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CAMALEXIN BIOSYNTHESIS IN ARABIDOPSIS: A STUDY OF PUTATIVE INTERMEDIATES AND OF THE EFFECTS OF DIFFERENT PATHOGENS ON ITS PRODUCTION

by

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ABSTRACT

CAMALEXIN BIOSYNTHESIS IN ARABIDOPSIS: A STUDY OF PUTATIVE INTERMEDIATES AND OF THE EFFECTS OF DIFFERENT PATHOGENS ON ITS PRODUCTION

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The biosynthesis of camalexin was studied in order to evaluate the role of this phytoalexin in resistance of *Arabidopsis thaliana* to disease. Mutant Arabidopsis seedlings were screened for camalexin deficiency to search for mutants with biosynthetic blocks and phenotypes that would indicate the effects of camalexin deficiency on disease resistance. Some plants, which were camalexin-deficient in response to the fungus *Cochliobolus carbonum*, produced wild-type amounts of camalexin in response to the bacterium *Pseudomonas syringae* pv. *maculicola* (*Psm*). Radiolabeling studies with *pad* (phytoalexin-deficient) mutants suggested that some of these mutants produced more camalexin in response to *C. carbonum* than in response to *Psm*. Camalexin accumulation was compared in wild-type and *pad2* Arabidopsis inoculated with different pathogens. Wild-type plants produced comparable amounts of camalexin in response to both pathogens, and *pad2* plants produced little in response to either pathogen. Camalexin usually accumulated more rapidly in response to *C. carbonum* than in response to *Psm*, although some exceptions reinforced the importance of evaluating camalexin production at more than one point in time when

assessing the plant's ability to synthesize it. No camalexin was detected in plants inoculated with 10^6 to 10^7 colony-forming units per milliliter of *Pseudomonas syringae* pv. *syringae*. Attempts to characterize inducible compounds led to the isolation of indole-3-carboxaldehyde, a putative intermediate, in fungal-inoculated wild-type and *pad2* plants. Kinetic and radiolabeling studies indicated that it is a possible biosynthetic intermediate but that a pathway independent of indole-3-carboxaldehyde may operate as well. A study of twenty-four ecotypes of *A. thaliana* revealed quantitative differences in camalexin production and in susceptibility to the fungus *Alternaria brassicicola*. However, no firm correlation between resistance and camalexin production was found, possibly due to the relative insensitivity of *A. brassicicola* to camalexin.

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Chapter 1. General Introduction

A pathogen's entry into plant tissue has two possible outcomes. One is a compatible interaction, in which colonization is successful and the plant's susceptibility is manifested macroscopically a few days later by pathogen development--with or without widespread necrosis--and, eventually, by death of the plant. The other possible outcome is an incompatible interaction, in which colonization is unsuccessful and the plant's resistance is manifested in about 24 hours by a hypersensitive response, or HR, of which the macroscopic evidence is small necrotic lesions at the infection site (Hammond-Kosack and Jones, 1996; O'Connell et al., 1985). Compatible and incompatible interactions are accompanied by biochemical changes. These changes can include the accumulation of salicylic acid, other phenolic compounds, and pathogenesis-related (PR) proteins; production of active oxygen species; calcium influx; and lignin deposition (Bell, 1981; Ebel, 1986; Hammond-Kosack and Jones, 1996; Nicholson and Hammerschmidt, 1992; Nürnberger et al., 1994; Paxton and Groth, 1994).

Another change that frequently occurs in infected plants is the production of phytoalexins, commonly defined as "low-molecular weight, antimicrobial compounds synthesized by and accumulating in plants following infection by microorganisms" (Paxton, 1981). The concept of phytoalexins (from the Greek *phyto*, plant; + *alexein*, to push away) was first proposed by

Müller and Börger in 1940 to explain their finding that if a potato tuber slice were inoculated with an incompatible race of *Phytophthora infestans* (the causal agent of potato blight), a compatible race subsequently inoculated would not grow. The incompatible race had elicited the production of an antifungal compound (Deverall, 1982; Harborne, 1988). Almost 20 years after the postulation of their existence, the phytoalexin pisatin was isolated and crystallized from pea pods (Cruickshank and Perrin, 1960). Since then, over 350 phytoalexins have been found in plants from about 30 different plant families, including the potato phytoalexins (rishitin and lubimin) whose existence had been postulated by Müller and Börger (Harborne, 1988; Kuc, 1995).

General Properties of Phytoalexins.

The chemical structures of phytoalexins vary and include stilbenes, isoflavonoids, and sesquiterpenes. In general, plants of a given family produce phytoalexins of the same basic structure(s): members of the Solanaceae produce sesquiterpenoid phytoalexins, while members of the Fabaceae (Leguminosae) produce isoflavonoid or pterocarpan phytoalexins (Ebel, 1986; Harborne, 1988; Kuc, 1995).

The biosynthetic pathways of phytoalexins originate from the biosynthetic pathways of primary metabolites such as carotenoids and amino acids (Kuc, 1995). The sesquiterpenoid phytoalexins of the Solanaceae, for example, are synthesized via the isoprenoid pathway responsible for sterol

biosynthesis. The phytoalexin pathway branches off from the sterol pathway at farnesyl pyrophosphate, from which either squalene (a sterol precursor) or sesquiterpenoid phytoalexin precursors are made (Kuc, 1995).

This branching-off from primary biosynthetic pathways occurs after infection. Phytoalexins are usually absent in healthy plants or present only in trace amounts (Harborne, 1988). The presence of trace amounts in some uninfected plants may indicate that the plants have been subjected to some sort of stress, which is almost impossible for plants to avoid in nature (Harborne, 1988).

As their presence in uninfected plants may indicate, phytoalexins appear in some respects to be a fairly non-specific response to stress. In addition to pathogens, abiotic stress--heavy metals, cold stress, or ultraviolet light--can elicit phytoalexin production and fall into the category of "abiotic elicitors" (Ebel, 1986; Kuc, 1995). Among pathogens (biotic elicitors), it is typical for many kinds of fungi, bacteria, or viruses to be capable of inducing the biosynthesis of a particular phytoalexin (Kuc, 1995). The wide range of elicitors suggests either that phytoalexins are a very nonspecific response to stress, or that these elicitors have a common mode of action. According to Ebel (1986), it has been suggested that the different elicitors may all cause cell death and, consequently, responses similar to those following cell death in an HR. It is also possible that phytoalexin synthesis is a general

response to alterations in metabolism after infection or abiotic stress (Kuc, 1995). However, phytoalexin production is not a completely non-specific response because not all stresses induce phytoalexin production. Wounding of potato tubers does not induce synthesis of rishitin or lubimin (Kuc, 1995).

Due to efforts to find a specific mechanism of action, a major focus in the realm of biotic elicitors has been on chemical constituents of pathogens. Efforts to characterize the active components of elicitors have led to the identification of oligosaccharides and peptides from fungi (Ebel, 1986; Albersheim and Valenti, 1978; Nürnberger et al., 1994) and also from plants (Ebel, 1986).

Just as they can be induced by many different pathogens, so can phytoalexins be effective against many pathogens. In general, the antimicrobial spectrum of any given phytoalexin is very broad. These compounds are usually active against many kinds of fungi, bacteria, and viruses (Harborne, 1988). However, they do not affect all pathogens, and those affected differ in sensitivity (Harborne, 1988). Variations in sensitivity were illustrated in a study by Cruickshank (1962) on the antimicrobial activity of pisatin against 50 fungal and 24 bacterial species. About half of the bacterial species were highly sensitive to pisatin, while the others were unaffected. Among the fungi tested, most were sensitive to pisatin at fairly low concentrations, but a few (*Aspergillus nidulans* and species of *Pellicularia* and

Fusarium) had intermediate sensitivity, and a few (*Mycosphaerella pinodes*, *Pellicularia filamentosa*, *Fusarium solani*, and *Ascochyta pinodella* and *A. pisi*) were not sensitive at all.

Sensitivity to phytoalexins *in vivo* is manifested by a reduction in fungal spore germination, bacterial colony-forming units, or virus titer and spread (Smith, 1982). Sensitivity to phytoalexins *in vitro* typically is measured by reduction of bacterial or fungal growth in liquid media containing the phytoalexin, reduction of fungal mycelial growth on agar impregnated with the phytoalexin, decrease in spore germination, or decrease in germ-tube growth (Smith, 1982). The study by Cruickshank (1961), for example, evaluated bacterial sensitivity on the basis of growth inhibition in liquid cultures containing pisatin. Fungal sensitivity was determined by reduction of mycelial growth on agar containing pisatin. Because phytoalexins are small organic molecules and usually are easily separated by thin-layer chromatography (TLC), another common bioassay is to spray a thin-layer chromatogram with fungal spores or bacterial cells in a nutrient broth, incubate the chromatogram in a humid environment to allow the spores/cells to grow, and look for inhibition of growth at the site of the putative phytoalexin (Harborne, 1988; Homan and Fuchs, 1970; Smith, 1982).

Some phytoalexins are toxic to pathogens, while others are inhibitory. Pisatin, for example, is fungistatic but not

fungitoxic: spores of *Sclerotinia fructicola* did not germinate in the presence of 0.28 mM pisatin, but after being thoroughly washed of the pisatin, some spores were able to germinate (Cruickshank and Perrin, 1960).

Another characteristic of phytoalexins is the localization of their production at the site of infection or stress. According to Deverall (1982), Müller and Börger found that if half a potato tuber slice were inoculated with an incompatible race of *P. infestans*, a subsequent inoculation of the entire slice with a compatible race would lead to growth of the latter only on the half that had not been previously inoculated. The unknown phytoalexin clearly did not diffuse over long distances, although it did diffuse into neighboring cells: if a thin layer of the tuber slice were removed and the area below inoculated with a compatible race, no growth occurred in the area just below the site of inoculation with the incompatible race.

The Role of Phytoalexins in Disease Resistance.

The antimicrobial nature, localization, and *de novo* synthesis of phytoalexins upon infection suggest that these compounds may play a role in disease resistance and have led to considerable interest in their potential use in agriculture. Currently, with farmers trying to reduce pesticide use and consumers expressing concern about pesticides in their food, there is a growing interest in protecting crops by exploiting the natural defenses of

plants. If phytoalexins do help plants to resist various diseases and stresses, perhaps some crops could become more resistant to certain diseases if transformed with the genes for biosynthesis of a phytoalexin. Also, as pathogens do differ in their sensitivities to some phytoalexins (Cruickshank, 1961), some of these compounds may prove to be good selective fungicides, or their molecular structures may provide clues as to the kinds of molecules that would be effective against certain pathogens (Pedras et al., 1997). However, because some phytoalexins are phytotoxic as well as fungitoxic (Glazener and Van Etten, 1978; Hargreaves, 1980), genetic engineering of plants may be a safer way to use phytoalexins in agriculture.

A transformation of tobacco with the genes required for production of the grape phytoalexin resveratrol (Hain et al., 1993) suggests that introducing genes for phytoalexin biosynthesis into plants is feasible. Transformed plants were more resistant to the fungus *Botrytis cinerea* than wild-type plants or transformants with a plasmid lacking the resveratrol biosynthesis genes (Hain et al, 1993). However, one success story (of questionable success, since the severity of disease in nontransformed plants was low enough that the large amount of disease reduction in transformed plants would have been unnoticeable in the field) is hardly enough to convince the general public to purchase genetically engineered produce, or to convince grant-giving agencies to fund the cloning of phytoalexin biosynthetic genes and

transformation of vegetables. More evidence of the importance of phytoalexins in disease resistance is necessary, and so far, the evidence has been inconclusive.

If phytoalexins are important in disease resistance, a pathogen's ability to colonize a plant should depend partly on its ability to detoxify or metabolize the plant's phytoalexin(s). A compatible pathogen should be able to detoxify the phytoalexin(s), while an incompatible pathogen should not. This colonization/detoxification relationship has been demonstrated in at least one case: the maize pathogen *Cochliobolus heterostrophus*, which normally cannot infect pea, was able to do so after it had been transformed with a gene for pisatin demethylase (pdm), which allows detoxification of pisatin (Schäfer et al., 1989). Isolates of the pea pathogen *Nectria haematococca* , after being transformed with the pdm gene, also became more virulent on pea (Ciufetti and VanEtten, 1996). However, additional work (Wasmann and VanEtten, 1996) demonstrated that after disruption of the inserted gene, the gene-disruption mutants were still more virulent than the original pdm-deficient isolates, suggesting that successful infection depended on something other than pisatin detoxification.

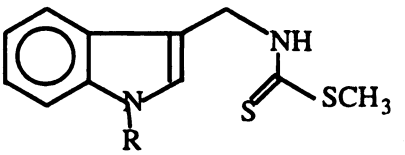
Timing of synthesis is another indicator of whether phytoalexins have a role in disease resistance, since they should be produced shortly after infection in order to stop the spread of a pathogen. In the resveratrol-transformed tobacco mentioned previously, the leaves with the fewest

lesions, besides having higher levels of resveratrol than leaves with more lesions, accumulated the phytoalexin more rapidly than the more-diseased leaves (Hain et al., 1993). In soybean roots infected with *Phytophthora megasperma* f.sp. *glycinea,* the phytoalexin glyceollin accumulated to lower concentrations in response to an incompatible race than in response to a compatible one, but it accumulated more rapidly (Hahn et al., 1985). In sorghum resistant to *Colletotrichum graminicola*, phytoalexin accumulation begins a few hours after fungal penetration, suggesting that the sorghum phytoalexin does play a role in disease resistance (Snyder and Nicholson, 1990). The timing of phytoalexin production in some other plants, however, is too late to suggest a critical role in disease resistance. In parsley cell suspension cultures, mRNA for parsley phytoalexins was first detected 12 to 18 hours after inoculation, well after initiation of mRNA transcripts for other defense responses such as the pathogenesis-related protein PR-1 (Schmelzer et al., 1989). The variation in timing of phytoalexin production suggests that the relative importance of a phytoalexin in disease resistance varies with the phytoalexin, and perhaps with the plant as well.

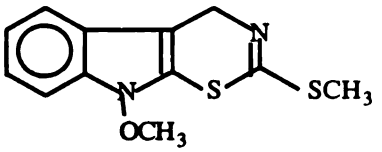
Phytoalexins of the Brassicaceae: General Properties and Role in Disease Resistance.

This thesis describes work on camalexin, a phytoalexin of the Brassicaceae (Cruciferae). The cruciferous

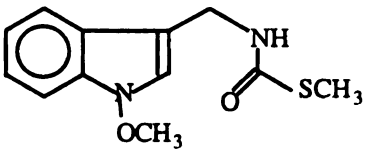
phytoalexins are relatively new to the world of plant research. The first report of these compounds was in 1986 by Takasugi and co-workers, who isolated four compounds (methoxybrassinin, brassinin, cyclobrassinin, and methoxybrassitin, shown in Figure 1) from *Brassica pekinensis*, or Chinese cabbage (Gross, 1993).



R=H, brassinin; R=OCH_3,methoxybrassinin



cyclobrassinin



methoxybrassitin

Figure 1. A few cruciferous phytoalexins.

These compounds had typical characteristics of phytoalexins, as they were induced by abiotic and biotic elicitors (ultraviolet light and the bacterial pathogen *Pseudomonas cichorii*), and they had a broad antimicrobial spectrum, being antifungal to over 31 plant pathogenic fungi (Takasugi et al., 1988). As shown in Figure 1, they had similar structures consisting of an indole ring with a sulfur-containing moiety. These were the first sulfur-containing phytoalexins isolated. About 24 phytoalexins from the Brassicaceae have now been isolated from a number of plants, including *Raphanus sativus* var. *hortensis* (Japanese radish), *Brassica campestris* ssp. *rapa* (turnip), and *Brassica napus*

(canola) (Gross, 1993; Pedras et al., 1997). All consist of an indole ring with a sulfur-containing substituent (Gross, 1993; Pedras et al., 1997).

Within the Brassicaceae, no phytoalexins have demonstrated a clear role in disease resistance. Pedras et al. (1997) found a correlation between the virulence of *Leptosphaeria maculans* (the causal agent of blackleg disease of crucifers) and the extent to which it could metabolize brassinin. Since brassinin is a precursor of spirobrassinin and cyclobrassinin, the ability to detoxify brassinin could be a strategy to stop phytoalexin biosynthesis before encountering a triple phytoalexin threat (Pedras et al., 1997).

Studies on the role of brassilexin (Figure 2) in disease resistance revealed that when Brassica species were inoculated with *L. maculans*, the resistant species *Brassica juncea*, *Brassica carinata*, and *Brassica nigra* accumulated more brassilexin than did the susceptible *B. napus* (Rouxel et al., 1990).

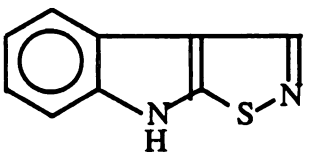


Figure 2. Brassilexin

In response to cupric chloride treatment, *B. juncea* accumulated more brassilexin than *B. napus*, and the onset of accumulation in *B. juncea* (6 hours) was earlier than in *B. napus* (18 hours) (Rouxel et al., 1989). Assuming similar

kinetics of brassilexin accumulation in response to fungal inoculation, the more rapid accumulation of brassilexin in the resistant species suggests that this phytoalexin could have a role in disease resistance and that resistance depends partly on rapid accumulation of brassilexin. When resistant and susceptible species were crossed, resistance in the F2 generation correlated well with high brassilexin accumulation after cupric chloride treatment (Rouxel et al., 1990). Further studies (Rouxel et al., 1991) demonstrated, however, that some resistant species accumulated very little brassilexin, indicating that brassilexin was not the key determinant in disease resistance.

General Properties of Camalexin.

Camalexin, which has the structure 3-thiazol-2'-yl-indole (Figure 3), was first detected by Conn et al. (1988) in leaves of *Camelina sativa* inoculated with the fungus *Alternaria brassicae*. A further study by Jejelowo et al.(1991) of the isolated compound, whose structure was unknown at the time, demonstrated that it had the typical properties of a phytoalexin: it could be extracted with organic solvents (methanol and chloroform) and separated by TLC; it was produced following inoculation of leaves with *A. brassicae*; and production of the antimicrobial compound was restricted to the area underneath and immediately surrounding the droplets of inoculum. On leaves, antimicrobial activity was demonstrated by a decrease in fungal germination and

germ-tube growth. Bioassays of thin-layer chromatograms revealed zones of inhibition at bands found later to correspond to camalexin and methoxycamalexin.

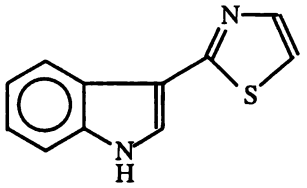


Figure 3. Camalexin

The structure of camalexin was determined by Browne et al. (1991). The structure of the phytoalexin produced by *Arabidopsis thaliana* was subsequently determined by Tsuji et al. (1992) to be the same compound. Recently, camalexin has been isolated from two other cruciferous plants, *Capsella bursa-pastoris* (shepherd's purse; Jimenez et al., 1997), and *Arabis lyrata* (M. Zook, in press). It is easily recognized on thin-layer chromatograms by its purple fluorescence under long-wave and short-wave ultraviolet (UV) light at an R_f of about 0.6 in chloroform-methanol (9:1 or 19:1, v/v).

Like other phytoalexins, camalexin is induced by many microorganisms and abiotic elicitors. The microorganisms that induce camalexin production include the fungi *Alternaria brassicae* (Conn et al., 1988; Jejelowo et al., 1991), *Rhizoctonia solani* (Conn et al., 1994), and *Cochliobolus carbonum* (Glazebrook et al., 1997); the bacteria *Xanthomonas campestris* pv. *campestris* (Zhao and Last, 1996; Zhou et al., 1998), *Pseudomonas syringae* pv. *syringae* (Tsuji et al.,

1992), *P.syringae* pv. *maculicola* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997; Zhao and Last, 1996; Zhou et al., 1998); and *P.syringae* pv. tomato (Glazebrook and Ausubel, 1994; Zhou et al., 1998). Among viruses, turnip crinkle virus (Dempsey, 1996; Dempsey et al., 1997) and cauliflower mosaic virus (Callaway et al., 1996) elicit camalexin production. Camalexin is also induced by silver nitrate (Tsuji et al., 1992; Zhao and Last, 1996; Zhou et al., 1998), starvation for certain amino acids (methionine or branched-chain amino acids), the herbicide acifluorfen, and α-aminobutyric acid, a putative chemical inducer of resistance (Zhao et al., 1998). Little is known on the mechanisms of camalexin induction. Despite the wide range of biotic and abiotic elicitors, camalexin biosynthesis is not a general response to all forms of stress, since heat shock does not induce camalexin (Zhao et al., 1998). Salicylic acid (SA), a key component in the plant signal transduction pathway, is required, because SA-deficient Arabidopsis plants produced less camalexin than wild-type (Zhao and Last, 1996). The different elicitors may somehow stimulate SA production, or activate a signal that in turn activates SA. Another possibility, since one inducer, acifluorfen, generates free radicals, is that oxidative stress is a key player in camalexin elicitation (Zhao et al., 1998). Active oxygen species (peroxides, hydroxy radicals, or superoxide anions) frequently are produced in response to infection

(Hammond-Kosack and Jones, 1996; Zhao et al., 1998), and so it is possible that the different elicitors all act by stimulating the production of such molecules, which in turn induce camalexin biosynthesis. Amino acid deprivation may induce camalexin for the same reason, since such treatment may cause chloroplast damage, with consequent oxidative stress due to the lack of molecules to absorb and dissipate light energy (Zhao et al., 1998).

Camalexin has a broad but not entirely non-specific antimicrobial spectrum. Fungi seem to be more sensitive than bacteria to camalexin: in liquid cultures, the threshold concentration toxic to *Pseudomonas syringae* pv. *phaseolicola*, *P. syringae* pv.*maculicola*, and *Xanthomonas campestris* pv. *campestris* was 250-500 µg/ml, while the threshold concentration for *Fusarium oxysporum* and *Saccharomyces cerevisiae* was 20-50 µg/ml (Rogers et al., 1996). The lower inhibitory concentrations for fungi corroborate the findings of Jejelowo et al. (1991) that the hyphal tips of *Alternaria brassicae* swelled and burst in aqueous solutions containing 20 µg/ml or more of camalexin. Similar differences in fungal and bacterial sensitivities have been found with TLC plate bioassays: the minimum amount of camalexin to inhibit *P. syringae* pv. *syringae* was 4 times the minimum amount required to inhibit the fungus *Cladosporium cucumerinum* (Tsuji et al., 1992). The antimicrobial properties of camalexin seem to be due to membrane disruption, since addition of inhibitory concentrations of camalexin to bacterial cultures

caused electrolyte leakage (Rogers et al., 1996). Recently, camalexin was found to be highly toxic to a line of breast cancer cells (Moody et al. 1997); its toxicity in this latter case may be due again to membrane disruption.

The role of camalexin in disease resistance.

As with the other cruciferous phytoalexins, the role of camalexin in disease resistance is unclear. Many studies have demonstrated a relationship between camalexin accumulation and resistance. Arabidopsis plants transformed with a fusion product of the reporter gene ß-glucuronidase (GUS) and the promoter for the tobacco Tnt1 retrotransposon, whose expression has been correlated with disease resistance in tobacco, expressed the GUS gene (an indication of Tnt1 expression) in response to the abiotic elicitor cupric chloride, and these plants also produced camalexin under these conditions (Mhiri et al., 1997). Camalexin production was correlated with resistance of Arabidopsis to turnip crinkle virus, or TCV (Dempsey, 1996; Dempsey et al., 1997). In genetic crosses with a resistant line of the Dijon ecotype of Arabidopsis, camalexin production consistently segregated with the HRT (hypersensitive response to TCV) locus, which is required for TCV resistance, and after inoculation with TCV, resistant lines of the Dijon ecotype produced significantly more camalexin than susceptible lines (Dempsey, 1996; Dempsey et al., 1997). Inoculation of the Columbia ecotype of Arabidopsis with cauliflower mosaic virus (CaMV) or cucumber

mosaic virus (CMV), both compatible pathogens, did not lead to camalexin production (Zhao and Last, 1996). However, the En-2 ecotype of Arabidopsis, which is resistant to CaMV, did produce camalexin following inoculation (Callaway et al., 1996).

Although the En-2 ecotype produced camalexin without displaying a visible hypersensitive response (HR), most studies have demonstrated a strong correlation between camalexin production and an HR. Since the HR is a sign of an incompatible interaction, the observation of this relationship supports a role for camalexin in disease resistance. The studies by Dempsey et al. (1996, 1997) just described, indicated a link between camalexin production, resistance, and the ability to produce an HR. The accumulation of camalexin was correlated with an HR in response to infection by the incompatible pathogen *Pseudomonas syringae* pv. *syringae* (*Pss*) (Tsuji et al., 1992). Bacterial growth reached a maximum before camalexin levels reached a maximum, and bacterial growth then decreased while camalexin levels continued to increase. Camalexin was not produced upon inoculation with mutants of *Pss*, which were nonpathogenic due to loss of a *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) gene, which is required for bacteria to cause an HR and disease on plants.(Tsuji et al., 1992).

Further strengthening the correlation between camalexin production and the HR is the finding that regulation of camalexin production appears to be controlled by the ACD2

(accelerated cell death) locus, which controls the onset of the HR and the size of HR-type lesions (Greenberg et al., 1994). After bacterial inoculation or mechanical wounding (which usually does not induce high production of camalexin), *acd2* mutants had camalexin concentrations identical to those of bacterial-inoculated wild-type plants, as well as high concentrations of salicylic acid and mRNA's for a number of defense-associated genes (Greenberg et al., 1994).

Studies with the bacterial pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*), to which Arabidopsis is resistant, further support the connection between camalexin production and an HR. On Arabidopsis, *Xcc* does not cause an HR and can grow to relatively high titers *in planta*; resistance is judged by the absence of symptoms (Tsuji et al., 1991). Inoculation with *Xcc* at 10^8 colony-forming units per milliliter (cfu/ml)--a much higher inoculum concentration than would be found in nature--elicits no camalexin (Tsuji et al., 1991), although inoculation at 10^9 cfu/ml does (Zhao and Last, 1996). These results suggest that camalexin is not always produced in an encounter with a pathogen to which Arabidopsis is resistant. Without a hypersensitive response to the pathogen, camalexin is not produced, unless other stressing conditions (such as unusually high inoculum levels) are involved. The results both strengthen the camalexin/HR correlation and weaken the case for a primary role for camalexin in disease resistance.

As with most phytoalexins (Kuc, 1995), camalexin

accumulation is not associated exclusively with incompatible interactions. Inoculation with *Pseudomonas syringae* pv. *maculicola*, a compatible pathogen, leads to high camalexin production (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997; Rogers et al., 1996; Zhao and Last, 1996; Zhou et al., 1998).

It is difficult to determine the relative contribution of camalexin to inhibiting pathogen spread *in planta*. Since Tsuji et al. (1992) found that *Pss* growth decreased before camalexin levels peaked, it is reasonable to assume that the bacterial growth was inhibited in part by the increasing concentration of camalexin. However, the fact that bacterial growth did not increase as camalexin concentration decreased indicates that camalexin is not the sole factor controlling *Pss* spread *in planta*. It is not impossible that degradation products of camalexin are antibacterial and that the metabolism of camalexin contributes to the ability of Arabidopsis to prevent further infection.

If camalexin contributes significantly to inhibiting pathogen spread in a plant, the pathogens capable of detoxifying camalexin should spread and grow more readily than the pathogens that cannot detoxify camalexin, just as *N. haematococca* transformed with a pisatin demethylase gene caused limited infection on pea (Ciufetti and VanEtten, 1996). In the case of camalexin, however, the opposite has been demonstrated in a couple of cases. Mycelial growth of *Leptosphaeria maculans* was not inhibited on agar containing

camalexin, and the camalexin was not metabolized, suggesting that it is so nontoxic toward *L. maculans* that the fungus does not need camalexin detoxification mechanisms to be a successful pathogen. The fungus *Rhizoctonia solani*, although inhibited *in vitro* by 50 µg/ml camalexin and capable of metabolizing camalexin to 3 non-inhibitory compounds (Pedras and Khan, 1997), was a poor colonizer of *Camelina sativa* roots *in vivo* (Conn et al., 1994). The fact that camalexin, along with three other antimicrobial compounds, was isolated from the roots in that study, indicates that *R. solani* did not metabolize significant amounts of camalexin. Perhaps other factors--PR proteins, accumulation of phenolics, or the other antimicrobial compounds--stopped the spread of *R. solani* before detoxification of camalexin became a requirement for being able to infect.

Despite the fact that camalexin is not required for resistance to some pathogens and that its production is not sufficient to stop the spread of other pathogens, some of the studies just mentioned suggest that it may be part of the plant defense arsenal. Why is it produced in some incompatible interactions, along with all of the other responses of the HR? Do its antimicrobial properties aid in inhibiting pathogen growth?

The Use of Camalexin-Deficient Mutants to Study the Role of Camalexin in Disease Resistance.

A classic way to determine the function of a compound in an organism is to find mutants deficient in that compound and compare their phenotypes to those of wild-type organisms. The isolation of camalexin-deficient mutants of Arabidopsis by Glazebrook and Ausubel (1994) made this type of analysis possible for camalexin in Arabidopsis. To date, five of these *pad* (*p*hytoalexin-*d*eficient) mutants, each representing a mutation in a different gene, have been found (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997).

One aspect of disease resistance examined with these mutants was the effect of camalexin on gene-for-gene resistance to bacterial pathogens, which requires recognition of an avirulence (*avr*) gene due to a corresponding resistance gene in the plant. The plant response usually involves an HR, biochemical changes typical of incompatible interactions, and restriction of pathogen growth (Whalen et al., 1988). The *pad* mutants demonstrated that accumulating little or no camalexin does not affect gene-for-gene resistance to avirulent bacteria. In all five mutants, after inoculation with the compatible pathogen *Pseudomonas syringae* pv. *maculicola* or with an isogenic counterpart bearing an *avr* gene, the difference between final population densities of virulent and avirulent pathogens was about the same as in wild-type plants (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). Camalexin accumulation, therefore, does not

affect the ability of resistance genes to function. These results help to explain why inoculation with Xcc, for which resistance is controlled by a single gene (Tsuji et al., 1991), elicits no camalexin production in wild-type plants. Resistance genes and camalexin can function independently of one another.

Although the *pad* mutants still responded to *avr*-gene-bearing bacteria by restricting growth *in planta*, three of the mutants--*pad1*, *pad2*, and *pad4*--allowed more growth of the compatible bacteria than did the wild-type plants, suggesting that camalexin does somehow help to control the extent to which a compatible pathogen can grow *in planta* (Glazebrook et al., 1997). Camalexin may play a more important role in resistance to eukaryotic than to prokaryotic pathogens, since four of the *pad* mutants were more susceptible than wild-type plants to the oomycete *Peronospora parasitica* (Glazebrook et al., 1997).

Biosynthesis of Camalexin and Other Cruciferous Phytoalexins.

Another way to determine the role of a compound in an organism is to manipulate the genes for its biosynthesis and then to observe the effect of inactivating or overexpressing key biosynthetic enzymes or of introducing those enzymes into another organism. This approach, however, requires knowing the biosynthetic pathway of the compound. Since none of the biosynthetic pathways of cruciferous phytoalexins were known

until 1994 (Monde et al., 1994; Gross et al., 1994), and many have not yet been elucidated, camalexin was a potential model system for the biosynthesis of phytoalexins in the Brassicaceae. These would all be expected to have similar biosynthetic origins, due to their similarity of structure.

Determining the biosynthesis of camalexin could have additional benefits for agriculture. If camalexin could aid in disease resistance, introducing the genes for its biosynthesis into other plants--probably other plants in the same family, of which there are many economically important members--might make the plants more resistant to certain diseases. Also, if camalexin is truly an anticancer compound (Moody et al., 1997), it might be possible to clone the genes for its biosynthesis, overexpress them in plants, and thus facilitate isolating large quantities of this compound for medicinal purposes. These possibilities are a bit far-fetched, but understanding basic mechanisms in biology can lead to many benefits besides intellectual satisfaction, and the results are sometimes far more astonishing than imagined, as demonstrated by the development of *Agrobacterium*-based genetic engineering from studies of crown galls (Chilton, 1983).

Mutants, besides providing phenotypic evidence of the role of the compound that they lack, can be useful in determining biosynthetic pathways. The inability to synthesize a compound, if due to the lack of an enzyme catalyzing a certain step of the pathway, can lead to the

accumulation of intermediates prior to that step (Swain; 1965). With a large number of mutants, each representing a mutation in a different gene, all steps of the pathway could conceivably be determined, if different intermediates were found to accumulate in each mutant. The *pad* mutants initially seemed like potential sources of such information, and work with some other mutants (Tsuji et al., 1993; Zhao and Last, 1996) has helped to understand the biosynthesis of camalexin (see below).

What, then, is known about the biosynthetic pathways of camalexin or the other cruciferous phytoalexins? The indole ring suggests that these compounds originate from tryptophan. In fact, the radish phytoalexins brassinin, spirobrassinin, and cyclobrassinin do seem to originate from tryptophan (Monde et al., 1994). The more immediate precursor is glucobrassicin, a tryptophan-derived glucosinolate (Rausch et al., 1983). Spirobrassinin in kohlrabi (*Brassica oleracea* var. *gongylodes)* also was found to originate from tryptophan and methionine (Gross et al., 1994). The phytoalexins cyclobrassinone and 1-methoxyspirobrassinin, also isolated from kohlrabi, seem to originate from tryptophan and methionine as well (Gross et al., 1994). In addition, two other minor phytoalexins of kohlrabi, methoxybrassitin and methoxybrassinin, incorporated labeled tryptophan and methionine (Gross et al., 1994).

Camalexin, however, appears to be synthesized by a somewhat different pathway. Because anthranilate (the

product of the first committed step toward tryptophan biosynthesis in the shikimic acid pathway) was incorporated more efficiently into camalexin than tryptophan, it appeared that the camalexin biosynthetic pathway branched off from an intermediate between anthranilate and tryptophan (Tsuji et al., 1993). These results were further supported by the finding that of three tryptophan-deficient (*trp*) mutants tested, the only one that did not synthesize wild-type levels of camalexin was a mutant deficient in anthranilate synthase (*trp1-100*); those deficient in tryptophan synthase (*trp3-1*, deficient in tryptophan synthase α; and *trp2-1*, deficient in tryptophan synthase ß) were not affected (Tsuji et al., 1993). The absence of indole glucosinolates in etiolated seedlings of *Camelina sativa* (Schraudolf, 1968) also suggests that camalexin is not synthesized along the same pathway as the radish phytoalexins. It may be that indole glucosinolates were not detected in *C. sativa* in Schraudolf's (1968) study because of the developmental stage of the plant, or because they were being rapidly converted into something else. However, it may be that camalexin biosynthesis is partly the result of an inability of *C. sativa* to make other phytoalexins, due to the lack of indole glucosinolates.

It seemed possible that the indole ring of camalexin could be formed from a pathway branching off from a tryptophan pathway intermediate such as indole-3-glycerol phosphate (Tsuji et al., 1993), as shown in Figure 4.

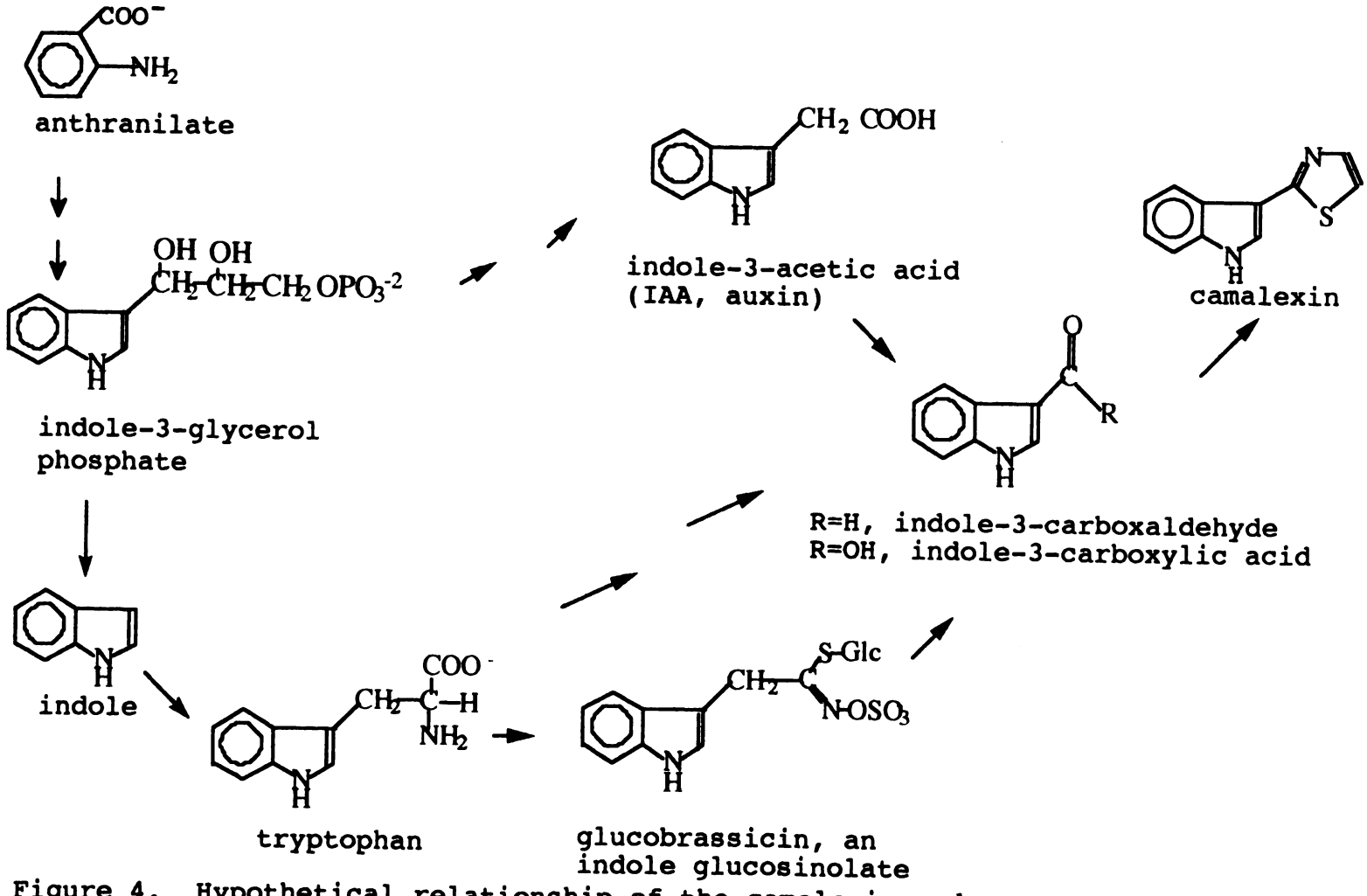


Figure 4. Hypothetical relationship of the camalexin and tryptophan biosynthetic pathways.

This hypothesis was quite reasonable in light of the findings by Wright et al. (1991) that indole-3-acetic acid (IAA) in maize is not synthesized from tryptophan. In that study, tryptophan-deficient mutants, although they produced much less tryptophan than wild-type seedlings, produced much more IAA, and they contained higher concentrations of anthranilate and indole than did wild-type plants. These results suggest that the IAA biosynthetic pathway, at least in maize, branches away from the tryptophan biosynthetic pathway at or before indole. The possible independence of this pathway from the tryptophan pathway suggested a few possibilities for tryptophan-independent intermediates of camalexin. A number of indole derivatives have been found in plants, including indole-3-carboxaldehyde and indole-3-carboxylic acid (Figure 4) (Müller, 1961; Melchior, 1957).

Browne et al. (1991) proposed that camalexin could be produced by a condensation reaction between indole-3-carboxaldehyde and cysteine. The former has been isolated in fairly high quantities in some plants, including cabbage, another crucifer (Devys and Barbier, 1991; Jones and Taylor, 1957). It could be formed in several ways. Indole-3-carboxaldehyde can be formed by oxidation of IAA (Stutz, 1958), photolysis of IAA (van Denffer and Fischer, 1952; Melchior, 1957; Meyer and Pohl, 1956; Ray and Curry, 1958), and also from photolysis or oxidation of tryptophan (Fischer, 1954; Melchior, 1957; Müller, 1961).It could also be formed from indole glucosinolates (Devys and Barbier, 1991).

Although *C. sativa* does not produce indole glucosinolates, Arabidopsis does, and it may be that the camalexin pathway in Arabidopsis differs from the pathway in *C. sativa*, or that more than one pathway to camalexin exists. The latter possibility is described by Bu'lock's (1965) model of the "metabolic grid," which depicts multiple pathways to the same compound.

Given the number of sources for the biogenesis of indole-3-carboxaldehyde, it seemed that a possible biosynthetic pathway for camalexin could involve the intermediates shown in Figure 5. Nucleophilic attack of indole-3-carboxaldehyde by cysteine would lead to an indole-cysteine adduct. Under low pH conditions, the oxygen bonded to the carbonyl carbon could be removed by protonation and subsequent loss of a water molecule, which would allow the carbocation to bind to the nitrogen atom in cysteine--thus effecting closure of a precursor to the thiazole ring. This indole-3-thiazoline carboxylic acid could be oxidized to indole-3-thiazolidine carboxylic acid, and oxidized and decarboxylated to produce camalexin (Zook and Hammerschmidt, 1997). Decarboxylation of the cysteine carboxyl group could also occur prior to the formation of the double bonds in the thiazole ring (J. Kagan, pers. comm.).

Project Description.

This project began as an effort to find intermediates in camalexin biosynthesis by characterizing the *pad* mutants.

