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Inducible expression of a Nep1-like protein serves as a model trigger system of camalexin biosynthesis

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ABSTRACT

Camalexin, the major Arabidopsis phytoalexin, is synthesized in response to a great variety of pathogens. Specific pathogen-associated molecular patterns, such as Nep1-like proteins from oomycetes act as signals triggering the transcriptional activation of the camalexin biosynthetic genes. PaNie, a Nep1-like protein from *Pythium aphanidermatum* was expressed in Arabidopsis under the control of an ethanol-inducible promoter. This system was developed as a tool to study the regulation of camalexin biosynthesis. It allowed induction of camalexin preceded by strong transcriptional activation of the tryptophan and camalexin biosynthetic genes. In flowers and green siliques PaNie expression elicited only minor camalexin formation, indicating low capability for phytoalexin synthesis in reproductive organs in contrast to leaf and stem tissue.

1. Introduction

In Arabidopsis the phytoalexin camalexin is synthesized *de novo* in response to a great variety of pathogens (Glawischnig, 2007). Infection triggers a complex signalling cascade resulting in strong transcriptional upregulation of the camalexin biosynthetic genes *CYP71A13* and *CYP71B15* (Nafisi et al., 2007; Schuhegger et al., 2006; Zhou et al., 1999). In this signalling cascade a number of MAP kinases are involved: MPK3, MPK6 (Ren et al., 2008), and MPK4, which physically interacts with WRKY33, a transcriptional activator of *CYP71A13* and *CYP71B15* (Qiu et al., 2008). Camalexin is synthesized from tryptophan via *CYP79B2/B3* (Glawischnig et al., 2004). As the cellular tryptophan pool is only in the micromolar range (Müller and Weiler, 2000) camalexin formation is coupled with high induction of the tryptophan biosynthetic genes, such as *ASA1* and *TSA* (Schuhegger et al., 2007; Zhao and Last, 1996).

Phytoalexins are typically synthesized locally in proximity to the site of pathogen infection (Kuc, 1995). Therefore, the amplitude of the camalexin response strongly depends on the spatial distribution of the growing pathogens. Microorganisms can actively interact with the defence mechanisms of the plant and the kinetic of camalexin induction in response to pathogen application, either by spraying or infiltration, might vary dependent on the growth phase and metabolic state. Some pathogens actively degrade camalexin (Pedras and Khan, 2000). These problems are avoided by triggering camalexin formation with heavy metals, such as silver nitrate (Tsuji et al., 1993). However, the response to this abiotic treatment is not identical with a response to pathogen infection. In addition, after silver nitrate spraying spots of cells undergoing hypersensitive response are observed which are surrounded by healthy tissue. The consequence is that gene expression and metabolites are analyzed as a patchwork of healthy and dying cells.

Nep1-like proteins (NLP), comprise a family of microbial virulence factors, which are phylogenetically widely distributed (Gijzen and Nürnberger, 2006; Pemberton and Salmond, 2004). Their mechanism of action has been investigated in detail with NLP_{Pp}, NLP_{Ps}, and PaNie (NLP_{Pya}) identified in the oomycetes *Phytophthora parasitica*, *Phytophthora sojae*, and *Pythium aphanidermatum*, respectively (Qutob et al., 2006; Veit et al., 2001). In addition to their role as toxin-like virulence factors, NLPs are efficient triggers of plant innate immune responses. A signalling cascade is induced, which involves reactive oxygen intermediates and ethylene synthesis and results in callose deposition and programmed cell death. As a component of these defence reactions high concentrations of camalexin are induced, which are observed after 8 h in response to infiltration with NLP_{Ps} or PaNie (Qutob et al., 2006).

Here, we investigated the induction of camalexin biosynthesis in plants, which heterologously express PaNie under the control of an ethanol inducible promoter. In vegetative tissue this system allows simple and reproducible camalexin expression upon pathogen-associated molecular pattern (PAMP) treatment. Reproductive organs showed low competence for camalexin synthesis.

