Arabidopsis NAD-dependent isocitrate dehydrogenase mutants

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NAD-dependent isocitrate dehydrogenase mutants of Arabidopsis thaliana suggest the enzyme is not limiting for nitrogen assimilation

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ABSTRACT

NAD-dependent isocitrate dehydrogenase (IDH) is a TCA cycle enzyme that produces 2-oxoglutarate, an organic acid required by the glutamine synthetase/glutamate synthase cycle to assimilate ammonium. Three Arabidopsis IDH mutants have been characterized, corresponding to an insertion into a different IDH gene (At5g03290, idhv; At4g35260, idhi; At2g17130, idhii). Analysis of IDH mRNA and protein show that each mutant is lacking the corresponding gene products. Leaf IDH activity is reduced by 92%, 60% and 43% for idhv, idhi and idhii respectively. These mutants do not have any developmental or growth phenotype and the reduction of IDH activity does not impact on NADP-dependent isocitrate dehydrogenase activity. Soil-grown mutants do not exhibit any alterations in day-time sucrose, glucose, fructose, citrate, ammonium and total soluble amino acid levels. However, GC-MS metabolic profiling analyses indicate that certain free amino acids are reduced in comparison to the wild-type. These data suggest that IDH activity is not limiting for TCA cycle functioning and nitrogen assimilation. On the other hand, liquid-culture grown mutants give a reduced growth phenotype, a large increase in organic acid (citrate is increased 35-fold), hexose-phosphate and sugar contents, while ammonium and free amino acids are moderately increased with respect to wild-type cultures. However, no significant changes in 2-oxoglutarate levels were observed. Under these non-physiological growth conditions, pyridine nucleotide levels remained relatively constant between the WT and the idhv line although some small but significant alterations were measured in idhii (lower NADH and higher NADPH levels). On the other hand, soil-grown idhv plants exhibited a reduction in NAD and NADPH contents.
INTRODUCTION

Mitochondrial metabolism is an important source of carbon skeletons, via the TCA cycle, that are used in anabolic processes such as porphyrin and amino acid synthesis. In photosynthetic tissues, respiratory decarboxylation is lower in the light than in the dark (Kirschbaum and Farquhar, 1987; Rebeille et al., 1988; Tcherkez et al., 2005). It is believed that the modulation of the TCA cycle is mediated by the regulation of certain enzymes such as the pyruvate dehydrogenase (PDH) complex and the different NAD-dependent dehydrogenases of the cycle (Tovar-Méndez et al., 2003; Fernie et al., 2004a). The PDH complex appears to be an important regulatory point for carbon entry into the cycle and it can be inactivated in the light (Budde and Randall 1990) by the phosphorylation of the E1α subunit (for a review see Tovar-Méndez et al., 2003). Recent isotopic discrimination experiments suggest that PDH activity is reduced by 27% while the CO₂ liberated by the action of the TCA cycle is inhibited by up to 95% in the light (Tcherkez et al., 2005). Whilst the exact degree of reduction of activity of the TCA cycle in the light remains controversial (Nunes-Nesi et al., 2007), inhibition is clearly apparent to some level. The fact that the genes encoding the enzymes of the pathway are only subject to minor diurnal oscillation (Blasing et al., 2005; Urbanczyk-Wochniak et al., 2005) suggests that this inhibition is mediated at the post-translational level. One possible explanation could be that it reflects the effect of high NADH levels on the activity of mitochondrial NAD-dependent dehydrogenases. Indeed, in other organisms, NAD-dependent isocitrate dehydrogenase (IDH) is believed to be a key regulatory step of the TCA cycle due to its low extractable activity (and lower maximal capacity than other TCA cycle enzymes) and its regulation by key metabolites such as NADH, AMP, and ADP (Nichols et al., 1994; Zhao and McAlister-Henn, 1997). Given these facts the conversion of isocitrate to 2-oxoglutarate via the activity of IDH is often regarded as a major controlling point in plant mitochondria (Wiskich and Dry, 1985, Cornu et al., 1996; McIntosh, 1997; Igamberdiev and Gardeström, 2003). The reaction catalysed by IDH transforms isocitrate to 2-oxoglutarate which can subsequently either be reduced in the mitochondria to give succinyl-CoA or exported to the plastids where it can be used in nitrogen metabolism by the glutamine synthetase/glutamate synthase (GS-GOGAT) cycle to produce glutamate (see Gálvez et al 1999). Since TCA cycle activity is modified in the light, C-skeletons for ammonium assimilation could come from a second pathway involving the action of the cytosol-located aconitase and NADP-dependent isocitrate dehydrogenase (ICDH) (Chen and Gadal, 1990). Indeed, it has been shown that, in the light, citrate is the major organic acid exported from the
mitochondria (Hanning et al., 1999). However, potato (Kruse et al., 1998) and tobacco plants (see Gálvez et al., 1999) containing only 6-10% ICDH activity show neither growth nor developmental phenotypes suggesting the activity to be non-essential. Intriguingly, this observation seems to hold true across kingdoms since it proved necessary to remove both cytosolic and mitochondrial ICDH in addition to IDH in order to obtain yeast glutamate auxotrophs (Zhao and McAlister-Henn, 1996). However, it should be noted that yeast cells lacking IDH activity are unable to grow on C-sources such as ethanol and acetate (Cupp and McAlister-Henn, 1991 and 1992).

In yeast, two genes encode IDH subunits (Cupp and McAlister-Henn, 1991 and 1992): IDH2 has been termed the catalytic subunit while IDH1 is involved in regulating enzymatic activity (Lin and McAlister-Henn, 2003). Active yeast IDH is an octomer composed of IDH1-IDH2 dimers. In plants such as Arabidopsis and tobacco, IDH is a heteromeric enzyme made up of at least one ‘catalytic’ and a ‘regulatory’ subunit (Lancien et al., 1998; Lemaitre and Hodges, 2006). The Arabidopsis genome contains five IDH genes (Lin et al., 2004; Lemaitre and Hodges, 2006) coding different subunits while only three genes have been found in tobacco (Lancien et al., 1998) and rice (Abiko et al., 2005). It has been proposed from expression analyses that IDH could be the source of carbon skeletons for the assimilation of ammonium in tobacco (Lancien et al., 1999) and rice (Abiko et al., 2005). Recently, an Arabidopsis knock-out mutant for AtIDHII was partially analysed. This plant contained only 55% of the IDH activity found in its corresponding wild type accession, however, this did not result in a growth or developmental phenotype. Further characterisation revealed that both mitochondrial respiration and leaf photosynthesis were also invariant between the mutant and wild type genotypes (Lin et al., 2004). In contrast, the characterization of a range of transgenic plants exhibiting reduced activity of TCA cycle enzymes including citrate synthase (Landschütze et al., 1995), aconitase (Carrari et al., 2003) and NAD-dependent malate dehydrogenase (Nunes-Nesi et al., 2005) showed that the TCA cycle was of a major importance during the vegetative to generative transition, and suggested a co-ordinate control of the major pathways of energy metabolism in actively photosynthesizing tissues (see also Raghavendra and Padmasree, 2003; Matsuo and Okobata, 2006).