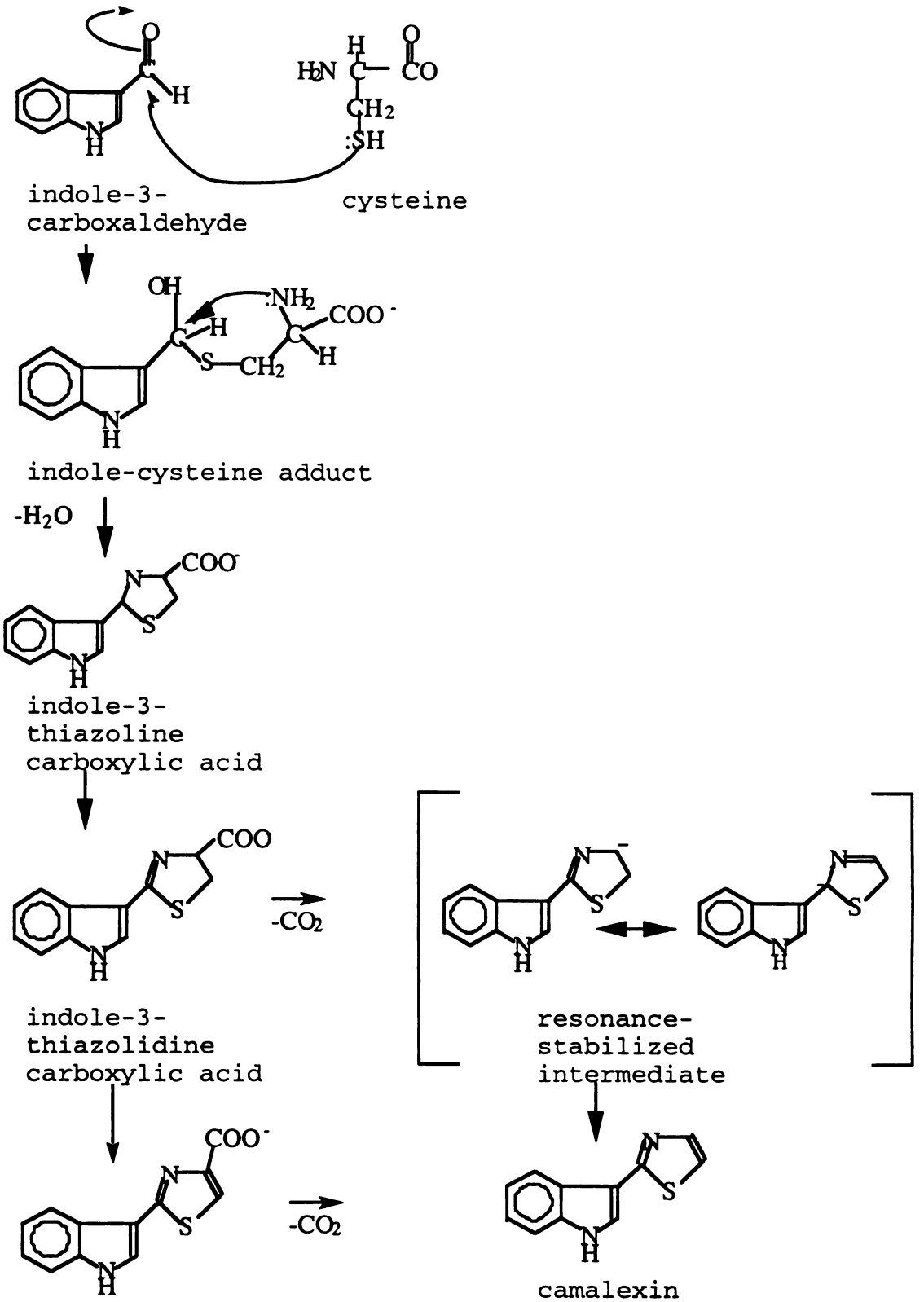


Figure 5. Two possible routes to camalexin.

Radiolabeling and fluorimetry studies of leaves elicited with the ascomycete *Cochliobolus carbonum* revealed no obvious intermediates accumulating in the *pad* mutants; however, they did reveal that some of these supposedly camalexin-deficient mutants produced fairly high amounts of camalexin. As these mutants had been identified by inoculation with the bacterial pathogen *Pseudomonas syringae* pv. *maculicola*, these results suggested that camalexin accumulation in Arabidopsis might vary with the infecting microorganism. Consequently, the project turned into an effort to compare patterns of camalexin accumulation in wild-type and *pad2* plants inoculated with *Cochliobolus carbonum* or the bacterial pathogens *Pseudomonas syringae* pv. *maculicola* or *P. syringae* pv. *syringae*. In the process of isolating camalexin (by thin-layer chromatography) from samples used for these studies, additional inducible compounds were seen on thin-layer chromatograms. Attempts to characterize these compounds led to the identification of indole-3-carboxaldehyde in fungal-inoculated leaves of wild-type and *pad2* plants. Radiolabeling studies with labeled anthranilate were then done to determine whether this putative biosynthetic intermediate really was on the pathway between anthranilate and camalexin. Finally, as an alternative to studying the phenotypes of mutants in order to understand the role of camalexin in disease resistance, a number of ecotypes of wild-type Arabidopsis were screened for differences in ability to produce camalexin, and efforts were made to

correlate those differences with the ability to respond to the fungal pathogen *Alternaria brassicicola*.

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Chapter 2. Screen for Camalexin-Deficient Mutants of Arabidopsis and Radiolabeling Studies With Phytoalexin-Deficient (*pad*) Mutants

Introduction

The initial approach to determining the biosynthetic pathway of camalexin and the role of camalexin in disease resistance was to study mutants. A simple way to assess the importance of a compound to an organism is to study the phenotype of that organism lacking that particular compound. The change in phenotype can be used to deduce the effect(s) of having the compound. For example, the *cpr1* mutant of Arabidopsis, which constitutively expresses the pathogenesis-related protein PR-1, is slightly stunted and has smaller leaves, suggesting that in the absence of a means to turn off the PR-1 gene, normal growth is reduced (Bowling et al., 1994).

Mutants also frequently accumulate biosynthetic precursors of the compound, if the mutation blocks a step of the biosynthetic pathway such that the precursors cannot be converted into the end product (Bu'lock, 1965; Pridham and Swain, 1965; Smith and Yanofsky, 1963). These accumulating precursors can be detected by supplying plants with labeled precursors (either heavy isotopes or radioisotopes), and studying the compound(s) into which the isotopic label was incorporated. This approach was used by Wright et al. (1991)

to determine that indole-3-acetic acid (IAA, or auxin) in maize was synthesized from indole and not tryptophan. Maize tryptophan auxotrophs, when fed deuterated water (D_2O), incorporated much more label into anthranilate than into tryptophan. In addition, these mutants produced more IAA than wild-type plants.

Besides acting as a source of accumulating precursors, mutants can sometimes help to support or reject hypotheses about whether a compound *x* is a precursor of another compound *y*. If a mutant is deficient in an enzyme needed to make compound *x*, but that mutant can still make compound *y*, then compound *x* is unlikely to be an intermediate in the biosynthetic pathway to *y*. This approach was used to help determine that camalexin is not synthesized from tryptophan. Since tryptophan-deficient mutants produced wild-type amounts of camalexin (Tsuji et al., 1993), tryptophan appears not to be a precursor of camalexin.

Given the hypothetical pathway of camalexin biosynthesis (Figure 5) and its hypothetical relationship to the tryptophan biosynthetic pathway (Figure 4), some of the compounds that might be expected to accumulate in camalexin-deficient mutants would be indole-3-glycerol phosphate, indole-3-carboxylic acid, indole-3-carboxaldehyde, and the indole-3-thiazoline and indole-3-thiazolidine carboxylic acids. The initial approach to finding these mutants was to screen for camalexin deficiency in an ethyl methane sulfonate (EMS)-mutagenized population of Arabidopsis seeds, and to

identify accumulating intermediates in those mutants by feeding radioactive anthranilate, a known camalexin precursor, and characterizing the compounds into which it was incorporated. The agent used to elicit camalexin production was the ascomycete *Cochliobolus carbonum*, a maize pathogen that elicits an incompatible response (although it does not cause a visible hypersensitive response) on Arabidopsis. The choice of elicitor was based on the results of Samantha Teplitsky, a high school student who worked in the Hammerschmidt lab in the summer of 1991 and found that *C. carbonum* induced higher amounts of camalexin than previously-used elicitors (silver nitrate and *Pseudomonas syringae* pv. *syringae*, an incompatible bacterial pathogen).

The mutant screen was facilitated by the fact that camalexin has a very specific fluorescence spectrum, and that it diffuses into droplets of spore inoculum directly above the tissue in which it is made. The ability of camalexin to diffuse into inoculum droplets was noted by Conn et al. (1988), when inoculating *Camelina sativa* and *Capsella bursa-pastoris* with *Alternaria brassicae*. Phytoalexins from other plants, such as pisatin in *Pisum sativum* (pea) (Cruickshank and Perrin, 1961) and medicarpin in *Medicago sativa* (alfalfa) (Harborne, 1988) have also been isolated from inoculum droplets. This method simplifies extraction and separation of phytoalexins, as it is not necessary to separate them from pigments, and fewer compounds are present in droplets than in leaf tissue (Cruickshank and Perrin, 1961; Harborne, 1988).

In the case of the mutant screen, the ability of camalexin to diffuse into inoculum droplets made it possible to collect those droplets and evaluate the mutants' ability to produce camalexin by measuring their fluorescence. This method was simple and bypassed the more time-consuming procedure of leaf tissue extraction and thin-layer chromatography.

Under normal circumstances, radiolabeling studies with mutants would have been greatly delayed by the need to backcross putative mutants to wild-type plants to ensure that camalexin-deficient offspring were isogenic with the wild-type plants, differing only in genes for camalexin biosynthesis. However, near the start of this project, three camalexin-deficient mutants were isolated by Dr. Jane Glazebrook through a screen for plants that produced little camalexin when inoculated with *Pseudomonas syringae* pv. *maculicola*, a compatible pathogen (Glazebrook and Ausubel, 1994). Dr. Glazebrook kindly provided seed from the *pad* (*p*hytoalexin-*d*eficient) mutants and agreed to test putative mutants from this project for camalexin deficiency in her screen, and to backcross confirmed mutants to wild-type plants for genetic analysis. As the *pad* mutants had already been backcrossed, the search for biosynthetic intermediates by radiolabeling began with these, of which one (*pad3*) was a null mutant producing no camalexin, and two (*pad1* and *pad2*) were leaky mutants producing 20 % and 10 % of wild-type levels of camalexin, respectively (Glazebrook and Ausubel, 1994).

Materials and Methods

Reagents and Chemicals.

Camalexin came primarily from a supply isolated from Arabidopsis by Jun Tsuji (Tsuji et al., 1992). Some of the camalexin used for standard curves in fluorimetric analysis was synthesized according to the method of Ayer et al. (1991) and purified as explained in Chapter 3. Radioactive anthranilate (^{14}C, uniformly labeled on the benzene ring,) was purchased from Sigma. The specific activity indicated on the label was 1-25 mCi/mmol, and the exact specific activity was never determined. All other chemicals used for radiolabeling and mutant screening were of reagent grade or better.

Plant Material and Growth Conditions.

The M2 generation of ethyl methane sulfonate (EMS)-mutagenized seeds of the Columbia ecotype of Arabidopsis (marker: glabrous; Col-gl), obtained from Lehle, were screened for camalexin deficiency. For radiolabeling studies, seeds of the third backcross of the *pad1*, *pad2*, and *pad3* mutants were generously provided by Dr. Glazebrook. These mutants were compared initially to the Columbia-0 (Col-0) ecotype. About halfway through these studies, a change was made to the Col-gl ecotype, which was used because it was a better point of comparison for the seeds being planted for

the mutant screen, which were grown from mutagenized Col-gl seeds. The Col-0 and Col-gl ecotypes produced comparable amounts of camalexin.

Seeds were grown in clay pots 14.5 cm in diameter, containing a mixture of perlite and Baccto® High Porosity Professional Planting Mix on a 2-cm-thick layer of perlite. The soil was wetted with fertilizer (half-strength Hoagland's solution, Appendix A) and hand-compacted to provide a level surface, and a 1-2 mm layer of fine vermiculite was sprinkled over the surface.

Seeds were washed in 5-ml glass screwcap tubes with water plus a small drop of Triton X-100 detergent, rinsed (by adding water to the tubes and removing it by aspiration) until no foaming was seen, and distributed over the vermiculite in a quasi-grid pattern with a Rainin P200 automatic pipettor. The plastic tip used on the pipettor was cut to make an opening wide enough to take up seeds. To ensure that many plants would grow in each pot,2 or 3 seeds were planted in each spot, either by ejecting 2 or 3 seeds at a time with the pipettor, or by distributing seeds twice over the vermiculite. The latter approach, although generally less desirable because it was more time-consuming, was used for growing putative mutants, as it was easier to identify the plant from which leaves came if the plants were not clustered too closely together. Pots were covered with plastic wrap to maintain humidity; the trays holding the pots were filled with a layer of water 3 cm deep; and the pots

were kept in a growth chamber with a 16- to 18-hour light regime, at about 21 °C.

Seeds usually germinated 2 to 3 days after planting, at which time the plastic wrap over the pots was slit. After another 2 to 4 days, the plastic was removed, and about 20 ml of half-strength Hoagland's solution was poured onto the surface of the soil to fertilize the seedlings. Fertilization was repeated every week, with the amounts increasing to about 150 ml per pot as the seedlings grew. A water height of about 3 cm was maintained in the trays for about the first 10 days to ensure that the seedlings had plenty of water. As the seedlings grew, the amount of water in the trays was reduced, since the roots were bigger and more capable of obtaining water and nutrients (and of becoming oxygen-depleted in an overly moist environment). Three- or four-week-old plants were watered only when the water in the trays had almost disappeared. Water was then added only to cover the bottom of the tray.

Fungal cultures and inoculation procedures.

Cochliobolus carbonum was grown on V-8 agar (per liter: 200 ml V-8 juice, 2 g calcium carbonate, 14 g agar) on an 18- to 24-hour light regime. Spores 1 to 2 weeks old were used to inoculate Arabidopsis leaves. The inoculum was prepared by flooding a plate of spores twice with non-sterile deionized water and filtering the spores through two layers of cheesecloth. Few precautions with sterility were taken at

this point, as the concentration of spores was far greater than the concentration of any contaminant was likely to be. The main objective was to find plants that could not produce camalexin, regardless of the eliciting conditions. In addition, the leaves to be inoculated were not sterile.

Inoculum concentration was not measured in mutant screening, but as the amount of camalexin produced appeared to increase with increasing spore concentration, based on casual observations and published results (Jejelowo et al., 1991), the inoculum was made as thick as possible and probably ranged from $5x10^5$ to $1x10^6$ spores/ml. For the radiolabeling studies, spore concentrations ranged from $3.6x10^5$ to $9.6x10^5$ spores/ml.

Rosette leaves from 3- to 4-week-old plants were excised with a razor blade and placed in covered 15-cm diameter Petri dishes lined with Whatman filter paper (#1 or #4) moistened with water to maintain a humid environment. Leaves were placed with the adaxial side down and inoculated on the abaxial side. The spore suspension was stirred frequently during the inoculation, as the spores rapidly settled to the bottom of the container. For the mutant screen, as the objective was simply to determine if camalexin could be produced, leaves were inoculated with the maximum amount of inoculum that would stay on the leaf. For the radiolabeling studies, leaves were inoculated with 0.1 ml of spores, distributed with a P1000 automatic pipettor whose plastic tip was cut to facilitate taking up spores. Inoculating with a

fixed volume ensured greater reproducibility of results. The amount of camalexin produced in leaves is greatest directly beneath the inoculum droplet (Jejelowo et al., 1991). Consequently, if the same amount of inoculum was used on each leaf and the covered surface of each leaf was roughly the same, the amounts of camalexin and precursors produced should have been fairly similar from one experiment to the next.

Extraction of camalexin.

For the mutant screen, camalexin did not need to be extracted from inoculum droplets. However, when putative mutants were found, the droplets from a few pairs of leaves were pooled and extracted with ethyl acetate (see Chapter 3) and compared on thin-layer chromatograms to extracts from wild-type leaves. The general method of leaf tissue extraction consisted of boiling leaves for 20 minutes in 80 % methanol or 100 % methanol, a method frequently used for plant tissue extraction because methanol penetrates tissues quickly (Harborne, 1973). In the radiolabeling studies,6 leaves per sample were placed in 12 ml of 80 % methanol/20 % water and heated in a water bath until the volume was reduced by about one-half. The sample was filtered through cheesecloth to remove leaves and boiling chips. The volume of extract was sometimes increased by 50 % by adding water (final volume approximately 9 ml), and it was then extracted 3 times with an equal volume of chloroform. The chloroform layers were pooled and dried over sodium sulfate, and the

chloroform was removed by drying at 45-50 °C on a rotary evaporator (Büchi). Samples were transferred to small test tubes by rinsing the round bottom flasks with two 250-µl aliquots of methanol, dried under nitrogen, and redissolved in 50 µl of methanol for thin-layer chromatography.

Thin-layer chromatography (TLC) of extracts.

Samples (10 or 20 µl) were loaded onto a 20x20 cm glass-backed silica-gel TLC plate (Fisher Redi-Plate). Although initially it was not done, eventually the plates were always activated by drying at 80-100 °C for 20 minutes. Plates were pre-developed in chloroform-methanol (3:2 or 1:1, v/v) if precleaning was needed. Samples were loaded as thin bands with 10-µl micropipets (VWR Scientific). Standards of camalexin, indole-3-carboxaldehyde, and indole-3-carboxylic acid were also loaded. Plates were developed in chloroform-methanol (9:1 or 19:1, v/v). This development was sometimes preceded by development with chloroform to help separate the less-polar leaf pigments from other compounds.

Mutant Screen.

Plants were screened for camalexin deficiency when 3 to 4 weeks old: at or near the time of flower production, but before the leaves began to senesce and turn purple. In each pot, 10 to 15 plants were chosen at random and marked with a numbered tag on a wooden toothpick. Two leaves were excised from each plant and inoculated. After 36 to 48 hours,

droplets were collected with a Pasteur pipet and transferred to 13x100 mm glass tubes. Deionized water (2 ml) was added to each tube, and the samples were mixed by vortexing. Fluorescence was measured on a Hitachi F-2000 fluorimeter (wavelength of excitation 330 nm; wavelength of emission 393 nm). The wavelengths of excitation and emission were supposedly fairly specific to camalexin (M. Zook, pers. comm.). Thus, other fluorescent compounds that might have been present, such as sinapic acid, were not likely to create false positive results. Readings were given, as is common in fluorescence spectroscopy, in relative intensities, based on the following formula:

$$F=K\ I_0\ c\ l\ \varepsilon\ \emptyset$$

where F=relative fluorescence intensity; K=a constant defined for the instrument; I_0=intensity of the light entering the flow cell; c=sample concentration; l=path length of flow cell; ε=extinction coefficient of sample; and $\emptyset$=quantum yield (ratio of optical energy absorbed to total fluorescent energy emitted).

No standard curve of fluorescence versus camalexin concentration was prepared at the time of the mutant screening. The screen was intended to be qualitative, and plants were considered putative mutants if relative intensities were below 200. Wild-type relative intensities were typically over 1000 (Tables 1-3). Eventually, a few samples from *pad3* and wild-type plants were always inoculated

and evaluated with the samples from putative mutants, to determine the range of values that represented camalexin deficiency and wild-type camalexin production.

If samples had readings in the *pad3* range, the plants from which the leaves had been taken were saved and allowed to go to seed. (As Arabidopsis is self-pollinating, the seed contained only the genes of the original plant of interest.) Eventually, plants with readings between *pad* 3 and wild-type readings were saved for seed, in case the intermediate readings were due to a heterozygous trait for camalexin deficiency. The seed was collected by shaking the siliques over a wire mesh, retrieving the seeds that fell through onto paper, and storing them in vials or microfuge tubes over desiccant at 4 °C. Seeds were planted (sometimes by the method described previously, but more frequently by sprinkling them over the pot surface without washing, as aspirating water off of seeds could cause a high loss of seed) and grown under the conditions previously described. Ten to forty of these M3 seedlings were chosen at random and numbered, and 2 leaves from each seedling were screened for camalexin deficiency as previously described. If all or most of the samples had low readings, the plants were considered to be camalexin-deficient mutants, and the M3 or M4 seed (the latter if no M3 seed were left over from planting) was sent to Dr. Glazebrook for further analysis. If the M3 seedlings had readings that ranged from low to high, the mutant was considered a potential camalexin-deficient heterozygote, and

the seed from plants giving low readings was saved for replanting and retesting, or sent directly to Dr. Glazebrook if the readings fell into a convincing 1:2:1 ratio of low to intermediate to high readings (a good indication, based on classical Mendelian genetics, that recombination between 2 genes Aa and Aa had occurred and given rise to a 1:2:1 ratio of aa:Aa:AA).

Radiolabeling studies.

Leaves of 3- to 4-week-old wild-type and *pad* seedlings were excised, placed in Petri dishes lined with moistened filter paper, and inoculated with 0.1 ml of water or *C. carbonum* spores. After the desired incubation time, the leaves were blotted dry with a Kimwipe and leaves placed in trimmed 0.7-ml microfuge tubes containing 50 µl of ^{14}C-anthranilic acid diluted with water so that the activity in each tube was 0.035-0.058 µCi (0.21-0.35 µCi per 6-leaf sample). Initially, labeled anthranilate was fed to leaves after a 24-hour incubation period. That was the time of maximum camalexin accumulation in leaves inoculated with *Pseudomonas syringae* pv. *syringae* (Tsuji et al., 1991), and high concentrations of camalexin had been found at 24 hours in preliminary non-radioactive extractions of *C. carbonum*-inoculated leaves. It seemed, according to standard procedures for biosynthetic studies (Bu'lock, 1965), that the time of maximum accumulation would be the best time to detect incorporation into camalexin and intermediates in wild-type,

and into biosynthetic blocks in the mutants. To ensure that the leaves would be able to take up solution, the petiole was cut under water just before placement in the tube. After the solution was taken up (2-4 hours), the tube was marked with a dot to indicate that uptake was completed, and 50 µl of water were added to prevent the leaf from becoming desiccated. When all leaves had finished taking up solution, they were extracted as described above. After separation by TLC, the R_f's of the standards were recorded; and the plates were sprayed with EN^{3}HANCE spray (a fluor from NEN Research Products that aids in visualizing low-activity bands on film), wrapped in Borden™ plastic wrap, and laid onto film for 7-10 days.

Results

Mutant screen.

Corey Sonnett, an undergraduate assistant in the lab, and Dr. Michael Zook isolated mutant 2120. This mutant was found by Dr. Glazebrook to be heterozygous for camalexin deficiency, with the homozygous form of the gene being a recessive trait. The camalexin-deficient homozygous recessive progeny from this mutant were bred for further analysis, and this mutation has been named *pad5* (Glazebrook et al., 1997). Due to the realization that some mutants might be heterozygous for genes involved in camalexin

production, the checks for putative heterozygotes mentioned in the Materials and Methods were implemented.

As a result of this more careful screening, two putative heterozygotes, mutants 4420 and 4440, were identified (Table 1). These were identified by comparing their relative fluorescence intensities to those of *pad3* and wild-type, and by grouping them according to the different ranges of relative intensities noted. Later, to quantitate those values, a standard curve of relative intensity versus camalexin concentration was determined (Figure 6). For both mutants, the average amount of camalexin in plants with intermediate relative intensities was about twice the amount in plants with low intensities and about half that in plants with high intensities. The number of plants in each category, for mutant 4440, was 10 low to 18 intermediate to 10 high readings: almost a 1:2:1 Mendelian ratio. Even the plants with high relative intensities produced less than half the amount of camalexin produced by wild-type. Although the segregation ratios for mutant 4420 were less impressive (5 low to 10 intermediate to 14 high readings), the categories were reasonably distinct, and it is possible that the number of plants screened was too small to observe Mendelian patterns of segregation. However, when these mutants (identified by their response to inoculation with *C. carbonum)*, were inoculated with *Pseudomonas syringae* pv. *maculicola* strain ES4326 (*Psm* ES4326), they produced wild-type amounts of camalexin (J. Glazebrook, pers. comm.).

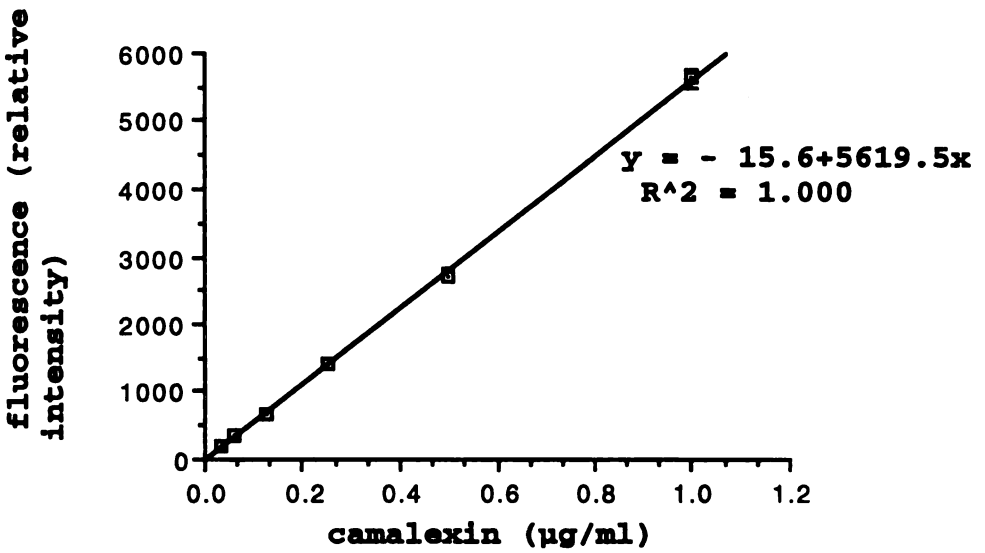

Figure 6. Standard curve of camalexin concentration versus fluorescence (excitation wavelength 330 nm, emission wavelength 393 nm). Data represent means plus standard errors of relative intensities (duplicate readings) of pure synthetic camalexin dissolved in methanol and diluted with water (maximum methanol concentration 0.5%).

Table 1. Camalexin in 2 putative camalexin-deficient heterozygotes. First row of numbers in each cell represents mean plus standard error of relative fluorescence intensity; second row denotes number of seedlings screened; and third row denotes mean plus standard error of camalexin concentration in micrograms per leaf, based on the standard curve in Figure 6. Fifth and sixth columns denote camalexin concentrations in wild-type and *pad3* leaves inoculated at the same time as the putative mutants.

mutant	camalexin in "low" samples	camalexin in "mid" samples	camalexin in "high" samples	camalexin in *pad3*	camalexin in wild-type
4420	524.4±47.6 (N=5) 0.0961±0.0085	1194±85 (N=10) 0.215±0.015	2824±291 (N=14) 0.505±0.052	476.4±46.7 (N=10) 0.0876±0.0083	6836±1164 (N=5) 1.22±0.21
4440	431.8±61.5 (N=10) 0.0796±0.0109	1066±39 (N=18) 0.192±0.007	2040±105 (N=10) 0.366±0.019	143.4±12.7 (N=10) 0.279±0.002	7158±1176 (N=4) 1.28±0.21

Two putative camalexin-deficient mutants that were not heterozygotes--plants 4648 and 4512--were also isolated. Table 2 demonstrates that mutant 4648 produced about 55 % as much camalexin as *pad3* produced on the day that 4648 was screened, and about as much as *pad3* produced on other occasions. Mutant 4512, although it produced 4-5 times more camalexin than *pad3*, was considered a mutant because it produced only 14 % as much camalexin as wild-type.

It should be noted that the amount of camalexin produced by wild-type and *pad3* leaves varied at each screening (Tables 1-3). Camalexin concentrations varied by a factor of 2 to 3 in wild-type leaves, and by a factor of 2 to 5 in *pad3* leaves. It is rather interesting that *pad3*, supposedly a null mutant, produced small amounts of camalexin. This result was confirmed by Kaitlyn Hwang, a high school student who worked in the Hammerschmidt lab during the summer of 1995 and found that *pad3*, 48 hours after inoculation with the fungus *Colletotrichum lagenarium*, produced small amounts of camalexin that could be detected by high-performance liquid chromatography. Perhaps the fluorescence in *pad3* was partly due to other fluorescent compounds whose spectra slightly overlapped the camalexin spectrum. The readings were not due to instrument background, as inoculation with water alone gave even lower readings (Table 3).

Table 2. Camalexin in putative camalexin-deficient mutants 4648 and 4512, and in *pad1* and *pad2*. Third and fourth columns represent camalexin concentrations for wild-type and *pad3* leaves inoculated at the same time as the putative mutants. See Table 1 for definitions of numbers in each row.

mutant	camalexin in mutant	camalexin in wild-type	camalexin in *pad3*
4648	237.0±10.2 (N=31) 0.0450±0.0018	>9999 (out of fluorimeter range) (N=10) >1.78	431.0±16.5 (N=7) 0.0795±0.0029
4512	449.9±41.6 (N=39) 0.0828±0.0074	3225±757 (N=4) 0.577±0.135	97.41±41.55 (N=10) 0.0201±0.074
pad2	524.9±20.8 (N=5) 0.0962±0.0037	8107±900 (N=4) 1.44±0.16	229.9±35.8 (N=3) 0.0437±0.0064
pad1	9724±1101 (N=6) 1.73±0.20	22240±3166 (N=3) 3.96±0.56	596.9±24.3 (N=10) 0.109±0.004

Table 3. Comparison of camalexin concentrations in inoculum droplets from fungal- and water-inoculated leaves of wild-type, *pad2*, and *pad3* plants. The mean plus or minus the standard error of relative fluorescence intensities is shown for the number of seedlings tested (N).

sample	camalexin in droplets of fungal-inoculated sample(relative intensity)	camalexin in droplets of water-inoculated sample(relative intensity)
wild-type	8107±900 (N=4)	16.48±10.29 (N=3)
pad2	524.9±20.8 (N=5)	21.46±8.73 (N=4)
pad3	229.9±35.8 (N=3)	17.40±8.50 (N=3)

Mutant 4648 produced no camalexin in response to *Psm* ES4326 (Glazebrook et al., 1997). Complementation tests demonstrated that this mutant was in the same complementation group as *pad3*, and it was named *pad3-2* (Glazebrook et al., 1997).

Mutant 4512, however, like mutants 4420 and 4440, produced wild-type amounts of camalexin in response to *Psm* ES4326. Apparently, some of the mutants were responding differently to the different pathogens. This suspicion had already arisen with the *pad* mutants, due to the suggestion of undergraduate assistant Corey Sonnett to screen those fluorimetrically. An initial check of several inoculated *pad1* leaves indicated that they produced wild-type amounts of camalexin; a more careful screen with more samples (Table 2) reinforced those findings. Only one sample had a relative intensity that did not exceed the range that the fluorimeter could read (0-9999); other samples had to be diluted by a

factor of 1 or 2 to obtain a measurable intensity. The average amount of camalexin produced by *pad1* on that occasion was higher than the amount produced by wild-type on other days when plants were screened, and higher than the maximum produced by wild-type in some of time courses described in Chapter 3. In contrast, *pad2* produced roughly 15 % of wild-type amounts of camalexin (Table 2), an amount similar to what had been found upon inoculation with *Psm* ES4326 (Glazebrook and Ausubel, 1994).

Radiolabeling studies.

The focus of the radiolabeling studies was on putative intermediates in the organic (chloroform) layer of leaf tissue extracts. The hypothetical camalexin intermediates could be in either the aqueous or organic phase, depending on the pH of the extract. The amino group on the indole moiety, if it became protonated, would cause the compound to partition to some extent into the aqueous phase; or if the pH were above the pK_a of the compounds, the carboxyl groups would become deprotonated, and the compounds would partition into the aqueous phase. At a lower pH, however, indole-derived compounds would be more likely to partition into the organic phase. Also, if any camalexin intermediates were constitutive and stored as glycosides or other conjugates, these would be in the aqueous phase. Because anthranilate is a precursor of many polar compounds, which would be in the aqueous phase and would generate very complex chromatograms,

it was simpler, and possibly less misleading, to focus on the organic phases. A few attempts were made to chromatograph the aqueous phases in a couple of different solvent systems (butanol-acetic acid-water 4:1:1, v/v; and chloroform-ethyl acetate-formic acid 35:55:10, v/v). However, the resolution was poor, and the chromatograms contained numerous bands. Analysis of the aqueous phases was quickly abandoned, apart from extracts of inoculum droplets on ^{35}S-cysteine-labeled leaves that will be described later.

Since the camalexin biosynthetic pathway is pathogen-induced, putative intermediates were expected to appear as bands on autoradiograms of TLC plate analyses of extracts of fungal-inoculated samples. It was also possible that constitutive plant compounds would be biosynthetic intermediates, since phytoalexin biosynthetic pathways are derived from primary metabolic pathways (Kuc, 1995). Evidence of a constitutive intermediate could be a band that would be present in a water-inoculated control but absent or very faint in the fungal-inoculated sample due to its being used in camalexin biosynthesis. Other intermediates could be constitutive but have their synthesis stimulated in response to infection. In that case, a band would be present in both water-and fungal-inoculated samples but darker in the inoculated sample. Still other intermediates could be nonconstitutive and formed *de novo* in response to infection, in which case the bands would be absent in water-inoculated and present in fungal-inoculated samples.

In two radiolabeling experiments (Figures 7 and 8), no obvious intermediates were detected in *pad1*, *pad2*, or *pad3*. In other words, no compounds consistently behaved like one of the types of intermediates described above. In most of the samples extracted in late June of 1994 (Figure 7), a band with an R_f of 0.13 was present. In wild-type and *pad3*, this band was darker in the control than in the inoculated samples, suggesting that it was a constitutive intermediate converted into camalexin after infection. In *pad1*, however, this band was darker in the inoculated than in the control sample, as if it were a constitutive intermediate whose production was stimulated after infection. The relative darkness of the band in control and inoculated samples varied between experiments as well. In samples extracted in mid-June of 1994 (Figure 8), a band with an R_f of 0.13-0.15 (presumably the same compound as the one seen at that approximate position in Figure 7) was less dark in the *pad1* control than in the inoculated sample. It was absent in *pad3*, suggesting this time that if it were an intermediate, it was formed *de novo* instead of being constitutive. Although it was again darker in the wild-type control than in the inoculated sample, the majority of the information from the two experiments was contradictory. The compound at the R_f of 0.13-0.15 seemed likely to be unrelated to camalexin biosynthesis because the amount of label incorporated varied so much.

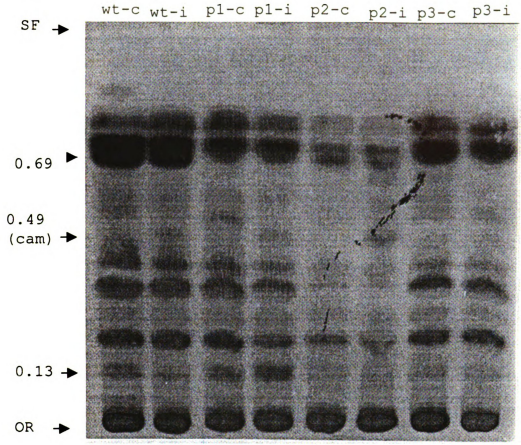

Figure 7. Autoradiogram of TLC plate: tissue extracts of wild-type (wt), *pad1* (p1), *pad2* (p2), and *pad3* (p3) leaves, fed ^{14}C-anthranilate 24 hours after inoculation with water (c) or *Cochliobolus carbonum* (i), 6/27/94-6/28/94. Samples were dissolved in methanol and loaded onto a 20x20 cm glass-backed silica TLC plate, which was developed in chloroform followed by chloroform-methanol (19:1, v/v). Arrows at left denote the origin (OR), solvent front (SF), and bands discussed in the text. Numbers at left denote R_f values of indicated bands. The total distance traveled by the solvent front was 15 cm. Other abbreviations: cam=camalexin.

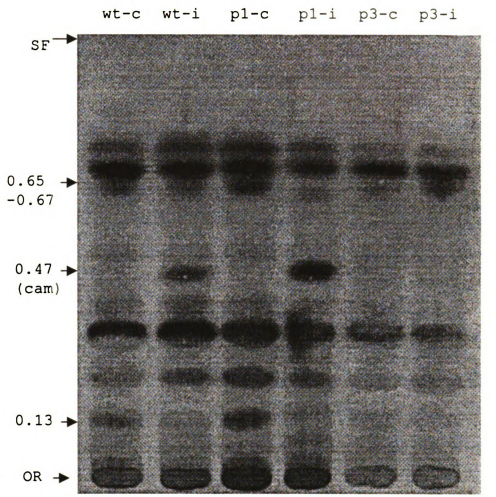

Figure 8. Autoradiogram of TLC plate: tissue extracts of wild-type, *pad1,* and *pad3* leaves fed ^{14}C-anthranilate 24 hours after inoculation with *C. carbonum*, 6/15/94-6/16/94. Abbreviations and TLC conditions are as indicated in Figure 7. Note the intensity of the camalexin(cam) band in the extract of inoculated *pad1* (p-1 i) leaves.

A band with an R_f of 0.65-0.67 (the approximate R_f of indole in this solvent) was darker in control than in inoculated wild-type and *pad1* in Figure 8, again suggesting a constitutive intermediate. However, since in Figure 7 this band (R_f=0.69) was darker in inoculated *pad1* than in the control, the results seemed again too variable to be related to camalexin biosynthesis.

Interestingly, relatively high incorporation of anthranilate into camalexin was observed in fungal-inoculated leaves of *pad1* (Figures 7 and 8) and *pad2* (Figure 7). These results suggested that the *pad1* and *pad2* mutants might be mutated not in camalexin biosynthetic enzymes but in the ability to recognize certain pathogens. Although they synthesized 30 % and 10 % of wild-type levels of camalexin, respectively, in response to *Psm* ES4326 infection (J. Glazebrook, pers. comm.), they appeared to synthesize more in response to *C. carbonum* infection.

Since *pad1* and *pad2* appeared to be regulatory mutants, the focus of the labeling studies turned to *pad3*, in which no camalexin had been detected in previous experiments. Previous experiments (Figures 7 and 8) indicated no incorporation of anthranilate into camalexin in *pad3*. No intermediates were detected in *pad3* in those experiments (Figures 7 and 8), but it seemed possible that they might accumulate prior to the onset of camalexin production and then be diverted into other biosynthetic pathways. Searching

for intermediates at various points in time is a technique frequently used to establish a biosynthetic pathway. One of the best-known examples is the work of Melvin Calvin to establish the path of carbon in photosynthesis (Taiz and Zeiger, 1992). Time course analysis was also used to determine the biosynthesis of indole alkaloids in periwinkle (*Vinca rosea*)(Scott et al., 1971). To search for intermediates that might accumulate before camalexin was produced, wild-type and *pad3* leaves were fed ^{14}C-anthranilate 3-18 hours after inoculation. In one such experiment (Figure 9), label was incorporated at 3 hours into a green fluorescent band with an R_f of 0.50. This band was darker in inoculated *pad3* than in inoculated wild-type leaves, which suggested that it was a camalexin intermediate accumulating due to a blocked biosynthetic pathway. In wild-type leaves, the band disappeared at 6 hours, when a camalexin band was first detectable, which suggested that it was being converted into camalexin. However, the results for these time courses were again quite variable. In the first one done (results not shown), the band seen at 3 hours was about equally intense in control and inoculated wild-type samples, and it was absent in *pad3*. In a third time course, camalexin was present at the earliest time point (3 hours) and the sought-after compound was not. This inducible compound was then not investigated further.

The unusually early appearance of camalexin could have

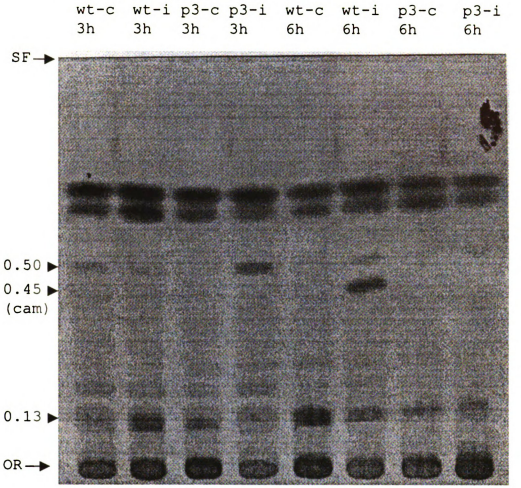

Figure 9. Autoradiogram of TLC plate: tissue extracts of wild-type and *pad3* leaves fed ^{14}C-anthranilate 3 and 6 hours after inoculation with *C. carbonum*, 10/9/94. Abbreviations and TLC conditions are as indicated in Figure 7.

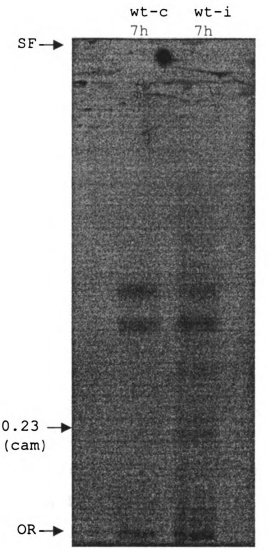

Figure 10. Autoradiogram of TLC plate: extracts of inoculum droplets from wild-type leaves fed ^{35}S-cysteine and then inoculated with *C. carbonum*, 3/29/95. Droplets were extracted 7 hours after inoculation. Samples were dissolved in methanol and loaded onto a 20x20 glass-backed silica plate, which was developed in chloroform-ethyl acetate-formic acid (35:55:10, v/v/v). Abbreviations are as indicated in Figure 7.

been due to the inoculated leaves synthesizing camalexin during the time when they were being fed ^{14}C-anthranilate. Since the feeding period lasted about 4 hours, the total time after incubation would have been 7 hours. This would have been enough time for camalexin synthesis to occur. From then on, radioactive solutions were fed to leaves prior to inoculation.

