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Anaerobic Induction of Alanine Aminotransferase in Barley Root Tissue[1]

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ABSTRACT

Alanine aminotransferase, otherwise called glutamate-pyruvate aminotransferase (GPT), activity increases up to fourfold during several days of anaerobic induction in barley (*Hordeum vulgare* L.) roots, reaching a maximum activity of 13 international units per gram fresh weight. This increase in activity paralleled the increase in alcohol dehydrogenase activity in the same root tissue. Upon return to aerobic conditions, the induced GPT activity declined with an apparent half-life of 2 days. The isozyme profile of GPT in barley root tissue comprised one band of activity; in maize there were three bands of activity, the bands with greater mobility had much lower activity. Native polyacrylamide gel electrophoresis indicated that the induction of GPT activity results from an increase in the level of activity of these bands; no other activities were detected. When root tissue was induced under different levels of hypoxia (0%, 2%, 5%, and 21% O_2), changes in GPT activity were found to increase with lower levels of oxygen. Comparisons of GPT induction in barley, maize (*Zea mays*), rye, (*Secale cereale*) and wheat (*Triticum aestivum*) indicate that this enzyme is induced in the root tissue of all of these cereals; however, anaerobic root conditions do not result in the induction of GPT activity in leaf tissue. The dependence of GPT induction on high levels of nitrate in the media was tested by comparing activity levels in Hoagland solution and a nitrate-free nutrient solution. GPT activity was induced to similar levels under both conditions. These results indicate that alanine aminotransferase shows a very similar pattern of induction to alcohol dehydrogenase in barley root tissue and may be important in anaerobic glycolysis.

The flooding of soils can subject plant roots to considerable periods of anaerobiosis (5). This condition precludes aerobic respiration so that root survival becomes dependant on fermentative metabolism. Under these conditions, the synthesis of most cellular proteins is suppressed and a subset of anaerobic proteins are synthesized (18). These anaerobic proteins include ADH,[3] pyruvate decarboxylase (14), LDH (11), and several other glycolytic enzymes (12, 13). During periods of O_2 deficiency, plants produce a number of glycolytic end products including ethanol, lactate, various organic acids, and amino acids (2, 3).

Although large amounts of ethanol are produced by anaerobic roots, one of the major products of anaerobic metabolism in root tissue is alanine. Effer and Ranson (4) showed that the levels of alanine increased fourfold after 12 h of anaerobic treatment in buckwheat seedlings. Smith and apRees (21) and Hoffman *et al.* (11) have shown by radiolabeling studies that pea and barley roots produce large amounts of alanine under anaerobic conditions. The *in vivo* NMR data of Roberts *et al.* (17) also demonstrate that, even during the initial stages of anaerobiosis (0–120 min), alanine begins to accumulate in root tissue.

Pyruvate is converted to alanine in plant cells by alanine aminotransferase (EC 2.6.1.2) (GPT, 7). The occurrence of this enzyme has been reported in a number of different plants and plant tissues (1, 9, 15, 22). Hatch and Mau (10) found that a number of C_4 pathway species contain exceptionally high activities of aspartate and alanine aminotransferases. Two major isozymes of alanine aminotransferase were found in leaf tissue, one isozyme was associated with mesophyll cells, the other isozyme with bundle sheath cells (10). Biekmann and Feierabend (1) demonstrated that alanine aminotransferase activity in green leaves was predominantly localized in leaf microbodies and to a minor extent in the mitochondria. Lillo (15) has shown that the alanine aminotransferase activity undergoes diurnal fluctuations, as do several other enzymes.