Here we investigate the function of IDH in Arabidopsis thaliana by the analysis of mutants affected in different IDH genes. The aim of this work was to understand the importance of IDH activity in the regulation of the TCA cycle and in producing the 2-oxoglutarate for ammonium assimilation. Three mutants affecting the expression of IDH genes (AtIDHI (At4g35260), AtIDHII (At2g17130) and AtIDHV (At5g03290), see Lemaitre...
and Hodges, 2006) were isolated, characterised and analysed with respect to their growth and development, their metabolic profiles (sugars, organic acids and amino acids) and their pyridine nucleotide levels under different growth conditions. The obtained results are discussed in the context of current models of the regulation of respiratory metabolism and nitrogen assimilation.

RESULTS
Isolation of IDH insertion mutants

Three IDH mutants have been isolated and characterized, each coming from a different mutant library (see Fig. 1). A single AtIDHI (At4g35260) T-DNA insertion line (DYC 179) was identified in the Arabidopsis mutant collection of INRA Versailles (Bouchez et al., 1993) by sequential PCR screening of the available genomic DNA pools. A homozygous mutant for the insertion (idhi) was selected by PCR from T3 generation seed. The structure and the localisation of the T-DNA insertion was deduced from a sequence analysis of the flanking PCR fragments using AtIDHI and T-DNA specific primers. The insertion was located to the last intron of the AtIDHI gene (Fig. 1) and it led to a small deletion of 71bp, ten of these nucleotides being situated at the end of the 3rd exon. The second AtIDH mutant (idhv) was identified in the SAIL library (Sessions et al., 2002). Seeds (line 806-A06) were obtained and a homozygous line was isolated by PCR. The T-DNA insertion was located to the fifth intron of AtIDHV (At5g03290), encoding the major catalytic subunit in leaves (see Lemaitre and Hodges, 2006). Again, the analysis of PCR generated flanking regions showed a single T-DNA insertion and a 27bp deletion in the intron (Fig. 1). The third AtIDH mutant (idhii) was identified as N100075 in the Ds-IMA (Institute of Molecular Agrobiology, Singapore) transposon tagged library (Parinov et al., 1999). The insertion was located to the first exon of the AtIDHII gene (At2g17130), 77bp downstream from the ATG initiation codon (Fig. 1). The insertion also led to a 2bp deletion in the exon. This mutant has been described previously by Lin et al., 2004. The analysed homozygous line was obtained by crossing T4 seed grown plants with the above-mentioned idhi mutant line (in an attempt to generate double mutants) and the consequent selfing of the resulting heterozygous AtIDHII/AtIDHI plants. No double mutants were obtained. Each homozygous mutant line appeared to contain a single insertion as judged by selection marker gene segregation analyses of selfed heterozygous plants that gave a ratio of 1 sensitive to 3 resistant plantlets.
IDH mRNA, protein and activity in the mutant lines compared to wild-type plants

To evaluate the impact of the insertions on the expression of the respective AtIDH genes, RT-PCR analyses were carried out with total RNA extracts from rosette leaves using AtIDH gene-specific primers (Fig. 2). The primers were chosen to amplify the entire coding region of each type of mRNA. This approach showed that the different mutant lines lacked IDH transcripts corresponding to the gene containing the insertion when compared to the respective WT plants (Fig. 2). To estimate the impact of the reduced IDH gene expression on IDH protein levels Western blot analyses were performed on mitochondria-enriched soluble protein extracts from rosette leaves taken from each mutant line and compared to the equivalent WT leaf extract. This was carried out after protein separation by SDS-PAGE (Fig. 3A) and by 2D gel electrophoresis (Fig. 3B), the IDH protein being detected with antibodies raised against recombinant tobacco IDHA (Lancien et al., 1998). These antibodies cross-react with each type of IDH subunit (catalytic and regulatory) and detect two major protein bands on SDS-PAGE; the lower one corresponding to the catalytic subunit(s). The identity of the bands being confirmed by the absence of the lower IDH band in leaf extracts from the idhv mutant (Fig. 3A). Furthermore, a reduced amount of the regulatory subunits (upper protein band) is apparent in this mutant in comparison to the WT. The Western blot analysis of the two regulatory subunit mutants (idhi and idhii) using SDS-PAGE revealed the presence of the catalytic subunit(s) and a reduced level (idhi) or an insignificant (idhii) difference in the upper IDH protein band (Fig. 3A). However, the analysis of IDH protein by 2D electrophoresis/Western blot analyses showed that each mutant line was missing certain IDH protein spots. It can be seen that the IDH antibodies cross-react with several proteins (separated between pH 5 and 8) that have the expected MM of an IDH subunit (Fig. 3B WT). Interestingly, different protein spots appear to be lacking in each mutant line. In idhv leaf extracts, four major protein spots localised between pH 6 and 6.3 are missing (see box in Fig. 3B, idhv). In the idhi extracts, two protein spots have disappeared (between pH 7 and 7.4) while two different protein spots are lacking in the idhii leaf extracts (between pH 5.8 and 6) (see Fig. 3B, idhi and idhii respectively). These observations clearly show that each IDH mutant line is lacking different IDH proteins (as expected if the insertion disrupts the expression of a specific IDH gene). The exact reason for the presence on the 2D gels of several protein spots corresponding to a single gene product is however currently unknown.

In order to evaluate the impact of the absence of a specific IDH subunit on IDH activity, the in vitro IDH activity was measured using mitochondrial enriched fractions of
rosette leaves. This analysis revealed that idhi leaves contained 40% of WT IDH activity, idhii leaves exhibited an inhibition of 43% (as already reported by Lin et al., 2004) and the idhv mutant contained only 8% of the WT leaf IDH activity (Table I). The NADP-dependent isocitrate dehydrogenase (ICDH) activity was unaltered in the same extracts used to measure IDH activity (Table I). Furthermore, total ICDH activity was measured in crude soluble proteins extracts from rosette leaves of soil-grown plants. This ICDH activity reflects the major leaf isoform that is located to the cytosolic (Galvez et al., 1994; Chen, 1998). No differences in total soluble ICDH activity were observed between the idhv and Co extracts and between the idhi and WS extracts (Table II). Therefore, the reduction in IDH activity does not bring about a compensatory increase in the NADP-dependent isoforms.

**Growth and development of the AtIDH mutants**

Previous analyses of mutants affected in TCA cycle enzymes have shown effects on flower development (Landschütze et al., 1995), altered growth kinetics (Carrari et al., 2003) and enhanced fruit biomass (Nunes-Nesi et al., 2005). The different AtIDH mutants and their corresponding wild-type counterparts were grown under different conditions to investigate the effect of differing IDH activities on plant growth and development. When the three mutant lines were germinated and grown in the greenhouse under non-limiting conditions of light, water and mineral nutrition, no observable differences were detected in germination, plant growth, development or flowering time. An absence of growth phenotype was also seen when the idhv mutant (only 8% of the WT IDH activity) was cultured in a controlled growth chamber under either high (500 µE.m⁻².s⁻¹) or low light (75 µE.m⁻².s⁻¹). Since IDH has been reported to be a key TCA cycle enzyme and perhaps involved in the synthesis of 2-oxoglutarate for ammonium assimilation (Gálvez et al., 1999), it was decided to undertake a metabolic analysis of each mutant to better understand the impact of reduced IDH activity on carbon and nitrogen metabolisms.