Although it might have been reasonable to resume pursuit of the putative intermediate after changing the sequence of leaf feeding and inoculation, this question was temporarily abandoned to test a hypothesis that *pad3* might be mutated in the ability to incorporate sulfur-containing compounds, which would not be detectable by ^{14}C-labeling. Wild-type and *pad3* leaves were fed either ^{14}C-anthranilic acid or ^{35}S-cysteine and inoculated with *C. carbonum*. Inoculum droplets were extracted 0, 3, and 7 hours after inoculation. One inducible band near the origin was detected after a 7-hour incubation in cysteine-fed wild-type leaves. The fact that the wild-type plants appeared to be a better source of intermediates than a null mutant was one of the final factors in the decision to abandon the labeling studies with the mutants. To see whether the inducible band might represent several poorly-resolved compounds, wild-type leaves were again fed ^{35}S-cysteine. Inoculum droplets were extracted 7 hours after inoculation. The extracts were divided between 2 TLC plates, of which one was developed in ethyl acetate-methanol

(24:1,v/v) and one was developed in chloroform-ethyl acetate-formic acid (35:55:10, v/v). Several inducible bands were present on both plates, but more bands were present on the plate developed in chloroform-ethyl acetate-formic acid (Figure 10). One attempt was made to characterize these compounds in a similar, nonradioactive experiment with larger quantities of leaves. When high-performance liquid chromatography (HPLC) did not resolve the compounds well enough for characterization, the labeling project was abandoned.

Discussion

Both the mutant screen and the radiolabeling studies had unexpected and rather disappointing results: putative mutants that were camalexin-deficient in response to *C. carbonum* inoculation were not camalexin-deficient in response to *Psm* ES4326 inoculation, and the *pad* mutants did not contain obvious biosynthetic blocks based on TLC analysis. To confuse matters further, *pad1* appeared to produce wild-type amounts of camalexin based on fluorescence intensity (Table 2) and radiolabeling data (Figures 7 and 8), and *pad2*, although it produced low amounts of camalexin based on fluorescence intensity (Table 2), seemed to incorporate fairly high amounts of ^{14}C-anthranilate into camalexin (Figure 7).

Several explanations are possible for the discrepancies in results for the mutants that appeared camalexin-deficient

in response to *C. carbonum* inoculation and not in response to *Psm* ES4326 inoculation. One is that the *C. carbonum* screen was based on the camalexin content of inoculum droplets, while the *Psm* ES4326 screen was based on the camalexin content of leaf tissue. There may have been more camalexin in the *C. carbonum*-inoculated leaves than in the droplets. It was determined later, through dose-response analyses (see Chapter 3), that although leaves inoculated with 10^5 spores/ml of *C. carbonum* contained about the same total amount of camalexin as leaves inoculated with 10^6 spores/ml, the partitioning of camalexin between leaf tissue and droplets differed greatly: at 10^5 spores/ml, most of the camalexin was in the droplet, while at 10^6 spores/ml, most of the camalexin was in the leaf. The reason for this difference is uncertain, but it may be due to non-specific binding of camalexin to spores on the leaf surface (R. Hammerschmidt, pers. comm.) The inoculum concentration used in the mutant screen was never measured, but the spore suspensions were always very dark and probably (based on subsequent experience) closer to 10^6 than to 10^5 spores/ml. Perhaps the camalexin produced after inoculation remained in the leaf tissue and was not detected in the screen. This possibility helps to account for the many M2 plants that appeared to be camalexin-deficient but produced M3 seedlings with wild-type concentrations of camalexin.

It is also possible that the kinetics of camalexin

production varied between wild-type plants and some of the putative mutants. It was determined later (see Chapters 3 and 4) that in wild-type plants alone, the time at which camalexin concentrations start to increase after inoculation with *C. carbonum*, and the time of maximum accumulation, can vary from 12 to 48 hours. Perhaps some of the putative mutants produced camalexin more rapidly than wild-type in the *C. carbonum* screen and were examined after the camalexin was metabolized to other compounds. Alternatively, they produced camalexin more slowly and were examined before camalexin concentrations increased significantly. This hypothesis is supported by more recent studies of camalexin production in tryptophan-deficient (*trp*) mutants (Zhao and Last, 1996). Whereas the *trp1-100* mutant previously was found to produce significantly less camalexin than wild-type plants 18 hours after inoculation (Tsuji et al., 1993), it produced as much camalexin as the wild-type 24 hours after inoculation (Zhao and Last, 1996).

Another possible explanation for discrepancies between results of the *C. carbonum* and the *Psm* ES4326 screens (one that would also provide an alternative explanation for the results with the *trp1-100* mutant just mentioned) is that some of the mutants were mutated not in camalexin biosynthetic genes, but in genes required for recognition of certain pathogens. As mentioned in Chapter 1, the mechanism for induction of phytoalexin synthesis may be a signal or signals from oligosaccharides or polypeptides from plants (Ebel,

1986) or from fungal cell walls (Ebel, 1986; Albersheim and Valenti, 1978; Nürnberger et al., 1994). These signals appear to be very specific: in parsley cell suspension cultures, phytoalexins were elicited by a 13-amino acid polypeptide from cell walls of *Phytophthora megasperma* f. sp. *glycinea*, and a putative receptor binding site was found (Nürnberger et al., 1994). Mutants 4440, 4420, and 4512 may have had an intact camalexin biosynthetic pathway but had a mutation in a gene involved in recognizing and responding to a signal from *C. carbonum*. This gene may not have been required for responding to *Psm* ES4326--which would not be surprising, as bacteria, having modes of infection different from those of fungi, would be expected to produce different signals that would be recognized by different genes or gene products. Zhao and Last's (1996) results for the *trp1-100* mutant may be explainable by this reason, as much as by the consideration of kinetics of accumulation, as the *trp1-100* mutant in their studies was inoculated with *Psm* ES4326, whereas in the study by Tsuji et al. (1993), it was inoculated with silver nitrate. It may be that the *trp1-100* mutant responds more rapidly to bacterial infection than to abiotic elicitation.

Both the fluorimetric and the radiolabeling studies suggest that *pad1* may be a regulatory mutant, although this suggestion has not been verified. A more-recently isolated *pad* mutant, *pad4*, has demonstrated that at least one PAD gene has a regulatory and not a biosynthetic function. In

response to *Psm* ES4326, *pad4* produced 10 % as much camalexin as wild-type, whereas it produced wild-type amounts of camalexin in response to *C. carbonum* (Glazebrook et al., 1997). Recent work by Zhou et al. (1998) demonstrated that *pad* 4 also produced wild-type levels of camalexin in response to *Xanthomonas campestris* pv. *campestris* strain BP109, silver nitrate, and *Psm* ES4326 carrying the avirulence gene *avr*Rpt2. A role for the *PAD4* gene was proposed, based on the fact that the *pad4* mutation led to low salicylic acid production in *Psm* ES4326-inoculated plants. Salicylic acid (SA) plays an important role in the induction of plant responses to infection (Vernooij et al., 1994; Zhou et al., 1998). The *PAD*4 gene may affect camalexin production by encoding a gene needed to activate SA production. Since SA concentrations were normal in plants inoculated with *Psm* ES4326 bearing *avr* Rpt2, it appears that more than one signal transduction pathway to camalexin production exists in Arabidopsis. Following abiotic elicitation or infection by fungi or avirulent pathogens, a signal transduction pathway not involving *PAD4* is activated. The genes that are activated can, in turn, activate production of SA, which leads to camalexin production (Zhou et al., 1998). In contrast, infection by *Psm* ES4326 requires *PAD4* in order for SA production to be activated.

In light of the findings of Zhou et al.(1998), it is easier to understand the ambiguous results in camalexin production with *pad1* in the fluorimetric and radiolabeling

analysis, and with *pad2* in the radiolabeling analysis. These mutants may respond differently to signals from *C. carbonum* than to signals from *Psm* ES4326. It is also easier to find reasons for why mutants 4420, 4440, and 4512 appeared camalexin-deficient in response to *C. carbonum* but not in response to *Psm* ES4326. The fact that camalexin is produced in response to chemicals causing amino acid starvation or oxidative stress (Zhao et al., 1998), suggests that many regulatory genes could be involved in its biosynthesis, in which case a number of mutations, unrelated to camalexin biosynthesis, could lead to camalexin deficiency.

As far as the radiolabeling studies are concerned, it may be unfair to invoke only regulatory mutations to explain the absence of obvious intermediates in the *pad* mutants. A number of other reasons may be involved, not the least of which would be inexperience on the part of the author. One possible reason is that the solvent system used in the thin-layer chromatography did not separate bands well enough to allow identification of inducible bands. In light of the labeling experiments described in Chapter 4, in which camalexin was poorly separated from another compound, this explanation is not unreasonable. A single experiment with *pad3* also supports this explanation: an inducible compound was detected in a large-scale extract (60 leaves) of *pad3* extracted 54 hours after inoculation. On a TLC plate developed in a more polar solvent (chloroform-methanol 9:1, v/v; followed by ethyl acetate-methanol 24:1, v/v) the

compound appeared as a fluorescent blue band just below camalexin. It was not characterized. Further studies of *pad3* may therefore yield some useful results.

Another possibility is that the inducible compounds were in the aqueous and not the organic phase, and that they were overlooked because only the organic phase was studied. The need to study the correct phase was demonstrated by Jimenez et al. (1997), who found camalexin and other phytoalexins in *Capsella bursa-pastoris* (shepherd's purse), but only in the basic fraction of ethyl acetate extracts.

Considering the variable kinetics of camalexin accumulation, it is also possible that some of the information on the autoradiograms was misinterpreted. The band with an R_f of 0.13 that was seen in Figures 7 and 8, and whose intensity varied between the two experiments represented in those figures, may have been a constitutive intermediate. In one experiment (Figure 7), camalexin accumulated slowly in *pad1*. Consequently, the majority of the compound was not converted into camalexin when the leaves were extracted. In the second experiment (Figure 8), camalexin accumulated quickly, and the majority of the compound was converted into camalexin at the time of extraction. This could explain why the camalexin band is darker in Figure 8 than in Figure 7. A similar argument can be made for the wild-type leaves in these figures.

Given all the possible explanations for the lack of biosynthetic intermediates found in the *pad* mutants, it may

have been a mistake not to pursue the search with revised tactics such as different TLC solvents, HPLC, or studies of different phases. All of these tactics would be worth attempting in future work. It is possible that the inducible band found in *pad* 3, if studied further, will prove to be a biosynthetic intermediate. Considering the ambiguous results of the mutant screen and radiolabeling studies, however, the most likely explanation for the lack of intermediates was that the mutants were not biosynthetic but regulatory mutants. Possibly the inducible band in *pad3* had nothing to do with camalexin biosynthesis. It seemed time to abandon the search for biosynthetic intermediates and mutants, and to examine more closely the differences in camalexin production between fungal- and bacterial-inoculated plants, the results of which are described in Chapter 3.

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Chapter 3. Comparison of Patterns of Camalexin Accumulation in Response to the Fungal Pathogen *Cochliobolus carbonum* and the Bacterial Pathogens *Pseudomonas syringae* pv. *maculicola* and *Pseudomonas syringae* pv. *syringae*.

Introduction

The studies described in Chapter 2 suggested that some camalexin-deficient mutants of Arabidopsis were not biosynthetic mutants, but regulatory mutants that produced different amounts of camalexin in response to *Cochliobolus carbonum* and *Pseudomonas syringae* pv. *maculicola (Psm)* strain ES4326. It seemed possible that the differences in camalexin production were due to different responses to fungal and bacterial infection. Fungi and bacteria are distinct organisms with distinct modes of plant infection. Whereas fungi can infect plant cells by direct penetration as well as by entry through wounds or natural openings, bacteria enter cells only through wounds or natural openings (Agrios, 1997). Bacteria are also much smaller than fungi and have chemically different cell membranes. Therefore, it would be expected that the signals or elicitors produced by fungi and bacteria would differ and that in the infected plant, different genes would be required to recognize and respond to the various signals. The recent studies on the *pad4* Arabidopsis mutant support this model. As described in Chapter 1, the *PAD4* gene

affects camalexin biosynthesis by controlling production of salicylic acid, which is in some way necessary for initiating camalexin biosynthesis (Zhou et al., 1998). Because more than one signal transduction pathway appears to be present, some elicitors may activate a signal transduction pathway that does not go via *PAD4* when triggering salicylic acid production (Glazebrook et al., 1997; Zhou et al., 1998).

To determine whether some of the mutants might be signal transduction mutants, camalexin accumulation over a 3-day period was compared in fungal- and bacterial-inoculated wild-type and *pad2* plants. As it was not certain if *pad2* was a biosynthetic or a regulatory mutant, it was possible that closer study of its patterns of camalexin accumulation in response to different inducers would lead to finding biosynthetic intermediates in large-scale extracts. These experiments thus had the potential to provide information on both the biology and the chemistry of camalexin biosynthesis.

Optimum conditions for measuring camalexin levels in *Psm* ES4326-inoculated leaves, such as inoculum concentration and time of maximum accumulation of camalexin, have been reported (Glazebrook and Ausubel, 1994). As these conditions were not previously established for *C. carbonum*, this part of the project began by studying the kinetics of *C. carbonum*-elicited camalexin accumulation in order to determine the time of maximum accumulation. Optimal spore inoculum concentration was also determined. Camalexin accumulation was then compared in wild-type and *pad2* leaves inoculated

with *C. carbonum* or *Psm* ES4326. To determine if the patterns of accumulation reflected differences between responses to fungi and bacteria in general, or between responses to compatible and incompatible pathogens (*Psm* ES4326 being a compatible pathogen, and *C. carbonum* being an incompatible pathogen), camalexin accumulation was also compared in wild-type and *pad2* plants inoculated with *C. carbonum* or the incompatible bacterial pathogen *Pseudomonas syringae* pv. *syringae* (*Pss*).

Materials and Methods

Reagents and chemicals.

Camalexin was synthesized according to the method of Ayer et al. (1991) in the laboratory of Dr. W. Reusch at Michigan State University. Some synthetic camalexin was also generously provided by Dr. Alois Fürstner of the Max-Planck-Institut für Kohlenforschung (Mülheim/Ruhr, Germany). Indole-3-carboxaldehyde was purchased from Aldrich or Sigma, and anthranilic acid and indole-3-carboxylic acid were purchased from Aldrich. Solvents used for high-performance liquid chromatography (HPLC) were of HPLC grade. All other chemicals were of reagent grade or better.

Synthesis and purification of camalexin.

The yield of camalexin in the synthesis (approximately 0.5 %) was considerably lower than the 68-76 % yield reported

by Ayer et al. (1991). When the reaction mixture was checked by thin-layer chromatography (TLC) in chloroform-methanol (9:1, v/v), the major band had an R_f value higher than that of camalexin, although the latter was also present. The major band, although never characterized, was probably acetylcamalexin, which Ayer et al.(1991) found to comprise about 6 % of the products. The predominance of acetylcamalexin was probably due to failure to quench the reaction mixture with water prior to work-up with ethyl acetate (Ayer et al., 1991). Another factor may have been the water bath temperatures of 45-50 °C used for rotary evaporation during work-up. At such temperatures, excess indole may have reacted with ethyl acetate to produce acetylcamalexin (J. Kagan, pers. comm.). (When this synthesis was attempted by other workers, acetylcamalexin was also the major product, and the yield of camalexin was higher when work-up was done in ether without heating [J. Kagan, pers. comm.].)

The synthetic camalexin was purified by preparative TLC on Fisherbrand 1000 µm silica plates in chloroform-methanol (9:1 or 19:1, v/v). The camalexin band was eluted with acetone or ethyl acetate. The solvent was removed under vacuum at 45-50 °C. The residue was transferred to a vial, dried, and weighed. For TLC, the residue was redissolved in methanol and used without further purification. For high-performance liquid chromatography (HPLC), the residue was redissolved in isopropanol-hexane (7:93, v/v), combined with

some plant-produced camalexin, and purified on an Alltech Econosphere silica column (pore size: 5 µm, column dimensions: 4.6 mm inner diameter and 250 mm length) attached to a Waters HPLC (pump model 501, injector model U6K, UV detector model 486). A wavelength of 215 nm was used for detection because camalexin has an absorbance maximum at this wavelength (Hammerschmidt et al., 1993). In a mobile phase of isopropanol-hexane (7:93, v/v) with flow rate of 1 ml/minute, camalexin had a retention time of 9 to 9.5 minutes (retention times varied on different days, due to slight variations in solvent composition). Fractions corresponding to camalexin peaks were collected. The solvent was removed under vacuum. The residue was weighed, redissolved in a known volume of isopropanol-hexane (7:93, v/v), and aliquoted into vials. These aliquots were used for standard curves in HPLC and fluorimetric analysis.

Plant material and growth conditions.

Wild-type and *pad2* Arabidopsis plants were grown as described in Chapter 2. For wild-type plants, the glabrous strain of the Columbia ecotype (Col-gl) was used, since this strain had also been used for the radiolabeling studies (Chapter 2). For the time courses done with *Pss*, the Col-0 ecotype was used.

Fungal and bacterial cultures.

C. carbonum was grown as described in Chapter 2. For the time courses comparing camalexin accumulation in *C. carbonum*- and and *Pss*-inoculated leaves, the fungus was grown on V-8 agar of a slightly different composition (per liter: 160 ml V-8 juice, 1 g calcium carbonate, 14 g agar). *Psm* ES4326, a strain pathogenic on many ecotypes of Arabidopsis (Dong et al., 1991), was provided by Dr. Glazebrook (University of Maryland, College Park). The culture was transferred from a plate or a slant to a plate of King's B agar (per liter: 1.5 g dibasic potassium phosphate, 1.5 g magnesium sulfate heptahydrate, 20 g peptone, 10 ml glycerol, 15 g agar), an indicator of the presence of pseudomonads because of the production of fluorescent pigments. Cells from this plate were used to inoculate a 50-ml flask containing about 15 ml of LB (Luria-Bertani medium: 10 g tryptone, 5 g yeast extract, 10 g sodium chloride per liter), which was then incubated at room temperature on a shaker (Lab-Line Instruments, Model 3590) at about 120 rpm. *Pss* strain D20 (Tsuji et al., 1992) was grown on LB agar amended with nalidixic acid, and cells from these plates were used to inoculate 50-ml flasks containing about 15 ml of LB broth. Fourteen-hour-old broth cultures of *Psm* ES4326 and *Pss* D20 were centrifuged on a benchtop centrifuge (International Chemical Centrifuge, Schaar & Co.) at about two-thirds of the maximum speed. Cells were washed 2 or 3 times with 10 mM magnesium sulfate. After resuspension of the washed cells in

10 mM magnesium sulfate, the optical density at 600 nm (OD_{600}) was measured on a Zeiss spectrophotometer. Cells were diluted (again in 10 mM magnesium sulfate) so that the final concentration would correspond to an OD_{600} of 0.02. To determine the number of colony-forming units per ml of inoculum (cfu/ml), a portion of the final cell suspension was serially diluted, and dilutions were plated onto LB agar. The number of cfu/ml ranged from $1.1x10^6$ to $1.2x10^7$.

Leaf inoculation.

For the time courses establishing approximate kinetics of camalexin accumulation, one fungal-inoculated sample of 30 or 45 leaves and one water-inoculated control, with an equal number of leaves, was prepared for each timepoint. For subsequent experiments, only 15-20 leaves per sample were inoculated, and all samples were prepared in duplicate or triplicate. In these latter experiments, water controls were extracted only at 72 hours, or at 24 and 72 hours. Three- to four-week-old leaves were excised and in covered Petri dishes, as described in Chapter 2. An effort was made to use leaves from several pots of plants for each timepoint, and to ensure that a fungal-inoculated sample and the corresponding water-inoculated control contained leaves from the same plants. Each leaf was inoculated with 0.1 ml of water or a suspension of 8- to 14-day old *C. carbonum* spores, prepared as described in Chapter 2 (see Materials and Methods). Spore

inoculum concentration (determined with a Bausch & Lomb hemacytometer) ranged from $6x10^5$-$7x10^5$ spores/ml in the studies of the approximate kinetics of camalexin accumulation. For dose-response experiments, spore concentrations of 0, $2x10^4$, $2x10^5$, and $2x10^6$ spores/ml were prepared by serial dilution of a suspension of the highest concentration. For subsequent experiments, the spore concentration was 1.4-2.0 $x10^5$ spores/ml. Following inoculation, leaves were incubated at room temperature in the covered Petri dishes.

For bacterial inoculation, the cells, grown and diluted as described above, were infiltrated into intact leaves with the blunt end of a 3 ml plastic syringe. Some leaves were inoculated with 10 mM magnesium sulfate as controls. For each sample, 15-20 leaves were inoculated. Plants were covered with wet sheets of plastic or with plastic bags about 12 hours prior to inoculation, to cause the stomata to open and make the leaves easier to infiltrate.

Extractions.

On fungal-inoculated leaves (15-45 per sample), droplets of water or inoculum were collected with a Pasteur pipet and extracted by vortexing in a test tube 3 times with equal volumes of ethyl acetate (the volume of the aqueous phase was increased by adding about 50 % the original volume of water, to reduce the percentage of sample lost to flask walls). The

organic phases were pooled, evaporated to dryness at 40 °C on a Büchi rotary evaporator, and redissolved in 1 ml methanol (two 0.5-ml aliquots) to transfer to 10-ml glass tubes. The tubes were capped with aluminum foil, sealed with Parafilm, and then stored at -20 °C.

The leaves were extracted by a method similar to that used by Hammerschmidt et al. (1993). They were boiled for 20 minutes in 80 % methanol (about 1.5 ml per leaf, which seemed enough to ensure an efficient extraction without generating too much solvent waste), and then stored at 4°C in foil-capped flasks sealed with Parafilm until a convenient time for extraction with chloroform. In the time courses comparing camalexin accumulation in response to *Psm* ES4326 and *C. carbonum*, or in response to *Pss* D20 and *C. carbonum*, fungal-inoculated leaves and inoculum droplets were combined and boiled together in 80 % methanol. The intention was to make the extraction of these samples as similar as possible to the extraction of the bacterial-inoculated leaves which did not contain inoculum droplets.

The methanol extract was concentrated on the rotary evaporator at 40-45 °C until the sample looked turbid (an indication that the majority of the methanol was removed), resuspended in water so that the final volume was 1.5 times the volume obtained by concentration, and then extracted in a separatory funnel 3 times with an equal volume of chloroform. When the approximate kinetics of camalexin accumulation were being determined, the aqueous phases were then extracted

three times with equal volumes of ethyl acetate to look for residual camalexin and potential camalexin precursors.

After drying with anhydrous sodium sulfate, the organic phases were evaporated to dryness on the rotary evaporator, redissolved in 1-1.5 ml of methanol, and transferred to 10-ml tubes and stored at -20°C, as described for droplet extracts.

This extraction procedure was time-consuming, and it was not possible to process all samples in 1-2 days. Therefore, an effort was made to standardize the time frame within which samples were extracted. For example, in the time courses comparing camalexin accumulation in *C. carbonum*- and *Psm*-inoculated leaves, fungal- and bacterial-inoculated samples from a given timepoint and replicate were extracted with chloroform on the same day. Also, the period between the initial methanol extraction and the work-up was kept roughly the same for all timepoints, so that if replicate "B" of the 24-hour samples was extracted 3 days after boiling in methanol, replicate "B" of the 48-hour samples was also extracted 3 days after boiling in methanol.

Determination of the presence of camalexin and other inducible compounds.

Camalexin was separated by TLC as described in Chapter 2. Extracts were dissolved in 30-45 µl of methanol. Usually, 20 µl of the extract (two 10-µl aliquots with a glass capillary micropipet) were loaded. For the dose-response experiments, the entire sample was loaded, and for

heavily-pigmented leaf tissue extracts, 10 µl were loaded. A camalexin standard (2.5-5 µg) was loaded onto each plate. For TLC of extracts from the preliminary time courses of camalexin accumulation (Tables 5 and 6, Figures 12A and 12B), standards of indole-3-carboxaldehyde (0.07 mg/ml in methanol), indole-3-carboxylic acid (0.67 mg/ml in methanol), and anthranilic acid (0.5 or 0.2 mg/ml in methanol) were loaded as well. Extracts from those experiments consisted of more leaf tissue than extracts from previous experiments (fivefold increase) or later ones (two- to threefold increase). They consequently had the potential to contain detectable amounts of camalexin intermediates. Therefore, it was of interest to see whether any bands comigrated with known or hypothetical intermediates.

Plates were usually developed in chloroform-methanol (9:1 or 19:1, v/v), sometimes preceded by a development in chloroform to improve separation from pigments. The large-scale extracts were separated in several different TLC solvent systems in order to find one that separated camalexin well from pigments and one that resolved polar putative intermediates.

Camalexin was visualized under long-wave and short-wave ultraviolet (UV) light as a fluorescent purple band with an R_f of 0.3-0.5 in chloroform-methanol (19:1, v/v) and 0.5-0.7 in chloroform-methanol (9:1, v/v). Indole-3-carboxaldehyde was visualized under short-wave UV light as a dark, light-absorbing band that was best seen by shining a hand-held UV

lamp onto the front of the TLC plate and viewing the plate from the back while in a dark room. Indole-3-carboxylic acid appeared under short-wave UV light as a fluorescent purple band, and anthranilic acid fluoresced purple under both short-wave and long-wave UV light. The R_f values of all standards used are summarized for different solvent systems in Table 5. Plates were photographed as described in Chapter 5.

Preparation of samples for HPLC analysis.

The camalexin bands on the TLC plates were eluted with 3 ml of ethyl acetate, and the eluates were dried under nitrogen at 40-50 °C. To elute samples, bands at the R_f of the camalexin standard were scraped with a spatula onto weighing paper and transferred to 20-ml glass scintillation vials, which were stored at -20 °C if they could not be eluted within several hours of scraping. Samples were stored in this manner for only 1-3 days before being eluted, as earlier attempts to scrape and quantitate samples had demonstrated that after 2 months, the majority of the camalexin on scraped silica gel degraded. In contrast, about 80 % of the camalexin remained on TLC plates stored for the same length of time. The sample was transferred to a 15-ml sintered glass funnel (fine frit) set in a 250-ml filtering flask. The vial was rinsed with 1.5 ml of ethyl acetate; and the rinsate was then added to the funnel. After the flask

had been gently swirled to mix silica and solvent, vacuum was applied, and the solvent was collected in a 10-ml tube set directly underneath the funnel inside the sidearm flask. The vial was rinsed again with 1 ml of ethyl acetate, which was added to the funnel and collected in the same tube after being swirled in the funnel. The funnel was rinsed with 0.5 ml of ethyl acetate, to retrieve any camalexin that might have been splashed near the upper portion of the funnel during the elution, and this rinsate was also collected. The tube of eluate was dried under nitrogen (Meyer N-Evap) at 40-50 °C. The sintered glass funnel was rinsed with 7-15 ml of ethyl acetate between samples.

After drying, samples were sealed and stored at -20 °C until analysis. They were quite stable under those conditions: Samples analyzed once and then dried and stored yielded very similar results in HPLC analysis 2 months later (8-15 % decrease in OD_{215}).

Quantitation of camalexin by HPLC.

The HPLC analysis was done under the conditions described for camalexin purification. Samples were dissolved in 100-200 µl of mobile phase, and 10-20 µl were injected with a Hamilton 25-µl syringe. The syringe was rinsed with methanol and mobile phase between injections, and mobile phase was injected between each sample to ensure that no residue from the previous sample remained. Because the retention time of camalexin varied between 9 and 9.5 minutes

on different days, due to slight variations in mobile phase composition, a standard was injected at the start of each analysis to verify the retention time. The putative camalexin peak from the standard, and from 1-3 injected samples, were collected, concentrated, and co-spotted with a standard on 4x5 cm TLC plates to verify the identity of the peak. Samples usually were injected only once. The injection error was checked on 2 occasions. For samples dissolved in 200 µl or more of mobile phase, peak heights differed by 0-9 % between 2 injections. For samples dissolved in 100 µl of mobile phase, peak heights differed by 0-27 % between 2 injections. The greater variation in the second case may be due to rapid evaporation of smaller volumes, since peak heights were always greater on the second injection.

Camalexin was quantitated by the equation obtained from the standard curve in Figure 11A. For the time courses comparing camalexin accumulation in response to *C. carbonum* and *P.syringae* pv. *syringae*, camalexin was quantitated by the equation obtained from a standard curve run 2 years later to correct for decreased intensity of the detector bulb (Figure 11B).

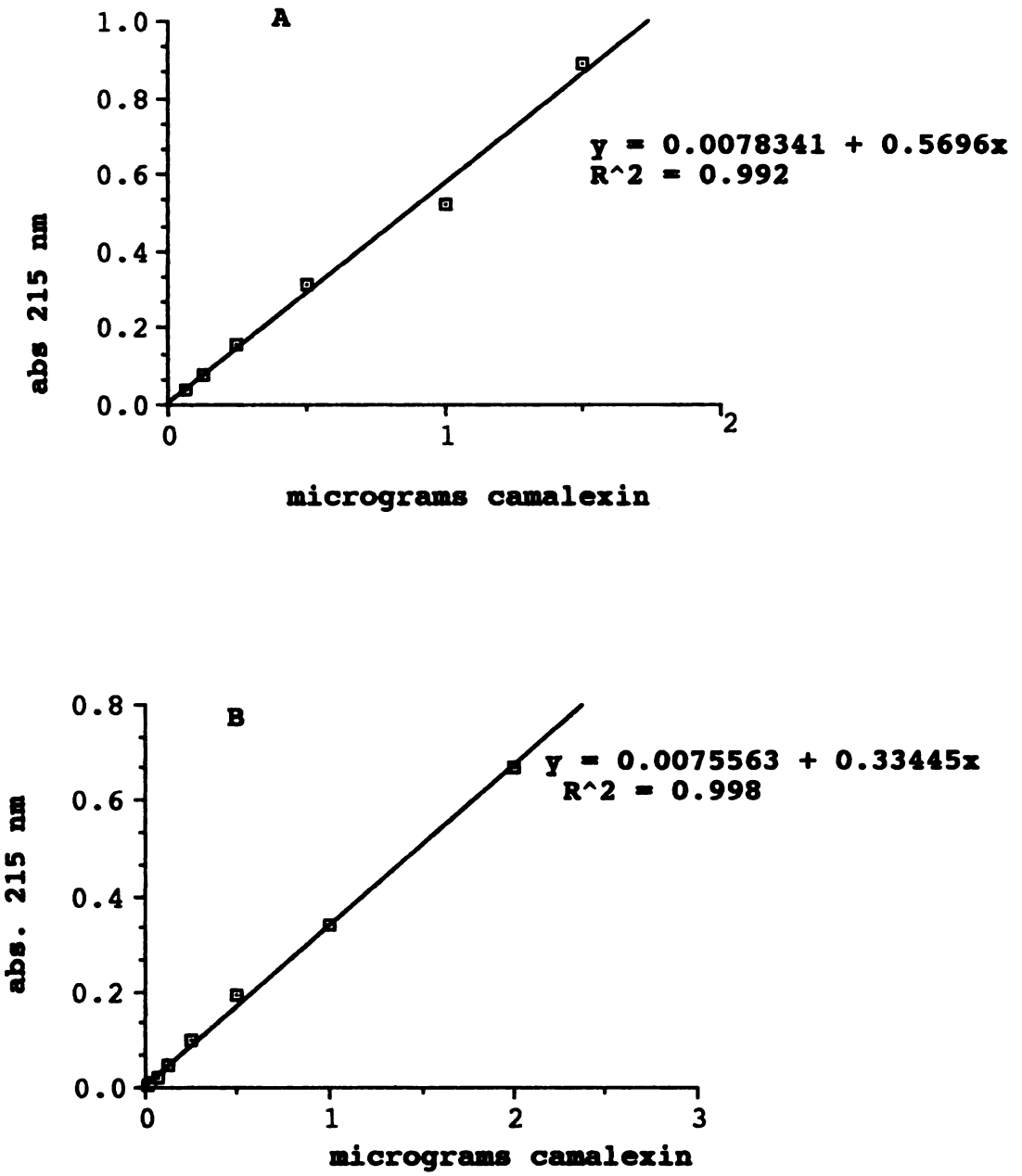


Figure 11. Standard curve of camalexin concentration versus absorbance at 215 nm, 12/15/95 (A) and 1/21/98 (B). Data represent means of duplicate HPLC injections of dilutions of pure synthetic camalexin in mobile phase.

Results

Determination of a standard curve of camalexin concentration versus absorbance.

The standard curves obtained for camalexin concentration versus OD_{215} are shown in Figure 11. The "known" amount of camalexin in the standards was based on an aliquot from 600 µg of HPLC-purified camalexin. Since the analytical balance used to weigh the standard was accurate only to 4 decimal places (0.0001 g, or 100 µg) the accuracy of the concentrations in the standard curve is questionable, and numerical discrepancies with the results of other workers may be due partly to initial inaccuracy in weighing the standard. However, the precision of the standard curves was satisfactory, judging by comparison of Figure 11A to a standard curve done 2 months later with a separate aliquot of that standard (y=0.03074+0.48916x; data not shown). As mentioned in the Materials and Methods, the equation obtained from Figure 11A was used to calculate camalexin concentration for most of the experiments described in this chapter, and the equation obtained from Figure 11B was used for the time courses of camalexin accumulation in *Pss* D20 and *C. carbonum*.

Determination of optimal separation of camalexin and putative intermediates by TLC.

The time courses depicted in Figures 12A and 12B were done, in part, to look for biosynthetic intermediates. A few

inducible bands were seen on TLC plates in various solvent systems. The R_f values of those bands in some of the solvent systems tried are listed in Table 4. Most were not studied further. Attempts to characterize one of these (R_f=0.4-0.5 in chloroform-methanol 9:1, [v/v]) led to the experiments described in Chapter 4.

The R_f values of the standards in those solvent systems are listed in Table 5. These should be taken only as an approximation. R_f values varied with the extent to which the plate was activated prior to use, usually being higher on less-activated plates. They could vary on a single plate, with camalexin bands running higher at the edges than at the center. The type of silica plate (glass- or plastic-backed) could cause the relative R_f values of standards to be reversed with respect to each other or increased (see also Chapter 4). The amount of standard loaded also made a difference. R_f values were usually higher when more standard was loaded. Finally, the size of the TLC plate affected R_f calculations: those obtained from small (5x5 cm) TLC plates were particularly rough approximations, since the error in distance measurement increased as the distance measured decreased.

Separation of camalexin from pigments was fairly good in ethyl acetate-hexane (1:1, v/v), but the best separation was

Table 4. R_f values and descriptions of inducible bands in different TLC solvents. Abbreviations: SWUV=short-wave ultraviolet light; LWUV=long-wave ultraviolet light, h=hours; $CHCl_3$=chloroform, MeOH=methanol, HOAc=acetic acid, EtOAc=ethyl acetate.

TLC solvent (v/v composition)	R_f values and descriptions of inducible bands
$CHCl_3$-MeOH 9:1	0.15 at 12 and 24 hours post-inoculation fluorescent purple under LWUV and SWUV 0.1 at 24, 48, and 72 hours post-inoculation fluorescent purple under LWUV 0.4-0.5 at 24, 48, and 72 hours post-inoculation fluorescent purple under LWUV
$CHCl_3$ followed by $CHCl_3$-MeOH 19:1	0.3-0.4 at 24, 48, and 72 hours post-inoculation fluorescent purple under LWUV and SWUV 0.2 at 24, 48, and 72 hours post-inoculation fluorescent purple under LWUV
toluene followed by 2 developments in $CHCl_3$-HOAc 9:1 (1 experiment)	0.43 at 12 hours post-inoculation appearance not recorded; probably fluorescent purple

Table 5. R_f values of camalexin, indole-3-carboxaldehyde, indole-3-carboxylic acid, and anthranilic acid developed on silica TLC plates in various solvent systems. Some R_f values obtained from experiments in Chapter 4 are included to make the table more useful. R_f's are listed with only 1 significant digit if they varied greatly among experiments. Abbreviations are as in Table 4.

TLC solvent (v/v composition)	R_f : camalexin	R_f: indole-3-carboxaldehyde	R_f: indole-3-carboxylic acid	R_f: anthranilic acid
$CHCl_3$-MeOH 9:1	0.5-0.6	0.5-0.6 (just below camalexin)	0.09-0.2	0.05-0.2
$CHCl_3$ followed by $CHCl_3$-MeOH 19:1	0.3-0.6	0.3-0.5 (just below camalexin)	0.04-0.05	0.04-0.07[a] 0.4[b]
$CHCl_3$-HOAc 9:1, 2 developments (1 experiment)	0.31	0.45	0.63	0.82
$CHCl_3$-EtOAc-HOAc 35:55:10	0.07[b,c]	0.2-0.5[b,c]	0.2-0.6[b,c]	0.6[b,c]
$CHCl_3$-MeOH 19:1 followed by $CHCl_3$-EtOAc-HOAc 35:55:10 (1 experiment)	0.93[a]	0.69[a]	0.58[a] and 0.67[a] (possibly protonated and unprotonated forms)	not determined
EtOAc-MeOH 24:1 (1 experiment)	0.60[b]	0.58[b]	0.62[b]	not determined

[a]glass-backed 20x20 cm plate used; [b]plastic-backed 5x5cm plate used; [c]standards well-separated from each other on a plate

Table 5 (cont'd.)

TLC solvent (v/v composition)	R_f: camalexin	R_f: indole-3-carbox-aldehyde	R_f: indole-3-carboxylic acid	R_f: anthra-nilic acid
EtOAc-hexane 1:1 (1 experiment)	0.33	0.27	0.07	0.027
$CHCl_3$-EtOAc-HOAc 35:55:10 followed by $CHCl_3$ -HOAc 9:1 (1 experiment)	0.14[b]	0.33[b]	0.42[b]	not deter-mined

[a]glass-backed 20x20 cm plate used; [b]plastic-backed 5x5cm plate used; [c]on a single plate, standards well-separated from each other

achieved by development in chloroform followed by chloroform-methanol (19:1, v/v). This solvent was used to separate leaf extracts in all subsequent experiments. Occasionally, if camalexin and pigments were poorly separated, the camalexin band was eluted and developed again by TLC.

For ethyl acetate extracts of droplets and leaf tissue, which contained more polar compounds than the chloroform extracts, fairly good separations were achieved in chloroform-acetic acid (9:1, v/v) and chloroform-ethyl acetate-acetic acid (35:55:10, v/v). In both solvent systems, the relative polarities of camalexin, indole-3-carboxaldehyde, and indole-3-carboxylic acid were reversed from what was observed in chloroform-methanol (19:1, v/v; see Table 5). All 3 standards were well separated. Ethyl acetate-methanol (24:1, v/v) resolved the standards poorly: indole-3-carboxaldehyde and camalexin had nearly-identical R_f values. Chloroform-ethyl acetate-formic acid (35:55:10, v/v) yielded many bands, but as this solvent system can break indole rings (M. Zook, pers. comm.), those bands may have represented solvent-induced artefacts.

The most-commonly used solvent in the subsequent experiments was chloroform-methanol (9:1, v/v) because the migration of camalexin and the other standards was similar to what was seen for leaf tissue extracts in chloroform-methanol (19:1, v/v).

Preliminary time courses of camalexin accumulation in wild-type Arabidopsis.

The other purpose of the time courses just described was to establish the optimum time of camalexin accumulation in *C. carbonum*-inoculated leaves, in order to know when to extract leaves in the future studies of camalexin accumulation in *C. carbonum*- and *Psm*-inoculated leaves. Figure 12, and tables 6 and 7, demonstrate that the kinetics of camalexin accumulation were quite variable. In one experiment (Figure 12A), camalexin concentrations increased over 100 % between 24 and 48 hours after inoculation, and in the second experiment (Figure 12B), concentrations reached a maximum at 24 hours and then decreased about 33 % by 72 hours. In both experiments, however, camalexin concentrations at all time points were high enough to be easily detected by HPLC. About 5 % to 10 % of a sample eluted from a TLC plate (1.25 % to 5 % of the original extract) gave large peaks at a sensitivity of 0.25 absorbance units full scale (AUFS). Therefore, any time from 12-72 hours post-inoculation was suitable to extract leaves in a time course. Since camalexin concentrations in *Psm* ES4326-inoculated leaves peaked at about 40 hours and remained fairly high through 72 hours (Glazebrook and Ausubel, 1994), it was possible to compare the kinetics of camalexin accumulation within a time frame of 24 to 72 hours. Also, for about the same amount of work required to prepare a single 45-leaf sample, it would be possible to prepare three 15-leaf samples, which would yield

Table 6. Time course of camalexin accumulation in wild-type (wt) leaves inoculated with *Cochliobolus carbonum* (inoc) or water (ctrl), 8/25/95-8/27/95. Leaves of *pad2* were extracted at one timepoint to compare camalexin concentrations to those of wild-type. The inoculum concentration was 6.9×10^5 spores/ml, and 45 leaves per sample were used for wild-type samples. The *pad2* samples consisted of 20 leaves each. Standard errors are not shown because the samples were not replicated.

hours after inoculation	µg camalexin in leaves	µg camalexin in droplets	total µg camalexin in sample	µg camalexin per leaf
12-wt ctrl	0	0.54	0.54	0.012
12-wt inoc	14.0	21.9	35.9	0.798
24-wt ctrl	0	0.079	0.079	0.0018
24-wt inoc	11.6	24.6	36.2	0.805
48-wt ctrl	1.8	0.81	2.6	0.058
48-wt inoc	24.5	47.0	71.5	1.59
12-*pad2* ctrl	0.19	0.064	0.25	0.012
12-*pad2* inoc	0.870	1.39	2.26	0.113

Table 7. Time course of camalexin accumulation in wild-type leaves inoculated with *C. carbonum*, 10/17/95-10/20/95. Abbreviations are as for Table 6. Leaves were inoculated with $6x10^5$ spores/ml, and 30 leaves per sample were used.

sample	µg camalexin in leaves	µg camalexin in droplets	total µg camalexin	µg camalexin per leaf
24-wt-ctrl	0	0.26	0.26	0.0088
24 -wt-inoc	13.5	23.0	36.5	1.22
36- wt-ctrl	0	0.28	0.28	0.0093
36- wt-inoc	12.5	18.9	31.4	1.05
48- wt-ctrl	0.12	0.23	0.36	0.012
48- wt-inoc	17.1	13.9	31.0	1.03
72-wt-ctrl	1.0	0	1.0	0.034
wt-inoc 72	14.9	9.41	24.3	0.810

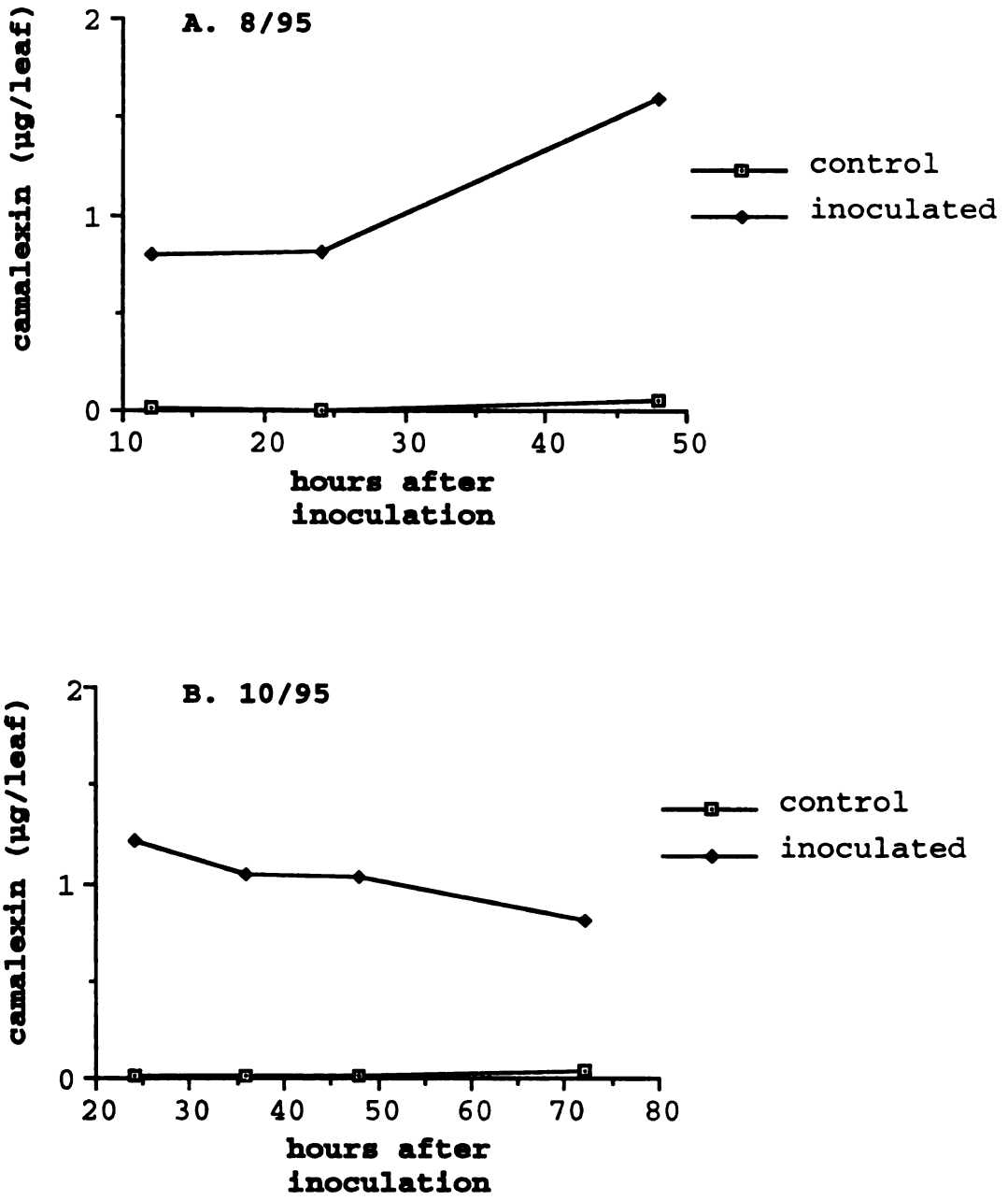


Figure 12. Time course of camalexin accumulation in wild-type leaves inoculated with *Cochliobolus carbonum* or water (control), 8/25/95-8/27/95 (A) and 10/17/95-10/20/95 (B).

measurable amounts of camalexin and provide the statistical reliability of replication.

