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Induction of Arabidopsis Tryptophan Pathway Enzymes and Camalexin by Amino Acid Starvation, Oxidative Stress, and an Abiotic Elicitor

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The tryptophan (Trp) biosynthetic pathway leads to the production of many secondary metabolites with diverse functions, and its regulation is predicted to respond to the needs for both protein synthesis and secondary metabolism. We have tested the response of the Trp pathway enzymes and three other amino acid biosynthetic enzymes to starvation for aromatic amino acids, branched-chain amino acids, or methionine. The Trp pathway enzymes and cytosolic glutamine synthetase were induced under all of the amino acid starvation test conditions, whereas methionine synthase and acetolactate synthase were not. The mRNAs for two stress-inducible enzymes unrelated to amino acid biosynthesis and accumulation of the indolic phytoalexin camalexin were also induced by amino acid starvation. These results suggest that regulation of the Trp pathway enzymes under amino acid deprivation conditions is largely a stress response to allow for increased biosynthesis of secondary metabolites. Consistent with this hypothesis, treatments with the oxidative stress-inducing herbicide acifluorfen and the abiotic elicitor α-amino butyric acid induced responses similar to those induced by the amino acid starvation treatments. The role of salicylic acid in herbicide-mediated Trp and camalexin induction was investigated.

INTRODUCTION

The study of the regulation of amino acid biosynthetic pathways in bacteria and fungi has led to the discovery of unique regulatory mechanisms in these organisms. Allosteric regulation by end product inhibition is one important pathway-specific regulatory mechanism for various biosynthetic pathways in bacteria and fungi (Herrmann, 1983). In bacteria, repression and attenuation in response to end product availability transcriptionally regulate the biosynthetic genes and are important for pathway-specific regulation (Yanofsky and Crawford, 1987). In fungi, in addition to pathway-specific control, a cross-pathway regulatory mechanism known as general control or cross-pathway control is well documented for the regulation of amino acid biosynthesis (Hinnebusch, 1992; Sachs, 1996). For example, starvation of yeast for one amino acid results in increased steady state levels of mRNAs for at least three dozen enzymes of amino acid and purine biosynthesis in a dozen or more pathways.

What mechanisms do plants use to regulate synthesis of amino acids? As occurs in microbes, feedback inhibition plays a role in the regulation of amino acid accumulation in plants. For example, a feedback-insensitive mutant of anthranilate synthase α (ASA), the first enzyme in the tryptophan (Trp) biosynthetic pathway, accumulates three times more free Trp than does the wild type (Li and Last, 1996; Kreps et al., 1996). A cross-pathway regulatory mechanism, reminiscent of that found in fungi, has been proposed for amino acid and purine biosynthesis in plants (Guyer et al., 1995). Starvation for His by the imidazoleglycerol phosphate dehydratase inhibitor IRL1803 led to the induction of mRNAs of enzymes involved in the biosynthesis of not only His but also aromatic amino acids, Lys, and purines. However, only the two Trp pathway enzymes tested were found to be strongly induced by starvation for aromatic or branched-chain amino acids, which were caused by glyphosate and primisulfuron treatment, respectively (Guyer et al., 1995). This observation is in contrast to general control in fungi, in which starvation for different amino acids resulted in the induction of a similar set of genes (Hinnebusch, 1992).

Several questions remain to be answered about the observed responses to amino acid starvation–inducing treatments. What feature of the Trp pathway causes its enzymes

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to be induced under amino acid starvation conditions that do not affect other biosynthetic pathway enzymes? Are other Trp pathway enzymes induced by these treatments? Are genes unrelated to amino acid and purine biosynthesis induced by amino acid starvation? Are there phytotoxic treatments that induce a similar response without triggering amino acid starvation?

Unlike microorganisms, plant amino acid biosynthetic pathways lead to the production of various secondary products that function as growth regulators, in defense against pathogens and other environmental stresses, and as structural components (reviewed in Conn, 1995; Kutchan, 1995). The Arabidopsis Trp pathway, for example, leads to the biosynthesis of Trp, the plant hormone auxin, the phytoalexin camalexin, and other secondary compounds (Kutchan, 1995; Radwanski and Last, 1995), as shown in Figure 1. It is plausible that under some stress-inducing conditions, the regulation of these amino acid pathways may be dominated by the need for secondary metabolites derived from the pathway rather than for protein synthesis. In support of this hypothesis, it is known that the mRNAs encoding ASA and anthranilate synthase β (ASB) are induced by wounding and pathogen infection (Niyogi and Fink, 1992; Niyogi et al., 1993). In addition, the proteins of the Trp pathway appear to be coordinately regulated with the accumulation of the indolic phytoalexin camalexin after bacterial pathogen and abiotic elicitor treatment and in spontaneous lesions of *accelerated cell death2* (*acd2*) mutants (Zhao and Last, 1996). It is not known whether the induction of the Trp pathway by pathogen attack and amino acid starvation is mediated by the same trigger molecules or signal transduction pathways.

Salicylic acid (SA) appears to be an important signal molecule in the induction of pathogenesis-related (PR) proteins, systemic acquired resistance (SAR), camalexin, and the Trp pathway (Yalpani et al., 1991; Ryals et al., 1995; Zhao and Last, 1996). NahG plants, which cannot accumulate a significant amount of SA because of expression of the bacterial *nahG* gene encoding salicylate hydroxylase, are blocked in the induction of PR proteins and SAR upon pathogen infection (Bowling et al., 1994; Delaney et al., 1994). The role of SA in the induction of PR proteins and SAR appears to be different from that of camalexin and the Trp pathway. The induction of Trp pathway enzymes and camalexin was attenuated but not blocked in NahG plants treated with *Pseudomonas syringae* pv *maculicola.* Treatment of plants with SA or 2,6-dichloroisonicotinic acid, which is sufficient to induce PR proteins and SAR (Uknes et al., 1992; Ryals et al., 1995), failed to induce the Trp pathway enzymes and camalexin (Zhao and Last, 1996). In addition, the mutant *npr1*, which is defective in the induction of PR proteins and SAR (Cao et al., 1994; Delaney et al., 1995), induces wild-type levels of camalexin and the Trp pathway enzymes (Zhao and Last, 1996). Is there a role for SA in the induction of the Trp pathway by amino acid starvation?

