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Evidence for cross-pathway regulation of metabolic gene expression in plants

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ABSTRACT **In *Arabidopsis thaliana*, blocking histidine biosynthesis with a specific inhibitor of imidazoleglycerol-phosphate dehydratase caused increased expression of eight genes involved in the biosynthesis of aromatic amino acids, histidine, lysine, and purines. A decrease in expression of glutamine synthetase was also observed. Addition of histidine eliminated the gene-regulating effects of the inhibitor, demonstrating that the changes in gene expression resulted from histidine-pathway blockage. These results show that plants are capable of cross-pathway metabolic regulation.**

Integration among diverse metabolic pathways occurs in both prokaryotes and eukaryotes (1, 2). In several fungi, genes encoding enzymes in unrelated primary anabolic pathways are induced by an amino acid deficiency (3–8). This mechanism has been extensively studied in the yeast *Saccharomyces cerevisiae*, where it is known as general control (2). In this system, starvation for any one of a number of amino acids is known to cause 2- to 10-fold transcriptional activation of genes encoding at least 35 enzymes in 12 biosynthetic pathways, including aromatic amino acids, branched-chain amino acids, lysine, threonine, methionine, glutamine, histidine, arginine, and amino acyl-tRNA synthetases.

In plants the molecular biology of primary metabolism is a rapidly emerging discipline (9). To date, only one report of derepressed gene expression in response to pathway inhibition has appeared; blocking aromatic amino acid biosynthesis with the herbicide glyphosate causes a severalfold increase in activity of 2-keto-3-arabinoheptulosonate 7-phosphate synthase (DHS), the committed step in the shikimate pathway (10). Here, we show that specifically inhibiting a single biosynthetic pathway can induce expression of genes not only in that pathway but also in multiple unrelated pathways.

MATERIALS AND METHODS

Plant Growth. *Arabidopsis* seeds (Ecotype Columbia) were germinated on GM medium (Murashige–Skoog salts at 4.3 g/liter, Mes at 0.5 g/liter, 1% sucrose, thiamine at 10 μg/liter, pyridoxine at 5 μg/liter, nicotinic acid at 5 μg/liter, *myo*-inositol at 1 mg/liter, pH 5.8) containing agar at 8 g/liter and transferred to flasks containing liquid GM medium 1 week after germination (5 seedlings per 250-ml flask containing 50 ml of medium). Flasks were agitated (100 rpm) at 20°C in 16 hr of light (≈100 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 8 hr of dark. After 1 week of growth in liquid, inhibitors or supplements were added at the following final concentrations: IRL 1803 {3-hydroxy-3-[2H-(1,2,4)triazole-3-yl]-cyclohexyl-phosphonic acid}, 30 ppm (130 μM); glyphosate (Crescent Chemical, Hauppauge, NY), 120 ppm (712 μM); primisulfuron (CIBA-Geigy), 10 ppb (21 nM); acifluorfen (Crescent Chemical), 100 nM; histidine, 1 mM. Concentrations of herbicides were chosen that strongly inhibited seedling growth. In liquid culture, tissue was chlorotic at 84 hr after each inhibitor was added and became necrotic later. Glyphosate and primisulfuron-treated samples were harvested at 12, 36, and 60 hr after treatment.

Amino Acid Analysis. Free amino acids were extracted from equal samples of fresh weight 100 mg in water/$CHCl_3$/methanol as described (11). A known amount of the amino acid analog norleucine was added to each tissue sample before extraction to correct for recovery. HPLC separation and quantitation were done after derivitization with phenylisothiocyanate by the use of an Applied Biosystems amino acid analyzer.

RNA Gel Blot Analysis. Tissue was harvested by freezing in liquid N_2 at the times indicated. RNA preparations and gel blot hybridizations were done as described (12). Each gel lane contained 10 μg of total RNA; equal gel loading was confirmed by staining with ethidium bromide incorporated into each sample at gel loading.

