The Plant Journal (2007) **49**, 1108–1121 doi: 10.1111/j.1365-313X.2006.03023.x

Alanine aminotransferase catalyses the breakdown of alanine after hypoxia in *Arabidopsis thaliana*

Yo Miyashita[1], Rudy Dolferus[2], Kathleen P. Ismond[1] and Allen G. Good[1,*]
[1]*Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada, and*
[2]*CSIRO Division of Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia*

Received 22 September 2006; revised 8 November 2006; accepted 14 November 2006.
*For correspondence (fax +1 780 492 9234; e-mail allen.good@ualberta.ca).

Summary

Alanine aminotransferase (AlaAT) catalyses the reversible transfer of an amino group from glutamate to pyruvate to form 2-oxoglutarate and alanine. The regulation of *AlaAT* in several plant species has been studied in response to low-oxygen stress, light and nitrogen application. In this study, induction of Arabidopsis *AlaAT1* and *AlaAT2* during hypoxia was observed at the transcriptional level, and an increase in enzyme activity was detected in hypoxically treated roots. In addition, the tissue-specific expression of *AlaAT1* and *AlaAT2* was analysed using promoter:GUS fusions. The GUS staining patterns indicated that both *AlaAT* genes are expressed predominantly in vascular tissues. We manipulated *AlaAT* expression to determine the relative importance of this enzyme in low-oxygen stress tolerance and nitrogen metabolism. This was done by analysing T-DNA mutants and over-expressing barley *AlaAT* in Arabidopsis. The *AlaAT1* knockout mutant (*alaat1-1*) showed a dramatic reduction in AlaAT activity, suggesting that AlaAT1 is the major AlaAT isozyme in Arabidopsis. Over-expression of barley *AlaAT* significantly increased the AlaAT activity in the transgenic plants. These plants were analysed for metabolic changes over a period of hypoxic stress and during their subsequent recovery. The results showed that *alaat1-1* plants accumulate more alanine than wild-type plants during the early phase of hypoxia, and the decline in accumulated alanine was delayed in the *alaat1-1* line during the post-hypoxia recovery period. When alanine was supplied as the nitrogen source, *alaat1-1* plants utilized alanine less efficiently than wild-type plants did. These results indicate that the primary role of AlaAT1 is to break down alanine when it is in excess. Therefore, AlaAT appears to be crucial for the rapid conversion of alanine to pyruvate during recovery from low-oxygen stress.

Keywords: alanine aminotransferase, *AlaAT*, nitrogen metabolism, anaerobic stress.

Introduction

Transient flooding often causes oxygen deprivation in the rhizosphere. Such oxygen limitation adversely affects plant performance, as plant roots require sufficient oxygen supply to attain their full functions. Plants respond to low-oxygen stress by activating the pathway of anaerobic metabolism. The *de novo* synthesis of approximately 20 anaerobic polypeptides (ANPs) (Sachs *et al.*, 1980) triggers the metabolic shift from oxidative phosphorylation to anaerobic fermentation. These ANPs, including the enzymes of glycolysis and anaerobic fermentation pathways, allow a limited amount of ATP synthesis in an oxygen-independent manner, typically resulting in the production of ethanol and lactate (Sachs *et al.*, 1996; Vartapetian and Jackson, 1997). The parallel expression of genes encoding enzymes involved in nitrogen assimilation and amino acid metabolism also indicates that diverse metabolic pathways operate under low-oxygen conditions (Dennis *et al.*, 2000; Liao and Lin, 2001). The production and accumulation of certain amino acids is indeed another common response of plants subjected to low-oxygen stress (Reggiani and Bertani, 2003; Ricard *et al.*, 1994; Streeter and Thompson, 1972).

Alanine aminotransferase (AlaAT; E.C. 2.6.1.2) converts pyruvate and glutamate to alanine and 2-oxoglutarate. In doing so, it is involved in both carbon and nitrogen metabolism in plants. Arabidopsis contains four AlaAT homologues. Two of these homologues encode GGAT1

and GGAT2, which have both AlaAT activity and glutamate:glyoxylate aminotransferase (GGAT) activity (Igarashi *et al.*, 2003; Liepman and Olsen, 2003). The presence of a peroxisomal targeting signal in both GGAT1 and GGAT2 suggests that they are localized in the peroxisomes (Liepman and Olsen, 2003), and subcellular fractionation analysis has found GGAT activity to be localized exclusively within Arabidopsis peroxisomes (Igarashi *et al.*, 2003; Liepman and Olsen, 2003). GGATs appear to play important roles in photorespiration and amino acid metabolism (Igarashi *et al.*, 2003; Liepman and Olsen, 2003). The other two genes, *AlaAT1* and *AlaAT2*, have not been characterized in detail. *In silico* predictions suggest that AlaAT2 is mitochondrially localized while AlaAT1 is cytosolic (Liepman and Olsen, 2003). Cytosolic localization of a GFP–AlaAT1 fusion protein was observed in *Nicotiana tabacum* BY-2 cells (Igarashi *et al.*, 2003).

The induction of *AlaAT* during hypoxia has been reported for several plant species including *Hordeum vulgare* (barley) (Good and Crosby, 1989; Muench and Good, 1994), *Zea mays* (maize) (Muench *et al.*, 1998) and *Glycine max* (soybean) (Sousa and Sodek, 2003). Recent microarray studies have also demonstrated up-regulation of *AlaAT1* in Arabidopsis during hypoxia (Klok *et al.*, 2002; Loreti *et al.*, 2005). Furthermore, induction of *AlaAT* by other stresses has been observed; for example, *AlaAT* expression is regulated by light and nitrogen stress in the leaves of *Panicum miliaceum* (Son *et al.*, 1991, 1992). *AlaAT* was also up-regulated during recovery from nitrogen stress in maize (Muench *et al.*, 1998). It is not unusual for enzymes to be involved in more than one stress response. Alcohol dehydrogenase (ADH) is likewise induced by several stresses, including wounding, low-oxygen, cold, ABA and heat (Dolferus *et al.*, 1994a).

AlaAT expression has been detected in various tissues and organs. In addition to *AlaAT* expression in roots and leaf tissues, which is observed in most plant species, *AlaAT* expression has been detected in the inner endosperm tissues of developing seeds of *Oryza sativa* (rice) (Kikuchi *et al.*, 1999) and floral organs in Arabidopsis (Igarashi *et al.*, 2003). The versatile expression profile of *AlaAT* suggests the involvement of this enzyme in various physiological processes throughout the life cycle of plants.

Under hypoxic conditions, alanine is produced in relatively large amounts and becomes one of the most abundant amino acids in plant roots (Fan *et al.*, 1997; Good and Muench, 1993; Reggiani *et al.*, 1985; Sousa and Sodek, 2003; Streeter and Thompson, 1972). However, the mechanism of alanine accumulation and its ameliorating effect on low-oxygen stress have not been elucidated. It has been suggested that AlaAT is the key enzyme responsible for hypoxic alanine production based on its induction profiles in response to low-oxygen stress (Good and Crosby, 1989; Muench and Good, 1994). However, Sousa and Sodek (2003) point out that most of the increase in AlaAT activity takes place after alanine production ceases, suggesting that the major role of AlaAT occurs during the recovery from low-oxygen stress. While the purpose of hypoxic alanine production remains unclear, evidence suggests that it is important in the survival and nitrogen metabolism of plants under hypoxic conditions. In the single-cell alga *Selenastrum minutum*, most of the external ammonium assimilated under hypoxic conditions was incorporated into alanine (Vanlerberghe *et al.*, 1991). Thus, alanine production under hypoxia is a mechanism by which the alga increases its nitrogen pool in preparation for the return to normal oxygen conditions. Supporting these findings is the observation that the anaerobic tolerance of rice coleoptiles depends upon the assimilation of inorganic nitrogen into alanine and other amino acids (Fan *et al.*, 1997). In the legume *Medicago truncatula*, an inhibitor of azaserine (GOGAT) that indirectly blocks alanine synthesis impairs germination and seedling development under anoxic conditions (Ricoult *et al.*, 2005).

We are interested in studying the roles of hypoxically inducible genes to improve flooding tolerance in plants. We have previously demonstrated that increasing metabolic flux through the ethanol fermentation pathway improves low-oxygen stress tolerance in Arabidopsis (Ismond *et al.*, 2003). In this study, we have over-expressed barley *AlaAT* to assess the potential benefits of altering *AlaAT* expression under low-oxygen conditions. We have also analysed an *AlaAT1* T-DNA knockout mutant to characterize the role of this enzyme in hypoxic alanine production and plant survival during low-oxygen stress. Our results suggest that hypoxic alanine synthesis does not require AlaAT activity; but indicate that AlaAT activity contributes to the rapid conversion of accumulated alanine to pyruvate during the post-hypoxia recovery period. Moreover, we have looked at the tissue-specific expression of both *AlaAT1* and *AlaAT2* using promoter:GUS fusions. Here we describe our findings regarding the regulation and functional characterization of AlaAT in Arabidopsis.

Results

Arabidopsis AlaAT *gene family and their expression profiles*

The Arabidopsis *AlaAT* gene family consists of four *AlaAT* genes (Igarashi *et al.*, 2003; Liepman and Olsen, 2003). These four genes are subdivided into two groups, genes encoding AlaAT (*AlaAT1*, At1g17290; *AlaAT2*, At1g72330) and those encoding GGAT (*GGAT1*, At1g23310; *GGAT2*, At1g70580). Arabidopsis *AlaAT1* and *AlaAT2* have high sequence identity to the previously characterized *AlaAT* genes of other plant species such as barley (Muench and Good, 1994), maize (Muench *et al.*, 1998) and rice (Kikuchi *et al.*, 1999). Igarashi *et al.* (2003) have analysed the expression profile of each member of the Arabidopsis *AlaAT* gene family using real-time RT-PCR and have shown expression of *AlaAT1* in both

leaves and roots, as well as strong expression of *GGAT1* in leaves. To confirm similar gene expression profiles in our growth system (see Experimental procedures), we examined expression levels of each gene using total RNA isolated from 17-day-old seedlings. Strong expression of *AlaAT1* was seen in both the roots and shoots, whereas weak expression of *AlaAT2* was detected only in the shoots (Figure 1). The expression of *GGAT1* was observed in shoot tissue but not in root tissue (Figure 1). These results indicate that *AlaAT1* is the major gene expressed in roots among the four members of the Arabidopsis *AlaAT* gene family.