**Metabolic analyses**

We first decided to investigate the sugar and nitrogen status of the different IDH mutants. Sugar (glucose, fructose and sucrose) and citrate contents were measured before and after illumination and during the day period since it is known that these metabolites show diurnal changes in their extractable amounts (Blasing et al., 2005; Urbanczyk-Wochniak et
al., 2005). It was hypothesised that perhaps the reduction in IDH activity might impact on the diurnal accumulation of these C-metabolites. Figure 4 shows the results concerning the idhv mutant that contains a severe reduction in leaf IDH activity of 92%. Both the WT and mutant plants exhibited an increase in sucrose, glucose and fructose levels during the light period that declined at the end of the day (Fig. 4A). However, no differences could be detected in rosette leaf sugar levels during the day between the idhv mutant and the WT rosette leaves. Similar observations were found for the idhi and idhii mutants (data not shown). Surprisingly, leaf citrate levels were not modified in idhv mutant leaves when compared to the WT (Fig. 4B), and this was also the case for the other two IDH mutants (data not shown). Soluble free amino acids and ammonium ion contents were also invariant between the idhv mutant and Co (Table III).

To gain further information about carbon and nitrogen metabolites, metabolic profiling analyses were carried out on leaf extracts from the three mutants and their respective control plants using an established gas chromatography-mass spectrometry (GC-MS) protocol (Fernie et al., 2004b). These studies revealed no evidence of major alterations in sugar and organic acid levels including 2-oxoglutarate between the mutant and wild-type plants (Table IV). However, succinate, arabinose, ribose, xylose and glucose appeared to be significantly reduced in idhv leaves, while maltose was found to be decreased in the idhii mutant leaves (Table IV). In contrast, the levels of several amino acids were significantly decreased in the mutants when compared to the wild-type leaves (Table IV). This trend was more pronounced in the idhv mutant (lacking 92% of IDH activity) where significantly reduced soluble amino acids levels were detected for arginine, aspartate, cysteine, glycine, isoleucine, leucine, threonine and tyrosine (Table IV). This apparent reduction in amino acid levels did not impact on the measured total soluble amino acid content since the major amino acids (Glu, and Gln) were not altered significantly.

**Phenotypic and metabolic analyses of the Arabidopsis IDH mutants grown in liquid culture**

With the aim to purify Arabidopsis mitochondria, the different lines were grown in a liquid medium under low light (75 µE m⁻² s⁻¹), and with a continuous shaking at 150 rpm. The three mutant lines and their respective wild-type counterparts could develop only in the presence of added sucrose, indicating that they were not autotrophic. When grown under such non-physiological conditions, the three mutant lines exhibited a reduced growth phenotype
when compared to the wild-type plants. This was most pronounced for the idhv mutant (Fig. 5). The growth phenotype was observed at each of the tested sucrose concentrations (from 0.1% to 2%, data not shown). Furthermore, the phenotype did not appear to be dependent on the submergence of the plants, since it was detected when the plants were grown under varying volumes of liquid culture (see Fig. 5, for idhv).

As for the greenhouse grown plants, glucose, fructose, sucrose and citrate levels were measured in the leaves of the liquid culture grown idhv and wild-type plants. Major differences were seen in the sucrose and citrate levels between the idhv mutant and the WT, and the mutant also contained higher levels of glucose and sucrose (data not shown). These quantitative measurements were confirmed by the metabolic profiling (using GC-MS) of leaf extracts from the liquid culture grown plants (Figs. 6 and 7). These analyses showed that hexose-phosphate levels were also modified in the mutant leaves (approximately 3-6 fold increase depending on the mutant examined). In contrast to the soil grown plants, the liquid culture idhv mutants were greatly affected in their levels of TCA cycle organic acids. Indeed, isocitrate and citrate levels were found to be 25- and 35-fold higher, with aconitate (5.7-fold), fumarate and malate (2-fold) and succinate (1.4-fold) also increased when compared to the WT plants (Fig. 6). Similar differences were seen in the idhii mutant, however the increase in citrate (5.3-fold), isocitrate (4.3-fold), and aconitate (2.3-fold) were lower when compared to idhv, although similar changes were observed for the other TCA cycle organic acids. Surprisingly, 2-oxoglutarate levels were only slightly reduced in the idhv mutant line and doubled in the idhii plant extracts when compared to their wild-type controls (Fig. 6), however these differences were not statistically significant.

The liquid culture grown idh mutants also showed differences in the levels of certain amino acids when compared to their WT counterparts (Fig. 7). In contrast to the soil grown plants, the mutants mainly exhibited increased free amino acids levels (compare Table IV and Fig. 7). Significant increases were measured for alanine, aspartate, cysteine, glycine, leucine, proline, serine and valine when idhv extracts were compared to those of the WT (Fig. 7). This gave rise to an 18% increase in total free amino acids in the leaves of the idhv mutant as well as a significant 45% increase in free ammonium ions (Table III). The small change in total amino acid levels can be explained by the fact that only minor amino acids where altered while the two major amino acids (Glu (84 µmol/gFW in WT extracts) and Gln (79.6 µmol/gFW in WT extracts) accounting for 89% of the total amount of amino acids (183.6 µmol/gFW in WT extracts)) were not significantly modified in the mutant extracts. On the
other hand, arginine, asparagine and lysine appeared to be reduced in \textit{idhv} while only asparagine was seen to be decreased in \textit{idhii} plant extracts. On the whole, the changes in \textit{idhv} and \textit{idhii} plants were quite similar with the exception of those in arginine, GABA, glutamate, lysine and tyrosine (which appeared to increase in \textit{idhii} plants).

**Pyridine nucleotide contents**

It might be expected that there are pronounced effects on redox balance in liquid culture grown plants. Therefore, NAD(P) and NAD(P)H levels were measured in WT and mutant plant rosette leaf extracts, and compared to those found in soil-grown plants. When \textit{idhv} plants were grown in liquid culture, the only notable difference was detected in total NADP+NADPH levels due to lower amounts of NADPH when compared to the WT extracts. No significant changes were seen in the NAD+NADH pools. On the other hand, \textit{idhii} extracts contained lower levels of NADH as well as an altered NADP/NADPH balance (Table V). Surprisingly, the \textit{idhv} mutants exhibited more pronounced alterations in pyridine nucleotide levels when grown under soil conditions (lower NAD and NADPH levels compared to WT extracts). The \textit{idhi} plants only showed an increase in NADH levels under soil conditions, with no changes in NAD, NADP and NADPH contents (Table V).

**Total NADP-dependent isocitrate dehydrogenase levels**

The total extractable ICDH activity was measured in crude extracts from the different liquid culture grown plants (Table II). The \textit{idhv} mutant contained a similar ICDH activity to the Co control. On the other hand, \textit{idhii} leaves exhibited a significant increase in total ICDH activity when compared to the control, WSLer line.