Nucleic Acid Probes. Enzyme and corresponding probe were as follows: acetohydroxy acid synthase (AHAS); 1.5 kb cDNA—corresponding to nt 800–2300 (approximately) of the sequence in GenBank, accession no. X51514 (13); 5′-phosphoriboxyl-5-aminoimidazole synthetase (AIRS); PCR-amplified cDNA probe ≈1.1 kb in length—nt 218–1785 of GenBank, accession no. L12457 (14); anthranilate synthase β subunit (ASB); PCR-amplified cDNA probe corresponding to nt 129–1006 of *ASB1*—GenBank accession no. L22585 (15); chorismate mutase (CM); 1.2-kb full-length cDNA—GenBank accession no. Z26519 (16); dihydrodipicolinate synthase (DHPS); 250-bp PCR product amplified from genomic DNA with degenerate primers to conserved regions of the maize (17) and wheat (18) cDNAs; DHS; nt 138–1160 of *DHS2*—GenBank accession no. M74820 (19) (similar signals were obtained with DHS1 or DHS2 probes); *enol*pyruvylshikimate phosphate synthase (EPSPS); 1.5-kb PCR-amplified cDNA fragment (20); glutamine synthetase (GS); ≈1.7-kb PCR-amplified genomic fragment corresponding to nt 122–1226 from the published cDNA sequence—GenBank accession no. S69727 (21); histidinol dehydrogenase (HDH); partial cDNA clone obtained by cross-hybridization with the cabbage cDNA (22); imidazoleglycerol-phosphate dehydratase (IGPD); full-length cDNA of IGPD-1, which cross-hybridizes to both IGPD-encoding genes—GenBank accession no. U02689 (23) [gene-specific probes from either IGPD-1 or IGPD-2 (unpublished data) yielded similar signals]; phosphoribosylanthranilate transferase (PAT), PCR-amplified cDNA probe ≈850 bp in length; nt 1243–2713 of *PAT1*—GenBank accession no. M96073 (23). All PCR-amplified probes were sequenced to confirm their identity.

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Abbreviations: IGPD, imidazoleglycerol-phosphate dehydratase; DHS, 2-keto-3-arabinoheptulosonate 7-phosphate synthase; GS, glutamine synthetase; ASB, anthranilate synthase β subunit; DHPS, dihydrodipicolinate synthase; EPSPS, *enol*pyruvylshikimate phosphate synthase; HDH, histidinol dehydrogenase; PAT, phosphoribosylanthranilate transferase; AIRS, 5′-phosphoribosyl-5-aminoimidazole synthetase; CM, chorismate mutase; AHAS, acetohydroxy acid synthase.

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RESULTS AND DISCUSSION

Recently, specific inhibitors of IGPD have been described that are herbicidal (24, 25). IRL 1803 is a mechanism-based inhibitor of IGPD that mimics its substrate, imidazoleglycerol phosphate. In cell cultures, IRL 1803 potently inhibits growth in a manner that can be specifically overcome by histidine addition; pools of other amino acids tested (branched chain; branched chain plus aromatics; or a mixture of lysine, methionine, and threonine) had no effect on growth inhibition (25). In addition, IRL 1803 has been shown to reduce free histidine levels in *Xanthium* sp. (25). We tested the effects of IRL 1803 on free histidine levels in *Arabidopsis* seedlings. The plants were cultured in a minimal liquid medium to ensure immediate, uniform exposure to the inhibitor. After 60 hr of treatment with inhibitor, amino acid analysis revealed that young plants had virtually undetectable levels of free histidine (Fig. 1). A distinctive peak appeared, migrating close to histidine, that may represent accumulation of a biosynthetic intermediate. The distinctive metabolite and histidine resolved into two