In this report, we show that different amino acid starvation treatments induce accumulation of the five Trp biosynthetic enzymes tested and camalexin. Cytosolic glutamine synthetase (GS) was also induced. Genes unrelated to amino acid biosynthesis, such as the stress-inducible glutathione *S*-transferase (GST) and chalcone synthase (CHS), were induced by amino acid starvation, whereas the amino acid biosynthetic enzymes methionine synthase (MTS) and acetolactate synthase (ALS) were not induced. A similar induction pattern was observed when plants were treated with the abiotic elicitor α-amino butyric acid (α-AB) or the oxidative stress-inducing herbicide acifluorfen. The induction of camalexin was attenuated but not abolished in the NahG plants.

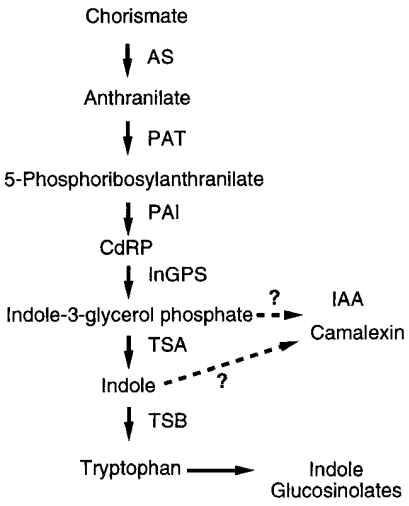

Figure 1. Trp Biosynthetic Pathway and Secondary Metabolites.

AS, anthranilate synthase; PAT, phosphoribosylanthranilate transferase; PAI, phosphoribosylanthranilate isomerase; InGPS, indole-3-glycerolphosphate synthase; TSA, Trp synthase α subunit; TSB, Trp synthase β subunit; CdRP, 1-(*O*-carboxyphenylamino)-1-deoxyribulose-5-phosphate; IAA, indole-3-acetic acid. Uncertainty regarding the branch point from the Trp pathway to IAA or camalexin is indicated by question marks above the arrows leading to IAA and camalexin.

RESULTS

Induction of the Trp Pathway Enzymes and Camalexin during Amino Acid Starvation

It was shown previously that the mRNAs for ASB and phosphoribosylanthranilate transferase (PAT) are upregulated under conditions that induce starvation for His and aromatic or branched-chain amino acids. In contrast, other genes for amino acid and purine biosynthetic genes were induced either by starvation for His alone or not upregulated at all (Guyer et al., 1995). We followed up on these observations

in several ways. First, we tested whether the unusual regulation of the Trp pathway was also observed during Met starvation induced by treatment with a mixture of Lys and Thr, which acts by inhibiting aspartate kinase activity (reviewed in Galili, 1995). Second, to test whether the published results of Guyer et al. (1995) were influenced by stress caused by growth of seedlings in a submerged liquid culture system, we developed a system for treating sterile Petri plate–grown 2-week-old seedlings with inhibitors (see Methods). Third, we asked whether enzymes throughout the Trp pathway were upregulated in response to amino acid starvation. Finally, we analyzed the accumulation of camalexin and expression of several genes unrelated to amino acid biosynthesis under these conditions.

Under our treatment conditions, the plants began to become chlorotic 3 or 4 days after glyphosate or chlorsulfuron treatment and were completely chlorotic 6 days after treatment, as illustrated in Figure 2. In contrast, the plants treated with Lys plus Thr usually appeared healthier, becoming chlorotic only 5 or 6 days after treatment (Figure 2). The herbicidal effects of glyphosate and chlorsulfuron can be partially blocked by supplementing them with aromatic amino acids or branched-chain amino acids, respectively. These results indicate that the observed chlorosis and tissue death result from amino acid starvation.

As shown in Figures 3A to 3C, all four of the Trp pathway enzymes tested, ASA, PAT, and tryptophan synthase α (TSA) and β (TSB), were induced by the three starvation conditions. In addition, camalexin accumulation was stimulated after application of chlorsulfuron and Lys plus Thr. This result is consistent with the coordinate regulation of Trp enzymes and camalexin previously demonstrated for Arabidopsis plants infected with bacterial pathogens (Zhao and Last, 1996). However, we did not observe camalexin accumulation in glyphosate-treated plants (Figure 3A). This is presumably because glyphosate inhibits 5-enolpyruvylshikimate 3-phosphate synthase, the penultimate enzyme of chorismate synthesis (Steinrücken and Amrhein, 1980), which is required for Trp and camalexin biosynthesis. The induction of the Trp pathway enzymes and camalexin was suppressed by the addition of the starved amino acids (Figures 3A to 3C), suggesting that the observed induction results from amino acid starvation.

Regulation of mRNA Accumulation by Amino Acid Starvation

Induction of the Trp pathway proteins was accompanied by an ~10-fold increase in the accumulation of *ASA*, *PAT*, *TSA*, and *TSB* mRNAs, as illustrated for *TSB* in Figures 4A and 4B. In contrast, two other amino acid biosynthetic enzymes were not induced by all three of the amino acid starvation treatments. For example, the mRNA for MTS either remained at a level similar to the control or increased only slightly in some experiments (Figure 4). The mRNA encoding *ALS*, the committing enzyme for branched-chain amino acid biosynthesis, was not induced by glyphosate or Lys plus Thr treatments (Figures 4A and 4B). Interestingly, chlorsulfuron treatment did cause a modest (approximately twofold) but reproducible increase in *ALS* mRNA (Figure 4A), suggesting the possibility of pathway-specific regulation at the transcriptional level. In contrast, the mRNA recognized by a presumed cytosolic glutamine synthetase (*GSKB*) probe was induced by all three treatments (Figure 4; data not shown). These results indicate that under our conditions, various amino acid biosynthetic genes are differentially regulated by treatments that cause amino acid starvation.

