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Coordinate Regulation of the Tryptophan Biosynthetic Pathway and Indolic Phytoalexin Accumulation in Arabidopsis

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Little is known about the mechanisms that couple regulation of secondary metabolic pathways to the synthesis of primary metabolic precursors. Camalexin, an indolic secondary metabolite, appears to be the major phytoalexin in Arabidopsis. It was previously shown that camalexin accumulation is caused by infection with plant pathogens, by abiotic elicitors, and in spontaneous lesions in the accelerated cell death mutant *acd2*. We demonstrate that the accumulation of this phytoalexin is accompanied by the induction of the mRNAs and proteins for all of the tryptophan biosynthetic enzymes tested. A strong correlation was observed between the magnitude of camalexin accumulation and the induction of tryptophan biosynthetic proteins, indicating coordinate regulation of these processes. Production of disease symptoms is not sufficient for the response because systemic infection with cauliflower mosaic virus or cucumber mosaic virus did not induce the tryptophan pathway enzymes or camalexin accumulation. Salicylic acid appears to be required, but unlike other documented pathogenesis-related proteins, it is not sufficient for the coordinate induction. Results with *trp* mutants suggest that the tryptophan pathway is not rate limiting for camalexin accumulation. Taken together, these results are consistent with the hypothesis that the regulation of the tryptophan pathway in plants responds to needs for biosynthesis of secondary metabolites.

INTRODUCTION

Plants produce hundreds of thousands of secondary metabolites with diverse functions, including regulation of plant growth, protection against biotic and abiotic stress, conferment of structural integrity, and attraction of pollinating animals. Most of the known secondary metabolites arise from a handful of primary metabolic pathways (reviewed in Conn, 1995; Kutchan, 1995). For example, the tryptophan pathway leads to the biosynthesis of many secondary metabolites, including the auxin indole-3-acetic acid, indole glucosinolates, anthranilate-derived alkaloids, tryptamine derivatives, such as gramine, and tryptophan-derived chemotherapeutic monoterpenoid indolic alkaloids (Kutchan, 1995; Radwanski and Last, 1995). Given the importance of alkaloids and other secondary metabolites to plants and in promoting human health, there is a great deal of interest in genetic engineering of these pathways in plants. This will require the ability to coordinate the production of precursors from primary metabolic pathways with the enzymes that produce secondary metabolites. Almost nothing is known about how plants regulate their primary metabolic pathways to accommodate the biosynthesis of these diverse secondary metabolites.

Structurally diverse antimicrobial secondary metabolites are produced by plants in response to infection by microorganisms. Synthesis of these compounds, called phytoalexins, is believed to be one mechanism that plants employ to defend against infection (Darvill and Albersheim, 1984; Paxton et al., 1994). Synthesis of camalexin (3-thiazol-2′-yl-indole), identified as the major phytoalexin in Arabidopsis (Tsuji et al., 1992), is induced by infection with virulent or avirulent strains of *Pseudomonas syringae* pv *tomato* or *P. s.* pv *maculicola* (Tsuji et al., 1992; Glazebrook and Ausubel, 1994) or treatment with the abiotic elicitor silver nitrate (Tsuji et al., 1993). Camalexin has been shown to inhibit the in vitro growth of pathogenic fungi and bacteria (Tsuji et al., 1992; Glazebrook and Ausubel, 1994). Results from the study of Arabidopsis *pad* mutants defective in camalexin biosynthesis suggest that this phytoalexin may be important in limiting the growth of virulent strains of *P. syringae* (Glazebrook and Ausubel, 1994). The indole ring of camalexin is proposed to be derived from the tryptophan pathway, as shown in Figure 1 (Tsuji et al., 1992, 1993).

In recent years, genetic, molecular, and biochemical tools have become available to study the regulation of the Arabidopsis tryptophan biosynthetic enzymes (Last and Fink, 1988; Radwanski and Last, 1995; Radwanski et al., 1995; Zhao and Last, 1995). These tools and the facile quantification of

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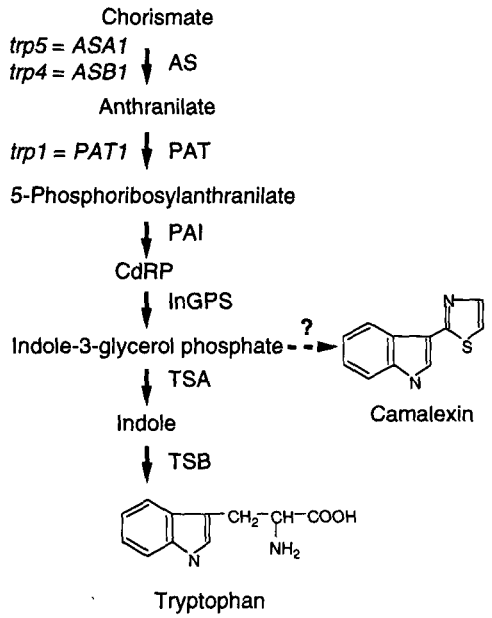

Figure 1. The Arabidopsis Tryptophan Biosynthetic Pathway and Proposed Branch Point for Camalexin Biosynthesis.