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2. Results and discussion

2.1. Inducible expression of PaNie (NLP_{Pya}) in Arabidopsis

The elicitor PaNie from *P. aphanidermatum* triggers programmed cell death and a multitude of defence responses, including camalexin formation in Arabidopsis leaves within a few hours (Qutob et al., 2006; Veit et al., 2001). PaNie has been applied by infiltration of Arabidopsis leaves. This induction has to be technically performed with great care to allow reproducible results and it has to be ensured that the protein has not been degraded. In order to avoid such problems, PaNie was expressed as a transgene in Arabidopsis under the control of the ethanol inducible promoter AlcA (Maizel and Weigel, 2004; Roslan et al., 2001).

The first 21 amino acids at the N-terminus of PaNie presumably constitute an export targeting sequence. Transgenic lines for expression of three different proteins were generated: (i) native PaNie ($PaNie_L$), (ii) PaNie without N-terminal targeting sequence ($PaNie_{Del}$), and (iii) PaNie with the oomycete-derived targeting sequence replaced by the extensin export targeting sequence from carrot (*Daucus carota*) (Chen and Varner, 1985) ($PaNie_{Dc}$). The plants were monitored for symptoms of cell death 40 h after ethanol treatment. While plants, which carry the native or N-terminally truncated PaNie remained phenotypically wildtype (Fig. 1C and D), a number of lines expressing PaNie with the plant export targeting sequence developed severe symptoms (Fig. 1A). These results implied that the oomycete export signal is not correctly transmitted in the plant and a plant-derived targeting sequence is a prerequisite for protein export.

2.2. Camalexin biosynthesis in response to $PaNie_{Dc}$ expression

In Alc::$PaNie_{Dc}$ plants camalexin formation was observed in rosette leaves 6 h after spraying with ethanol (Fig. 2). With three independent batches of plants, 24 h after spraying with 2% ethanol, camalexin concentrations in μg per g fr. wt of 16.1 ± 4.0, 15.9 ± 1.6, and 8.2 ± 4.3 were obtained (Fig. 2, Supplementary Figs. S1 and S2). These values exceeded levels obtained in response to silver nitrate spraying, ranging at 5.3 ± 1.7/3.7 ± 1.2 μg/g fr. wt (Glawischnig et al., 2004; Nafisi et al., 2007) or infiltration with PaNie protein (Qutob et al., 2006; dry weight data provided therein equals 3.6 ± 1.6 μg/g fr. wt (unpublished)). After 24 h no significant degradation of accumulated camalexin was observed (Supplementary Fig. S2).

Glucosinolates are mainly involved in defence against herbivores but show also activity against pathogens (Halkier and

