs or iGSs were detected in myb28/29 or cyp79B2/B3 mutants, confirming their functions in aGS and iGS biosynthesis, respectively. Moreover, the iGS profile detected in myb28/29 and the aGS profile detected in cyp79B2/B3 were similar to those in Col-0 (Fig. 4a,b). Although the I3M concentration was also significantly reduced in the pen2-1 mutant, the 4MO13M concentration was dramatically increased after Agrobacterium infection (Fig. 4a). This observation is consistent with previous reports (Bednarek et al., 2009; Clay et al., 2009), which suggest that PEN2-mediated iGS hydrolysis functions in Agrobacterium-infected Arabidopsis seedlings.

In Arabidopsis seedlings, aGSs are represented by two main groups: the methylthioalkyl GSs and the methylsulfinylalkyl GSs (Fig. 2b and Table S5). We found that two methylsulfinylalkyl GSs, 4MSOB and 8-methylsulfinyloctyl GS (8MSOO), and three methylthioalkyl GSs, 4MTB, 7-methylthioheptyl GS (7MTH) and 8MTO, were most abundant in Col-0 seedlings (Fig. S3d). At 3 dpi, only the concentrations of 4MSOB, 4MTB and 8MTO were decreased, but no significant change was observed for 8MSOO and 7MTH (Figs S3d and 4b). Furthermore, a time course experiment with Col-0 seedlings showed that most GSs did not change significantly from 8 to 48 hpi, except for I3M, which was significantly reduced.
at 48 hpi (Fig. 4c,d). We also observed a reduction in 4MSOB and 8MTO concentrations, but the differences were not statistically significant, probably as a result of variations amongst biological replicates (Fig. 4d).

Exogenous applications of iGS and aGS products impact Agrobacterium transient transformation efficiency

Our GS profile data from Arabidopsis seedlings suggest that PEN2-mediated iGS hydrolysis is activated in Arabidopsis seedlings on Agrobacterium infection (Fig. 4), which is consistent with a negative role of PEN2 myrosinase in the transient transformation process (Fig. 3). Thus, we next directly treated the seedlings with 13M during Agrobacterium infection, and found that the transient transformation efficiency was reduced in a dose-dependent manner relative to methanol-treated control seedlings (Fig. 5a,b). As exogenous application of 13M does not influence the number of viable Agrobacterium cells grown in either co-cultivation medium or associated with infected seedlings at 1 or 3 dpi (Figs S5a,b, see Supporting Information) and has no impact on the GUS enzymatic activity in vitro (Fig. S4a, see Supporting Information), we suggest that the reduced transient transformation efficiency on 13M application is probably caused by its impact on the host plant.

Although we did not observe a clear statistical difference in the transient transformation efficiencies between the myb28/29 mutant and Col-0, the mutant showed a tendency to a lower transient transformation efficiency in general (Fig. 3b). Thus, we tested whether the most prevalent hydrolysis products from aGSs, the ITCs (Wittstock and Burow, 2010), may have a direct impact on Agrobacterium-mediated transient transformation efficiencies. We tested the effect of ITCs derived from 4MSOB, 7MTH, 8MTO and 8MSOO on the transient transformation efficiency of Col-0 seedlings by the application of ITCs in the co-cultivation medium at 200 and 300 μM. The results showed that application of the aliphatic ITCs resulted in different effects, with the transient transformation efficiency being inhibited when the seedlings were treated with ITCs of 4MSOB (85% reduction) or 8MSOO (74% reduction) (Fig. 5c,d), and enhanced when the seedlings were treated with ITCs of 7MTH (3–7-fold increase) and 8MTO (>1.5-fold increase). It is notable that exogenous application of these aGS-ITCs caused 10%–20% reduction in GUS enzymatic activity in vitro (Fig. S4b). Thus, we conclude that 7MTH and 8MTO indeed play positive roles in promoting transient transformation efficiency, despite their modest effects in inhibiting GUS enzymatic activity per se. However, the reduced transient GUS expression of infected seedlings treated with 4MSOB-ITC and 8MSOO-ITC may be partly caused by their direct effects in inhibiting GUS enzymatic activity.

