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Expression of antimicrobial peptides under control of a camalexin-biosynthetic promoter confers enhanced resistance against *Pseudomonas syringae*

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ABSTRACT

In *Arabidopsis thaliana* phytoalexin biosynthesis is tightly regulated. The camalexin biosynthetic gene *CYP71B15*/*PAD3* is highly expressed in response to pathogens and specific abiotic triggers, while constitutive expression is very low. Based on this property we expressed artificial antimicrobial peptides under control of the *CYP71B15* promoter avoiding potential toxic effects to the plant related to constitutive expression. Significant and substantial growth inhibition of *Pseudomonas syringae* was observed, demonstrating that expression of these peptides under control of a phytoalexin promoter is an effective approach for enhancement of resistance against bacterial pathogens.

1. Introduction

Plant protection is mainly based on the application of chemicals (Montesinos, 2007) and resistance against new pesticides develops very quickly within microbe populations. Antimicrobial peptides (AMPs) are seen as an appropriate alternative (Marshall and Arenas, 2003; Perron et al., 2006; Hancock and Sahl, 2006; Baltzer and Brown, 2011; Sumi et al., 2015; Xiao et al., 2015). They are widely spread among mammals, amphibians, insects and plants (Wang et al., 2009) underlining their antimicrobial efficiency and importance in evolution (Thomma et al., 2002; Zasloff, 2002). Natural AMPs often show a broad activity spectrum against important pathogens, but some are also highly toxic to human and plant cells (Marcos et al., 2008; Maróti et al., 2011; Wilmes et al., 2011; Seo et al., 2012). Several families of AMPs have been identified in plants (Garcia-Olmedo et al., 1998; Montesinos, 2007; Pelegrini et al., 2011). Though their primary sequences are very divers, shared structural properties exist, such as small size and a clustering of cationic and hydrophobic amino acids (Boman, 2003; Fjell et al., 2012). Due to these properties, the classic mode of action of AMPs involves their ability to incorporate into microbial membranes, to form pores and cause cell membrane damage (Epand and Vogel, 1999; Brogden, 2005; Schmidt and Wong, 2013). However, in the last decade intracellular targets of AMPs were also identified (Pinto da Costa et al., 2015; Guilhelmelli et al., 2013). In order to obtain AMPs showing high activity against pathogens and low toxicity against human and plant cells, artificial AMPs were rationally designed, and production in tobacco was established (Zeitler et al., 2013a,b). A set of in total 60 synthetic peptides (SPs) was generated belonging to four structural groups. Several of these peptides inhibited the growth of bacterial pathogens, such as *Pseudomonas syringae*, *Xanthomonas vesicatoria* and *Clostridium herbarum*, *in vitro* at concentrations below 1 μg/ml with no toxic side effects to human cells or plant protoplasts.

In response to pathogen infection and specific abiotic treatments many plants synthesize phytoalexins, low molecular weight antimicrobial compounds, which are largely absent in healthy tissue (Jeandet et al., 2014). Characteristic for *Arabidopsis* is camalexin, which became a model compound to study phytoalexin biosynthesis, regulation, and relevance for pathogen resistance *in vivo* (for review see: Rauhut and Glawischnig, 2009). Essential for camalexin formation is CYP71B15/Phytoalexin Deficient 3 (PAD3), which catalyzes two specific biosynthetic steps (Glazebrook and Ausubel, 1994; Zhou et al., 1999; Schuhegger et al., 2006; Böttcher et al., 2009). *CYP71B15* is tightly transcriptionally regulated. While constitutive expression is very low, *CYP71B15* transcript levels increase several orders of magnitude

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in response to pathogen perception. This was e.g. observed for infection with virulent and avirulet *P. syringae* strains or the fungus *A. alternata* or for inducible expression of a NEP1-like protein from *Pythium aphanidermatum* (Zhou et al., 1999; Schuhegger et al., 2007; Rauhut et al., 2009). As shown by qRT-PCR data of dissected leaf tissue, this induction is strictly localized to tissue in which an infection is detected by the plant (Schuhegger et al., 2007). These properties suggest the *CYP71B15* promoter as an appropriate tool to express transgenes in *Arabidopsis*, which are effective against pathogens, but potentially harmful to plant cells, if expressed constitutively.

