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RESEARCH PAPER

Metabolism of γ-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat

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Abstract

Amino acid homeostasis was investigated in frost-resistant barley seedlings under either cold- or freezing-stress conditions. Total free amino acid content varied only slightly, but a substantial conversion of glutamate to γ-aminobutyric acid (GABA) was found that was proportional to the severity of the stress. Cold acclimation caused a significant increase in amino acid pools, and induced the expression of the GABA-shunt genes. As a consequence, GABA accumulated to a higher extent during the subsequent exposure to lower temperature. A different picture was obtained with a frost-sensitive genotype, in which glutamate decarboxylation occurred during the stress as well, but the activation of the GABA shunt seemed not to take place, and free glutamate was almost depleted. Analogous results were found in frost-resistant and frost-sensitive wheat cultivars. Feeding non-hardened plants with exogenous glutamate resulted in increased GABA accumulation under low temperature. The possibility that glutamate decarboxylation and GABA metabolism would play a role in frost tolerance is discussed.

Key words: Amino acids, cold hardening, barley, freezing, GABA shunt, γ-aminobutyric acid, substrate availability, temperature, wheat.

Introduction

Overwintering plant species have evolved an adaptive mechanism that allows them to survive through the cold season, the so-called hardening process. This response occurs when plants are exposed to mild stress conditions, i.e. low but non-freezing temperatures, and contributes significantly to frost resistance, a major component of the ability to withstand winter freezing (Beck *et al*., 2004). Cold adaptation is associated with many physiological and metabolic processes, which require changes at both molecular and biochemical levels, and lead to a complete rearrangement of cell metabolism (Kaye and Guy, 1995). Cold resistance may thus be considered a multigenic trait involving many genes that may have either redundant or additive effects. Since 1985, when it was first shown that variations in gene expression do occur during cold acclimation (Guy *et al*., 1985), a major goal has been to identify cold-responsive (*cor*) genes and to determine whether they have roles in freezing tolerance. A great number of *cor* genes have been isolated and characterized from a variety of plant species, but only some of them code for proteins whose biochemical function is known, or can be at least predicted based on sequence similarity (Jung *et al*., 2003). The review of available information focused on the occurrence of several interconnected signal transduction cascades, partially shared with the cell response to different stress conditions. Some integrated models depicting temperature perception, signal transduction, transcriptional activation, and components of temperature stress tolerance have been hypothesized recently (Knight and

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Abbreviations: DAG, days after germination; GABA, γ-aminobutyric acid; GABA-T, γ-aminobutyric acid transaminase; GAD, glutamate decarboxylase; ROS, reactive oxygen species; SSADH, succinic semialdehyde dehydrogenase.

Knight, 2001; Shinozaki *et al*., 2003; Sung *et al*., 2003; Verslues and Zhu, 2005). In several cases, the analysis of plants either overexpressing or antisense for a given *cor* gene demonstrated that the accumulation of protecting molecules or the degradation of dangerous metabolites is associated with cold and/or frost tolerance (Iba, 2002). However, the exact function of many stress-related gene products still remains unclear. Moreover, changes in lipid composition and accumulation of sugars, that are likely to contribute to low temperature tolerance, do not necessarily rely on changes in gene expression, but may be brought about, completely or in part, by post-translational modulation of enzymes involved in their metabolism (Cossins *et al*., 2002; Stitt and Hurry, 2002). Yet, to understand completely how the cold-acclimation response is activated by low temperature will require considerable efforts.

The synthesis of osmoprotectants able to accumulate even at the highest concentrations without interfering with physiological processes, collectively known as compatible osmolytes, plays a central role in the response of the cell to frost and several other abiotic stress conditions (Yancey *et al*., 1982; Yancey, 2004). In plants, the distribution of these low-molecular-weight compounds, such as mono- and disaccharides (glucose, fructose, sucrose, and trehalose), quaternary amines (glycine betaine), polyols (mannitol, sorbitol), and amino acids (mainly proline), is species specific. They act both by avoiding cell dehydration through their contribution to osmotic adjustment, and by stabilizing the quaternary structure of proteins and membranes (Yancey, 2005). At least in the case of proline, a role as radical scavenger has also been reported (Yoshiba *et al*., 1997). Several genes involved in the biosynthesis of such osmolytes have been cloned and used to obtain stress-resistant plants through genetic engineering of the corresponding metabolic pathways. Among these, are those coding for choline mono-oxygenase and betaine aldehyde dehydrogenase, which control the biosynthesis of glycine betaine (Nuccio *et al*., 1999), and for δ^1-pyrroline-5-carboxylate synthetase and proline dehydrogenase, whose reciprocal expression is responsible for proline homeostasis (Kavi Kishor *et al*., 2005).

Among the numerous compounds that seem to serve as osmoprotectants in plants is the four-carbon, non-protein amino acid γ-aminobutyric acid (GABA), which under stress conditions may represent a significant fraction of the free amino acid pool (Bown and Shelp, 1989). Intracellular levels of GABA are typically low, but they rapidly increase in response to several abiotic stresses, such as hypoxia, drought, cold, heat shock, and mechanical stimulation (Bown and Shelp, 1997). In this context, given also some peculiar properties like the zwitterionic form at neutral pH and high solubility, a role as a compatible osmolyte has been hypothesized (Shelp *et al*., 1999). GABA is synthesized by a cytosol-localized glutamate decarboxylase (EC 4.1.1.15; GAD), a Ca^{2+}-calmodulin-dependent protein (Baum *et al*., 1993) that can thus be rapidly activated during various stress situations which elicit changes in cytosolic Ca^{2+} concentration (Snedden *et al*., 1996). Following irreversible glutamate decarboxylation, GABA may be further metabolized to succinic acid in the so-called GABA shunt, a short pathway bypassing two steps in the Krebs cycle. The GABA shunt takes place in the mitochondrion by means of two enzymes, a GABA transaminase using either α-ketoglutarate or pyruvate as amino acceptor (GABA-T; EC 2.6.1.19), and a succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.16) (Shelp *et al*., 1999). Although the GABA shunt is widely distributed in most prokaryotes and eukaryotes, it is only in animals that a major role for GABA, as the predominant inhibitory neurotransmitter of the brain, has been well established. During the last decade some experimental evidence has been described that suggests a possible involvement of GABA in several physiological processes in plants (Bouché and Fromm, 2004). Because of its role in neurotransmission, GABA could be produced to deter insect feeding and interfere with their development (MacGregor *et al*., 2003). GABA might function as an endogenous signalling molecule as well, since there are indications that glutamate/GABA receptors do exist in plants as well (Kang and Turano, 2003). The finding that proline transporters (e.g. AtProT2 and LeProT1) are able to recognize GABA as a substrate (Schwacke *et al*., 1999; Grallath *et al*., 2005) has been invoked to strengthen its role as osmoprotectant further. GABA levels increased under long-term conditions that limit glutamine production, reduce protein synthesis or enhance protein degradation, thus GABA might be a temporary store of nitrogen, and play a role as a sensor of nitrogen status and C:N balance, or a long-distance inter-organ signal molecule (Beuve *et al*., 2004). Moreover, because GAD activity is low pH-dependent and consumes H^+, GABA synthesis might function as a pH-stat, being activated under conditions that cause cytosolic acidification (Crawford *et al*., 1994). On the other hand, Arabidopsis *pollen–pistil interaction2* mutant resulting in growth inhibition and misguidance of pollen tubes in pistils was shown to lack a functional GABA-T; thus a gradient of GABA concentration seems essential for the growth and guidance of pollen tubes (Palanivelu *et al*., 2003). Moreover, SSADH was found to be negatively regulated by ATP and NADH, suggesting a control of the GABA shunt by mitochondrial energy charge and reducing potential (Breitkreutz and Shelp, 1995). The disruption of the unique SSADH gene in *A. thaliana* resulted in plants undergoing necrotic cell death caused by an abnormal accumulation of reactive oxygen species (ROS). Such behaviour might rely upon the ability of the GABA shunt to supply NADH and/or succinate to mitochondrial metabolism under conditions that inhibit the TCA cycle, impair respiration, and enhance the production of ROS (Bouché *et al*., 2003). Novel evidence for an intriguing link between the GABA