Impact of exogenous applications of aGS products on Agrobacterium growth

To determine whether the different impacts of aGS-ITCs on transformation efficiency may contribute to differences in Agrobacterium growth during co-cultivation, we measured the viable
Agrobacterium cell number in both the co-cultivation medium and cells associated with seedlings at 1 and 3 dpi. The aGS-ITC treatments did not affect the viable Agrobacterium cell number in the co-cultivation medium at either 1 or 3 dpi, except for 4MSOB, which caused a modest reduction in viable cell number at 1 dpi (Fig. S5d). However, no difference in the seedling-associated Agrobacterium cell number was observed for seedlings treated with 4MSOB or dimethylsulfoxide (DMSO) control at 1 or 3 dpi (Fig. S5c), suggesting that the reduced transient transformation efficiency of the Col-0 seedlings treated with 4MSOB (Fig. 5c,d) could not be attributed to the number of Agrobacterium cells. Similarly, the reduced transient transformation efficiency of the Col-0 seedlings treated with 8MSOO (Fig. 5c,d) is probably not impacted by Agrobacterium growth, as no reduced Agrobacterium cell number was observed in the medium or in planta in the presence of 8MSOO (Fig. S5c,d). However, the seedling-associated Agrobacterium cells were significantly higher in number when treated with 7MTH-ITC or 8MTO-ITC than mock control at 1 and 3 dpi when a higher concentration (300 μM) was applied (Fig. S5c,d). It is notable that more viable Agrobacterium cells were only observed in planta, but not in co-cultivation medium, which contains one to two orders more viable Agrobacterium cells than those in planta. Thus, 7MTH-ITC and 8MTO-ITC do not have an impact on Agrobacterium growth, but can facilitate Agrobacterium association with Col-0 seedlings and enhance transformation efficiencies.

Camalexin, but not GSs, contribute to Agrobacterium-mediated tumour formation on Arabidopsis inflorescence stalks

We next determined whether GS and camalexin biosynthesis pathways also impact tumorigenesis, the later stage of infection. Surprisingly, tumours of cyp79B2/cyp79B3 were significantly smaller than, and those on myb28/myb29 showed no difference from, those of wild-type Col-0 at 4 weeks after inoculation of young inflorescence stalks (Fig. 6a). The pad3-1 mutant developed significantly larger crown galls relative to the wild-type. These results differed from the transient transformation efficiencies detected in cyp79B2/cyp79B3 (increased) and pad3-1 (unchanged; Fig. 3). The quadruple mutant qko (cyp79B2/cyp79B3/myb28/myb29) (devoid of GSs and camalexin) developed very small tumours or none at all (Fig. 6b), which is similar to cyp79B2/cyp79B3. Because the tumorigenesis efficiency...
is a result of the combination of cell proliferation ability and transformation efficiency, including steps of T-DNA translocation from the bacterium into plant cells, T-DNA trafficking from the cytoplasm into the nucleus, T-DNA integration and T-DNA gene expression, we tested the callus induction ability of these mutants using *Arabidopsis* root explants following the methods described previously (Hwang and Gelvin, 2004). We did not observe any significant difference between Col-0 and the tested mutants including *cyp79B2/B3*, *myb28/29* and *pad3-1* (Fig. S6a, see Supporting Information). Thus, cell proliferation ability is unlikely to be the key reason for the reduced tumour size of the *cyp79B2/cyp79B3* mutant and the increased crown gall development of *pad3-1*.

We found a 6.9-fold higher concentration of aGSs in tumours than in mock-inoculated inflorescence stalks at 28 dpi, but no significant difference for iGSs (Fig. 7a). In tumours, the major aGSs were 3-methylsulfinylpropyl GS (3MSOP), 5-methylsulfinylpentyl GS (5MSOP), 4MTB and 8MSOO, whereas I3M, 4MOI3M and 1MOI3M were the major iGSs (Fig. 7b). The myrosinase enzyme activity was significantly lower in crown galls than in mock-inoculated tissues (Fig. 7c), which suggests that crown gall tissues may not activate GS hydrolysis like non-inoculated plant tissues.

*Fig. 6* Tumorigenesis assay on inflorescence stalks of *Arabidopsis* Col-0 and mutants lacking either camalexin and/or glucosinolates (GS). (a) The tumour weight of the biosynthesis mutants for aliphatic GS (*myb28/29*), indole GS (*cyp79B2/B3*) and camalexin (*pad3-1*). (b) The tumour weight of the GS-free mutants (*qko*). The mean fresh weight ± standard error of the mean (SEM) of crown galls was calculated of three to six independent experiments with a minimum of 11 plants per experiment. Significant differences from the control plants are indicated [one-way analysis of variance (ANOVA) with Dunnett’s test, ***P < 0.001].

*Fig. 7* Glucosinolate (GS) and camalexin content in crown galls. Mean amounts of total aliphatic GSs (aGSs) and indole GSs (iGSs) (a) and the main aGSs and iGSs (b) in crown galls and mock-inoculated inflorescence stalk tissue. (c) Mean myrosinase enzyme activity in crown galls and mock-inoculated inflorescence stalks of three independent biological replicates with two samples in each experiment. (d) Camalexin content of *Arabidopsis* inflorescence stalks inoculated with Agrobacterium or mock-inoculated at three different time points. The mean values of three to four independent biological replicates ± standard error of the mean (SEM) are shown. Each replicate consisted of material from more than 10 individual plants. dpi, days post-infection; DW, dry weight; hpi, hours post-infection.
Camalexin accumulates after Agrobacterium infection and inhibits transient transformation in Arabidopsis seedlings when exogenously applied