**Respiratory activities**

The uncoupled respiration of TCA cycle substrates (citrate, succinate and malate) and NADH by mitochondria-enriched leaf extracts of liquid culture grown plants was investigated using a Clarke-type oxygen electrode. These experiments indicated that there were no differences in \textit{in vitro} respiratory capacity between the \textit{idhv} mutant and the WT, whatever the substrate tested (data not shown). Whilst at first sight surprising, this result is not without precedence since reductions in activity of several enzymes of the TCA cycle produced only a minimal effect on the respiration rate (see Lin et al., 2004; Nunes-Nesi et al., 2005).
DISCUSSION

The aim of this work was to determine the importance of IDH in plant metabolism by isolating, characterizing and analyzing *Arabidopsis* knock-out mutants of IDH genes that gave rise to altered IDH activities. The *Arabidopsis* genome encodes five different IDH subunits (Lin et al., 2004; Lemaitre and Hodges, 2006), that have been proposed to have regulatory (3 genes) or catalytic (2 genes) functions. We have analyzed three IDH mutants, each having insertions in a different IDH gene. In these mutants, the corresponding mRNA was not detectable (Fig. 2), IDH protein levels were modified (Fig. 3A and 3B) and leaf IDH activity was reduced by 43 % to 92 % (depending on the mutant studied). The highest inhibition of IDH activity was measured in the *idhv* mutant line where the T-DNA is inserted into At5g03290, encoding a ‘catalytic’ subunit. It appeared that the reduction of IDH activity in each mutant correlated well to the expression level of the corresponding IDH gene in the wild-type context (see Lemaitre and Hodges, 2006). Our data show that IDHI, IDHII or IDHV are not essential to obtain IDH activity. This has been reported already for IDHII (Lin et al., 2004). Therefore, a single ‘catalytic’ subunit and a single ‘regulatory’ subunit is the minimal requirement to obtain *in planta* leaf IDH activity, thus confirming a hypothesis already put forward after the complementation of yeast IDH mutants with tobacco IDH-encoding cDNA (Lancien et al., 1998).

The quasi-absence in the *idhv* mutant extract of the faster migrating protein band after SDS-PAGE confirmed that this protein is the major catalytic IDH subunit (encoded by At5g03290) of *Arabidopsis* leaves. The western blot analysis of IDH proteins separated by 2D electrophoresis showed the presence of several forms for each IDH subunit. This could be explained by several phenomena including post transcriptional modifications such as protein phosphorylation, or alternative splicing of RNA. Indeed, phosphorylation of potato IDH has been reported (Bykova et al., 2003) and we have retained potato tuber IDH on affinity columns designed to enrich fractions in phosphorylated proteins (unpublished data). Furthermore, the tissue dependent, alternative splicing of RNA encoding the mammalian IDH β subunit has been described (Weiss et al., 2000). In the TAIR database, a splice variant of *AtIDHII* (At2g17130.2) is catalogued that has a lower calculated pI value and a smaller MM compared to At2g17130.1. Such differences could explain the two IDHII protein spots detected in Fig. 3B (the more acidic protein appearing to have a lower MM). However, more
work is required to determine the exact origin and functional significance of the multiple protein spots for the different IDH subunits.

The major aim of this work was to elucidate the importance of IDH activity on cell functioning with particular attention being paid to carbon and nitrogen metabolisms. Indeed, it has been proposed that IDH is a major source of 2-oxoglutarate, the C-skeleton required for the functioning of the GS-GOGAT cycle (Lancien et al., 1999; Abiko et al., 2006). To our surprise, the three idh mutants did not exhibit a modified growth and development phenotype when grown under controlled conditions. This seems to indicate that IDH is not limiting for plant growth since plants containing only 8% of wild-type leaf IDH activity (idhv) had an unaffected developmental phenotype. Indeed, these plants did not exhibit changes in either citrate levels (Fig. 4B, Table IV) or sugar levels during the day (Fig. 4A, Table IV), thus suggesting that C flow through the glycolytic pathway and the TCA cycle were unaffected by the deficiency of IDH activity. The severe reduction in IDH activity did not alter the diurnal changes in sugar levels (Fig. 4A). Unlike potato plants containing 6% of normal citrate synthase activity (Landschütze et al., 1995), the idh mutants were additionally not affected in the reproductive phase of their life cycle. However, it should be noted that the repression of citrate synthase activity in the potato did not alter vegetative growth, as with our IDH mutants. Again, the idh mutants differ from the tomato aconitase mutant (Aco-1), since the plants having lower aconitase activities (40%) exhibited a stunted phenotype at early growth stages, higher sucrose levels during the day and modified TCA cycle metabolite levels (higher isocitrate and citrate levels, lower succinate, fumarate and 2-oxoglutarate levels) (Carrari et al., 2003). However, the idh and Aco-1 mutants were affected in amino acid content. Indeed, the metabolite profiling of our idh mutants showed that under controlled ‘normal’ growth conditions, these plants appeared to contain lower relative soluble amino acid levels when compared to their respective WT plants (Table IV). This effect was more pronounced in the idhv mutant, where nearly half of the amino acids showed significantly reduced relative amounts. However, the two major amino acids (Glu and Gln) found in Arabidopsis leaf extracts (89% of the total soluble amino acids/gFW, data not shown) were not affected and therefore the total soluble amino acid content was not significantly reduced (see Table III). Decreases in certain amino acids were also seen in the Aco-1 tomato mutant (including Asp, Leu, Gln) but others such as Gly differed in the opposite direction when compared to idhv. The reason(s) for such changes in amino acid profiles between idhv and WT leaves is not easy to explain. No particular class of amino acid was affected. More surprisingly, Glu and Gln levels were not significantly modified in the different idh mutant lines (Table IV), indicating
that IDH is not essential (or limiting) in the production of the 2-oxoglutarate for ammonium assimilation via the GS-GOGAT cycle. Indeed, the idh mutants were not significantly affected in their 2-oxoglutarate contents (Table IV). This could be due to the non-limiting IDH activity for TCA cycle functioning or due to the non-exclusive role of IDH in 2-oxoglutarate production. Several isoforms of ICDH exist in leaves and they could also play a role in synthesizing this key organic acid (Hodges et al., 2003). It should be noted that total leaf NADP-ICDH activity and the activity associated with the mitochondrial-enriched extracts were unaltered in the idhv mutant under these growth conditions (Tables I and II). Chen and Gadal (1990) proposed the cytosolic ICDH to be a key player in GS-GOGAT functioning. However, potato (Kruse et al., 1998) and tobacco (our unpublished data) plants containing only 8-10% of WT ICDH activity showed no growth phenotype, no differences in amino acid levels and only small affects on the accumulation of citrate and isocitrate. It is thus probable that the 2-oxoglutarate production for N-assimilation does not come from a privileged pathway. Such a situation has already been proposed in yeast where IDH, cytosolic and mitochondrial ICDH must be absent to generate Glu auxotrophic strains (Zhao and McAlister-Henn, 1996).