We are interested in the regulation of genes in response to anaerobic stress. In anaerobic root tissue, an increase in ethanol and lactate is matched by an increase in the activity of ADH and LDH. Since under anaerobic conditions pyruvate is converted to alanine, we were interested in whether alanine aminotransferase activity also increased under anoxic conditions. We demonstrate that GPT activity does increase in activity in parallel with ADH activity. This increase in activity does not result in any change in GPT isozyme patterns as shown by native PAGE.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Barley seeds (*Hordeum vulgare* L. cv Himalaya) were surface-sterilized in 1% NaOCl (w/v) for 20 min, rinsed with water, and planted in moist perlite. After 4 d of growth at 20°C, individual seedlings were removed from the perlite and inserted through a hole in a foam stopper. Plants in their

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[3] Abbreviations: ADH, alcohol dehydrogenase; GPT, alanine aminotransferase; LDH, lactate dehydrogenase; TEMED, *N,N,N',N'*,-tetramethylethylenediamene.

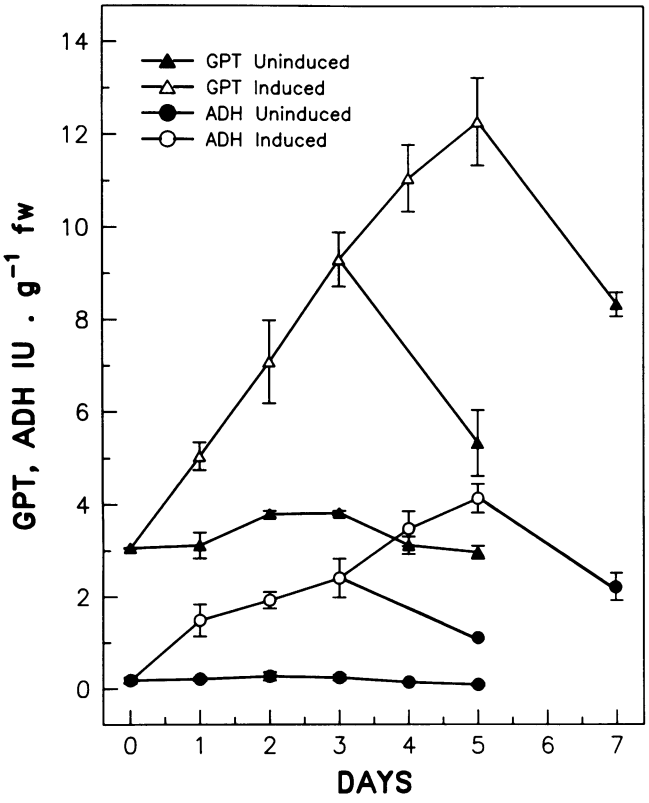

Figure 1. Induction of alanine aminotransferase (GPT) and alcohol dehydrogenase (ADH) activity in hypoxic barley roots. Plants were sparged with air until 2 weeks old; N_2 then replaced air for half of the plants. GPT and ADH activities are the means of three plants; bars represent the standard error. A portion of the plants that had been induced for 3 or 5 d was transferred to tanks sparged with air and tested for activity after 2 d. fw, fresh weight.