Camalexin is a phytoalexin that inhibits the growth of various plant pathogens and often negatively impacts pathogen infection (Ahuja et al., 2012; Rogers et al., 1996). It is intriguing to note that the pad3-1 mutant exhibited similar transient transformation efficiency to the wild-type during the seedling stage, but was highly susceptible to tumorigenesis (Figs 3 and 6a). One plausible reason is that the camalexin concentration may be different during different developmental stages. Thus, we measured the camalexin concentrations in Arabidopsis seedlings during the course of Agrobacterium infection and at later stages of tumorigenesis. Camalexin could not be detected in seedlings before infection (0 dpi) and the concentration gradually increased during the infection time, with 0.24 \( \mu \text{g/g FW} \) (1.2 \( \mu \text{M} \)) at 1 dpi, 1.64 \( \mu \text{g/g FW} \) (8.2 \( \mu \text{M} \)) at 2 dpi and 3.44 \( \mu \text{g/g FW} \) (17.2 \( \mu \text{M} \)) at 3 dpi (Fig. 8a), and correlated with the gene expression profile (Fig. 2a andDatasheet S2). In crown galls, the camalexin concentration was also lower at 3 hpi, but significantly higher at 6 dpi and further increased at 28 dpi (Fig. 7d).

We then treated the Col-0 seedlings with increasing concentrations of camalexin in co-cultivation medium during Agrobacterium infection to determine the impact of camalexin on the transient transformation efficiency during the seedling stage. The GUS signal was dramatically reduced in seedlings treated with 10 \( \mu \text{M} \) camalexin, and almost no transiently expressed GUS activity could be detected on application of 100 \( \mu \text{M} \) camalexin (Fig. 8b,c). As the exogenous application of camalexin did not affect or only slightly inhibited GUS enzymatic activity in vitro with higher dosage (Fig. S4c), the negative impact of camalexin on transient GUS expression in infected seedlings is probably the result of reduced transient transformation efficiency rather than affecting GUS enzymatic activity per se. Because the application of camalexin did not inhibit Agrobacterium cell growth in co-cultivation medium or associated with infected seedlings at 1 or 3 dpi (Fig. S5e,f), the Agrobacterium cell number has no impact on the reduced transient transformation efficiency of camalexin-treated seedlings. However, although no difference in callus induction ability of root explants could be detected with the camalexin-deficient mutant pad3-1, exogenous application of camalexin can reduce the callus induction rate of root explants and transient transformation efficiencies of seedlings, both in a dose-dependent manner (Figs 8c and Fig. S6b). These results suggest that camalexin exhibits negative effects on both transformation efficiency and cell proliferation when the camalexin concentration reaches the threshold of \( \sim 10 \mu \text{M} \), which can be reached in seedlings at 2 dpi (Fig. 8a) and in wounded/infected inflorescence stalks at 6 dpi (Fig. 7d). Taken together, these results suggest that camalexin plays a negative role in both the early stage of transformation and the later stage of tumorigenesis, which may be partly a result of the reduced cell proliferation activity of plant cells when the camalexin concentration is high.

**DISCUSSION**

In this study, the transcriptome analysis of Agrobacterium-infected seedlings and crown galls of Arabidopsis plants led to the discovery of DEGs involved in GS and camalexin biosynthesis pathways.
during the *Agrobacterium* infection process. Furthermore, functional studies suggested a potential role of iGS hydrolysis products in seedling defence against *Agrobacterium* infection at an early stage, whereas camalexin plays a key role in the inhibition of tumour development at a later stage of the *Agrobacterium* transformation process.

Although the GS-related GO terms were enriched in both shoots and roots of seedlings at 24 hpi (Tables S3 and S4), the biosynthesis of iGSs and aGSs was differentially regulated. The induction of genes in I3M modification (CYP81F2, IGMT1 and IGMT2 which convert I3M into 4MOI3M) as early as 2 hpi (Fig. 2a and Datasheet S2) was correlated with a reduced content of I3M at later stages of infection (2 and 3 dpi; Fig. 4a,c). Previous studies have suggested that the reduction in I3M is an early response to pathogen infection, and that PEN2-mediated iGS hydrolysis provides defense signalling (Bednarek et al., 2009; Clay et al., 2009). This is also supported by our findings of higher transient transformation efficiencies of *Arabidopsis* mutant seedlings deficient in iGS biosynthesis and hydrolysis (Fig. 3). Furthermore, the transient transformation efficiency was decreased when Col-0 seedlings were treated with I3M (Fig. 5a,b). I3M did not inhibit agrobacterial growth in co-cultivation medium or in planta (Fig. S5a,b), and an in vitro study has shown that *Agrobacterium* is more resistant to iGS than to aGS hydrolysis products (Aires et al., 2009). Thus, the reduction in transformation efficiency by iGS was not a result of the restriction of agrobacterial growth, but probably caused by the immune response triggered by iGS hydrolysis. However, we do not exclude the possibility that iGS hydrolysis may also impact other physiological functions or signal transduction of *A. tumefaciens* and its infected plant cells, which may then affect the transient transformation efficiency. Such studies are beyond the scope of this work and await future investigations.