Some camalexin was present in the controls (Tables 6 and 7). It was barely detectable in 45-leaf samples, which explains why no camalexin had been detected in the 6- to 15-leaf samples used in the past, or by fluorimetric analysis of pairs of leaves (Table 3). It may be that inoculation with water creates a certain amount of stress, as no camalexin was detected in one experiment in which 60 non-inoculated leaves were extracted 54 hours after being excised (data not shown).

Interestingly, the amount of camalexin in spore droplets was sometimes higher than the amount in leaves (Tables 6 and 7). This result was noted again in the dose-response experiments described in the next section.

Effects of *C. carbonum* inoculum concentration on camalexin production (dose-response studies).

At $2x10^4$ spores of *C. carbonum* per ml, little or no camalexin was produced (Tables 8 and 9, Figures 13 and 14). The maximum amount of camalexin was produced following inoculation with $2x10^5$ spores/ml in one experiment (Figure 13, Table 8) and with $2x10^6$ spores/ml in the other experiment (Figure 14, Table 9). The yields of camalexin at both inoculum concentrations were high enough to be detected easily by HPLC and differed by only 20 %. Apparently, camalexin production approaches or attains a saturation point

Table 8. Effects of spore inoculum concentration on camalexin production, 11/3/95. Numbers represent means plus standard errors of 3 replicates, consisting of 15 leaves each.

inoculum concentration (spores/ml)	camalexin (µg/leaf) in leaves	camalexin (µg/leaf) in droplets	camalexin (µg/leaf) in leaves and droplets combined
0 (water-inoculated control)	0.014±0.004	0	0.014±0.004
$2.1x10^4$	0.003±0.003	0.019±0.003	0.022±0.005
$2.1x10^5$	0.243±0.003	0.602±0.054	0.845±0.056
$2.1x10^6$	0.567±0.018	0.116±0.012	0.683±0.029

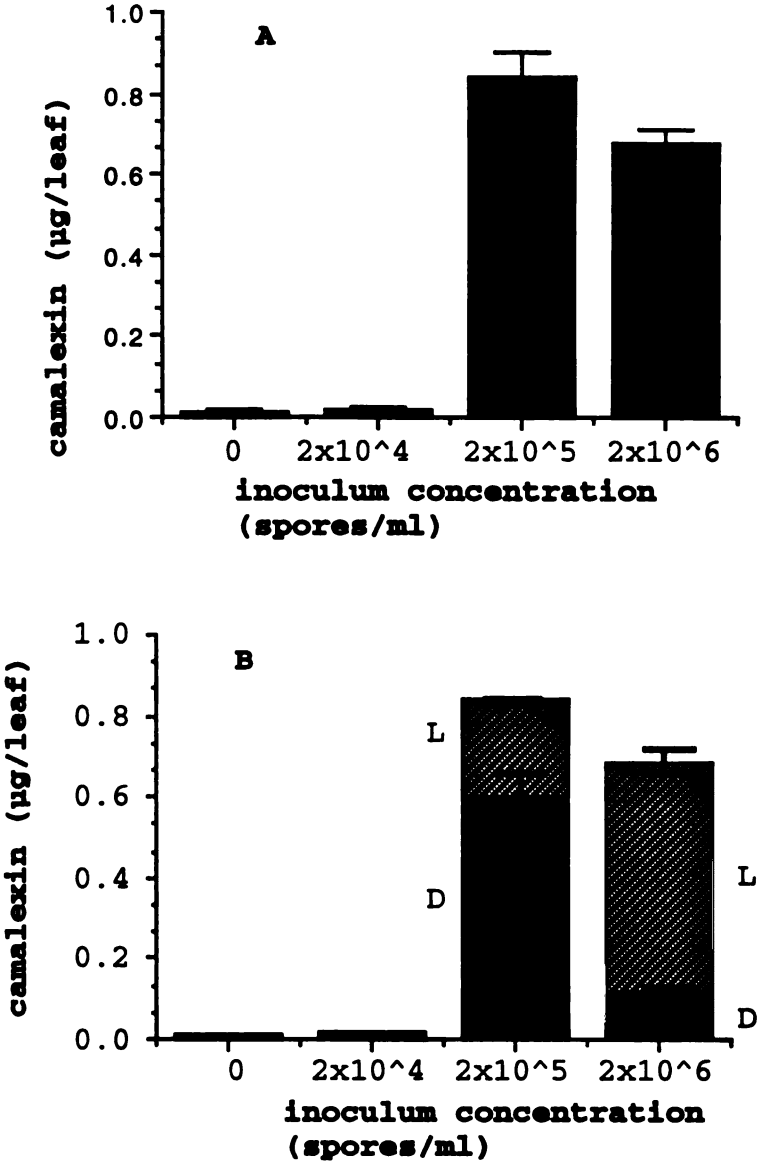


Figure 13. Effect of concentration of *Cochliobolus carbonum* spores on camalexin production, 11/3/95 (see also Table 8). Standard error bars are shown. Leaves of wild-type Arabidopsis were inoculated with 12-day-old spores at the concentrations indicated on the horizontal axis, and inoculum droplets and leaves were collected 40 hours after inoculation and extracted separately. Graph A displays the total camalexin in leaves and droplets, and graph B displays the relative contributions of leaves (L) and droplets (D) to the total.

Table 9. Effects of spore inoculum concentration on camalexin production, 11/13/95. Numbers represent means plus standard errors of 3 replicates (15 leaves each), except that only 2 samples with $2.5x10^4$ spores/ml were extracted.

inoculum concentration (spores/ml)	camalexin (µg/leaf) in leaves	camalexin (µg/leaf) in droplets	camalexin (µg/leaf) in leaves and droplets combined
0 (water-inoculated control)	0	0	0
$2.5x10^4$	0	0.0020±0.0006	0.0020±0.0006
$2.5x10^5$	0.411±0.004	0.743±0.324	1.15±0.33
$2.5x10^6$	0.896±0.044	0.533±0.338	1.43±0.35

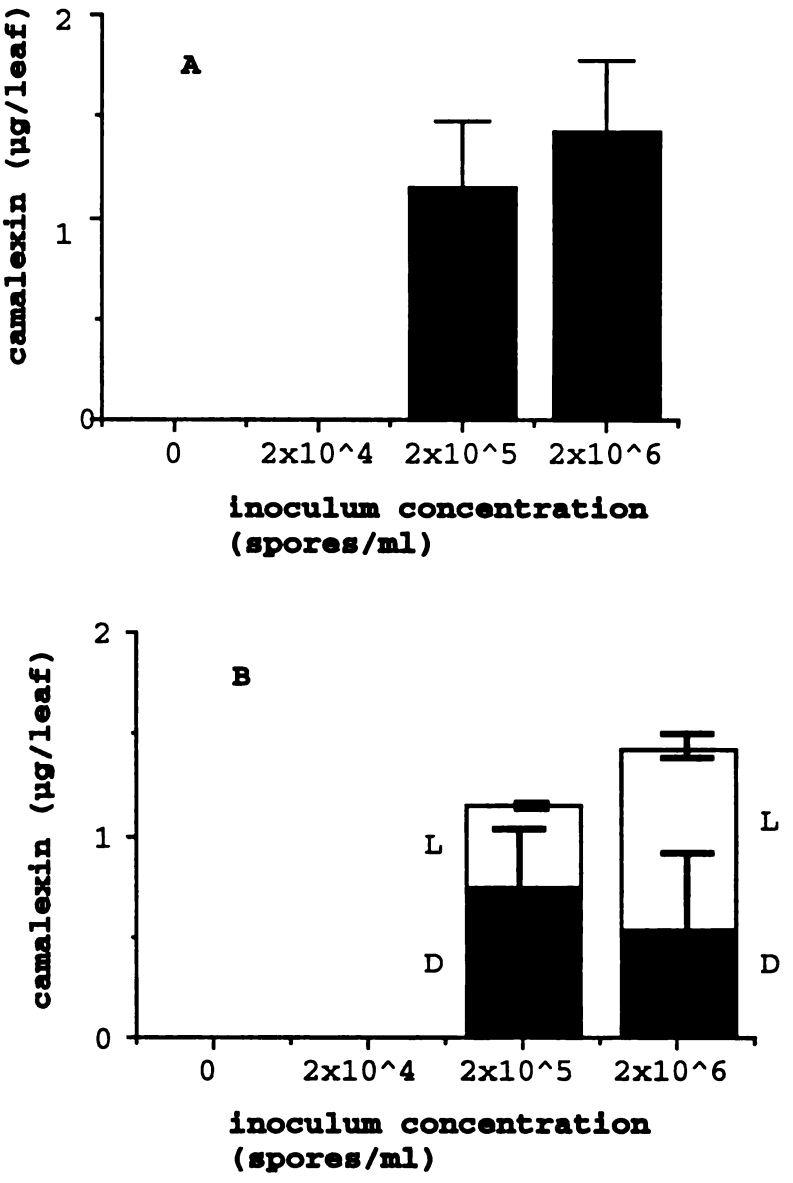


Figure 14. Effect of concentration of *C. carbonum* spores on camalexin production, 11/13/95 (see also Table 9). Graphs A and B are labeled as in Figure 13. Experimental procedures were identical to those described for Figure 13. Standard error bars are shown.

between 10^5 and 10^6 spores/ml of inoculum. A concentration of $1\text{-}2x10^5$ spores/ml was used in subsequent experiments, as it provided the most camalexin for the least work. Spores obtained from 1 or 2 plates of *C. carbonum* cultures had a concentration of about $2x10^5$ spores/ml, while obtaining a concentration of $2x10^6$ spores/ml required using more plates and often centrifuging to concentrate the spores.

Although the total amount of camalexin produced was similar in response to 10^5 and 10^6 spores/ml, the relative amount in leaves and inoculum droplets was very different. At 10^5 spores/ml, 29 % to 36 % of the camalexin was in the leaves, while 67 % to 71 % was in the droplets (Tables 8 and 9; Figures 13 and 14). At 10^6 spores/ml, 63 % to 83 % of the camalexin was in the leaves, and only 17 % to 37 % was in the droplets. The reason for this inoculum-dependent partitioning of camalexin was not investigated. As mentioned in Chapter 2 (see Discussion), it may be that camalexin binds nonspecifically to spores and that at higher spore concentrations with many spores available for binding, less diffuses into the inoculum droplets.

Effects of *C. carbonum* and *Pseudomonas syringae* pv. *maculicola* (*Psm* ES4326) on camalexin accumulation in wild-type and *pad2* Arabidopsis.

In 3 time courses, *Psm*-inoculated *pad2* plants accumulated 13-21 % as much camalexin as *Psm*-inoculated

wild-type plants (Tables 10-12, Figures 15-17). These results agree fairly well with the results of other workers that *pad2* accumulates about 10 % of wild-type levels of camalexin (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). The different values may reflect differences in methods of quantitation.

In *pad2* leaves inoculated with *C. carbonum*, maximum levels of camalexin were 7 % to 12 % of wild-type maximum levels in 2 experiments (Tables 10 and 11, Figures 15 and 16), indicating that this mutant responded similarly to *Psm* ES4326 and *C. carbonum*. In the third time course, *pad2* leaves accumulated 85 % as much camalexin as wild-type leaves (Table 12, Figure 17). Since the maximum amount produced by *pad2* in this case was less than the amount produced by *Psm*-inoculated *pad2* in the previous time course (Table 11, Figure 16), *pad2* did not accumulate unusually high amounts of camalexin on this occasion. Rather, the wild-type accumulated unusually low amounts.

In wild-type leaves, camalexin accumulated to higher concentrations in response to *Psm* ES4326 than in response to *C. carbonum* (Tables 10-12, Figures 15-17). This difference may reflect a difference in response to compatible and incompatible pathogens, since a similar trend has been observed in other plant-pathogen systems (Hahn et al., 1985; Keen and Kennedy, 1974; Storck and Sacristan, 1994). The *pad2* leaves also tended to accumulate more camalexin in response to *Psm* than in response to *C. carbonum*, with one

Table 10. Time course of camalexin accumulation in wild-type and *pad2* leaves inoculated with *C. carbonum* or *Pseudomonas syringae* pv. *maculicola* strain ES4326 (*Psm* ES4326), 12/31/95-1/3/96. Leaves and droplets of the *C. carbonum*-inoculated plants were extracted separately, and the eluates from TLC plates were combined for HPLC analysis. Not all of the *C. carbonum* leaf extract was used; therefore, results for *C. carbonum* are an underestimate of the total amount of camalexin in the samples. Results for *C. carbonum*-inoculated samples, except for the 72-hour *pad2* samples, are from 1 replicate, and so standard errors are not shown. Results for *Psm* ES4326-inoculated samples represent means plus standard errors of 2 replicates. Inoculum concentrations were 1.4×10^5 spores/ml for *C. carbonum*, and 5.8×10^6 cfu/ml for *Psm* ES4326. Controls (ctrl) for the *C. carbonum* treatments consisted of water-inoculated leaves; controls for the *Psm* treatments consisted of leaves infiltrated with 10 mM magnesium sulfate.

hours after inoculation	Camalexin produced in response to *C. carbonum* (µg/leaf)		Camalexin produced in response to *Psm* ES4326 (µg/leaf)	
	wild-type	*pad2*	wild-type	*pad2*
24	0.320	0.019	0.0992 ±0.0328	0.0096 ±0.0054
48	0.292	0.040	0.637 ±0.115	0.114 ±0.039
72	0.206	0.004 ±0.004	0.892 ±0.064	0.072 ±0.022
72-ctrl	0.015	sample lost	0.007	0

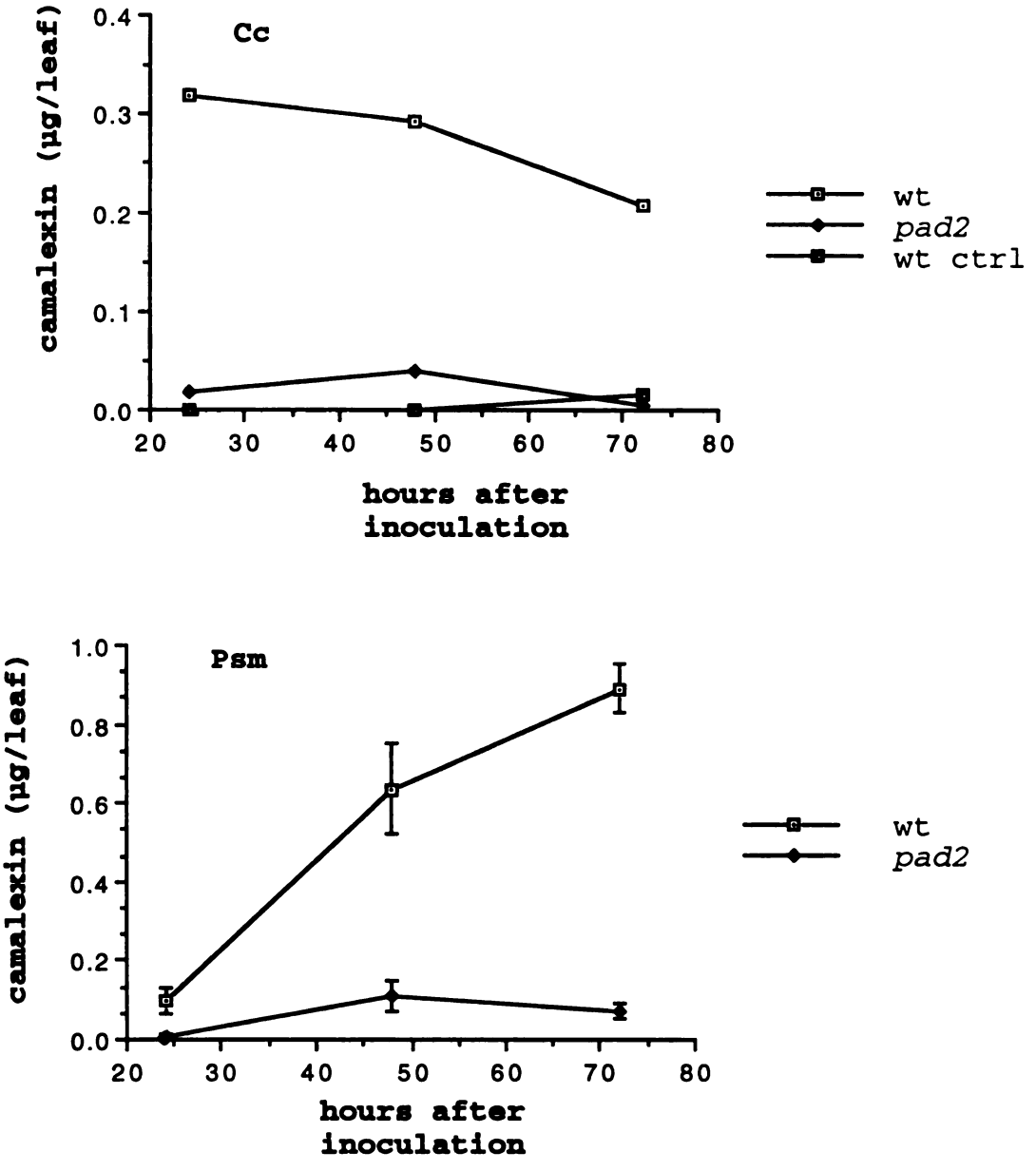


Figure 15. Time course of camalexin accumulation in wild-type (wt) and *pad2* leaves inoculated with *C. carbonum* (Cc, top graph), water (ctrl), or *Pseudomonas syringae* pv. *maculicola* strain ES4326 (Psm, bottom graph), 12/31/95-1/3/96. No camalexin was detected in controls for *Psm*-inoculated samples. Standard error bars are shown for *Psm*-inoculated samples. See Table 10 for data.

Table 11. Time course of camalexin accumulation in wild-type and *pad2* leaves inoculated with *C. carbonum* or *Psm* ES4326, 1/20/96-1/23/96. Leaves and droplets of the *C. carbonum*-inoculated plants were combined for extraction. Numbers represent means plus standard errors of 3 replicates except for the controls (ctrl), which were prepared as described in Table 10 and consisted of 1 sample each. Inoculum concentrations were $2x10^5$ spores/ml for *C. carbonum*, and $1.1x10^7$ cfu/ml for *Psm* ES4326.

hours after inoculation	Camalexin produced in response to *C. carbonum* (µg/leaf)		Camalexin produced in response to *Psm* ES4326 (µg/leaf)	
	wild-type	*pad2*	wild-type	*pad2*
0	0.002	0	0	0
24	0.454 ±0.009	0.040 ±0.012	0.056 ±0.001	0.0062 ±0.0005
48	0.589 ±0.098	0.039 ±0.006	0.597 ±0.078	0.16 ±0.03
72	0.350 ±0.043	0.032 ±0.008	0.763 ±0.058	0.066 ± 0.011
72-ctrl	0	0	0	0

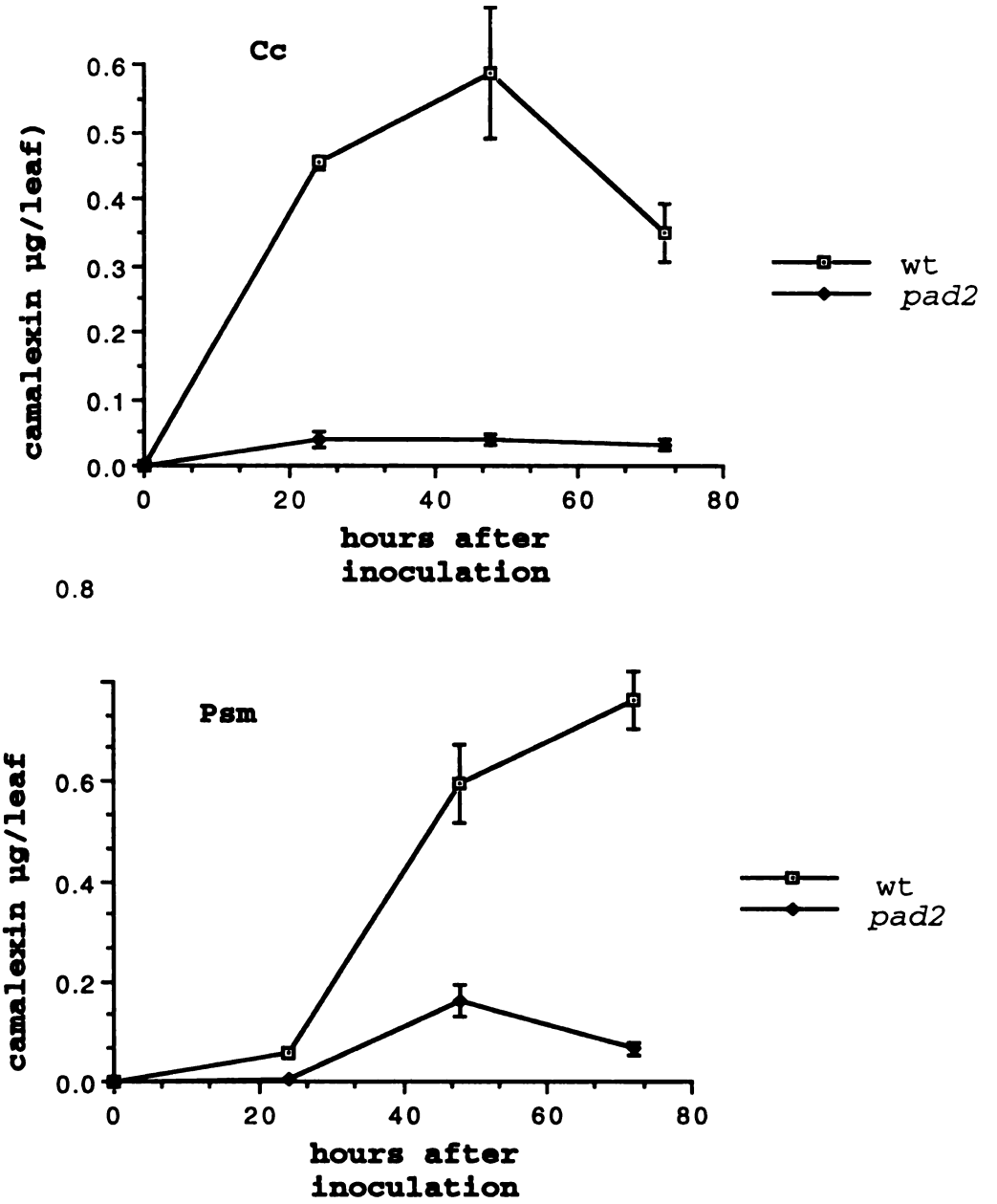


Figure 16. Time course of camalexin accumulation in wild-type and *pad2* leaves inoculated with *C. carbonum* (top graph) or *Psm* ES4326 (bottom graph), 1/20/96-1/23/96 (see Table 11 for data). Abbreviations are as in Figure 15. Controls contained no detectable camalexin. Standard error bars are shown.

Table 12. Time course of camalexin accumulation in wild-type and *pad2* leaves inoculated with *C. carbonum* or *Psm* ES4326, 4/5/96-4/8/96. Numbers represent means plus standard errors of 3 replicates for the *C. carbonum*-inoculated samples, and 2 replicates for the *Psm*-inoculated samples. Inoculum concentrations were 2.0×10^5 spores/ml for *C. carbonum* and 1.2×10^7 cfu/ml for *Psm* ES4326. Controls (ctrl) were prepared as described for Table 10.

hours after inoculation	Camalexin produced in response to *C. carbonum* (µg/leaf)		Camalexin produced in response to *Psm* ES4326 (µg/leaf)	
	wild-type	*pad2*	wild-type	*pad2*
0	0	0	0	0
24	0.072±0.008	0.137±0.017	0.002±0.002	0.064±0.063
48	0.16±0.06	0.093±0.020	0.37±0.08	0.026±0.001
72	0.12±0.05	0.077±0.017	0.36±0.22	0.029±0.003
72-ctrl	0	0	0	0

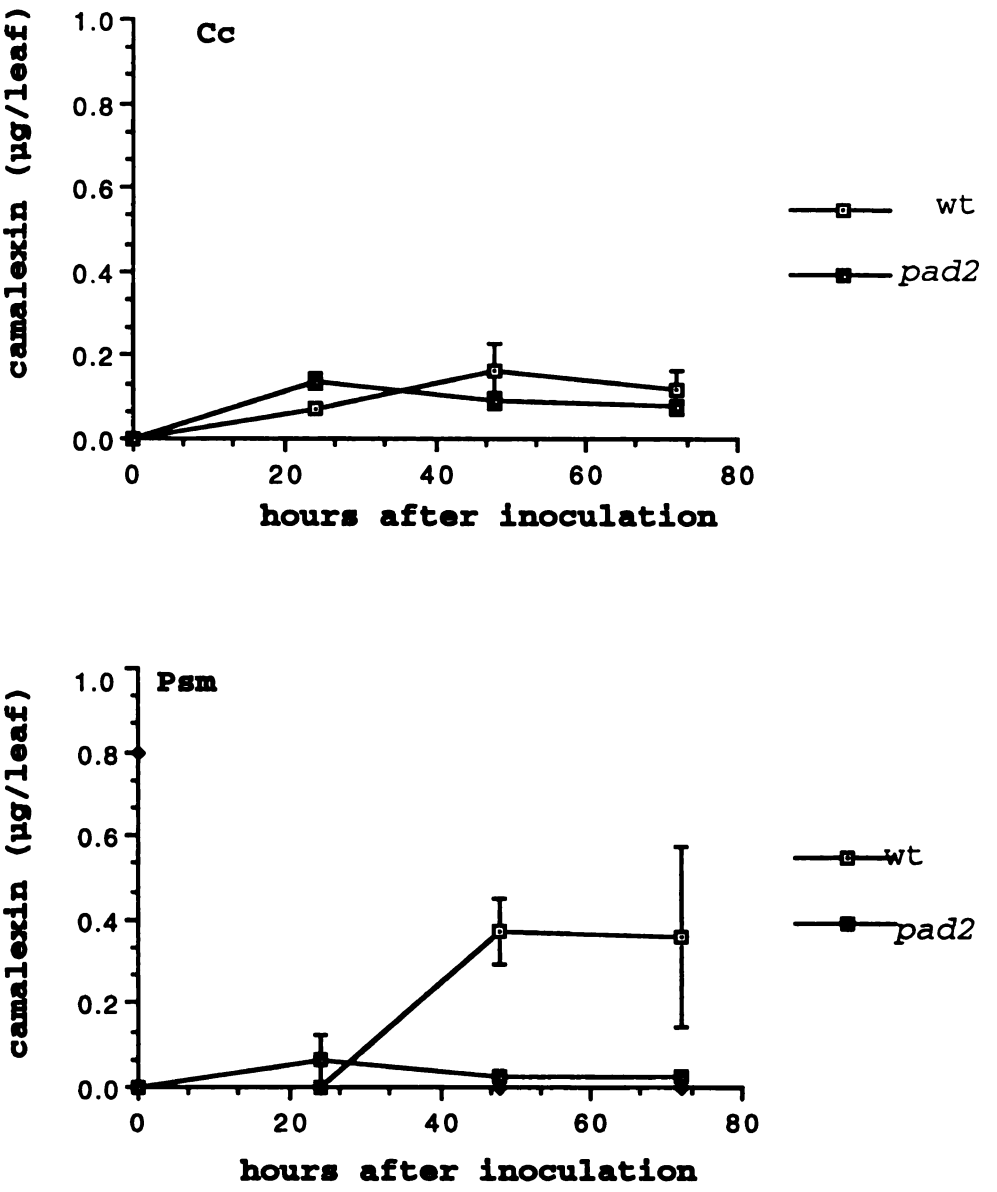


Figure 17. Time course of camalexin accumulation in wild-type and *pad2* leaves inoculated with *C. carbonum* (top graph) or *Psm* ES4236 (bottom graph), 4/7/96-4/10/96 (see Table 12 for data). Abbreviations are as in Figure 15. Water-inoculated controls contained no detectable camalexin. Standard error bars are shown.

exception (Table 12, Figure 17).

The maximum amount of camalexin accumulated, for *Psm*-inoculated leaves, ranged from 0.37-0.89 µg/leaf (Tables 10-12). These numbers correspond to 35-53 µg per gram of fresh weight (gfw), which is within almost the same range (22-50 µg/gfw) as those determined in similar experiments by Glazebrook et al. (1997). The fact that the numbers are lower than those determined fluorimetrically by Zhao and Last (1996) (70-80 µg/gfw) may, again, reflect differences in method of quantitation.

Some patterns in the kinetics of camalexin accumulation were observed. In general, camalexin accumulated more rapidly in the *C. carbonum*- than in the *Psm*-inoculated leaves (Tables 10 and 11, Figures 15 and 16). Twenty-four hours after inoculation, concentrations in *C. carbonum*-inoculated leaves were at or near their maximum, whereas they were barely detectable in *Psm*-inoculated leaves until 48 hours. This pattern was not invariable, and the kinetics of accumulation in wild-type and *pad2* leaves sometimes differed. There was a case in which *C. carbonum*-inoculated wild-type leaves produced very little camalexin until 48 hours after inoculation (Figure 17). In contrast, *pad2* concentrations peaked at 24 hours, so that *pad2* appeared to produce more camalexin than wild-type. This variability in kinetics underlined the need to evaluate camalexin production at several timepoints.

Effects of *C. carbonum* and *Pseudomonas syringae* pv. *syringae* on camalexin accumulation in wild-type and *pad2* Arabidopsis.

Very little camalexin was produced in response to *Pss* D20 in 1 time course (Table 13, Figure 18) and none was produced in response to *Pss* D20 in another time course (Table 14, Figure 19). This result was completely unexpected because *Pss* D20 had been found by Tsuji et al. (1992) to elicit high concentrations of camalexin.

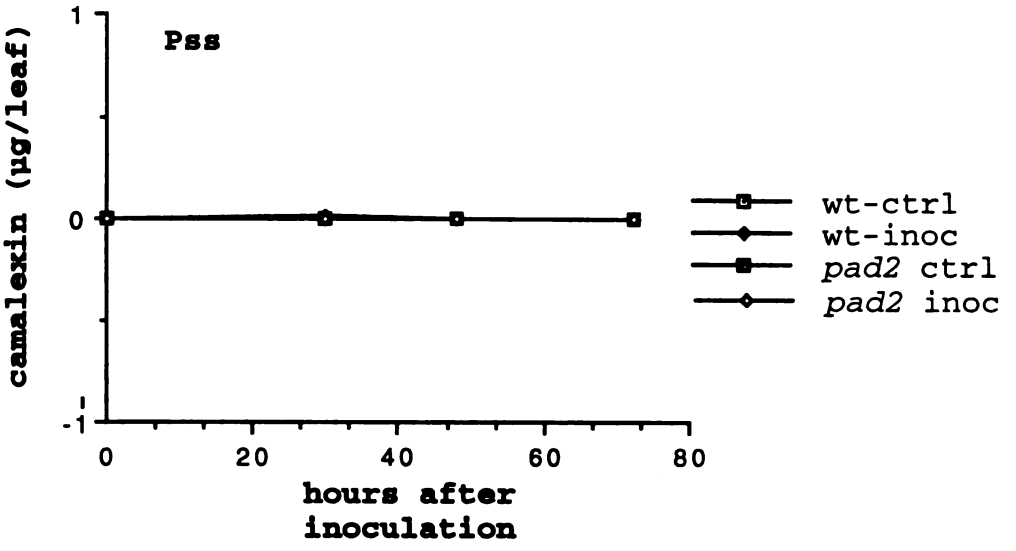
In *C. carbonum*-inoculated leaves, patterns of camalexin accumulation were similar to those observed for *C. carbonum*-inoculated leaves in the studies with *Psm* ES4326. Wild-type leaves accumulated more camalexin than *pad2* leaves (Tables 13 and 14, Figures 18 and 19), and concentrations were appreciably high 24 to 30 hours after inoculation. The concentration of camalexin in wild-type decreased dramatically in one experiment (Figure 19), which could raise questions about its metabolic fate.

Discussion

In summary, these time courses and dose-response experiments helped to clarify some biological aspects of camalexin accumulation, and to explain some of the ambiguous results of the mutant screen and radiolabeling studies described in Chapter 2. The inoculum-dependent partitioning of camalexin between leaves and inoculum droplets at different spore

Table 13. Time course of camalexin accumulation in wild-type and *pad2* leaves inoculated (inoc) with *C. carbonum* or *Pseudomonas syringae* pv. *syringae* (*Pss*) strain D20, 7/23/97-7/26/97. Numbers represent means plus standard errors of 3 replicates, except for the 24- and 72-hour *C. carbonum*-inoculated samples,which consisted of 2 replicates. Inoculum concentrations were 2.0×10^5 spores/ml for *C. carbonum* and 1.2×10^7 cfu/ml for *Pss* D20. Controls (ctrl) were prepared as described for Table 10.

hours after inoculation	Camalexin produced in response to *C. carbonum* (µg/leaf)		Camalexin produced in response to *Pss* D20 (µg/leaf)	
	wild-type	*pad2*	wild-type	*pad2*
0	0	0	0	0
24 ctrl	0.0001 ±0.0001	0.0001 ±0.0001	0	0
24 inoc	0.387 ±0.031	0.0606 ±0.0067	0.0068 ±0.0008	0.0005 ±0.0005
48 inoc	0.453 ±0.051	0.0460 ±0.0101	0.0023 ±0.0021	0
72 ctrl	0.0001 ±0.0001	0	0	0
72 inoc	0.557 ±0.095	0.0418 ±0.018	0	0



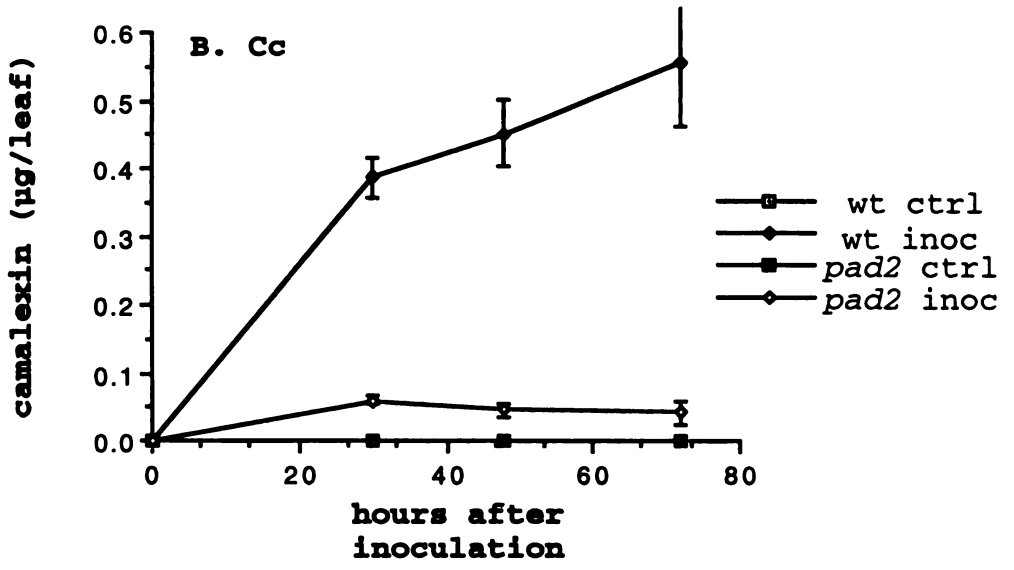


Figure 18. Camalexin accumulation in wild-type (wt) and *pad2* leaves inoculated (inoc) with *Pseudomonas syringae* pv. *syringae*, (Pss, top graph) or *Cochliobolus carbonum* (Cc, bottom graph) 7/23/97-7/26/97 (see Table 13 for data). Standard error bars are shown. Controls (ctrl) were prepared as described for Table 10.

Table 14. Time course of camalexin accumulation in wild-type and *pad2* leaves inoculated (inoc) with *C. carbonum* or *Pss* D20, 9/21/97-9/25/97. Numbers represent means plus standard errors of 3 replicates. Inoculum concentrations were $2.0x10^5$ spores/ml for *C. carbonum* and $1.1x10^6$ cfu/ml for *Pss* D20. Controls (ctrl) were prepared as described for Table 10.

hours after inoculation	Camalexin produced in response to *C. carbonum* (µg/leaf)		Camalexin produced in response to *Pss* D20 (µg/leaf)	
	wild-type	*pad2*	wild-type	*pad2*
0	0	0	0	0
24 ctrl	0	0	0	0
24 inoc	0.280 ±0.030	0.0374 ±0.0101	0	0
48 inoc	0.111 ±0.011	0.0239± 0.0096	0	0.0008 ±0.0008
72 ctrl	0	0	0	0.0002 ±0.0002
72 inoc	0.117 ±0.0370	0.0162 ±0.00741	0	0

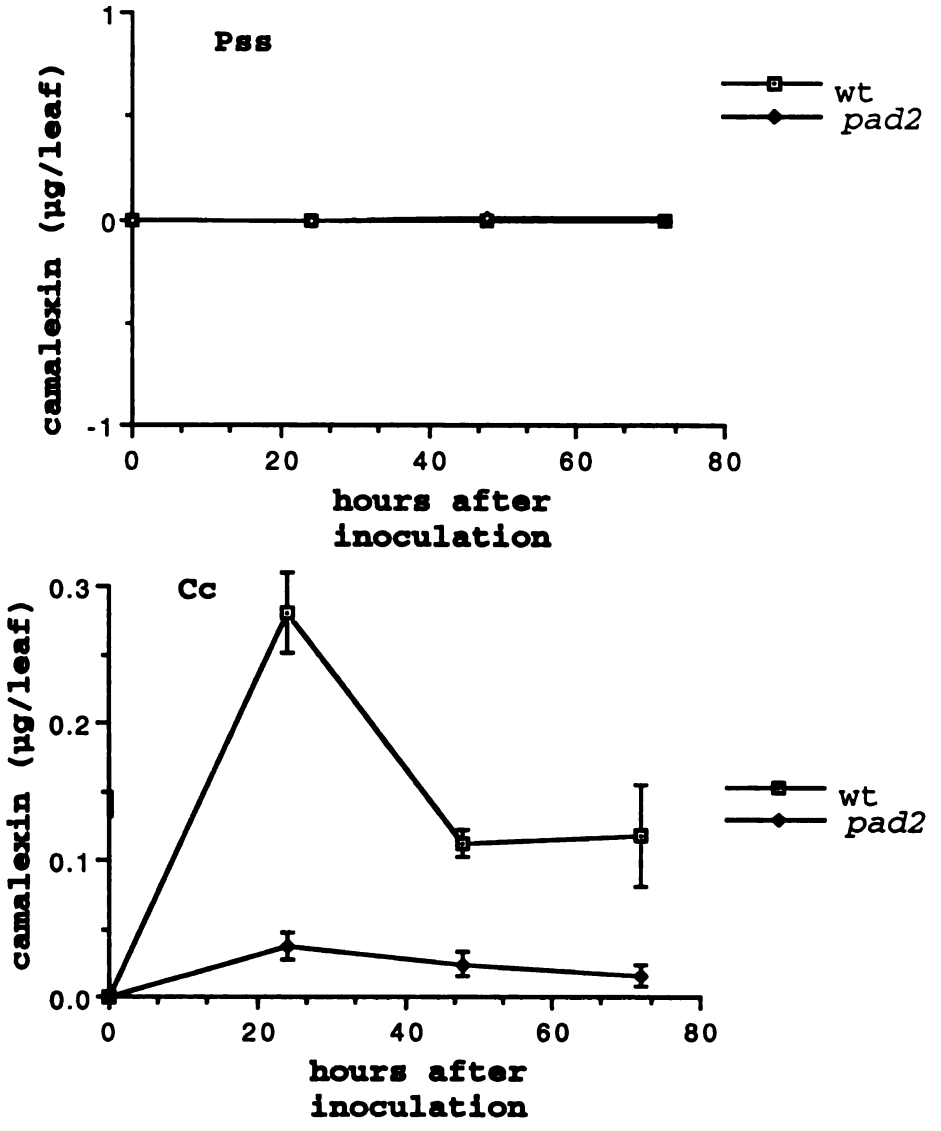


Figure 19. Time course of camalexin accumulation in wild-type and *pad2* leaves inoculated with *Pss* D20 (top graph) or *C. carbonum* (bottom graph), 9/21/97 -9/24/97 (see Table 14 for data). Abbreviations are as in Figure 18. No camalexin was detectable in controls. Standard error bars are shown.

concentrations provided a possible explanation for why some putative camalexin-deficient mutants proved not to be mutants when retested. If the initial inoculum concentration was near 10^6 spores/ml, the majority of the camalexin would have remained in the leaves, making relative fluorescence intensities of the droplets misleadingly low. These results could explain in part the variable results of the radiolabeling studies, since leaves were fed ^{14}C-anthranilate after being inoculated, and the inoculum droplet was removed. In general, the spore concentration was high enough that most of the camalexin probably remained in the leaf. However, the low incorporation of anthranilate into camalexin on some occasions may have been partly the result of low amounts of camalexin in the leaf. These dose-response analyses demonstrated that to trust the results of only droplets or leaves, inoculum concentration had to be standardized. Had this been known at the time of the mutant screen, some false leads might have been avoided.