InGPS, indole-3-glycerolphosphate synthase; CdRP, 1-(*O*-carboxyphenylamino)-1-deoxyribulose-5-phosphate. Relevant mutants and the affected genes are indicated at left. The question mark indicates that there is no information that unambiguously defines the branch point from the tryptophan pathway to camalexin.

camalexin make this a convenient model for studying coordinate regulation of primary and secondary metabolism. It was previously shown that the mRNAs for both subunits of the committing enzyme of tryptophan biosynthesis, anthranilate synthase α and β (ASA and ASB, respectively), are induced by pathogen treatment (Niyogi and Fink, 1992; Niyogi et al., 1993), suggesting that induction of anthranilate synthase is necessary for increased synthesis of secondary metabolites (Niyogi and Fink, 1992; Niyogi et al., 1993). However, the published studies did not address a number of questions regarding the coordination of the tryptophan pathway with biosynthesis of secondary metabolites. For example, how tight is this coordinate regulation between the tryptophan pathway and the biosynthesis of its secondary metabolites? Is induction of camalexin and tryptophan pathway enzymes mediated through known signal transduction pathways or novel mechanisms? Are genes for the remaining enzymes in the pathway also upregulated in response to pathogens? Does increased anthranilate synthase mRNA cause enhanced protein accumulation?

There are relatively few documented instances of coregulation of primary and secondary metabolic pathways in plants. One such example is the coordinate induction of phenylalanine ammonia-lyase with 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase, the committing enzyme of the shikimate pathway, in response to wounding in potato or tomato and to elicitor treatment of parsley cells (Dyer et al., 1989; Henstrand et al., 1992). Similarly, fungal elicitor treatment of tobacco cells causes the coordinate induction of 3-hydroxy-3-methylglutaryl-coA reductase (HMG-CoAR), sesquiterpene cyclase, and the sesquiterpene capsidiol (Chappell and Nable, 1987; Vögeli and Chappell, 1988). In neither case are the responsible regulatory mechanisms known.

The importance of salicylic acid (SA) as a signal molecule in the induction of pathogenesis-related (PR) proteins and systemic aquired resistance (SAR) is well established (Yalpani et al., 1991; Ryals et al., 1995). For example, treatment of plants with SA or 2,6-dichloroisonicotinic acid (INA) is sufficient to induce PR proteins and SAR (Uknes et al., 1992; Ryals et al., 1995). Furthermore, NahG plants, which cannot accumulate significant amounts of SA due to the expression of the bacterial *nahG* gene encoding bacterial salicylate hydroxylase, fail to induce PR proteins and SAR upon pathogen infection (Bowling et al., 1994; Delaney et al., 1994). The mutants *npr1* (for nonexpresser of PR genes) and *nim1* (for noninducible immunity) have been shown to be defective in the induction of PR proteins and SAR (Cao et al., 1994; Delaney et al., 1995).

In this report, we show that *P. syringae* infection or abiotic elicitor treatment, but not infection with virulent viruses, induces camalexin coordinately with the mRNAs and proteins of the tryptophan pathway enzymes in Arabidopsis. In contrast to the known PR genes, SA appears to be required but is not sufficient for the coordinate induction. Arabidopsis *trp* mutants defective at different steps of the pathway accumulate similar levels of camalexin as the wild type, suggesting that the control of camalexin biosynthesis lies beyond the tryptophan biosynthetic pathway.

RESULTS

Regulation of mRNA Accumulation by *P. s. maculicola* Infection

P. s. maculicola infection was previously shown to induce the mRNAs for ASA and ASB, subunits of the first enzyme of the tryptophan pathway (Niyogi and Fink, 1992; Niyogi et al., 1993). To test whether other tryptophan biosynthetic enzymes are also induced by *P. s. maculicola*, we infiltrated Arabidopsis leaves with the virulent strain ES4326 of *P. s. maculicola*. Although it took 2 days for the leaves to show visible disease symptoms, Figure 2A shows that the mRNAs encoding the enzymes ASA, phosphoribosylanthranilate transferase (PAT), and tryptophan synthase β (TSB) were induced more than fivefold within 1 day postinfiltration (DPI) and decreased thereafter. Similar results were obtained for phosphoribosylanthranilate isomerase (PAI) and tryptophan synthase α subunit (TSA) mRNAs, indicating that the entire pathway is pathogen inducible. This was not due to a general upregulation of amino acid biosynthetic enzymes, because methionine synthase (MTS) (Figure 2A) and acetolactate synthase (Niyogi and Fink, 1992; J. Zhao, data

not shown) mRNAs were not induced by infection. To compare the induction of tryptophan biosynthetic proteins by *P. s. maculicola* to a known stress-inducible gene, RNA gel blots were probed with the glutathione *S*-transferase 1 (*GST1*) cDNA (Conklin and Last, 1995). GST mRNA was induced more than ninefold at 1 DPI and then decreased (Figure 2A). Therefore, regulation of the tryptophan biosynthetic genes is more similar to a stress-inducible GST mRNA than to the other amino acid enzymes tested.