However, the *myb28/29* mutant (which lacks aGSs) did not show a statistically significant difference in either transient transformation efficiency or tumour development (Figs 3 and 6a). Beekwilder et al. (2008) have shown higher iGS concentrations in this mutant than in Col-0 after insect attack, but such an increase was not found in our seedling assay systems, as *myb28/29* mutant seedlings contained no aGSs and similar iGS amounts to wild-type Col-0 (Fig. 4a). Thus, we suggest that iGSs do not have a negative impact on the transformation efficiency of the *myb28/29* mutant.

To this end, the biological significance of the reduced expression of aGS biosynthetic genes in *Arabidopsis* seedlings after *Agrobacterium* infection and during crown gall development remains unclear. The global reduction in gene expression of aGS biosynthesis was reflected in the reduction in certain aGS amounts at 3 dpi (Figs 2b and 4b,d). The aGSs with side chains of four carbon atoms (4C; 4MTB and 4MSOB), 7C (7MTH) and 8C (8MTO and 8MSOO) were most abundant in 7–10-day-old *Arabidopsis* seedlings (Fig. S3d). However, only 4MSOB, 4MTB and 8MTO were significantly reduced after *Agrobacterium* infection (Fig. 4b), which suggests a selective regulation of 4C and 8C aGS biosynthesis.

It is interesting to note that exogenous applications of different aliphatic ITCs caused different impacts on the *Agrobacterium* transformation efficiencies in *Arabidopsis* seedlings. The transient transformation efficiencies were inhibited by exogenous applications of 4MSOB-ITC and 8MSOO-ITC, but enhanced with 7MTH-ITC and 8MTO-ITC treatments (Fig. 5c,d). Although 7MTH-ITC and 8MTO-ITC caused about 10% reduction in the GUS enzymatic activity per se (Fig. 5b), exogenous applications of 7MTH and 8MTO can still enhance transient GUS expression of infected seedlings. Thus, 7MTH and 8MTO indeed play positive roles in the promotion of transient transformation efficiency. However, 4MSOB-ITC and 8MSOO-ITC caused about 10%–20% reduction in GUS enzymatic activity *in vitro* (Fig. 5b), but more than 70% reduction in transformation efficiency, as reflected by the transient GUS expression levels. This indicates that 4MSOB-ITC and 8MSOO-ITC play a negative role in transient transformation, and the reduced transient GUS expression of infected seedlings may be partly a result of their negative influence on GUS enzymatic activity.

ITCs are known to be toxic to various pathogens, and a previous *in vitro* assay has shown that 4MSOB-ITC inhibits *Agrobacterium* cell growth (Aires et al., 2009). Consistent with this finding, our results also showed that 4MSOB-ITC caused a modest reduction in viable cell number at 1 dpi (Fig. S5d). However, all other tested ITC compounds did not affect *Agrobacterium* growth in cocultivation medium (Fig. S5c,d). Interestingly, the presence of 7MTH-ITC and 8MTO-ITC facilitated *Agrobacterium* association to Col-0 seedlings and enhanced transformation efficiencies, even though it did not affect *Agrobacterium* cell growth in cocultivation medium. Because several *Arabidopsis* mutants with enhanced defence responses showed reduced *Agrobacterium* binding to their root explants (Gaspar et al., 2004; Sardesai et al., 2013), we propose that 7MTH-ITC and 8MTO-ITC suppress the defence responses of Col-0 seedlings, which then become more susceptible to *Agrobacterium* attachment, thereby promoting transformation efficiency.