The variability in the kinetics of camalexin accumulation demonstrated the need to evaluate camalexin production at several timepoints in order to determine whether a plant was camalexin-deficient. Had camalexin in *C. carbonum*-inoculated leaves been measured only 24 hours post-inoculation in the experiment depicted in Figure 17, *pad2* would have seemed to be an overproducer, and the wild-type would have appeared camalexin-deficient. As discussed in Chapter 2, this variability in kinetics may explain the

results of Zhao and Last (1996), who found that the *trp1-100* mutant, which was found by Tsuji et al. (1993) to be camalexin-deficient when extracted 18 hours after elicitation with silver nitrate, produced wild-type amounts of camalexin 24 hours after inoculation with *Psm* ES4326 (Zhao and Last, 1996). The difference in results may be due to differences in response to biotic and abiotic elicitors of phytoalexin synthesis, but it may also be that the silver nitrate-elicited leaves would have accumulated more camalexin over a longer incubation period.

The time courses comparing the responses of wild-type and *pad2* to different pathogens confirmed that *pad2* responded similarly to *C. carbonum* and *Psm* ES4326. Whereas *pad4*, as explained in Chapter 2, is a signal transduction mutant affected in the ability to trigger salicylic acid production (Glazebrook et al., 1997; Zhou et al., 1998), *pad2* may be a biosynthetic mutant, albeit a somewhat leaky one (Figure 17).

The tendency for camalexin to accumulate more rapidly in response to *C. carbonum* may again be due to differences in response to compatible and incompatible pathogens, rather than differences in response to fungal and bacterial pathogens. It was hoped that by directly comparing the kinetics of accumulation in *C. carbonum* and *Pss*, an incompatible bacterial pathogen, it would be possible to determine whether the plants responded similarly to incompatible fungal and bacterial pathogens, or whether they responded differently to fungal and bacterial infection. The

lack of camalexin production in response to *Pss* prevented making such comparisons. However, studies by Zhou et al. (1998) with *Psm* ES4326 transformed with the avirulence gene *avrRpt2* demonstrated that camalexin did accumulate more rapidly in response to this incompatible bacterial pathogen than in response to the compatible one. In light of those results, it seems likely that the more rapid accumulation of camalexin in response to *C. carbonum* was a response to an incompatible pathogen.

The lack of camalexin production in response to *Pss* D20 may reflect the problem of using a low inoculum concentration (10^6-10^7 cfu/ml, compared to 10^8 used by Tsuji et al. [1992]). No sign of a hypersensitive response (HR) was seen on the inoculated leaves, although there was some yellowing. It may be that when the inoculum concentration is too low to cause visible necrosis, no camalexin is produced. The inoculum concentration was kept low because in trial extractions with leaves inoculated with Pss cells diluted to an OD_{600} of 0.1 or 0.2 (five- to tenfold greater concentration than what was used in these experiments), very little camalexin was detected. It was thought at that time that the leaves had mounted such a rapid HR (demonstrated by large necrotic spots), that the cells died before they could produce camalexin, and the spread of the pathogen was stopped before more leaf cells could respond with phytoalexin synthesis. A lower inoculum concentration was hopefully a way to

circumvent the problem associated with a rapid HR. Perhaps a visible HR of some intermediate magnitude was necessary for camalexin production. It is also possible that camalexin does not play an important role in resistance to *Pss*.

This part of the project demonstrated that camalexin deficiency, and discrepancies in the results of inoculation with pathogens, could be due to many factors besides regulatory mutations. It also provided, via TLC analysis, evidence for inducible compounds which led to the study described in the next chapter.

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Chapter 4. Isolation of Indole-3-Carboxaldehyde from Arabidopsis Leaves Inoculated with *Cochliobolus carbonum*, and Exploration of its Possible Role as an Intermediate in Camalexin Biosynthesis

Introduction

The time courses described in Chapter 3 had a dual purpose: to look for differences in camalexin production in response to fungal or bacterial infection, and to look for camalexin biosynthetic intermediates. When thin-layer chromatograms were examined under long-wave ultraviolet (UV) light to locate camalexin bands, other purple- or blue-fluorescent bands were also observed (Chapter 3, Table 4). Since these bands were present in extracts of *C. carbonum*- and *Psm* ES4326-inoculated leaves but not in the mock-inoculated controls, they were potential biosynthetic intermediates. One attempt was made to characterize one inducible compound that was present in extracts of *C. carbonum*-inoculated leaves and had an R_f value slightly less than that of camalexin. When this compound was eluted from several TLC plates and analyzed by HPLC, no peaks of noteworthy size were detected. Sample degradation on TLC plates was not a likely reason since preliminary TLC of the eluates had confirmed that the R_f values were unchanged. However, as the compound was obtained from several samples,

each equivalent to about 7 leaves, it was probable that more sample was needed to characterize the compound.

Consequently, fresh fungal-inoculated plant material was extracted on a larger scale. The extraction procedure described in Chapters 2 and 3 was altered because of a growing concern that if camalexin biosynthesis did involve volatile compounds like indole or aldehydes, extracting at high temperatures could lead to loss of compounds or condensations between compounds. The new extraction procedure led to the isolation of indole-3-carboxaldehyde (Figures 4 and 5) from fungal-inoculated wild-type and *pad2* leaves.

This result added support to the hypothesis that camalexin is formed by a condensation between indole-3-carboxaldehyde and cysteine (Browne et al., 1991; see also Figure 5). Because of the recent confirmation that cysteine is an intermediate (Zook and Hammerschmidt, 1997), the discovery of indole-3-carboxaldehyde in inoculated leaves provided support for this step in the pathway. Indole-3-carboxaldehyde is present in many plants as an oxidation or photolysis product of indole-3-acetic acid (IAA) and tryptophan (see Chapter 1), and so it could be a constitutive intermediate.

If indole-3-carboxaldehyde were an intermediate in camalexin biosynthesis, the concentration of indole-3-carboxaldehyde in inoculated plants was likely to increase soon after infection, reach a maximum, and then decrease as

it was converted into camalexin. Time course studies with wild-type and *pad2* leaves confirmed that the kinetics of indole-3-carboxaldehyde accumulation followed this pattern.

These time courses, however, provided only correlative evidence. Without following the fate of radioactive indole-3-carboxaldehyde in inoculated leaves, it was not possible to know whether the aldehyde was being converted into camalexin or was being used for other, unrelated compounds. Because radioactive indole-3-carboxaldehyde was not, to our knowledge, commercially available, a different approach was necessary.

One method sometimes used to determine whether a compound is a biosynthetic intermediate in a pathway, when a radiolabeled form of the compound is not available, is to feed the nonradioactive (cold) form along with a radiolabeled precursor and see whether specific incorporation (ratio of moles of product to moles of radioactive compound administered) into products of the pathway decreases (Wolf, 1964). Radiolabeling studies with the *pad* mutants (Chapter 2) had reaffirmed the findings of Tsuji et al. (1993) that ^{14}C-anthranilate was incorporated into camalexin. Incorporation of ^{14}C-anthranilate into camalexin was expected to decrease in leaves fed ^{14}C-anthranilate mixed with cold anthranilate, since the leaves would make camalexin from both the radioactive and nonradioactive molecules. If indole-3-carboxaldehyde were an intermediate between anthranilate and

camalexin, the incorporation of ^{14}C-anthranilate into camalexin should decrease even more in leaves fed ^{14}C-anthranilate mixed with cold indole-3-carboxaldehyde, since those leaves would have a more immediate precursor from which to make camalexin and should be less likely to make it from the more remote precursor. This method has been used to verify the role of putative intermediates in the biosyntheses of other compounds, including ethylene (Adams and Yang, 1979), dimethylsulfonio-propionate (DMSP) (Hanson et al., 1994) and quercetin (Watkin et al., 1957). Therefore, an attempt was made to see whether dilution of radioactive anthranilate with cold indole-3-carboxaldehyde decreased the efficiency of incorporation of anthranilate into camalexin.

Materials and Methods.

Plant material, fungal cultures, and inoculations.

Wild-type plants (Columbia-0 ecotype) and *pad2* plants were grown as described in Chapter 2. Leaves of 3- to 4-week-old seedlings were used in all experiments. Both wild-type and *pad2* plants were used for the initial searches for intermediates and kinetic studies. Only wild-type plants were used for the radiolabeling studies. *Cochliobolus carbonum* was grown as described in Chapter 2, on V-8 agar (per liter: 160 ml V-8 juice, 1 g calcium carbonate, 14 g agar). Plants were inoculated as described in Chapter 2.

Reagents and chemicals.

^{14}C-anthranilic acid (uniformly labeled on the ring), with a specific activity of 60 mCi/mmol, was purchased from ARC. Indole-3-carboxaldehyde was purchased from Sigma. Indole-3-carboxylic acid and anthranilic acid were purchased from Aldrich. The anthranilic acid was recrystallized from ethanol for the radiolabeling studies. Even after recrystallization, some material remained at the origin on TLC plates; however, the size and intensity of the material at the origin were greatly reduced.

Camalexin was purified by preparative TLC (glass-backed silica plates from Whatman, 1000 µm thickness) of a mixture obtained from Arabidopsis and from two syntheses: one done in January 1994 in the laboratory of Dr. W. Reusch at Michigan State University, and one done in May 1996 in the laboratory of Dr. J. Kagan at the University of Illinois at Chicago. Both syntheses followed the method of Ayer et al. (1992), except that in the later synthesis, tetrahydrofuran was used as a solvent instead of benzene. Some synthetic camalexin was also generously provided by Dr. Alois Fürstner of the Max-Planck-Institut für Kohlenforschung (Mülheim/Ruhr, Germany). Small amounts of these various sources of camalexin were prep TLC-purified at various stages of this project. The camalexin used in the radiolabeling study was isolated from preparative TLC plates and recrystallized from hexanes-acetone (4:1, v/v) to give a final yield of 20 mg.

Solvents used for HPLC were of HPLC grade. All other

chemicals were of reagent grade or better.

Sample size and incubation.

Confirmation of the presence of indole-3-carboxaldehyde in inoculated leaves came from three experiments. Sample size and duration of incubation varied with each experiment as information was gathered from the previous one. For the extractions that led unexpectedly to isolation of indole-3-carboxaldehyde, 78 leaves per sample were used, and leaves were extracted 55 hours after inoculation. In a later attempt to confirm the presence of indole-3-carboxaldehyde, leaves were extracted 24 to 29 hours after inoculation, to see whether the aldehyde was present when camalexin concentrations normally were increasing. About 400 *C. carbonum*-inoculated leaves (11.7 g) were used for one wild-type sample, and about 300 leaves (5.8 g) were used for one *pad2* sample. As a rough estimate of the amount of aldehyde produced without infection, water-inoculated controls of 50 wild-type and 60 *pad2* leaves were prepared. In a third experiment to compare results between replicates and to assess better the concentrations of aldehyde in control tissue, wild-type and *pad2* fungal- and water-inoculated samples, prepared in triplicate, all consisted of 75 to 98 leaves. Twenty-four hours after inoculation, droplets were collected from leaves and frozen, and leaves were frozen in liquid nitrogen and stored at -20 °C. Samples were extracted 4 days later.

The yield of indole-3-carboxaldehyde for some samples in the latter experiment was high enough that it was considered sufficient to use 40 leaves per sample for nonradioactive kinetic studies. Leaves were taken from several pots to minimize the chance that variation between pots would influence results. All samples were prepared in triplicate. Inoculum droplets and leaves were collected and frozen 3, 6, 12, 24, and 36 hours after inoculation. Water-inoculated controls were collected at 6 and 24 hours. Non-inoculated leaves were collected for extraction immediately after inoculation of the other leaves to determine aldehyde concentrations just before infection. Samples were stored dry at -20 °C up to 6 weeks prior to extraction.

For radiolabeling studies, samples (prepared in duplicate) consisted of 5 leaves each, except for two 6-leaf samples (collected before it was realized that there would not be enough leaves to use 6 per sample throughout the experiment) and four 4-leaf samples (the result of running out of leaves). Leaves were inoculated after taking up one of three radioactive solutions (see below). Samples were frozen 3,6,9 and 24 hours after inoculation. Non-inoculated zero-hour samples were frozen with as much water as would have been on inoculated leaves. Water-inoculated controls were collected and frozen at every timepoint. Samples were stored up to 3 weeks at -80 °C before being extracted.

Radiolabeling of leaves.

The accumulation of indole-3-carboxaldehyde and camalexin was followed over a 24-hour period in water- or *C. carbonum*-inoculated leaves to which one of the following solutions had been fed:

a) ^{14}C-anthranilate (1.7nmol/leaf)

b) ^{14}C-anthranilate (1.7 nmol/leaf) plus unlabeled anthranilate (17 nmol/leaf; 336 µM, added as a 4.28 mg/ml solution in ethanol)

c) ^{14}C-anthranilate (1.7 nmol/leaf) plus unlabeled indole-3-carboxaldehyde (17 nmol/leaf; 336 µM, added as a 5 mg/ml solution in dimethylsulfoxide)

A tenfold dilution of radioactive precursor seemed likely to be enough to note changes in ^{14}C-anthranilate incorporation without encouraging aberrant biosynthetic pathways due to an unexpectedly large pool of a compound, as has been documented in other cases (Leete, 1991). To determine whether the final concentrations of ethanol and dimethylsulfoxide (DMSO) were phytotoxic, cold versions of solutions a, b, and c were fed to leaves, as well as solutions containing twice those volumes of anthranilate and indole-3-carboxaldehyde. No macroscopic signs of phytotoxicity were observed.

The ^{14}C-anthranilate in all three solutions was diluted with sterile deionized water to an activity of about 0.1 µCi per 50 µl (the amount of solution fed to each leaf). The activity of each solution was determined by counting two

10-µl aliquots in 5 ml of Safety-Solve scintillation fluid on a Packard 1500 Tri-Carb scintillation counter (95 % efficiency for ^{14}C).

Feeding of the radioactive solutions was as described in Chapter 2. Leaves were inoculated following solution uptake. To ensure that the light regime was the same for each treatment, lights were left on during most of the experiment. The 3, 6, and 9-hour timepoints were constantly in the light. The 24-hour timepoints were exposed to about 18 hours of light.

Extractions.

All steps of extractions were done at temperatures below 35 °C. Leaves were extracted according to a modification of the Bligh-Dyer technique for extracting lipids (Bligh and Dyer, 1959), using the proportions of chloroform, methanol, and water determined by Kates (1972). In the kinetic studies, to compensate for smaller sample size, all volumes were doubled. For the initial aldehyde-seeking experiments, leaves were frozen in liquid nitrogen and ground with a mortar and pestle in chloroform-methanol (1:2 v/v; 1 ml chloroform/1 g tissue). Water (0.8 ml/g tissue) was added upon or after transfer to a beaker..The homogenate was vacuum-filtered through a Whatman #1 or #4 filter, and the residue was again ground with chloroform-methanol-water 1:2:0.8 and filtered. The residue in the funnel was washed with half as much chloroform-methanol 1:2 (v/v) as was used

in tissue homogenization. The filtrate was transferred to a separatory funnel, to which chloroform and water were added to give a final chloroform-methanol-water ratio of 1:1:0.9 (v/v/v). The chloroform layer was discarded. The aqueous phase was extracted with 2 equal volumes of ethyl acetate. The ethyl acetate layers were combined, dried under vacuum or nitrogen, and stored at -20 °C. In the radiolabeling study, to compensate for small sample size and keep the final volumes small, leaves were ground in 15-ml plastic centrifuge tubes with a glass rod. The second homogenization in chloroform-methanol-water was omitted as well.

Inoculum droplets, if extracted separately from leaves (as was done for the latter two aldehyde-isolation experiments and for the kinetic studies), were extracted essentially according to the procedure for inoculum droplet extraction described in Chapter 3.

For the radiolabeling study, and for the experiment in which indole-3-carboxaldehyde was first isolated, the extraction procedure was similar to the leaf tissue extraction procedure described above. The main difference was that for the radiolabeling study and the first aldehyde-isolation experiment, inoculum droplets were extracted with the leaves. Chloroform and methanol were added so that the volume ratios of chloroform, methanol, and inoculum droplets were 1:2:0.8 (v/v/v). The leaf mass was not taken into account because the mass of the droplets (0.1 g per leaf for 0.1-ml droplets) far exceeded the mass of the leaves. The

chloroform layer, instead of being discarded, was dried at 30-50 °C and saved for TLC, in addition to the ethyl acetate extract of the aqueous layer.

Identification and quantitation of camalexin and indole-3-carboxaldehyde.

Extracted samples were separated by TLC, as described in Chapter 2. The majority of the separations were done on glass-backed silica plates (Analtech) 250 µm thick. Cold samples extracted to calculate recovery of camalexin and indole-3-carboxaldehyde (see below) were separated on plastic-backed plates 200 µm thick. The plastic-backed plates were not used for the radioactive samples because separation of camalexin, indole-3-carboxaldehyde, and anthranilate was not as good as on glass-backed plates. Plates were developed in chloroform-methanol 9:1 or 19:1, with a prior development in chloroform if samples contained large amounts of pigment. Camalexin and indole-3-carboxaldehyde were visualized under UV light (see Chapter 3). Plates were photographed under long-wave and short-wave UV light (see Chapter 5).

Indole-3-carboxaldehyde was quantitated by HPLC after eluting from TLC plates with ethyl acetate, (described in Chapter 3). HPLC operating conditions were as described in Chapter 3, except that the mobile phase was isopropanol-hexane (8:92, 9:91, or 10:90 [v/v]). Composition was adjusted to minimize peak tailing, which varied with

analyses. The typical retention time of indole-3-carboxaldehyde was 13 to 14 minutes on an old column and 20 to 22 minutes on a new column. The identity of the peak was confirmed by injection of a standard and by TLC of fractions collected at the retention times of the standard. Because the aldehyde had a low solubility in the mobile phase, as the long retention times indicated, samples were dissolved not in mobile phase but in hexane-isopropanol 70:30 (v/v) or 80:20 (v/v). Aldehyde concentrations in samples were calculated by measuring the area of the triangulated peaks (Johnson and Stevenson, 1978) and calculating concentrations from a standard curve of peak area versus concentration (Figure 21).

For kinetic studies and recovery determinations, camalexin and indole-3-carboxaldehyde were eluted for HPLC as one band. The identity of each peak was verified by TLC of collected, concentrated peaks (see Chapter 3). Camalexin had a retention time of 6.5 to 8.5 minutes, depending on the day and the polarity of the mobile phase. The camalexin peak was broader in isopropanol-hexane 8:92 or 10:90 (v/v) than in isopropanol-hexane 7:93 (v/v). Consequently, camalexin was quantitated by measuring peak area and comparing to the results of the standard curve used for the *C. carbonum*/*P.s. syringae* time courses (Chapter 3, Figure 11B). The standard curve for concentration of camalexin was recalculated to quantitate camalexin based on peak area (Figure 22).

Determination of percent incorporation of ^{14}C-anthranilate into camalexin, indole-3-carboxaldehyde, and other compounds.

After separation of samples by TLC, the radioactive plates were wrapped in plastic wrap (Borden), and laid onto 8x10" X-ray film (Kodak). After 4 weeks in a drawer, plates were developed. Bands on the film were located on the TLC plates by laying plastic wrap over the film, setting the film over a light source, and tracing over the bands. The traced reproductions of the film were then laid over the TLC plates, and the bands were outlined on the plates, scraped, and counted for 2 minutes in 5 ml of Safety-Solve scintillation fluid on a Packard Tri-Carb 1500 scintillation counter (efficiency for ^{14}C: 95 %). Incorporation of anthranilic acid into those compounds was calculated by converting the number of counts per minute (cpm) to degradations per minute (dpm, determined by efficiency of counting instrument) and dividing this number by the initial dpm in the administered solutions.

Camalexin and indole-3-carboxaldehyde bands, which were visible on the back of plates illuminated by short-wave UV light, were outlined on the back of the plate with a permanent marker. The outlines made it possible, once the radioactive bands on the plate had been scraped, to see whether the putative camalexin and aldehyde bands in the autoradiogram coincided with the bands seen under UV light.

Calculation of recovery of camalexin and indole-3-carboxaldehyde.

Because aldehydes are generally unstable and readily undergo aldol condensations, the reliability of the extraction procedure was tested by extracting samples containing known amounts of indole-3-carboxaldehyde. To determine recovery of the aldehyde in the absence of plant components, trial extractions were done with aqueous solutions of indole-3-carboxaldehyde (7.5 µg per 29.7 ml, prepared by adding 30 µl of a 0.25 mg/ml stock in isopropanol to 29.7 ml of water. Trial extractions were also done with leaf tissue and with droplets spiked with 40 µl (0.4 µg) of an aldehyde solution (a 1 mg/ml stock in tetrahydrofuran that was diluted 1:100 in water).

For the radiolabeling studies, samples collected during the time course were spiked with both camalexin and indole-3-carboxaldehyde, to minimize the loss of compounds to tube walls and leaf debris. As the extraction procedures were different from what had been done for the other experiments, preliminary nonradioactive extractions were done with leaves spiked with camalexin and indole-3-carboxaldehyde, to determine the percent recovery of both compounds.

Leaves were not spiked with indole-3-carboxaldehyde if fed the solution of anthranilate diluted with aldehyde, since preliminary extractions demonstrated that such leaves contained easily-detectable amounts of aldehyde (Table 17A). Leaves not fed aldehyde were spiked with 10 µg per 5-leaf

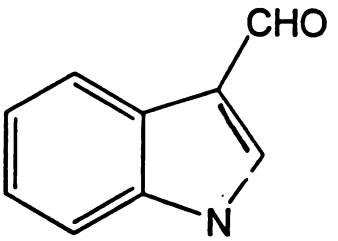
sample, added as a 0.5 mg/ml solution in ethanol (prepared from a 5 mg/ml stock in dimethylsulfoxide). Camalexin was added as a 0.56 mg/ml solution in methanol (16.8 µg/5 leaves).

Results

Isolation of indole-3-carboxaldehyde from wild-type and *pad2* leaves.

Indole-3-carboxaldehyde (molecular weight 145.0 g) was isolated on two occasions from wild-type but not *pad2* leaves, and on one occasion from inoculum droplets on *pad2* leaves. The putative aldehyde was visualized under short-wave ultraviolet light as a dark, UV light-absorbing band at the R_f of a standard. The eluted compound was purified by HPLC. The putative indole-3-carboxaldehyde peak comigrated with an aldehyde standard on TLC (chloroform-methanol 9:1, v/v). The aldehyde peak in droplets of *pad2* leaves was collected from repeated injections of sample on the HPLC (mobile phase: isopropanol-hexane 8.5:91.5, v/v), dried under nitrogen, and sent for mass spectroscopic analysis.

The mass spectrum of the putative indole-3-carboxaldehyde (Figure 20) contained the expected peak at m/z (charge-to-mass ratio)=145. The peak at m/z=144 indicated the loss of a hydrogen atom from the carbonyl group, which is characteristic of the mass spectrum of aldehydes (Hill, 1966; D. Gage, pers. comm.) The peak at m/z=116 suggested the loss



Indole-3-carboxaldehyde (M.W.=145.0g)

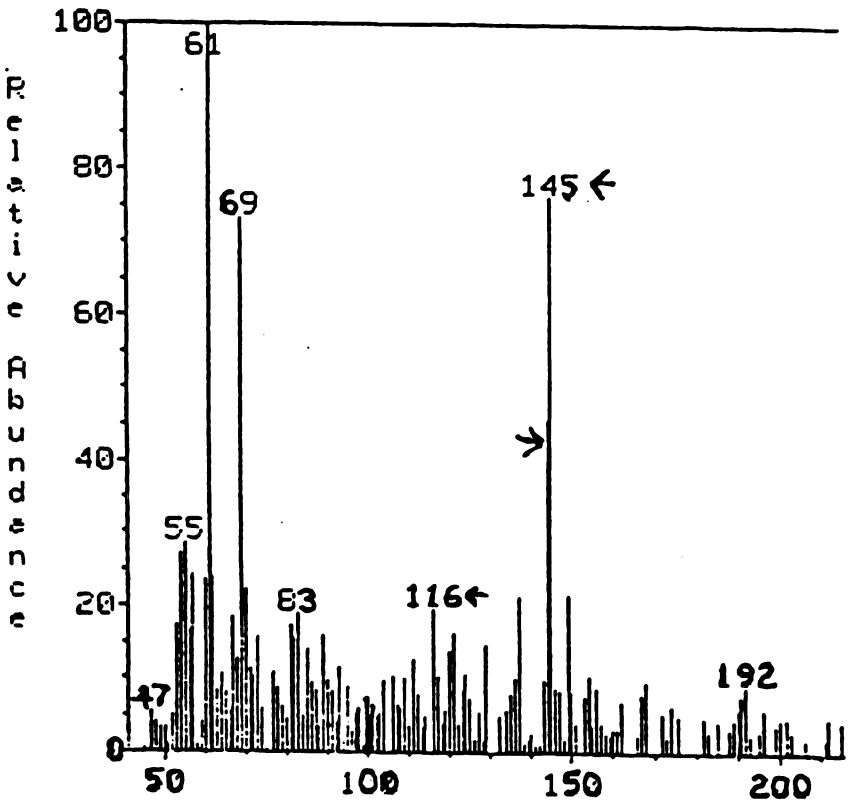


Figure 20. Mass spectrum of indole-3-carboxaldehyde isolated from pad2 inoculated with C. carbonum. Arrows denote characteristic peaks.

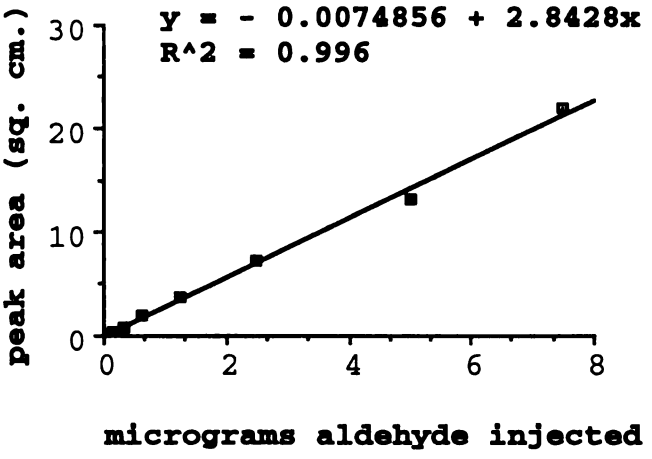


Figure 21. Standard curve of peak area versus micrograms of indole-3-carboxaldehyde, 12/17/96. Points are means of 2 HPLC injections of a pure standard. Peak area is based on measurement of peak height and width at a recording chart sensitivity of 1.0 AUFS.

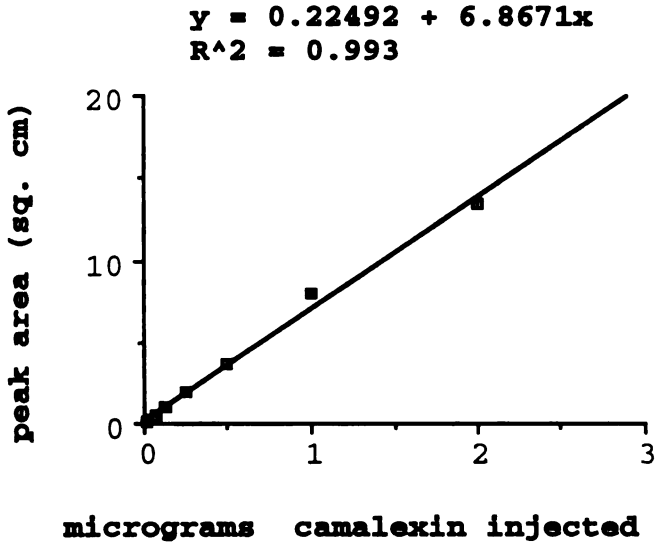


Figure 22. Standard curve of peak area versus micrograms of camalexin, 1/21/98. Points represent means of 3 HPLC injections of a pure camalexin standard. Area is based on peak height and width at a recording chart sensitivity of 0.25 absorbance units full scale (AUFS).

of the carbonyl group (CHO, 29 mass units), which is also typical of aldehydes (Hill, 1966). The presence of the characteristic peaks seemed sufficient proof of the presence of indole-3-carboxaldehyde and provided further support for the results of HPLC and TLC analysis. The fact that the aldehyde was sometimes detected only in wild-type, and sometimes only in *pad* 2, was attributed to instability of the aldehyde.

Recovery of camalexin and indole-3-carboxaldehyde.

The average percent recovery of indole-3-carboxaldehyde under various extraction conditions is presented in Tables 15-17. Table 15 demonstrates that in the absence of plant material, indole-3-carboxaldehyde is quite stable. Recovery was relatively high (over 60 %), and for the 0.25 µg/ml solutions tested, recovery may have been a bit higher because the aldehyde had a very low solubility in isopropanol and may not have been completely dissolved. The high recovery in a solution kept at room temperature for 9 hours indicated that degradation was unlikely to occur while leaves were being fed radiolabeled solutions. No other compounds were seen under UV light, and nothing was seen under UV light in TLC's of the lyophilized aqueous phases. Hence, it did not seem likely that the aldehyde was very prone to oxidation to indole-3-carboxylic acid, or to decarbonylation to indole. Also, it would not be necessary to study the aqueous phases to find residual aldehyde.

Table 15. Recovery of indole-3-carboxaldehyde in the absence of plant components. Only one replicate of the first sample was extracted. For the second sample (0.25µg/ml), 3 replicates were extracted, and means plus standard errors are shown.

sample	µg aldehyde recov-ered	µg aldehyde added	% recovery
aqueous solution of indole-3-carboxaldehyde (33.9 µg/ml) extracted after 9 hours at room temperature	66.3	80.8	82.1
aqueous solution of indole-3-carboxaldehyde (0.25 µg/µl)extracted with ethyl acetate	0.587± 0.102	7.5	64.6± 11.2

The results of Table 16 were obtained under the extraction conditions for the nonradioactive studies (40-leaf samples homogenized in a mortar and pestle); and the results of Tables 17A and 17B were obtained under the extraction conditions for the radiolabeling studies. The low recoveries in Table 16 may have been partly due to spiking with a small amount of standard (2.8-5 % of what was used to spike samples in Table 17B), but as the concentration of indole-3-carboxaldehyde in plants is also low, the small amount of spike probably provided a more accurate picture of recovery in extractions of unspiked leaves. The samples may have degraded partially while frozen, since the results for samples analyzed seven months later were more variable and cold be quite low. In calculating recovery of indole-3-carboxaldehyde, values from the most recent recovery-determination analyses were used.

One very unexpected result of the trial extractions for radiolabeling was that the majority of the aldehyde was in the chloroform fraction, although it was evaporated at high temperatures conducive to condensation reactions. This result suggested that the aldehyde was more stable than expected. In that case, the absence of indole-3-carboxaldehyde in the studies described in chapters 2 and 3, thought to be due to work done at high temperatures (boiling in 80 % methanol, or concentrating at 45 °C), may have been due to enzymatic degradation. Since the leaves were put into cold 80 % methanol and gradually heated up to boiling, there

Table 16. Percent recovery of indole-3-carboxaldehyde from leaves spiked with a standard and extracted in the manner of the nonradioactive studies of the kinetics of indole-3-carboxaldehyde accumulation. Means plus standard errors of 2 or 3 replicates (number indicated by N) are shown.

sample	mean µg aldehyde recovered	µg aldehyde added	% recovery
wild-type leaves (no droplets), extracted 5/97 and analyzed 6/97	0.145±0.015 (N=3)	0.4	36±3
wild-type leaves, extracted 5/97 and analyzed 12/97	0.0690±0.0240 (N=2)	0.4	17± 6
droplets, extracted 5/97 and analyzed 12/97	0.146± 0.032 (N=2)	0.4	34± 14

Table 17A. Percent recovery of indole-3-carboxaldehyde from leaves fed a nonradioactive solution of indole-3-carboxaldehyde. Abbreviations: EtOAc=ethyl acetate, $CHCl_3$=chloroform.

sample	µg aldehyde in EtOAc phase	µg aldehyde in $CHCl_3$ phase	µg aldehyde fed	% recovery in $CHCl_3$ phase	% recovery in both phases combined
one 6-leaf sample, 5/6/98	1.44	5.34	14.5	36.8	46.8
two 5-leaf samples, 5/6/98	1.16	5.04	12.1	41.6	51.2±2.6
three 6-leaf samples, 2/23/98	0.770+0.027	3.80±0.22	10.2	37.3	44.8±0.2

Table 17B. Recovery of indole-3-carboxaldehyde from leaves spiked with aldehyde at time of extraction. Abbreviations as in Table 17A.

sample	µg aldehyde in EtOAc phase	µg aldehyde in $CHCl_3$ phase	µg alde-hyde in spike	% recovery in $CHCl_3$ phase	%recovery in both phases combined
two 5-leaf samples, 5/25/98	0.640±0.154	5.67± 0.98	10.0	56.7	63.1±8.3
two 4-leaf samples, 5/25/98	0.538±0.074	3.10±0.66	8.0	38.7	45.4±7.4
three 6-leaf samples, 5/6/98	1.77±0.17	7.39±0.12	14.5	51.0	63.1±1.7
three 6-leaf samples, 2/11/98	1.27±0.46	7.57±0.81	10.2	74.2	86.6±10.1

may have been time for stress-related responses to occur, such as the release of degradative enzymes that could oxidize the aldehyde. Putting leaves directly into boiling 80 % methanol would have quickly inactivated all enzymes (R. Hammerschmidt, pers. comm.; Harborne, 1973). The effectiveness of a liquid nitrogen extraction was perhaps due not to the low temperatures, but to the prevention of enzymatic degradation by immediate freezing of leaves.

Table 17A demonstrates that the recovery of aldehyde was lower in leaves fed the solution than in leaves spiked with the solution (45-51 % vs. 63 %; see Table 16). This difference in yield suggests that some of the aldehyde is utilized by the leaves when it is fed to them. Despite the lower recovery, the aldehyde was easily detected on thin-layer chromatograms of extracts of wild-type plants.

The organic (chloroform) phase obtained in extraction was heavily pigmented, making it very difficult to separate camalexin and indole-3-carboxaldehyde from pigments on TLC. To see whether the amount of camalexin in the chloroform phase might be negligible, two analyses were made of the amount of camalexin in each phase. The results are listed in Table 18 (below).

Table 18. Relative amounts of camalexin (expressed as percentages of the total) in the phases obtained from the liquid nitrogen extraction method used for most nonradioactive samples in this chapter. For each sample, 5-7 g of leaves per sample were extracted. Camalexin was quantitated by HPLC (isopropanol-hexane 7:93, v/v).

phase from extraction	% recovery wild-type 10/1	% recovery wild-type 10/4	% recovery *pad2* 10/1	% recovery *pad2* 10/4
chloroform phase	13.2	8.57	36.7	22.9
chloroform extract of aqueous phase	6.68	4.54	5.35	14.3
droplets	80.1	86.9	58.1	63.0

Although *pad2* contained 20 % of its camalexin in the chloroform phase, separating pigments from compounds of interest was so difficult that a 20 % error due to lack of chloroform analysis seemed about as accurate as the amount of error involved in trying to separate camalexin from pigments and assuming high recovery when half the camalexin might remain hidden by chlorophylls. Therefore, the chloroform phases in the nonradioactive studies were not analyzed.

Considering the relatively low amount (<25%) of camalexin found in the chloroform phases at that time (Table 18), it was surprising to find the majority of the camalexin in the chloroform phase in trial extractions done just prior to radiolabeling (Table 19). The difference may be due to extracting droplets separately from leaves in the extractions described in Table 18. Had the droplets been ground in

Table 19. Percent recovery (mean plus standard error) of camalexin (cam.) from leaves spiked with a camalexin standard and extracted in the manner of extractions done in the radiolabeling experiment. In columns 2-4, each row of numbers denotes a separate replicate. Abbreviations are as in Tables 17A and 17B.

sample	µg cam. in EtOAc phase	µg cam. in $CHCl_3$ phase	µg cam. added	mean% recovery
two 5-leaf samples, 5/25/98	0.201 0.0292	7.12 9.52	14.0 14.0	60.2±8.0
two 5-leaf samples, 5/6/98	0 0	10.53 8.94	16.8	58.0±4.8
three 6-leaf samples, 2/98	0 0 0.024	5.4 3.6 6.6	8.0 8.0 8.0	65±11
two 4-leaf samples, 5/25/98	0.094 0.035	2.90 4.44	11.2 11.2	33.3±6.6

chloroform and methanol, the camalexin that diffused into the inoculum droplet might have dissolved in the organic phase.

Kinetics of accumulation of indole-3-carboxaldehyde and camalexin.

Indole-3-carboxaldehyde, although present prior to inoculation, increased after inoculation with *C. carbonum* (Figure 23A, Table 23B). Concentrations reached a maximum at 6 hours, when camalexin was first detectable (Figure 23B, Table 23A), and decreased rapidly afterwards. The kinetics were as expected for an intermediate (Hanson et al., 1994), since it appeared to accumulate prior to the onset of camalexin accumulation, and then to decrease--presumably because it was being converted into camalexin. Patterns of accumulation were similar in two time courses (Figures 23 and 24). However, the maximum amount of indole-3-carboxaldehyde produced in the first time course, when corrected for recovery (Table 22), was only 10 % of the maximum amount of camalexin produced (Table 23). Unless recovery of indole-3-carboxaldehyde was extremely poor, or conversion into camalexin was very rapid, it seems that not enough indole-3-carboxaldehyde was produced to account for the amount of camalexin produced. In *pad2*, the ratio of nanomoles of camalexin to nanomoles of aldehyde was not as high (Table 23), but the amount of camalexin produced still exceeded the amount of indole-3-carboxaldehyde produced. However, the lower ratio of nanomoles of camalexin to nanomoles of

Table 20A. Time course of accumulation of camalexin in wild-type and *pad2* leaves inoculated with water (ctrl) or *C. carbonum* (inoc); 3/23/97-3/24/97. Means plus standard errors are shown.

hours after inoculation	camalexin in wild-type (µg/leaf)			camalexin in *pad2* (µg/leaf)		
	leaves	droplets	total	leaves	droplets	total
0	0	0	0	0	0	0
3-inoc	0	0	0	0	0	0
6-ctrl	0	0	0	0	0	0
6-inoc	0.00507± 0.00237	0	0.00507± 0.00237	0	0	0
12-inoc	0	0.212± 0.023	0.212± 0.023	0	0.00675± 0.00082	0.00675± 0.00082
24-ctrl	0	0.00282± 0.00282	0.00282± 0.00282	0	0	0
24-inoc	0.00453± 0.00082	0.536± 0.264	0.541± 0.263	0	0.0258± 0.0105	0.0258± 0.0105
36-inoc	0.00195± 0.00195	0.325± 0.142	0.327± 0.142	0.00188± 0.00188	0.00913± 0.00159	0.0110± 0.0021

Table 20B. Time course of accumulation of indole-3-carboxaldehyde wild-type and *pad2* leaves inoculated with water (ctrl) or *C. carbonum* (inoc); 3/23/97-3/24/97.

hours after inocula-tion	wild-type			*pad2*		
	leaves	droplets	total	leaves	droplets	total
0	0	---	0	3.19±1.07	---	3.19±1.07
3-inoc	4.47±0.50	1.63±0.18	6.11±0.64	3.45±1.77	1.13±0.33	4.53±0.18
6-ctrl	0.395±0.395	0.332±0.332	0.707±0.354	1.99±0.39	0	1.99±0.39
6-inoc	2.27±0.15	7.26± 0.47	9.53±0.384	2.21±0.23	2.87±0.75	5.08±0.53
12-inoc	1.74±0.25	3.12±0.69	4.87±0.44	1.19±0.17	2.27±0.33	3.46±0.30
24-ctrl	3.31±1.21	0.565±0.286	3.87±1.35	1.41±0.21	0	1.41±0.21
24-inoc	1.80±0.42	0	1.80±0.42	0.561±0.281	0	0.561±0.281
36-inoc	1.36±0.32	0	1.36±0.32	0	0	0

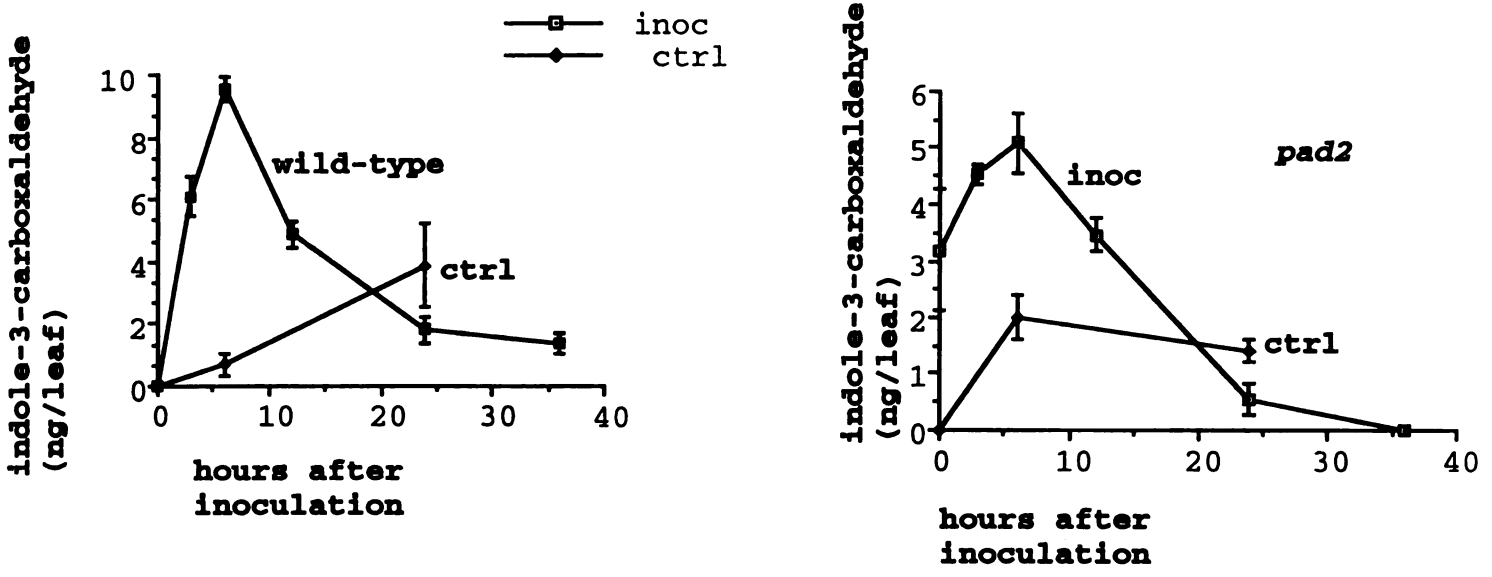


Figure 23A. Time course of accumulation of indole-3-carboxaldehyde in wild-type and *pad2* leaves inoculated with *C. carbonum* (inoc)or water (ctrl) 3/23/97-3/24/97. Standard error bars are shown.