Infection with *P. s. maculicola* Induces Tryptophan Biosynthetic Proteins and Camalexin

Immunoblot analysis was used to test whether increased mRNAs led to the induction of tryptophan biosynthetic enzymes at the protein level. Figure 3A shows that the levels of ASA, TSA, and TSB proteins were induced three- to fivefold at 1 DPI. In contrast to mRNA levels, which decreased within 2 DPI, protein remained elevated for ~3 days and only started to decrease at 4 DPI (Figure 3A), most likely due to severe tissue damage caused by the pathogen. PAT and PAI proteins were also induced three- to fivefold. The accumulation of camalexin correlated with the induction of tryptophan pathway proteins, with ~25 μg/g fresh weight at 1 DPI and reaching a maximum of ~70 μg/g fresh weight at 2 DPI (Figure 3B).

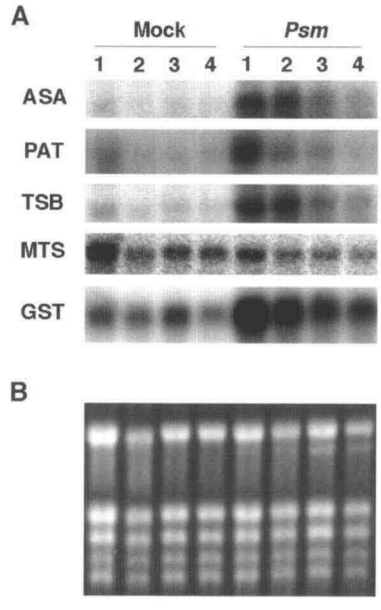

Figure 2. Regulation of mRNAs by Infection with ES4326 of *P. s. maculicola*.

Arabidopsis rosette leaves were hand infiltrated with either ES4326 of *P. s. maculicola* in 10 mM $MgSO_4$ (*Psm*) or with 10 mM $MgSO_4$ (Mock).

(A) RNA gel blots probed with *ASA1*, *PAT1*, *TSB1*, *MTS1*, and *GST1* cDNA probes.

(B) Ethidium bromide staining of RNA used for RNA gel blots in **(A)**.

Days postinfiltration are indicated above each lane. Similar results were obtained in two other independent experiments. These samples were also probed with *PAI2* and *TSA1* DNAs. These mRNAs were undetectable in control samples and were induced after pathogen treatment.

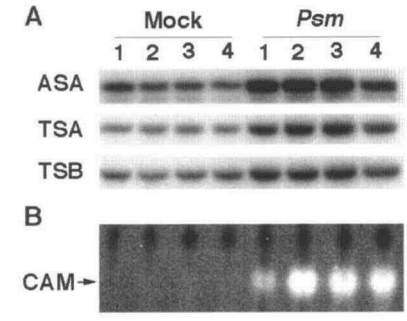

Figure 3. Induction of Tryptophan Biosynthetic Proteins and Camalexin by Infection with ES4326 of *P. s. maculicola*.

(A) Equal amounts of total protein from the tissues used in Figure 2 were loaded onto each lane for immunoblots with antibodies raised against ASA, TSA, and TSB.

(B) Camalexin (CAM) samples from 25 mg of leaf tissue as given in **(A)** were analyzed by thin-layer chromatography.

Days after infiltration are indicated above each lane. Similar results were obtained in five independent experiments.

Induction of the Tryptophan Biosynthetic Enzymes by Silver Nitrate and in *acd2-2*

The results with *P. s. maculicola* show that camalexin accumulation in response to pathogen infection is accompanied by induction of the tryptophan pathway enzymes. We took advantage of the fact that the abiotic elicitor silver nitrate is an effective inducer of camalexin (Tsuji et al., 1993) to test how tightly the induction of tryptophan pathway proteins is coupled to phytoalexin accumulation. Indeed, the levels of ASA, TSA, and TSB proteins increased after elicitor treatment along with camalexin, as shown in Figure 4. As was the case with the *P. s. maculicola* treatment, the change in protein accumulation is reflected in increased mRNA for all enzymes tested; ASA, PAT, TSA, and TSB mRNAs were induced to a maximal level within 12 hr after the treatment and decreased thereafter, as seen in Figure 5A (and data not shown). Consistent with the results from infection with *P. s. maculicola* (Figure 2A), silver nitrate treatment caused upregulation of the stress-inducible GST mRNA but not of the mRNAs encoding the amino acid biosynthetic enzymes MTS (Figure 5A) and acetolactate synthase (J. Zhao, data not shown).

Camalexin is also known to accumulate in spontaneously necrotic tissues but not in the healthy leaves of the accelerated cell death mutant *acd2-2* (Greenberg et al., 1994). If camalexin accumulation is coordinately regulated with the

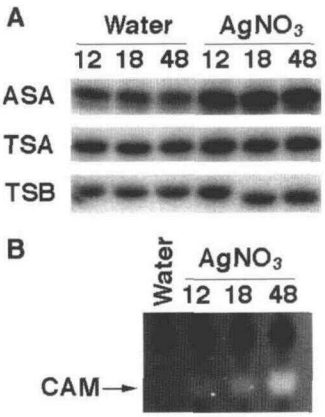

Figure 4. Induction of the Tryptophan Pathway Proteins and Camalexin by Silver Nitrate Treatment.