Expression of AlaAT *genes is induced by low-oxygen stress*

The hypoxic induction of Arabidopsis *AlaAT1* has been shown previously in microarray studies (Klok *et al.*, 2002; Liu *et al.*, 2005; Loreti *et al.*, 2005). These experiments demonstrated that low-oxygen stress rapidly induced *AlaAT1* expression upon onset of the stress (Figure 2a). A dramatic induction of the well-characterized anaerobically induced gene, *ADH1*, was also observed in the microarray experiment (Figure 2a). We analysed the expression profiles of the *AlaAT* gene family in response to low-oxygen stress. To do this, we performed semi-quantitative RT-PCR on total RNA samples isolated from plants subjected to various lengths of hypoxic stress (Figure 2b). In the roots, induction of both *AlaAT1* and *AlaAT2* was observed after 2 h of hypoxia, and peaked at 8 h (>fourfold increase). In the shoots, this up-regulation occurred more rapidly and was obvious for both genes after 2 h of hypoxic stress treatment. These results demonstrate that both *AlaAT* genes are induced during hypoxia. For *GGAT1*, there was no detectable message in the root tissue; however, induction was observed in shoot tissue after 8 h of hypoxia. The induction of *GGAT1* in shoot tissue contrasts with what was observed in the microarray experiments (Figure 2a). As the different analyses were conducted at similar time points (6 h vs. 8 h), the reason for the discrepancy between these results is unclear. Expression of GGAT2 was virtually undetectable at all time points in roots and shoots.

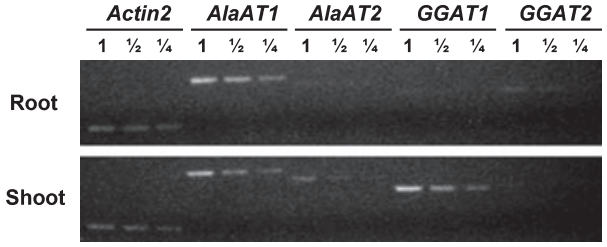

Figure 1. Semi-quantitative RT-PCR of the *AlaAT* gene family.
Total RNA isolated from shoots and roots of 17-day-old plants were used. PCR was carried out with two- or fourfold diluted aliquots of the synthesized first-strand cDNA (lanes 1/2 and 1/4, respectively), as well as undiluted aliquots (lane 1).

AlaAT *genes are preferentially expressed in vascular tissues*

Transgenic plants expressing the β-glucuronidase (GUS) reporter gene under the control of the *AlaAT1* and *AlaAT2* promoters were stained for GUS activity under both aerobic and hypoxic conditions. Under aerobic conditions, *pAlaAT1:*GUS transgenic plants showed strong GUS expression in the vascular tissues of both roots and shoots (Figure 3a,e). GUS staining in the roots was noticeably fainter in the sections closer to the tip of the roots, and was absent in root tips as well as in the lateral root primordia (Figure 3a,b). In the leaves, preferential staining of vascular tissues was observed, but staining was also seen throughout the young rosette leaves (Figure 3e). *pAlaAT2:*GUS transgenic plants showed similar expression patterns in the shoots, but the coloration was fainter (Figure 3k). In addition, cotyledons and the first pair of rosette leaves were not stained intensely (Figure 3k). In the roots, vascular GUS expression was limited to the upper portions of the primary root (data not shown). Typically, GUS expression was absent in most of the root system, including the lateral roots, the lateral root primordia and the root tips (Figure 3g,h). Non-transgenic plants showed no background GUS activity (data not shown).

In hypoxically treated plants, similar GUS staining patterns to those of untreated plants were observed for both *pAlaAT1:*GUS and *pAlaAT2:*GUS transgenic plants (Figure 3c,i). To detect low-level GUS activity, GUS staining was also performed overnight (14 h duration). When stained for this longer time period, more intense vascular staining of the roots was apparent for *pAlaAT1:*GUS plants (Figure 3d), but it was still faint for *pAlaAT2:*GUS plants (Figure 3j). The root tips remained unstained for both types of transgenic plants. In the shoots, GUS expression occurred predominantly in the vascular tissues, but weak staining was also detected in non-vascular leaf tissue for both *pAlaAT1:*GUS and *pAlaAT2:*GUS plants. For better resolution, the leaves of 20-day-old plants stained overnight are shown (Figure 3f,l).

To test whether the promoter fragments used in this study were capable of expressing GUS in a similar way to the native promoters, we confirmed that the hypoxic induction patterns of GUS were similar to those of *AlaAT1* or *AlaAT2* (Figure S1). The results indicate that the expression patterns of GUS shown in Figure 3 reflect the tissue specificity of the two endogenous *AlaAT* genes.

Isolation of an alaat1-1 *mutant and transgenic plants over-expressing a barley* AlaAT *gene*

We screened Salk T-DNA insertion lines (Alonso *et al.*, 2003) in search of knockout mutants for *AlaAT1* and *AlaAT2* (for the complete list of the Salk lines screened in this study, see Table S1). We identified one mutant line containing a T-DNA insertion in *AlaAT1* between the 8th and 9th exons (Figure 4a). Left border sequences were found on both sides of

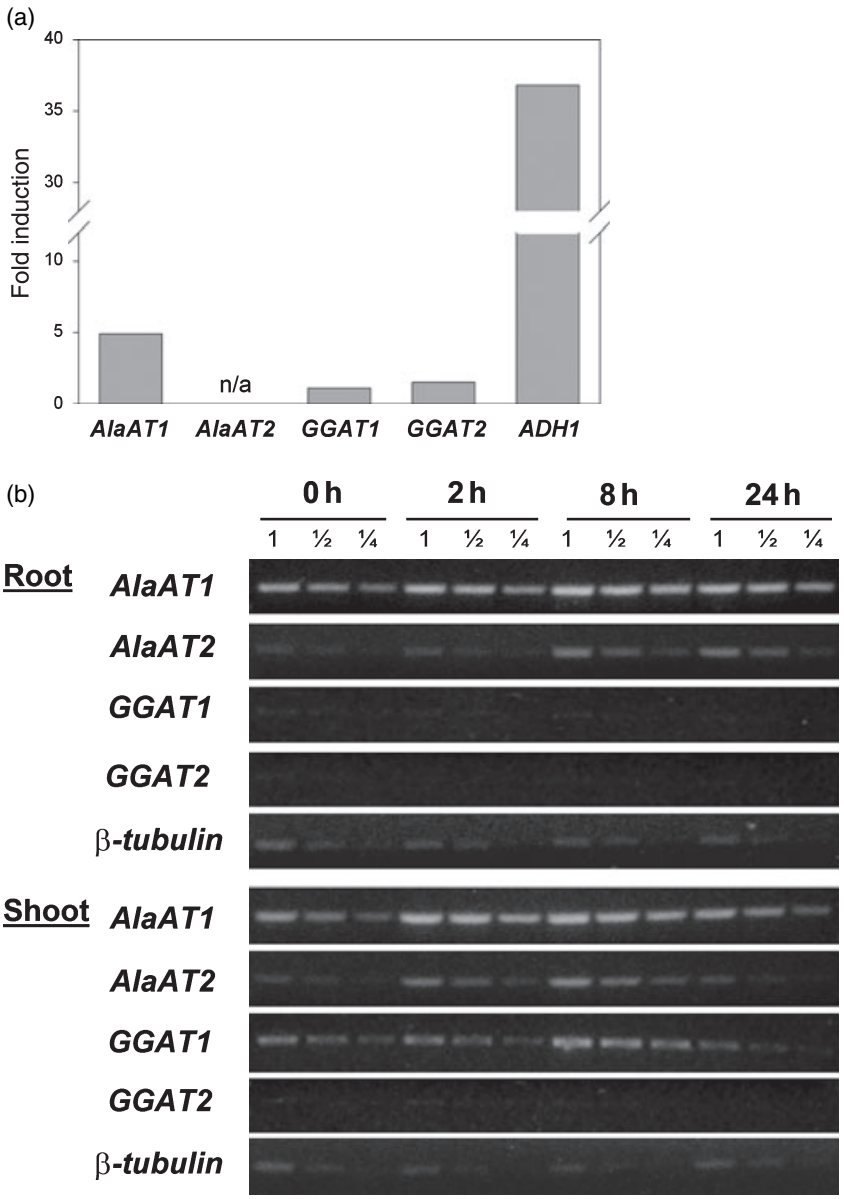

Figure 2. Hypoxic induction profiles of *AlaAT* genes.
(a) Microarray experiments showing the induction of *AlaAT1* and *ADH1* in response to anoxia. The data are taken from the microarray experiment performed by Loreti *et al.* (2005). RNA samples for the microarray were extracted from anoxically treated (6 h) and untreated 4-day-old Arabidopsis seedlings. The expression levels of each gene were compared between the two treatments. n/a, not available.
(b) Semi-quantitative RT-PCR showing the increased expressions of *AlaAT* transcripts in roots and shoots in response to hypoxic stress. Total RNA was extracted from plants that had been hypoxically treated for the indicated period. PCR was carried out with two- or fourfold diluted aliquots of the synthesized first-strand cDNA (lanes 1/2 and 1/4, respectively) as well as undiluted aliquots (lane 1).

the insertion junction, suggesting the insertion of inverted repeats of the T-DNA. RT-PCR analysis indicated the absence of normal *AlaAT1* transcript in this line (Figure 4b), thus we designated the mutant line *alaat1-1*. An aberrant product was amplified when using total RNA isolated from the shoots of *alaat1-1*, and appeared to be derived from non-specific annealing of the primer to a T-DNA region, based on sequence results (data not shown). Western blot analysis confirmed the absence of AlaAT protein in the *alaat1-1* line (Figure 4c). To confirm the number of loci containing T-DNA insertions in *alaat1-1*, the knockout mutant was crossed with non-transgenic plants. Segregation of the kanamycin resistance gene, *NPTII*, was followed by PCR in the F_2 population as *NPTII* was silenced in the mutant line. *NPTII* segregated in a 3:1 ratio, and was detected only in plants containing the T-DNA insertion within *AlaAT1* (data not shown), suggesting that the *alaat1-1* line contained the T-DNA insertion in only one locus.

For *AlaAT2*, the only T-DNA insertions found were in the promoter region of the gene (Table S1). These insertions did not affect the expression of *AlaAT2* under either aerobic or hypoxic conditions (data not shown). Thus, no *AlaAT2* mutants were identified.