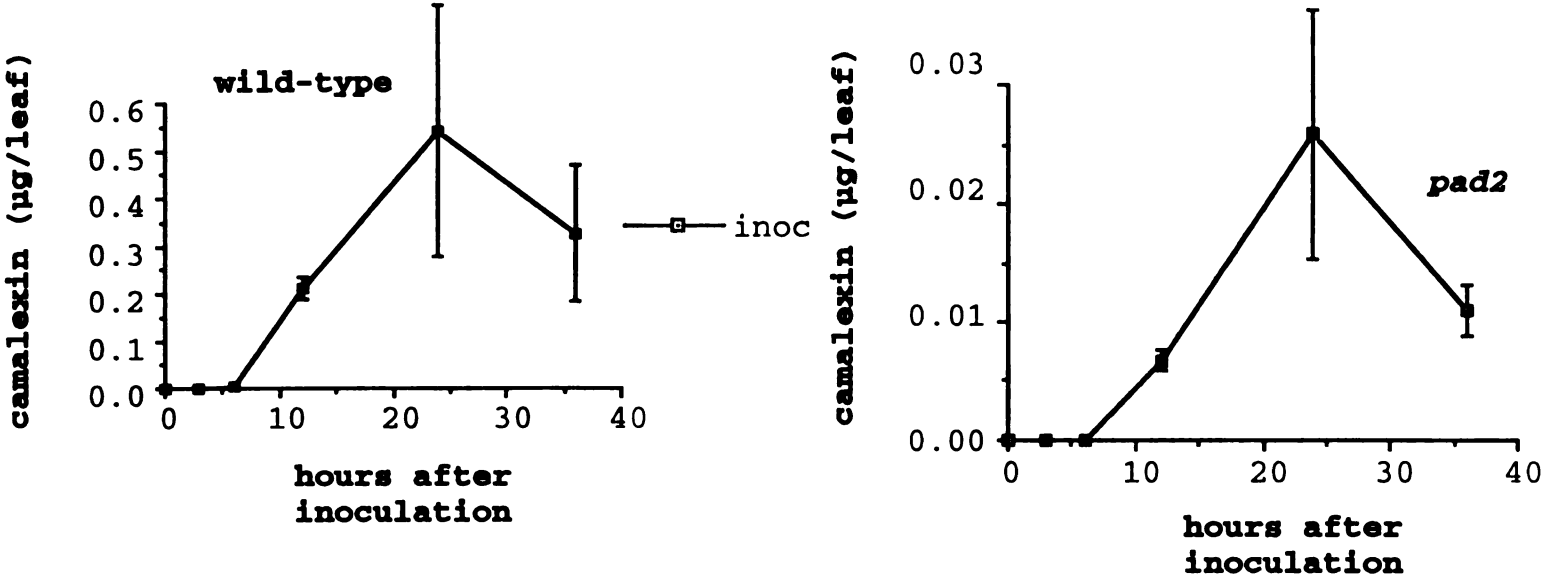


Figure 23B. Time course of accumulation of camalexin in wild-type and *pad2* leaves inoculated with *C. carbonum* (inoc), 3/23/97-3/24/97. No significant amount of camalexin was detected in the water-inoculated controls. Standard error bars are shown.

Table 21. Time course of accumulation of camalexin (A) and indole-3-carboxaldehyde (B) in wild-type and *pad2* leaves inoculated with water (ctrl) or C. carbonum (inoc); 5/31/97-6/1/97. Leaves and droplets were extracted separately and combined for TLC.

A.

hours after inocula-tion	camalexin in wild-type (µg/leaf)	camalexin in *pad2* (µg/leaf)
0	0	0
3-inoc	0	0
6-ctrl	0	0
6-inoc	0.0103± 0.0035	0
12-inoc	0.0254±0.00654	0.00189±0.00159
24-ctrl	0	0
24-inoc	0.0220±0.0035	0
36-inoc	0.0173±0.0032	0.00159±0.00125

B.

hours after inocula-tion	aldehyde in wild-type (ng/leaf)	aldehyde in *pad2* (ng/leaf)
0	0	0
3-inoc	3.71±1.25	0.940±0.484
6-ctrl	0	0
6-inoc	3.41±0.467	0.997±0.501
12-inoc	1.43±0.325	0
24-ctrl	0	0
24-inoc	0	0
36-ctrl	0	0

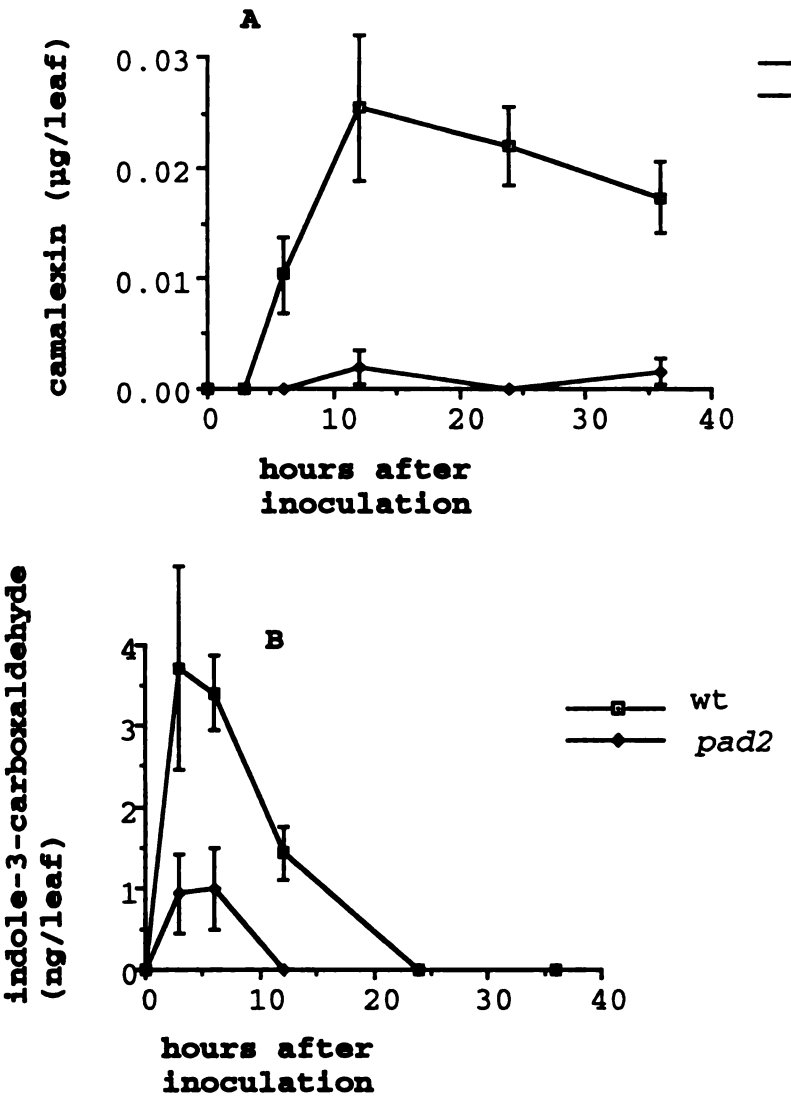


Figure 24. Time course of accumulation of camalexin (A) and indole-3-carboxaldehyde (B) in wild-type (wt) and *pad2* Arabidopsis leaves inoculated with *C.carbonum*, 5/31/97-6/1/97 (see Table 21 for data). Standard error bars are shown. No camalexin or aldehyde were detected in water-inoculated controls.

Table 22. Accumulation of indole-3-carboxaldehyde (ng/leaf, corrected for recovery) in wild-type and *pad2* leaves inoculated with *C. carbonum* or water, 3/23/97-3/24/97. Abbreviations are as in Tables 20A and 20B. Calculations for recovery were based on the results of Table 16.

time (hours)	wild-type			*pad2*		
	leaves	droplets	total	leaves	droplets	total
0	0	---	0	8.9	---	8.9
3-inoc	12.4	4.4	16.8	9.6	3.0	12.6
6-ctrl	1.1	0.90	2.0	5.5	0	5.5
6-inoc	6.3	19.6	25.9	6.1	7.8	13.9
12-inoc	4.8	8.4	13.2	3.3	6.1	9.4
24-ctrl	9.2	1.5	10.7	3.9	0	3.9
24-inoc	5.0	0	5.0	1.6	0	1.6
36-inoc	3.8	0	3.8	0	0	0

Table 23. Comparison of nanomoles of indole-3-carboxaldehyde (corrected for recovery) and camalexin (% recovery based on the results of Table 18) produced in wild-type and *pad2* leaves inoculated with *C. carbonum*. Numbers for camalexin are based on the results of Table 18A for total camalexin (droplets+leaves). Numbers for indole-3-carboxaldehyde are based on the results of Table 16.

hours after inocula-tion	wild-type		*pad2*	
	nmol aldehyde	nmol camalexin	nmol aldehyde	nmol camalexin
0	0	0	0.061	0
3-inoc	0.116	0	0.0869	0
6-ctrl	0.014	0	0.038	0
6-inoc	0.179	0.0284	0.0959	0
12-inoc	0.0910	1.19	0.065	0.0480
24-ctrl	0.0737	0.0158	0.027	0
24-inoc	0.0345	3.03	0.011	0.183
36-inoc	0.0262	1.84	0	0.078

aldehyde suggests that the aldehyde and camalexin do have a precursor-product relationship that is stoichiometrically fairly sound in *pad2*. It may be that more than one pathway to camalexin exists, and that *pad2* is camalexin-deficient because it lacks that alternative pathway. Possibly the wild-type plants utilize several camalexin biosynthetic pathways simultaneously, in a manner similar to what is proposed in Bu'Lock's (1965) discussion of the "metabolic grid" by which some compounds come from many different pathways. Examples of compounds synthesized in this manner are the tryptamine alkaloids of the grass *Phalaris tuberosa* (Baxter and Slaytor, 1972). Based on the incorporation of various putative precursors, at least 5 different biosynthetic routes were possible.

The fact that *pad2* accumulated two-thirds of wild-type amounts of indole-3-carboxaldehyde, but only one-tenth as much camalexin (Tables 22 and 23), suggests that indole-3-carboxaldehyde is not a key regulatory step in the pathway, if it is a step at all. Possibly, too, the *pad2* mutant is blocked at a biosynthetic intermediate between indole-3-carboxaldehyde and camalexin (M. Zook, pers. comm.).

Potential phytotoxicity of radioactive solutions.

No visible signs of phytotoxicity were observed in trial feedings of nonradioactive solutions. However, the rapid wilting of some of the leaves during the radioactive feeding may indicate that the solutions were somewhat phytotoxic, and

that the effect was more pronounced at the unexpectedly higher temperature of the room on that occasion.

Radiolabeling.

Because anthranilate is a precursor of many compounds in addition to camalexin (Dewick, 1995), an attempt was made to standardize the amount of time that elapsed between completion of solution uptake and leaf inoculation. Otherwise, there would be a risk that the anthranilate would be shunted into primary metabolic pathways, such as tryptophan biosynthesis, and that less would be available for camalexin biosynthesis. However, it was not possible to inoculate leaves a fixed number of hours after they had taken up the radioactive solution. The large number of leaves to check for completion of solution uptake (378 total), and the fact that the leaves took up the solutions more rapidly than usual (perhaps due to larger size and a warmer room) made it too difficult to inoculate before all leaves had finished taking up solution. The time lag between leaf feeding and inoculation is depicted in Table 24. It is not impossible that variations in time lapses prior to inoculation affected pools of available anthranilate and anthranilate-derived compounds, which, in turn, may have introduced unexpected variables in the patterns of incorporation seen.

Table 24. Time lapses during labeling of leaves.

hours after inoculation	time between start of feeding and removal from solution (hours)			time between removal from solution and inoculation (hours)		
solution	a	b	c	a	b	c
0	3-6	6-8	6-9	0	0	0
3	6-9	8-10	6-9	6-7	6-8	8
6	6-9	8-10	6-9	6-7	6-8	8
9	7-12	8-10	6-9	4	6-8	8
24	7-12	8-10	6-9	4	6-8	8

Many leaves wilted during the feeding period, due to not taking up the solution or taking it up rapidly and then wilting before water was added to the tube. Consequently, sample size was reduced from 6 to 5 leaves per sample, which meant that yields of compounds were lower than anticipated.

Appearance of thin-layer chromatograms and incorporation of ^{14}C-anthranilate into camalexin and indole-3-carboxaldehyde.

Thin-layer chromatograms of both the ethyl acetate and the chloroform extracts contained many bands per sample (Figures 25-30). Camalexin was easily identified in the samples, due to the spike added to each sample at the time of extraction. Indole-3-carboxaldehyde was identifiable in most samples for the same reason. However, in samples fed solution (c) (^{14}C-anthranilate diluted with nonradioactive indole-3-carboxaldehyde), the aldehyde was detectable only in

the zero-hour samples (one of which had been spiked by mistake). The absence of aldehyde in the other "c" samples was surprising because in trial feedings, when leaves were fed nonradioactive anthranilate diluted with indole-3-carboxaldehyde, the aldehyde was detectable on thin-layer chromatograms of the extracts, and the recovery was 45-50 % (Table 17A). However, in those trial feedings, leaves were always extracted immediately after solution uptake. Therefore, the fate of the fed aldehyde over a 24-hour incubation period was not known. It may be that the aldehyde was used in other metabolic pathways, or that it was oxidized to indole-3-carboxylic acid (Müller, 1961).

On autoradiograms, bands at the R_f's of camalexin and indole-3-carboxaldehyde were present in some samples, indicating that anthranilate had been incorporated into both compounds. Surprisingly, a band at the R_f of camalexin was present in the zero- and three-hour samples (Figure 25) and in some controls (Figure 26). Because a band just below camalexin was observed in some samples (Figure 26, sample 6b-i, and Figures 27 and 28, controls), it seemed possible that this latter compound sometimes comigrated with camalexin. To determine whether a different TLC solvent would resolve the compounds, the unused portions of some extracts (one replicate of each of treatments a, b, and c) were first developed on TLC plates in the usual solvent system. The camalexin bands were eluted with ethyl acetate,

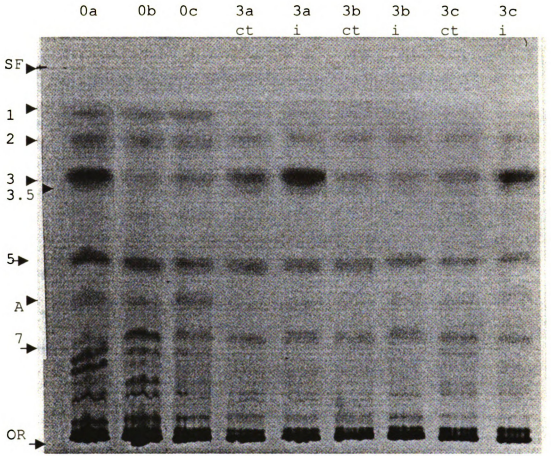


Figure 25. Autoradiogram of TLC plate: tissue extracts of wild-type Arabidopsis leaves, 0 and 3 hours after inoculation with *C. carbonum* (i) or water (ct). Before inoculation, leaves were fed one of the following solutions: ^{14}C-anthranilate (a), ^{14}C-anthranilate+cold anthranilate (b), or ^{14}C-anthranilate+cold indole-3-carboxaldehyde. Bands are numbered as in Figures 31-38 (see those figures for R_f-values. Other abbreviations: SF=solvent front (total distance traveled by solvent was 15 cm); OR=origin; A=indole-3-carboxaldehyde.

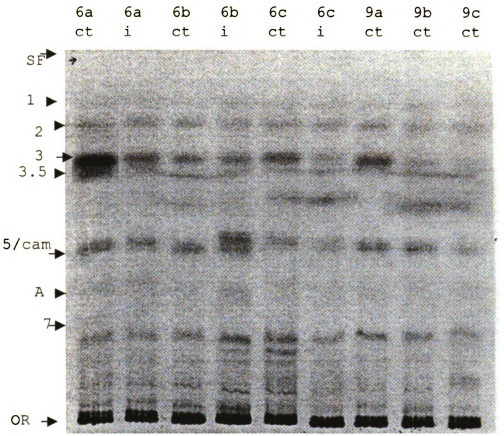


Figure 26. Autoradiogram of TLC plate:extracts of wild-type Arabidopsis leaves extracted 6 and 9 hours after inoculation with *C. carbonum* or water. Abbreviations are as in Figure 25. *C. carbonum*-inoculated 9-hour samples are shown in Figure 27.

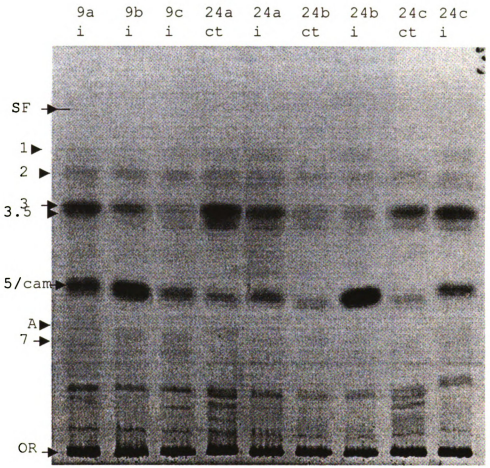


Figure 27. Autoradiogram of TLC plate: extracts of wild-type Arabidopsis leaves extracted 9 and 24 hours after inoculation with water or *C. carbonum*. Abbreviations are as in Figure 25.

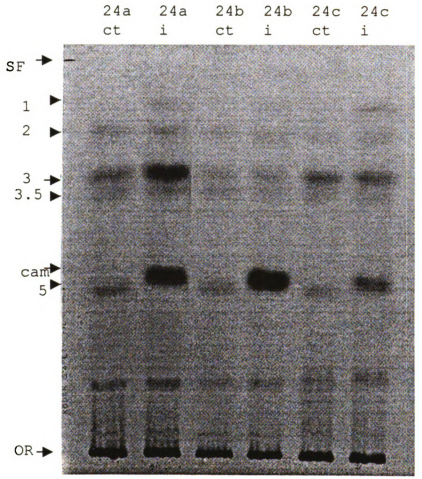


Figure 28. Autoradiogram of TLC plate; tissue extracts of wild-type Arabidopsis leaves, 24 hours after inoculation with water or *C. carbonum*. Abbreviations are as in Figure 25. These extracts were from a separate replicate of the experiment shown in Figures 25-27.

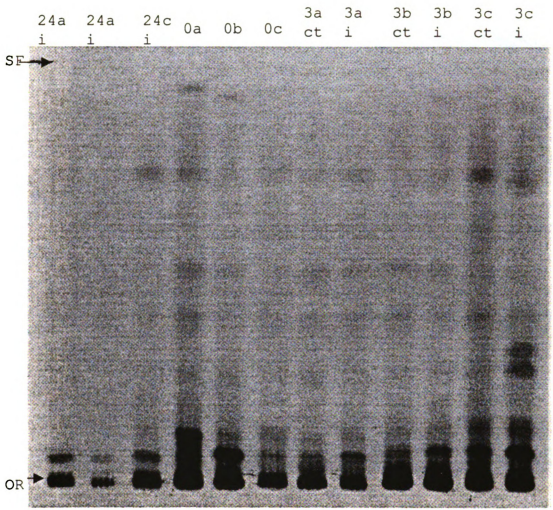


Figure 29. Autoradiogram of TLC plate: ethyl acetate extracts of leaves extracted 0, 3, 6, and 24 hours after inoculation with *C. carbonum* or water. Bands are not numbered because none were analyzed, due to the complexity of the autoradiogram. Abbreviations are as in Figure 25. The 24-hour samples are from one replicate of the experiment, and the other samples are from a separate replicate. Sample 24a was accidentally loaded onto 2 lanes, and sample 24b was consequently spotted on a separate plate.

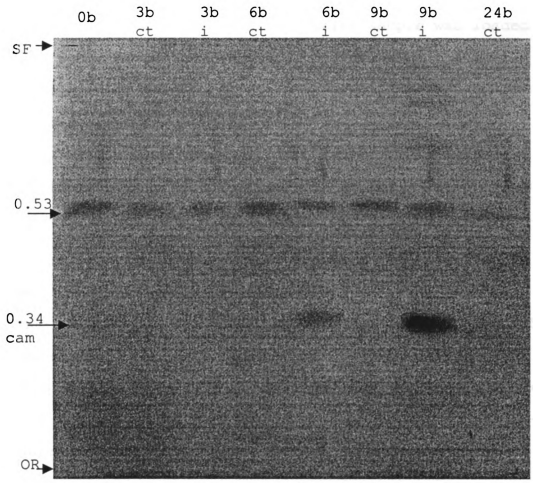


Figure 30. Autoradiogram of TLC plate: camalexin bands eluted and redeveloped in chloroform-acetic acid 94:6 (v/v). R_f values of camalexin (cam) bands and of the other band (possibly corresponding to band 5 in Figures 25-28) are indicated next to the arrows. Samples were from leaves fed ^{14}C-anthranilate diluted with cold anthranilate. Abbreviations are as in Figure 25.

as described in Chapter 3. The eluted samples were dissolved in 45 µl of ethyl acetate, and the entire sample was loaded onto glass-backed silica TLC plates 250 µm thick. Plates were developed in chloroform-acetic acid 94:6 (v/v) and laid onto film for 3 weeks. As Figure 30 demonstrates, two compounds were present in the 6- and 9-hour *C. carbonum*-inoculated samples. In the other samples, only one compound was present, and it had a much higher R_f value than camalexin. Therefore, it seemed safe to conclude that the putative camalexin bands in controls, and at very early timepoints, were due to comigration of another compound (possibly band 5) with camalexin.

For indole-3-carboxaldehyde, co-chromatography with the aldehyde spike, or (in the case of the aldehyde samples) co-migration with spiked samples, was considered sufficient proof that the band at that R_f was indeed indole-3-carboxaldehyde, since in the kinetic studies, no other compound had been detected by HPLC at the R_f of the aldehyde.

It was probable that some of the other bands consisted of more than one superposed band. Anthranilate derivatives are generally polar, and in a nonpolar solvent like chloroform-methanol 19:1 (v/v), those compounds would tend to stay near the origin and be poorly separated from one another. They could have represented many compounds unrelated to camalexin biosynthesis. Possibilities would include indole derivatives like tryptamine, indole-3-butyric

acid, indole-3-glyoxylic acid, indole-3-pyruvic acid, or ascorbigen (Müller, 1961; Robinson, 1962). These compounds, due to their relatively high polarity, were more likely to be present in the ethyl acetate than in the chloroform phases of leaf extracts. The large number of faint bands on one autoradiogram of a TLC of ethyl acetate extracts (Figure 29) demonstrates that anthranilate was incorporated into many compounds. Because the chromatograms of ethyl acetate extracts were very complex and total incorporation into the ethyl acetate phases varied greatly, no bands were counted from these plates.

On TLC plates of chloroform extracts, 7 different bands were scraped in each sample: camalexin (R_f=0.51), indole-3-carboxaldehyde (R_f=0.37-0.39), and 5 bands with R_f-values of 0.81, 0.71, 0.67, 0.46, and 0.27, respectively. These bands were numbered 2, 3, 3.5, 5, and 7, respectively. Band 1 and 4, which had an R_f value of about 0.88, was so faint and incorporated so few cpm of radioactivity that it was not analyzed. Band 4 occasionally appeared as a very faint band below band 3.5 (so called because it was so close to band 3), but the appearance was so sporadic that it was not analyzed. Band 6 corresponded to indole-3-carboxaldehyde.

About 5 other bands closer to the origin appeared consistently in the chloroform extracts (Figures 25-31), but these were not further analyzed because they were not as well resolved as bands 1-7.

Total percent incorporation is given in Table 25 and Figure 31. The incorporation of ^{14}C-anthranilate into these different compounds is depicted in Figures 32-39 and Tables 26-33.

An unexpected result of this study was that out of 3 treatments, incorporation into camalexin was highest in leaves fed ^{14}C-anthranilic acid diluted with cold anthranilic acid (Table 26, Figures 32 and 33). Based on the results of other biosynthetic studies (Adams and Yang, 1983), incorporation into camalexin was expected to decrease in leaves fed that solution, since leaves would be making camalexin from both radioactive and non-radioactive anthranilate. However, higher incorporation into diluted compounds is not unprecedented. Similar results were found in studies with the flavonoid phloridzin, in which feeding a cold form of a putative precursor led to higher overall incorporation (Hutchinson et al., 1959). It may be that the increased supply of anthranilate to leaves caused an overall increase in metabolism. Thus, the uptake of radioactive anthranilate was more efficient than it would have been otherwise. Because more anthranilate was available, more camalexin was made. If more camalexin were made due to the increased anthranilate pool, the overall recovery of camalexin in the extraction may have been better. Thus, it is possible that not much more radioactive camalexin was made in these leaves than in the leaves fed the other solutions, but that the recovery of radioactive camalexin was improved.

Incorporation of ^{14}C-anthranilate into camalexin was lowered slightly by dilution with unlabeled indole-3-carboxaldehyde. However, as the decrease was within the span of the error bars for leaves fed undiluted anthranilate, it is difficult to conclude that indole-3-carboxaldehyde significantly lowered the percent incorporation into camalexin.

Analysis of the effects of indole-3-carboxaldehyde was complicated by the fact that the total incorporation into aldehyde-fed leaves was less than in leaves fed the other two solutions (Table 25). This observation suggests that the aldehyde may indeed be phytotoxic at the concentrations in which it was fed, despite a lack of macroscopic symptoms. If so, differences in incorporation into camalexin may reflect not differences in the availability of biosynthetic precursors, but differences in the solutions which were fed to the leaves.

The phytotoxicity hypothesis aside, when the two replicates are considered separately (Table 26, Figure 33), differences in kinetics of camalexin accumulation are apparent. In one replicate, camalexin reached a maximum 9 hours after inoculation in leaves fed undiluted ^{14}C-anthranilic acid. At this time, the percent incorporation into camalexin in aldehyde-fed leaves was slightly lower (0.287 % versus 0.296 %). At 24 hours post-inoculation, incorporation into camalexin was lower than at 9 hours in leaves fed only anthranilic acid, but in leaves fed

anthranilic acid diluted with aldehyde, incorporation into camalexin increased between 9 and 24 hours. Perhaps incorporation into camalexin decreased in leaves fed only anthranilic acid, due to the radioactive camalexin being diluted by nonradioactive camalexin synthesized from endogenous precursors. However, because the results for a second replicate are clearly different (incorporation at 24 hours being lower in aldehyde-fed leaves being lower than in leaves fed undiluted ^{14}C-anthranilate), this conclusion is not well supported. The differences observed between treatments may simply reflect the fact that total incorporation into aldehyde-fed leaves was lower. Again, it may be that variation in incubation times prior to inoculation created differences in metabolite pools that caused artificial differences between treatments. Also, the variations may reflect differences in rate of penetration of *C. carbonum* on individual leaves. Leaves on which penetration was slower would begin to produce camalexin later than leaves on which penetration was rapid. With such small sample sizes (one-eighth the number of leaves used for the kinetic studies), slight variations in camalexin concentrations among leaves could be a significant percentage of the total amount of camalexin produced.

The patterns of incorporation of anthranilate into indole-3-carboxaldehyde (Figure 28, Table 33) do not provide definitive answers on its role in camalexin biosynthesis. Incorporation was very low. In leaves fed ^{14}C-anthranilate

alone, there was a small peak 6 hours after inoculation, suggesting that aldehyde had accumulated to a maximum before being converted into camalexin, as had been seen in the kinetic studies. The decrease between 0 and 3 hours could be due to the aldehyde being used in other pathways or being oxidized to indole-3-carboxylic acid (Müller, 1961). However, the difference in incorporation between controls and fungal-inoculated samples is so small that it would be difficult to conclude that the kinetics of accumulation in the fungal-inoculated samples represent the accumulation of a compound in an inducible pathway.

It is also possible, as discussed earlier, that indole-3-carboxaldehyde is one possible intermediate of camalexin biosynthesis, but that other pathways exist. A possible intermediate is indole-3-carboxylic acid (Figure 4). A band at the approximate Rf of the carboxylic acid was present on autoradiograms, but it was not analyzed because it was poorly resolved. Incorporation of indole-3-carboxylic acid into camalexin may be worth examining in the future.

None of the other bands examined appeared to be obvious precursors of camalexin. The incorporation into bands 2 and 7 is very low (Tables 28 and 32, Figures 34 and 38), and the incorporation into bands 3 and 3.5 (Tables 29 and 30, Figures 35 and 36) varied so much between replicates that it is difficult to draw conclusions from those results. The results of incorporation into band 5 (Table 31, Figure 37), the band that ran just below camalexin or sometimes

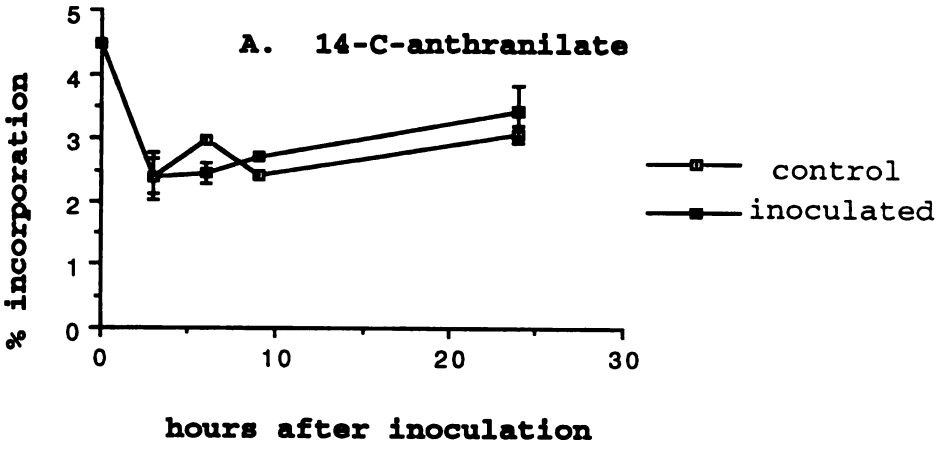
comigrated with it, are incomplete because the camalexin band may have contained some of that compound. If so, the eluted band 5 samples did not contain the entire yield of the compound, and the differences in the amount of compound obtained from the TLC plate may have varied erratically between samples.

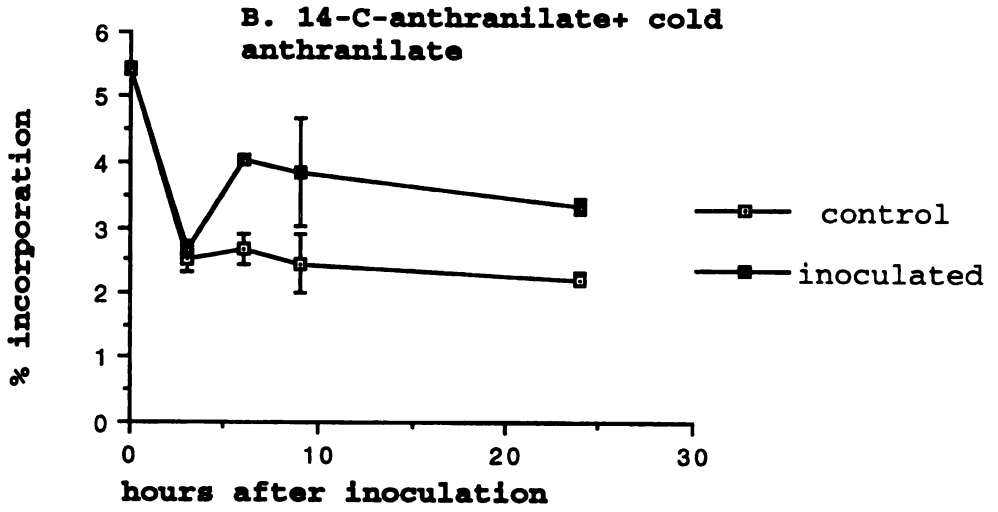
In summary, although the kinetic studies of accumulation of indole-3-carboxaldehyde and camalexin supported the role of indole-3-carboxaldehyde as an intermediate, the radiolabeling studies did not provide an unambiguous answer. The slight decrease in incorporation into camalexin could simply be a reflection of the lower overall incorporation into leaves fed indole-3-carboxaldehyde. Clear resolution of this quesiton may require the synthesis of radioactive indole-3-carboxaldehyde to feed as a precursor. It may be worthwhile to examine other putative intermediates instead, such as indole-3-carboxylic acid. Since the kinetics of accumulation support the role of the aldehyde as a camalexin precursor but stoichiometry and radiolabeling data do not support it strongly, it may be that more than one pathway to camalexin operates. The carboxylic acid is one possible alternative precursor. Unlike *Camelina sativa*, another camalexin-producing crucifer, Arabidopsis makes indole glucosinolates, which have been shown to be precursors of other cruciferous phytoalexins (Monde et al., 1994). Perhaps Arabidopsis can utilize glucobrassicin (Figure 4) as a camalexin precursor.

Table 25. Percent incorporation (% inc) into chloroform extracts of wild-type Arabidopsis leaves fed one of solutions A, B, or C and then inoculated with water (ctrl) or *C. carbonum* (inoc). Leaves were fed ^{14}C-anthranilate alone or with a tenfold higher concentration (on a per mole basis) of nonradioactive (cold) anthranilate or indole-3-carboxaldehyde. Results for the individual replicates are shown in the "% inc" columns; the mean and standard error are shown in the "mean % inc." columns.

hours after inoculation	A. ^{14}C-anthranilate		B. ^{14}C-anthranilate +cold anthranilate		C. ^{14}C-anthranilate+cold indole-3-carboxaldehyde	
	% inc.	mean % inc.	% inc.	mean % inc.	% inc.	mean % inc.
0	4.547	4.48± 0.08	5.535	5.43± 0.11	3.406	3.06± 0.35
	4.397		5.321		2.711	
3 ctrl	2.114	2.40± 0.29	2.286	2.52± 0.225	2.763	2.60± 0.165
	2.691		2.744		2.426	
3 inoc	2.778	2.40± 0.38	2.790	2.67± 0.120	2.508	2.98± 0.46
	2.006		2.547		3.436	
6 ctrl	2.968	2.99± 0.02	2.425	2.66± 0.24	2.910	3.36± 0.46
	3.007		2.902		3.818	
6 inoc	2.281	2.44±0.16	4.050	4.02± 0.02	2.114	2.40± 0.29
	2.602		4.001		2.688	
9 ctrl	2.359	2.42± 0.06	1.991	2.44± 0.45	2.087	2.34± 0.26
	2.489		2.889		2.605	
9 inoc	2.742	2.72± 0.02	3.031	3.84± 0.82	1.877	1.86± 0.02
	2.695		4.660		1.828	
24 ctrl	3.199	3.07± 0.13	2.319	2.21± 0.11	2.802	2.68± 0.12
	2.942		2.101		2.568	
24 inoc	3.027	3.42± 0.40	3.230	3.34± 0.10	2.434	2.60± 0.16
	3.817		3.445		2.764	

Figure 31. Average percent incorporation into chloroform extracts of leaves inoculated with water (control) or *C. carbonum* after being fed labeled anthranilate (graph A) or labeled anthranilate diluted with cold anthranilate (graph B) or cold indole-3-carboxaldehyde (graph C). See Table 25 for data and solution preparation. Means plus standard errors are shown.





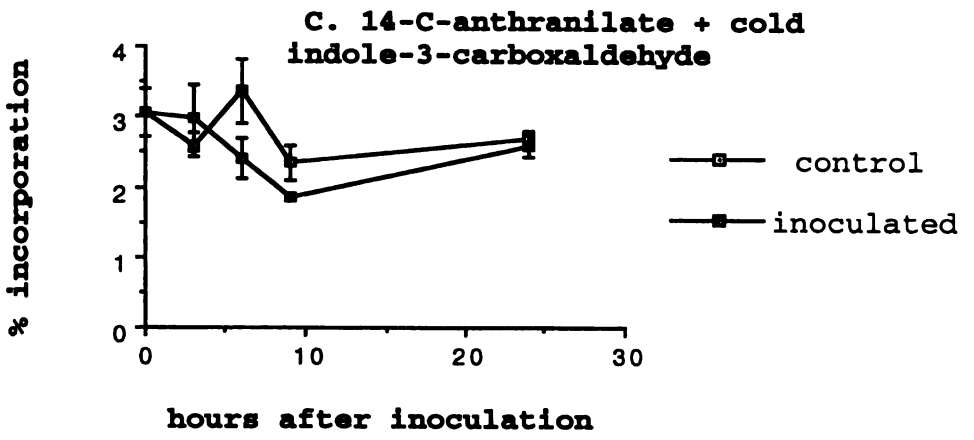


Figure 31 (caption on facing page).

Table 26. Percent incorporation into camalexin in wild-type Arabidopsis leaves fed one of solutions A, B, or C and then inoculated with water or *C. carbonum*. Abbreviations and solution preparations are as in Table 25. Zeroes indicate that the zone scraped at the R_f of camalexin had an activity less than 100 cpm (roughly twice background on the scintillation counter).

hours after inoculation	solution A (^{14}C-anthranilate)		solution B (^{14}C-anthranilate +cold anthranilate)		solution C (^{14}C-anthranilate +cold indole-3-carboxaldehyde)	
	% inc.	mean % inc.	% inc.	mean % inc.	% inc.	mean % inc.
0	0.0742	0.0371± 0.0371	0.0815	0.0714± 0.0101	0.0748	0.0374± 0.0374
	0		0.0613		0	
3 ctrl	0	0	0	0	0.0450	0.0225± 0.0225
	0		0		0	
3 inoc	0	0	0	0	0	0
	0		0		0	
6 ctrl	0	0	0	0	0	0.0229± 0.0229
	0		0		0.0459	
6 inoc	0	0	0.241	0.186± 0.055	0	0.0225± 0.0225
	0		0.131		0.0450	
9 ctrl	0	0	0	0	0	0
	0		0		0	
9 inoc	0.462	0.296± 0.166	1.09	1.28± 0.18	0.287	0.201± 0.086
	0.130		1.46		0.116	
24 ctrl	0	0	0	0	0	0
	0		0		0	
24 inoc	0.230	0.591± 0.361	3.05	3.00± 0.05	0.399	0.338± 0.061
	0.952		2.96		0.277	

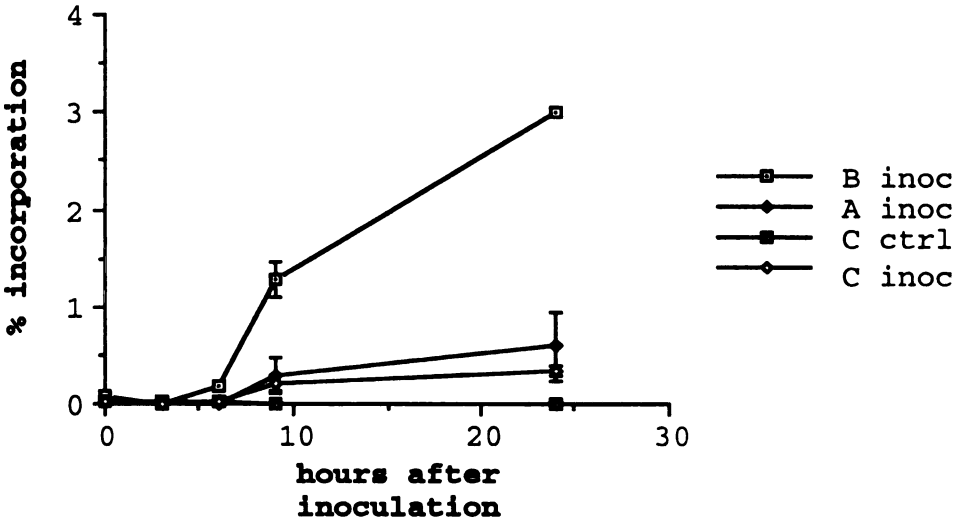


Figure 32. Time course of incorporation of labeled anthranilate into camalexin in leaves fed one of solutions A, B, or C (see Figure 31) and then inoculated with water (ctrl) or C. carbonum (inoc). No incorporation into camalexin was detected in the controls of leaves fed solutions A and B. Standard error bars are shown.