Arabidopsis plants were sprayed with 10 mM $AgNO_3$ in 0.02% Silwet L-77 ($AgNO_3$) or with 0.02% Silwet L-77 (Water). Plants were severely damaged by 2 days after the $AgNO_3$ treatment, making the analysis difficult beyond that point.

(A) Immunoblots probed with anti-ASA, anti-TSA, or anti-TSB antibodies.

(B) Analysis of camalexin (CAM) by thin-layer chromatography.

Numbers above each lane indicate hours after the treatment. Similar results were obtained in three independent experiments. Maximal induction of ASA protein was approximately twofold, whereas TSA and TSB proteins were induced ~1.5-fold.

tryptophan enzymes, induction of these proteins should be observed in necrotic leaves of *acd2-2*. Figure 6 shows that this is the case.

Virulent Viruses Uncouple Disease Symptoms and Camalexin Accumulation

Although the results described above are consistent with specific induction of the tryptophan pathway in response to bacterial infection and abiotic elicitation, severe tissue trauma was associated with both of these inducing conditions. To test whether induction of the tryptophan pathway and camalexin synthesis are obligatorily associated with disease symptoms, we infected Arabidopsis with virulent viral pathogens. The Arabidopsis plants infected with cauliflower mosaic virus (CaMV) showed typical mosaic symptoms of vein clearing and stunted growth (Leisner and Howell, 1992); infection with cucumber mosaic virus (CMV) caused extremely stunted growth (Hellwald and Palukaitis, 1995). Figure 6 shows that despite the disease symptoms, no obvious changes in the levels of ASA, TSA, and TSB proteins or camalexin accumulation were observed in CaMV- and CMV-infected leaves. These results strengthen the observation that there is a strong relationship between phytoalexin accumulation and upregulation of the tryptophan pathway.

A Strong Correlation between Camalexin Accumulation and Induction of Tryptophan Enzymes

If camalexin synthesis and tryptophan biosynthetic enzymes are coordinately regulated, we would expect to see a tight correlation between these two processes. To test this hypothesis, we took advantage of the observation that the amount of camalexin accumulation varies in response to different eliciting conditions (Glazebrook and Ausubel, 1994). Figure 7 shows that there is a surprisingly good association between ASA protein induction and camalexin levels for these varied treatments. Similar correlations were obtained for the induction of TSA and TSB with phytoalexin accumulation (data not shown). Taken together, our results support the hypothesis that the tryptophan pathway is coordinately regulated with camalexin accumulation. It is interesting that although *Xanthomonas campestris* pv *campestris* induced very little phytoalexin at an OD_{600} of 0.02, as previously reported (Tsuji et al., 1992), we observed significant production with a 10-fold higher inoculum of *X. c. campestris* (Figure 7).

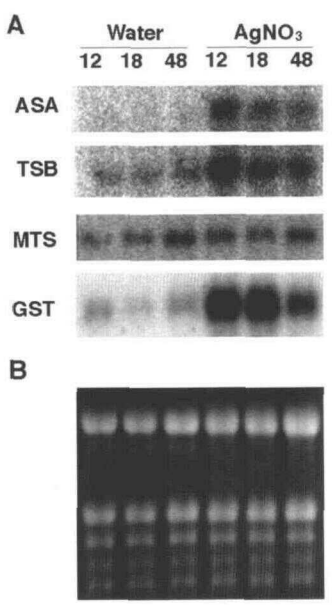

Figure 5. Regulation of mRNA Accumulation by Silver Nitrate Treatment.

The same tissues described in Figure 4 were used to isolate total RNA.

(A) RNA gel blots probed with *ASA1*, *TSB1*, *MTS1*, and *GST1* cDNA probes.

(B) Ethidium bromide staining of RNA used for the RNA gel blots in **(A)**.

Numbers above each lane indicate hours after the treatment. Similar results were obtained from two independent experiments. These samples were also probed with *PAI2* and *TSA1* DNAs. These mRNAs were undetectable in control samples and were induced following silver nitrate treatment.

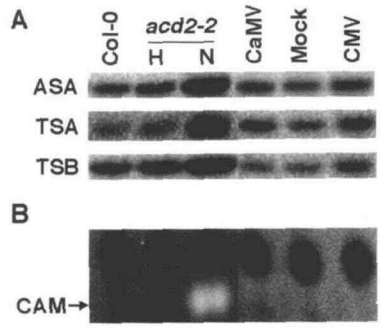

Figure 6. Analysis of Tryptophan Biosynthetic Proteins and Camalexin in *acd2-2* and Virus-Infected Leaves.

Healthy (H) and necrotic (N) leaves of 4-week-old *acd2-2* plants and Arabidopsis ecotype Columbia (Col-0) plants infected with CaMV and CMV were analyzed.

(A) Immunoblot analysis of the tryptophan pathway enzymes.

(B) Thin-layer chromatography analysis of camalexin (CAM).

Col-0, 4-week-old Col-0 plants that served as controls for *acd2-2* plants; Mock, plants treated similarly to the virus-infected plants, except the viruses were omitted. Similar results were obtained in two independent experiments.