Unlike the observations in *Arabidopsis* at an early *Agrobacterium* infection stage, where the total amounts of iGS and aGS were not increased relative to the mock control, crown galls hyperaccumulated particularly the aGSs 3MSOP and 8MSOO. In contrast, the content of iGSs was low and similar to the concentrations in mock-infected inflorescence stalks (Fig. 7a,b). Because all genes in aGS biosynthesis were suppressed during tumour development (Fig. S2b), the accumulation of aGSs in crown galls probably comes from long-distance transport via the *Arabidopsis* phloem (Chen et al., 2001). A similar observation was reported for *Arabidopsis* phloem-feeding and chewing insects, which specifically caused a significant increase in the amounts of aGSs, but not...
iGSs (Yan and Chen, 2007). The accumulated aGSs are probably not degraded because of low myrosinase enzyme activity in crown galls (Fig. 7c) and may not play a major role at the tumour stage. However, cyp79B2/B3 galls (Fig. 7c) and may not play a major role at the tumour stage. not degraded because of low myrosinase enzyme activity in crown iGSs (Yan and Chen, 2007). The accumulated aGSs are probably 1966 cyp79B2/B3 growth phenotype in the 2002), which is consistent with our observation of no detectable ing the IAA precursor indole-3-acetamide (IAM) (Sugawara et al. double mutant plants contain wild-type levels of indole-3-acetic acid (IAA) and grow in a similar manner to the wild-type under normal growth conditions (Stepanova et al., 2011; Zhao et al., 2002), which is consistent with our observation of no detectable growth phenotype in this mutant. The reduced IAA contents and growth phenotype in the cyp79B2/B3 mutant are temperature and light dependent, and can be restored to wild-type levels by supplying the IAA precursor indole-3-acetamide (IAM) (Sugawara et al., 2009; Zhao et al., 2002). This suggests that the auxin signalling and biosynthesis pathways remain active in the cyp79B2/B3 mutant. It is known that modifications in the biosynthetic pathway to iGS affects auxin production, because more precursors are available for IAA biosynthesis when the iGS pathway is interrupted (Malka and Cheng, 2017). In Arabidopsis crown galls, IAA can be produced via the agrobacterial enzymes IaaM and IaaH, in addition to plant-specific pathways (Britton et al., 2008). Thus, in crown galls of the cyp79b2/b3 double mutant, IAA may be over-produced and cause growth inhibition of the tumour because the iGS pathway is interrupted. An example for this hypothesis is the sur1 mutant, which produces less iGS, but increasing amounts of IAM, which causes growth inhibition of sur1 (Boerjan et al., 1995; Malka and Cheng, 2017). Calli induced in cyp79B2/B3 do not express the agro bacterial IAA biosynthesis pathway; therefore, IAA is only produced by the plant-specific pathway, does not over-accumulate and therefore callus growth is wild-type-like. Therefore, we propose that the mis-regulation of endogenous free IAA could be a possible reason for the small tumour in the cyp79B2/B3 mutant, although a possible role of iGS or some indole-3-acetaldoxime (IAOx)-derived metabolite in promoting tumour formation cannot be excluded.

However, despite the finding that the camalexin biosynthesis genes CYP71A12, CYP71A13 and PAD3 were up-regulated at 24 hpi, no significant change in the transient transformation efficiency could be observed in these mutants relative to Col-0 (Fig. 3), probably because of the low levels of camalexin produced in seedlings at the early stage of infection (Fig. 8a). However, Col-0 seedlings showed reduced transient transformation efficiency when camalexin was present in the co-cultivation medium at 10 μM (Fig. 8b,c), the concentration neither influencing GUS activity per se (Fig. 54c) nor inhibiting bacterial growth in medium or the association with seedlings (Figs 55e and 5f). Interestingly, exogenous application of camalexin can also reduce the callus induction ability of Col-0 root explants at 10 μM or higher in a dose-dependent manner (Figs 8c and 56b). Thus, we suggest that larger tumour development in the pad3-1 mutant (Fig. 6a) is caused by both higher T-DNA transformation efficiency at an early stage and increased cell proliferation at a later stage of tumour development because of the lack of camalexin.

In conclusion, this study provides transcriptomic data revealing both common and distinct genes expressed in shoots and roots of Arabidopsis seedlings in response to Agrobacterium infection. Focusing on the roles of GS and camalexin, we showed that iGS hydrolysis plays a defensive role at the early stage of Agrobacterium infection and causes reduced transient transformation efficiency, whereas camalexin accumulates during tumour development to restrict tumorigenesis. The findings that 1M and camalexin inhibit, and that 7MTH-ITC and 8MTO-ITC promote, transient transformation efficiency in Arabidopsis seedlings may be applicable to crown gall control and improve Agrobacterium-mediated transformation efficiency, respectively. Because GSs and camalexin are found mainly in certain Brassicaceae species, it remains to be tested whether these compounds can function in other crop species. It is worth studying the underlying mechanisms of these chemicals in regulating Agrobacterium transformation.

EXPERIMENTAL PROCEDURES

Plant materials

Arabidopsis thaliana ecotype Col-0 was used for the seedling transformation assay, transcriptome assay and Agrobacterium inoculation assay. GS mutants in the Col-0 background, including myb28/myb29 (SALK_136312 x GABI_868E02), cyp81f2-1 (SALK_073776), cyp81f2-2 (SALK_123882), myb51-1 (SM_3_16332), myb51-2 (SALK_059765), cyp79B2/cyp79B3 (Zhao et al., 2002), pen2-1 (Lipka et al., 2005) and pen2-2 (GABI-KAT 134CO4), the camalexin mutants, including cyp71A12 (GABI-KAT 127-H03), cyp71A13-1 (SALK_105136), cyp71A13-3 (SALK_128994) and pad3-1 (CS3805), and the cyp79b2/b3/myb28/29 quadruple (qko) mutant, completely free of GSs and camalexin, were used in the transient seedling transformation assay as described.