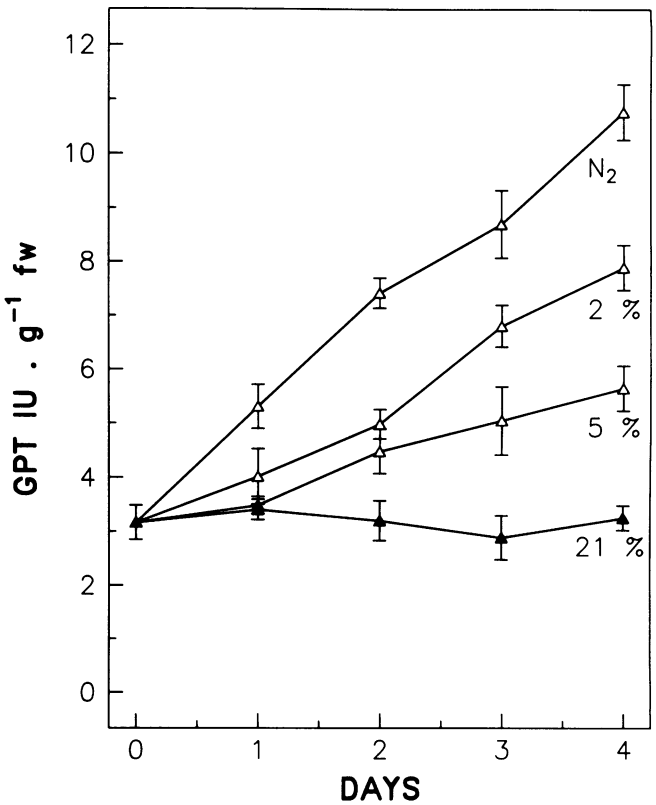

Figure 2. Induction of GPT activity under different O_2 concentration in the nutrient medium. Roots were sparged with various gas mixtures from premixed tanks and the O_2 levels in the nutrient solution monitored daily. Data points are the mean of three plants, bars represent the standard error. fw, fresh weight.

foam plugs were placed in a Plexiglas board cut to fit a 20-L fish tank. Plants were grown hydroponically with roots in darkness in half-strength Hoagland solution sparged continuously with air for 10 to 20 d. Hypoxic conditions were achieved by sparging the tank with N_2 or N_2 mixed with O_2. Growth chamber conditions were: day, 16 h, 20°C: night 8 h, 20°C; 250 μmol m^2 s^{-1} PPFD. Other cereals (*Zea mays* cv Spancross, *Triticum aestivum* cv Neepawa, *Secale cereale* cv Prima) were grown in the same way.

Extraction and Assay of ADH and GPT

Extractions were carried out at ice temperature. Roots were rinsed in distilled H_2O, briefly blotted, weighed, and ground with sand in a mortar and pestle in 0.1 M Tris-HCl (pH 8.0) containing 10 mM DTT, 15% (v/v) glycerol (EB). The brei was centrifuged for 3 min in a microcentrifuge and the supernatant assayed for enzyme activity. ADH assays were performed in the ethanol → acetaldehyde direction as previously described (8). GPT was assayed in the alanine → pyruvate direction by coupling the reaction to NADH reduction of lactate dehydrogenase as described by Lillo (15). The reaction mixture contained, in a final volume of 1 mL, 25 mM L-alanine, 5 mM 2-oxoglutarate, 0.1 mM NADH, 100 mM Tris-HCL (pH 8.0), 5 units of lactate dehydrogenase (Sigma L2375), and 50 μL of enzyme extract. The reaction was started by adding the 2-oxoglutarate and the assay temperature was 23°C. ADH and GPT activities are reported in international units (μmol/min).

Table I. *Induction of Alanine Aminotransferase Activity in Cereals*

All plants were sparged with air until 2 weeks of age. Roots were then sparged with air or N_2 for 5 d. N_2 leaf samples were from plants where the roots had been sparged with N_2.

	Root		Leaf	
	O_2	N_2	O_2	N_2
	IU/g fresh weight			
Maize	3.00 ± 0.46	6.84 ± 0.80	4.23 ± 0.81	3.75 ± 0.63
Barley	3.16 ± 0.37	11.97 ± 0.93	16.50 ± 1.19	14.37 ± 1.72
Rye	2.16 ± 0.24	7.56 ± 0.11	9.52 ± 1.49	6.58 ± 1.23
Wheat	3.09 ± 0.29	6.46 ± 0.86	17.52 ± 1.38	11.72 ± 0.82

Gel Electrophoresis

Nondenaturing electrophoresis was performed in slab gels (1.5 mm thickness) using 6% to 10% gradient gels. The 6% running gel buffer contained (w/v); 6.0% acrylamide-0.16% bisacrylamide, 5% (w/v) sucrose, and 0.37 M Tris-HCl (pH 8.9). The 10% running gel buffer contained (w/v); 10.0% acrylamide-0.26% bisacrylamide, 17% (w/v) sucrose, and