shunt, its by-product γ-hydroxybutyric acid (rising from the activity of a succinic semialdehyde reductase), and ROS was also provided recently (Fait *et al*., 2005). However, in most cases GABA accumulation can be simply associated with the cited physiological processes, and the speculated links are far from being definitely proved. Overall, the exact role of the GABA shunt in plants still awaits elucidation.

Here the occurrence of a quantitative glutamate decarboxylation is described in hardened barley and wheat seedlings when exposed to either cold- or freezing-stress conditions. GABA synthesis was limited by glutamate availability that increased during cold acclimation and was higher in cold-resistant genotypes. Also the expression of the GABA-shunt enzymes was different in frost-resistant and frost-sensitive varieties, suggesting that GAD activity might contribute to frost tolerance in acclimated plants.

Materials and methods

Plant materials, growth conditions, and stress treatments

Hordeum vulgare L. and *Triticum aestivum* L. varieties with different levels of cold resistance were used: the winter barley Nure and wheat Cheyenne (CNN) as frost-resistant genotypes, the spring barley Tremois and wheat Chinese Spring (CS) as frost-sensitive genotypes. Some CS/CNN chromosome substitution lines, genetic stocks in which a specific chromosome pair of the frost-sensitive CS is replaced by the homologous chromosomes of the frost-resistant CNN (Sutka, 1981), were also tested. In particular, the lines CS/CNN 5A and CS/CNN 5D that contain the chromosomes carrying QTLs with the highest influence on the cold tolerance trait (Sutka and Snape, 1989), and the line CS/CNN 1D, a negative control, were evaluated. Seeds were germinated in peat pots and grown to the first-leaf stage at +22 °C (9 h light at 160 μmol photons m^{-2} s^{-1})/+16 °C (15 h dark).

Seedlings were subjected to low temperature stress in the dark, following or not following cold hardening. Unacclimated plants were treated 8 d after germination (DAG). Acclimation was obtained through exposure to +3 °C (8 h light)/+1.5 °C (16 h dark) for a further 21 d. Temperatures of −3 °C and −8 °C were chosen as representative of cold- and freezing-stress conditions, respectively. After a gradual (about 2 °C h^{-1}) decrease of temperature from 3 °C to −3 °C, cold stress was applied for up to 16 h. Further freezing at −8 °C, by means of an analogous gradual decrease of temperature, was achieved for up to 21 h, as shown in Fig. 1. No ice inoculation was done; however, in all cases the treatment at −8 °C resulted in extensive freezing, with the leaf surface covered by hoar-frost, and having a rigid texture. When appropriate, after a gradual increase of temperature, plants were allowed to recover under the same conditions as for seed germination.

To test the effect of exogenously supplied amino acids, plants were grown in pots of sand at 22 °C as described, in the presence of 5–50 mmol kg^{-1} sand of either glutamic acid (brought to pH 6.0 with KOH) or glutamine, then exposed as above to −3 °C for up to 16 h. In order to rule out the possibility of osmotic effects, controls in which seedlings were treated with 50 mmol kg^{-1} KCl were also performed. Plants were then allowed to recover as above.

Total RNA extraction and semi-quantitative RT-PCR analysis

Frozen leaves were ground in liquid nitrogen to a fine powder, and total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Aliquots corresponding to 1 μg total RNA were reverse-transcribed with Superscript II (Invitrogen) using oligo(dT) primers. Tentative consensus sequences coding for barley GABA-shunt enzymes were identified in the TIGR database by high similarity level in sequence comparison with *Arabidopsis* GABA-shunt genes, which have been cloned and characterized (Baum *et al.*, 1993; Busch and

