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Ascorbate content of wheat leaves is not determined by maximal L-galactono-1,4-lactone dehydrogenase (GalLDH) activity under drought stress

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ABSTRACT

Although ascorbic acid (AA) is a high-abundance metabolite, relatively little is known about the factors controlling its accumulation in leaves. To address this issue, we examined the role of L-galactono-1,4-lactone dehydrogenase (GalLDH), the enzyme which catalyses the last step of this pathway, in the control of AA content under optimal and stress conditions. In a range of species, no clear relationship between AA content and leaf GalLDH protein and activity was found under optimal growth conditions. To explore the effect of drought stress on GalLDH activity and protein content, wheat (*Triticum aestivum* L.) was selected for detailed analysis, using two cultivars that differ in their constitutive AA level. In well-watered plants, the AA content of cv Buck Chambergo (BCH) was over twice that of cv Cooperativa Maipún (CM) but dehydroascorbic acid content was similar in both cv. In agreement with this, dehydroascorbate reductase and glutathione reductase activities were higher in cv BCH than in cv CM, indicating a higher capacity for AA regeneration. Neither leaf DHA content nor activities of AA regenerating enzymes were modified by drought. Although drought caused a substantial increase in GalLDH protein and activity in the low AA cv CM, this treatment had no effect on these parameters in cv BCH. Notably, leaf AA content was unaffected by drought in either cv. These results suggest that GalLDH protein and activity cannot be used as an indicator for changes in the capacity for ascorbate biosynthesis and that AA biosynthesis is constrained by other factors under stress. This can be explained by the importance of regeneration in maintaining AA levels and possibly also by redox regulation of GalLDH.

Key-words: ascorbic acid; dehydroascorbate; drought; L-galactono-1,4-lactone; L-galactono-1,4-lactone dehydrogenase; mitochondria; oxidative damage; redox regulation; wheat.

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Abbreviations: AA, reduced ascorbic acid; APX, ascorbate peroxidase; BCH, Buck Chambergo; CM, Cooperativa Maipún; cv, cultivar; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GL, L-galactono-1,4-lactone; GalLDH, L-galactono-1,4-lactone dehydrogenase; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; PAR: photosynthetically active radiation.

INTRODUCTION

Although ascorbic acid (AA) plays many important functions in plant biology (Noctor & Foyer 1998; Arrigoni & de Tullio 2000; Smirnoff & Wheeler 2000), little is known about the factors that control its synthesis and accumulation in different tissues. This metabolite is one of the most abundant in green leaves, where in favourable conditions it represents about 10% of the total soluble carbohydrate pool (Noctor & Foyer 1998; Smirnoff & Wheeler 2000).

There is a general consensus that the biosynthetic pathway proposed by Wheeler, Jones & Smirnoff (1998) represents a major pathway of AA production in plants. However, other routes have been demonstrated (Davey *et al.* 1999) and several biosynthetic pathways may coexist (Davey *et al.* 1999; Agius *et al.* 2003; Wolucka & van Montagu 2003; Lorence *et al.* 2004). Although L-gulono-1,4-lactone has been suggested to be a precursor of ascorbate in suspension cultured cells (Wolucka & van Montagu 2003), there has been no characterization of a leaf enzyme catalysing this conversion. Therefore, regardless of the early steps of AA biosynthesis, the proven final step in all schemes demonstrated to date is catalysed by L-galactono-1,4-lactone dehydrogenase (GalLDH; EC 1.3.2.3; Ôba *et al.* 1995; Østergaard *et al.* 1997; Pallanca & Smirnoff 1999; Siendones *et al.* 1999; Bartoli, Pastori & Foyer 2000), an enzyme located on the inner mitochondrial membrane.