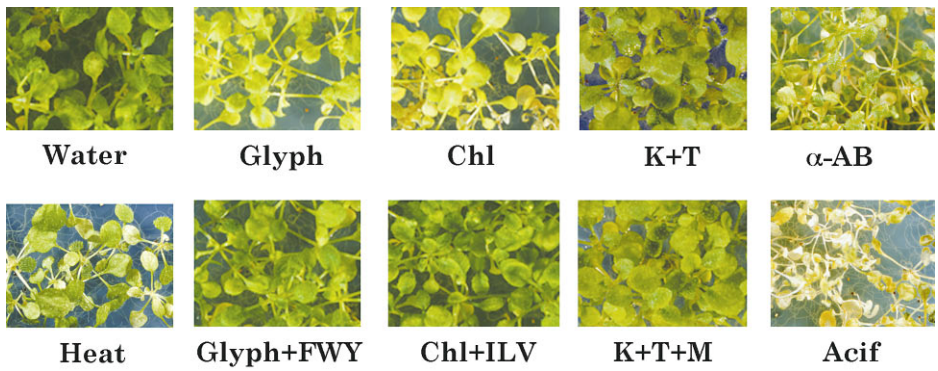

Figure 2. Effects of Inhibitor Treatments and Heat Shock on Arabidopsis.

Arabidopsis plants were grown on sterile nutrient agar plates for 2 weeks. These plates were treated as indicated. Photographs were taken 6 days after the following treatments: the water control, glyphosate (Glyph), glyphosate plus aromatic amino acids (Glyph+FWY), chlorsulfuron (Chl), chlorsulfuron plus branched-chain amino acids (Chl+ILV), α-amino butyric acid (α-AB), or acifluorfen (Acif). Photographs were taken 5 days after treatment with Lys plus Thr (K+T) or Lys plus Thr plus Met (K+T+M). Plants were photographed 4 days after heat shock (Heat).

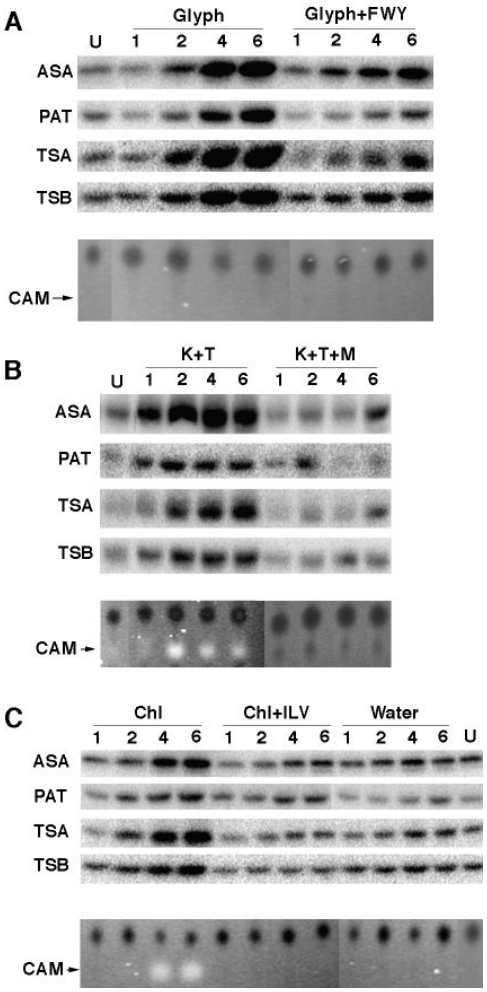

Figure 3. Induction of Trp Biosynthetic Enzymes and Camalexin Accumulation by Amino Acid Starvation.

Arabidopsis plants were grown on sterile nutrient agar plates for 2 weeks, followed by the indicated treatments. The Trp pathway proteins and camalexin were analyzed by immunoblotting or thin-layer chromatography, respectively.

(A) Treatment with glyphosate (Glyph) or glyphosate plus aromatic amino acids (Glyph+FWY).

(B) Treatment with Lys plus Thr (K+T) or Lys plus Thr plus Met (K+T+M).

(C) Treatment with chlorsulfuron (Chl), chlorsulfuron plus branched-chain amino acids (Chl+ILV), or the water control (Water).

The position of camalexin (CAM) is indicated by arrows. The number of days after application is indicated above each lane. Similar results were obtained in four independent experiments. U, untreated plants.

It was surprising to observe camalexin accumulation after amino acid starvation, because phytoalexins are traditionally thought of as pathogen-inducible antimicrobial metabolites. This suggests that amino acid starvation is mimicking some aspect of pathogen infection. To test this hypothesis, we examined the accumulation of *GST* and *CHS* mRNAs. Both of these transcripts are known to be inducible by biotic and abiotic stresses (Conklin and Last, 1995; Dixon and Paiva, 1995; Zhao and Last, 1996). Consistent with our hypothesis, *GST* mRNA was massively induced by all three starvation conditions (Figure 4). Interestingly, although *CHS* mRNA was induced after all three treatments, glyphosate caused a much larger response (~20 fold) than did chlorsulfuron or Lys plus Thr (approximately four- to eightfold), suggesting that *CHS* is differentially regulated by different starvation conditions (Figure 4). The larger response of *CHS* to glyphosate treatment might be caused by the depletion of a Phe-derived substrate or products of the CHS enzyme.

Induction of the Trp Pathway Enzymes and Camalexin by α-AB and Acifluorfen

These results suggest that the observed induction of gene expression and phytoalexin accumulation might be a stress response. This predicts that the camalexin and Trp regulon and cytosolic GS should be induced by other chemical agents that cause stress without producing amino acid starvation. To test this hypothesis, Arabidopsis plants were treated with α-AB, an abiotic elicitor that induces pathogen-responsive genes, and acifluorfen, a herbicide that inhibits heme and chlorophyll biosynthesis, causing the generation of free radicals and oxidative stress in plants (Matringe et al., 1989). α-AB and acifluorfen treatment clearly caused stress, because the plants became chlorotic (Figure 2). As shown in Figure 5, both treatments induced Trp protein (Figure 5A) and camalexin (Figure 5B) accumulation. Thus, chemical treatments that are not known to cause amino acid starvation can induce the Trp pathway and camalexin in our assay system. The rapid induction kinetics observed with α-AB and acifluorfen compared with amino acid starvation treatments suggest a direct mechanism for inducing Trp and camalexin, rather than causing amino acid starvation.