SA Is Required but Not Sufficient for the Coordinate Induction

Given that the tryptophan pathway and camalexin accumulation are pathogen inducible, it was of interest to ask whether SA plays a role. Although treatment of Arabidopsis with SA or INA is sufficient to induce PR proteins (Uknes et al., 1992), our results indicate that these treatments cause production of <2% of the amount of camalexin observed with *P. s. maculicola* (<1 μg/g fresh weight). Consistent with the camalexin results, no significant induction of tryptophan biosynthetic enzymes was observed for either treatment (data not shown). NahG plants (Bowling et al., 1994; Delaney et al., 1994) were infiltrated with the ES4326 strain of *P. s. maculicola* to determine whether SA is necessary for the induction of camalexin and tryptophan biosynthetic enzymes. As shown in Figure 8A, upon pathogen infection, the NahG plants accumulated only 20% as much ASA, TSB, and GST mRNAs as the untransformed ecotype Columbia (Col-0). This inhibition was less than that seen for PR-1 mRNA, which was undetectable in NahG plants, as expected (Delaney et al., 1994). Consistent with the mRNA results, the induction of ASA, TSA, and TSB proteins and camalexin was also reduced significantly in NahG plants, as seen in Figure 9. Similar results were obtained with two independent NahG transgenic lines, suggesting that the effect is due to the expression of *nahG* and not to gene disruption by T-DNA. Taken together with the lack of induction after SA and INA treatment, these results indicate that SA is necessary but not sufficient for the upregulation of camalexin and the tryptophan pathway enzymes.

The induction of PR proteins and SAR by SA, INA, or pathogens is blocked in the *npr1* (Cao et al., 1994) and *nim1* mutants (Delaney et al., 1995), whose gene product(s) is proposed to function downstream of SA in the induction of PR proteins. To study the role of *NPR1* in the induction of the tryptophan pathway enzymes and camalexin, we infected *npr1* with *P. s. maculicola*. As shown in Figure 9, the induction in the mutant is similar to that of the wild type, suggesting that *NPR1* is not required for the induction.

Effect of Tryptophan Enzyme Activity on the Accumulation of Camalexin

The coordinate induction of the tryptophan biosynthetic pathway and camalexin accumulation suggests that the increased flux through this pathway might be needed for maximal phytoalexin induction. If this is the case, mutants of the tryptophan pathway would be expected to have altered phytoalexin accumulation. To test this idea, we measured camalexin accumulation in Arabidopsis *trp* mutants. The mutant *trp5-2* is less sensitive to feedback inhibition by tryptophan, causing a threefold higher accumulation of free tryptophan than in the wild type (Li and Last, 1996). The other two mutants tested have reduced activity of enzymes in the pathway: the prototrophic *trp1-100* mutant has <1% of the wild-type PAT activity

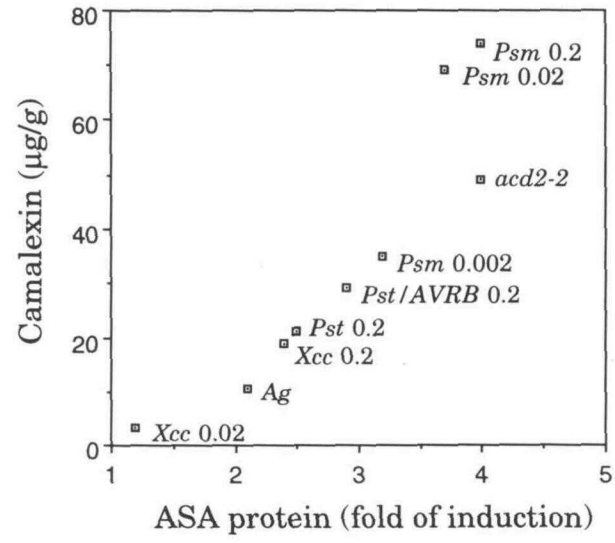

Figure 7. Coordinate Induction of ASA Protein and Camalexin.

Col-0 plants were treated with *P. s. maculicola* ES4326 (*Psm*), *P. s. tomato* DC3000 (*Pst*), *P. s. tomato* DC3000/*AVRB1* (*Pst/AVRB*), *X. c. campestris* (*Xcc*), or silver nitrate (Ag). Bacterial concentrations are indicated as OD_{600} units. The maximal induction of ASA protein and camalexin in different treatments is plotted on the graph. *acd2-2* indicates the induction of ASA and camalexin in the spontaneously necrotic leaves. The coefficient of linear regression (R^2) = 0.84.

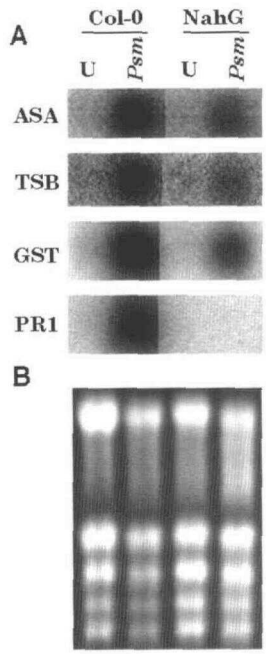

Figure 8. Regulation of mRNA Accumulation by *P. s. maculicola* in NahG Plants.