When the idh mutants were grown under non-physiological conditions (liquid medium supplied with sucrose, and under low light), they showed a modified growth phenotype (Fig. 5). Similar conditions (except for day length) have been used to investigate the effect of nitrogen on the genome reprogramming of primary and secondary plant metabolisms (Scheible et al., 2004). Under our liquid culture conditions, the addition of sucrose was necessary for plant growth, thus indicating that they could not grow by photosynthesis alone. The metabolic analyses showed that the decrease of IDH activity appeared to become limiting for the C flux towards the TCA cycle and led to an accumulation of citrate and isocitrate in the leaves (Fig. 6). This bottle-neck impacted on glycolytic metabolites (hexose-phosphates) and sugar metabolism (Fig. 6). The increase in succinate, fumarate and malate levels suggest that organic acids are fed into the Krebs via cytosolic by-pass pathways. However, under these non-physiological conditions, no significant differences in 2-oxoglutarate levels were found (Fig. 6) most probably indicating that IDH activity has a limited impact on total 2-oxoglutarate levels, and that 2-oxoglutarate can be produced probably by an ICDH activity that was not modified in the idhv plants and increased in the idhii extracts (Table II). It is known that ICDH activity is inhibited by NADPH (Igamberdiev and Gardeström, 2003) and therefore the in planta activity might be reduced by changes in pyridine nucleotide reduction state brought about by the lower IDH activity and the liquid culture conditions. Surprisingly,
pyridine nucleotide levels (NADP and NADPH) were not greatly altered between the mutant and WT leaves (Table V), and therefore they would not be expected to inhibit leaf ICDH activity in the mutants.

In addition, the metabolite profiling of the *idh* mutants showed that the relative amounts of certain leaf soluble amino acids accumulated under liquid culture conditions with respect to the WT plants (Fig. 7). This apparent increase in soluble amino acids might reflect the lower growth rate of the mutant plants, where amino acids are produced but not utilized for protein synthesis. However, this observation could be due to the increase in sugar levels found in the mutant leaves (Fig. 6), since it has already been reported that high sucrose levels activate the biosynthetic pathways of minor amino acids (Morcuende et al., 1998).

We first believed that O$_2$ availability might be involved in generating the growth phenotype. Indeed, anoxia is known to affect respiration and mitochondrial enzymes (see Bologa et al., 2003; Geigenberger, 2003) due to the stimulation of fermentation pathways and a decrease in ATP-utilizing pathways. However, when the plants were grown on little or completely submerged in liquid medium, the decreased growth phenotype was still observed (Fig. 5). We also suspected that the high sucrose content of the medium could be implicated in producing the phenotype, since high sugar levels are known to inhibit photosynthesis (Koch, 1996) and act in many signalling pathways (Jang and Sheen, 1994; Smeekens, 2000). However, differences in growth were still observed when the sucrose concentration was varied (from 0.1% to 2%) so this is clearly not the predominant factor behind this change. In photosynthetically active plants grown on soil, reductant and ATP can be synthesized by the chloroplasts and the major role of leaf mitochondria could be to ensure efficient photosynthesis via its participation in photorespiration and redox balancing (Scheibe et al., 2005; Sweetlove et al., 2006). When grown in liquid culture, the light intensity was very low, and the leaf sugar concentration was 10-fold higher than under soil conditions. We suggest that under such conditions, plants are not photosynthetically active, therefore the reducing power and ATP necessary for growth and development must be provided by the mitochondria. This would be anticipated to increase the flux of C through glycolysis and the TCA cycle. Therefore, the decrease in IDH activity of the mutants becomes limiting for the required TCA cycle activity and thus lead to a decrease in growth and development (and an accumulation in organic acids and sugars).
MATERIALS AND METHODS

Plant cultures

Wild-type and mutant Arabidopsis thaliana plants were grown under several culture conditions. For mutant isolation and characterisation, plants were germinated from sterilised seed on agar plates containing Murashige and Skoog (M-5519, Sigma) and 2% sucrose.Plantlets were transferred to soil either in the greenhouse or controlled growth rooms and allowed to develop to seed. For metabolite studies carried out at Orsay (France), plants were grown in a controlled growth room under a short day (8h) photoperiod of 500 µE m⁻² s⁻¹ at 17°C and a hygrometry of 65 %. Plants were grown also in liquid medium under short day, low light (75 µE m⁻² s⁻¹) at 18°C in transparent, closed pots under constant agitation (150 rpm).

For metabolic profiling studies, plants were grown in soil in a culture room under 250 µE m⁻² s⁻¹ and a long day photoperiod (Golm, Germany) or in liquid medium (as described above).

idh mutant lines

The three idh mutant lines studied in this work originated from different mutant libraries. The 806-A06 line containing an insertion in AtIDHV, came from the SAIL library (Sessions et al., 2002), the DYC 179 line with an insertion in AtIDHI was from the INRA Versailles library (Bouchez et al., 1993) and the N100075 line with an insertion in AtIDHII was from the Ds-IMA (Institut of Molecular Agrobiology) library (Parinov et al., 1999).

Identification of homozygous idh mutant lines

Mutants containing a homozygous insertion in the respective AtIDH gene were identified by PCR using genomic DNA extracted from leaves of T3 or T4 generation plants (grown on a selection marker antibiotic). Genomic DNA was extracted from 1cm² of a rosette leaf ground with sand in 200mM Tris-HCl pH 7.5, 250mM NaCl, 25mM EDTA and 0.5% SDS. After a 5 min incubation at room temperature and centrifugation (1 min, 11000 g), 350 µL of isopropanol was added to the supernatant, incubated for 15 min and centrifuged at 11000 g for 5 min. The resulting pellet was dissolved in 10mM Tris-HCl pH 7.0 and 1mM EDTA. PCR was performed with the following AtIDH specific primers:

IDHV: 5’ : TCGAATCTTCTTTTGGGAGC ; 3’ : GAATCTTCTTGCCTTACGGCAG
IDHI: 5’ : ATTACGTGTTCCCGCTCTGC; 3’ : AGGCGCCAACATACGTAGC
*IDHII*: 5’ : AAGCATCAGTCACACGTCGG ; 3’ : GCTCTGAGGCAGTTTTCAC
and either a T-DNA (TAG5 (for *IDHI*) : CTACAAATTGCCTTTTCTTATCGCA; SYNLB3 (for *IDHV*) : GCATCTGAATTTCATAACCAATCTCG) or a Ds transposon (for *IDHIII* : CGATACCGTATTTATCCGTTC) specific primer. The hybridization temperature was 50°C and 35 PCR cycles were carried out.

