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Molecules of interest

Camalexin

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Abstract

Camalexin (3-thiazol-2′-yl-indole) is the characteristic phytoalexin of *Arabidopsis thaliana*, which is induced by a great variety of plant pathogens. While particular pathogens, as well as a human tumour cell line, were growth inhibited by camalexin, some fungi show resistance due to active degradation. Camalexin originates from tryptophan and its biosynthesis involves the cytochrome P450 enzymes CYP79B2 and CYP71B15 (PAD3). Camalexin induction is a complex process, for which triggering by reactive oxygen species (ROS), salicylic acid signalling, and the glutathione status are important. Targets of the signalling cascade are the tryptophan and camalexin biosynthetic genes, which are strongly transcriptionally upregulated at the sites of pathogen infection. The important knowledge on camalexin, which is reviewed in this paper, will help to establish camalexin as a model for the investigation of the significance of phytoalexins in response pathogen challenge.

Keywords: Camalexin; *Arabidopsis thaliana*; Phytoalexin; Tryptophan; Indole-3-acetaldoxime; Cytochrome P450; Salicylic acid; Reactive oxygen species

1. Introduction

In response to pathogen attack many plants rapidly induce the biosynthesis of low molecular weight antimicrobial compounds. These so called phytoalexins show great structural diversity and are often restricted to a limited number of plant species. Camalexin (3-thiazol-2′-yl-indole) has attracted particular attention as integral part of the network of plant defence reactions in *Arabidopsis thaliana*. Several camalexin biosynthetic and regulatory genes have been identified. Large transcriptomic data sets are available for the response of *Arabidopsis* to a wide range of plant pathogens, which allow to follow the induction of camalexin related genes. Unless other species are specifically stated, the work described in this review was conducted with *A. thaliana*.

2. Identification of camalexin

3-Thiazol-2′-yl-indole was detected and isolated from leaves of the crucifer *Camelina sativa* infected with *Alternaria brassicae* and was termed "camalexin" (Browne et al., 1991). *A. thaliana* leaves challenged with *Pseudomonas syringae* also accumulated 3-thiazol-2′-yl-indole (Tsuji et al., 1992). In addition, it was identified in *A. lyrata* and *Capsella bursa-pastoris* (Jimenez et al., 1997; Zook et al., 1998). The purified phytoalexin showed strong blue fluorescence, a characteristic feature that allows easy detection in TLC assays as well as sensitive quantification with fluorescence detectors after HPLC separation. Different synthesis protocols for camalexin were established, starting from indole (Ayer et al., 1992), 2-trimethylsilyl thiazole (Fürstner and Ernst, 1995), and 1-(*tert*-butoxycarbonyl)indole-3-carboxaldehyde (Dzurilla et al., 2001).

The infection of *A. thaliana* leaves with both biotrophic and necrotrophic plant pathogens induces camalexin formation. These pathogens include bacteria (Glazebrook and Ausubel, 1994; Tsuji et al., 1992), viruses (Callaway et al., 1996; Dempsey et al., 1997), fungi (Glazebrook

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et al., 1997; Thomma et al., 1999; Zook and Hammerschmidt, 1997), and oomycetes (Roetschi et al., 2001). The camalexin biosynthetic capacity is not restricted to leaves, as also roots infected with the oomycete *Pythium sylvaticum* accumulated camalexin (Bednarek et al., 2005). In addition, a number of abiotic treatments, presumably triggering the formation of reactive oxygen species (ROS), induced camalexin biosynthesis. These include application of heavy metals ions (Tsuji et al., 1993), ROS-inducing chemicals, such as acifluorfen (Zhao et al., 1998), UV-B irradiation (Mert-Türk et al., 2003), the toxin fusaric acid (Bouizgarne et al., 2006) and the volatile allo-ocimene (Kishimoto et al., 2006). Usually, camalexin only accumulates when also lesion formation is observed. However, recently Raacke et al. (2006) detected camalexin several days after induction with an autoclaved yeast suspension in leaves that did not show apparent damage.