To characterize the responses of other genes to α-AB and acifluorfen treatments, we performed RNA gel blot hybridization analysis. Figure 6A shows that the mRNAs for *TSB*, *GS*, *CHS*, and *GST* were induced by α-AB and acifluorfen, as were *ASA* and *PAT* (data not shown). In contrast, the levels of *MTS* (Figure 6A) and *ALS* mRNAs (data not shown) were reduced only somewhat. Taken together, the responses of all of the genes tested to α-AB and acifluorfen treatments were similar to those caused by amino acid starvation. These results are consistent with the hypothesis that upregulation of the Trp and camalexin regulon and cytosolic GS after amino acid starvation is a result of secondary stress.

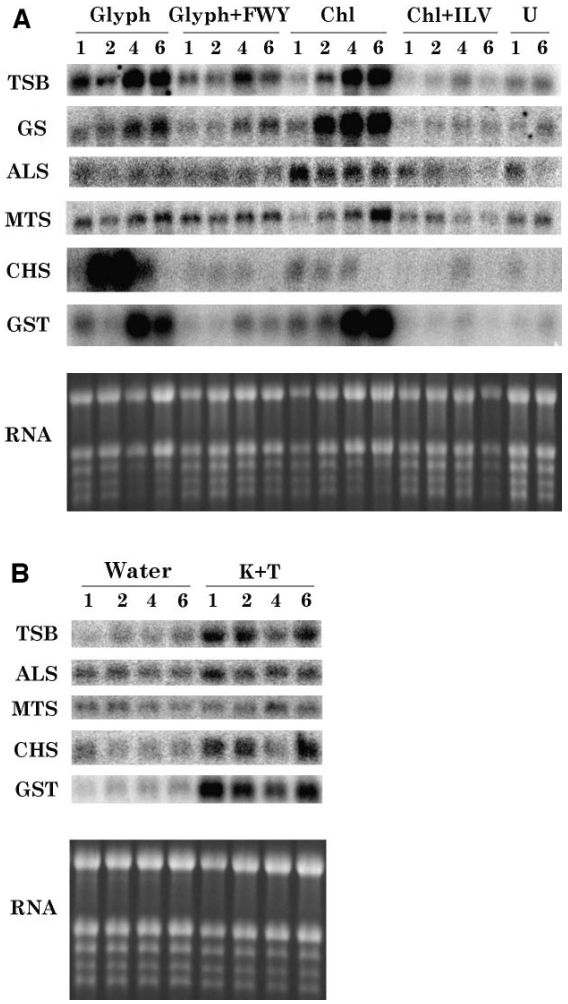

Figure 4. Regulation of mRNA Accumulation by Amino Acid Starvation.

Total RNA was isolated from the tissue samples used for Figure 3, 5 μg of total RNA was loaded in each lane, and RNA gel blots were hybridized with *TSB1* (TSB), *GSKB* (GS), *ALS*, *MTS1* (MTS), *CHS*, and *GST1* (GST) cDNA probes.
(A) Treatments with glyphosate (Glyph), glyphosate plus aromatic amino acids (Glyph+FWY), chlorsulfuron (Chl), or chlorsulfuron plus branched-chain amino acids (Chl+ILV).
(B) Treatments with Lys plus Thr (K+T) or the water control (Water). The number of days after application is indicated above each lane. Similar results were obtained in three independent experiments. U, untreated plants; RNA, ethidium bromide staining of the RNA gel used for RNA gel blots.

Heat Shock Treatment

To test whether all abiotic stress–provoking treatments induce camalexin and Trp, we analyzed the response of plants to heat shock treatment. Under our conditions, the plants appeared to be healthy for 3 days after initiation of the treatment, but they abruptly turned chlorotic on the fourth day (Figure 2). Figure 7 shows that ASA, TSA, and TSB protein concentrations were reduced slowly during the first 3 days of heat shock and then decreased dramatically by the fourth day, mirroring the appearance of visible symptoms. As predicted, the accumulation of heat shock protein 21 (HSP21) was induced (Figure 7). Similar to the lack of Trp protein induction, camalexin was not induced by heat shock (data not shown). These results extend our previous observations that compatible cauliflower mosaic virus and cucumber mosaic virus, both of which cause vein clearing and chlorosis, did not lead to the induction of camalexin or Trp proteins (Zhao and Last, 1996). The fact that not all stress conditions induce the Trp pathway and camalexin accumulation suggests that specific signals and not just general stress are needed for the induction.

Does SA Play a Role in Induction by Herbicidal Treatments?

SA appears to be required for maximal induction of camalexin and Trp proteins after infection with the bacterial pathogen *P. s. maculicola* ES4326 (Zhao and Last, 1996). If the response to chemical treatments requires SA, then the induction of camalexin and Trp proteins should be affected in NahG plants. To test this hypothesis, we subjected NahG and wild-type plants to the chemical treatments described

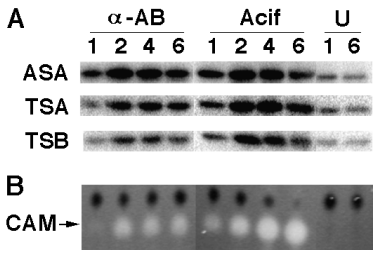

Figure 5. Induction of the Trp Biosynthetic Enzymes and Camalexin by α-AB and Acifluorfen.

Arabidopsis plants were grown on sterile nutrient agar plates for 2 weeks followed by treatment with α-AB or acifluorfen (Acif).
(A) Immunoblot analysis of Trp pathway proteins. The PAT protein was also induced by these treatments (data not shown).
(B) Thin-layer chromatographic analysis of camalexin (CAM).
The number of days after application of each chemical is indicated above each lane. Similar results were obtained in four independent experiments. U, untreated plants.

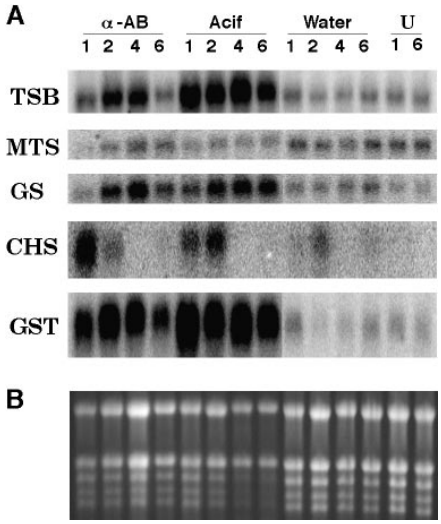

Figure 6. Regulation of mRNA Accumulation by α-AB and Acifluorfen Treatments.