In this work we expressed selected artificial antimicrobial peptides under control of the *CYP71B15* promoter. While the plants developed normally, resistance against *P. syringae* was substantially increased. This provides a proof of concept that expression of antimicrobial peptides under control of a phytoalexin-specific promoter is an effective tool to reduce bacterial infection in plants.

2. Results

2.1. Generation of CYP71B15p::SP plants

The *CYP71B15* promoter (Schuhegger et al., 2006) was cloned into a gateway-compatible vector, which was originally designed for expression of tagged ribosomal protein RPL18 in translatomics analysis (Mustroph et al., 2009). After RPL18 excision, sequences encoding AMPs could be directly cloned into the resulting KpnI/XbaI restriction sites as hybridized oligonucleotides, which were designed according to the desired peptide sequence, taking codon usage in *Arabidopsis* into account. Five different peptides were selected for expression, based on *in vitro*-properties and for coverage of three structural different subgroups: SP1, SP10-2, SP10-5, SP13-6 and SP15 (Zeitler et al., 2013b). *A. thaliana* Col-0 was transformed with the resulting expression plasmids and primary transformants were identified by kanamycin resistance and PCR specific for the SP construct.

High dosages of UV light highly reproducibly trigger the biosynthesis of cruciferous phytoalexins (Pedras et al., 2009). Therefore, SP expression levels in transgenic plants were analyzed with and without UV challenge. For each peptide, lines were identified in which expression of SPs was dramatically increased in response to induction (Fig. 1). These lines were selected for analysis of pathogen resistance.

2.2. CYP71B15p::SP plants show substantially enhanced resistance against P. syringae

In order to analyze the effectiveness of the five different antimicrobial peptides *in planta* for which *CYP71B15p::SP* lines were generated, infection studies with *P. syringae* DC3000 were performed. In response to virulent *P. syringae*, *CYP71B15* transcript levels are highly elevated 24 h after infection (Zhou et al., 1999). We determined *SP* transcript levels in *CYP71B15p::SP* 24 h after infiltration and observed substantial induction for most lines (Fig. 1). 48 h after infiltration the number of bacteria per leaf area was determined. For all lines tested significant and substantial bacterial growth inhibition was observed (Fig. 2). For example, for line SP10-5 #20 bacterial count was reduced to approx. 5% of wild-type level.

To analyze these lines for resistance against a fungal pathogen, *Alternaria brassicicola*, for which camalexin gene induction is documented (Narusaka et al., 2003), was applied as a model. To analyze SP gene induction, plants were spray-inoculated with a spore suspension and after 2 d, SP transcript levels were determined (Supplemental Fig. 1). Resistance was analyzed by a drop infection approach. Up to three leaves were inoculated with a drop of spore suspension and after 5 d incubation, lesion sizes and the percentage of infected leaves (lesion numbers) were determined (Fig. 3). For line SP10-2 #26 a reduction of lesion number to approx. 71% of wild-type was detected (Fig. 3B), while the lesion size was largely unchanged (Fig. 3C). For all other lines no significant effects were observed. These results are in accordance with *in vitro* data showing that the investigated SPs generally were more effective against bacterial than against fungal pathogens (Zeitler et al., 2013b).

3. Discussion

In this proof-of-concept study we tested, whether expression of artificial antimicrobial peptides under control of a phytoalexin-specific promoter enhances resistance against pathogens. Therefore a set of peptides was expressed in *Arabidopsis* under control of a camalexin-specific promoter, which is known to be highly active in tissue infected with pathogens. The effects on the fungus