Figure 33. Time course of incorporation of labeled anthranilate into camalexin in each of 2 replicates of leaves fed one of solutions A, B, or C and then inoculated with water (ctrl) or *C. carbonum* (inoc). Standard error bars are shown. See Figure 31 for solution preparations.

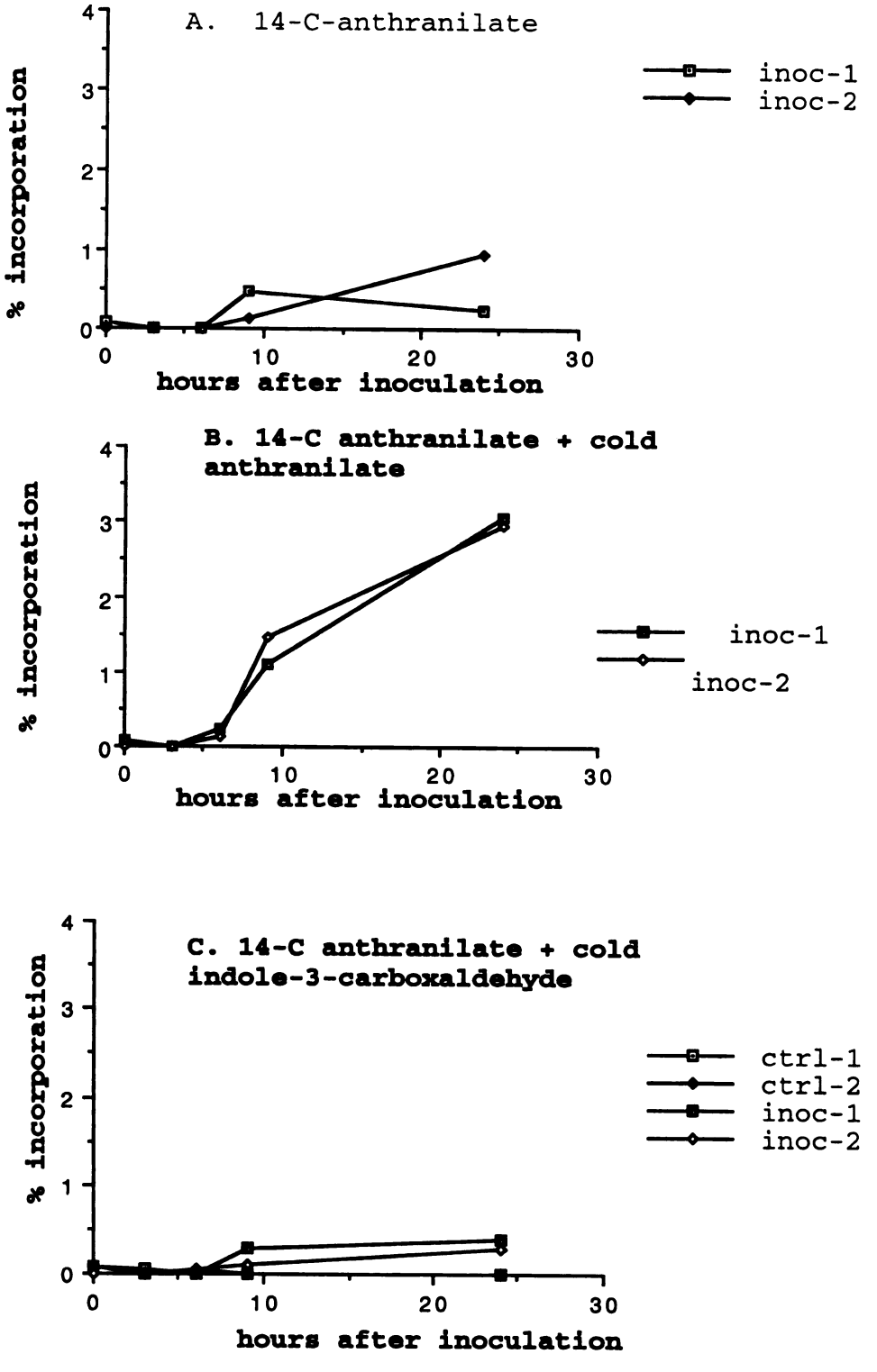


Figure 33 (caption on facing page).

Table 27. Percent incorporation into indole-3-carboxaldehyde in wild-type Arabidopsis leaves fed one of solutions A, B, or C and then inoculated with water or *C. carbonum*. Abbreviations and solution preparations are as in Table 25. Zeroes indicate that the zone scraped at this R_f had an activity below 100 cpm (roughly twice background on the scintillation counter).

hours after inoculation	solution A (^{14}C-anthranilate)		solution B (^{14}C-anthranilate+cold anthranilate)		solution C (^{14}C-anthranilate+cold indole-3-carboxaldehyde)	
	% inc.	mean % inc.	% inc.	mean % inc.	% inc.	mean % inc.
0	0.244	0.259± 0.016	0.240	0.191± 0.049	0.343	0.201± 0.142
	0.275		0.142		0.0592	
3 ctrl	0.0800	0.0766± 0.0034	0.0777	0.0712± 0.0066	0.147	0.150± 0.003
	0.0732		0.0646		0.153	
3 inoc	0.0786	0.0797± 0.0012	0.0884	0.0668± 0.0217	0.136	0.170± 0.034
	0.0809		0.0451		0.204	
6 ctrl	0.0730	0.0678± 0.0052	0	0.0308± 0.0308	0	0.0991± 0.0991
	0.0626		0.0617		0.198	
6 inoc	0.0822	0.0932± 0.0111	0	0.0436± 0.0436	0	0.0648± 0.0648
	0.104		0.0872		0.130	
9 ctrl	0.0619	0.0548± 0.0071	0	0	0.0916	0.0458± 0.0458
	0.0477		0		0	
9 inoc	0.0504	0.0500± 0.0004	0.0809	0.0765± 0.0044	0.0801	0.0401± 0.0401
	0.0496		0.0721		0	
24 ctrl	0.0566	0.0530± 0.0036	0	0	0	0
	0.0494		0		0	
24 inoc	0.0656	0.0645± 0.0011	0.0990	0.106± 0.007	0.0775	0.0819± 0.0044
	0.0634		0.112		0.0863	

Figure 34. Time course of incorporation of 14-C anthranilate into indole-3-carboxaldehyde in leaves fed one of the indicated solutions (see Figure 31 for solution preparation) and then inoculated with water (control) or *C. carbonum*. Standard error bars are shown.

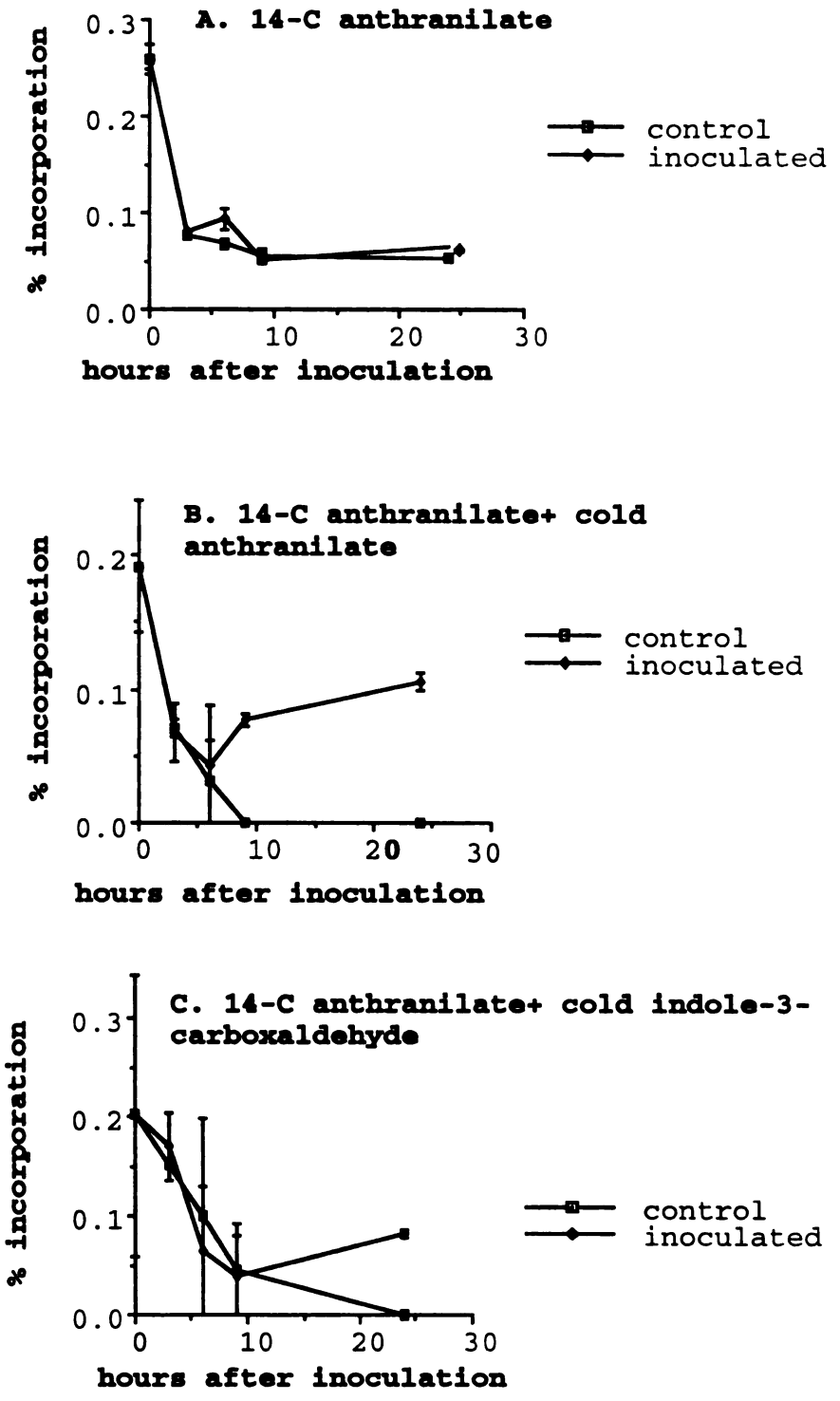


Figure 34 (caption on facing page).

Table 28. Average percent incorporation (mean plus standard error of 2 replicates) into band 2 (R_f=0.81) into leaves fed one of solutions A, B, or C and then inoculated with water or *C. carbonum*. Abbreviations and solution preparations are as in Table 25. Zeroes indicate that the zone scraped at this Rf had an activity below 100 cpm (roughly twice background on the scintillation counter).

hours after inoculation	% inc. solution A (^{14}C-anthranilate)	% inc. solution B (^{14}C-anthranilate + cold anthranilate)	% inc solution C (^{14}C-anthranilate + cold indole-3-carboxaldehyde)
0	0.117±0.030	0.127±0.0098	0.0817±0.0157
3 ctrl	0.0981±0.0003	0.0716±0.0004	0.0654±0.0090
3 inoc	0.0734±0.0124	0.0658±0.0006	0.0602±0.0102
6 ctrl	0.0787±0.0030	0.0576±0.0023	0.0681±0.0002
6 inoc	0.0624±0.0060	0.0734±0.0015	0.0695±0.0040
9 ctrl	0.0594±0.0055	0.0631±0.0002	0.0587±0.0112
9 inoc	0.0898±0.0020	0.0691±0.0015	0.0469±0.0052
24 ctrl	0.0589±0.0154	0.0446±0.00146	0.0427±0.0110
24 inoc	0.0610±0.0003	0.0489±0.0031	0.0518±0.0052

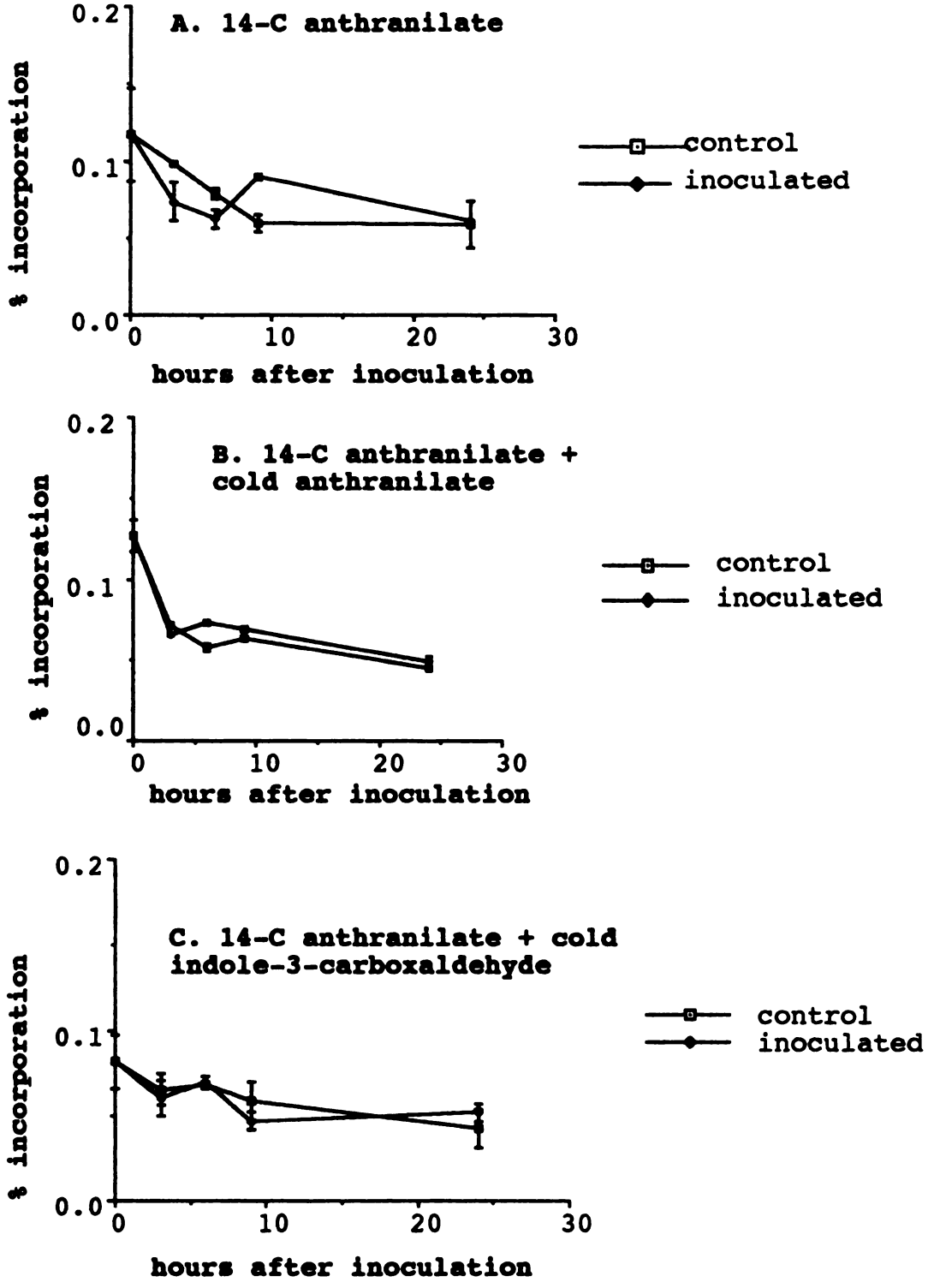


Figure 35. Incorporation of 14-C anthranilate into band 2 (Rf=0.81) over time. Graphs labeled as in Figure 31.

Table 29. Average percent incorporation (mean plus standard error) into band 3 (R_f=0.71, possibly indole) in leaves fed one of solutions A, B, or C and then inoculated with water or *C. carbonum*. Abbreviations and solution preparations are as in Table 25.

hours after inocu-lation	% inc. solution A (^{14}C-anthranilate)	% inc solution B (^{14}C-anthranilate + cold anthranilate)	% inc. solution C (^{14}C-anthranilate + cold indole-3-carboxalde-hyde)
0	0.202±0.131	0.0740±0.0305	0.0538±0.0538
3 ctrl	0.169±0.016	0.0731±0.0003	0.0906±0.0283
3 inoc	0.337±0.198	0.0837±0.0107	0.202±0.092
6 ctrl	0.330±0.167	0.0763±0.0173	0.147±0.032
6 inoc	0.114±0.021	0.149±0.024	0.0708±0.0029
9 ctrl	0.144±0.0358	0.0506±0.0028	0.0735±0.0295
9 inoc	0.259±0.094	0.146±0.026	0.0810±0.0084
24 ctrl	0.263±0.151	0.0600±0.0110	0.168±0.045
24 inoc	0.317±0.0664	0.0877±0.0184	0.216±0.086

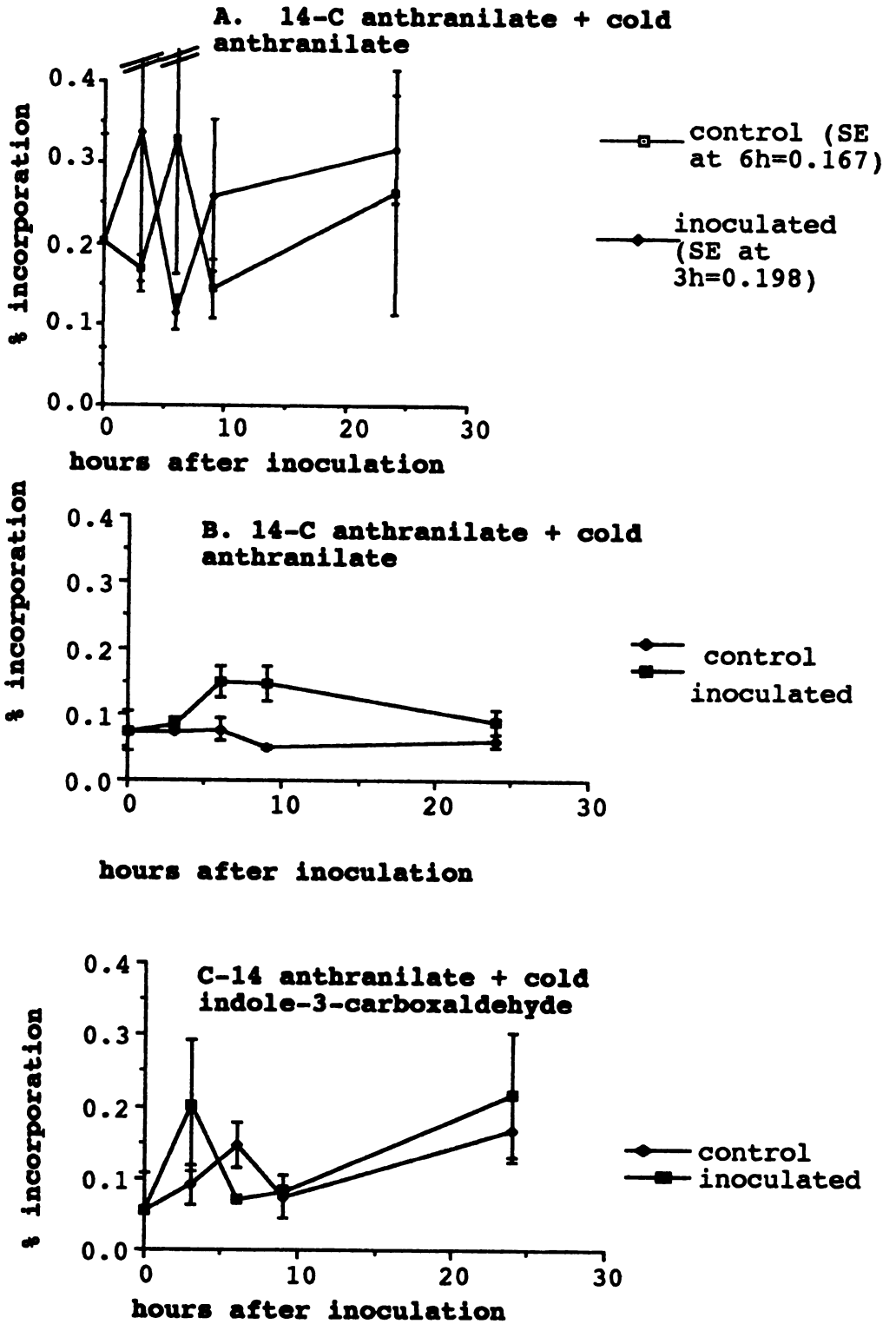


Figure 36. Percent incorporation of 14-C anthranilate into band 3 (Rf=0.7) over time.

Table 30. Average percent incorporation (mean plus standard error of 2 replicates) into band 3.5 (R_f=0.67) in leaves fed one of solutions A, B, or C and then inoculated with water or *C. carbonum*. Abbreviations and solution preparations are as in Table 25. Zeroes indicate that the zone scraped at this Rf had an activity below 100 cpm (roughly twice background on the scintillation counter).

hours after inoculation	% inc. ^{14}C-anthranilate	% inc. ^{14}C-anthranilate+cold anthranilate	% inc. ^{14}C-anthranilate+cold indole-3-carboxaldehyde
0	0.0733±0.0419	0.0372±0.0054	0.0158±0.0158
3 ctrl	0.0705±0.0138	0.0360±0.0016	0.0355±0.0083
3 inoc	0.0782±0.0208	0.0428±0.0140	0.0693±0.0361
6 ctrl	0.124±0.053	0.0290±0.0008	0.0595±0.0230
6 inoc	0.0670±0.0170	0.0821±0.0381	0.0269±0.0269
9 ctrl	0.0427 ±0.0009	0	0.0264±0.0264
9 inoc	0.0847±0.0015	0.0551±0.0073	0.0347±0.0056
24 ctrl	0.154±0.086	0.0548±0.0069	0.0913±0.0311
24 inoc	0.0139±0.0029	0.0640±0.0127	0.101±0.024

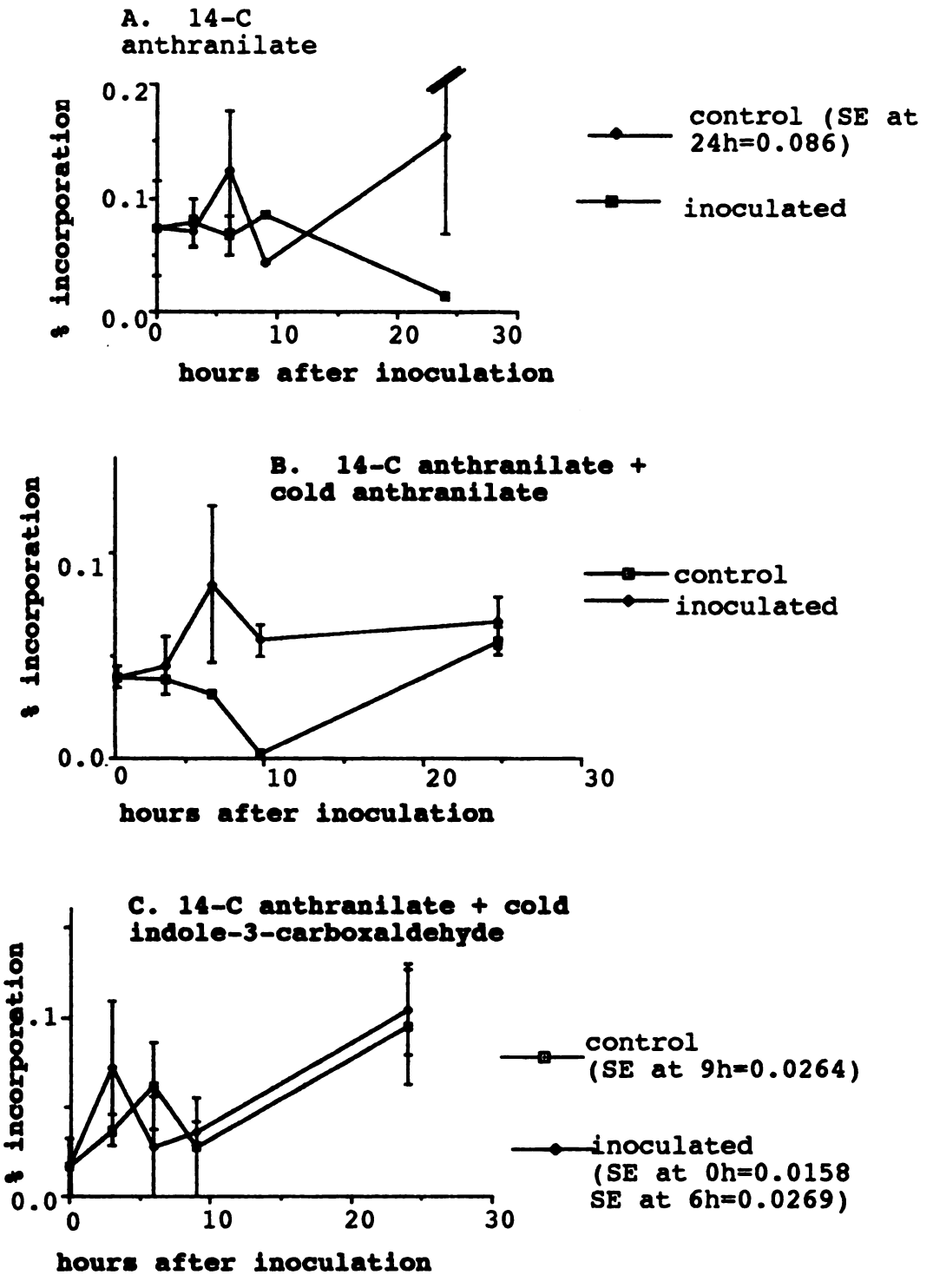


Figure 37. Percent incorporation of 14-C anthranilate into band 3.5 (Rf=0.67) over time. Graphs labeled as in figure 31.

Table 31. Average percent incorporation (mean plus standard error of 2 replicates) into band 5 (R_f=0.46) in leaves fed one of solutions A, B, or C and then inoculated with water or *C. carbonum*. Abbreviations and solution preparations are as in Table 25. Zeroes indicate that the zone scraped at this R_f had an activity below 100 cpm (roughly twice background on the scintillation counter).

hours after inoculation	% inc. solution A (^{14}C-anthranilate)	% inc. solution B (^{14}C-anthranilate+ cold anthranilate)	% inc. solution C (^{14}C-anthranilate)
0	0.174±0.008	0.228±0.023	0.125±0.035
3 ctrl	0.127±0.002	0.118±0.008	0.0837±0.0019
3 inoc	0.122±0.0018	0.126±0.012	0.0848±0.0036
6 ctrl	0.141±0.002	0.0905±0.0274	0.0838±0.0076
6 inoc	0.0896±0.0371	0.124±0.062	0.0695±0.0082
9 ctrl	0.149±0.004	0.0857±0.0003	0.0689±0.0005
9 inoc	0.0817±0.0358	0.0321±0.0321	0
24 ctrl	0.103±0.001	0.0776±0.0029	0.0564±0.0105
24 inoc	0.0327±0.0051	0	0.0142±0.0142

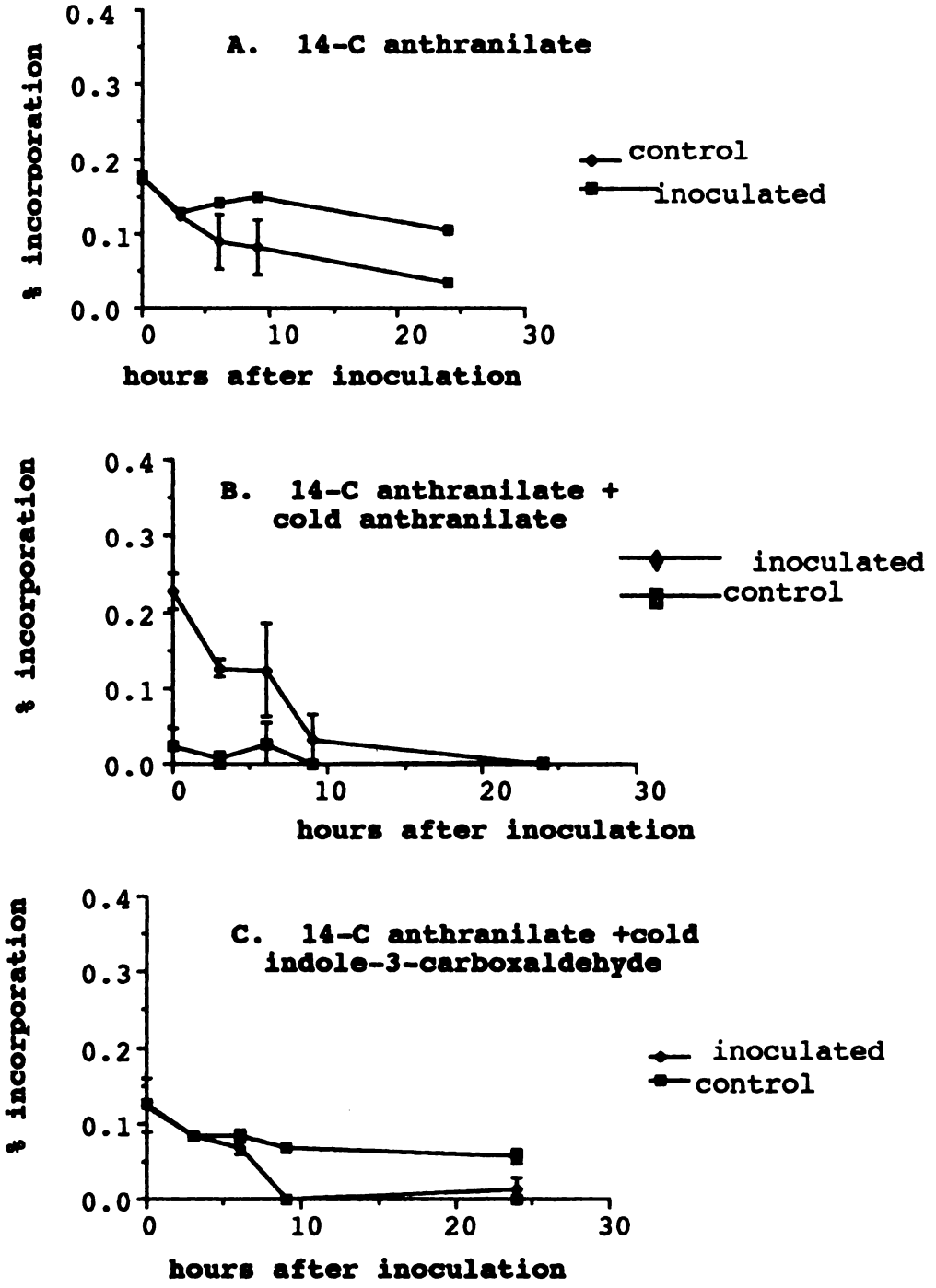


Figure 38. Percent incorporation into band 5 (Rf=0.45) over time. Graphs labeled as in Figure 31.

Table 32. Average percent incorporation (mean plus standard error of 2 replicates) into band 7 (Rf=0.27) in leaves fed one of solutions A, B, or C and then inoculated with water or *C. carbonum*. Zeroes indicate that the zone scraped at this R_f had an activity below 100 cpm (roughly twice background on the scintillation counter). Abbreviations and solution preparations are as in table 25.

hours after inoculation	% inc. solution A (^{14}C-anthranilate)	% inc. solution B (^{14}C-anthranilate + cold anthranilate)	% inc. solution C (^{14}C-anthranilate + cold indole-3-carboxaldehyde)
0	0.177±0.073	0.215±0.089	0.0596±0.0596
3 ctrl	0.0835±0.0010	0.0733±0.0102	0.0865±0.0050
3 inoc	0.0769±0.0096	0.0735±0.0123	0.120±0.028
6 ctrl	0.0536±0.0174	0.0390±0.0390	0.0628±0.0269
6 inoc	0.0284±0.0284	0.0351±0.0351	0.0422±0.0422
9 ctrl	0	0	0
9 inoc	0	0	0
24 ctrl	0	0	0
24 inoc	0.0137±0.0137	0	0.0154±0.0154

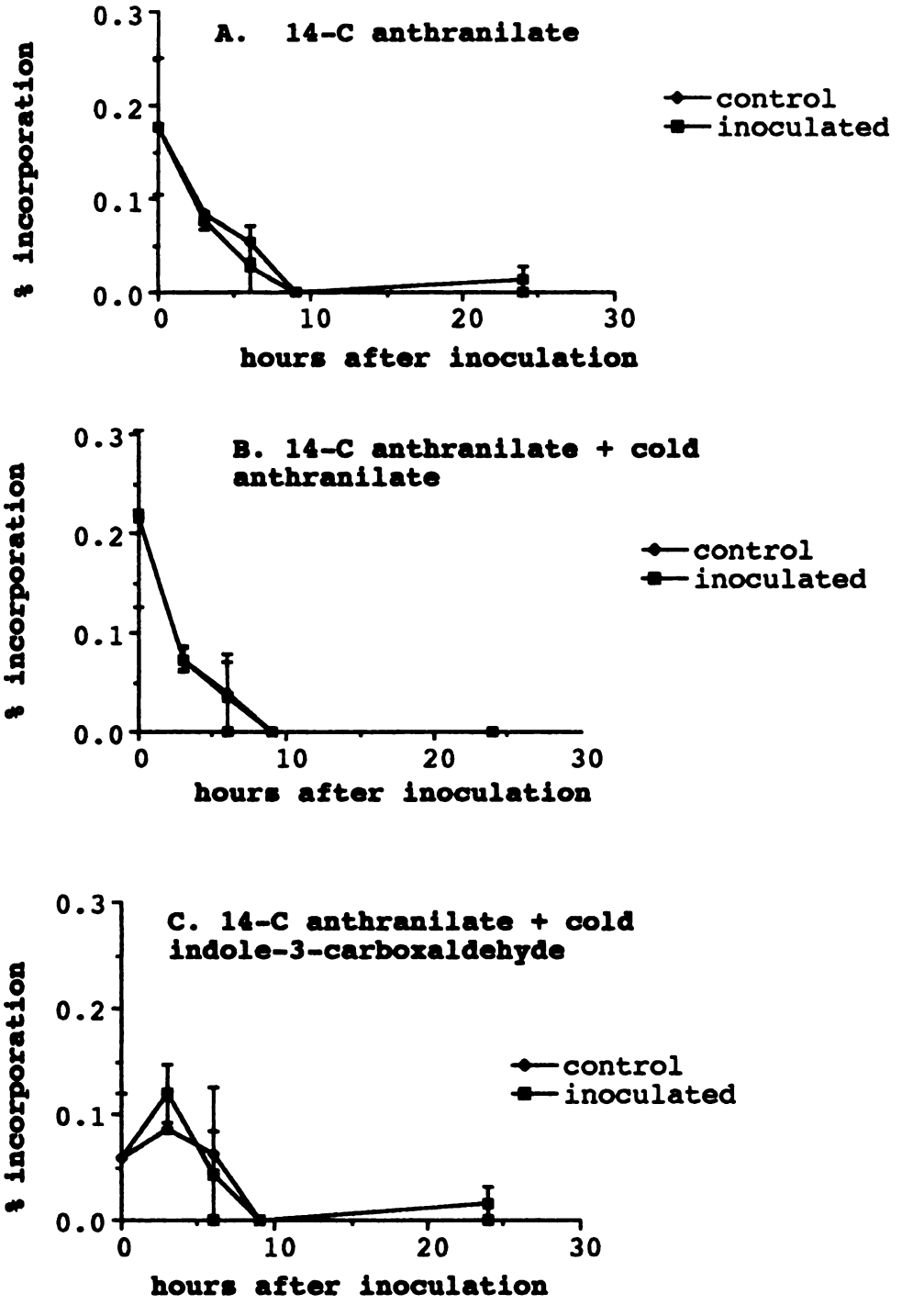


Figure 39. Percent incorporation into band 7 (Rf=0.27) over time. Graphs labeled as in Figure 31.

This part of the project demonstrated the difficulty of determining biosynthetic precursors in radiolabeling experiments, and perhaps above all else, the difficulty of balancing the need to work with small amounts of radioactivity and yet obtain measurable amounts of product. Given the difficulty of obtaining and interpreting the results, it was a pleasure to turn to the work described in the following chapter, partly because it provided hope for finding biosynthetic precursors.

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Chapter 5. Comparison of camalexin production and resistance to the fungus *Alternaria brassicicola* among twenty-four ecotypes of *Arabidopsis thaliana*.

Introduction

As explained in Chapter 1, the *pad* mutants initially seemed ideal plants to obtain information on camalexin biosynthesis and its role in disease resistance. The *pad* phenotypes would illustrate the relative importance of camalexin in disease resistance. The biosynthetic blocks due to the *pad* mutations would yield camalexin intermediates. However, no putative intermediates had been found in the pad mutants, except for indole-3-carboxaldehyde, whose kinetics of accumulation were similar in wild-type and in *pad2* plants.

The latest studies of *pad4* (Glazebrook et al., 1997; Zhou et al., 1998) demonstrated that the *PAD4* gene controlled the ability of Arabidopsis to produce salicylic acid, a compound necessary for potentiating camalexin production (Zhao and Last, 1996; Zhou et al., 1998) in one of at least two signal transduction pathways. A study of the *pad* mutants indicated that camalexin plays a part in restricting growth of some bacterial pathogens and in imparting resistance to the oomycete *Peronospora parasitica* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). It was possible that the other *pad* mutations might be regulatory as well, and that conclusions being drawn about the role of camalexin in

disease resistance might reflect the effects not of biosynthetic blocks, but of mutations in other genes regulating signal transduction. What other methods, or other sources of biosynthetic blocks, might be available to evaluate the role of camalexin in disease resistance?

It seemed possible that natural variation in Arabidopsis populations would provide answers. Many ecotypes of Arabidopsis thaliana (plants of the same species grown in different environments) have been collected and made commercially available. Although these ecotypes are similar enough to be considered the same species, they differ genetically in some respects because of selection pressures imposed by the different environments. It is generally expected that plants of the same species in different locations will differ genetically (Gerson and Kelsey, 1998). These differences frequently include variation in resistance or susceptibility to certain pathogens. For example, different ecotypes of Arabidopsis differ in their ability to resist infection by the slime mold *Plasmodiophora brassicae* (Fuchs and Sacristan, 1996), cauliflower mosaic virus (Callaway et al., 1996; Leisner and Howell, 1992) turnip crinkle virus (Dempsey, 1996; Dempsey et al., 1997), the bacterium *Xanthomonas campestris* pv. *campestris* (Tsuji et al., 1991) the oomycete *Albugo candida* (Holub et al., 1995), and the oomycete *Peronospora parasitica* (Mauch-Mani et al., 1993). In those cases, resistance to a pathogen was due to the presence of one or more genes, which some ecotypes had

and others did not.

Plant species in different environments also differ in the kinds of compounds that they produce. The same pine species, in different locations, will qualitatively and quantitatively produce different piperidine alkaloids (Gerson and Kelsey, 1998). Some oat species do not produce avenacins, which are saponins that provide resistance to the wheat pathogen *Gaeumannomyces graminis* var. *tritici*, and these avenacin-deficient species are susceptible to that pathogen (Osbourn et al., 1994). Since camalexin is produced by other genera in the Brassicaceae, the ability to produce it is a relatively ancestral trait, and an absence of camalexin among some ecotypes of *Arabidopsis thaliana* could represent a relatively recent evolutionary divergence within the species. The absence of camalexin would conceivably reflect differences in selection pressures of each habitat. Since camalexin is not effective against all pathogens (Rogers et al., 1996; Pedras et al., 1997), and sulfur is a scarce element in plants, it is reasonable to think that genes for camalexin production would be lost in environments where it served no purpose. However, genes for production of precursors might remain if any conferred a selective advantage in a habitat.

It seemed reasonable, therefore, to look for variation in camalexin production among Arabidopsis ecotypes and to see whether any differences would be correlated with differences in response to a given pathogen. A few outcomes were

possible, assuming variation in response to the pathogen. Resistant and susceptible plants might produce similar amounts of camalexin, or differing amounts that did not correlate with any type of response. Alternatively, there might be a pattern of either the resistant or the susceptible plants accumulating more camalexin. Those plants accumulating less camalexin might accumulate precursors--antimicrobial ones providing resistance, or non-antimicrobial ones that would not impede or contribute to resistance.

For this study, 24 ecotypes of *Arabidopsis thaliana* were screened for the ability to produce camalexin when inoculated with *Cochliobolus carbonum*. This fungus was chosen because it induced high concentrations of camalexin in the Columbia ecotype (see Chapter 2, tables 2 and 3; and Chapter 3, Tables 6-11). Because *C. carbonum* is an incompatible pathogen of Arabidopsis, and phytoalexin production frequently occurs in incompatible interactions, it seemed like a good neutral inducer to use in testing the general ability of other ecotypes to make camalexin. A compatible pathogen might not induce phytoalexin production, or induce a slower initial rate of accumulation. At the same time that plants were tested for their general ability to produce camalexin, they were inoculated with the fungus *Alternaria brassicicola*, a common crucifer pathogen that causes disease primarily on species of the genus *Brassica* but also on other genera (Ellis, 1968). Symptoms of the different ecotypes were evaluated, and correlations were sought between resistance to

A. brassicicola and ability to produce camalexin.

Materials and Methods

Reagents and Chemicals.