**RT-PCR**

RT-PCR was used to determine the presence of ‘full-length’ coding region AtIDH mRNA in the different *idh* mutant lines. 1 µg of total leaf mRNA was used to amplify the corresponding cDNA (Promega 1X buffer, 0.4mM dNTP, 250 µM oligonucleotides, 10mM DTT and 1 U of Reverse Transcriptase (M-MLV, RNase H minus, Promega) during 1 h at 42°C. PCR was performed using the following conditions: 45s at 94°C, 45s at 50°C, 1minkb at 72°C during 30 cycles. The following primers were used:

*IDHV*: 5’ CGACCATGGCAACTCTCTTCCCTGGC 3’ GAATCTTCTTGCCTTACGGCAG
*IDHI*: 5’ CACCCATGGTGACGCTGATCCCCGGA 3’ AGGCGCCAACATACGTAGC
*IDHIII*: 5’CAACCATGGTGACGTTAATCCCCGGA 3’ GCTGTGAGGCAGTTTTCAC

**Protein extractions**

Total soluble leaf proteins were extracted from 100-150 mg of frozen leaf material, ground in liquid nitrogen in an extraction buffer (100mM phosphate, 14mM β-mercaptoethanol, 5mM MgCl2) and centrifuged at 12000 g for 15 min. The supernatant was used as the crude protein extract for ICDH activity measurements. Mitochondrial-enriched proteins were extracted from 1-4 g of leaves ground with glass beads at 4°C in an extraction buffer (100mM HEPES, pH 7.4, 0.6M sorbitol, 2mM EDTA, 0.2% BSA and a protease inhibitor cocktail (Complete, Promega)). The extract was filtered (0.25 µm tissue), centrifuged at 2000 g, 10 min, and the resulting supernatant was further centrifuged at 12000 g, 20 min. The pellet was suspended in extraction buffer. The final soluble extract was obtained after 3 freeze/thaw cycles and a centrifugation at 20000 g for 8 min. The extract was then diluted 2-fold with glycerol (to give a final glycerol concentration of 50%).

**Protein analyses**

50 µg of soluble leaf mitochondrial-enriched proteins were separated by SDS-PAGE (12% acrylamide) according to Laemmli (1970) or 100 µg were separated by 2D
electrophoresis according to (Lemaitre, 2005). The 1st dimension separated the denatured proteins by their pI (range pH 5 to 8) and the 2nd dimension by their MM (SDS-PAGE, 10% acrylamide). In each case, the proteins were transferred to nitrocellulose membranes as described by Towbin et al (1979). IDH protein was detected by ECL after incubation with IDH antiserum (diluted 2000-fold) raised against recombinant IDHA from tobacco (Lancien et al., 1998) and secondary antibodies raised against rabbit proteins and coupled to a peroxidise activity (dilution 50000-fold).

Isocitrate dehydrogenase activity and mitochondrial respiratory capacity

The IDH activity of mitochondrial-enriched protein extracts was measured with a spectrophotometer by following the absorption change at 340nm due to the production of NADH during the reaction. This was carried out in a buffer containing 50mM HEPES, pH 7.4, 5mM MnCl₂, 10mM isocitrate, 10mM NADH and 20% glycerol. NADP-dependent isocitrate dehydrogenase activity was measured as described in Gálvez et al. (1994).

Uncoupled respiratory activities were measured using the mitochondrial enriched extracts (before the freeze/thaw cycles) and a Clarke-type oxygen electrode. Oxygen production was measured at 25°C using 100 µg of protein extract resuspended in 100mM HEPES, 0.6 M sorbitol, 7.5 mM MgCl₂, and 0.2% BSA. The substrate concentrations used were 2mM for NADH, succinate, malate and pyruvate and 4mM for citrate in the presence of 2µM CCCP.

Metabolite analyses

Sugar, free amino acid and ammonium ion analyses were carried out using ground leaf extracts treated with 2% sulfosalicylic acid and centrifuged at 12 000 g, 15 min at 4°C. Free amino acids were quantified by the method of Rosen (1957) using Gln as the reference compound. Free ammonium ions were measured by the phenol hypochlorite assay according to the Berthelot reaction (after 20 min of colorimetric reaction, NH₄⁺ content was measured spectrophotometrically at 635nm). Sucrose, glucose and fructose levels were measured using the “Sucrose/D-Glucose/D-Fructose” kit from Roche according to the manufacturer’s instructions. Leaf extractions and metabolic profiling by GC-MS were carried out following the protocols described in Wagner et al. (2006) and Roessner et al. (2001), respectively.
Pyridine nucleotide analyses
NAD⁺ and NADP pool sizes and reduction state were measured in acid and alkaline extracts using the protocol described in Queval and Noctor, 2007. The assays involve the phenazine methosulfate-catalyzed reduction of dichlorophenolindophenol in the presence of ethanol and alcohol dehydrogenase (for NAD⁺ and NADH) or glucose 6-phosphate and glucose 6-phosphate dehydrogenase (for NADP⁺ and NADPH). Reduced and oxidized forms are distinguished by preferential destruction in acid or base.

Statistical analysis
Where two observations are described in the text as different, this means that they were determined to be statistically different by performing Student’s t tests using the algorithm incorporated into Microsoft Excel 7.0 (Microsoft Corp., Seattle)

LITERATURE CITED


Budde RJ, Randal DD (1990) Pea leaf mitochondrial pyruvate dehydrogenase complex is inactivated in vivo in a light-dependent manner. Proc Natl Acad Sci USA 87: 673-676


Landschütze V, Willmitzer L, Müller-Röber B (1995) Inhibition of flower formation by antisense repression of mitochondrial citrate synthase in transgenic potato plants leads to a specific disintegration of the ovary tissues of flowers. EMBO J 14: 660-666


FIGURE LEGENDS

**Figure 1.** Localisation of the insertion within the *AtIDH* mutant lines studied in this work. Exons are given as arrows and introns as bent lines. The mutants originated from the following mutant libraries: *idhv* (SAIL), *idhi* (Versailles) and *idhii* (Ds-IMA).

In the DNA sequences, the right (RB) and left (LB) borders of the insertions are given by the arrows. The deleted nucleotides are underlined. The exons are in capitals and the introns (or 5’ UTR regions) are in small letters.

**Figure 2.** RT-PCR analysis of *AtIDH* expression in leaves of the three insertion mutant lines. The presence of ‘full-length’ IDH transcripts was investigated by RT-PCR using total RNA extracted from rosette leaves of the different IDH (*idhv*, *idhi* and *idhii*) mutant lines (m) and their respective control plants (WT). The PCR was carried out using IDH gene specific primers.

**Figure 3.** Western blot analyses of IDH protein in leaves of the three *AtIDH* insertion mutant lines. Soluble proteins from mitochondrial enriched leaf extracts were separated by (A) SDS-PAGE (12% acrylamide) and (B) by 2D electrophoresis (isoelectric focusing (pH 5-8) and SDS-PAGE). IDH subunits were detected using antibodies raised against the recombinant tobacco IDH (Lancien et al., 1998). WT, wild-type control extracts; *idhv*, *idhi* and *idhii*, IDH mutant line extracts. The boxes show the absence of IDH proteins in the different mutant lines. The IDH proteins migrated in between the 36.5 kDa and 48.5 kDa molecular weight markers, thus indicating an approximate MM of 38-40 kDa.

**Figure 4.** Day time changes in sugar and citrate levels in growth chamber grown WT and *idhv* rosette leaves. (A) Sucrose, glucose and fructose levels and (B) citrate levels were measured at different times of the day (1h before and after illumination, and during the 8h light period of 500 µEm^-2^-s^-1^). Measurements were carried out on 3 independent plants and standard deviations are given. Open columns (WT), closed columns (*idhv*).

**Figure 5.** The effect of liquid culture volume on the growth of WT and *idhv* plants. Seeds were allowed to germinate and develop in different volumes of liquid culture medium (10-70 ml) containing 2% sucrose (constant agitation, 75 µEm^-2^-s^-1^, 8h day-16h night).
**Figure 6.** Comparison of the relative levels of sugars and organic acids between the liquid culture *idhv* (black) and *idhii* (white) mutants grown under short-day conditions. Data were normalised to the mean values of the wild-type plant extracts (see hashed line, each metabolite = 1). Values presented are mean +/- SE of determinations on six independent cultures, an asterisk indicates values determined to be significantly different from the control, WT values by means of t-tests (P<0.05). Measurements were carried out as described in the Materials and Methods by GC-MS.