To produce transgenic plants with higher levels of AlaAT activity, we over-expressed a barley *AlaAT* cDNA under the control of the CaMV 35S promoter. Twenty independent transgenic lines were carried through to the T_3 and T_4 generations. Segregation of *NPTII* indicated that, of the lines selected (Ox*AlaAT#10*, *#15*, *#18* and *#20*; prefix 'Ox' for over-expression) based on the preliminary enzyme activity assay,

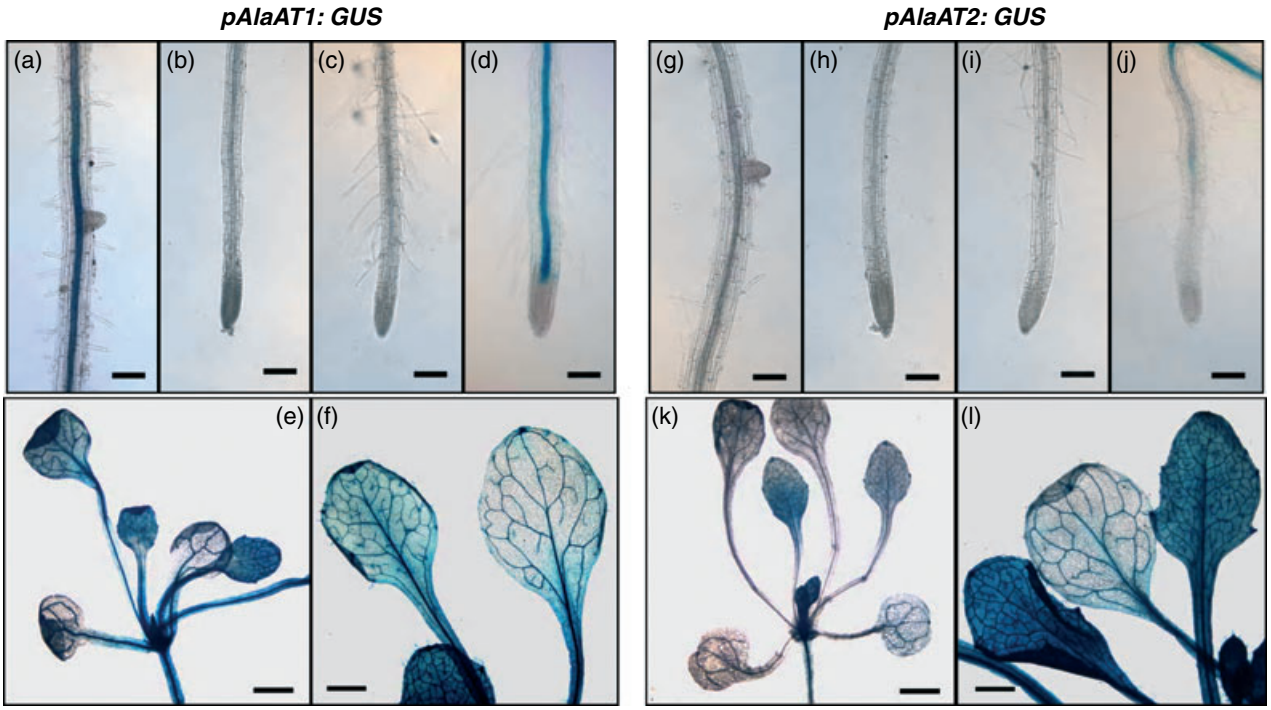

Figure 3. Tissue-specific expression of *AlaAT* genes.
Transgenic plants expressing GUS under the control of the *AlaAT1* or *AlaAT2* promoters were stained for GUS activity. Untreated 14-day-old plants stained for 2 h are shown unless otherwise indicated.
(a,b) Root of transgenic *pAlaAT1:*GUS plant. (c) Root of hypoxically treated plant. (d) Root of 20-day-old plant stained for 14 h. (e) Shoot of transgenic *pAlaAT1:*GUS plant. (f) Leaves of 20-day-old plant stained for 14 h.
(g,h) Root of transgenic *pAlaAT2:*GUS plant. (i) Root of hypoxically treated plant. (j) Root of 20-day-old plant stained for 14 h. (k) Shoot of transgenic *pAlaAT2:*GUS plant. (l) Leaves of 20-day-old plant stained for 14h. The bars in (a–d) and (g–j) are 0.1 mm; those in (e,f,k,l) are 1.0 mm.

all except *OxAlaAT#18* contained a single insertion of the transgene. The Ox*AlaAT#18* line appeared to have the transgene inserted in two loci (data not shown). We confirmed the presence of the barley *AlaAT* transcript by RT-PCR (Figure 4d) and the protein by Western blot analysis (Figure 4e). These results indicated that the transgene was actively transcribed and translated in the selected Ox*AlaAT* lines. However, the expression in *OxAlaAT#15* was not uniform between different tissue types despite using a constitutive promoter. This may indicate a tissue-specific position effect.

*Manipulated AlaAT enzyme activity in alaat1-1 and Ox*AlaAT *lines*

We measured AlaAT enzyme activity in the *alaat1-1* mutant and the Ox*AlaAT* lines under both aerobic and hypoxic conditions (Figure 5). Under aerobic conditions, the enzyme activity in the roots of *alaat1-1* plants was negligible, and that in the shoots was reduced to 20–30% of wild-type (WT) activity. Hypoxic induction of AlaAT activity was only seen in the roots of WT plants after 24 h of hypoxia (approximately 1.6-fold). The results indicate that the T-DNA insertion in the AlaAT1 gene results in a dramatic decrease in overall AlaAT activity. Furthermore, the absence of activity in the roots of the *alaat1-1* mutant suggests that AlaAT1 is the isozyme responsible for nearly all AlaAT activity in the roots of Arabidopsis. The low-level AlaAT activity detectable in the shoots of *alaat1-1* could be attributed to GGAT1, which has been shown to be responsible for some AlaAT activity (approximately 40%) in photosynthesizing tissues (Igarashi *et al.*, 2003).

The three selected Ox*AlaAT* lines showed significant increases in AlaAT activity (Figure 5). Under aerobic conditions, approximately 3.5–7-fold increases in activity were seen in the roots of the Ox*AlaAT* plants. AlaAT activity levels were similarly elevated in the shoots of the Ox*AlaAT* lines (approximately 4.5–10-fold increases). When subjected to hypoxic stress, AlaAT activity in the roots increased approximately 1.5-fold in WT plants, whereas smaller inductions were observed in the roots of the Ox*AlaAT* lines (<1.2-fold increase). In the shoots, hypoxic induction of AlaAT activity was not evident in WT plants, and activity decreased in the *OxAlaAT* lines under hypoxia, perhaps as a result of a hypoxically triggered reduction in CaMV 35S promoter activity.

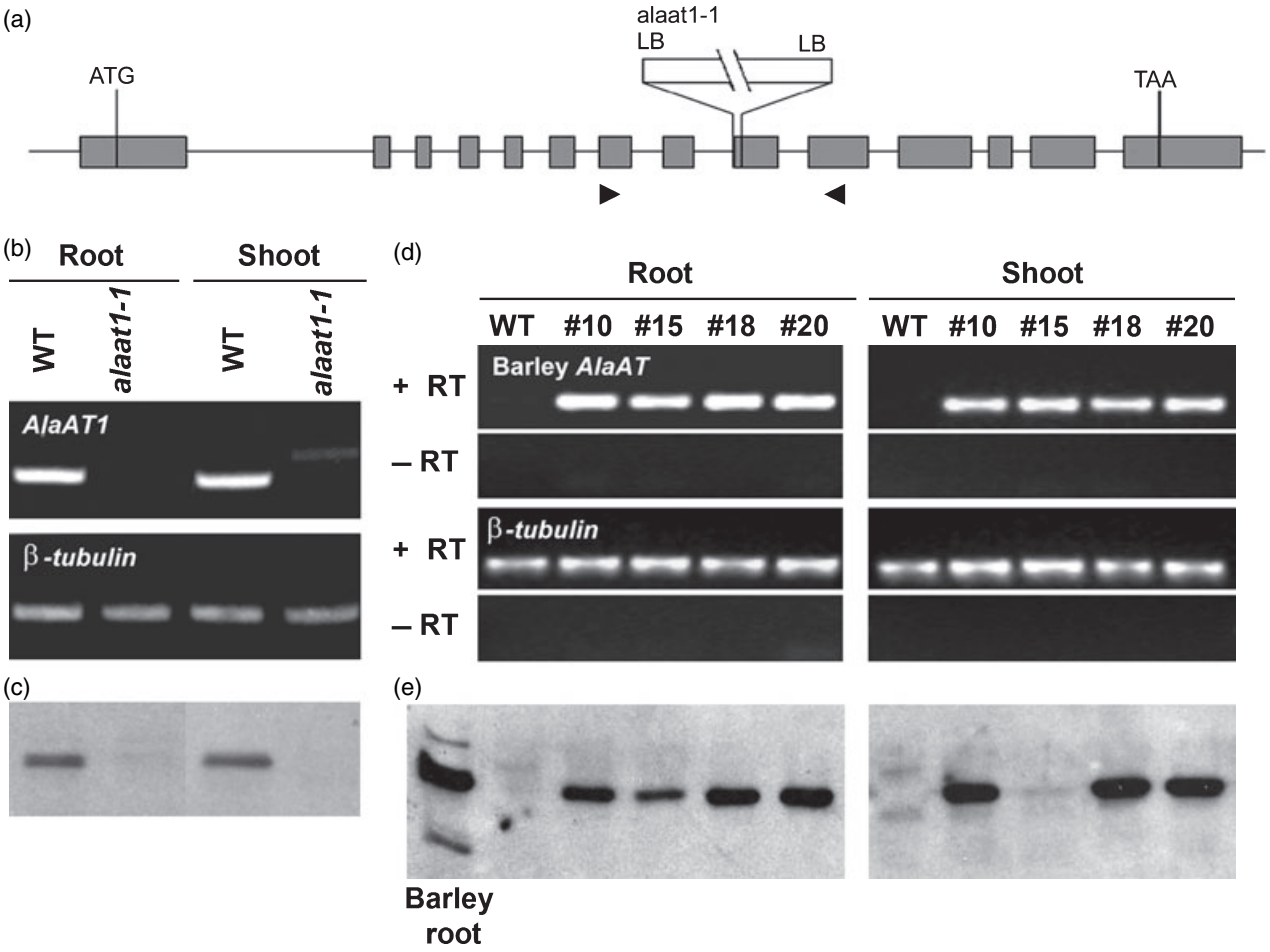

Figure 4. Characterization of *alaat1-1* mutant and *AlaAT*-over-expressing lines.
(a) The gene structure of *AlaAT1* and the site of T-DNA insertion in the knockout mutant (*alaat1-1*). Exon regions of *AlaAT1* are shown by shaded boxes.
(b) RT-PCR analysis showing the absence of normal *AlaAT1* transcript in *alaat1-1*. Total RNA was isolated from roots and shoots of *alaat1-1* and WT plants. PCR was carried out using synthesized first-strand cDNA with primers indicated as black triangles in (a).
(c) Western blot analysis of *alaat1-1* and WT. Proteins extracted from shoots and roots of *alaat1-1* and WT plants were used: 10 μg protein were loaded into each lane.
(d) RT-PCR analysis showing the expression of barley *AlaAT* in Ox*AlaAT* lines. Total RNA was isolated from roots and shoots of Ox*AlaAT* and WT plants. PCR was carried out using synthesized first-strand cDNA (+RT) or DNase I-treated RNA samples (–RT) and gene-specific primers.
(e) Western blot analysis of Ox*AlaAT* lines and WT. Proteins extracted from shoots and roots were used. For barley root and WT, 10 μg proteins were used, whereas 2 μg proteins were used for the Ox*AlaAT* lines. Lanes correspond to those shown in (d).