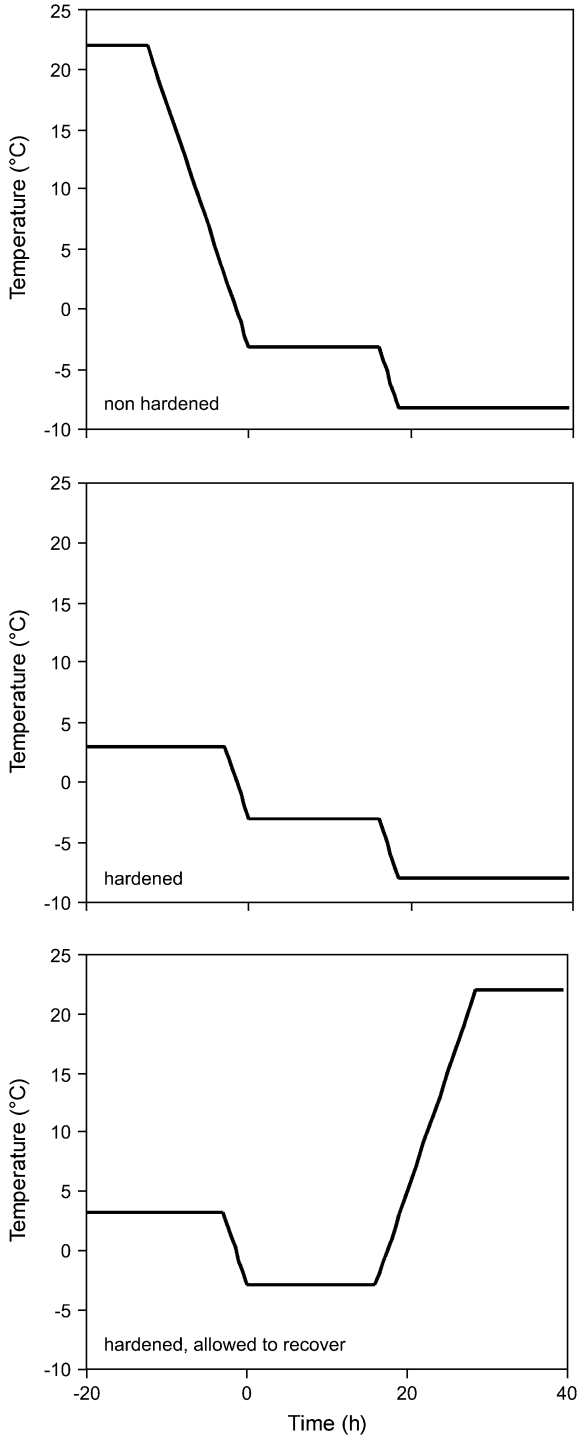

Fig. 1. Temperature profiles during the exposure of seedlings to either cold or freezing conditions.

Fromm, 1999; Van Cauwenberghe *et al.*, 2002). To ensure amplification of target genes, the following RT-PCR primers were chosen in conserved regions, thus obtaining a global expression analysis.

GAD: 5′-TGCCGGAGAACTCGATCCCCAAG-3′ and 5′-CGGTTCTGGAGCTCGGTGGTGAC-3′, expected product size 200 bp;
GABA-T: 5′-ATTCACAGCTGGATGGCAGAGC-3′ and 5′-AGGAAGATGGAAGCGCCAGTAGTG-3′, 500 bp;
SSADH: 5′-CTACGACGGGAAGACCATCGAG-3′ and 5′-ATGCTGGTCCAACCTTTCTGG-3′, 400 bp.

The identity of amplified products was verified by sequencing. Semi-quantitative RT-PCR was used to determine differential expression of the three genes. The exponential phase of amplification was determined for each of them by analysing the gel electrophoresis pattern of the PCR products generated with different numbers of cycles. Primers for the constitutively expressed ribosomal protein 12 (RPL12) were used to normalize the result (Baldi *et al.*, 2001). RT-PCR products were resolved in 2% agarose gels run with half-strength TRIS-borate-EDTA buffer and stained with ethidium bromide.

Amino acids extraction, separation, and quantification

For amino acid analysis, three pools of 30–35 leaves each were sampled for a given treatment. The material was ground with liquid nitrogen, and the powder was resuspended in 1 ml g^{-1} of a 3% (w/v) solution of 5-sulphosalicylic acid. Following centrifugation for 10 min at 12 000 *g*, 1 ml aliquots of the supernatant were brought to dryness at room temperature in a centrifugal vacuum concentrator (Eppendorf, model 5301). Residues were reconstituted with 0.1 ml of 2.5 N NaOH, resulting in a pH value of 10.2±0.2, and immediately analysed. Samples were mixed with the same volume of *o*-phthaldialdehyde solution [0.5 M in 0.5 M sodium borate buffer, pH 10.0, containing 0.5 M β-mercaptoethanol and 10% (v/v) methanol]. After exactly 60 s, 20 µl of derivatized samples were injected onto a 4.6×250 mm Zorbax ODS column (Rockland Technologies, Newport, DE, USA) equilibrated with 59% solvent A [50 mM sodium phosphate–50 mM sodium acetate buffer, pH 7.5, containing 2% (v/v) of both methanol and tetrahydrofuran] and 41% solvent B (65% methanol). Elution proceeded at a flow rate of 60 ml h^{-1} using a computer-controlled (Data System 450; Kontron, Munchen, Germany) complex gradient from 41% to 100% solvent B as previously described (Forlani *et al.*, 2000), monitoring the eluate at 340 nm. This procedure allowed complete resolution of equimolar mixtures of derivatizable amino acids (all the 20 protein amino acids except Pro and Cys), with a detection limit of about 0.1 nmol. Peaks were integrated by area, with variation coefficients ranging from 0.8% to 3.2%. Proline and total amino acid content were quantified by the ninhydrin method, as described by Bates *et al.* (1973). Results were expressed as µmol g^{-1} FW, and means ±standard deviation over the three replications were presented. Data were analysed by using standard statistical procedures for analysis of variance and *t*-test. Where differences are reported, they are at the 99% confidence level (*, $P < 0.01$).

Results

Amino acid homeostasis during cold stress in barley

In order to evaluate whether changes in amino acid pools may play a role in the plant response to either cold- or freezing-stress conditions, free amino acid levels were determined in frost-resistant barley seedlings during treatments at low temperatures. When plants were directly exposed to −3 °C, a severe treatment for 16 h did not affect total amino acid content, that was only slightly increased when plants were subjected to further periods at a freezing temperature (−8 °C) (Table 1). However, if the single contribution of each compound is considered, some significant variations were indeed found. Unexpectedly, the intracellular level of proline did not show any fluctuation, representing in all cases about one-quarter of all the amino acids. On the contrary, a strong reduction was evident in −3 °C-treated plants for glutamate and, to a lesser extent, for glutamine. This reduction was paralleled by a 16-fold increase in the content of GABA that, under non-stressing conditions, was only a minor component. The amount of GABA was further enhanced in seedlings treated at −8 °C. When −3 °C-treated plants were allowed to recover,

Table 1. *Free amino acid content in non-acclimated frost-resistant barley (*H. vulgare *L. cv. Nure) seedlings before and after exposure to cold and freezing conditions*

Eight days after germination, plants were subjected to cold stress for 16 h at −3 °C. Some seedlings were further treated for 21 h at −8 °C, as shown in Fig. 1. Treatments were carried out in the dark. Some of the plants treated at −3 °C were then allowed to recover for 48 h under the same conditions as for seed germination. Plants treated at −8 °C did not survive. Amino acid pools were quantified by reverse-phase HPLC following derivatization with *o*PDA; Pro and total amino acid content were measured by the ninhydrin method. Cys was undetectable. Three replications were done for each treatment, and means ±standard deviation over replicates are reported. All pools in −8 °C-treated plants were significantly ($P < 0.01$) different from those in −3 °C-treated seedlings. Significant differences between pools in −3 °C- or −8 °C-treated plants and those in untreated controls (8 DAG) are marked with an asterisk.