3. Camalexin-related compounds

Many indolic metabolites are known to be synthesized in plants. The auxin indole-3-acetic acid (IAA) is essential and ubiquitous. In the Brassicaceae IAA biosynthesis is interlinked with the biosynthesis of indolic compounds involved in defence (for review, see Kriechbaumer and Glawischnig, 2005). Most importantly, these include indole glucosinolates (for review, see Halkier and Gershenzon, 2006) and indolic phytoalexins (for review, see Pedras et al., 2000). Like camalexin, many of the phytoalexins are substituted with sulphur and nitrogen-containing side chains and are restricted to a limited number of species. In addition to camalexin, the *Arabidopsis*-relative *Camelina sativa* synthesizes 6-methoxy-camalexin (Browne et al., 1991). In *Capsella bursa-pastoris* camalexin and its 6-methoxy- and *N*-methyl-derivatives were identified (Jimenez et al., 1997).

The presence of indolic compounds in *A. thaliana*, other than indole glucosinolates and camalexin, was surveyed in a number of metabolomic approaches. Leaves, treated with *P. syringae* (Hagemeier et al., 2001; Tan et al., 2004) and extracts from roots infected with *Pythium sylvaticum* (Tan et al., 2004) were analyzed. Indole-3-carbaldehyde and indole-3-carboxylic acid, two potential camalexin biosynthetic intermediates were identified. Root exudates are a particularly rich source of indolic metabolites (Bednarek et al., 2005; Narasimhan et al., 2003; Walker et al., 2003) Besides the camalexin precursor dihydrocamalexic acid, indole-3-propanoic acid, indole-3-acetonitrile, indole-3-carbaldehyde, and indole-3-carboxylic acid, as well as their methylated, methoxylated, or glycosylated derivatives are released by the root. Narasimhan et al. (2003) also detected the release of a camalexin glucoside and phytoalexins, such as brassilexin and sinalexin. These have so far not been reported in *Arabidopsis* leaves, but are known from other members of the Brassicaceae (Pedras et al., 2000). It is likely that the indolic compounds are biosynthetically related (Fig. 1). In *Brassica* species, for some of these metabolites, indole-3-acetaldoxime (IAOx) was described as biosynthetic intermediate (Pedras et al., 2001).

4. Camalexin biosynthesis

The biosynthetic origin of camalexin (Fig. 1) was addressed by in vivo feeding. The thiazole ring, apart from the bridging C-2′ atom, originates from cysteine (Zook and Hammerschmidt, 1997). The tryptophan biosynthetic intermediates anthranilate and indole are precursors of the indole ring (Tsuji et al., 1993; Zook, 1998). Also, label from the tryptophan metabolite indole-3-acetaldoxime (IAOx)