GalLDH is encoded by a single gene in *Arabidopsis*, cauliflower, sweet potato and tobacco (Østergaard *et al.* 1997; Imai *et al.* 1998; Yabuta *et al.* 2000). Antisense suppression of GalLDH in synchronous BY-2 tobacco cell cultures resulted in a decline in enzyme activity and a 30% reduction in AA levels (Tabata *et al.* 2001). This had a pro-

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nounced negative effect on cell division, growth and cell structure. Little is known about the factors that regulate the expression and activity of GalLDH and how this might contribute to maintenance of the AA pool (Smirnoff, Running & Gatzek 2004). Light appears to be a trigger for expression of GalLDH transcripts over a 5 d period in tobacco leaves (Tabata, Takaoka & Esaka 2002) and conversion of exogenously supplied L-galactono-1,4-lactone (GL) to AA over 24 h was increased by light treatment of barley leaf slices (Smirnoff 2000). However, there is some indication of interspecific variation with regard to diurnal regulation of GalLDH transcript abundance, since this was observed in *Arabidopsis* (Tamaoki *et al.* 2003), but not in tobacco (Pignocchi *et al.* 2003).

Isolated mitochondria produce copious amounts of AA when supplied with the substrate, GL, using the respiratory chain cytochrome c as the electron acceptor (Siendones et al. 1999; Bartoli et al. 2000) and we have shown that AA synthesis is geared to mitochondrial metabolism in Arabidopsis. Specifically, GalLDH is bound to mitochondrial complex I in Arabidopsis and regulated by the activity of the electron transport chain (Heazlewood, Howell & Millar 2003; Millar et al. 2003). From this finding, we predicted that GalLDH activity would exert a high degree of control over the rest of the pathway, a property which would not normally be expected of the terminal step in metabolite biosynthesis. Because of the tight regulation of GalLDH activity by the mitochondrial electron transport chain, a key inference is that maximal extractable activity and amount of GalLDH protein would not be good markers for AA accumulation. However, a recent study has shown a correlation between mitochondrial AA content and maximal extractable GalLDH activity, following Botrytis cinerea inoculation of tomato leaves (Kuźniak & Skłodowska 2004).

In light of these findings, we wished to investigate further the relationship between GalLDH protein, activity and AA abundance, in order to determine whether the protein level is regulated. We prepared a polyclonal antiserum to GalLDH and described the relationship between leaf AA content and GalLDH in a number of species. We then concentrated specifically on the response of these parameters to water stress in wheat (*Triticum aestivum* L.), owing to the availability of a range of cultivars that vary in their AA content and water stress tolerance. We report results from the two cultivars that represented the extremes of variation. Since it has been shown that maize and tobacco plants constitutively expressing dehydroascorbate reductase have higher levels of leaf AA (Chen *et al.* 2003), we also examined the relationship between water stress and the role of GalLDH and regenerating enzymes in determining AA content in these cultivars.

MATERIALS AND METHODS

Plant material

For the survey of AA content and synthesis in different plant species, soybean (*Glycine max* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L. cv Buck Chambergo), sunflower (*Helianthus annuus* L.) and oat (*Avena sativa* L.) were grown at 25 ± 2 °C, 350 μmol. m^{-2} s^{-1} of photosynthetically active radiation (PAR) and a photoperiod of 10 h. The samples were taken from the first expanded leaf at 2 weeks after sowing. *Arabidopsis thaliana* L. was grown at 15 ± 5 °C and 100 μmol. m^{-2} s^{-1} PAR, and all the green leaves from mature plants were used. Leaves for each species were divided in two groups: one used for AA determinations immediately after extraction and the other for GalLDH extraction. These samples were frozen in liquid nitrogen and stored at −73 °C until required for GalLDH activity and content determinations.

For the light/dark experiments, wheat, *T. aestivum* L cv Cadenza plants were grown in soil for 2 weeks at 18 °C day/ 15 °C night with a 16-h day at 700 μmol /m^{-2} s^{-1} PAR. The third leaf was sampled at 3 and 6 h intervals over a complete 24-h period.