**Figure 7.** Comparison of the relative levels of amino acids and GABA between the liquid culture *idhv* (black) and *idhii* (white) mutants grown under short-day conditions. Data were normalised to the mean values of the wild-type plant extracts (see hashed line, each metabolite = 1). Values presented are mean +/- SE of determinations on six independent cultures, an asterisk indicates values determined to be significantly different from the control, WT values by means of t-tests (P<0.05). Measurements were carried out as described in the Materials and Methods by GC-MS.
Table I. *NAD- (IDH) and NADP-dependent (ICDH) isocitrate dehydrogenase activities*

Isocitrate dehydrogenase activities were measured in mitochondrial-enriched soluble protein fractions extracted from leaves of the different mutant lines (*idhi, idhii* and *idhv*) and their wild-type (WS, WSLe and Co) counterparts grown under short-day conditions.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>IDH Activity (pmolNADH/min/mg protein)</th>
<th>ICDH Activity (pmolNADPH/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS</td>
<td>14.4</td>
<td>6.7</td>
</tr>
<tr>
<td><em>idhi</em></td>
<td>5.8</td>
<td>6.0</td>
</tr>
<tr>
<td>WSLe</td>
<td>11.2</td>
<td>6.7</td>
</tr>
<tr>
<td><em>idhii</em></td>
<td>6.4</td>
<td>6.5</td>
</tr>
<tr>
<td>Co</td>
<td>16.7</td>
<td>6.7</td>
</tr>
<tr>
<td><em>idhv</em></td>
<td>1.4</td>
<td>6.1</td>
</tr>
</tbody>
</table>
Table II. *NADP-dependent isocitrate dehydrogenase activities*

Total ICDH activity was measured in total soluble protein extracts from leaves of the different mutant lines (*idhi*, *idhii* and *idhv*) and their wild-type (WS, WSLe and Co) counterparts grown in soil or in liquid culture under short-day conditions. Measurements were carried out on 3-4 independent plants (soil) or 3 plant cultures each containing 50 plantlets (Liquid Culture) and standard deviations are given. *a*Significantly different values (t-test P<0.05).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Activity, nmol NADPH/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil</td>
</tr>
<tr>
<td>WS</td>
<td>38.6±0.5</td>
</tr>
<tr>
<td><em>idhi</em></td>
<td>36.2±0.3</td>
</tr>
<tr>
<td>WSLe</td>
<td>nd</td>
</tr>
<tr>
<td><em>idhii</em></td>
<td>nd</td>
</tr>
<tr>
<td>Co</td>
<td>31.4±0.3</td>
</tr>
<tr>
<td><em>idhv</em></td>
<td>30.6±2.2</td>
</tr>
</tbody>
</table>

nd = not determined
Table III. *Free amino acid and ammonium levels in idhv and Co*

Measurements were carried out on 3-4 independent plants (soil) or 3 liquid cultures each containing 50 plantlets grown under short-day conditions and standard deviations are given.

*a* Significantly different values (t-test P<0.05)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Genotype</th>
<th>Free amino acids nmoles amine gFW⁻¹</th>
<th>Ammonium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>Co</td>
<td>15.0 ±3.35</td>
<td>1.79±0.03</td>
</tr>
<tr>
<td></td>
<td><em>idhv</em></td>
<td>16.4±2.03</td>
<td>1.75±0.08</td>
</tr>
<tr>
<td>Liquid</td>
<td>Co</td>
<td>130.4±5.5</td>
<td>38.4±1.9*</td>
</tr>
<tr>
<td>Culture</td>
<td><em>idhv</em></td>
<td>154.2±16.2</td>
<td>56.8±4.9*</td>
</tr>
</tbody>
</table>


**Table IV. Metabolic profiling of the different IDH mutants**

Plants were grown under long day conditions at Golm, Germany. Values are the mean ± SE of determinations made on six plants. The given values are relative to the respective WT controls (each metabolite = 1). aSignificantly different values (t-test P<0.05) with respect to the WT