aa	8 DAG		Treated −3 °C		Treated −8 °C		−3 °C, after recovery	
	µmol g^{-1} FW	%	µmol g^{-1} FW	%	µmol g^{-1} FW	%	µmol g^{-1} FW	%
Asp	0.426±0.001	8.5	0.551±0.016*	11.3	0.813±0.029*	10.5	0.802±0.054	7.2
Glu	1.013±0.019	20.1	0.526±0.013*	10.8	0.712±0.025*	9.2	1.097±0.072	9.8
Asn	1.121±0.034	22.3	0.934±0.025*	19.2	1.637±0.043*	21.2	1.672±0.128	15.0
Gln	0.432±0.018	8.6	0.203±0.009*	4.2	0.417±0.014	5.4	1.384±0.133	12.4
Ala	0.223±0.003	4.4	0.273±0.005*	5.6	0.328±0.026*	4.3	0.581±0.073	5.2
GABA	0.023±0.001	0.5	0.385±0.028*	7.9	0.688±0.057*	8.9	1.330±0.120	11.9
Pro	1.242±0.049	24.7	1.307±0.035	26.8	1.838±0.041*	23.8	1.950±0.135	17.5
Other	0.550±0.037	10.9	0.695±0.020*	14.3	1.282±0.063*	16.6	2.330±0.219	20.9
All	5.030±0.141	100.0	4.873±0.121	100.0	7.713±0.164*	100.0	11.147±0.847	100.0

a similar trend was maintained for at least 48 h, with a more pronounced increase in total amino acid content, and a higher contribution of GABA, that accounted for 12% of free amino acids (Table 1). As in a previous study (Rizza *et al*., 1994), −8 °C-treated plants failed to recover.

Since pretreatments at low, non-freezing temperatures are able to promote acclimatization of plants to cold, analogous experiments were performed in which 8-d-old seedlings were exposed to +3 °C for 3 weeks before stress treatments were applied. Acclimated plants, whose growth was almost completely arrested, showed a striking increase in total amino acid content (Table 2). Nevertheless, the percentage contribution of each compound remained essentially the same, with the only exception of asparagine, which was lowered concomitantly with a comparable increase in free glutamine. Interestingly, when hardened seedlings were exposed to the lower temperature, total amino acids did not vary further, but once again a remarkable accumulation of GABA was found. At −3 °C such an increase came along with a parallel decrease in free glutamine. At −8 °C, an even more conspicuous accumulation of GABA reflected a decrease in glutamate content also. Moreover, in both cases, an increase in alanine pools was evident that was proportional to the severity of the stress (Table 2). Alanine may in fact be synthesized as a by-product of the GABA shunt, as a result of the activity of GABA-T when pyruvate is used as the amino group acceptor (Shelp *et al*., 1999). Both −3 °C- and −8 °C-treated plants survived the treatment. Contrary to non-acclimated plants, when −3 °C-treated seedlings were allowed to recover, the intracellular concentration of all amino acids comprising GABA came back to the values scored for non-stressed, non-hardened seedlings (Tables 1, 2). This result seems, therefore, to suggest that the damage caused to non-acclimated plants by subzero yet permissive temperatures significantly impairs their ability to maintain amino acid homeostasis.

Time-course of GABA accumulation under cold and freezing conditions

In order to support the possibility that the GABA shunt would be activated in response to cold-stress conditions, the content of GABA and related amino acids was determined in barley plants at increasing times after the exposure to low temperature. In non-acclimated seedlings, GABA increased with time and with the severity of the stress from hardly detectable amounts up to 0.65–0.70 μmol g^{-1}. In the meantime, the content of glutamate decreased from 0.75 to 0.25 μmol g^{-1}, whereas free alanine remained at quite a constant level throughout (Fig. 2A). A dramatically different picture was obtained with hardened seedlings. The initial availability of a 3-fold higher level of glutamate most likely favoured GABA production that accumulated sharply up to 1.10 μmol g^{-1} as soon as the temperature lowered from −3 °C to −8 °C (Fig. 2B). Later on, a complex pattern was evident. The steady-state level of GABA decreased, with a concomitant increase of both glutamate and alanine. Then it increased again, concurrently to a new decrease in alanine and glutamic acid. This complex trend was confirmed when the whole experiment was repeated. Oscillating, complementary levels of alanine/glutamate and GABA indeed seem to suggest that in hardened plants, following an initial decarboxylation of glutamate, the GABA shunt may be activated, stoichiometrically converting the product into alanine and new glutamate. In such a way, GAD activity would be favoured by further substrate availability. On the contrary, in non-acclimated seedlings this conversion seems not to take place. Accordingly, GABA levels reached a maximum, and remained relatively constant thereafter.

Expression of GABA-shunt genes during acclimation and freezing

In order to strengthen such a hypothesis, the expression profile of the genes coding for GABA-shunt enzymes was

Table 2. *Free amino acid content in acclimated frost-resistant barley (*H. vulgare *L. cv. Nure) seedlings before and after exposure to cold and freezing conditions*

Eight days after germination, plants were acclimated by incubation for 21 d at +3 °C (8 h light)/+1.5 °C (16 h dark). Low temperature treatments, amino acid analysis, and replications were as in Table 1. All pools in −8 °C-treated plants were significantly (P <0.01) different from those in −3 °C-treated seedlings. Significant differences between pools in −3 °C- or −8 °C-treated plants and those in untreated controls (8 DAG) are marked with an asterisk. Both −3 °C- and −8 °C-treated plants were able to recover.