Arabidopsis ecotype Col-0 rosette leaves and those of NahG plants (Delaney et al., 1994) were infiltrated with *P. s. maculicola* ES4326 (*Psm*). Tissues 1 DPI were used to isolate total RNA. U, untreated control plants. **(A)** RNA gel blots probed with *ASA1*, *TSB1*, *GST1*, and *PR1* cDNA probes. **(B)** Ethidium bromide staining of RNA used for the RNA blots in **(A)**.

(Niyogi et al., 1993; Rose et al., 1997), and the auxotrophic *trp4-1 trp1-100* double mutant is defective in both ASB and PAT (Niyogi et al., 1993). To our surprise, despite the dramatic differences in these mutant enzyme activities, no major changes in camalexin accumulation were observed after infection with *P. s. maculicola* ES4326, as shown in Figure 10. These results suggest that the tryptophan biosynthetic enzymes are not rate limiting for phytoalexin accumulation. Apparently, important regulation occurs beyond the tryptophan biosynthetic pathway.

DISCUSSION

In this study, we have made three advances in our understanding of how the Arabidopsis tryptophan biosynthetic pathway is regulated in response to needs for secondary metabolites. First, our results suggest that induction of the tryptophan biosynthetic enzymes is coordinately regulated with the major indolic phytoalexin in Arabidopsis. Second, we found that SA is a necessary but insufficient component of the signal transduction pathway for coordinate induction. Finally, despite this tight coupling, it appears that the enzymes of the tryptophan pathway are not rate limiting for camalexin accumulation.

Several lines of evidence support the notion that the induction of the mRNAs and proteins of tryptophan biosynthesis is coordinately regulated with camalexin accumulation. Perhaps the most compelling result is that there is a strong correlation between the amount of camalexin and maximal induction of tryptophan pathway proteins (Figure 7). Second, protein induction was observed whenever camalexin accumulation was seen, regardless of the inducing condition. Finally, the kinetics of mRNA, protein, and camalexin accumulation are consistent with their functions: peak levels of tryptophan biosynthetic enzyme mRNAs and proteins always preceded the maximal accumulation of camalexin.

The induction of the tryptophan biosynthetic pathway is not due to a general increase in amino acid biosynthetic enzymes or in response to all stresses. The first line of evidence is that the mRNAs for amino acid biosynthetic enzymes from two other pathways are not induced by bacterial pathogens or elicitor (Figures 2A and 5A; Niyogi and Fink, 1992). This is consistent with the hypothesis that the induction of the tryptophan pathway is to accommodate increased biosynthesis of secondary metabolites. In addition to camalexin, infection by a pathogen and elicitor treatment have been shown to induce other secondary metabolites derived from the tryptophan pathway, including indole glucosinolates (Doughty et al., 1991) and anthranilate-derived alkaloids (Niemann, 1993; Bohlmann et al., 1995). Another important finding is that induction of the tryptophan pathway is not simply a general response to tissue damage, because infection by two different virulent viruses failed to induce the pathway enzymes. This is further supported by the fact that heat shock, while causing chlorosis and induction of the Hsp21 protein, does not induce the tryptophan pathway or camalexin accumulation (data not shown).

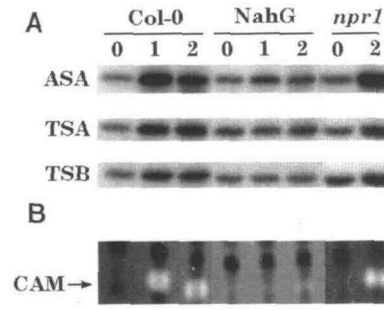

Figure 9. Induction of Tryptophan Biosynthetic Enzymes and Camalexin in NahG and *npr1* Plants.

Arabidopsis ecotype Col-0 rosette leaves and those of NahG and *npr1* plants were infiltrated with *P. s. maculicola* ES4326. Numbers above each lane indicate days after infiltration.
(A) Immunoblots probed with anti-ASA, anti-TSA, or anti-TSB antibodies.
(B) Thin-layer chromatography analysis of camalexin (CAM).
Similar results were obtained with an independent NahG line in the Landsberg *erecta* background (Bowling et al., 1994).

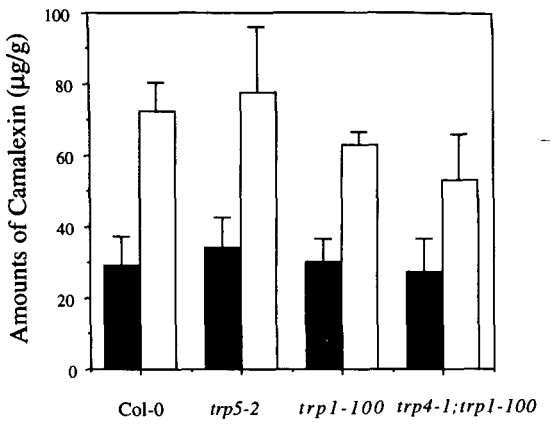

Figure 10. Induction of Camalexin by *P. s. maculicola* ES4326 in *trp* Mutants.