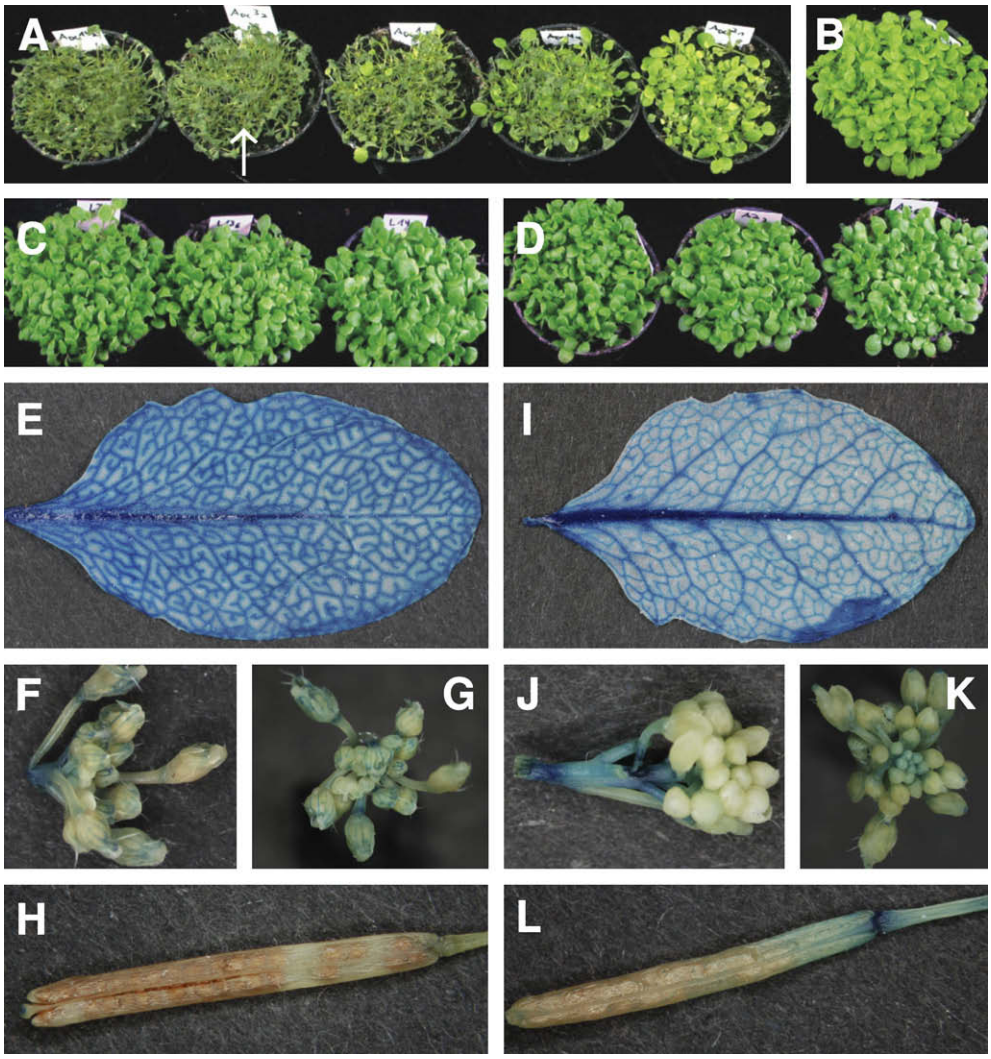

Fig. 1. Phenotype of Alc::$PaNie_{Dc}$ plants and tissue specificity of camalexin biosynthetic gene expression. A: Seedling phenotype of Alc::$PaNie_{Dc}$ lines 40 h after ethanol induction. Severe symptoms were observed. An arrow denotes a single insertion line selected for further studies. Col-0 (B), Alc::$PaNie_{Del}$ (C), or Alc::$PaNie_L$ (D) lines 40 h after ethanol spraying did not develop symptoms. Activity of *CYP79B2p:GUS* (E, F, G, H) or *CYP71B15p:GUS* (I, J, K, L) in Alc::$PaNie_{Dc}$ background 8 h after induction. E, I: rosette leaves; F, G, J, K: inflorescence; H, L: siliques. Low GUS activity in reproductive organs was observed.

Gershenzon, 2006; Tierens et al., 2001). Therefore, also a potential effect of PaNie$_{Dc}$ expression on glucosinolate composition was investigated, which was largely unchanged after ethanol spraying (Supplementary Fig. S3).

The transcriptional activation of tryptophan and camalexin biosynthetic genes in rosette leaves was analyzed by quantitative RT-PCR (Fig. 3). Strong transient activation, up to 400-fold, was observed and the time course of this induction matched between the different genes analyzed (Fig. 3). The tryptophan biosynthetic genes *ASA1* and *TSA* were induced more than 50-fold within 6 h after induction. The kinetics of *ASA1* and *TSA* induction suggests that camalexin biosynthetic genes are highly co-regulated with the tryptophan biosynthetic genes. This is in accordance with the analysis of published array data on the response of the Col-0 transcriptome to pathogen infection, using programs such as expression angler (Toufighi et al., 2005). This observed co-regulation suggests that in response to pathogen infection, camalexin is a major sink for tryptophan. Here, in response to PaNie we observed a camalexin synthesis rate between 6 and 8 h of 1.75 ± 0.96 μg h^{-1} g^{-1} (fr. wt).