For the survey of wheat cultivars with different AA content, 3-week-old plants were used. The following 10 cultivars were tested: cvs. Cooperativa Maipún, Buck Halcón, Buck Arriero, Buck Poncho, Buck Panadero, Buck Charrúa, Klein Pegaso, Buck Pronto, PoINTA Isla Verde, and Buck Chambergo. Plants were grown in the greenhouse in pots containing 5 kg of soil (five plants per pot) at an average maximum/minimum temperature of 28/18 °C, 700 μmol m^{-2} s^{-1} PAR with a photoperiod of 14 h.

For the drought stress experiments, plants were grown in a culture room at 25 ± 2 °C, 500 μmol m^{-2} s^{-1} of PAR and a photoperiod of 14 h. Drought was imposed on 3-week-old plants by withholding watering until the pots reached a soil water potential of −1.5 MPa. Leaf conductance was measured with a Licor Li-1600 Steady State Porometer (Li-Cor Inc., Lincoln, NE, USA). Water potential was measured with a Wescor HR33T Dew Point Hygrometer (Wescor, Logan, UT, USA) using C-52 chambers and PST-55 probes, for leaves and soil, respectively. Typically the soil reached −1.5 MPa in about 7 d, and thereafter pots were kept at this water potential for another 5 d by replacing the amount of water lost every day. The third leaf (counting from the base) was used for all measurements. Photosynthesis measurements were made at about 650 p.p.m. CO_2 and 350 μmol m^{-2} s^{-1} PAR by infra-red gas exchange (IRGA; Licor 6200). Net CO_2 uptake measurements in rehydrated leaves were performed after 24 h of re-watering droughted plants.

Production of GalLDH antiserum

Databases of expressed sequence tags (ESTs) from monocotyledonous plants were BLAST-searched for sequences with homology to *Brassica oleracea* and *Ipomoea batatas* GalLDH (accession numbers Z97060, AB017357, respectively). Initially, wheat sequences with significant homology were not identified but a maize EST (accession number AI737066) with 73.7% nucleotide identity to *I. batatas* GalLDH was identified and obtained from ZmDB (Gai *et al.* 2000). Sequencing of the insert agreed with the published EST data, and indicated that the clone contained

a partial GalLDH cDNA, encoding the C-terminus of the protein, which is predicted to be soluble. An *Eco* RI/*Xho* I restriction fragment from AI737066 encoding the C-terminal 145 amino acids and 150 bp 3′-untranslated region of maize GalLDH was ligated into *Eco* RI/*Sal* I-digested pMAL-c2 (New England Biolabs, Hitchin, Herts, UK), to give plasmid pMBP-GalLDH. This construct was transformed into *Escherichia coli* (*E. coli*) strain XL1Blue (Stratagene, La Jolla, CA, USA), and maltose-binding protein/GalLDH fusion protein purified according to manufacturers' instructions, using amylose affinity chromatography. The fusion protein was dialysed into phosphate-buffered saline and concentrated to 2 mg mL^{-1} prior to immunization of rabbits. This work was carried out in accordance with the Animals (Scientific Procedures) Act 1986, Project Licence No. PPL 70/4356.

Sodium dodecyl sulphate-polyacryalamide gel electrophoresis and western blotting

Membrane proteins for GalLDH content determinations were extracted as previously described (Bartoli *et al.* 2000) and mixed with an equal volume of 2× sample buffer containing 125 mM Tris-HCl pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol and 10% (v/v) mercaptoethanol. Samples were loaded in 12% (w/v) acrylamide gels at 12.5 mV gel^{-1} for 2.5 h and proteins electro transferred at 70 V for 2 h. Blots were blocked with 5% (w/v) non-fat dried milk dissolved in PBS-T [8.0 mM potassium phosphate buffer pH 7.4, 150 mM NaCl, 0.02% (v/v) Tween-20] for 24 h. Blots were probed sequentially with anti-maize GalLDH antiserum (1 : 5000 in PBS-T) and goat anti-rabbit IgG-horseradish peroxidase conjugate (1 : 50 000 in PBS-T), prior to visualization using a chemiluminescence detection kit (Renaissance™; DuPont, Boston, MA, USA) and Kodak X-OMAT films according to manufacturers' instructions. Relative protein content (in arbitrary units) was determined by densitometry of western blots.