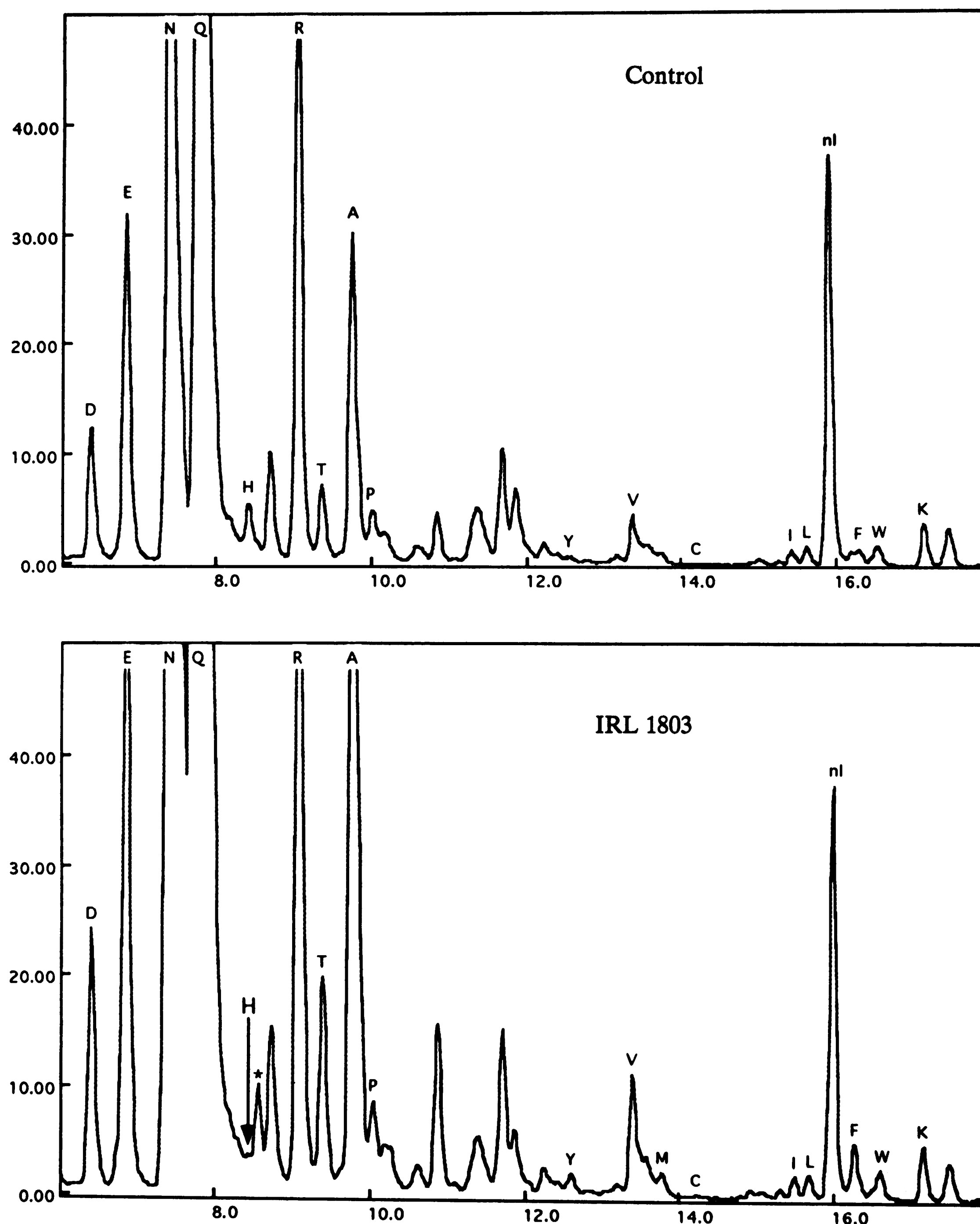

FIG. 1. Depletion of the free histidine pool by IRL 1803 treatment. HPLC chromatograms representing amino acid analysis of control plants and plants treated with IRL 1803 (130 μM, 60 hr). Position corresponding to depleted histidine peak in the IRL 1803-treated sample (determined by adding histidine to an aliquot of the sample before extraction) is marked with an arrow. A distinctive peak, perhaps corresponding to an accumulated intermediate, is marked with a star. Peaks corresponding to other amino acids are labeled with their standard single-letter abbreviations. Sample loadings were adjusted slightly to equalize heights of the norleucine (nl) peaks, thus allowing direct comparison between amino acid peak heights. The ordinate indicates absorbance, marked in arbitrary units; the abscissa indicates time in minutes.

peaks in samples treated with histidine. Therefore, the signal in IRL 1803-treated tissue clearly represents a metabolite distinct from histidine.