2.3. Which tissues are capable of synthesizing camalexin?

It is well established that camalexin formation can be triggered in rosette leaves (Glawischnig, 2007). Root pathogens, such as *Pythium sylvaticum* or *Plasmodiophora brassicae*, also induce camalexin biosynthesis (Bednarek et al., 2005; Siemens et al., 2008).

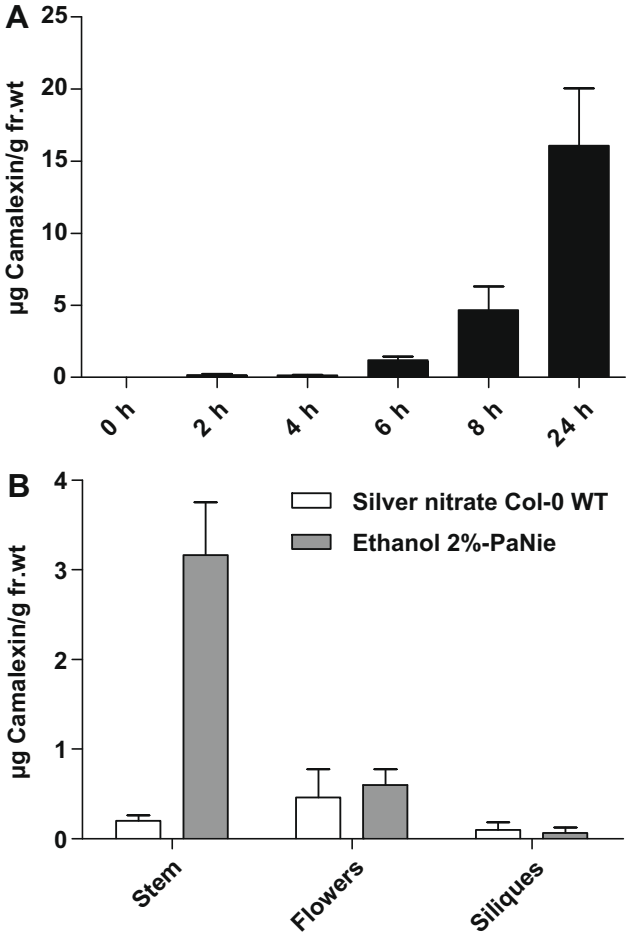

Fig. 2. Induction of camalexin synthesis. A: Camalexin formation in rosette leaves of Alc::PaNie$_{Dc}$ plants induced with ethanol. B: Camalexin formation in stem, flower, and silique of Col-0 plants 24 h after silver nitrate spraying or Alc::PaNie$_{Dc}$ plants 24 h after induction with ethanol.

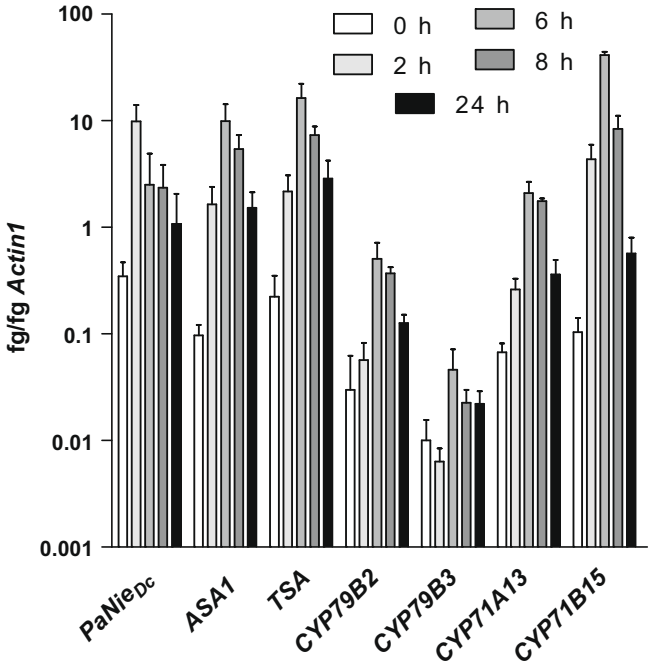

Fig. 3. Induction of tryptophan and camalexin biosynthetic genes in rosette leaves of Alc::PaNie$_{Dc}$ plants. The same plant material as in Fig. 2A was analyzed. Transcript concentrations relative to *Actin1* in response to ethanol spraying were determined by quantitative RT-PCR (n = 3).