Metabolic and physiological analysis of alaat1-1 and OxAlaAT lines

To investigate how the mutation in *AlaAT1* and over-expression of barley *AlaAT* affect metabolic profiles, we analysed the levels of several metabolites in *alaat1-1* and the Ox*AlaAT* lines (Figure 6). For these assays, plants were grown for 17 days and then subjected to hypoxia for various lengths of time. After 24 h of hypoxic treatment, plants were returned to aerobic conditions for up to 48 h. Plant samples taken at various time points during the hypoxic treatment and during the recovery period were used for metabolite assays.

For ethanol and lactate, *alaat1-1* and the WT plants showed almost identical patterns of accumulation in the external medium during hypoxic stress (Figure 6a). Upon return to aerobic conditions, both metabolites disappeared from the media at the same rate in both *alaat1-1* and WT. A rapid increase in alanine concentration under hypoxia was observed in both *alaat1-1* and WT plants (Figure 6a). Typically, alanine increased at a faster rate in the roots than in the shoots. In the roots of *alaat1-1*, alanine increased more rapidly than in WT plants, and significantly higher levels of alanine, relative to WT levels, were found after 2 h of hypoxia (1.34-fold). *alaat1-1* plants also contained higher levels of alanine after 24 h of hypoxia, but this difference was not statistically significant and could not be repeated in other independent experiments. Upon return to aerobic conditions, alanine concentrations gradually decreased. During the recovery period, the decrease in alanine occurred

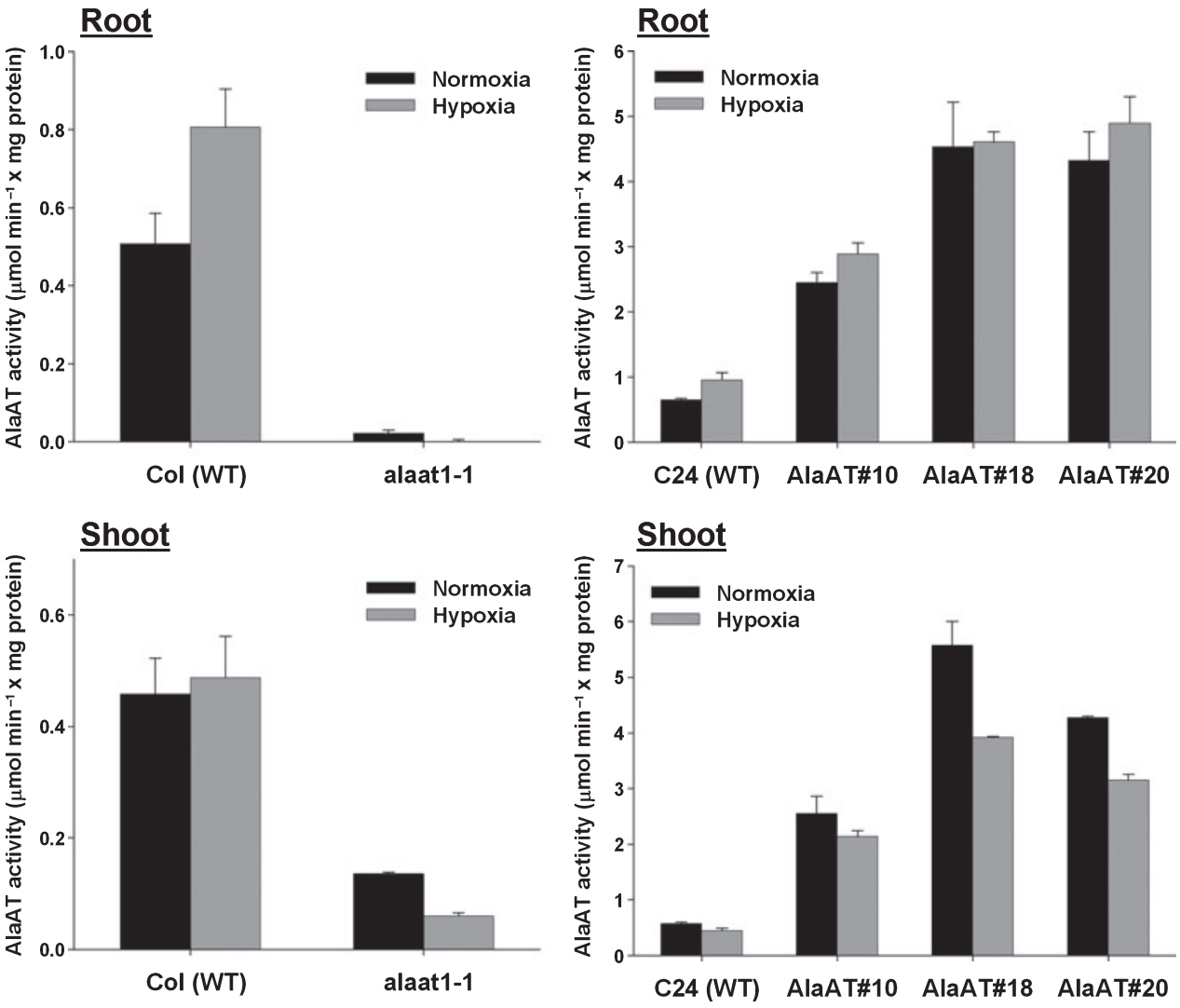

Figure 5. Alanine aminotransferase activity in the *alaat1*-1 mutant and *AlaAT*-over-expressing lines.
Seventeen-day-old plants that were treated for hypoxia (5% O_2 for 24 h) and untreated plants were used. AlaAT activity was measured spectrophotometrically using the enzyme extracts of roots and shoots. Results shown are the means of triplicate samples. The error bars represent SE. The experiments were repeated with similar results.

more slowly in *alaat1-1* than in WT plants, and significantly higher concentrations of alanine (1.59-fold) remained in the roots of *alaat1-1* at 24 h after return to normoxia. In the shoots, significantly higher levels of alanine (1.74-fold) were detected in *alaat1-1* plants compared with WT plants at 24 h after return to normoxia. The delay in the decline of alanine concentrations in *alaat1-1* plants was verified in separate experiments with additional time points over the 48 h recovery period (Figure 6b). The results showed that the decrease in alanine concentration upon return to normoxia did not start in *alaat1-1* until after the first 6 h of recovery, indicating the delay in alanine catabolism. In this experiment, the *alaat1-1* plants contained more alanine than WT at all times during the recovery period in both shoots and roots (Figure 6b).

OxAlaAT lines produced and accumulated ethanol and lactate in the external medium at similar levels to WT plants during hypoxia (Figure S2). The accumulated metabolites disappeared from the media during the post-hypoxic period. Alanine concentrations increased at a similar rate in the Ox*AlaAT* lines and WT plants during hypoxic treatment (Figure S2). During the recovery, one transgenic line, *OxAlaAT#10*, displayed a more rapid decrease of alanine in the roots; however, this was not observed in the other Ox*AlaAT* lines. The level of glutamate was generally unaffected throughout the time course of the experiment in both Ox*AlaAT* lines and WT plants, although the Ox*AlaAT* lines contained more glutamate during the recovery period.

To evaluate low-oxygen stress tolerance in *alaat1-1* and the Ox*AlaAT* lines, we subjected the plants to either anoxic