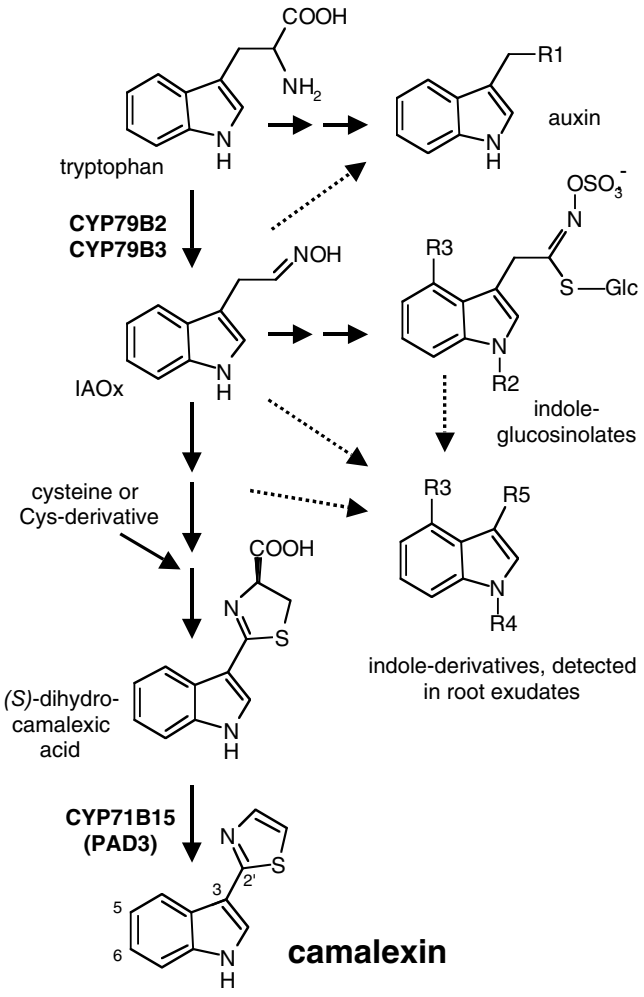

Fig. 1. The biosynthesis of camalexin and related indolic compounds in *Arabidopsis thaliana*. CYP79B2 and CYP79B3 catalyze the conversion of tryptophan to indole-3-acetaldoxime (IAOx), an intermediate of indole glucosinolate and camalexin biosynthesis. The last step in camalexin synthesis is the decarboxylation of dihydrocamalexic acid by CYP71B15 (PAD3). IAOx probably is also a precursor of indole-3-carbaldehyde (R5 = CHO), indole-3-caboxylic acid (R5 = COOH), and indole-3-acetonitrile (R5 = CH_2CN) derivatives, as well as auxin (IAA, the predominant auxin, R1 = COOH; indole propionic acid, R1 = CH_2COOH; indole butyric acid, R1 = $(CH_2)_2COOH$). R2 = H or OCH_3; R3 = H, OCH_3; R4 = H, CH_3, or OCH_3.

was well incorporated into camalexin, indicating that camalexin is synthesized via tryptophan and IAOx (Glawischnig et al., 2004). This is in accordance with IAOx being a precursor of brassinin and brassinin-derived phytoalexins (Pedras et al., 2001), structurally related to camalexin. Synthesis of IAOx from tryptophan is catalyzed by CYP79B2 and CYP79B3 (Hull et al., 2000; Mikkelsen et al., 2000). A *cyp79b2/cyp79b3* double knockout mutant was shown to be devoid of indole glucosinolates and synthesized reduced IAA levels in heat-stressed seedlings (Zhao et al., 2002) and root tips (Ljung et al., 2005). This double mutant was not able to accumulate camalexin, demonstrating that camalexin is synthesized from tryptophan-derived IAOx (Fig. 1) (Glawischnig et al., 2004). As tryptophan was only weakly incorporated and the tryptophan synthase mutants *trp 3-1* (*tsa1*) and *trp 2-1* (*tsb1*) did not show reduced camalexin synthesis, initially a tryptophan-independent biosynthesis was concluded (Tsuji et al., 1993; Zook, 1998). The cause of this puzzling weak tryptophan incorporation is not yet understood. Possibly, exogenous tryptophan is inaccessible to CYP79B2/B3, due to compartmentation or metabolic channelling of camalexin biosynthesis.

The IAOx-metabolizing steps in camalexin biosynthesis, which should involve additional cytochrome P450 reactions, have not yet been fully clarified. However, detection of camalexin synthesis in the *sur1* mutant excluded that camalexin is a metabolite of indole glucosinolates (Mikkelsen et al., 2004; Glawischnig et al., 2004). In a screen for camalexin deficient mutants, *pad3* (*phytoalexin deficient 3*) was isolated (Glazebrook and Ausubel, 1994) and the corresponding gene was cloned and shown to encode CYP71B15 (Zhou et al., 1999). In root exudates and induced leaves of *pad3* mutant dihydrocamalexic acid accumulated (Bednarek et al., 2005; Schuhegger et al., 2006). Dihydrocamalexic acid is the substrate of PAD3. CYP71B15 (PAD3), expressed in yeast, efficiently decarboxylated dihydrocamalexic acid to camalexin (Fig. 1) (Schuhegger et al., 2006). *Arabidopsis* microsomes isolated from leaves of transgenic plants overexpressing CYP71B15 and wild-type plants induced with silver nitrate were capable of the same reaction. Microsomes from induced leaves of *pad3* mutants did not turn over dihydrocamalexic acid. These data confirm that functional CYP71B15 is essential for the final step in camalexin biosynthesis (Schuhegger et al., 2006).

5. Regulation of camalexin biosynthesis

Induction of camalexin biosynthesis in response to biotrophic and necrotrophic pathogens is part of an elaborated network of defence mechanisms, which involves salicylic acid, jasmonate, and ethylene dependent signalling pathways. For a thorough review on these defence responses in relation to pathogen resistance refer to Glazebrook (2005). For camalexin induction salicylic acid signalling, the glutathione status, and in particular the generation of reactive oxygen species (ROS) are important. Targets of this signalling cascade are both the camalexin, as well as tryptophan biosynthetic genes, which are highly transcriptionally upregulated in a concerted fashion (Narusaka et al., 2003; Schuhegger et al., 2007; van Wees et al., 2003; Zhao and Last, 1996; Zhao et al., 1998).