In order to analyze, which mature tissues are capable of synthesizing camalexin, stem tissue, flowers, and siliques were harvested from 10-week-old Alc::PaNie$_{Dc}$ plants, induced with ethanol for 24 h, and analyzed for camalexin concentration (Fig. 2). The camalexin concentration in stem tissue was approx. 20% to 40% in comparison to leaves. In flowers and siliques respectively, only ~4–7% and 0.4–0.8% of the camalexin concentration present in leaves was detected. To analyze whether this effect is due to low induction of the PaNie$_{Dc}$ transgene in flowers and siliques *PaNie$_{Dc}$* transcript levels were quantified. In response to ethanol spraying after 2 h *PaNie$_{Dc}$* was induced in flowers and siliques, to ~1.1 and 0.2 fg per fg *Actin1*, respectively. Despite lower levels of transgene expression in stem (~0.4 fg per fg *Actin1*) in comparison to flowers, induction of the camalexin biosynthetic genes was much more efficient. This suggests that *PaNie$_{Dc}$* levels are not the limiting factor for camalexin synthesis in flowers of induced Alc::PaNie$_{Dc}$ plants. It cannot be ruled out that in siliques *PaNie$_{Dc}$* expression is below a threshold necessary for triggering camalexin synthesis.

Transcript concentrations of tryptophan and camalexin biosynthetic genes were monitored in stem tissue, flowers, and siliques 0, 2, and 6 h after ethanol spraying (Fig. 4). Transcript levels after induction correlated with the observed camalexin concentration (Fig. 2). Induction of the camalexin biosynthetic genes in flowers and siliques was minor, consistent with the low concentration of observed end product. In conclusion, flowers and siliques show low competence to transmit the PAMP signal into induction of phytoalexin synthesis.

Alc::PaNie$_{Dc}$ plants were crossed with plants expressing GUS under the control of the *CYP71B15* or *CYP79B2* promoter (Glawischnig et al., 2004; Mikkelsen et al., 2000; Schuhegger et al., 2006). GUS expression in response to ethanol spraying in these crosses allowed analyzing the spatial distribution of competence for camalexin biosynthesis in more detail. Rosette leaves were evenly competent for camalexin synthesis. This contrasts our observation in flowers and green siliques, where both silver nitrate application and PaNie expression trigger the synthesis of only minor amounts