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>idhv</th>
<th>idhi</th>
<th>idhii</th>
</tr>
</thead>
<tbody>
<tr>
<td>arginine</td>
<td>0.39 ± 0.00 a</td>
<td>0.57 ± 0.11 a</td>
<td>0.70 ± 0.09 a</td>
</tr>
<tr>
<td>asparagine</td>
<td>0.64 ± 0.15</td>
<td>0.43 ± 0.00 a</td>
<td>1.18 ± 0.37</td>
</tr>
<tr>
<td>aspartate</td>
<td>0.26 ± 0.06 a</td>
<td>0.86 ± 0.17</td>
<td>0.66 ± 0.15</td>
</tr>
<tr>
<td>cysteine</td>
<td>0.36 ± 0.07 a</td>
<td>1.89 ± 0.31 a</td>
<td>0.88 ± 0.21</td>
</tr>
<tr>
<td>GABA</td>
<td>0.56 ± 0.06</td>
<td>0.86 ± 0.12</td>
<td>1.27 ± 0.28</td>
</tr>
<tr>
<td>glutamate</td>
<td>1.00 ± 0.00</td>
<td>0.65 ± 0.11</td>
<td>0.78 ± 0.09</td>
</tr>
<tr>
<td>glutamine</td>
<td>0.67 ± 0.09</td>
<td>0.41 ± 0.14</td>
<td>0.69 ± 0.09</td>
</tr>
<tr>
<td>glycine</td>
<td>0.50 ± 0.13 a</td>
<td>0.73 ± 0.13</td>
<td>0.61 ± 0.32</td>
</tr>
<tr>
<td>isoleucine</td>
<td>0.40 ± 0.07 a</td>
<td>1.13 ± 0.25</td>
<td>1.42 ± 0.36</td>
</tr>
<tr>
<td>leucine</td>
<td>0.13 ± 0.04 a</td>
<td>1.23 ± 0.34</td>
<td>0.44 ± 0.07 a</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>1.00 ± 0.00</td>
<td>1.39 ± 0.43</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>proline</td>
<td>0.59 ± 0.25</td>
<td>0.90 ± 0.37</td>
<td>0.99 ± 0.28</td>
</tr>
<tr>
<td>serine</td>
<td>0.66 ± 0.19</td>
<td>0.55 ± 0.10 a</td>
<td>1.40 ± 0.29</td>
</tr>
<tr>
<td>threonine</td>
<td>0.50 ± 0.08 a</td>
<td>0.49 ± 0.09 a</td>
<td>1.09 ± 0.28</td>
</tr>
<tr>
<td>tyrosine</td>
<td>0.36 ± 0.03 a</td>
<td>0.86 ± 0.46</td>
<td>0.99 ± 0.21</td>
</tr>
<tr>
<td>citrate</td>
<td>0.87 ± 0.25</td>
<td>0.44 ± 0.16</td>
<td>5.54 ± 2.74</td>
</tr>
<tr>
<td>isocitrate</td>
<td>0.83 ± 0.21</td>
<td>0.42 ± 0.11</td>
<td>1.09 ± 0.36</td>
</tr>
<tr>
<td>2-oxoglutarate</td>
<td>0.89 ± 0.19</td>
<td>0.81 ± 0.15</td>
<td>1.05 ± 0.46</td>
</tr>
<tr>
<td>succinate</td>
<td>0.61 ± 0.06 a</td>
<td>0.87 ± 0.19</td>
<td>1.29 ± 0.24</td>
</tr>
<tr>
<td>fumarate</td>
<td>0.92 ± 0.24</td>
<td>0.67 ± 0.19</td>
<td>1.33 ± 0.26</td>
</tr>
<tr>
<td>malate</td>
<td>0.68 ± 0.29</td>
<td>0.70 ± 0.15</td>
<td>1.41 ± 0.38</td>
</tr>
<tr>
<td>maleate</td>
<td>0.72 ± 0.09</td>
<td>0.75 ± 0.14</td>
<td>1.10 ± 0.29</td>
</tr>
<tr>
<td>threonate</td>
<td>0.34 ± 0.07 a</td>
<td>1.87 ± 0.33 a</td>
<td>0.92 ± 0.23</td>
</tr>
<tr>
<td>dehydroascorbate</td>
<td>0.32 ± 0.11 a</td>
<td>0.61 ± 0.17</td>
<td>1.00 ± 0.30</td>
</tr>
<tr>
<td>arabinose</td>
<td>0.43 ± 0.06 a</td>
<td>0.86 ± 0.14</td>
<td>0.75 ± 0.11</td>
</tr>
<tr>
<td>fructose</td>
<td>1.01 ± 0.17</td>
<td>5.14 ± 2.08</td>
<td>1.04 ± 0.29</td>
</tr>
<tr>
<td>galactose</td>
<td>0.95 ± 0.18</td>
<td>0.90 ± 0.17</td>
<td>0.68 ± 0.14</td>
</tr>
<tr>
<td>glucose</td>
<td>0.55 ± 0.05 a</td>
<td>1.85 ± 0.39</td>
<td>1.21 ± 0.27</td>
</tr>
<tr>
<td>maltose</td>
<td>0.65 ± 0.09</td>
<td>1.03 ± 0.16</td>
<td>0.45 ± 0.06 a</td>
</tr>
<tr>
<td>mannitol</td>
<td>0.93 ± 0.25</td>
<td>1.09 ± 0.49</td>
<td>0.63 ± 0.13</td>
</tr>
<tr>
<td>mannose</td>
<td>1.36 ± 0.26</td>
<td>1.71 ± 0.50</td>
<td>0.66 ± 0.24</td>
</tr>
<tr>
<td>ribose</td>
<td>0.41 ± 0.09 a</td>
<td>0.97 ± 0.13</td>
<td>1.11 ± 0.25</td>
</tr>
<tr>
<td>sucrose</td>
<td>0.99 ± 0.14</td>
<td>1.34 ± 0.22</td>
<td>0.73 ± 0.11</td>
</tr>
<tr>
<td>trehalose</td>
<td>0.66 ± 0.14</td>
<td>1.40 ± 0.66</td>
<td>1.35 ± 0.97</td>
</tr>
<tr>
<td>xylose</td>
<td>0.36 ± 0.05 a</td>
<td>0.69 ± 0.14</td>
<td>1.10 ± 0.31</td>
</tr>
</tbody>
</table>
Table V. Pyridine nucleotide levels in IDH mutants and their wild-type counterparts

NAD, NADH, NADP and NADPH levels were measured in leaf extracts of different IDH mutants and their WT controls grown under short-day conditions in soil or liquid culture (LC). Measurements were carried out on 3-4 independent plants (soil) or 3 plant cultures each containing 50 plantlets (LC) and standard deviations are given.

 significantly different values (t-test P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Pyridine nucleotide content, nmol/gFW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAD</td>
</tr>
<tr>
<td>Co</td>
<td>23.8±2.1</td>
</tr>
<tr>
<td>idhv</td>
<td>14.2±1.0</td>
</tr>
<tr>
<td>Soil WS</td>
<td>16.3±0.9</td>
</tr>
<tr>
<td>idhi</td>
<td>18.3±2.1</td>
</tr>
<tr>
<td>Co</td>
<td>15.6±1.7</td>
</tr>
<tr>
<td>idhv</td>
<td>17.7±3.4</td>
</tr>
<tr>
<td>LC WSLe</td>
<td>17.6±1.1</td>
</tr>
<tr>
<td>idhii</td>
<td>17.8±2.7</td>
</tr>
</tbody>
</table>
A. Localisation of the insertion in the fifth intron of the AtIDHV gene (SAIL line 806-A06)

```
1741 ttccactgtt ttccacatgtg gaatatgcga ctgataatct tgagatacct gcagCTTGTG
1801 AAAAACCCAG CACTTTTTGA TGTCTTGGTG ATGCCAAACC TGTACGGTGA CATTATTAGC
1861 GATCTGTGTG CCGGATTGGT TGGAGGACTA GGATTGACTC CAAGgtaaaa acacttgttc
1921 cttcatcttg
tgtaatagt
g atctttagta
tgattatgag
tcatacatc
ttaaggcctt
1981 attatcctttg acgcagtTGT AATATTGGAG AAGATGGTGT AGCCCTTGCT GAAGCAGTTC
```

B. Localisation of the insertion in the third intron of the AtIDH1 gene (Versailles line DYC179)

```
1681 agagcagttaa cttcactgtt gctaaagtaa aacctttcat tactgtatgt aqtagcgtct
1741 tttgcttttt ccccccccccc ataaacagaa cttataaatg atttttgCAGG TAACGCCGAA
1801 TCTCTATGGG AATTTAGTTG CAAACACTGC TGCTGGTATT GCTGGAGGCA CAGGAGTCAT
1861 GCCTGgaggt aaaaaaacat cctgtattga tattctctcg agctatacat tagtggataa
1921 agaata gttc taaaatttat acatctgcga cattaaatac aaaaaCAGGA AATGTTGGGG
1981 CTGACCATGC GGTATTCGAG CAAGGTGCAT CAGCAGGAAA TGTTGGGAAA GACAAGATAG
```

C. Localisation of the insertion in the first exon of the At1DH2 gene (Ds IMA line N100075)

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361 taaacaataa aagttataga cttaaaagtaa acgcttttct tgttttttct tcgttccaaa
421 aagtgcgaac tctttccat ttccttttct tgcagctatg ttcagaga tgctgatctc
481 gatcaaaaat gcaagaaag AGTGTCTGCC AATCGTTTTC TCTACTGAAG AATCTTCGTA
541 GCATCGCTTC CGGTTCCAAA ATCCAGACC GGATCCCTCC TTACATGCCC AGACCAGGTT
601 AGGGAAACC AGAACCACTT AGCTTAAATC CCAGGAGCAG AATTGTTGCC TGTTAACA
```

The right (RB) and left (LB) borders of the insertions are given by the arrows. The deleted nucleotides are underlined. The exons are in capitals and the introns (or 5' UTR regions) are in small letters.

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6