Agrobacterium transformation of Arabidopsis seedlings and GUS assays

The virulent A. tumefaciens wild-type strain C58 was used for the infection of Col-0 seedlings. Seeds were germinated in 2 mL of half-strength Murashige and Skoog (MS) (Basal Salt Mixture, PhytoTechnology Laboratories, Kansas City, Kansas, USA) liquid medium (half-strength MS salt supplemented with 0.5% sucrose (w/v), pH 5.7) in each well of a six-well plate.
Germination and growth took place in a growth room at 22 °C under a 16-h light–dark cycle (100 μmol/m²/s). Virulence of A. tumefaciens was pre-induced by 200 μM acetosyringone in AB-MES (AB Minimal Medium plus MES salt, pH 5.5) (Wu et al., 2014) at 25 °C for 16 h prior to infection. The Arabidopsis seedlings were infected with pre-induced A. tumefaciens C58 cells at an optical density at 600 nm (OD600) = 0.02 in half-strength MS medium. If the removal of agrobacterial cells was necessary, cocultivation medium was removed after the chosen infection time and replaced with 2 mL of freshly prepared half-strength MS medium containing 100 μM timentin, and incubated for recovery before analysis.

For the monitoring of the transient transformation efficiency, the T-DNA vector pBISN1 carrying the gusA-intron genes (Narasimhulu et al., 1996) was transformed into A. tumefaciens strain C58 for infection of Arabidopsis seedlings. GUS staining and activity assays were carried out as described at the chosen infection time (Salinas and Sánchez-Serrano, 2006; Wu et al., 2014). In brief, seedlings were stained by incubation in GUS staining solution containing 5-bromo-4-chloro-3-indolyl glucuronic acid (X-Gluc) and incubated at 37 °C in the dark overnight, followed by destaining in 90% ethanol (EtOH). For the GUS activity assay, liquid nitrogen-frozen seedlings from each well were ground into a fine powder to extract total protein. The GUS activity in 20 μg of protein per 200-μL reaction was quantified with the fluorochrome substrate 4-methylumbelliferyl-β-D-glucuronic acid (MUG). The fluorescence intensity (excitation, 365 nm; emission, 455 nm; filter at 430 nm) was measured using a Microplate Reader (BioTek, Taipei, Taiwan) at 37 °C for 1 h. GUS activity was normalized to the protein amount and 4-methylumbelliferone standard curve. For statistical analysis, one-way analysis of variance (ANOVA) with Dunnnett’s test was performed. To determine the effects of GS-derived metabolites and camalexin on GUS enzyme activity in vitro, the selected compounds and DMSO control were each incubated with 5 ng of recombinant GUS protein (Sigma-Aldrich, St. Louis, MO, USA) in GUS extraction buffer containing 1 mM MUG. The reaction mixture was measured for GUS activity at 37 °C for 1 h.

Transcriptome analysis

For gene expression profiling of Agrobacterium-infected seedlings, the shoots and roots of Col-0 seedlings (infected or mock control) were separated by cutting with a micro-scissor and immediately frozen in liquid nitrogen. Total RNA was extracted according to the protocol (pH 4.5)/chloroform protocol, followed by gene expression analysis with Affymetrix ATH1 chips (Affymetrix, Santa Clara, CA, USA). The chips of three biological repeats were normalized by the MA50.0 algorithm using GeneSpring software (Agilent Technologies, Santa Clara, CA, USA), and the genes with an intensity higher than the background value (value > 75), which passed the asymptotic unbiased test with Benjamini–Hochberg test correction (FDR, P < 0.05), were selected for further analysis. The fold changes were determined from the signals of infected plant tissues versus mock infection controls under the same conditions, and two-fold changes were used as cut-off to determine Agrobacterium-responsive genes. GOBU software (Lin et al., 2006) was used to analyse GO. The significant GO items were calculated with elim Fisher’s exact test (P < 0.01) based on gene counts (Alexa et al., 2006).

Crown gall growth assay

For the crown gall growth assay, the A. thaliana wild-type Col-0 and mutants were cultivated in growth cabinets (Percival, CLF, Wertingen, Germany) under short-day conditions at 22 °C (8 h of 80–100 μmol/m²/s light; Osram 400 W, Power Star HQI-E 400W/DV, 380–780 nm) (Wuerzburg, Germany) and 16 °C during the dark period (16 h) with a relative humidity of 50%–60%. Tumour development was induced by streaking virulent A. tumefaciens strain C58 into a wound of 1.5 cm in length, scratched into the base of young 5-cm-long inflorescence stalks. Tissue was harvested 28 days after infection using a scalpel and a binocular. Wounded, but uninfected, tumour-free inflorescence stalk sections of the same age served as reference tissues.