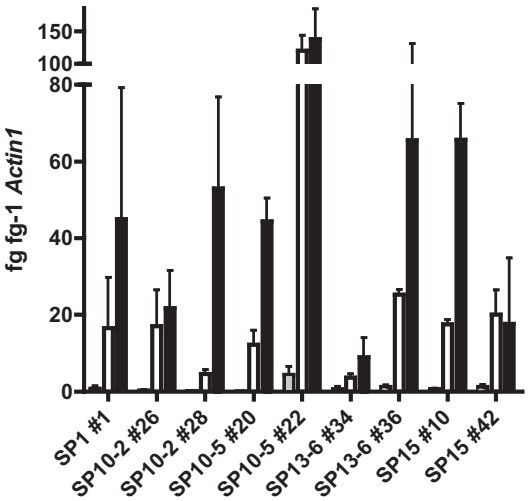

Fig. 1. Validation of *Arabidopsis CYP71B15p::SP* expression lines: *SP* transcript abundance in UV-treated (white bars), *P. syringae*-infected (black bars), and untreated (gray bars) rosette leaves (in fg *SP* per fg *Actin1*; $n = 3$).

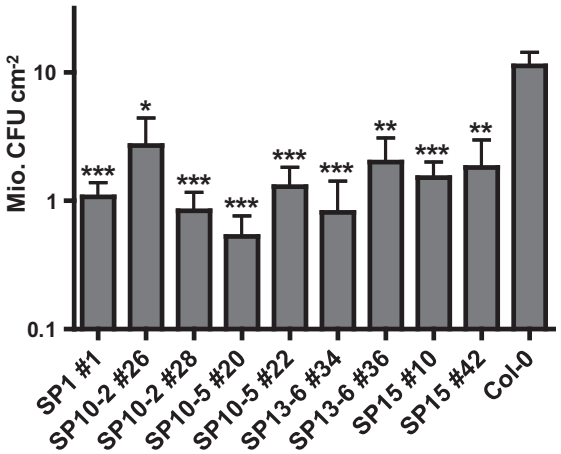

Fig. 2. Enhanced resistance of *CYP71B15p::SP* lines against *P. syringae*. Mio. colony-forming units (CFU) cm^{-2} were determined 48 h after infiltration. Significant reduction with respect to Col-0 was observed (*t*-test after log-transformation; $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 10^{-4}$).