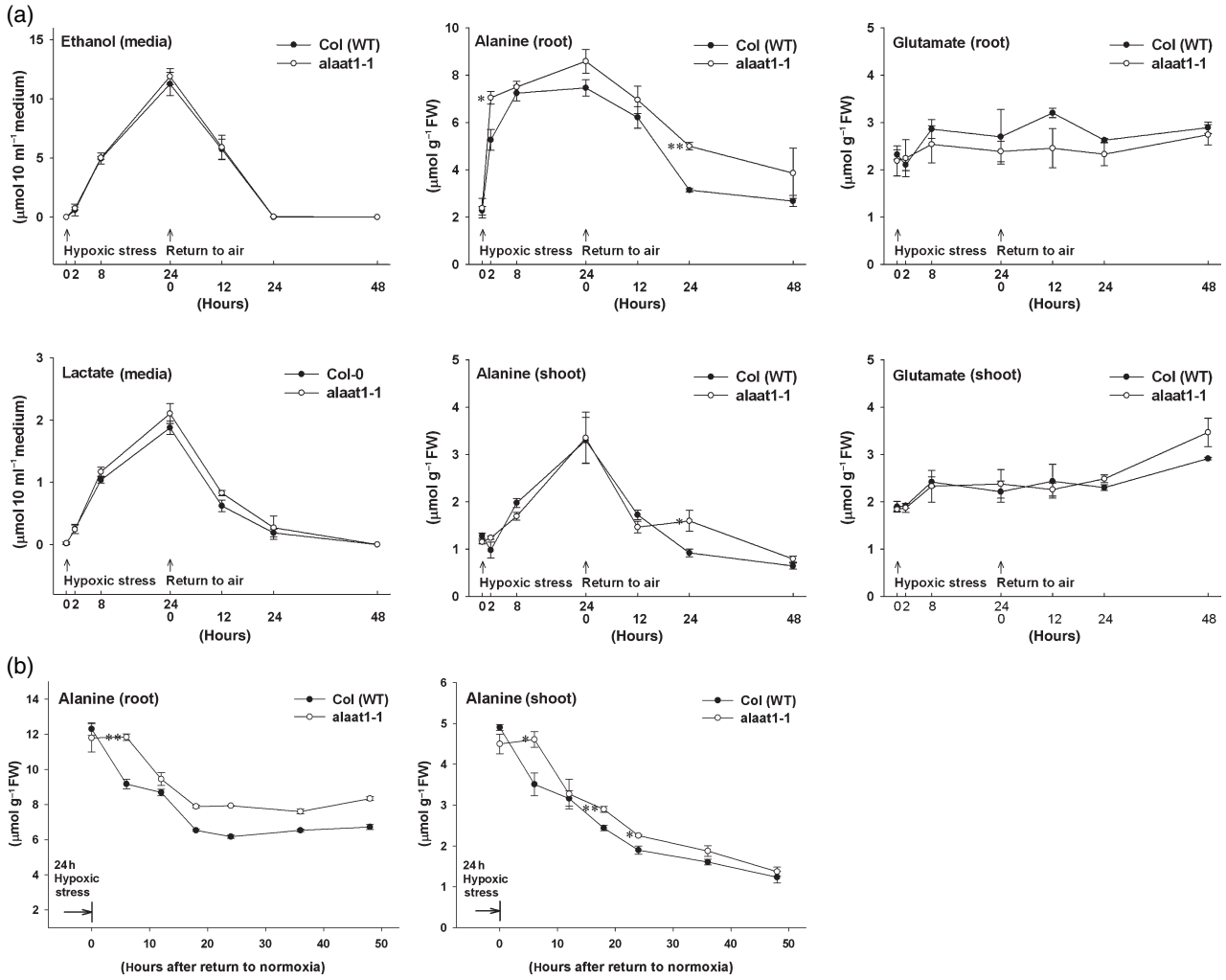

Figure 6. Metabolic profiles of the *alaat1-1* mutant over the period of hypoxic treatment and subsequent recovery.
(a) Changes in the metabolites during over the period of hypoxia and post-hypoxia. Plants were subjected to hypoxia (5% O_2) and the metabolites were extracted from the plants at four time points during the stress treatment (0, 2, 8 and 24 h). After 24 h of treatment, hypoxically treated plants were returned to normoxic conditions, and the samples were taken at three time points (12, 24 and 48 h).
(b) Delayed decline in alanine concentration in *alaat1-1* during the post-hypoxia recovery period. Plants were first hypoxically treated for 24 h, and samples were taken at seven time points over the 48 h recovery period to analyse the alanine decrease. The level of each metabolite was enzymatically determined. Results shown are the means of triplicate samples. The error bars represent SE.
*$P < 0.05$ and **$P < 0.01$ (Student's *t*-test) for comparison of *alaat1-1* with WT. The experiments were repeated with similar results.

(100% N_2) or hypoxic conditions (0.1% oxygen, 99.9% N_2) for various lengths of time, and then scored the survival of the primary root tip based on its ability to maintain growth when returned to normoxic conditions. The decline in viability of the root tips over the period of anoxic stress was similar in *alaat1-1* and WT plants (Figure S3). The root tips of Ox*AlaAT* lines also behaved in a similar way to the WT plants (Figure S3). Hypoxic stress treatment yielded similar results. The shoot and root weights of plants that recovered from anoxic or hypoxic conditions were not significantly different between *alaat1-1* and WT plants or between Ox*AlaAT* lines and WT plants (data not shown).

The ability to utilize externally supplied alanine is affected in the alaat1-1 *mutant*

As the metabolite assays suggested that AlaAT generally converts alanine to pyruvate *in vivo*, we hypothesized that the *alaat1-1* knockout would not be able to utilize alanine as a nitrogen source as effectively as WT plants. To test this hypothesis, plants were grown on MS medium containing various concentrations of inorganic nitrogen (KNO_3:NH_4NO_3 = 1:1) with or without supplementation of alanine. When grown without alanine supplementation, no differences were observed between *alaat1-1* and WT plants;

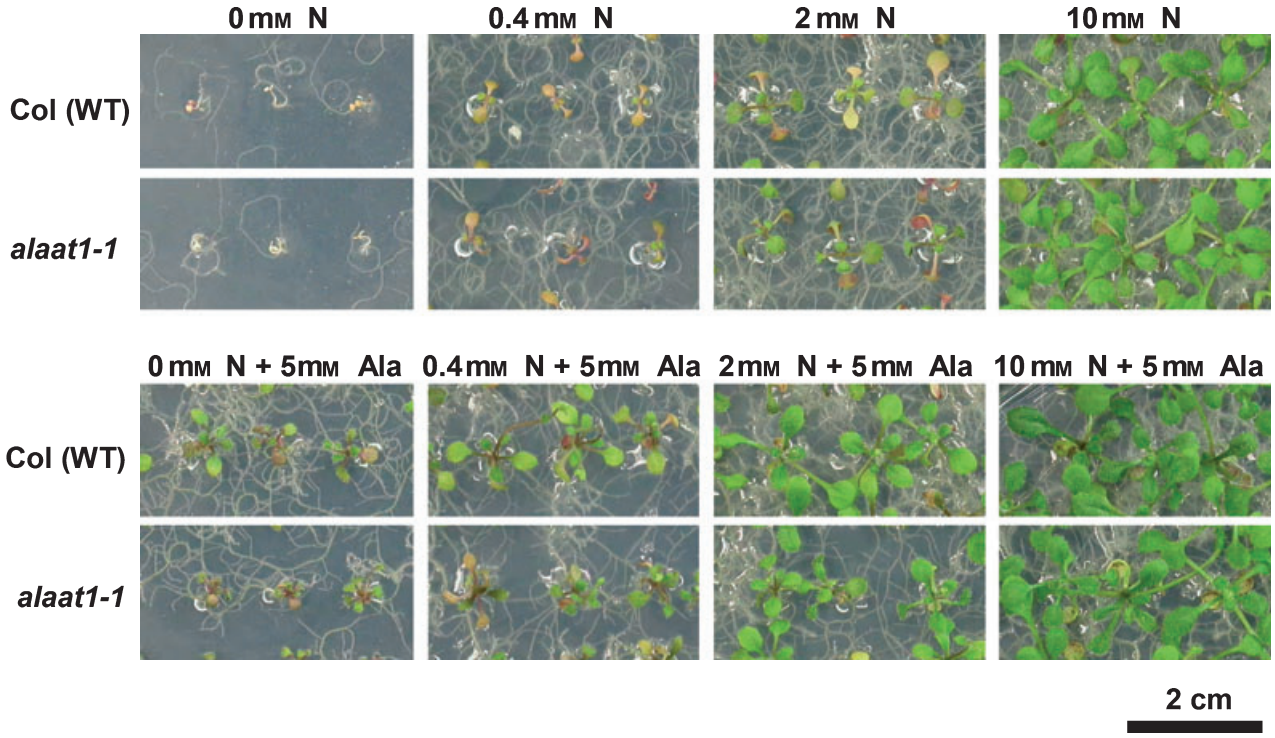

Figure 7. Reduced growth of the *alaat1-1* mutant on alanine supplementation media.
Plants were grown for 2 weeks on nitrogen-controlled 0.5 x MS medium with or without 5 mM alanine supplementation.

Table 1 Fresh weights (FW) of plants grown on alanine supplementation media FW (mg) of twenty 2-week-old plants grown on nitrogen-controlled 0.5 x MS medium with or without supplementation of 5 mM alanine were measured. Values shown are the means of triplicate plates ± SE

	Alanine concentration (mM)	0 mM nitrogen	0.4 mM nitrogen	2 mM nitrogen	10 mM nitrogen
Col (WT)	0	4.0 ± 0.2	25.3 ± 0.6	87.4 ± 5.9	237.7 ± 9.8
alaat1-1		4.0 ± 0.8	25.0 ± 0.7	82.5 ± 2.0	232.3 ± 11.9
Col (WT)	5	84.6 ± 0.8	133.1 ± 2.1	177.0 ± 2.7	245.9 ± 2.8
alaat1-1		** 57.6 ± 2.0	** 99.4 ± 4.3	**134.2 ± 3.7	225.3 ± 11.6
C24 (WT)	0	3.3 ± 0.3	26.6 ± 0.2	95.1 ± 6.5	175.0 ± 15.4
Ox*AlaAT#20*		3.9 ± 0.1	26.2 ± 1.2	87.6 ± 4.7	198.2 ± 11.8
C24 (WT)	5	81.3 ± 1.9	97.9 ± 4.8	129.8 ± 3.3	215.6 ± 13.1
Ox*AlaAT#20*		74.9 ± 2.3	101.2 ± 4.5	141.1 ± 6.3	212.4 ± 2.6

**$P < 0.01$ (Student's *t*-test) for comparison of *alaat1-1* with WT. The experiments were repeated with similar results.

however, when grown on low-nitrogen medium with alanine supplementation, *alaat1-1* plants showed reduced growth compared to WT plants (Figure 7, Table 1). On alanine-supplemented media, WT plants grew approximately 1.3–1.5-fold larger than *alaat1-1* plants. It should be noted, however, that the *alaat1-1* line was still capable of utilizing the supplied alanine as indicated by the improved growth of *alaat1-1* plants on low-nitrogen medium when supplemented with alanine. The Ox*AlaAT* lines did not show any differences relative to WT plants when grown on these media. The results from a representative line (*OxAlaAT#20*) are shown in Table 1.

Discussion

The hypoxic induction of *AlaAT* is a well-known response of plants subjected to low-oxygen stress. The varied physiological roles of AlaAT are suggested by the diverse induction profiles of the genes by several different stresses and by the presence of multiple isoforms in distinct subcellular compartments, including the cytosol, mitochondria and peroxisome (Biekmann and Feierabend, 1982; Liepman and Olsen, 2004). In Arabidopsis, four AlaAT isozymes, each encoded by a member of the *AlaAT* gene family, have been described previously (Igarashi *et al.*, 2003; Liepman and Olsen, 2003).

In this study, we looked at the regulation and expression of *AlaAT1* and *AlaAT2*, and attempted to elucidate the role of AlaAT through functional analysis of an *alaat1-1* and transgenic lines over-expressing barley *AlaAT* in Arabidopsis.