In addition to *pad3*, phytoalexin deficient mutants in response to *P. syringae* (*pad1*, *pad2*, *pad4*, and *pad5*) were identified (Glazebrook and Ausubel, 1994; Glazebrook et al., 1996). Other mutants affected in camalexin induction include *bos2* (Veronese et al., 2004) and *ald1*, which is defective in an aminotransferase (Song et al., 2004). The *pad4* mutant synthesizes reduced camalexin levels in response to *P. syringae*. *PAD4* encodes a lipase-like protein (Jirage et al., 1999), which is important for salicylic acid signalling acting upstream of salicylic acid (Zhou et al., 1998). The importance of this *PAD4* signalling pathway is strongly dependent on the pathogen interaction analyzed. *PAD4* played a minor role for camalexin induction in response to infection with the necrotroph fungus *Botrytis cinerea* (Ferrari et al., 2003). Here also, salicylic acid-dependent signalling was relevant for resistance, but was largely independent of *PAD4*. In addition, salicylic acid induction mutants were isolated, which were not impaired in camalexin accumulation (Nawrath and Metraux, 1999).

Recently, *PAD2* was cloned and shown to encode the γ-glutamylcysteine synthetase 1 (GSH1, CAD2), which catalyzes the first step in glutathione biosynthesis (Parisy et al., 2007). This points out the involvement of glutathione in camalexin formation, either as regulatory component or possibly as biosynthetic precursor of the thiazole ring of camalexin.

For the oomycetes *Phytophthora* and *Pythium* Nep1-like proteins (necrosis and ethylene-inducing peptide 1-like proteins) are the initial triggers of camalexin synthesis and formation of reactive oxygen species (ROS) (Qutob et al., 2007). ROS appear to be of general relevance for camalexin formation. Chemical induction of ROS, such as by application of acifluorfen, coincided with camalexin synthesis. In a screen for enhanced susceptibility to *Alternaria brassicicola* the *esa1* mutant was identified, which showed delayed camalexin induction (Tierens et al., 2002). Particularly in response to ROS inducing agents reduced camalexin levels were synthesized. Therefore it was suggested that ESA1 is involved in transduction of signals generated by ROS (Tierens et al., 2002). This crucial role for *ESA1* was confirmed by the inability of *esa1* mutants to synthesize camalexin in response to *Leptosphaeria maculans* (Bohman et al., 2004). An additional mutant that exhibits greatly reduced camalexin accumulation is *ups1*, which was isolated on the basis of diminished expression of a tryptophan biosynthetic enzyme. Also UPS1 was suggested to be a component of ROS signal transduction. The *ups1* mutant was defective in activation of ROS-induced genes (Denby et al., 2005). The architecture of this ROS-induced camalexin signalling cascade is not yet clarified. In addition to salicylic acid, phytoprostanes might play a role in the transmission of the signal (Loeffler et al., 2005).

Phytoalexins are typically synthesized locally in proximity to the site of pathogen infection (Kuc, 1995). The spatial distribution of camalexin formation was analyzed after infection with *Botrytis cinerea* (Kliebenstein et al., 2005) and *Alternaria alternata* (Schuhegger et al., 2007). High camalexin concentrations were observed at the infection site (*Alternaria*) or in proximity to the lesions induced by *Botrytis*, while leaf areas that did not show disease symptoms were camalexin deficient. This localized camalexin accumulation corresponded to strong localized induction of tryptophan and camalexin biosynthetic genes (Schuhegger et al., 2006, 2007). Interestingly, *CYP79B3* was induced only to a minor extent (Schuhegger et al., 2007). Generally, after infection with various pathogens induction of *CYP79B2* exceeded *CYP79B3* induction, which is in accordance with a reduced camalexin content of *cyp79b2* knockout mutants in response to silver nitrate or *B. cinerea* infection (Glawischnig et al., 2004; Kliebenstein et al., 2005). This indicates that primarily *CYP79B2* expression is adapted for camalexin formation, while the main function of *CYP79B3* is rather in indole glucosinolate biosynthesis.