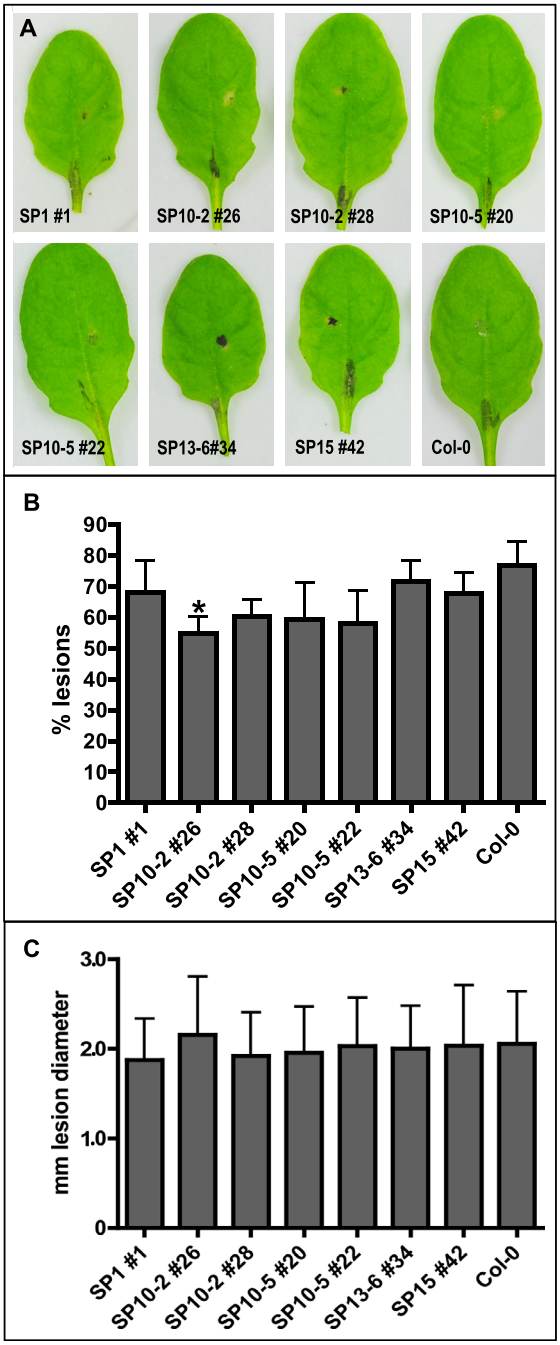

Fig. 3. Infection of *CYP71B15p::SP* lines with *A. brassicicola*. (A) Lesion phenotype. Representative rosette leaves are shown (black marker for tagging of infected leaves was applied). No morphological differences were detected between SP lines and Col-0. (B) Quantification of numbers of leaves infected per total number ($n = 3–6$). Line SP10-2 #26 showed reduction with respect to Col-0 (t-test; $^{*}p = 0.014$). (C) Lesion diameters ($n = 37–157$); means and standard deviations are indicated.

A. brassicicola were minor (Fig. 3), even for SP15 line 42, for which we observed high *SP* transcript levels (Supplemental Fig. 1). In contrast, the tested antimicrobial peptides effectively inhibited growth of *P. syringae* (Fig. 2). *In vitro*, substantial differences in the inhibition of *P. syringae* pv tomato (Zeitler et al., 2013b) were detected for these AMPs, ranging from MIC concentrations of 0.25 μg/ml for SP10-5 to 40 μg/ml for SP15. Surprisingly, here we observed similar *in vivo* growth inhibition for lines SP10-5 #22 and SP15 #10 (Fig. 2). For both lines high *SP* transcript levels were detected in *P. syringae* infected leaves.

Possibly, in response to infection, all lines synthesize sufficiently high local peptide concentrations to exceed the respective MIC concentrations. Alternatively, the peptides have different modes of action. While SP1, SP10-2, SP10-5, and SP13-6 directly "attack" the pathogens, the phytotoxic SP15 destroys the plant cells at the side of infection similar to a hypersensitive response (Zeitler et al., 2013b). In summary, expression of antimicrobial peptides under control of the *CYP71B15* promoter robustly inhibited growth of *P. syringae*.

Applying the *CYP71B15* promoter provided the advantage that constitutive expression is very low (Fig. 1) and growth and leaf morphology apparently was not visibly affected (Fig. 3A, Supplemental Fig. 1). Other inducible promoters have been used previously for the expression of AMPs, including e.g. a heat-shock inducible promoter (Company et al., 2014). For expression of an antifungal defensin, an *Agrobacterium* promoter was applied, which is responsive to auxin and wounding and therefore expressed at infection sites but also in vascular tissue (Langen et al., 2006). Similarly, a wound-inducible promoter from poplar was successfully used for the expression of cecropin A-melittin (Yevtushenko et al., 2005). The *CYP71B15* promoter used in this study shows only minor wound induction (Schuhegger et al., 2006), but is highly inducible by PAMPs, such as NEP1-like proteins (Rauhut et al., 2009). Consequently, SP expression can be induced upon perception of the pathogen independent of wounding. Also for biotechnological production of AMPs or other potentially toxic peptides or proteins in *Arabidopsis*, expression under control of the *CYP71B15* promoter could be effective in combination with high dosages of UV-light as a simple and effective trigger applicable to whole batches of plants.

We propose that the presented strategy expressing SPs under control of a phytoalexin biosynthetic promoter can be applied to crops in which bacterial pathogens are important pests. As some AMPs have also specific intracellular targets, development of resistances against AMPs expressed in plants is a challenge, which has to be carefully evaluated. Here the use of artificial, in contrast to naturally occurring peptides could possibly minimize ecological and health risks related to spreading resistances. Especially, if SPs target microbial membranes as generalized targets, this bares a lower risk of resistance development (Peschel and Sahl, 2006).

In a number of cases, antimicrobial peptides have already been expressed in crops to combat eukaryotic, but also bacterial pathogens (Jung and Kang, 2014). For example, cecropin B expression in rice reduced infection with *Xanthomonas oryzae* causing bacterial leaf blight (Sharma et al., 2000). Here, expression of SPs under control of the promoter for a rice diterpenoid phytoalexin biosynthetic gene might prove efficient against this disease.