In *Saccharomyces cerevisiae*, one of the original demonstrations of general control used 3-amino-1,2,4-triazole, an inhibitor of IGPD in microbes (26), to starve cells for histidine (6). 3-Amino-1,2,4-triazole is also a herbicide but acts by an undetermined mode-of-action unrelated to histidine biosynthesis (27). Because IRL 1803 does exert its herbicidal effect by specifically inhibiting histidine biosynthesis, we wished to investigate its effects on expression of histidine biosynthetic genes and to determine whether it could exert a cross-pathway gene-inducing effect in plants similar to that of 3-amino-1,2,4-triazole in *S. cerevisiae.* As analyzed by RNA gel blot, an increase in the steady-state level of HDH (the last enzyme of the histidine biosynthetic pathway) mRNA was apparent within 24 hr after adding IRL 1803 (Fig. 2). IGPD mRNA did not reproducibly increase over control levels (Fig. 3).

Steady-state mRNA levels from the following genes were analyzed after treatment with IRL 1803 (Fig. 3): AHAS, the committed step in branched-chain amino acid biosynthesis; AIRS, the imidazole ring-closing step in *de novo* purine biosynthesis; ASB, the committed step in tryptophan biosynthesis; CM, the committed step in tyrosine and phenylalanine biosynthesis; DHPS, the committed step in lysine biosynthesis; DHS; EPSPS, the target for glyphosate in aromatic amino acid biosynthesis; GS, a key enzyme in nitrogen assimilation; HDH; IGPD; and PAT, the second step in tryptophan biosynthesis. In addition to HDH, the genes encoding AIRS, ASB, CM, DHPS, DHS, EPSPS, and PAT were significantly induced by treatment with IRL 1803. Expression of AHAS and IGPD was not changed, while GS was markedly repressed. Of these pathways, only purine biosynthesis shares a known metabolic link with histidine biosynthesis (28).

To rule out the possibility that IRL 1803 fortuitously altered gene expression by a mechanism independent of its effect on histidine biosynthesis, we treated plants with a mixture of the inhibitor plus histidine. In all cases, histidine addition blocked the changes in gene expression seen with inhibitor alone (Fig. 3).

Interestingly, free pools of some but not all amino acids (alanine, aspartate, glutamate, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine) increased between 1.5- and 2-fold in response to histidine starvation (Fig. 1). These changes may have resulted from increased activities of pathway-limiting enzymes that occur due to gene induction.

To investigate whether starvation for other amino acids had similar effects on gene expression, we treated seedlings with glyphosate or primisulfuron, herbicides that block aromatic and branched-chain amino acid biosynthesis, respectively (29, 30). As with IRL 1803, each compound significantly induced ASB and PAT; however, maximum induction occurred 12 hr after addition of either inhibitor (Fig. 4). GS mRNA was clearly decreased in both treatments but to a lesser extent than in histidine-starved tissue. Other biosynthetic genes for which expression was markedly altered by IRL 1803 treatment showed little response to either glyphosate (Fig. 4*A*) or primisulfuron (Fig. 4*B*).

To determine whether the changes in gene expression occurring after herbicide treatment were merely part of a broad response to lethal stress, we analyzed the effects of

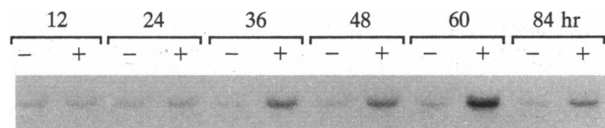

FIG. 2. Time course of HDH induction by IRL 1803. Gel blot of total RNA from plants treated with (+) or without (−) IRL 1803 at 30 ppm for the times indicated. The decrease in expression at 84 hr may be due to necrosis.

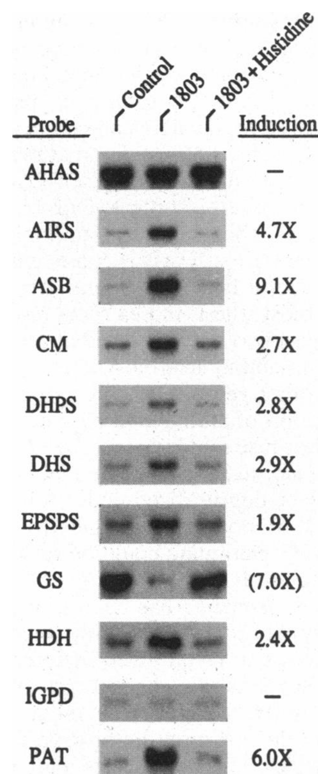

FIG. 3. Alteration of gene expression by IRL 1803. Gel blots of total RNA from plants treated as indicated, hybridized to nucleic acid probes corresponding to described genes. Samples were harvested at 60 hr after addition(s). Fold-induction (or repression) by IRL 1803 relative to control is listed at right.