AA and DHA determinations

The content of reduced AA was measured as described by Foyer, Rowell & Walker (1983). DHA content was calculated as the difference between total and reduced AA. Total AA was measured after incubation of the sample in dithiothreitol (2.4 μM) for 15 min.

Preparation of leaf homogenates and enzyme assays

For measurements of MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1), APX (EC 1.11.1.11) and GR (EC 1.6.4.2) activities, leaves were ground in a medium containing 0.1 M bicine (pH 7.5), 1 mM ethylenediaminetetraacetic acid, 10% (v/v) glycerol, 4 mM cysteine, and protease inhibitors (25 μM phenylmethylsulfonyl fluoride and 2 μM leupeptin). For APX determinations, AA was added to homogenates to give a final concentration of 0.5 mM. Homogenates were filtered through a 20-μm mesh and centrifuged at 10 000 *g* for 10 min. Supernatants were used for the determinations of enzyme activities.

Extraction and measurement of GalLDH (EC 1.3.2.3) activity was carried out as described previously (Bartoli *et al.* 2000). MDHAR and DHAR were measured essentially as described by De Gara *et al.* (2000). For DHAR measurements the reaction mixture consisted of 50 mM phosphate buffer (pH 6.5), 0.2 mM DHA, 2.5 mM reduced glutathione, and 50–100 μg of leaf protein. APX and GR were measured as previously described (Bartoli *et al.* 1999).

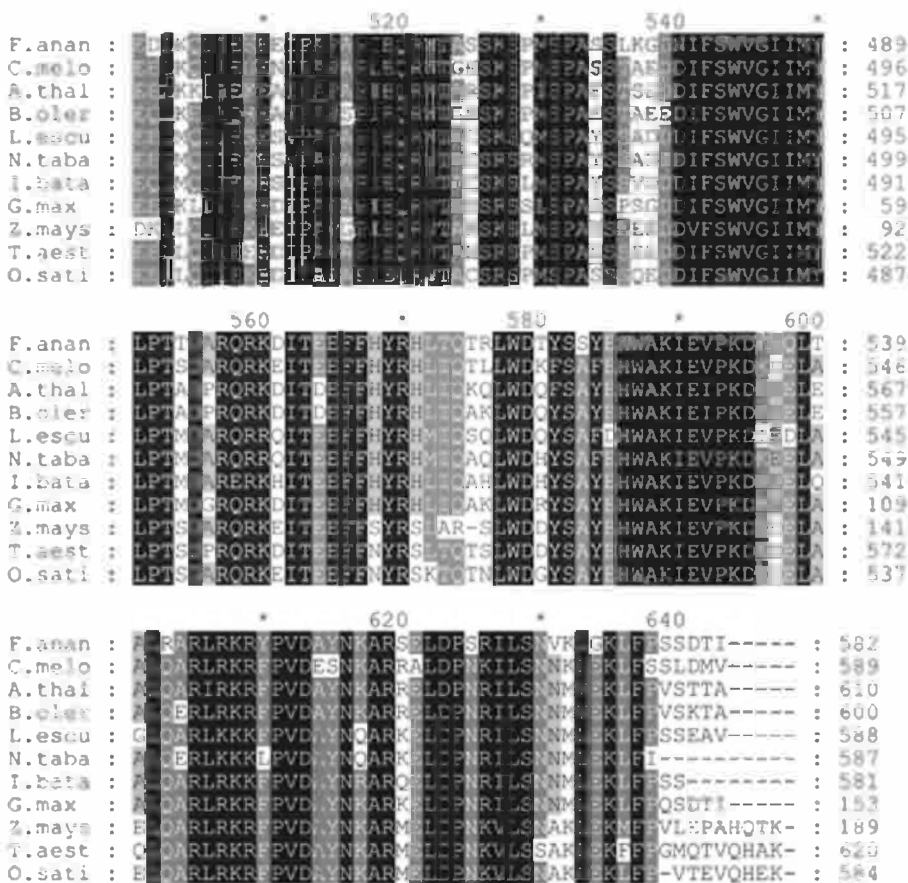
RESULTS AND DISCUSSION

Relationships between GalLDH protein, activity and AA content in the leaves of different plant species