The same tissue samples described in Figure 5 were used to isolate total RNA. Five micrograms of total RNA from each sample was loaded in each lane.

(A) RNA gel blots hybridized with *TSB1* (TSB), *MTS1* (MTS), *GSKB* (GS), *CHS*, and *GST1* (GST) cDNA probes.

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

The number of days after application of each treatment is indicated above each lane. Similar results were obtained in two independent experiments. Acif, acifluorfen; U, untreated plants; Water, water control.

above and analyzed the levels of ASA, TSA, and TSB proteins. As seen in Figure 8, the Trp proteins accumulated to ~60 to 80% of wild-type levels in NahG plants after these chemical treatments. However, the basal levels of the Trp proteins were also lower in the SA-deficient plants. Thus, NahG expression does not appear to have a dramatic effect on fold of induction of Trp proteins in response to amino acid starvation.

To test more carefully whether the degradation of SA affects Trp protein regulation, we analyzed the kinetics of induction in NahG and wild-type plants after the application of Lys plus Thr or α-AB. As described above for plants subjected to 3 days of treatment (Figure 8), the accumulation of ASA, TSA, and TSB was lower in NahG at all time points tested. However, when the level of ASA protein was normalized to that of the untreated plants, we observed no difference in induction kinetics between NahG and Columbia (Col-0) wild-type plants, as shown in Figure 9A. Comparable results were obtained for TSA and TSB protein induction (C.C. Williams and R.L. Last, unpublished results).

These similar induction kinetics could be explained in two different ways: (1) SA plays no role in modulating gene expression in response to these chemical treatments; (2) so much SA is produced in response to these treatments that salicylate hydroxylase levels in these NahG plants cannot maintain SA levels low enough to block SA-mediated induction of gene expression. To test the second hypothesis, we decided to determine whether chemically treated NahG plants would be able to induce *PR-2* gene expression, a sensitive indicator of SA accumulation. Both α-AB (Figure 10A) and Lys plus Thr–treated (data not shown) wild-type plants accumulated *PR-2* mRNA within 1 day, whereas expression was blocked in the NahG plants. Similar results were observed when other chemical treatments were used (K.J. Denby and R.L. Last, unpublished results). These results argue that the α-AB and Lys plus Thr treatments induced SA accumulation in wild-type plants and that this accumulation of SA is necessary for *PR-2* gene induction. Our data are consistent with the hypothesis that although NahG expression reduces both the basal and induced levels of Trp protein accumulation, SA is not required for chemically induced changes of Trp pathway gene expression.

Previous results from this laboratory demonstrated a strong SA dependence for increased camalexin accumulation during infection with virulent *P. s. maculicola* ES4326 (Zhao and Last, 1996). In this study, we tested whether chemical treatment–induced camalexin accumulation, shown in Figures 3 and 5, also required SA by analyzing the kinetics of induction in NahG and wild-type plants. As shown in Figure 9B, NahG plants produced less camalexin than did wild-type plants in response to both α-AB and Lys plus Thr treatments. However, unlike the dramatic effects of NahG after bacterial treatment (Zhao and Last, 1996), NahG plants had ~50% of the level of camalexin as that seen in wild-type plants. Because camalexin levels were too low to be quantified in untreated plants, we could not evaluate the fold induction for camalexin after chemical treatments. Thus, it is possible that the ~50% reduction in NahG camalexin levels was due to the effects of SA on basal level, as seen for Trp pathway proteins (Figures 8 and 9A).

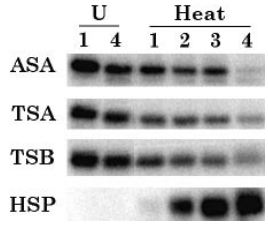

Figure 7. Regulation of the Trp Pathway Enzymes and HSP21 by Heat Shock Treatment.

The results of immunoblot analysis of the Trp pathway proteins and HSP21 (HSP) are shown. Arabidopsis plants were grown on sterile nutrient agar plates for 2 weeks. Control plates were maintained at 22°C (lane labeled U). Treated plates were transferred to 40°C (Heat), and the number of days of treatment is indicated above each lane. Similar results were obtained in two independent experiments.

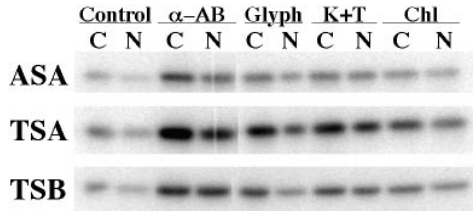

Figure 8. Accumulation of the Trp Pathway Enzymes in NahG Plants.

Untransformed Col-0 wild-type (C) and NahG (N) plants were grown on sterile nutrient agar plates for 2 weeks. These were treated with α-AB, glyphosate (Glyph), Lys plus Thr (K+T), or chlorsulfuron (Chl). The Trp pathway proteins were analyzed by immunoblotting. Similar results were obtained in three independent experiments with the Columbia NahG plants and in three experiments with an NahG line in the Landsberg *erecta* ecotype.

DISCUSSION

Cross-pathway regulation of amino acid and purine biosynthetic pathways in Arabidopsis was previously proposed to occur based on results obtained by inducing His starvation after treatment of seedlings with the imidazoleglycerol phosphate dehydratase inhibitor IRL1803 (Guyer et al., 1995). In that study, the authors observed increases in steady state mRNAs for five enzymes of aromatic amino acid biosynthesis, the Lys pathway enzyme dihydrodipicolinate synthase, and the purine biosynthetic enzyme 5′-phosphoribosyl-5-aminoimidizole synthetase, in addition to one of the two His biosynthetic genes analyzed. Surprisingly, this widespread induction of amino acid biosynthetic enzymes was not seen after starvation for aromatic or branched-chain amino acids (Guyer et al., 1995). In fact, the two Trp biosynthetic enzymes tested (TSB and PAT) had the only mRNAs whose steady state levels increased under these conditions. These results suggest that (1) His starvation, or some other less direct effect of IRL1803 treatment, is unique in activating expression of genes for varied metabolic enzymes; (2) the Trp biosynthetic enzymes are unusually sensitive to induction under amino acid starvation conditions.