aa	Hardened		Treated −3 °C		Treated −8 °C		−3 °C, after recovery	
	μmol g^{-1} FW	%	μmol g^{-1} FW	%	μmol g^{-1} FW	%	μmol g^{-1} FW	%
Asp	1.266±0.077	8.4	1.463±0.014	8.8	0.833±0.015*	5.7	0.343±0.023	5.1
Glu	3.077±0.201	20.3	3.391±0.084	20.5	2.262±0.087*	15.6	1.113±0.053	16.5
Asn	2.583±0.199	17.1	2.827±0.024	17.1	2.165±0.011	14.9	1.625±0.058	24.1
Gln	2.132±0.361	14.0	1.607±0.063*	9.7	1.061±0.076*	7.3	0.307±0.029	4.6
Ala	0.881±0.046	5.8	1.446±0.020*	8.7	1.670±0.128*	11.5	0.236±0.010	3.5
GABA	0.133±0.005	0.9	0.552±0.012*	3.3	1.869±0.090*	12.9	0.095±0.015	1.4
Pro	3.333±0.125	22.1	3.619±0.038	21.9	3.270±0.041	22.5	2.406±0.107	35.7
Other	1.717±0.037	11.4	1.659±0.025	10.0	1.376±0.018*	9.5	0.608±0.043	9.0
All	15.123±0.966	100.0	16.563±0.177	100.0	14.507±0.223	100.0	6.733±0.286	100.0

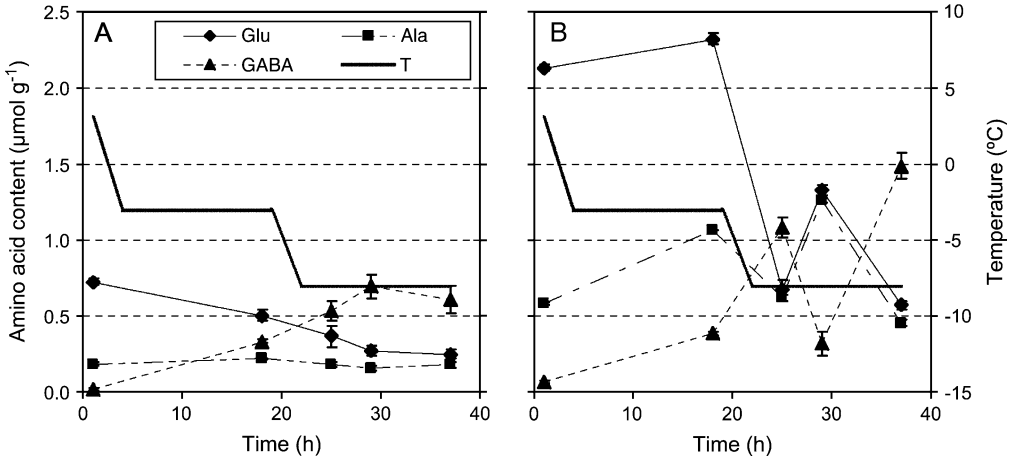

Fig. 2. Time-course of GABA shunt-related free amino acids in seedlings of non-acclimated (A) or acclimated (B) frost-resistant barley. Measurements were made in triplicate during the exposure of plants to low temperatures as indicated.

analysed by means of semi-quantitative RT-PCR. In *A. thaliana* only a single *SSADH* is known, while for GABA-T and GAD several isoforms exist (Bouché and Fromm, 2004). Homologous tentative consensus sequences were identified in barley by a high similarity level in sequence comparison. A few homologues were found for *GABA-T* and *GAD*, thus RT-PCR primers were chosen in conserved regions to obtain a global expression analysis. All genes showed a basal level of expression. When non-acclimated seedlings were exposed to −8 °C, no induction was evident. Conversely, the low expression was further depressed, mainly for *GAD* (Fig. 3A). On the contrary, although GABA levels at the end of the hardening period showed only a slight increase (Tables 1, 2), cold acclimation resulted in a strong induction of all three genes (Fig. 3B), whose levels of expression remained high when acclimated plants were subsequently exposed to frost conditions. Interestingly, a differential behaviour was found between frost-resistant and frost-sensitive barley cultivars. In the frost-sensitive genotype Tremois, even though the three genes showed a similar induction during hardening, after exposure to frost, their expression dropped progressively to basal levels, being barely detectable after 18 h of freezing. Data thus suggest that acclimation induces the expression of GABA-shunt genes in barley, but that only in the frost-resistant cultivar is it maintained during subsequent freezing.

Restriction of GABA synthesis due to the amount of available glutamate

Because the synthesis of GABA may be restrained by glutamate availability, and previous reports had shown that its production seems to be regulated by the steady-state level of the substrate (Scott-Taggart *et al.*, 1999), the relationship between glutamate content and GABA accumulation was addressed in glutamate-fed seedlings. However, the exogenous supply of either glutamate or glutamine resulted in their rapid and quantitative conversion to asparagine, whose levels increased up to 5-fold, representing about 60% of total amino acid content (data not shown). A significant increase was evident also for glutamine, whereas the pool of free glutamate was only slightly affected. This notwithstanding, when non-acclimated, glutamate-treated plants were exposed to −3 °C, the resulting amount of intracellular GABA was significantly higher than in non-fed seedlings (Fig. 4). In both cases free glutamate was almost completely depleted, thus strengthening the possibility that the rate of GAD activity may indeed be affected by substrate levels.

Links between GABA metabolism and frost tolerance

The induction of the GABA shunt during cold hardening could imply its involvement in the response of cells to freezing. However, it may represent a consequence of cold-stress conditions as well, without any functional role in plant protection. In order to shed further light on this aspect and to assess a possible relationship between GABA accumulation and frost tolerance, a genetic approach was carried out. Amino acid pools before and after acclimation, as well as following subsequent freezing, were compared in two barley and two wheat cultivars showing different levels of frost resistance. Data obtained with the frost-sensitive barley cultivar Tremois, the frost-resistant wheat Cheyenne (CNN), and the frost-sensitive wheat Chinese Spring (CS) are summarized in Tables 3, 4, and 5, respectively. In both species, seedlings of the resistant genotype grown for 8-d showed a slightly higher amino acid content than the sensitive one. However, differences in glutamate pools were a bit higher and, at least in barley, these were even more striking if the sum of glutamate and glutamine is considered. A similar trend was also found following cold acclimation, since free amino acids showed an overall

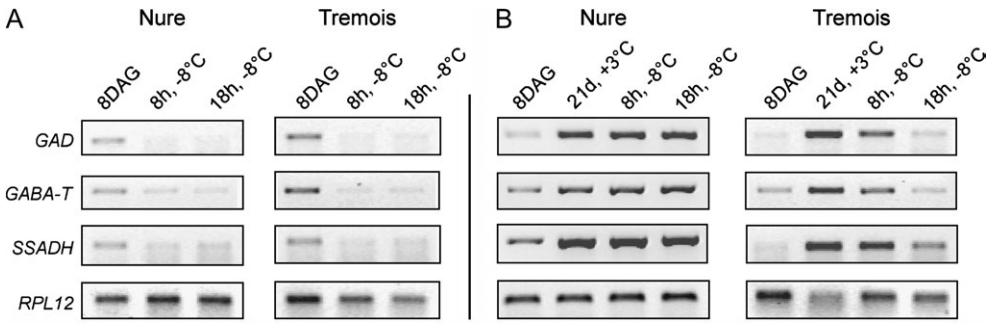

Fig. 3. Cold induction of GABA-shunt genes in barley. Freezing-stress conditions were imposed on either non-acclimated (A) or acclimated (B) plants. RT-PCR analysis was performed as detailed in the Materials and methods. Primers for the constitutive *LR12* gene (Baldi *et al*., 2001) were used to normalize the result.