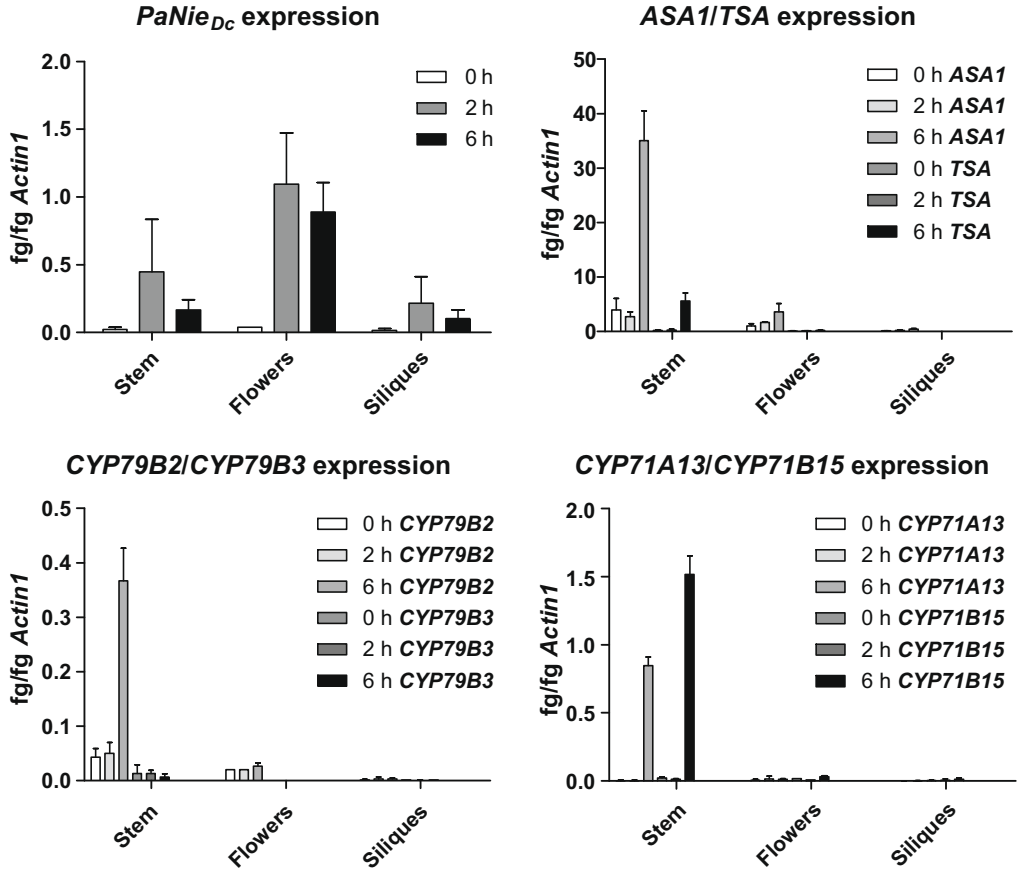

Fig. 4. Induction of tryptophan and camalexin biosynthetic genes in stem, flower, and silique tissue of Alc::PaNie$_{Dc}$ plants induced with ethanol. Transcript concentrations relative to *Actin1* were determined by quantitative RT-PCR ($n = 3$).

of camalexin (Fig. 2). Similarly, *CYP79B2p::GUS* and *CYP71B15p::GUS* expression was low in induced flowers and siliques (Fig. 1).

Probably components of the camalexin-linked signalling cascade, which remain to be elucidated, are low expressed in reproductive organs. One has to keep in mind that camalexin is also toxic for the plant (Rogers et al., 1996). While loss of some leaf tissue during defence against pathogens is tolerated, camalexin synthesis in flowers or seeds might impair reproductive fitness. Therefore, it would be not a useful defence strategy against infection of these organs. High concentrations of glucosinolates are accumulated in flowers, siliques, and particularly in seeds (Brown et al., 2003; Petersen et al., 2002). In contrast to camalexin, glucosinolates are converted to biologically active degradation products primarily when they come into contact with myrosinases after tissue disruption (Halkier and Gershenzon, 2006). Possibly, in flowers and siliques glucosinolates are therefore advantageous as defence molecules and lack of the phytoalexin is compensated by high concentrations of phytoanticipins (VanEtten et al., 1994).

3. Conclusion

The quantity of camalexin synthesis in response to pathogen infection depends on the metabolic state and the mode of application of the microorganism. Here, we developed a system that allows very simple handling and reproducible time-dependent induction of the camalexin biosynthetic genes. It can be provided as a helpful tool for the analysis of camalexin signalling, e.g. to be used in genetic screens. Candidate components of the signalling cascade triggering camalexin biosynthesis can be evaluated by crossing corresponding mutants with Alc::PaNie$_{Dc}$ plants. The reproducibility of the system will facilitate the detection of alterations on transcript, protein, and metabolite levels.