Camalexin was from the source used for the radiolabeling experiments described in Chapter 4. The concentration of a sample was checked by HPLC, using the parameters described in Chapter 3, and the sample was redissolved in methanol to give a 0.5 mg/ml solution. The solution was stored at -20 °C to prevent evaporation or degradation. Some of the indole-3-carboxaldehyde used was purchased from Sigma Chemical Company and used without further purification. Some was purchased from Aldrich Chemical Company and recrystallized from 95 % ethanol. Indole-3-carboxylic acid was from Aldrich. Solvents used for HPLC were of HPLC grade. The methanol used for TLC was of HPLC grade, since a casual check demonstrated that separation of compounds was better if the methanol component of the TLC solvent was of HPLC grade. All other chemicals were of reagent grade or better.

Plant Material and Plant Growth Conditions.

The following Arabidopsis ecotypes were purchased from Lehle Seed Company (Tucson, AZ): Aua/Rhon, Bensheim, C24, Cape Verde, Columbia-0, Columbia-glabrous, Dijon G, Estland, Greenville, Kendalville, Landsberg erecta, Muhlen, Niederzenz, Nossen, RLD, RLD1, S96, Turk Lake, Wassilewskija.

Ecotypes Ksk-1, Kas-1, Wei-1, and WA-1 were provided by S. Somerville (Carnegie Institute, Washington). Ecotype Anna was provided by Jeff Conner (Kellogg Biological Station, Hickory Corners, Michigan). Seeds were stored in desiccant at room temperature, except for Columbia-0 and Columbia-gl, which were stored at 4 °C.

Seeds were grown in clay pots (14.5 cm or 10 cm in diameter) containing Baccto® High Porosity Professional Planting Mix. The soil was wetted with Peters Professional Fertilizer (2.49 g/l, as specified on the package) and hand-compacted to provide a level surface, and a 1- to 2-mm layer of fine vermiculite was sprinkled over the surface. Seeds were transferred to small dishes of water and distributed over the vermiculite surface with a Rainin P200 automatic pipettor, using pipet tips whose ends had been cut to make them wide enough to take up seeds. Pots were covered with plastic wrap until the seeds germinated, as described in Chapter 2. Plants were grown in a growth chamber kept at 21 °C day and night, with a 16-hour light regime.

Fungal cultures and inoculations.

Cochliobolus carbonum and *Alternaria brassicicola* were grown on V-8 agar (see Chapter 4) at room temperature under a 24-hour light regime. Inoculations with these cultures were always done on the same day, using 7- to 14-day-old spores. For *C. carbonum* inoculations, leaves were excised with a razor blade and laid, adaxial side down, in Petri dishes

lined with moistened filter paper. Leaves were inoculated with 0.1 ml of spores ($2.0x10^5$ spores/ml). For *A. brassicicola* inoculations, a spore suspension was sprayed onto intact plants with a commercial mister. The suspension was prepared in water containing 0.05 % Tween-20, which acted as a surfactant and created finer and more uniform droplets. Each pot received roughly the same amount of inoculum. To maintain a humid environment conducive to fungal infection, pots were then covered with plastic bags that had been wetted on the inside with water and held over a steam bath to ensure that the inside was uniformly moist. The plastic bags were arranged to cover the pots fairly snugly, again to maintain a humid environment. Plants were left covered in this way for 24 hours to allow the fungus ample time to infect (minimum of 18 hours required [R. Hammerschmidt, pers. comm.]). A 3-cm slit was then made in each bag, to allow the plants to adjust gradually to the outside environment. The slit was widened, usually 17 hours later, to about 7 cm. The 7-cm slit was widened 4-7 hours later (44-48 hours after inoculation) to a hole the size of the pot diameter. Symptoms were first recorded about 60 hours (2.5 days) after inoculation. Bags were usually removed at this time. Symptoms were then recorded once a day for an additional 3 to 7 days, so that the record of symptoms spanned 2.5-9.5 days post-inoculation (dpi).

Cytological analysis of *A. brassicicola* infection.

Leaves of one susceptible and two resistant ecotypes, and of one ecotype with an intermediate degree of resistance, were excised 19, 48, and 72 hours after inoculation. Leaves were placed in glass Petri dishes lined with 2 layers of Whatman #1 or #4 filter paper saturated with a fixative consisting of ethanol-acetic acid 3:1 (v/v). This solution, by diffusing into the leaves, usually removed pigments in 2 to 5 days. Additional solution was pipetted over leaves once or twice a day to keep them from drying out. Excess solution, usually quite green, was sometimes removed prior to adding fresh solution. Doing so helped to speed the pigment-removal (clearing) process, probably because it prevented the equilibration of pigments remaining in the leaves with pigments in the surrounding solution. After a few days, ethanol alone was added to the leaves, as the concentration of residual acetic acid in the filter paper and leaves was quite high by then. Prior to being mounted for microscopy, leaves were rinsed with 3-5 aliquots of deionized water (changed every 3-12 hours over a 1- to 2 -day period) to remove the ethanol and acetic acid. Leaves were then transferred to glass slides, blotted dry with tissue paper, mounted in glycerol, and stored at 4 °C until photographed for microscopy. Light micrographs were done on a Leica microscope at a magnification of 400x, using Nomarski differential interference contrast (DIC) optics.

Extraction of camalexin from inoculated leaves.

For each sample of leaves inoculated with *C. carbonum*, inoculum droplets from 10 leaves were pooled and extracted as described in Chapter 3. Samples were stored dry at 4 °C or -20 °C until analysis.

To examine camalexin production in response to *A. brassicicola*, 0.1-0.7 g of leaves (6-75 leaves) were excised 48 hours after inoculation (a time when camalexin was almost always present in high concentrations in *C. carbonum*-inoculated plants [Chapter 3]). Because the leaves were sprayed with spores instead of being inoculated with a known droplet volume, the area of infected tissue was less consistent, and consequently, it seemed reasonable to quantitate camalexin based on micrograms of camalexin per gram of fresh weight (µg/gfw) instead of micrograms per leaf. The leaves were weighed and boiled in 80 % methanol (added such that the ratio of solvent volume to leaf weight was 150:1) for 20 minutes. Samples were usually stored at 4 °C for 24-60 hours and then extracted in the manner of leaf tissue extractions described in chapter 3.

Thin-layer chromatography (TLC) of extracts.

Droplet extracts were redissolved in 35 µl of ethyl acetate, and the entire sample was loaded (in 5-µl aliquots) onto plastic- or glass-backed silica-gel TLC plates (silica thickness: 200 µm on plastic-backed plates [Selecto Scientific], and 250 µm on glass-backed plates [Analtech]).

In one case, leaf extracts were dissolved in 50 µl of chloroform, and the entire sample was loaded. Because so many pigments dissolved in the chloroform, it was not certain that the camalexin was well-separated from pigments. Consequently, in subsequent experiments, samples were dissolved in 35 to 60 µl of methanol, depending on the amount of leaf tissue from which the extract was made.

Known amounts of standards of camalexin, indole-3-carboxaldehyde, and indole-3-carboxylic acid (usually 5 µg, 1.25-5 µg, and 2.5-5 µg, respectively) were also loaded. Plates, which had been washed and activated prior to use, were developed once in chloroform and then twice in chloroform-methanol 19:1. Camalexin, indole-3-carboxaldehyde, and indole-3-carboxylic acid were identified in samples by seeing which bands comigrated with the appropriate standards. The plates were photographed with Kodak slide film (100 ASA) under long-wave or short-wave ultraviolet light, with an Olympus OM-1 camera. A Kodak Wratten filter for blue light was taped over the camera lens. The camera was mounted onto a camera stand or a round ring stand clamp and held about 52 cm above the plate. A hand-held UV lamp was attached with a clamp about 7 cm above the TLC plate. To obtain a sharper image of bands, the camera was focused on the scored lanes on TLC plates, and the lens was then raised 4 mm for photographing under long-wave UV light, and 20 mm for photographing under short-wave UV light. An exposure time of about 1 minute was used for long-wave UV

light exposures. This exposure time was sufficient for short-wave UV exposures of fluorescent-backed TLC plates. For non-fluorescent-backed plates, an exposure time of 2.5 minutes was necessary.

Bioassays for evaluation of camalexin production in response to *C. carbonum* and *A. brassicicola*.

To compare camalexin production among the different ecotypes, and to identify other antimicrobial compounds, TLC plates were sprayed with a suspension of *Cladosporium cucumerinum* spores (usually 3-3.5x10^6 spores per ml) in half-strength potato-dextrose broth (per liter: 100 g potatoes, 5 g dextrose) and incubated in a humid chamber for 3 days, so that spores grew everywhere except in fungal-inhibitory areas such as camalexin bands. TLC plates containing extracts of *A. brassicicola*-inoculated leaves were sprayed with *A. brassicicola* (5x10^5 to 1x10^6 spores/ml). Cultures used for bioassays were 7-11 days old, but an effort was made to use 7-day-old cultures, as the younger cultures contained more viable spores (R. Hammerschmidt, pers. comm.).

Quantitation of camalexin.

Camalexin was quantitated from the bioassayed plates according to the method of Lazarovits et al. (1982), who found that the radii of zones of inhibition at the site of a compound on a TLC plate were directly proportional to the logarithm of the mass of the compound present. The zones of

inhibition at the R_f of camalexin were traced onto white paper and scanned with a Hewlett-Packard ScanJet 4c scanner (resolution of 150 dots per inch in both the horizontal and vertical directions). The area in pixels was determined with the Jandel Sigma-Scan program. These numbers were converted into square centimeters (cm^2) based on the number of pixels in standards of known size. Since the area of a circle is equal to πr^2 (π=3.14159), the radius (r) of the zone of inhibition was calculated by the formula $r=(A/\pi)^{1/2}$. The mass of camalexin was determined from a standard curve (Figure 40) of inhibitory zone radius versus $\log_{10}$ of mass of a known amount of camalexin.

RESULTS

Phenotypic characterization of responses to *Alternaria brassicicola*.

In general, symptoms of *A. brassicicola* infection consisted of circular brown or gray lesions (1-20 per leaf), ranging in size from less than 0.5 mm to 1.5 mm in diameter. The larger lesions were sometimes irregularly shaped and possibly consisted of several smaller lesions that had coalesced. Leaves with lesions were sometimes chlorotic in the entire area in which the lesions were found, but sometimes the chlorosis was confined to a yellow halo around the lesion, which appears to be fairly typical of *A.*

brassicicola infection (Ellis, 1968; Conn et al., 1988). A condition termed "spreading chlorosis" was sometimes noted on susceptible plants, in which a chlorotic zone began around the leaf midrib and radiated outward, with no fixed boundaries. Sometimes leaves did not become chlorotic around the lesions, and the lack of chlorosis was considered a sign of resistance. Leaves without lesions were sometimes chlorotic as well. This might be partly due to the inoculation procedure, as control plants inoculated with water + 0.05 % Tween-20 sometimes had some chlorotic leaves. However, most chlorosis was from the infection, since there was more chlorosis in infected than in control leaves.

On some plants, leaves with lesions wilted and became necrotic 3 to 7 days post inoculation (dpi). Even on plants that appeared to have no symptoms of infection, it was common to find that at least one of the oldest leaves (usually close to the ground, and often closely surrounded by neighboring plants and hence easily overlooked) was dead 3 to 5 dpi. Apparently, the older leaves are more susceptible than the younger leaves to *A. brassicicola* infection. Another symptom of infection was the formation of a lesion spanning the cross-sectional diameter of the petiole. Leaves with such lesions usually died. Since *A. brassicicola* is a necrotrophic fungus (Boyd et al., 1994), quickly strangling the plant would speed up its ability to acquire nutrients.

Few of the ecotypes tested had specific characteristic disease phenotypes. Leaves could have spreading chlorosis

and large lesions in one experiment, and small lesions with chlorosis only around the lesions in another experiment. Consequently, the ecotypes in Table 33 were grouped by degree of resistance (high, intermediate, or low). Any consistent phenotypic traits were noted, but as these were fairly rare, it seemed artificial to use those as categories.

For each ecotype, the severity of infection and degree of resistance were judged partly by the percentage of leaves with visible symptoms. The number of plants was counted at the start of symptom observation, and the total number of leaves was calculated based on the assumption that each plant had at least five obvious rosette leaves. Since plants seldom bolted before being inoculated, cauline leaves were not counted. Disease severity was also judged by whether the number of diseased leaves increased over time. Plants that had symptoms on 15 % of the leaves at the start of the observation period and did not develop more symptoms could have been responding with an HR and stopping the spread of the fungus. In contrast, if the number of diseased leaves increased, it seemed that the plants might be less capable of stopping the spread of the pathogen.

Plants with a high degree of resistance tended to have few chlorotic leaves, if any, and fewer than 10 % of the leaves had lesions. Sometimes the only symptoms were the dead rosette leaves. Plants with a moderate degree of resistance had lesions on 10 % to 40 % of leaves. The symptoms sometimes looked rather severe. That is, the

lesions and chlorotic/necrotic areas could be quite large. However, if relatively few leaves displayed symptoms, it did not seem right to rate the plant as not resistant. The "intermediate" category included plants with gradually increasing numbers of diseased leaves, and plants with symptoms that varied greatly from one experiment to the next. Since those plants had a wide range of possible responses to infection, it was difficult to rate them as resistant or susceptible.

Plants with a low degree of resistance had large lesions on many leaves at the start of the observation period. An increase in the number of diseased leaves (up to 50-100 %) was common. Necrosis, wilting, and death would begin 3-4 days after inoculation. Leaves often had the "spreading chlorosis" mentioned before.

The disease phenotypes of the 24 ecotypes are shown in Table 33, with additional comments about symptoms unique to certain ecotypes. For ecotypes with greatly varying degrees of susceptibility, those variations are noted.

The degree of resistance or susceptibility in each ecotype depended partly on the light regime and the concentration of inoculum used. When plants were grown under less light, some of the normally resistant ecotypes, such as Bensheim, had severe symptoms. These plants were somewhat stunted at the outset and probably did not have enough reserves for normal metabolism, let alone infection conditions. At a higher concentration of inoculum ($3.5x10^5$

Table 33. Ecotypes studied for camalexin production and *A. brassicicola* resistance: abbreviation, origin, and disease phenotype.

Ecotype name and common abbreviation, if known	Place of origin, if known	degree of resistance	additional traits
Anna	USA (MI)	high	displayed symptoms in only 1 out of 6 experi-ments
Aua/Rhön (Aa)	Germany	intermediate	---
Bensheim (Be)	Germany	high	---
C24	---	low	large lesions, usually at margins
Cape Verde (Cvi-0)	Cape Verde Islands	intermediate	large lesions and spreading chlorosis
Columbia-0 (Col-0; no genetic markers)	USA	intermediate	variable symptoms (<5% to 15% diseased leaves)
Columbia-glabrous (Col-gl; glabrous leaves used as a genetic marker)	USA	low	large lesions at margins and rapid development of wilting and necrosis
Dijon G (Di-G)	France (natural habitat: botanic garden)	low	---
Estland (Est)	Estland, former USSR	intermediate	---
Greenville (Gre)	USA (MI)	high	---
Kas-1	India	low	---
Kendalville	USA (MI)	high	---
Ksk-1		high	---

Table 33 (cont'd).

Landsberg erecta (La-er)	Germany	intermediate	---
Muehlen (Mh)	Poland	high	---
Niederzenz (Nie)	Germany	intermediate to low	lesions usually small (<0.5-1 mm in diameter) and numerous (5-10 per diseased leaf)
Nossen	---	high	---
RLD	Russia	high	leaves sometimes developed lesions but seldom became chlorotic
RLD 1	Russia	intermediate to high	large necrotic zones following lesion development
S96	Koornneef Group, Nether-lands	high	leaves sometimes appeared more chlorotic than on most ecotypes due to leaf morphology (leaves more spread out than on most ecotypes)
Turk Lake	USA (MI)	high	very seldom had any symptoms
WA-1	Poland	intermediate	large circular lesions
Wassilewskija (Ws)	Russia	intermediate	---
Wei-1	Switzerland	intermediate to low	round lesions about 1.5 mm in diameter

spores/ml), almost all of the ecotypes tested (Mühlen, RLD1, RLD, Columbia-0, Greenville, Kendalville, Estland, S96, Aua/Rhön, Nossen, and Anna) with the exception of Anna, had severe symptoms. At a lower inoculum concentration ($1.0x10^5$ spores/ml), all plants tested (Anna, Columbia-0, Columbia-glabrous, Cape Verde, C24, Ksk-1, Landsberg erecta, Niederzenz, Wei-1, Wa-1, Wassilewskija, and Kas-1) appeared resistant except for Niederzenz, Wei-1, and Wassilewskija. Symptoms could vary considerably from one experiment to the next. Dijon G, although it consistently had lesions and chlorotic leaves, sometimes had large lesions within chlorotic areas and sometimes had many small necrotic lesions less than 0.5mm in diameter.

Camalexin production in response to *C. carbonum*.

All ecotypes were capable of producing camalexin (Table 34). The Dijon G ecotype appeared to produce less than the others, and on some occasions, it produced none. However, no ecotype consistently failed to produce camalexin. Although the amount produced was not always enough to inhibit *Cladosporium cucumerinum in a bioassay*, it could usually, in those cases, be detected under ultraviolet light (Figure 43A). The Col-0 ecotype sometimes produced very little camalexin. Given the results of the time courses described in Chapter 3, this variability in the amount of camalexin produced may reflect variation in the kinetics of camalexin accumulation from one experiment to the next. Inoculum

droplets were always collected 24 hours after inoculation, and as was seen in one time course (Chapter 3, Figure 12), camalexin concentrations are sometimes still low then. Because the spore concentration was one at which the majority of the camalexin should have diffused into the droplets (Chapter 3, Tables 8 and 9), differences in camalexin production were not likely to be due to variable partitioning between droplets and leaves.

A few zones of inhibition besides camalexin were observed on some TLC plates after bioassaying with *C. cucumerinum*. However, these were not consistently present in all experiments, and even between replicates, their presence was inconsistent.

Although its identity was not verified, a compound at the approximate R_f of indole-3-carboxylic acid was observed in most extracts. Whether this compound was present as a precursor or metabolite of camalexin, or as a degradation product of other indole compounds in Arabidopsis, is unknown. No zones of inhibition were formed at the R_f of that compound, demonstrating that if it was indole-3-carboxylic acid and a precursor of camalexin, it was not antimicrobial at the concentrations in which it was present. The amount of indole-3-carboxylic acid standard loaded (2.5 µg) was also not inhibitory.

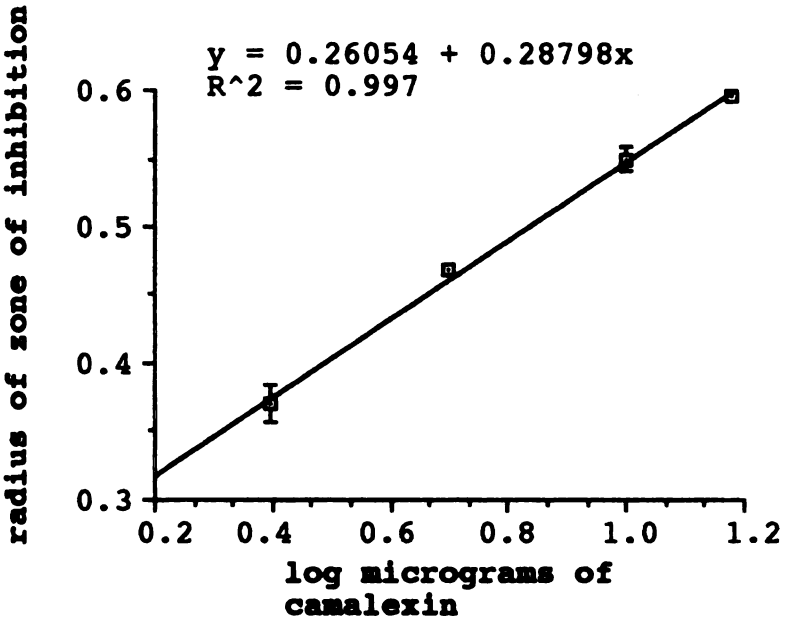

Figure 40. Relationship between log of micrograms of camalexin and radius of zone of inhibition on TLC plates bioassayed with *Cladosporium cucumerinum*,. The radii are the mean radii of zones of inhibition from two separate TLC plates onto which known amounts of camalexin were loaded.

Table 34. Camalexin production in response to inoculation with *C. carbonum*. Droplets of inoculum ($2x10^5$ spores/ml) were removed from leaves 24 hours after inoculation and extracted with ethyl acetate. Camalexin on TLC plates was quantitated by measuring the area of the zone of fungal inhibition after bioassay with *Cladosporium cucumerinum*. Numbers represent means plus standard errors of three computer scans of a traced zone of inhibition. The last column in the table denotes the amount of camalexin produced relative to the amount produced by the Col-0 ecotype extracted and bioassayed on the same dates. Where two extracts of Col-0 were extracted and bioassayed on the same date, the average of the results was used. Samples of Col-0 that were extracted on the same day and bioassayed on two different days were not averaged.

Symbols and abbreviations: A "*" denotes that the amount of camalexin in the Col-0 ecotype could not be used for comparison, due to not being extracted or to the lack of a measurable zone of inhibition. The letters "ND" indicate that the amount of camalexin produced was not detectable by a TLC plate bioassay (no zone of inhibition was formed). A "---" indicates that the amount of camalexin produced relative to Columbia-0 was not calculated because the amount produced was too low to quantitate.

Table 34.

ecotype	date of inoculation	date of bioassay	micrograms camalexin per 10 leaves	% of amount of camalexin produced by Col-0
Anna	7/7/98	7/21/98	0.659±0.000	*
			0.864± 0.000	*
	7/24/98	8/23/98	7.16±0.01	81.9
	8/6/98	9/1/98	1.35±0.00	*
	9/21/98	10/8/98	5.92±0.00	331
			5.18±0.01	289
Aua/Rhön	7/24/98	8/23/98	4.15±0.01	47.5
			2.54±0.00	29.1
	8/15/98	9/28/98	0.851±0.00	*
	10/24/98	11/11/98	87.0±0.0	82.3
			121±0	114
Bensheim	8/8/98	9/1/98	3.70±0.13	243
			ND	---
	8/19/98	9/28/98	4.59±0.00	425
			4.12±0.00	381
	10/4/98	10/16/98	489±0	91.2
			565±0	105
C-24	7/24/98	8/23/98	2.80±0.00	32.0
			3.58±0	41.0
	8/8/98	9/17/98	3.18±0.00	209
		9/1/98	1.38±0.01	*
Cape Verde	7/24/98	8/23/98	2.56 ±0.00	29.3
			0.788±0.001	9.02
	8/8/98	9/17/98	3.56±0.00	234
		9/1/98	12.5±0.0	*
Col-0	7/24/98	8/23/98	8.74±0.01	N.A.
	8/8/98	9/17/98	1.52±0.00	
	8/8/98	9/1/98	ND	
	8/15/98	9/28/98	ND	
	8/19/98	9/28/98	0.907±0.000	
	8/19/98		1.26±0.00	
	9/5/98/	9/28/98	5.88±0.02	
			4.81±0.00	
	9/21/98	10/8/98	1.76±0.00	
			1.82±0.00	
	10/4/98	10/16/98	551±0	
			521±0	
	10/24/98	11/11/98	113±0	
			98.3±0.1	

Table 34 (cont'd).

Columbia-gl	7/24/98	8/23/98	1.01±0.00	11.6
			2.42±0.00	27.7
	8/19/98	9/28/98	1.09±0.00	101
	9/21/98	10/8/98	2.74±0.00	153
			ND	---
Dijon G	7/7/98	7/21/98	0.585±0.000	*
			0.804±0.000	*
	7/24/98	8/23/98	ND	---
	8/8/98	9/17/98	ND	---
		9/1/98	1.53±0.00	*
	9/21/98	10/8/98	ND	---
Estland	8/19/98	9/28/98	0.747±0.000	69.2
			ND	---
	9/21/98	10/8/98	2.10±0.00	119
			1.92±0.00	107
Greenville	7/7/98	7/21/98	2.95±0.00	*
	7/24/98	8/23/98	8.75±0.01	100
	8/15/98	9/28/98	ND	---
	9/21/98	10/8/98	2.94±0.00	164
			3.62±0.00	202
	10/4/98	10/16/98	347±0	64.7
			224±0	41.8
Kas-1	7/24/98	8/23/98	5.31±0.01	60.8
	8/8/98	9/1/98	3.36±0.00	*
		9/17/98	4.76±0.01	313
	10/4/98	10/16/98	537±2	100.
			313±0	58.4
Kendalville	7/24/98	8/23/98	2.05±0.01	23.4
			3.06±0.00	35.0
	8/8/98	9/1/98	2.95±0.00	*
			ND	---
Ksk-1	8/8/98	9/1/98	1.87±0.00	*
	8/19/98	9/28/98	3.06±0.00	289
	9/21/98	10/8/98	3.70±0.00	207
			7.13±0.00	398
Landsberg erecta	7/24/98	8/23/98	1.85±0.00	21.2
			3.33±0.01	38.1
	8/8/98	9/17/98	ND	---
		9/1/98	2.96±0.00	*
Mühlen	7/24/98	8/23/98	10.4±0.0	119
	8/19/98	9/28/98	3.14±0.00	291
			0.728±0.000	67.4
	9/21/98	10/8/98	1.97±0.00	110.
			1.61±0.00	89.9

Table 34 (cont'd).

Niederzenz	7/24/98	8/23/98	4.51±0.00	51.6
			2.79±0.00	34.0
	8/8/98	9/1/98	1.76±0.00	*
	9/21/98	10/8/98	2.19±0.00	122
Nossen	7/24/98	8/23/98	1.56±0.01	17.8
			1.76±0.00	20.1
	8/8/98	9/17/98	1.66±0.00	109
		9/1/98	0.405±0.000	*
RLD	7/7/98	7/21/98	1.84±0.00	*
			2.16±0.00	*
	7/24/98	8/23/98	6.56±0.01	75.0
	8/8/98	9/1/98	1.30±0.00	*
		9/17/98	ND	---
	9/21/98	10/8/98	3.86±0.00	188
			2.77±0.00	155
RLD1	7/7/98	7/21/98	3.53±0.00	*
			2.04±0.00	*
	7/24/98	8/23/98	8.95±0.01	102
			4.48±0.00	512
	9/21/98	10/8/98	2.38±0.00	133
S96	7/24/98	9/1/98	3.24±0.00	37.1
			5.65±0.01	64.6
	8/8/98	9/17/98	2.09±0.00	202
		9/1/98	5.03±0.00	*
Turk Lake	8/8/98	9/1/98	ND	---
	9/5/98	9/28/98	5.31±0.00	99.4
			5.79±0.00	108
			6.77±0.02	127
WA-1	7/24/98	8/23/98	2.78±0.00	31.8
	8/19/98	9/28/98	ND	---
	9/21/98	10/8/98	2.60±0.00	145
Wassilew-skija	7/24/98	8/23/98	1.88±0.00	21.5
			ND	---
	8/15/98	9/28/98	ND	---
	10/4/98	10/16/98	560.±0	104
			410.±0	76.5
Wei-1	7/24/98	8/23/98	5.30±0.00	60.6
			2.80±0.00	32.0
	8/8/98	9/17/98	0.841±0.000	55.3
	8/19/98	9/28/98	2.06±0.00	191

Camalexin production in response to *A. brassicicola*.

Of ten ecotypes extracted after inoculation with *A. brassicicola*, only two (Kas-1 and RLD) produced enough camalexin to be detected on TLC plates. No zones of inhibition were formed in TLC plate bioassays. When the experiment was repeated with nine of those ecotypes, using about five times more leaves than in the first experiment, only Kas-1, Anna, and RLD produced enough camalexin to detect under UV light. After bioassay, faint zones of inhibition at the R_f of camalexin were detectable in extracts from Anna and Kas-1, but not from any other ecotype. Anna contained two other weakly antimicrobial compounds. The absence of zones of inhibition may reflect not only a lack of camalexin, but a lack of sensitivity of *A. brassicicola* to camalexin. The zone of inhibition around the standard was smaller and less clearly defined than it was on a plate containing the same concentration of standard and sprayed with *C. cucumerinum*.

Cytological analysis of infection in resistant and susceptible ecotypes.

About the same number of conidia per square centimeter were present on the surfaces of resistant and susceptible ecotypes (Figures 41 and 42). Therefore, differences in response to *A. brassicicola* were not due to differences in the number of spores available to cause infection. On the resistant ecotype Turk Lake, few signs of infection were seen

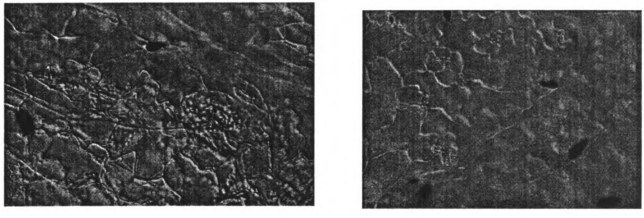

Figure 41. Light micrographs (400x magnification, Nomarski optics) of ecotype Kas-1(low degree of resistance) 19 hours (right) and 48 hours (left) after inoculation. Note the hypha in the cell at 48 hours, and the disorganized appearance of the cell.

Figure 42. Light micrographs (400x magnification, Nomarski optics) of ecotypes Turk Lake and RLD (high degree of resistance). Figure 42A, Turk Lake, 72 hours after inoculation with A. Brassicicola. Note the short germ tubes. Figure 42B, RLD, 19 hours after inoculation. Germ tubes are longer than germ tubes on Turk Lake, but few signs of penetration are visible. Figure 42C, RLD, 72 hours after inoculation. Penetration has occurred in the cell beneath the topmost conidium (upper middle, near the right), and the infected cell appears collapsed.

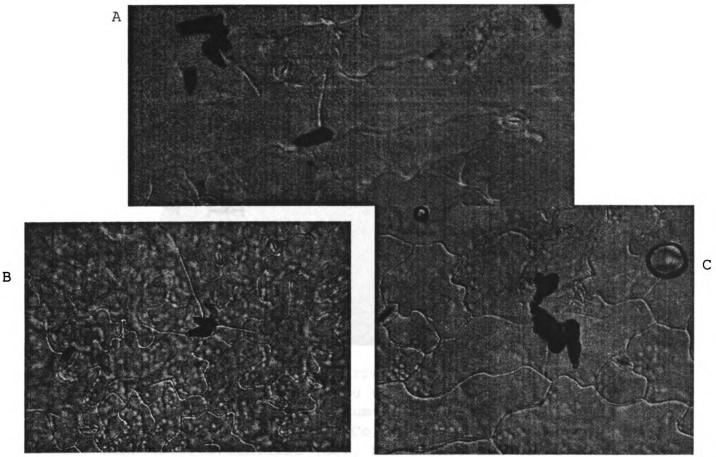

Figure 42.

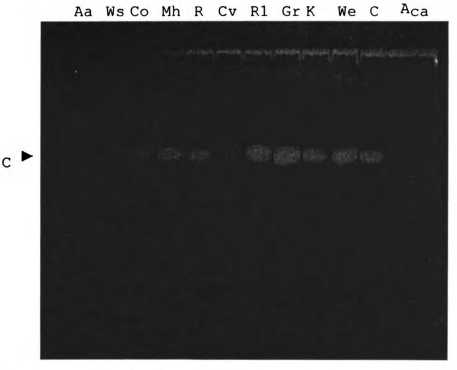

Figure 43a. Effects of *Cochliobolus carbonum* (neutral inducer) on camalexin production. TLC plate (viewed under short-wave UV light, of inoculum droplet extracts of *C. carbonum*-inoculated ecotypes Aua/Rhon (Aa), Wassilewskija (Ws), Columbia-0 (Co), Muhlen (Mh), RLD (R), Cape Verde (Cv), RLD1 (R1), Greenville (Gr), Kas-1 (K), and Wei-1 (We). The camalexin (C) band is shown by the arrow. Other abbreviations: A=indole-3-carboxaldehyde; ca=indole-3-carboxylic acid.

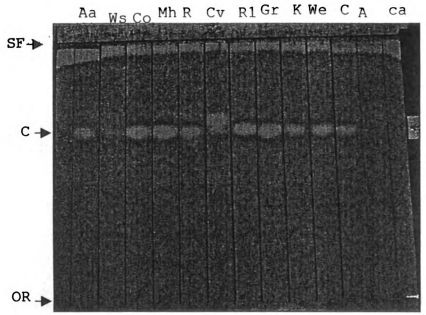

Figure 43b. Same TLC plate as in Figure 43a after a bioassay with *Cladosporium cucumerinum*. Plate was sprayed with *C. cucumerinum* spores (about $3x10^6$/ml) and incubated in a humid chamber for 3 days. Abbreviations are as in Figure 43a.

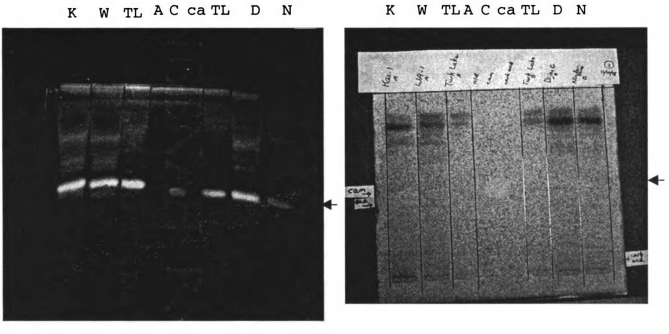

Figure 44. Effects of *Alternaria brassicicola* on camalexin production. Left, TLC plate of extracts of *A. brassicicola*-inoculated ecotypes Kas-1, WA-1, Turk Lake, Dijon G, and Niederzenz, viewed under short-wave UV light. Right, the same TLC plate after being sprayed with *A. brassicicola* spores and incubated in a moist chamber for 3 days. Camalexin is indicated by the arrow. Abbreviations as in Figure 43.

in leaf tissue. Conidia formed unusually short germ tubes, and penetration events were rare. On the resistant ecotype RLD, conidial germ tubes were about as long as on the susceptible ecotype Kas-1. However, fewer lesions and signs of browning were seen in RLD than in Kas-1 cells. Micrographs of RLD leaves viewed under UV light revealed the presence of a fluorescent orange material permeating the tissue, which may indicate the presence of phenolic compounds (R. Hammerschmidt, pers. comm.).

Discussion

These studies provided no earth-shattering conclusions about the role of camalexin in disease resistance. All ecotypes had the ability to produce camalexin. However, they produced little in response to *A. brassicicola*. The only ecotypes producing detectable amounts of camalexin in response to *A. brassicicola* were a highly susceptible one (Kas-1) and two highly resistant ones (RLD and Anna). Camalexin does not seem, therefore, to have a significant role in resistance to *A. brassicicola*. However, it is possible that it has a role in resistance to other pathogens.

The variations in symptoms demonstrated the need to consider the model of the disease triangle (Agrios, 1997) when evaluating disease resistance. Even if a plant has the capacity to resist infection, disease can develop if the environmental conditions are right and the pathogen is

aggressive enough. Similarly, in the case of the ecotypes studied here, differences in light regime or inoculum concentration led to aberrant disease phenotypes.

When disease phenotypes were evaluated under similar conditions between experiments, some general trends emerged, such that it was possible to classify plants in terms of high, intermediate, or low resistance. These categories encompassed a range of symptoms, which varied within each ecotype from one experiment to the next.

Microscopic analysis of infection revealed differences in resistance responses. Resistance in Turk Lake appeared to be due to inhibition of fungal growth at or near the leaf surface. In contrast, resistance in RLD appeared to be the result of cell response after penetration. It may be that the orange fluorescent material seen in RLD tissue represents phenolics or other defense-associated compounds forming in response to infection.

Similar differences in resistance responses were noted for the Col-0 and RLD ecotypes in response to *Peronospora parasitica* (Mauch-Mani et al., 1993). In RLD, an HR occurred near the site of penetration, and no spread of disease occurred. In Col-0, some spread of the pathogen occurred, demonstrated by a trail of necrotic flecks across the leaf (Mauch-Mani et al., 1993). These variations were thought to be due to multiple resistance genes. The variations in response to *A. brassicicola* seen in these studies may also be

the result of differential activation of multiple resistance genes.

Because no antimicrobial compounds besides camalexin were detected in TLC plate bioassays of *A. brassicicola*-inoculated leaves, it is difficult to determine the source of disease resistance in resistant ecotypes. It may be that the antimicrobial compounds in leaves were too labile to be detected, or that they were too polar to be extracted with chloroform. It is also possible that resistance to *A. brassicicola* depends primarily on the accumulation of the defensins which have been found in Arabidopsis inoculated with *A. brassicicola* (Penninckx et al., 1996) and which, being proteins, would not be isolated by the methods used in these studies.

This part of the project demonstrated the importance of studying more than one ecotype to evaluate resistance of a plant to a pathogen. It also demonstrated that camalexin is not a reliable marker for resistance to *A. brassicicola*, although it may be for resistance to other pathogens. The variations in camalexin production in response to *C. carbonum* demonstrated, as did the kinetic studies of chapter 3, that patterns of accumulation are not set in stone and that it is wise to study such patterns on more than one occasion before drawing conclusions about camalexin deficiency.

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Conclusions

The biosynthetic intermediates of camalexin, apart from anthranilate and cysteine, are still unknown. Indole-3-carboxaldehyde may be a precursor, but the radiolabeling data do not seem too supportive. The attempt to seek biosynthetic intermediates in camalexin-deficient mutants demonstrated that apparent camalexin deficiency can occur even in wild-type plants, depending on inoculum concentration, the use of leaves or droplets alone, and unknown factors causing camalexin to accumulate more slowly. The work with Arabidopsis ecotypes demonstrated that the choice of pathogen affects camalexin production, and it reaffirmed the variable kinetics of camalexin accumulation found in work with the *pad* mutants.

A few questions remain as a result of this project. The question of biosynthetic intermediates is an obvious one. Since indole-3-carboxaldehyde does not seem a strong candidate for an intermediate, it may be time to look more closely at the role of indole-3-carboxylic acid in camalexin biosynthesis. On the autoradiograms shown in chapter 4 (Figures 25-30), bands at the approximate R_f of indole-3-carboxylic acid were present, and the relative darkness of those bands did vary over time. It is possible that indole-3-carboxylic acid leads to camalexin by reduction to indole-3-carboxaldehyde or formation of an acetyl CoA thioester, as occurs in lignin biosynthesis. Indole is another possible

intermediate. Given the volatility of indole, the extraction procedure may need to be modified extensively. Indole-3-carboxaldehyde may be worth reinvestigating if the radiolabeled form can be made.

Both the time courses described in Chapter 3 and the large-scale extractions described at the beginning of Chapter 4 generated several inducible bands besides indole-3-carboxaldehyde on TLC plates. Characterizing those may reveal biosynthetic intermediates.

Since *pad* 2 does not appear to be a regulatory mutant, it may be a leaky biosynthetic mutant. Radiolabeling with *pad* 2 was only done with a 24-hour incubation period after feeding and inoculation. Perhaps a time course of camalexin accumulation in *pad* 2 leaves fed ^{14}C-anthranilate would yield intermediates at early time points.

Since camalexin concentrations in leaves sometimes decreased during 72-hour time courses, it would be interesting to determine the metabolic fate of camalexin. Some of the inducible bands on TLC plates may be metabolites and not biosynthetic precursors. Knowing the metabolites of camalexin would help to distinguish between potential precursors and metabolites in further radiolabeling studies. Also, those metabolites may have some role in disease resistance.

Ultimately, the purpose of determining the camalexin biosynthetic pathway is to understand its role in disease resistance. The work with Arabidopsis ecotypes may provide

answers without a firm knowledge of the biosynthetic pathway. Little correlation was found between camalexin production and resistance to *Alternaria brassicicola*, but it is possible that *A. brassicicola* is not a pathogen of those ecotypes in their natural habitat. It would be interesting to find out what pathogens--compatible and incompatible--are present in the natural habitats of the ecotypes studied, and to determine if resistance or susceptibility are correlated with camalexin production. If little camalexin is produced in response to incompatible pathogens, perhaps a search for other antimicrobial compounds, like those that appeared on some TLC plate bioassays, would help to explain resistance. The presence of a fluorescent orange compound in infected leaves of the RLD ecotype suggests that other compounds are being produced.

The relative insensitivity of *A. brassicicola* to camalexin raises the question of whether resistance to a pathogen is correlated with the pathogen's sensitivity to camalexin. This question could also be examined in a study of ecotype resistance to native pathogens.

It may also be helpful to study other compounds that Arabidopsis produces during infection. Looking only at camalexin production leads to a narrow picture of the responses of Arabidopsis to infection. A more complete view would require studying the types of other compounds produced (phenolics, defensins, or PR proteins) and the relationship between the timing of their production and camalexin

production. Eventually, it would be interesting to see compare results of such studies on Arabidopsis and on other camalexin-producing genera (*Camelina sativa* and *Capsella bursa-pastoris*).

Appendix A. Recipe for half-strength Hoagland's solution used to fertilize Arabidopsis plants in the experiments described in Chapters 2-4 (courtesy of J. Klug, Michigan State University).

Half-strength Hoagland's Solution

The following solutions are prepared.

Solution A:

calcium nitrate ($Ca[NO_3]\cdot 4H_2O$), 295.0 g/l
sequestrene (DTPA, 10% Fe), 38.44 g/l

Solution B:

potassium phosphate (KH_2PO_4), 34.25 g/l
potassium nitrate (KNO_3), 126.65 g/l
magnesium sulfate ($MgSO_4\cdot 7H_2O$), 62.5 g/l
zinc sulfate ($ZnSO_4\cdot 7H_2O$), 0.056 g/l
manganous sulfate ($MnSO_4\cdot H_2O$), 0.391 g/l
copper sulfate ($CuSO_4\cdot 4H_2O$), 0.021 g/l
boric acid (H_3BO_3), 0.725 g/l
molybdic acid ($MoO_3\cdot 2H_2O$), 0.005g/l

The pH of solution B is adjusted to about 4.6.

For 1 l of nutrient solution, 2 ml of solution A and 2 ml of solution B are mixed in 996 ml of water. The pH is then adjusted to 6.0-6.4 with 1N KOH (about 0.25 ml of KOH required to adjust pH for 1 liter of solution).

Stock solutions A and B should not be mixed unless diluted.