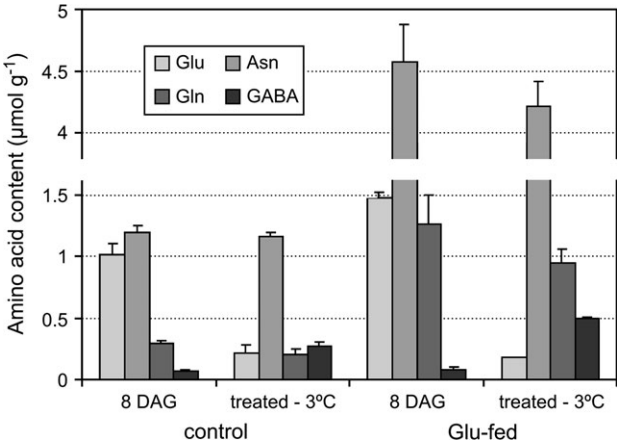

Fig. 4. GABA levels in glutamate-fed barley plants. Seedlings of the frost-resistant cultivar Nure were grown for 10 d in the presence of 10 mmol kg^{-1} of Glu in the substrate and then exposed to −3 °C for 16 h. The resulting intracellular pools of GABA and related amino acids were quantified and compared with those of untreated controls. Similar data were obtained with exogenously supplied glutamine (not shown).

2-fold increase, but the percentage contribution of each compound did not vary significantly. Following exposure to frost, the amount of total amino acids slightly decreased in barley, whereas in both wheat genotypes a further increase was found that relied almost entirely upon a sharp rise in free amide pools. With respect to GABA shunt-related amino acids, in frost-resistant cultivars, GABA (and alanine) rose to higher levels, most likely because of the initial higher amount of glutamate. Remarkably, while in frost-sensitive genotypes GABA synthesis caused an almost complete depletion of the substrate, in frost-resistant cultivars significant amounts of free glutamate were retained. These patterns do seem to strengthen a positive correlation between high rates of glutamate decarboxylation and the ability to cope with frost.

Taking this approach one step further, amino acid content was also measured in CS/CNN chromosome substitution lines. Substitutions at 5A and 5D were selected, because chromosome 5D and, mainly, 5A were shown to be involved in frost tolerance, carrying QTLs with the highest influence on the resistance trait (Sutka and Snape, 1989). Line CS/CNN 1D was used as a frost-susceptible control. Intracellular levels of free glutamate, glutamine, and GABA were thus evaluated in untreated, hardened, and frost-stressed seedlings (Fig. 5). A clear-cut similarity was evident between the frost-tolerant cultivar CNN and the substitution line carrying chromosome 5A, suggesting that chromosome 5A might regulate Glu/Gln availability during frost exposure and, as a consequence, the extent of GABA metabolism.

Discussion

To date, most of the studies mentioned concerning frost tolerance have been focused on changes associated with exposure to low, non-freezing temperatures, an event that naturally precedes winter freezing and allows plants to cope with more severe conditions. On the contrary, the ability of acclimated seedlings to induce further defence mechanisms at lower temperatures is still poorly investigated. Present results may thus represent an original contribution to understanding the response of the plant cell to freezing. As detailed in Materials and methods, −3 °C and −8 °C treatments were chosen as conditions representative of subzero non-freezing and freezing conditions, respectively. Under the experimental conditions employed, as suggested also by previous reports (Brush *et al*., 1994; Pearce and Fuller, 2001), ice formation was not evident at −3 °C, a temperature that allowed a complete recovery of both hardened and non-hardened barley and wheat plants. On the contrary, −8 °C treatments always resulted in extensive ice formation. Because no ice inoculation was done, some cells might be subjected to intracellular instead than extracellular freezing, thus leading randomly to lethal damage. However, the same is true in the field, where various defence mechanisms provide winter cultivars with the basis of an overall tolerance to subzero temperatures. Despite a possible variability in the damage, a noteworthy uniformity was found as to the survival rates: in accordance with previous data (Rizza *et al*., 1994),

Table 3. *Free amino acid content in frost-sensitive barley (*H. vulgare *cv. Tremois) seedlings before and after exposure to hardening and subsequent freezing conditions*

Eight days after germination, plants were acclimated for 21 d at +3 °C (8 h light)/+1.5 °C (16 h dark). Hardened plants were treated for 16 h at −3 °C and then subjected to freezing for 21 h at −8 °C. Significant differences between pools in acclimated versus untreated (8 DAG) plants, or in −8 °C-treated versus acclimated plants are marked (*, P <0.01). Plants did not survive the treatment at −8 °C.

aa	8 DAG		Acclimated plants		Treated −8 °C.	
	μmol g^{-1} FW	%	μmol g^{-1} FW	%	μmol g^{-1} FW	%
Asp	0.223±0.055	5.5	0.352±0.034	5.1	0.335±0.067	6.5
Glu	0.450±0.074	11.2	0.699±0.010*	10.2	0.022±0.004*	0.4
Asn	1.083±0.029	26.9	1.732±0.086*	25.2	1.212±0.109*	23.5
Gln	0.095±0.013	2.4	0.114±0.030	1.7	0.264±0.046*	5.1
Ala	0.121±0.026	3.0	0.140±0.028	2.0	0.317±0.036*	6.1
GABA	0.020±0.034	0.5	0.150±0.017*	2.2	0.926±0.098*	18.0
Pro	1.576±0.318	38.9	3.139±0.265*	45.6	1.522±0.122*	29.4
Other	0.467±0.032	11.6	0.547±0.041	8.0	0.566±0.130	11.0
All	4.033±0.127	100.0	6.873±0.295*	100.0	5.163±0.443*	100.0

Table 4. *Free amino acid content in frost-resistant wheat (*T. aestivum *cv. Cheyenne) seedlings before and after exposure to hardening and subsequent freezing conditions*