Although our results confirm that the entire Trp pathway is strongly responsive to conditions that trigger amino acid privation (Figures 3 and 4), several lines of evidence suggest that the observed induction may be a stress response rather than a direct result of amino acid limitation. First was the surprising observation that camalexin, a pathogen-induced antimicrobial phytoalexin thought to be derived from the Trp pathway (Tsuji et al., 1993), was induced by branched-chain amino acid and Met starvation (Figure 3). This suggests that there is some physiological relationship between stress induced by amino acid starvation and pathogen infection. Second, two stress-inducible genes unrelated to amino acid biosynthesis, *CHS* and *GST*, were upregulated by these amino acid starvation conditions, whereas the amino acid biosynthetic genes *ALS* and *MTS* did not respond (Figure 4). Third, acifluorfen and α-AB, which are not thought to directly cause amino acid limitation, triggered an even stronger induction of the Trp enzymes and camalexin than that produced by amino acid starvation. Treatment with the oxidizing gas ozone has also been shown to induce *PAT* mRNA (Conklin and Last, 1995) and the Trp proteins used in this study (D.J. Kliebenstein, J. Zhao, and C.C. Williams, unpublished results). Finally, results in this study strengthen the hypothesis that there is coordinate regulation of the enzymes of Trp biosynthesis and camalexin (Zhao and Last, 1996). In all cases in which the Trp enzymes were induced, camalexin accumulation was observed, with the only exception being glyphosate treatment, which blocks camalexin precursor synthesis. Conversely, heat shock treatment did not activate expression of either pathway.

Our working hypothesis is that induction of the synthesis of Trp enzymes and camalexin is triggered at least in part by oxidative stress. For example, ozone and acifluorfen are both documented to cause enhanced reactive oxygen stress in plants. Similarly, enhanced active oxygen species are documented in plant–pathogen interactions and after elicitor treatment. Amino acid starvation is likely to damage chloroplasts, causing photooxidative stress. Considering that the Trp pathway and camalexin are induced by such a wide variety of biotic and abiotic stress conditions, it remains to be determined whether any part of the induction of the Trp pathway caused by chlorsulfuron, glyphosate, or Lys plus Thr treatments is directly due to amino acid limitation.

Can the changes in expression of the seemingly diverse set of enzymes reported with IRL1803 (Guyer et al., 1995) be explained without invoking cross-pathway regulation in response to amino acid starvation? Of the eight genes found to be induced by this treatment, five encode aromatic amino acid biosynthetic activities, including the committing and penultimate steps of the general aromatic amino acid (shikimic acid) pathway, two Trp pathway enzymes, and the committing enzyme of Phe and Tyr biosynthesis. Aromatic amino acid biosynthetic enzymes, including those found to be IRL1803 inducible, are all known to be upregulated in response to biotic and abiotic stress. For example, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase is inducible by wounding, elicitation, and pathogenesis in solanaceous plants and in Arabidopsis (Dyer et al., 1989; Keith et al., 1991; Henstrand et al., 1992; Görlach et al., 1995). Chorismate mutase is increased by wounding in potato tubers (Kuroki and Conn, 1988) as well as by elicitation and pathogen treatment in Arabidopsis (Eberhard et al., 1996); along with 5-enolpyruvylshikimate 3-phosphate synthase and shikimate kinase, it is upregulated after elicitor treatment or *Phytophthora infestans* infection in tomato (Görlach et al., 1995). Finally, one of two characterized anthranilate synthase α subunit genes (*ASα1*) is massively induced after fungal elicitor treatment in cell cultures of the acridone alkaloid–producing plant *Ruta graveolens* (Bohlmann et al., 1995).