GS and camalexin analysis in Arabidopsis seedlings

Extraction and analysis of seedling GSs and camalexin were performed and modified as described previously (Glauser et al., 2012; Zandalinas et al., 2012). In total, 100 mg FW of Arabidopsis seedlings were homogenized and dissolved in 1 mL of 70% high-performance liquid chromatography (HPLC)-grade methanol containing 12.5 ng/mL salinbin (4-hydroxybenzyl GS) as an internal standard. The supernatants obtained were heated at 80 °C for 20 min and subjected to a UPLC-Synapt G1 high-definition mass spectrometry (HDMS) system (Waters, Taipei, Taiwan). GSs were separated on an Acquity CSH C18 column (length, 100 mm; 2.1 mm i.d.; 1.7 μm; Waters) at a flow rate of 400 μL/min. The GSs were eluted by solvent A (2% acetonitrile and 0.05% formic acid) and solvent B (100% acetonitrile and 0.05% formic acid) for 8 min in 10%–90% solvent B and 1 min in 45%–100% solvent B. The fractions were injected for MS analysis, and negative ion data were recorded in MS1 mode. The peak area was calculated by MassLynx software (Waters), and then normalized to nanomoles for GSs or micrograms for camalexin per gram FW. The GSs were quantified with the given references, including 13M for iGSs, 4MTB for methylthioalkyl GSs and 4MSOB for methylsulfinylalkyl GSs, purchased from AppliChem (Darmstadt, Germany). Camalexin was quantified with pure camalexin (Sigma-Aldrich, St. Louis, MO, USA).

GS and camalexin analysis in Arabidopsis inflorescence stalks

For GS analysis of infected Arabidopsis inflorescence stalks, 100 mg (FW) were lyophilized, thoroughly homogenized and extracted three times with 1 mL of 80% (v/v) methanol. For the first extraction step, benzyl GS (Phytoplan, Heidelberg, Germany) was added to each sample as internal standard. GSs were desulfonated as described previously (Agerbirk et al., 2001), and separated on a Grom-Sil B0 ODS 7 pH column (length, 60 mm; 4 mm i.d.; 4 μm; Alltech) (Wuerzburg, Germany) by HPLC (Agilent 1200, Waldbronn, Germany; flow rate, 0.25 mL/min). The desulfo GSs were eluted as follows: 0.3 min in 0%–5% solvent A (water), 7 min with 1.2 min hold in 5%–95% solvent B (methanol) and 3.5 min in 95%–5% solvent B. Desulfo-GSs were determined via UV diode array detection (229 nm), identified and quantified using particular response factors (aGSs, 1; iGSs, 0.26) (Gonzales-Megias and Müller, 2010).

Camalexin was extracted from lyophilized tissue (50 mg FW) by the addition of 400 μL of 85% methanol. The samples were thoroughly homogenized with a metal ball in a Mixer Mill 301 (Retsch, Haan, Germany). Wounded, but uninfected, tumour-free inflorescence stalk sections of the same age served as reference tissues.
GS derived metabolites and camalexin treatment for transient transformation assays and Agrobacterium cell counts

The selected aGS-ITCs (LKT Laboratories, St Paul, MN, USA) and camalexin were dissolved in DMSO, and I3M was dissolved in methanol. These compounds were added to the seedling co-cultivation medium for Agrobacterium infection and GUS assays, as described above.

For the measurement of the viable Agrobacterium cell number, the bacterial cells (CS8 strain carrying pBISNI) in co-cultivation medium and associated with seedlings at 1 and 3 dpi were collected. Six seedlings per well were washed by 2 mL of double-distilled H2O to remove unbound bacteria and ground by a mortar in 1 mL of 0.9% NaCl solution. The bacterial cells in medium or associated with seedlings were 10× serially diluted and then plated on 523 medium (Kado and Horinouchi, 1983) containing kanamycin, and incubated at 25 °C for 2 days to obtain colony-forming units (CFUs). The seedling-associated Agrobacterium cell number was further normalized to the plant fresh weight.

Myrosinase activity

Myrosinase activity was determined from 50–200 mg of frozen plant material, which was purified from internal substrate. Activity was measured by the photometric quantification of the released glucose from standardized amounts of externally added substrate according to the protocol developed by Travers-Martin et al. (2008).

Callus induction assay

Callus induction assay was performed and modified as described previously (Hwang and Gelvin, 2004). Col-0 and the tested mutants were grown on half-strength MS agar plates for 3 weeks, and the roots were cut into ~1-cm segments. About 60 root explants were transferred to agar plates containing callus induction medium (CIM), further incubated for 4 weeks, followed by counting of the number of developing calli and calculation of the rate of callus induction.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Fig. S1 T-DNA-encoded genes are expressed at detectable levels not before 24 h post-infection (hpi) of Arabidopsis seedlings. (a–d) Seven-day-old Col-0 seedlings were infected with Agrobacterium tumefaciens wild-type C58 harbouring a β-glucuronidase (GUS) construct, and analysed by GUS staining at 2–96 hpi. Representative images of infected seedlings at 24 hpi (b) or 48 hpi (c) of shoots and at 48 hpi of roots (d) are shown. (e) Percentage of shoots and roots with a GUS signal. (f–i) Col-0 seedlings were transferred into timentin-containing medium 2–72 h after infection with A. tumefaciens (infection time) and further incubated for another 24–94 h as indicated (recovery time), followed by GUS staining. Enlarged images show infected seedlings at 24 hpi (g) or 48 hpi (h) of shoots and at 24 hpi of roots (i). (j) Percentage of shoots and roots with a GUS signal. Scale bar: 1 mm (a, f); 1 mm (b–d, g–i).