Comparison of GalLDH peptide sequences from a range of plant species indicated that, with the exception of the putative mitochondrial transit peptide, GalLDH is highly conserved over the majority of the length of the protein. Conservation was particularly high in the C-terminal region (Fig. 1a). Given this high level of conservation, and since GalLDH is an integral membrane protein, the soluble C-terminal region of maize GalLDH was expressed in *E. coli* as a maltose binding protein fusion and used to raise a polyclonal antiserum. The antiserum recognized a single protein of *c.* 64 kDa in a range of plant species (Fig. 1b). This antiserum was also used to establish a relationship between GalLDH distribution and calcium oxalate crystal formation in a number of species (Kostman & Koscher 2003a,b), further demonstrating its specificity. An antiserum raised to sweet potato GalLDH (Ôba *et al.* 1995) that we have used previously in our studies of GalLDH in mitochondria (Bartoli *et al.* 2000), also recognized a protein of 64 kDa, lending support to the notion that this band represents GalLDH (data not shown). Since the peptide used to raise the antiserum is between 76 and 80% identical to GalLDH protein sequences from species represented on the blot, the intensity of the band in Fig. 1b is likely to give an approximate indication of the amount of GalLDH protein in different species. Assuming that the antiserum recognizes several conserved epitopes spanning the C-terminus, the abundance of GalLDH can be cautiously ranked as follows: maize > wheat > soybean > Arabidopsis > oat > sunflower. The relatively low protein content in oat suggests that the antiserum does not preferentially recognize monocot GalLDH.

While considerable variation in GalLDH activity and leaf AA content was observed in the different plant species, no clear relationship between these parameters could be determined (Fig. 2). Furthermore, neither GalLDH activity nor AA content correlated well with GalLDH protein, as estimated from Fig. 1b. These results demonstrate that, in the source leaves of plants grown under optimal growth conditions, GalLDH activity is not a major determinant of

(a)



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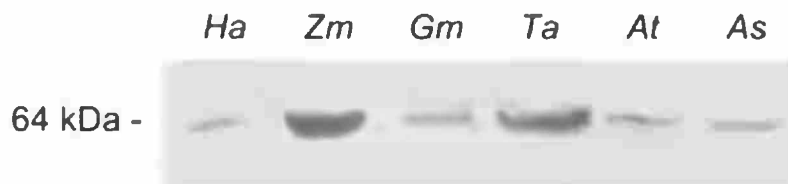

Figure 1. Comparison of GalLDH in diverse plant species. (a) GalLDH amino acid sequences were aligned using the GCG programme, LOCALPILEUP and the alignment was optimized using CLUSTALX and shaded using GENEDOC. Accession numbers used to create the alignment are: strawberry (*Fragaria ananassa*; F.anan) Q8L8A9; muskmelon (*Cucumis melo*; C.melo) Q9M4U9; *Arabidopsis thaliana* (A.thal) Q9SU56; cauliflower (*Brassica oleracea*; B.oler) O47881; tomato (*Lycopersicon esculentum*; L.escu) Q8LP11; tobacco (*Nicotiana tabacum*; N.tabac) Q9FXL9; sweet potato (*Ipomoea batatas*; I.bata) Q9ZWJ1; maize (*Zea mays*; Z.mays) AI737066; rice (*Oryza sativa*; O.sati) AK102697. For wheat (*Triticum aestivum*; T.aest) and soybean (*Glycine max*; G.max), expressed sequence tags with homology to GalLDH were assembled to produce contiguous sequences and the protein sequences derived by translation *in silico*. (b) Mitochondrial membrane proteins prepared as described in Bartoli *et al.* (2000) were extracted from leaves of: sunflower (*Helianthus annuus*; *Ha*), maize (*Zea mays*; *Zm*), soybean (*Glycine max*; *Gm*), wheat (*Triticum aestivum*; *Ta*), *Arabidopsis thaliana* (*At*) and oat (*Avena sativa*; *As*). Ten micrograms of each protein were separated in 12% (w/v) denaturing gels and transferred to nitrocellulose membranes. Membranes were probed with a polyclonal antiserum raised to maize GalLDH. No bands were detected when blots were probed with pre-immune serum (data not shown).