treatment with the herbicide acifluorfen, which inhibits chlorophyll and heme biosynthesis but does not directly affect amino acid or purine metabolism (31). Over a 60-hr time course, during which the tissue became chlorotic and failed to grow, none of the genes examined in Fig. 3 were induced. Either a constant mRNA level or a slight decrease was seen in each case (data not shown).

Why perturbation of histidine biosynthesis, in particular, results in such dramatic changes in gene expression is unclear. Plants could be especially sensitive to the status of the histidine pathway. Biosynthesis of histidine is energy-intensive; indeed,

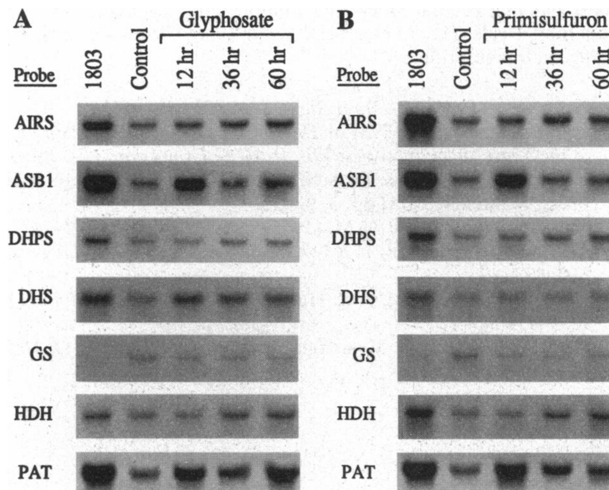

FIG. 4. Alteration of gene expression by glyphosate (*A*) and primisulfuron (*B*). Samples were harvested at the times indicated. The IRL 1803-treated controls were each harvested at 60 hr.

bacterial mutants resistant to feedback inhibition of histidine biosynthesis behave as functional adenine auxotrophs (32). Accumulation of a pathway-specific metabolic intermediate, such as imidazoleglycerol phosphate or imidazoleglycerol, could also be involved in signaling changes in gene expression.

Alternatively, the effects of histidine-pathway blockage may reflect a mechanism that is generally sensitive to amino acid starvation, such as general control. Our finding that some changes in gene expression can occur with inhibitors of other amino acid biosynthetic pathways is consistent with this latter model. Furthermore, it has been recently reported that inhibition of leucine biosynthesis in pea roots results in increased free pools of at least two amino acids that are not products of the branched-chain amino acid biosynthetic pathway (33).

In contrast to other genes that were expressed at elevated levels after inhibition of amino acid biosynthesis, GS expression decreased. Because glutamine is already present at very high levels in *Arabidopsis* seedlings (see Fig. 1), its biosynthesis could conceivably be deprioritized under conditions of amino acid starvation. It is not unreasonable to postulate that a reduced demand for glutamine could be reflected in changes in the steady-state levels of GS mRNA.

Teleologically, *S. cerevisiae* has been thought to possess a general control system to coordinate the biosynthesis of metabolites for which it is facultatively heterotrophic. Experimental evidence suggests that general control is required for optimal growth under conditions of amino acid imbalance (34). Most plants do not grow heterotrophically for amino acids and nucleotides; however, plants may encounter intracellular amino acid imbalances. For instance, infection with some pathogenic and parasitic bacteria can specifically inhibit individual amino acid pathways or deplete specific amino acid pools (35, 36). Physiological stresses may lead to an overabundance of a single amino acid; drought stress, for example, can induce accumulation of very high intracellular concentrations of proline (37). Excesses of individual amino acids in *S. cerevisiae* can also trigger the general control system (34).

Our results show that plants can manifest coordinate regulation among genes in unrelated metabolic pathways. Genetic analysis of the mechanism of gene induction will be required to determine the extent to which the plant response is analogous to similar responses in fungi. Further elucidation of the generality of this phenomenon in plants may yield clues to its physiological role in plant growth and development.

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