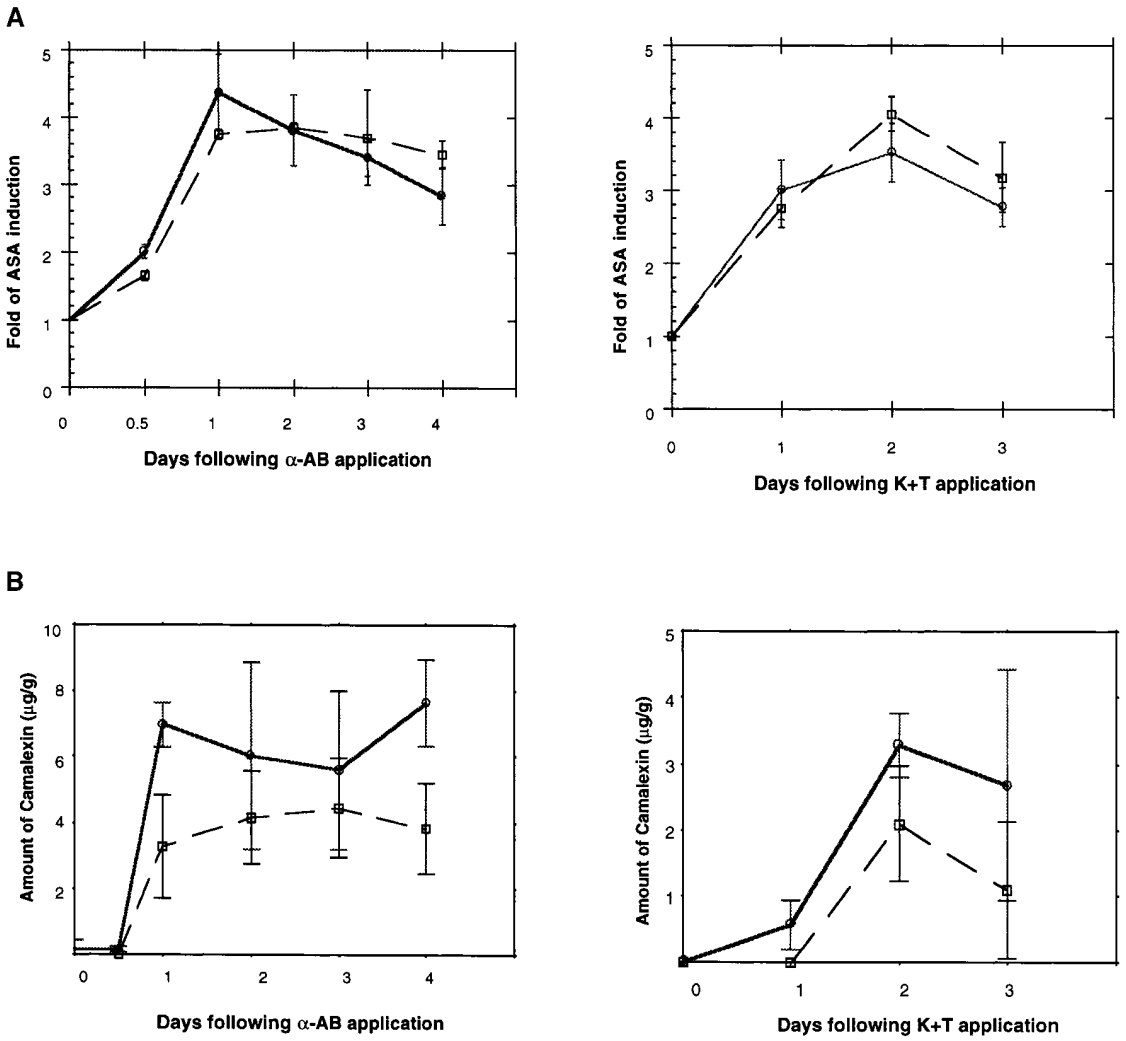

Figure 9. Kinetics of ASA Protein Induction and Camalexin Accumulation in Wild-Type and NahG Plants.

Untransformed Col-0 wild-type (circles) and NahG (squares) plants were grown on sterile nutrient agar plates for 2 weeks. Protein samples and camalexin extractions were performed 12 hr and 1, 2, 3, and 4 days after the application of α-AB, and 1, 2, and 3 days after the application of Lys plus Thr (K+T). Each datum is the mean from three or four independent samples, and standard deviations are indicated.
(A) Kinetics of ASA induction. The basal ASA levels from both untreated wild-type and NahG plants were independently normalized to a value of 1.
(B) Kinetics of camalexin accumulation. The amounts of camalexin accumulation after application of α-AB and Lys plus Thr were measured in micrograms per gram fresh weight of plant tissue.

Although less is known about stress induction of the Lys biosynthetic pathway, this amino acid is a precursor to the osmotic stress–inducible polyamine cadaverine and to several classes of alkaloids in plants as well (Hashimoto and Yamada, 1995; Ohmiya et al., 1995). Thus, it is plausible that the commiting enzyme dihydrodipicolinate synthase may be stress inducible. Induction of mRNAs for histidinol dehydrogenase (in the His pathway) and 5′-phosphoribosyl-5-aminoimidizole synthetase (in purine biosynthesis) may reflect pathway-specific regulation in response to His starvation rather than a cross-pathway mechanism. This is a reasonable hypothesis considering the close metabolic relationship between these two pathways: 5-aminoimidazole 4-carboxamide ribonucleotide, which is produced in the fifth step of the His pathway, is used as an intermediate in purine biosynthesis.

In fact, evidence for this sort of pathway-specific regulation was obtained in our studies in which starvation for branched-chain amino acids during chlorsulfuron treatment induced a rapid and strong induction of *ALS* mRNA (Figure

4). Feedback inhibition of enzyme activity by end product amino acids is another well-documented mechanism by which plants control accumulation of biosynthetically related amino acids (for recent examples, see Galili, 1995; Mourad and King, 1995; Singh and Shaner, 1995; Li and Last, 1996). Thus, as is seen in prokaryotes and fungi, it appears that plants regulate the activity of individual amino acid biosynthetic pathways at least in part through a combination of pathway-specific regulation of gene expression and feedback inhibition of enzyme activities. In this context, it is interesting to note that *CHS* mRNA, the committing enzyme of flavonoid biosynthesis, was strongly stimulated during aromatic amino acid starvation brought on by glyphosate treatment (Figure 4). This suggests that regulation of Phe-derived secondary metabolism responds to the availability of Phe or downstream metabolites.

We found some noteworthy differences between our results and those previously reported (Guyer et al., 1995). For example, whereas they reported that *GS* mRNA is downregulated after IRL1803, primisulfuron, or glyphosate treatments, we found the opposite result (Figure 4). In fact, *GS* mRNA identified with the *GSKB* probe was induced under all three amino acid starvation conditions as well as after treatment with α-AB and acifluorfen (Figure 6). We postulate that the difference in results reflects the fact that Guyer and colleagues used a probe for the chloroplast-localized GS2 isoform encoded by *GLN2* (GenBank accession number S69727) first described by Peterman and Goodman (1991), whereas our results were obtained with a probe for the presumed cytosolic isoform GSKB.

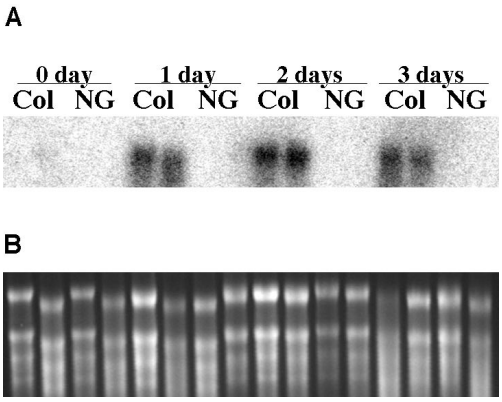

Figure 10. *PR-2* mRNA Expression in Wild-Type and NahG Plants after α-AB Treatment.

Untransformed Col-0 wild-type (Col) and NahG (NG) plants were grown on sterile nutrient agar plates for 2 weeks. Duplicate samples of total RNA were isolated 1, 2, or 3 days after the application of α-AB, and 5 μg of total RNA was loaded in each lane. Similar results were obtained in two independent experiments.

(A) RNA gel blot hybridized with the *PR-2* probe.