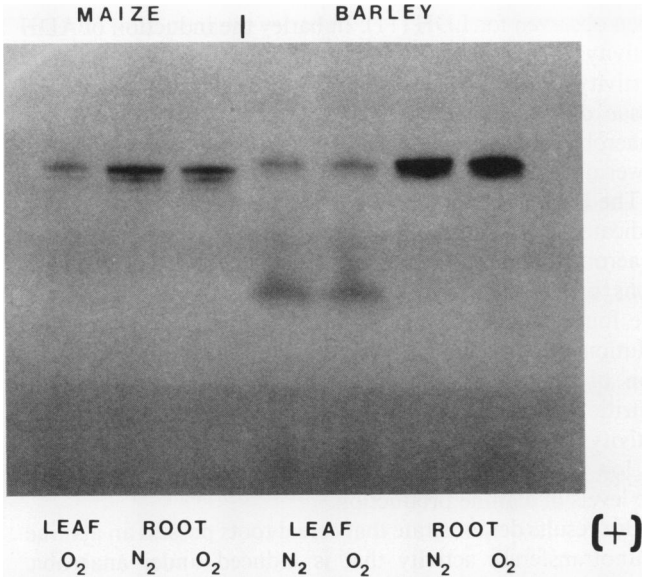

Figure 3. Native PAGE of cereal alanine aminotransferase activity. Samples are from uninduced (O_2) or induced (5 d N_2) root tissue. Leaf samples are also from uninduced or root induced (5 d N_2) plants. Each lane has the equivalent of 10 mg fresh weight of plant material loaded.

0.37 M Tris-HCl (pH 8.9). Both gel buffers were polymerized with 0.5 μL/mL TEMED and 0.32 mg/mL ammonium persulfate, and the gel was then immediately poured with a gradient former and peristaltic pump. The stacking gel was 5.0% acrylamide-0.13% bisacrylamide, 20% (w/v) sucrose, 60 mM Tris-HCl (pH 6.7), 0.6 μL/mL TEMED, and 5 μg/mL riboflavin, photo-polymerized for at least 3 h. Samples were homogenized in 10 mL of buffer/g fresh weight in EB. Typically, 90 μL of supernatant plus 10 μL of 90% glycerol containing 0.01% bromphenol blue was loaded. Gels were run at 4°C overnight. Gels were stained for GPT activity as described by Hatch and Mau (10). Briefly, gels were incubated for 30 min at 0°C in 50 mM Tris-HCl (pH 7.5) containing 20 units/mL LDH (Sigma). After warming to RT, the mixture was supplemented to 30 mM alanine, 10 mM 2-oxoglutarate, and 2 mM NADH. GPT activity from oxidized NADH resulted in nonfluorescent bands on a fluorescent background when viewed in UV light.

Nitrate-Free Nutrient Solution

Hoagland nutrient solution was prepared as described (23). The nitrate-free nutrient solution contained 4 mM $CaCl_2$, 2 mM $MgSO_4 \cdot 7H_2O$, 6 mM KCl, 1 mM $NaH_2PO_4 \cdot H_2O$, 0.07 g/L sequestrene 330 Fe, and 0.1 mL/L of the standard Hoagland micronutrient solution (21). The pH was then adjusted to 6.7 with NaOH.

RESULTS

Induction of GPT Activity

Alanine aminotransferase activity increased approximately fourfold after 5 d of induction (Fig. 1). No further induction in GPT activity was observed after that time. This increase in