Arabidopsis leaves were infiltrated with *P. s. maculicola* ES4326, and camalexin was measured for the first (filled bar) and second (open bar) day after infiltration. The numbers are from three independent experiments (duplicate measurements for each experiments), and standard errors are indicated.

We investigated the role of SA in this process because of its well-documented role as an inducer of gene expression in response to pathogen infection. Our results show that SA is required for the coordinate induction of camalexin and the tryptophan biosynthetic pathway, because NahG plants show an attenuated response. However, unlike the canonical PR proteins (Uknes et al., 1992), neither SA nor INA treatment is sufficient to induce camalexin and the tryptophan biosynthetic enzymes. Other lines of evidence support the idea that this process is regulated differently from PR proteins. For example, *cpr1*, a mutant with constitutively high PR protein expression due to a higher SA level, does not affect camalexin or tryptophan pathway protein accumulation (data not shown). *NPR1* has been identified as a component in the SA signal transduction pathway (Cao et al., 1994). However, the *npr1* mutant is not defective in the induction of camalexin and the tryptophan pathway enzymes, suggesting an *NPR1*-independent signal transduction pathway by SA. An alternative hypothesis is that this *npr1* harbors a leaky allele, and induction of the tryptophan pathway requires less NPR1 product than does *PR1* gene expression.

Based on these results, we propose a model in which both SA-dependent and SA-independent components are required for induction of tryptophan proteins and camalexin. In this model, SA induces both *NPR1*-dependent and *NPR1*-independent signal transduction pathways, and the *NPR1*-dependent process is required for the induction of PR proteins but not camalexin and the tryptophan pathway. Analysis of mutants altered in regulation of tryptophan pathway gene expression or camalexin synthesis is needed to test the validity of this model.

A surprising result is that *trp* mutations did not cause major changes in the amount of camalexin accumulation when plants were infected with *P. s. maculicola* (Figure 10). This is in contrast to the 50% decrease in camalexin accumulation seen for *trp1-100* after silver nitrate induction (Tsuji et al., 1993). Although our results with *P. s. maculicola* show a less dramatic decrease, both sets of data show that a mutation that abolishes detectable PAT enzyme activity has a modest effect on phytoalexin accumulation. Given that the tryptophan pathway leads to the biosynthesis of multiple secondary metabolites, it is reasonable that the primary metabolic pathway may not be the rate-limiting step for any one product.

The observation of coordinate regulation of primary and secondary metabolism, without the primary metabolic pathway being rate limiting, is reminiscent of results from studies of the coregulation of HMG-CoAR, the committing enzyme of isoprenoid biosynthesis, and terpenoids. After fungal elicitor treatment of tobacco cell suspension cultures, HMG-CoAR activity was increased coordinately with both susquiterpene cyclase activity and susquiterpenoid end products (Chappell and Nable, 1987; Vögeli and Chappell, 1988). Despite this parallel induction, published data suggest that HMG-CoAR activity is not rate limiting for accumulation of the susquiterpene capsidiol (Vögeli and Chappell, 1988).

Consistent with the idea that the observed induction of tryptophan pathway enzymes functions to maintain a sufficient level of pathway intermediates rather than as a rate-limiting step, the two subunits of tryptophan synthase, which are presumably not involved in the biosynthesis of camalexin (Tsuji et al., 1993), are also induced by pathogens and elicitor. A possible role for TSA and TSB induction is to accommodate the biosynthesis of other tryptophan-derived secondary metabolites such as the pathogen- and wound-inducible indole glucosinolates (Doughty et al., 1991; Bodnaryk, 1992). This hypothesis predicts that other tryptophan pathway-derived secondary metabolites are subject to the same regulation as camalexin and tryptophan.

The modest effect of *trp* mutations on camalexin accumulation may be explained by a variety of other hypotheses. For example, camalexin may be synthesized outside of the chloroplast, the site of tryptophan synthesis. In this scenario, the amount of tryptophan pathway intermediates available for synthesis of the phytoalexin may be limited by interorganellar transport. Another possibility is suggested by recent work on duplicate ASA genes in *Ruta graveolens*. Only one gene (*ASα1*) is induced upon treatment with fungal elicitor (Bohlman et al., 1995). In contrast to what is expected for an amino acid biosynthetic enzyme, Asα1 protein expressed in *Escherichia coli* is insensitive to feedback inhibition by tryptophan, whereas the constitutive Asα2 is inhibited by micromolar tryptophan (Bohlmann et al., 1996). Thus, it is likely that these two isoforms play very different roles in this alkaloid-producing plant: inducible Asα1 protein for secondary metabolism and Asα2 for amino acid synthesis. This suggests that the *trp1-100 trp4-1* double mutant may be affected in isoenzymes that are specifically important for the production of tryptophan rather than

secondary metabolites. However, this is unlikely to be the case for *trp1-100*, because it is defective in what appears to be the only gene encoding PAT in Arabidopsis (Rose et al., 1992). A final hypothesis, which runs counter to available data (Tsuji et al., 1993), is that the indole ring of camalexin may be derived from biosynthetic pathways other than tryptophan.