6. Natural variation

Natural variation of camalexin formation has been determined following infection with the necrotrophic fungus *Botrytis cinerea*, treatment with acifluorfen (Denby et al., 2004), *Pseudomonas syringae* DC3000/avrRps4 infection, and silver nitrate spraying (Schuhegger et al., 2007). All *Arabidopsis* accessions tested in these surveys synthesized camalexin. Additional data is available on *Cochliobolus carbonum* (Kagan and Hammerschmidt, 2002) and *Peronospora parasitica* (Mert-Türk et al., 2003) infection. Several fold quantitative differences were observed, but the relative camalexin concentration did not correlate between different treatments, e.g. Col-0 is a ''high-camalexin'' ecotype in response to acifluorfen treatment and a ''low-camalexin'' ecotype in response to *B. cinerea*, and *P. syringae* DC3000/avrRps4 infection. Taken these data together, the capacity to synthesize camalexin is dependent on the inducing agent. Therefore the natural variation of camalexin formation in different ecotypes is rather due to regulatory components, involving elicitor recognition and signal transduction, than to variation in catalytic efficiency of biosynthetic genes.

7. Biological activity and camalexin tolerance

Camalexin biosynthesis is induced by a great variety of plant pathogens. Nevertheless, the range of pathogens reported to be growth-inhibited by camalexin is much narrower. A valuable tool to address the biological role of camalexin is the comparison of resistance of camalexin deficient mutants versus wildtype (Glazebrook and Ausubel, 1994). Sensitivity of the biosynthetic mutant *pad3* and the respective wildtype Col-0 was compared. For this purpose it is important, whether the pathogen investigated triggers camalexin formation in the wildtype in concentrations toxic to the pathogen. Camalexin disrupts the integrity of bacterial membranes. 500 μg/ml camalexin inhibited proline uptake by *P. syringae* pv. *maculicula* ES 4326 and ion leakage was observed (Rogers et al., 1996). This rather high concentration probably is not reached *in situ* in the Col-0 ecotype after *P. syringae* infection, explaining the lack of increased sensitivity of *pad3* against this pathogen. In contrast, enhanced susceptibility of *pad3* mutants against *Alternaria brassicicola* and *Leptosphaeria maculans* was observed (Thomma et al., 1999; Bohman et al., 2004), which is in accordance with the inhibiting camalexin concentration that is 10 times lower for sensitive fungi than for gram-negative bacteria (Rogers et al., 1996). Accordingly, resistance to *A. brassicicola* was shown to correlate with high levels of camalexin in somatic hybrids of *Camelina sativa* and *Brassica oleracea* (Sigareva and Earle, 1999).

There are several reports that fungi can actively degrade camalexin to less toxic products. Different isolates of the root rot fungus *Rhizoctonia solani* degrade camalexin via 5-hydroxylation of the indole ring or via formation of an oxazoline derivative (Pedras and Khan, 1997, 2000). Biotransformation by the stem rot phytopathogen *Sclerotinia sclerotiorum* yielded camalexin derivatives glycosylated at N-1 or C-6 of the indole ring (Pedras and Ahiahonu, 2002). Resistant and sensitive isolates can be found within one pathogen species, as it was shown for *B. cinerea* (Kliebenstein et al., 2005). In addition, camalexin application can induce enhanced degradation of the indolic phytoalexin brassinin, as demonstrated for *Leptosphaeria maculans* (Pedras et al., 2005).

Camalexin is also toxic for the plant. A concentration of 100 μg/ml is sufficient to induce cell death in suspension cultures. However, camalexin is not the major cause of cell death for the plant during pathogen infection, as *pad3* mutant plants, when challenged with *P. syringae*, showed a cell death rate comparable to the wildtype (Rogers et al., 1996). Biological activity of camalexin is not restricted to microorganisms and plants. Remarkably, camalexin showed antiproliferative activity against a human breast cancer cell line (Mezencev et al., 2003) opening the possibility for a medical application of camalexin.

8. Perspective

Within the last few years our knowledge on phytoalexin synthesis in *Arabidopsis* has substantially increased and many factors involved in this process have been identified. The major challenge for the future will be to arrange the individual camalexin regulatory genes to specific regulatory cascades and to determine the specific cross-interactions that allow integration of different incoming signals to form

a network regulating camalexin synthesis. Camalexin induction will provide a useful model for understanding the formation and biological significance of phytoalexins.

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