4. Experimental

4.1. Generation and molecular analysis of CYP71B15p::SP plants

The CYP71B15 promoter sequence was amplified from *pCYP71B15p::GUS* (Schuhegger et al., 2006) using the primer pair GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTATACTGTGGCTATATA TG/GGGGACCACTTTGTACAAGAAAGCTGGGTTTCCTTGCCCTGTTCTT GTG, then introduced into pDONR223, and subsequently into pGATA:HF-RPL18 (Mustroph et al., 2009) via Gateway reactions. The resulting plasmid was digested with *Kpn*I/*Xba*I by which tagged RPL18 (Mustroph et al., 2009) was removed. For each peptide two oligonucleotides were designed, taking codon usage in *Arabidopsis* into account. These oligonucleotides were mixed at concentrations of 20 μM, incubated at 95 °C for 10 min, then hybridized by slow cooling to 25 °C, generating *Kpn*I/*Xba*I compatible overhangs and ligated into the open *Kpn*I/*Xba*I sites of the vector.

For a list of primers see Supplemental Table 1. Resulting expression clones were confirmed by sequencing and transferred into *A. thaliana* Col-0, via Agrobacterium mediated floral dip.

Plants were grown on soil in a growth chamber at a 12 h photoperiod (light intensity of 80–100 μmol m^{-2} s^{-1}) at 21 °C. Primary transformants were identified by kanamycin resistance and PCR with the primer pair ACGATTATTATTCACAAGCTACAG/TTTTTACAACGTGCACAACAGAAT. UV induction was carried out as in Mucha et al. (2015). Leaves were placed under a UV lamp (Desaga UVVIS, λ = 254 nm, 8 W) at a distance of 20 cm, radiated for 2 h and incubated additional 14 h. RNA extraction, cDNA synthesis and RT-qPCR, performed with the SYBRGreen/Light Cycler system (Roche), has been described previously (Schuhegger et al., 2007). For primers see Supplemental Table 1. *SP* expression in response to *P. syringae* and *A. brassicicola* infection (see below) was analyzed after 24 and 48 h, respectively.

4.2. Pathogen assays

P. syringae pathovar tomato DC 3000 was grown at 28 °C for 1 d in NYG (5 g/L peptone, 3 g/L yeast extract and 20 ml/L glycerol) medium containing rifampicin (50 μg/ml) and kanamycin (30 μg/ml). Cells were diluted in 10 mM $MgCl_2$ to a concentration of 1×10^5 cfu/ml. Three leaves each of 3 different plants for each line were cut and infiltrated at the abaxial side. The infiltrated leaves were placed on a wet tissue in a plate and incubated in ambient light (RT, 2 d). 4 leaf disks (r = 2 mm) of each leaf were punched out with a cork borer, collected in Eppendorf tubes with 500 μl $MgCl_2$/0.01% Silwet and incubated in a shaker (28 °C, 1 h). 100 μl of each sample were transferred to a 96-well plate and four consecutive serial 10× dilutions were made by mixing each 10 μl of bacterial suspension with 90 μl $MgCl_2$ (10 mM). From the 10^{-2}, 10^{-3} and 10^{-4} dilutions, each 20 μl were plated on NYG agar with 50 μg/ml rifampicin and 30 μg/ml kanamycin. After incubation at RT for 3 d, colonies were counted and the original titre (per cm^2 leaf area) was calculated.

For conidia production *A. brassicicola* was grown on oatmeal agar (2% oatmeal flakes and 1.5% agar) at RT for 3 weeks. Conidia were harvested by washing the plates with 60 mM KH_2PO_4 (pH 6)/0.5 mM glucose/0.001% tween 20, and conidia concentration was adjusted to 200 conidia/μl. For *A. brassicicola* pathogen assays plants were grown under controlled short day conditions (10 h light, 60% humidity, 20 °C) for 4 weeks. Up to 20 leaves per plant line (max. three leaves per plant) were inoculated with 3 μL drops of *A. brassicicola* conidial suspension. The plants were maintained under saturating humidity and infection sites were monitored 5 days after inoculation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2016.01.001.

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