METHODS

Plant Growth and Treatments

Plants (*Arabidopsis thaliana*) were grown under constant light (60 to 100 $\mu E\ m^{-1}\ sec^{-2}$) in sterile Cornell soil-less mix (Landry et al., 1995) at 22°C. Seed for NahG plants were supplied by Ciba-Geigy Corporation (Delaney et al., 1994) and X. Dong (Duke University, Durham, NC; Bowling et al., 1994). Strain ES4326 of *Pseudomonas syringae* pv *maculicola*, strains DC3000 and DC3000/*AVRB1* of *P. s.* pv *tomato*, and strain BP109 of *Xanthomonas campestris* pv *campestris* were grown overnight at 30°C in Luria-Bertoni or King's B medium (10 mg/mL protease peptone, 1.5 mg/mL K_2HPO_4, 15 mg/mL glycerol, 0.39 mg/mL $MgSO_4$, pH 7.0) with the appropriate antibiotics (100 μg/mL streptomycin for *P. s. maculicola* ES4326, 100 μg/mL rifampicin for *P. s. tomato* DC3000, 100 μg/mL rifampicin and 50 μg/mL kanamycin for *P. s. tomato* DC3000/pAVRB1). The bacterial cultures were washed with 10 mM $MgSO_4$ and diluted to an OD_{600} of 0.02 for infiltration, unless an alternative concentration is indicated. Rosette leaves of 3-week-old plants were infiltrated with bacterial solutions by syringe infiltration, as described previously (Glazebrook and Ausubel, 1994). Infiltrated leaves were harvested, ground to a powder in liquid nitrogen, and stored at −70°C.

Arabidopsis plants at the four true-leaf stage were infected with cauliflower mosaic virus (CaMV) isolate 1841 by agroinoculation, as described by Grimsley et al. (1986). Infection with isolate KNH of cucumber mosaic virus (CMV) was done as described using infectious CMV RNAs (Hellwald and Palukaitis, 1995). Only the Arabidopsis leaves showing disease symptoms were harvested for analysis.

Three-week-old Arabidopsis plants were sprayed with 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX) with or without 10 mM silver nitrate. The aboveground tissues were harvested at the indicated time points and stored at −70°C.

Immunoblot, RNA Isolation, and Filter Hybridization

Protein sample preparation, electrophoresis, immunoblotting, and radioactivity quantification with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) were done as described previously (Zhao and Last, 1995). Equal amounts of protein were loaded in each lane in a given experiment, and equivalent transfer was verified by Ponceau S staining of the nitrocellulose membrane. RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY). RNA gel blot hybridization was performed with 5 μg of total RNA from each sample, as described by Conklin and Last (1995). Radioactivity was measured with a PhosphorImager, and signals were normalized to the amount of total RNA, as assayed by ethidium bromide fluorescence (Conklin and Last, 1995). The anthranilate synthase (ASA) probe is a 1.1-kb EcoRI fragment of Arabidopsis, the *ASA1* cDNA (Niyogi and Fink, 1992). The phosphoribosylanthranilate transferase (PAT) probe is a 1.7-kb XhoI fragment of the *PAT1* cDNA (Rose et al., 1992). The tryptophan synthase β (TSB) probe is a 1.5-kb EcoRI fragment of the *TSB1* cDNA from pMBT3 (Berlyn et al., 1989). The methionine synthase (MTS) probe is a 2.0-kb SalI-NotI fragment of the *MTS* cDNA from expressed sequence tag clone 38H1T7. The glutathione *S*-transferase (GST) probe is a 0.9-kb BamHI-KpnI fragment of the *GST1* cDNA from expressed sequence tag clone ATTS1553.

Camalexin Analysis

Camalexin was determined using a modification of two published protocols (Tsuji et al., 1993; Glazebrook and Ausubel, 1994). Seven hundred microliters of 80% methanol was added to 70 mg of frozen tissue in a microcentrifuge tube. The samples were then heated at 80°C for 15 min and spun at room temperature for 5 min. Five hundred microliters of the supernatant was then transferred to a new tube, and the methanol evaporated in vacuo at room temperature. Each sample was adjusted to 100 μL with distilled H_2O and extracted twice with 100 μL of chloroform, and the pooled organic phase was evaporated. The dried pellet was resuspended in 15 μL of chloroform, loaded onto a silica thin-layer chromatography plate (VWR Scientific, New York, NY), and developed with chloroform/methanol at 9:1. Camalexin was visualized under a long-wave-length ultraviolet lamp and photographed using Kodak Etkar 1000 film with a Kodak type 47 filter. For quantitative studies, the silica gel containing camalexin was extracted twice with 1 mL of methanol, and camalexin was quantified in a Perkin-Elmer (Branchburg, NJ) MPF-44B spectrofluorometer (excitation at 315 nm, emission at 385 nm). Camalexin concentration was calculated by comparison with a standard curve obtained by using purified camalexin (kindly provided by J. Glazebrook, University of Maryland, College Park).

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