Growth, acclimation and freezing treatments, amino acid analysis, and replications were as in Tables 1 and 2. All pools in acclimated plants were significantly (P <0.01) different from those in untreated (8 DAG) seedlings. Significant differences between pools in −8 °C-treated versus acclimated plants are marked with an asterisk.

aa	8 DAG		Acclimated plants		Treated −8 °C	
	μmol g^{-1} FW	%	μmol g^{-1} FW	%	μmol g^{-1} FW	%
Asp	0.658±0.062	15.4	0.998±0.109*	12.1	0.904±0.090	7.3
Glu	1.256±0.106	29.4	1.867±0.092*	22.6	0.779±0.565	6.1
Asn	0.163±0.010	3.8	0.491±0.037*	6.0	3.215±0.219*	25.9
Gln	0.277±0.082	6.4	0.721±0.091*	8.7	1.745±0.249*	14.0
Ala	0.235±0.015	5.5	0.355±0.030*	4.3	0.956±0.191*	7.6
GABA	0.018±0.030	0.4	0.344±0.019*	4.2	1.479±0.243*	11.9
Pro	1.014±0.076	23.7	2.020±0.231*	24.4	2.181±0.266	17.5
Other	0.656±0.042	15.3	1.468±0.139*	17.8	1.200±0.470	9.6
All	4.277±0.273	100.0	8.263±0.710*	100.0	12.460±1.555	100.0

Table 5. *Free amino acid content in frost-sensitive wheat (*T. aestivum *cv. Chinese Spring) seedlings before and after exposure to hardening and subsequent freezing conditions*

Growth, acclimation and freezing treatments, amino acid analysis, and replications were as in Tables 1 and 2. Significant differences between pools in acclimated versus untreated (8 DAG) plants, or in −8 °C-treated versus acclimated plants are marked (*, P <0.01).

aa	8 DAG		Acclimated plants		Treated −8 °C	
	μmol g^{-1} FW	%	μmol g^{-1} FW	%	μmol g^{-1} FW	%
Asp	0.483±0.034	15.5	0.644±0.015*	12.2	0.718±0.128	8.5
Glu	0.750±0.061	24.1	1.200±0.090*	22.7	0.045±0.013*	0.5
Asn	0.193±0.042	6.2	0.378±0.174*	7.0	2.537±0.534*	29.8
Gln	0.199±0.010	6.4	0.328±0.072	6.2	1.009±0.206*	11.8
Ala	0.181±0.076	5.8	0.371±0.061	7.0	0.474±0.077	5.6
GABA	0.096±0.015	3.1	0.092±0.064	1.7	1.077±0.221*	12.7
Pro	0.626±0.039	20.1	1.275±0.041*	24.1	1.519±0.187	18.0
Other	0.588±0.038	18.9	1.005±0.046*	19.0	1.108±0.519	13.1
All	3.117±0.049	100.0	5.293±0.299*	100.0	8.487±1.459	100.0

almost 100% of acclimated plants of the frost-tolerant cultivar Nure survived −8 °C treatments, whereas almost 100% of non-acclimated plants and of acclimated seedlings of the frost-sensitive cultivar Tremois failed to recover.

In order to investigate whether changes in free proline content may occur during the response of barley to cold stress, as reported for other species (e.g. *A. thaliana*; Wanner and Junttila, 1999), amino acid levels were quantified following various temperature treatments. Several compatible osmolytes were indeed found to be produced under stress conditions in barley (Rhodes and Hanson, 1993; Murelli *et al*., 1995). Although some

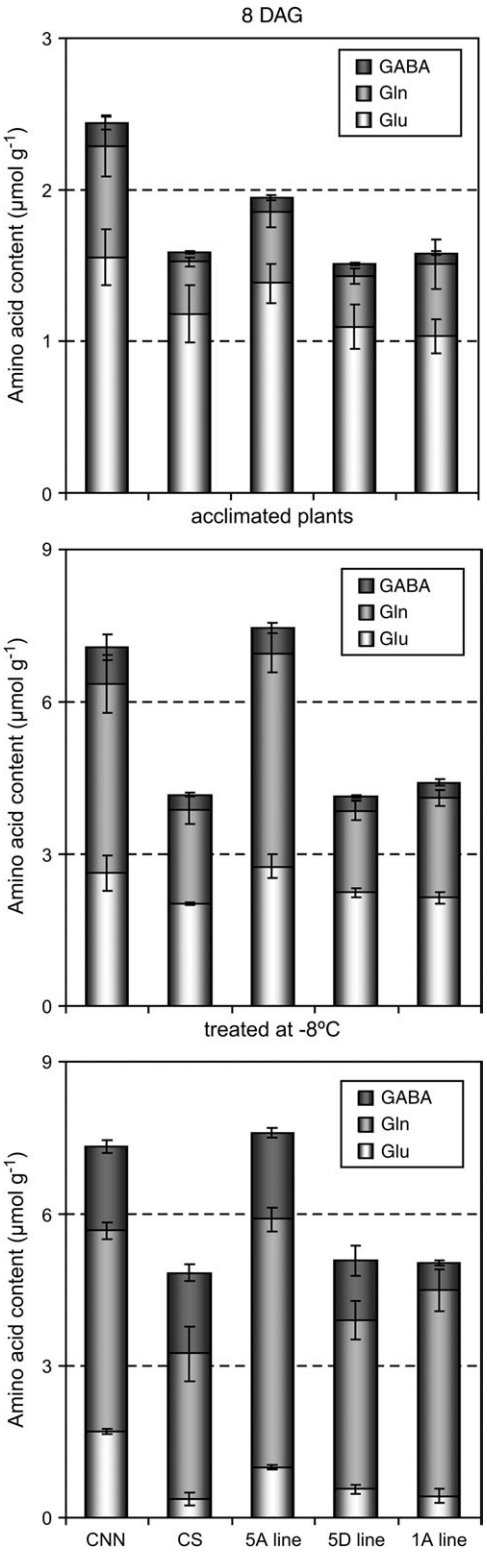

Fig. 5. Free glutamate, glutamine, and GABA levels in frost-resistant (Cheyenne, CNN) and frost-sensitive (Chinese Spring, CS) wheat cultivars, and in CS/CNN chromosome substitution lines. Eight days after germination, plants were acclimated for 21 d at +3 °C (8 h light)/ +1.5 °C (16 h dark). Hardened plants were then subjected to −3 °C for 16 h, and to −8 °C for 21 h, as shown in Fig. 1. Means ±standard deviation over three replications are given.

changes in the overall free amino acid content were evident, it was shown that proline did not accumulate during either cold acclimation or the subsequent exposure to frost, ruling out the possibility of its engagement in the defence response. However, results focused on GABA and related amino acids, whose levels were found to vary significantly, suggesting that a significant activation of GAD does occur early after exposure to cold. Since GAD is a Ca^{2+}/calmodulin-regulated enzyme (Snedden *et al*., 1996), the Ca^{2+} influx that occurs following a temperature drop (Monroy and Dhindsa, 1995) could be responsible for the activation of the enzyme under stress. In fact, a semi-quantitative RT-PCR analysis showed a constitutive, even if low, expression of GAD in barley seedlings. Moreover, the transcription of all three GABA-shunt enzymes was strongly induced during cold acclimation in both frost-resistant and frost-sensitive barley cultivars. Consistently, hardened seedlings exposed to frost conditions accumulated GABA to higher levels.