The transcriptional induction of *AlaAT* genes in response to low-oxygen conditions has been demonstrated in barley (Muench and Good, 1994), maize (Muench *et al.*, 1998) and *M. truncatula* (Ricoult *et al.*, 2005). In Arabidopsis, several microarray experiments have demonstrated up-regulation of *AlaAT1* in response to hypoxic stress; however, the response of *AlaAT2* to hypoxia remained uncertain (Klok *et al.*, 2002; Liu *et al.*, 2005; Loreti *et al.*, 2005). In one experiment, slight up-regulation of *AlaAT2* was detected by microarray analysis, but this could not be repeated with real-time RT-PCR (Liu *et al.*, 2005). In another study, the probes corresponding to *AlaAT2* were not present on the Affymetrix ATH1 chip used in the experiment (Loreti *et al.*, 2005). In the current study, we showed hypoxic induction of both *AlaAT1* and *AlaAT2* (Figure 2b). Our results demonstrate that transcript levels of both *AlaAT1* and *AlaAT2* increase quickly, plateau for a short period of time, and then slowly decrease. These induction profiles are similar to that of *ADH1* in Arabidopsis (Dolferus *et al.*, 1994a; Kürsteiner *et al.*, 2003). However, as with *ADH1*, the degree of transcriptional induction of the *AlaAT* genes was significantly higher than the concomitant increase in enzyme activity (Figures 2b and 5). In general, the increase in enzyme activity required relatively longer stress treatments (>24–48 h). The slight increase of AlaAT activity after 24 h of hypoxia (Figure 5) is consistent with the increases in enzyme activity observed after 24 h of hypoxic treatment in barley (Good and Crosby, 1989), maize (Muench *et al.*, 1998) and soybean (Sousa and Sodek, 2003). Moreover, the increase in AlaAT activity was limited to the roots, perhaps suggesting the inefficient translation of both *AlaAT* transcripts in the shoots under low-oxygen conditions.

The tissue specificity of both *AlaAT* transcripts was analysed by developing Arabidopsis transgenic plants expressing GUS under the control of either the *AlaAT1* or *AlaAT2* promoter. Analysis of the transgenic plants demonstrated that *AlaAT1* is expressed at higher levels in the vascular tissues of both shoots and roots, while *AlaAT2* is expressed predominantly in shoot vascular tissues. Similarly, *AlaAT* expression within vascular tissues has been shown in rice using GUS transcriptional fusions and *in situ* hybridization (Kikuchi *et al.*, 1999). In this study, the absence of expression of both Arabidopsis *AlaAT* transcripts within root tips is noteworthy when compared to the expression of rice *AlaAT* in the root tip (Kikuchi *et al.*, 1999) and that of other genes encoding ANPs, such as Arabidopsis *ADH1* (Dolferus *et al.*, 1994b). Furthermore, high expression of *AlaAT1* also occurs in pollen as shown in the AtGenExpress expression atlas (Schmid *et al.*, 2005). Pollen expression is also known for other ANPs, including pyruvate decarboxylase (PDC) and ADH, indicating the operation of an anaerobic-like metabolism in germinating pollen (Gass *et al.*, 2005). Hypoxic stress did not affect the GUS staining patterns, although apparent transcriptional induction of both *AlaAT* and GUS driven by either of the *AlaAT* promoters were confirmed by semi-quantitative RT-PCR (Figure S1). This inconsistency may be attributed to the inefficient translation of GUS mRNA, which could be due to the absence of *cis*-elements from the GUS transcript that are required for translation under low-oxygen conditions. Alternatively, this could result from the fact that GUS staining occurs in the same tissues under hypoxic and normoxic conditions, and any increase in staining intensity would be minor, as is the case for the increased AlaAT enzyme activity.

Changes in metabolite levels during periods of hypoxia or anoxia have been closely monitored in a number of previous studies. A pronounced increase in alanine has been well documented in plants subjected to low-oxygen stress (Fan *et al.*, 1997; Good and Muench, 1993; Sousa and Sodek, 2003; Streeter and Thompson, 1972). However, the metabolic changes upon return to aerobic conditions have not received the same attention. In this study, the products of anaerobic metabolism (ethanol, lactate and alanine) were measured during the post-hypoxic period. A steady decrease in alanine concentration was observed during the post-hypoxic period. Sousa and Sodek (2003) have also shown a similar but more rapid decrease in alanine concentration within 24 h post-hypoxia.

The involvement of AlaAT in hypoxic alanine production has long been speculated; however, compelling evidence has been lacking. We addressed this question by analysing the responses of *alaat1-1* and the Ox*AlaAT* lines to hypoxia. Under this stress condition, *alaat1-1* plants accumulated high levels of alanine, and the increase in alanine content was even more rapid in *alaat1-1* plants than in WT plants (Figure 6a). On the other hand, Ox*AlaAT* lines accumulated alanine as rapidly as WT plants in response to low-oxygen stress (Figure S2). These results refute the hypothesis that the main role of AlaAT under hypoxia is the production of alanine. Instead, the delayed decline in alanine concentration in *alaat1-1* plants upon return to normoxic conditions suggests that AlaAT activity is more important in the conversion of alanine during the post-hypoxic period (Figure 6a). Similarly, Sousa and Sodek (2003) concluded that the hypoxic induction of AlaAT is important to reduce elevated alanine levels to normal in a relatively short period of time upon return to normoxia. When subjected to hypoxic stress, plants are known to express genes whose products have functions during the subsequent recovery, 'anticipating' the return to normoxic conditions (Drew, 1997). The genes encoding enzymes responsible for the detoxification of reactive oxygen species generated during re-exposure to ambient oxygen (superoxide dismutase, peroxidase, ascorbate peroxidase, monodehydroascorbate reductase and

glutathione reductase) are good examples (Geigenberger, 2003; Klok *et al.*, 2002).

Manipulation of AlaAT activity did not alter the survival of the primary root tip under low-oxygen conditions. The root tips of *alaat1-1* plants and the over-expressing lines showed similar responses to anoxic or hypoxic stress as those of the corresponding WT plants (Figure 7). The results suggest that AlaAT activity is not necessary for root tip survival under low-oxygen stress. The absence of GUS staining in the root tips (Figure 3) also supports a similar conclusion.

As *alaat1-1* and Ox*AlaAT* lines are perfectly viable and phenotypically normal, the physiological role of AlaAT remains ambiguous. However, the reduced growth of *alaat1-1* plants on media supplemented with alanine suggests that AlaAT functions to convert excess alanine to pyruvate. This was also supported by the delayed decline of alanine in *alaat1-1* plants during the post-hypoxic period. A similar conditional phenotype was observed in a mutant for cytosolic aspartate aminotransferase (*aat2-2*), where addition of aspartate (20 mM) to the media impaired root development (Miesak and Coruzzi, 2002). The inability of the *aat2-2* mutant to negate the toxic effect of excess aspartate most likely caused the reduced growth of *aat2-2* plants (Miesak and Coruzzi, 2002). In this study, we tested the impact of alanine supplementation on low-nitrogen media, thereby forcing the plants to utilize the amino N of alanine for growth. The inefficient utilization of the supplied alanine in the *alaat1-1* line is the likely cause of the reduced growth of the *alaat1-1* plants. The results also indicate that alanine was less efficient as a source of nitrogen, when compared to inorganic nitrogen. Indeed, 5 mM alanine resulted in growth that was equivalent to the effect of approximately 1.8–2.0 mM of inorganic nitrogen (Table 1). It has been demonstrated that amino acids can be an important form of organic nitrogen utilized by plants (Lipson and Näsholm, 2001; Thornton and Robinson, 2005). Our experiment demonstrates that Arabidopsis possesses the ability to uptake and utilize externally supplied alanine; however, the ability of plant roots to utilize amino acids under agricultural conditions remains uncertain.

Although the activity of AlaAT2 appears to be minor, the predicted mitochondrial localization (Liepman and Olsen, 2003) suggests that this isozyme is important in the targeted organelle. Under low-oxygen stress, the membrane structure of mitochondria is affected and mitochondria play a key role in the perception and signalling of falling oxygen levels within the cell (Subbaiah and Sachs, 2003; Vartapetian *et al.*, 2003). Further characterization of the physiological role of AlaAT will require the identification and analysis of *AlaAT2* mutants as well as a double knockout mutant of the two *AlaAT* genes, as the phenotype of *alaat1-1* may have been diminished because of the redundant role of AlaAT2. Analysis of putative *AlaAT2* mutants identified by TILLING (Colbert *et al.*, 2001) is currently underway.

In summary, our results indicate that AlaAT1 primarily catalyses the conversion of alanine to pyruvate. The normal alanine level found in *alaat1-1* plants under aerobic conditions suggests that Arabidopsis has multiple pathways for production and breakdown of alanine, and normal levels of alanine can be maintained without a functional *AlaAT1* gene. Over-expression of *AlaAT* also did not alter alanine levels in the transgenic plants. This suggests that the AlaAT reaction is not the rate-limiting step for either alanine synthesis or breakdown. Given the vascular expression of both *AlaAT* transcripts, AlaAT activity may be important for the translocation of either alanine or pyruvate, and may facilitate maintenance of the carbon–nitrogen balance throughout plants.

Experimental procedures

Plant growth conditions and hypoxic stress treatment

Arabidopsis thaliana ecotypes Col-0 and C24 were used. Seeds were sterilized and sown on nylon mesh circles placed on top of 0.5 x MS medium containing 1% sucrose and 0.4% agar, unless otherwise indicated. Plants were grown under similar conditions as described by Ellis *et al.* (1999). Plants were grown on the nylon mesh plates for 10 days. Then, the plants grown on the nylon mesh were transferred from solid medium to liquid 0.5 x MS medium containing 1% sucrose, and were grown with gentle agitation on a rotary shaker for an additional 7 days. Tissues harvested from these 17-day-old plants were used for all RNA, protein and metabolite experiments. For the low-oxygen stress treatment, 16- or 17-day-old plants grown as described above were transferred to liquid 0.5 x MS medium containing 1% sucrose that had been first gassed for 15 min with 5% oxygen (balance nitrogen); 25 mg l^{-1} timentin was added to the liquid medium to prevent bacterial contamination. Four microfuge tubes cut in half, transversely, were placed inside the Petri dishes to slightly lift the lids and allow gas exchange. The plates were then stacked in anaerobic jars (Ismond *et al.*, 2003). The anaerobic jars were flushed with 5% oxygen for an additional 15 min, and left in the dark for the duration of the treatments. Controls were performed by transferring the plants to aerobic liquid 0.5 x MS medium containing 25 mg l^{-1} timentin and placing on an orbital shaker in the dark for 24 h.