the extent of AA accumulation. This might have been predicted, since ascorbate pool homeostasis is determined by many factors such as degradation, transport and utilization in addition to synthesis (Smirnoff, Conklin & Loewus 2001). However, we have shown previously that developmental variations in leaf AA accumulation are related to maximal extractable leaf GalLDH activity within the plant, such that young leaves have the highest GalLDH activity and AA content (Bartoli *et al.* 2000). Similarly, this relationship appears to hold during pathogen attack (Kuźniak & Skłodowska 2004). To explore the relationship between leaf AA accumulation and GalLDH protein and activity further, we examined the natural variation in leaf AA content in a range of commercial South American wheat cultivars (Table 1). Of these, cultivar Buck Chambergo (BCH) and cv. Cooperativa Maipún (CM) had the highest and lowest leaf AA content, with values of 17.7 ± 1.8 and 7.66 ± 1.8 mol g^{-1} DW, respectively.

In order to determine whether there was diurnal variation in AA content and GalLDH activity in wheat leaves, these parameters were measured over the day–night cycle, in the European cv Cadenza, which is commonly used for transformation (Fig. 3). Wheat leaf AA content was found to be relatively constant over the day–night cycle (Fig. 3), as was maximal extractable GalLDH activity. A similar situation was found in potato leaves (Imai, Kingston-Smith & Foyer 1999), but this is in contrast to species such as *Arabidopsis* and tobacco which show a pronounced decrease in AA content at night. The reasons for interspecific differences are unknown but might involve variation

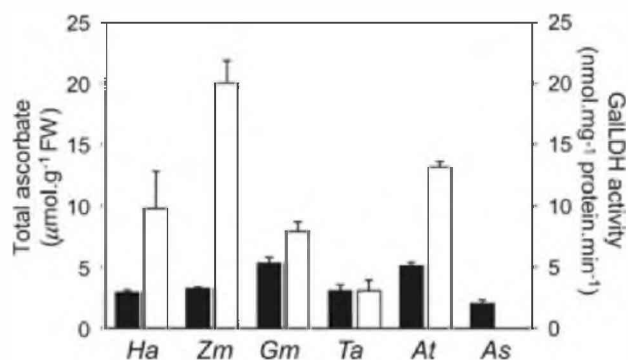

Figure 2. Relationship between total ascorbate and GalLDH activity and content in leaves of six plant species. Sunflower (*Helianthus annuus*; *Ha*), maize (*Zea mays*; *Zm*), soybean (*Glycine max*; *Gm*), wheat (*Triticum aestivum*; *Ta*), and oat (*Avena sativa*; *As*) were grown at 25 ± 2 °C, 350 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) and a photoperiod of 10 h. Samples were taken from the first expanded leaf 2 weeks after sowing. *Arabidopsis thaliana* (*At*) was grown at 15 ± 5 °C and 100 μmol m^{-2} s^{-1} PPFD, and all the green leaves from mature plants were used. Black bars: total ascorbate; open bars: GalLDH activity. The values are the mean ± standard error of three independent experiments. Note that GalLDH activity was not detectable in oat.

in synthesis and degradation between species, parameters which are also affected by compartmentation such as sequestration in the vacuole. The AA/DHA ratio was also constant over the day–night cycle in wheat (data not shown). In light of these findings, the relationship between AA content and GalLDH activity was explored under stress conditions. Water stress was chosen owing to its economic importance and the existing literature of responses of wheat leaf antioxidants to drought (Smirnoff & Colombé 1988; Menconi *et al.* 1995; Zhang & Kirkham 1996; Bartoli *et al.* 1999; Boo & Jung 1999; Tambussi *et al.* 2000).