Interestingly, a difference was found subsequently between frost-resistant and -sensitive genotypes. In the former, gene expression was maintained at high levels throughout, while in the latter at −8 °C mRNAs for GABA-shunt genes declined rapidly with time. As a consequence, in the frost-sensitive cultivar, GABA levels reached an intermediate amount, and did not vary further. On the contrary, an oscillatory, complementary trend shown by the intracellular pools of GABA and glutamate+alanine in the frost-resistant cultivar seems to confirm the activation of the pathway. GABA deamination by a pyruvate-dependent GABA-T would in fact yield alanine, that might be further used as a substrate by an alanine/α-ketoglutarate transaminase (EC 2.6.1.2), whereas an α-ketoglutarate-dependent GABA-T would recycle GABA directly into new glutamate (Bouché and Fromm, 2004), yielding an autocatalytic process sustaining continuous GAD activity. Moreover, increased GABA content in both non-acclimated plants of the frost-resistant barley cultivar and acclimated seedlings of the frost-sensitive genotype rebounded into an almost complete depletion of free glutamate. A correlation between the steady-state level of glutamate (and glutamine) when seedlings are exposed to low-temperature stress and the subsequent accumulation of GABA suggests that GAD activity is limited by the pool of the substrate. The occurrence of an overflow mechanism based upon substrate availability was strengthened by the results obtained with glutamate-fed plants, which synthesized higher GABA levels when directly exposed to stress. This is consistent with previous studies in which GAD activity was found to be regulated *in vivo* by glutamate concentration (Chung *et al*., 1992; Cholewa *et al*., 1997; Scott-Taggart *et al*., 1999), and may contribute to the higher rate of GABA production in hardened plants. In fact, acclimation resulted in a general, significant increase in amino acid content.

An overall increase in the steady-state level of free amino acids in response to cold exposure has been reported previously. In wheat, proline, glutamic acid, glutamine, alanine, aspartic acid, and asparagine were found to accumulate during cold acclimation (Kaldy and Freyman, 1984; Naidu *et al*., 1991). More recently, metabolomic profiling analyses in *Arabidopsis* showed a co-ordinated increase of amino acid pools in response to a cold shock treatment (Cook *et al*., 2004; Kaplan *et al*., 2004; Klotke *et al*., 2004). Such behaviour may either simply derive from a cold-induced reduction in the rate of protein synthesis or be connected to some, yet undiscovered, adaptive response. On the other hand, the activation of GABA metabolism following exposure to cold conditions, and higher rates of glutamate decarboxylation in acclimated seedlings exposed to frost, might imply the involvement of the GABA shunt in the mechanisms responsible for plant tolerance to low temperatures. A genetic approach was thus exploited to strengthen the occurrence of a causal connection between GABA production/accumulation (related to either the induction of the GABA-shunt genes, or to the amounts of available substrate) and frost resistance. In both barley and wheat, frost-resistant cultivars were shown to contain constitutively higher levels of GABA-related amino acids. In barley, the ability to maintain high levels of expression of GABA-shunt genes during freezing was found only in the winter, frost-resistant cultivar. Suitable wheat chromosome substitution lines revealed an association between steady-state levels of GABA precursors (and the rate of GABA synthesis, as a consequence) and chromosome 5A, which carries the genetic bases for frost tolerance (Sutka and Snape, 1989). These results seem therefore to support the possibility that a correlation may exist between frost tolerance and glutamate availability, GAD activity and the consequent synthesis of GABA, and that the GABA shunt could play a role in the plant response to freezing.

But what is the gain for the plant? Because GABA seems not to accumulate, but to be recycled to further fuel the GABA shunt (Fig. 2), its contribution to withstanding freezing, if any, should rely upon the maintenance of the carbon flow through the pathway. In fact, GABA is produced in both frost-tolerant and frost-sensitive cultivars, whereas the distinctive factor seems the ability to maintain high rates of GAD activity. This is evident at the trascriptional level (Fig. 3), and is further supported by the respective substrate pools: in the sensitive cultivar, at −8 °C the activity of GAD rapidly leads to glutamate depletion, whereas in the tolerant genotype substantial levels of the substrate can still fuel the GABA shunt 21 h after exposure to freezing conditions (Tables 3 and 2, respectively). As hypothesized previously (Shelp *et al*., 1999; Bouché and Fromm, 2004), a protective effect of the GABA shunt may depend upon the reaction catalysed by GAD. Because of its H^+-consuming properties, high rates of GAD activity could indeed act as a pH-stat mechanism counteracting cytosol acidification due to membrane leakage caused by frost (Crawford *et al*., 1994). More recently, new data emerged postulating a role for the GABA shunt in protecting the plant from oxidative stress (Bouché *et al*., 2003; Oliver and Solomon, 2004; Fait *et al*., 2005) that is well known to occur under low-temperature stress. However, although sound, these hypotheses have to be considered pure speculation. Useful elements in order to verify these aspects might come from measurement of intracellular pH and ROS production, for instance by means of fluorescent probes or other suitable techniques. However, technical drawbacks, mainly related to the need for measurements *in vivo* without a reversal of cold acclimation or freezing stress, have to date caused the failure of such approaches. To obtain more direct evidence supporting a role for the metabolism of GABA in the context of the plant response to cold stress, the sensitivity of plants fed with exogenous glutamate was also evaluated. However, as shown in Fig. 4, barley seedlings rapidly convert exogenously supplied glutamate to asparagine that seems the main form in which nitrogen is stored in the leaves. As a consequence, glutamate pools were only slightly increased, and inconsistent results were obtained (data not presented). Trials are currently in progress to obtain better experimental support. If confirmed, a new role for GABA in the response of plants to stress would emerge, possibly allowing new approaches for the assisted selection of increased frost resistance in crop species.

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