RT-PCR analysis

Total RNA was isolated from roots and shoots of 17-day-old plants using an RNeasy Plant Mini Kit (Qiagen; http://www.qiagen.com/). The isolated total RNA was treated with DNA-free DNase I (Ambion, http://www.ambion.com), and first-strand cDNA was synthesized using an oligo$(dT)_{12-18}$ primer (Invitrogen; http://www.invitrogen.com/) and Superscript II reverse transcriptase (Invitrogen). PCR was performed using the synthesized first-strand cDNA and the following gene-specific primers: for *AlaAT1*, 5′-CTACGGTGAGAAGGATCTTG-3′ and 5′-TGCTCTCACATCTTAGCTTCTG-3′; for *AlaAT2*, 5′-GGGCTATGGAGAAAAAGATATCT-3′ and 5′-AGTTCATCTTCATGGTCAGGTC-3′; for *GGAT1*, see Liepman and Olsen (2003); for *GGAT2*, 5′-GAGTTCGGAAAGAAGTCGCTGAG-3′ and 5′-GAGTTTGACACAGAGTAGGACCAG-3′; for barley *AlaAT*, 5′-ACCTGATGCATTCTATGCTCTT-3′ and 5′-GGTTGTATGTAATCCCACATGTT-3′; for *Actin2*, 5′-CACTATGTTCTCAGGTATCGCT-3′ and

5′-TTTTAAGCCTTTGATCTTGAGAGC-3′; for β-*Tubulin*, see Kang and Singh (2000).

Analysis for tissue-specific expression of AlaAT *transcripts*

To generate promoter: GUS fusion constructs, approximately 1000 bp upstream regions of *AlaAT1* and *AlaAT2* were amplified from Col-0 genomic DNA using high-fidelity Platinum Taq DNA polymerase (Invitrogen) and cloned into pCR2.1-TOPO (Invitrogen) by TA-cloning using the following primer sets: for the *AlaAT1* promoter, 5′-CCTCACTGCAAAACAGCAACA-3′ and 5′-TTGTGATTGTGTGGTATGGGA-3′; for the *AlaAT2* promoter, 5′-GCTTCGAAGAACTATTAGTCCCAA-3′ and 5′-CTGTGAATCAGCTTTATACTAGGAAC-3′. The inserts were sequenced to confirm cloning of the correct promoter regions. To introduce *Eco*RI and *Nco*I sites at the ends of the promoter fragments, the cloned promoter regions were amplified by PCR using the following primers containing the desired restriction enzyme recognition sequences: *Eco*RI-*AlaAT1*, 5′-AACCGGAATTCCCTCACTGCAAAACAGC-3′; *Nco*I-*AlaAT1*, 5′-TCTTGACCATGGGTGATTGTGTGGTATGG-3′; *Eco*RI-*AlaAT2*, 5′-AACCGGAATTCGCTTCGAAGAACTATTA-3′; *Nco*I-*AlaAT2*, 5′-TCCTGACCATGGGTGAATCAGCTTTATACT-3′. The amplified products were cut with the corresponding restriction enzymes and inserted into pCAMBIA1305.1 (http://www.cambia.org/daisy/cambia/home.html). The constructs were then introduced into Arabidopsis plants (ecotype Col-0) using a modified *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Transformed plants were selected based on hygromycin resistance, and a subset of T_3 homozygous lines that displayed an observable GUS expression pattern were then selected.

The expression of both *AlaAT* promoters was examined at various developmental stages under aerobic conditions and after 24 h hypoxia. Plants were grown on vertical 0.5 x MS plates (1% sucrose, 1% agar) for 9–20 days. Plants were then hypoxically treated (5% oxygen) for 24 h in the dark, while control plants were left in air for 24 h in the dark. *In vivo* GUS staining was performed as described previously (De Block and Debrouwer, 1992), with slight modifications to allow the staining of whole Arabidopsis seedlings. The plants were incubated at 37°C in the staining buffer (50 mM $NaHPO_4$, 2 mM potassium ferrocyanide, 2 mM potassium ferricynide, 0.2% Triton X-100 and 2 mM X-Gluc) for 2 h or overnight (14 h). A minimum of four independent transgenic lines were used for the histological studies. Untransformed plants were used as controls.

Development of AlaAT*-over-expressing transgenic lines*

For over-expression of *AlaAT*, the CaMV 35S promoter was used. The construct was created by introducing a barley AlaAT cDNA (Z26322) into the *Eco*RI/*Xba*I sites of plasmid pART7 (Gleave, 1992). The expression cartridge, consisting of the cDNA flanked by the CaMV 35S promoter and the octopine synthase gene (*ocs*) 3′ UTR, was transferred into the *Not*I site of the corresponding binary vector pART27. The construct was then introduced into Arabidopsis plants (ecotype C24) using a modified root transformation protocol (Dolferus *et al.*, 1994b). Transformed plants were selected based on kanamycin resistance, and homozygous T_3 or T_4 seeds were used for all experiments.

Isolation of the AlaAT1 *knockout line*

The *AlaAT1* knockout line was isolated from Salk T-DNA insertion lines (Alonso *et al.*, 2003) obtained from the Arabidopsis Biological Resource Centre (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm), using the PCR screening scheme described at http://signal.salk.edu. Primers specific for the left border sequence of T-DNA (LBb1; 5′-GCGTGGACCGCTTGCTGCAACT-3′) and for *AlaAT1* (5′-GCGTGCTTGGAAGATTCTGGA-3′ and 5′-GGAATGGAACTTTTTGTCAGGGA-3′) were used for the screening of plants homozygous for the T-DNA insertion. The primers for *AlaAT1* were also used for RT-PCR analysis on the *alaat1-1* line. For segregation analysis of the kanamycin resistance gene, primers for *NPTII* (5′-CGCTCAGAAGAACTCGTCAAGAA-3′ and 5′-TTTGTCAAGACCGACCTGTCC-3′) were used for PCR analysis.

Enzyme and metabolite assays

Plants that were hypoxically treated for 24 h or untreated plants were used. AlaAT enzyme extractions and activity measurements were conducted as previously described (Ismond *et al.*, 2003). Harvested tissues were ground in an extraction buffer (5 mM EDTA, 1 mM DTT, 10 mM cysteine, 0.1 mM PMSF, 5 μM leupeptin and 100 mM Tris, pH 7.5) with a pinch of sand and PVPP, and then centrifuged for 15 min (16 000 ***g***) to remove the cell debris. AlaAT activity was measured for the reverse reaction (alanine to pyruvate) in assay buffer (100 mM Tris, pH 8.0, 10 mM 2-oxoglutarate, 0.28 mM NADH, 70 mM alanine and 1.2 U ml^{-1} lactate dehydrogenase). Alanine was added last to initiate the reaction, and the concomitant decrease in absorbance at 340 nm by oxidation of NADH was monitored. Protein measurements for all extracts were performed using a Bio-Rad protein assay kit (http://www.bio-rad.com/) with BSA as a standard.

Aerobically (0 h treatment) and hypoxically treated plants were used for metabolite extractions. Samples were taken after 2, 8 or 24 h of hypoxic treatment. The stressed plants were subsequently returned to normoxic conditions after 24 h of hypoxic treatment, and samples were taken at 12, 24 and 48 h after the return to normoxia. Metabolite extractions were carried out as described previously (Good and Muench, 1993). Harvested tissues were flash-frozen in liquid nitrogen and immediately ground using a mortar and pestle in 0.6 M perchloric acid with a pinch of sand. The perchloric acid extracts were then neutralized with 3 M NaOH and stored at −70°C until used for the assay. Levels of pyruvate, lactate, ethanol, alanine and glutamate were measured by enzyme-linked assays using a 96-well ELISA plate reader (SpectraMax+) (Molecular Devices, Sunnyvale, CA, USA; http://www.moleculardevices.com) at room temperature (Ismond *et al.*, 2003). Samples were spiked with known amounts of metabolites to test the accuracy of the assay. Triplicate samples were used for all experiments, and the experiments were repeated at least twice for each time point.

Western blot analysis

Soluble proteins were extracted from shoots and roots using enzyme extraction buffer (see above) and then separated on a SDS polyacrylamide gel (7.5% running gel/4% stacking gel) using a Mini-Protein II electrophoresis cell (Bio-Rad). Proteins were then transferred to nitrocellulose membrane using a Mini Trans-Blot Cell (Bio-Rad; http://www.bio-rad.com) according to the manufacturer's specifications. Primary antibody raised against purified barley AlaAT2 (Good and Muench, 1992; Muench and Good, 1994) was used. Antibody staining was performed using an ECL Western detection kit (Amersham Biosciences; http://www5.amershambiosciences.com/) with Western blocking reagent from Roche Applied Science (http://www.roche-applied-science.com) instead of non-fat

dry milk to improve the specific binding of the primary antibody to the AlaAT proteins.

Root tip survival assay

To assess the anaerobic tolerance of the plants, a root tip survival assay was performed as described previously (Ellis *et al.*, 1999) with slight modifications. Sterilized seeds were sown on nylon mesh strips overlying 0.5 x MS medium solidified with 1 % agar and supplemented with 1% sucrose. Plates were placed upright to allow growth of the roots on the surface of the medium only. After 7 days, the nylon mesh strips supporting the seedlings were transferred to liquid 0.5 x MS medium containing 1% sucrose and 25 mg l^{-1} timentin that had been previously gassed with full nitrogen gas or 0.1% oxygen gas (balanced with nitrogen) for 15 min. Microfuge tubes that had been cut in half, transversely, were placed inside the Petri dishes to slightly lift the lids and ensure rapid gas exchange. The plates were then stacked into anaerobic jars and flushed with full nitrogen gas or 0.1% oxygen gas for an additional 15 min. The anaerobic jars were kept in the dark for the duration of hypoxic treatment. At the end of the stress treatment, 15 plants per plate were randomly selected and transferred to square Petri dishes containing 0.5 x MS medium with 1% agar and allowed to recover for 1 week. Root tip survival, based on the ability of the primary root tip to re-grow, was scored 2 days after transferring seedlings to the new square plates. At the end of the recovery period, shoot and root survival, as well as shoot and root weights, were recorded. Triplicate samples were used in all experiments and the experiments were repeated three times.

Alanine supplementation test

To assess the ability of the plants to utilize alanine as nitrogen source, plants were grown on controlled 0.5 x MS medium (0.6% agar) with varying concentrations of inorganic nitrogen (0–10 mM inorganic nitrogen, KNO_3:NH_4NO_3 = 1:1), with or without 5 mM alanine supplementation. Two sucrose concentrations, 3% and 0.3%, were also used. Twenty-two seeds of the Ox*AlaAT* lines or the *alaat1-1* mutant were co-plated with corresponding WT seeds on the same plates. After 2 weeks of growth, the plates were photographed and the fresh weight and dry weight of the above-ground tissues of 20 plants were measured. Triplicates were used in all experiments and the experiments were repeated three times.