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

These classes of GS are thought to have overlapping but distinct functions based on differences in regulation of gene expression during development and in response to light as well as from analysis of GS2-deficient mutants (reviewed in Lam et al., 1995). For example, the cytosolic isoforms are proposed to be involved in primary nitrogen assimilation and reassimilation of nitrogen liberated during seed germination, whereas GS2 plays an essential role in reassimilation of photorespiratory ammonium. Perhaps cytosolic GS activity is upregulated during the phytotoxic inhibitor treatments to facilitate reassimilation of nitrogen liberated after protein degradation, whereas loss of *GS2* mRNA results from the inactivation of chloroplasts and loss of photorespiration activity. It is plausible that amino acid starvation, oxidative stress, and abiotic elicitor treatments induce protein degradation and amino acid catabolism while inhibiting photorespiration.

The rapid and strong induction of the Trp pathway enzymes and camalexin by acifluorfen was unexpected because Guyer and co-workers reported that this oxidative stress–promoting herbicide did not induce the accumulation of mRNAs for the amino acid and purine biosynthetic enzymes tested (Guyer et al., 1995). This inconsistency might be caused by the differences in growth conditions used. Whereas only the roots of plants in this report were submerged in nutrient agar medium, Guyer et al. (1995) germinated the plants on nutrient agar and transferred them to flasks containing liquid nutrient solution. These plants were grown with shaking for 1 week, at which time different herbicides were added. Consistent with the idea that growth conditions affect induction responses, we observed that the induction of Trp pathway enzymes and camalexin is significantly lower for soil-grown Arabidopsis plants compared with plate-grown Arabidopsis plants after chlorsulfuron treatment (J. Zhao and R.L. Last, unpublished results).

Maximal induction of the Trp pathway and camalexin after *P. s. maculicola* infection appears to require SA, because the expression of salicylate hydroxylase in NahG transgenic plants greatly attenuated camalexin accumulation and also reduced Trp enzyme induction compared with the wild type (Zhao and Last, 1996). In contrast, the results described in this study indicate that SA is not required for regulating the fold induction of the Trp pathway enzymes by amino acid starvation, α-AB, or acifluorfen under our growth conditions (Figures 8 and 9). This interpretation is complicated somewhat by the observation that Petri plate–grown wild-type plants have a slightly elevated accumulation of Trp pathway enzymes compared with soil-grown plants. Under these conditions, NahG plants have a lower pretreatment level of expression of Trp enzymes than do untransformed controls. One plausible explanation is that the untreated Petri plate–grown plants experience a basal level of stress that activates the Trp pathway enzymes in an SA-dependent manner. In contrast, accumulation of camalexin was reproducibly attenuated in NahG transgenic plants after chemical treatments (Figure 9B). Unfortunately, we cannot evaluate

whether fold induction was affected in NahG versus wild-type plants because camalexin could not be detected in untreated Petri plate–grown plants. Because of these technical difficulties, it remains to be seen whether SA has different roles in regulating accumulation of Trp proteins and camalexin in the chemical stress–induced system under study in this report. It also remains to be resolved whether SA functions differently in Trp and camalexin responses after pathogen versus chemical treatments. These issues should be resolved with more sensitive assays for camalexin and by analysis of mutants altered in stress regulation of the Trp and camalexin regulon.

METHODS

Growth and Treatments of Arabidopsis Plants

Plants (*Arabidopsis thaliana* ecotype Columbia [Col-0]) were grown on sterile PN or PNS (Haughn and Somerville, 1986) Petri plates under constant cool-white fluorescent light (60 to 100 $\mu E\ m^{-2}\ sec^{-1}$) at 22°C. NahG plants in the Col-0 ecotype (Delaney et al., 1994) or the Landsberg *erecta* ecotype (Bowling et al., 1994) were used for several experiments. For chemical treatments, plants were flooded with 3 mL of the filter-sterilized chemical solution dissolved in water 2 weeks after plating. The solution was allowed to spread evenly over the 30 mL of agar medium by shaking and rotating the plate. Stock solutions of glyphosate, chlorsulfuron, acifluorfen, and α-amino butyric acid (α-AB) were applied at 15 mM, 6 μM, 0.3 mM, and 10 mM, respectively; Lys and Thr were used at 20 mM each, with Met at 40 mM; and aromatic and branched-chain amino acids were used at 5 mM each, with the exception of Tyr, which was added as a 2 mM solution. The final concentration of these chemicals would be 10 times lower than the above concentration, assuming rapid diffusion into the 30 mL of nutrient agar medium in each plate. The treated plants were then put back into the original growth chamber and grown under constant light. For heat shock treatment, 2-week-old plants were transferred to a growth chamber set at either 37 or 40°C. All tissue samples were harvested into liquid nitrogen and stored at −80°C.

Immunoblot Analysis 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. The HSP21 antiserum was as previously described (Chen et al., 1990). 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, and signals were normalized to the amount of total RNA, as assayed by ethidium bromide fluorescence (Conklin and Last, 1995).

The anthranilate synthase α (ASA) probe was a 1.1-kb EcoRI fragment of the *ASA1* cDNA (Niyogi and Fink, 1992). The phosphoribosylanthranilate transferase (*PAT*) probe was a 1.6-kb XhoI fragment of the *PAT1* cDNA (Rose et al., 1992). The tryptophan synthase β (*TSB*) probe was a 1.5-kb EcoRI fragment of the *TSB1* cDNA from pMBT3 (Berlyn et al., 1989). The acetolactate synthase (*ALS*) probe was a 3.3-kb NcoI-XbaI genomic DNA fragment from pGH1 (Niyogi and Fink, 1992). The methionine synthase (*MTS*) probe was a 2.0-kb SalI-NotI fragment of the *MTS* cDNA from expressed sequence tag (EST) clone 38H1T7. The glutathione *S*-transferase (*GST*) probe was a 0.9-kb BamHI-KpnI fragment of the *GST1* cDNA from EST clone ATTS1553. The glutamine synthetase *GSKB* probe was a 0.4-kb SalI-NotI fragment of the cDNA represented by EST 45G12T7. The *PR-2* probe was a 0.5-kb SalI-NotI fragment of the *PR-2* cDNA from EST clone 104P22T7. Camalexin was extracted and analyzed as described by Zhao and Last (1996).

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