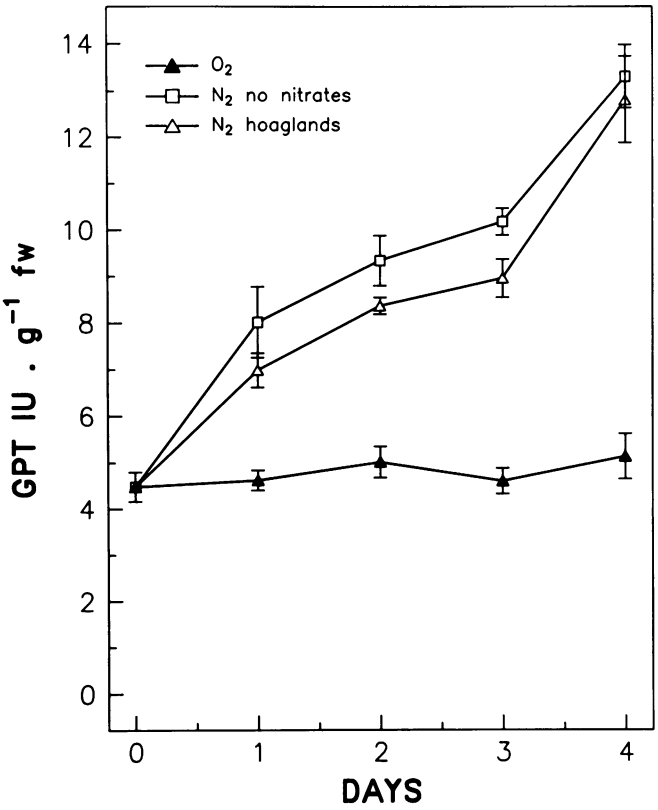

Figure 4. Induction of GPT activity in Hoagland solution and a N-free nutrient solution ("Materials and Methods"). Plants were sparged with air until 2 weeks old, then N_2 replaced air. The control plants were grown in Hoagland solution sparged with air. GPT activity are the means of three plants; bars represent the standard error. fw, fresh weight.

GPT activity paralleled the increase in ADH activity in the same root tissue; however, GPT activity was at least fourfold higher than the ADH activity (Fig. 1). It was necessary to maintain continuous hypoxia to retain the high level of GPT activity. When barley roots were returned to aerobic conditions, the level of GPT activity declined with an apparent half-life of 2 d. This decline in GPT activity was matched by a decline in ADH activity (Fig. 1) and is similar to what has been found for LDH (11).

Figure 2 demonstrates that GPT activity in barley roots is correlated with the degree of O_2 deficiency; the lower the level of O_2, the higher the level of GPT activity. Similar results have been found for the induction of LDH in barley root tissue (11). The induction of GPT activity also occurred in the roots of maize, wheat, and rye (Table I); however, the induction of barley GPT activity levels were higher than in any of the other three species. The level of GPT activity in leaf tissue was higher than in uninduced root tissue; however, anaerobic treatment of root tissue did not result in any increase in leaf GPT activity (Table I). Instead, plants with anoxic roots had lower levels of GPT activity in their leaf tissue.

Isozyme Profile of GPT

The isozyme profile of GPT is shown in Figure 3. Root tissue from either induced or uninduced barley plants had a

single band of GPT activity. The increase in root specific GPT activity resulted from an increase in activity of this single band; there was no evidence that, under hypoxia, different isozymes were expressed in comparison to aerobic conditions. In contrast, barley leaf tissue displayed two bands of activity, with the second band having much greater mobility than the root activity. Maize root and leaf tissue displayed three bands of activity, the bands with greater mobility having much lower levels of activity (Fig. 3). There was no change in the GPT activity pattern of maize root or leaf tissue when the root tissue was under anaerobic conditions.

GPT Induction under Low Nitrate Conditions

Hoffman *et al.* (11) have pointed out that the accumulation of alanine in hypoxic root tissue may depend on adequate N nutrition. The barley plants grown in these experiments were supplied with 7.5 mM NO_3^- in the nutrient solution. To test whether the induction of alanine aminotransferase was influenced by the level of NO_3^- in the nutrient solution we transferred plants to a nitrogen-free nutrient solution (see "Materials and Methods",) for 24 h, and then sparged plants with N_2 for 5 d. The data shown in Figure 4 demonstrate that the level of GPT activity increased similarly in Hoagland solution and in a N-free nutrient solution.