Acknowledgements

We would like to thank Mark Wolansky and Mary DePauw for technical assistance. This work was supported in part by a Natural Sciences and Engineering Research Council of Canada (NSERC) grant to A.G.G. R.D. was financially supported by the Cooperative Research Centre for Sustainable Rice Production (c/o New South Wales Institute of Agriculture).

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Semi-quantitative RT-PCR showing the hypoxic induction of GUS driven by *AlaAT* promoters.

Figure S2. Metabolic profiles of Ox*AlaAT* lines over the period of hypoxic treatment and subsequent recovery.

Figure S3. Root tip survival under anoxic conditions.

Table S1 Summary of the screening of Salk T-DNA lines.

This material is available as part of the online article from http://www.blackwell-synergy.com

References

Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Biekmann, S. and Feierabend, J. (1982) Subcellular distribution, multiple forms and development of glutamate-pyruvate (glyoxylate) aminotransferase in plant tissues. *Biochim. Biophys. Acta.* **721**, 268–279.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Colbert, T., Till, B.J., Tompa, R., Reynolds, S., Stein, M.N., Yeung, A.T., McCallum, C.M., Comai, L. and Henikoff, S. (2001) High-throughput screening for induced point mutations. *Plant Physiol.* **126**, 480–484.

De Block, M. and Debrouwer, D. (1992) *In-situ* enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free β-glucuronidase assay. *Plant J.* **2**, 261–266.

Dennis, E.S., Dolferus, R., Ellis, M.H., Rahman, M., Wu, Y., Hoeren, F.U., Grover, A., Ismond, K.P., Good, A.G. and Peacock, W.J. (2000) Molecular strategies for improving waterlogging tolerance in plants. *J. Exp. Bot.* **51**, 89–97.

Dolferus, R., De Bruxelles, G., Dennis, E.S. and Peacock, W.J. (1994a) Regulation of the *Arabidopsis Adh* gene by anaerobic and other environmental stresses. *Ann. Bot.* **74**, 301–308.

Dolferus, R., Jacobs, M., Peacock, W.J. and Dennis, E.S. (1994b) Differential interactions of promoter elements in stress responses of the *Arabidopsis ADH* gene. *Plant Physiol.* **105**, 1075–1087.

Drew, M.C. (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 223–250.

Ellis, M.H., Dennis, E.S. and Peacock, W.J. (1999) *Arabidopsis* roots and shoots have different mechanisms for hypoxic stress tolerance. *Plant Physiol.* **119**, 57–64.

Fan, T.W.M., Higashi, R.M., Frenkiel, T.A. and Lane, A.N. (1997) Anaerobic nitrate and ammonium metabolism in flood-tolerant rice coleoptiles. *J. Exp. Biol.* **48**, 1655–1666.

Gass, N., Glagotskaia, T., Mellema, S., Stuurman, J., Barone, M., Mandel, T., Roessner-Tunali, U. and Kuhlemeier, C. (2005) Pyruvate decarboxylase provides growing pollen tubes with a competitive advantage in petunia. *Plant Cell*, **17**, 2355–2368.

Geigenberger, P. (2003) Response of plant metabolism to too little oxygen. *Curr. Opin. Plant Biol.* **6**, 247–256.

Gleave, A.P. (1992) A versatile binary vector system with a T-DNA organisational structure conductive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20**, 1203–1207.

Good, A.G. and Crosby, W.L. (1989) Anaerobic induction of alanine aminotransferase in barley root tissue. *Plant Physiol.* **90**, 1305–1309.

Good, A.G. and Muench, D.G. (1992) Purification and characterization of an anaerobically induced alanine amino transferase from barley roots. *Plant Physiol.* **99**, 1520–1525.

Good, A.G. and Muench, D.G. (1993) Long-term anaerobic metabolism in root tissue – metabolic products of pyruvate metabolism. *Plant Physiol.* **101**, 1163–1168.

Igarashi, D., Miwa, T., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K. and Ohsumi, C. (2003) Identification of photorespiratory glutamate:glyoxylate aminotransferase (GGAT) gene in *Arabidopsis*. *Plant J.* **33**, 975–987.

Ismond, K.P., Dolferus, R., De Pauw, M., Dennis, E.S. and Good, A.G. (2003) Enhanced low oxygen survival in *Arabidopsis* through increased metabolic flux in the fermentative pathway. *Plant Physiol.* **132**, 1292–1302.

Kang, H.G. and Singh, K.B. (2000) Characterization of salicylic acid-responsive, *Arabidopsis* Dof domain proteins: overexpression of OBP3 leads to growth defects. *Plant J.* **21**, 329–339.

Kikuchi, H., Hirose, S., Toki, S., Akama, K. and Takaiwa, F. (1999) Molecular characterization of a gene for alanine aminotransferase from rice (*Oryza sativa*). *Plant Mol. Biol.* **39**, 149–159.

Klok, E.J., Wilson, I.W., Wilson, D., Chapman, S.C., Ewing, R.M., Somerville, S.C., Peacock, W.J., Dolferus, R. and Dennis, E.S. (2002) Expression profile analysis of the low-oxygen response in *Arabidopsis* root cultures. *Plant Cell*, **14**, 2481–2494.

Kürsteiner, O., Dupuis, I. and Kuhlemeier, C. (2003) The *pyruvate decarboxylase1* gene of *Arabidopsis* is required during anoxia but not other environmental stresses. *Plant Physiol.* **132**, 968–978.

Liao, C. and Lin, C. (2001) Physiological adaptation of crop plants to flooding stress. *Proc. Natl Sci. Counc. Repub. China [B]*, **25**, 148–157.

Liepman, A.H. and Olsen, L.J. (2003) Alanine aminotransferase homologs catalyze the glutamate:glyoxylate aminotransferase reaction in peroxisomes of *Arabidopsis*. *Plant Physiol.* **131**, 215–227.

Liepman, A.H. and Olsen, L.J. (2004) Genomic analysis of aminotransferases in *Arabidopsis thaliana*. *Crit. Rev. Plant Sci.* **23**, 73–89.

Lipson, D. and Näsholm, T. (2001) The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. *Oecologia*, **128**, 305–316.

Liu, F., VanToai, T., Moy, L.P., Bock, G., Linford, L.D. and Quackenbush, J. (2005) Global transcription profiling reveals comprehensive insights into hypoxic response in *Arabidopsis*. *Plant Physiol.* **137**, 1130–1138.

Loreti, E., Poggi, A., Novi, G., Alpi, A. and Perata, P. (2005) A genome-wide analysis of the effects of sucrose on gene expression in *Arabidopsis* seedlings under anoxia. *Plant Physiol.* **137**, 1130–1138.

Miesak, B. and Coruzzi, G.M. (2002) Molecular and physiological analysis of *Arabidopsis* mutants defective in cytosolic or chloroplastic aspartate aminotransferase. *Plant Physiol.* **129**, 650–660.

Muench, D.G. and Good, A.G. (1994) Hypoxically inducible barley alanine aminotransferase: cDNA cloning and expression analysis. *Plant Mol. Biol.* **24**, 417–427.

Muench, D.G., Christopher, M.E. and Good, A.G. (1998) Cloning and expression of a hypoxic and nitrogen inducible maize alanine aminotransferase gene. *Physiol. Plant.* **103**, 503–512.

Reggiani, R. and Bertani, A. (2003) Anaerobic amino acid metabolism. *Russian J. Plant Physiol.* **50**, 733–736.

Reggiani, R., Brambilla, L. and Bertani, A. (1985) Effects of exogenous nitrate on anaerobic metabolism in excised rice roots: 2. Fermentative activity and adenylic energy charge. *J. Exp. Bot.* **36**, 1698–1704.

Ricard, B., Couée, I., Raymond, P., Saglio, P.H., Saint-Ges, V. and Pradet, A. (1994) Plant metabolism under hypoxia and anoxia. *Plant Physiol. Biochem.* **32**, 1–10.

Ricoult, C., Cliquet, J.B. and Limami, A.M. (2005) Stimulation of alanine aminotransferase (AlaAT) gene expression and alanine accumulation in embryo axis of the model legume *Medicago truncatula* contribute to anoxia stress tolerance. *Physiol. Plant.* **123**, 30–39.

Sachs, M.M., Freeling, M. and Okimoto, R. (1980) The anaerobic proteins of maize. *Cell*, **20**, 761–767.

Sachs, M.M., Subbaiah, C.C. and Saab, I.N. (1996) Anaerobic gene expression and flooding tolerance in maize. *J. Exp. Bot.* **47**, 1–15.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D. and Lohmann, J.U. (2005) A gene expression map of *Arabidopsis thaliana* development. *Nature Genet.* **37**, 501–506.

Son, D., Jo, J. and Sugiyama, T. (1991) Purification and characterization of alanine aminotransferase from *Panicum miliaceum* leaves. *Arch. Biochem. Biophys.* **289**, 262–266.

Son, D., Kobe, A. and Sugiyama, T. (1992) Nitrogen-dependent regulation of the gene for alanine aminotransferase which is involved in the C4 pathway of *Panicum miliaceum*. *Plant Cell Physiol.* **33**, 507–509.

Sousa, C.A.F. and Sodek, L. (2003) Alanine metabolism and alanine aminotransferase activity in soybean (*Glycine max*) during hypoxia of the root system and subsequent return to normoxia. *Environ. Exp. Bot.* **50**, 1–8.

Streeter, J.G. and Thompson, J.F. (1972) Anaerobic accumulation of γ-aminobutyrate and alanine in radish leaves. *Plant Physiol.* **49**, 572–578.

Subbaiah, C.C and Sachs, M.M. (2003) Molecular and cellular adaptations of maize to flooding stress. *Ann. Bot.* **90**, 119–127.

Thornton, B. and Robinson, D. (2005) Uptake and assimilation of nitrogen from solutions containing multiple N sources. *Plant Cell Environ.* **28**, 813–821.

Vanlerberghe, G.C., Joy, K.W. and Turpin, D.H. (1991) Anaerobic metabolism in the N-limited green alga *Selenastrum minutum*. *Plant Physiol.* **95**, 655–658.

Vartapetian, B.B. and Jackson, M.B. (1997) Plant adaptations to anaerobic stress. *Ann. Bot.* **79**, 3–20.

Vartapetian, B.B., Andreeva, I.N., Generozova, I.P., Polyakova, L.P., Maslova, I.P., Dolgikh, Y.I. and Stepanova, A.Y. (2003) Functional electron microscopy in studies of plant response and adaptation to anaerobic stress. *Ann. Bot.* **91**, 155–172.