4. Experimental

4.1. Generation of PaNie expressing plants

For construction of full length PaNie, or PaNie lacking N-terminal 21 amino acids leader peptide was cloned into pBJ36_AlcA (http://www.weigelworld.org/resources/plasmids/Alc/pBJ36_AlcA) *via XhoI/BamHI*. The leader of the extensin gene (Dc) of *Daucus carota* (Chen and Varner, 1985) was amplified from carrot DNA using the primer pair tactctcgagatgggaagaattgctagagg/ttaccatggcagctgtggtttcggaagcc (5′ to 3′ orientation). PaNie was amplified as described previously (Veit et al., 2001) and then cloned into pBJ36_AlcA, containing the leader of the extensin gene (Dc) via *BamHI*. This construct was cloned into pMLBART_AlcR (http://www.weigelworld.org/resources/plasmids/Alc/pMLBART_AlcR) via *NotI*.

4.2. Plant growth conditions and reporter gene analysis

Alc::PaNie$_{Dc}$ plants were crossed with *CYP79B2p::GUS* and *CYP71B15p::GUS* plants (Mikkelsen et al., 2000; Schuhegger et al., 2006). Plants were grown in soil mixed with sand (3:1) in a growth chamber at 12 h light, 21 °C, 80–100 μmol of photons per m^2 per s and 40% relative humidity. GUS staining was performed as described previously (Glawischnig et al., 2004; Schuhegger et al., 2006).

4.3. Camalexin induction and analysis

The rosette leaves of 6-week-old plants or flowers, siliques, and stems of 10-week-old plants were sprayed with 5 mM $AgNO_3$ or 2% ethanol and incubated under a plastic hood. Spraying with ethanol concentrations $\geqslant 3\%$ resulted in a slight reduction of camalexin yield (Supplementary Fig. S1). Camalexin extraction and analysis was performed as described previously (Glawischnig et al., 2004). For each measurement 3–6 plants were used as biological replicates. Glucosinolate extraction was performed according to Petersen et al. (2001).

4.4. RNA extraction and quantitative real time PCR

For RNA extraction from leaves, flowers and stems (50–100 mg), NucleoSpin®RNA Plant-Kit (Macherey-Nagel, Düren) was used according to the manufacturer's instructions. Silique material (100 mg) was homogenized with RNA extraction buffer (25 mM Tris–HCl pH 8.0, 25 mM EDTA pH 8.0, 75 mM NaCl, 1% SDS), extracted with phenol/$CHCl_3$/isoamylalcohol (50:24:1), phenol/$CHCl_3$/isoamylalcohol (25:24:1) and $CHCl_3$/isoamylalcohol (24:1). RNA was precipitated with 0.25 vol 10 M LiCl, washed with 70% EtOH, dissolved in 200 µl DEPC-treated H_2O, precipitated with 2.5 vol EtOH/0.1 vol 3 M NaOAc pH 5.2, washed with 3 M NaOAc and 70% EtOH, and dissolved in 50 ml DEPC-treated H_2O.

Total RNA (0.5 µg) was used for cDNA synthesis according to the manufacturer's instructions (TaqMan, Roche, Mannheim, Germany). Quantitative real time PCR experiments were performed with a LightCycler instrument using the LightCycler® FastStart DNA Master^{PLUS}SYBRGreen I-Kit (*PaNie*$_{Dc}$, *ASA1*, *TSA*, *CYP79B2*, *CYP79B3*, *CYP71B15*) or the LightCycler® FastStart DNA Master SYBRGreen I-Kit (*Actin1*, *CYP71A13*) (Roche, Mannheim, Germany). Expression of the genes of interest was normalized to *Actin1*. For primer sequences see Supplementary Table ST 1. The annealing temperatures were as follows: *Actin1* (52 °C), *PaNie*$_{Dc}$ (54 °C), *ASA1* (54 °C), *TSA* (54 °C), *CYP79B2* (60 °C), *CYP79B3* (58 °C), *CYP71A13* (55 °C) and *CYP71B15* (54 °C). For *PaNie*$_{Dc}$ 0.2 µl DMSO, for *ASA1* 0.5 µl DMSO, for *CYP71A13* and *Actin1* 1.2 µl $MgCl_2$ (25 mM) were added to the reaction. For each measurement RNA preparations from three different plants were used as replicates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.12.010.

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