DISCUSSION

Alanine aminotransferase activity has been characterized in the leaves and cellular components of a number of different plant species (1, 9, 10). It is involved in alanine biosynthesis, and different GPT isozymes have been shown to be associated with the mesophyll and bundle sheath cells in several C_4 pathway plants (10). The observation that high levels of GPT activity compared with ADH or LDH activity were found in uninduced roots was not surprising, since GPT has functions in the plant cell other than just fermentative metabolism. However, the demonstration that GPT activity increases during hypoxia in a pattern similar to ADH indicates that this enzyme also undergoes a similar type of anaerobic induction (Fig. 1). This similarity in pattern of induction is further emphasized by the fact that lower levels of O_2 resulted in higher GPT activity (Fig. 2).

In contrast to ADH, where different genes are induced under anaerobic conditions (6, 8, 18), the induction of GPT activity in root tissue did not result in the differential increase in any specific band of GPT activity. Barley and maize root tissue and maize leaf were characterized by bands of activity that migrate very close together on native PAGE gels. In contrast, barley leaf material expressed a second band of activity of much higher mobility than found in root tissues (Fig. 3). Hatch and Mau (10) have shown that there are two GPT isozymes in a number of C_4 plants and that the isozyme present in bundle sheath cells corresponds to the higher mobility band. Surprisingly, we found that barley (a C_3 plant) had a pattern of GPT activity more akin to Hatch and Mau's (10) observations than did maize, a C_4 plant. The induction of GPT activity in all four species studied indicates that this induction is a general phenomenon found in the Gramineae. However, the levels of induction of this enzyme differ, as has been observed for LDH (11). In barley the induction of ADH activity in the root tissue results in an increase in ADH acrtivity in leaf tissue (16). However, GPT activity in leaf tissue did not increase when the plants roots were under anaerobic conditions. In fact, GPT activity levels were slightly lower under these conditions.

The large fluxes of ^{14}C to alanine from ^{14}C sugars (11, 21) indicate that large amounts of alanine may accumulate in anaerobic roots. However, under normal ecological conditions, one of the results of soil hypoxia is denitrification (5). We found that GPT activity is induced in both Hoagland solution and a N-free nutrient solution. Therefore, the induction of this enzyme is not dependent on the level of N nutrition during prolonged hypoxia. However, whereas GPT activity is induced in hypoxic roots under conditions of low N, low levels of glutamate (the amino donor) could reduce the levels of alanine production.

Our results demonstrate that cereal roots possess an alanine aminotransferase activity that is induced under anaerobic conditions similar to ADH and LDH induction. Previous work (11, 21) has demonstrated that ethanol glycolysis and alanine synthesis are major pathways of pyruvate metabolism in anaerobic root tissue. However, unlike ADH and LDH, the production of alanine by GPT does not regenerate NADH. Why, then, would root tissue produce such large amounts of alanine under anaerobic conditions? In contrast to ethanol, which diffuses out into the media, most of the alanine produced under anaerobic conditions is retained in the root tissue (11). Sakano and Tazawa (19, 20) have pointed out that the free amino acids in the vacuole of plant cells are relatively inactive in the metabolic sense and contribute to cytoplasmic homeostasis. However, during times of deficiency, these vacuolar amino acids can be transported across the tonoplast membrane into the cytoplasm and then utilized. They observed that alanine is the most rapidly transported amino acid (20). Perhaps under anaerobic conditions alanine is produced in large quantities but stored in the vacuole, where it is metabolically inactive until such time as it can be utilized by the cell. In this way the cell would retain the pyruvate carbons that would be lost if the cell were to produce ethanol which could diffuse out into the media. Although the level of GPT activity in anoxic root tissue suggests that alanine formation may be a major pathway in fermentative metabolism, the levels of substrates may limit the production of alanine in N-limited conditions. Further studies along this line are currently in progress in our laboratory.

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