Fig. S2 Gene expression profiles of the glucosinolate (GS) and camalexin biosynthesis pathways in Agrobacterium-infected inflorescence stalks. Biosynthesis pathways of indole glucosinolate (iGS) and camalexin (a) and of aliphatic glucosinolate (aGS) (b). Orange squares mark iGSs (a) and aGSs (b). The three squares next to the aGS pathway (b) present the fold changes (inoculated versus non-inoculated) of key genes at 3 h
post-infection (hpi) (left), 6 days post-infection (dpi) (middle) and 35 dpi (right). Differentially transcribed genes of four replicates are shown in red for up-regulation and green for down-regulation [false discovery rate (FDR), \(P < 0.05\)] as outlined in Lee et al. (2009), and the fold changes are presented as log2 values.

**Fig. S3** Glucosinolate (GS) profiles of *Agrobacterium*-infected *Arabidopsis* seedlings at 3 days post-infection (dpi). Mock (a) and infected (b) Col-0 seedlings were collected at 3 dpi for ultra-pressure liquid chromatography-mass spectrometry (UPLC-MS) analysis. The numbers marked above the peaks indicate the GS compound listed in Table S5, and sinalbin was used as an internal standard (IS). The peak area was calculated by MassLynx software (Waters, Taipei, Taiwan), and quantified by specific references. Concentrations of indole GSs (iGSs) (c) and aliphatic GSs (aGSs) (d) for mock (black) and *Agrobacterium*-infected (white) seedlings. Asterisks indicate significant changes compared with the mock sample (Student’s t-test, *\(P < 0.05\), \(n = 3\)). FW, fresh weight.

**Fig. S4** Effects of the glucosinolate (GS)-derived metabolites and camalexin on β-glucuronidase (GUS) enzyme activity. The recombinant GUS protein was incubated with indol-3-ylmethylglucosinolate (I3M) (a), aliphatic glucosinolate-isothiocyanates (aGS-ITCs) (b) and camalexin (c) for the assay of GUS enzymatic activity. The results are presented as the mean ± standard error of the mean (SEM) from three independent experiments (\(n = 9\)), and significant differences from the control groups methanol (MeOH) and dimethylsulfoxide (DMSO) are indicated [one-way analysis of variance (ANOVA) with Dunnett’s test, **\(P < 0.01\), ***\(P < 0.001\)].

**Fig. S5** Viable *Agrobacterium* cell numbers in co-cultivation medium and associated with the plant on indol-3-ylmethylglucosinolate (I3M) (a, b), aliphatic glucosinolate-isothiocyanate (aGS-ITC) (c, d) and camalexin (e, f) treatment at 1 and 3 days post-infection (dpi). *Agrobacterium* colony-forming units (CFU) obtained from six seedlings with similar size per well (*in planta*) (a, c, e) and from the medium per well (in medium) (b, d, f) at 1 and 3 dpi. *Agrobacterium* cell numbers associated with the seedlings were normalized to the plant fresh weight. Results are presented as the mean ± standard error of the mean (SEM) and asterisks indicate significant changes compared with the control groups methanol (MeOH) and dimethylsulfoxide (DMSO) [one-way analysis of variance (ANOVA) with Dunnett’s test, *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\)].

**Fig. S6** Root callus induction assays in the mutants lacking glucosinolates (GSs) and/or camalexin (a) and under camalexin-treated conditions (b). (a) Root explants from 3-week-old Col-0 and mutant plants were incubated on callus induction medium plates for 4 weeks. (b) Col-0 root explants were incubated on callus induction medium plates containing different concentrations of camalexin for 4 weeks. The number of root explants producing callus was counted under a dissection microscope. Results are presented as the mean ± standard error of the mean (SEM) from three experiments (\(n > 8\)), and asterisks indicate significant changes compared with the control Col-0 or dimethylsulfoxide (DMSO) treatment [one-way analysis of variance (ANOVA) with Dunnett’s test, **\(P < 0.01\), ***\(P < 0.001\)].

**Table S1** The enriched gene ontology (GO) items in shoots of C58-infected seedlings at 2 h post-infection (hpi).

**Table S2** The enriched gene ontology (GO) items in roots of C58-infected seedlings at 2 h post-infection (hpi).

**Table S3** The enriched gene ontology (GO) items in shoots of C58-infected seedlings at 24 h post-infection (hpi).

**Table S4** The enriched gene ontology (GO) items in roots of C58-infected seedlings at 24 h post-infection (hpi).

**Table S5** The detected glucosinolates and camalexin in *Arabidopsis* seedlings.

**Datasheet S1** The *Arabidopsis* gene list of *Agrobacterium*-responsive genes in shoots and roots at 2 and 24 h post-infection (hpi).

**Datasheet S2** Expression of the *Arabidopsis* Col-0 genes involved in glucosinolate and camalexin biosynthesis, as well as glucosinolate hydrolysis.