Table 1. Leaf ascorbate (AA) content of 3-week-old-plants of 10 commercial South American wheat cultivars

Cultivar	AA content (μmol g^{-1} DW)
Cooperativa Maipún	7.66 ± 1.8
Buck Halcón	7.95 ± 1.2
Buck Arriero	8.80 ± 1.2
Buck Poncho	10.1 ± 2.3
Buck Panadero	10.2 ± 1.9
Buck Charrúa	10.6 ± 1.6
Klein Pegaso	11.5 ± 2.1
Buck Pronto	12.8 ± 1.4
ProINTA Isla Verde	14.6 ± 0.8
Buck Chambergo	17.7 ± 1.7

Plants were grown in the greenhouse with an average maximum/minimum temperature of 28/18 °C, 700 μmol m^{-2} s^{-1} PAR with a photoperiod of 14 h. The values are the mean ± standard error of three independent experiments.

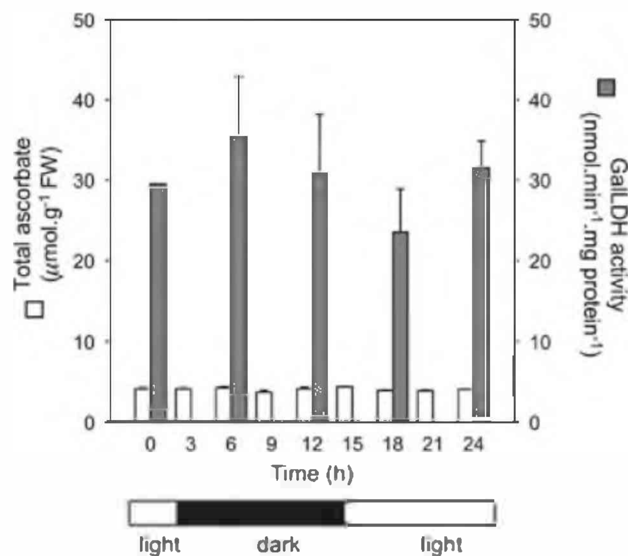

Figure 3. Ascorbate content and GalLDH activity during the day-night cycle in wheat. Wheat, *Triticum aestivum* L. cv. Cadenza was grown in soil for 2 weeks with a 16-h day length at 700 μmol m^{-2} s^{-1} PAR. Ascorbic acid (AA) and dehydroascorbate (DHA) were measured at 3 h intervals over a 24-h period. Open bars represent total AA. Reduced ascorbate accounted for 47–56% of the total ascorbate pool over the 24-h period. Maximal extractable GalLDH activity (grey bars) was measured at 6 h intervals. Values are means ± standard error of three measurements.

Drought-induced effects on ascorbate metabolism

The BCH and CM cultivars, which represented the extremes in the extent of AA content, were selected to study AA metabolism during the stress imposed by water deprivation. Stomatal conductance measurements of droughted plants revealed that the rate of water loss was similar in the two cultivars (Table 2). Moreover, the rates of photosynthetic CO_2 assimilation were decreased by a similar amount in the two cultivars under drought (Table 3). However, whereas BCH recovered to control rates of assimilation, photosynthesis in CM remained at a significantly lower value, when returned to water-replete conditions. We have recently reported that water stress increased oxidative damage to mitochondrial and peroxisomal proteins to a greater extent in CM than in BCH

Table 2. Effect of water deficit on stomatal conductance of two wheat cultivars with different AA content

	Leaf water conductance (mmol m^{-2} s^{-1})	
	Well-irrigated	Droughted
cv. Buck Chambergo	269 ± 18	67.4 ± 6.7
cv. Cooperativa Maipún	260 ± 20	75.2 ± 13.7

The values are the mean ± standard error of three independent experiments.

Table 3. Net CO_2 uptake displayed by leaves of two wheat cultivars under different watering regimes

	Net CO_2 uptake (μmol cm^{-2} s^{-1})		
	Control	Droughted	Rehydrated
Buck Chambergo	15.7 ± 0.3	6.9 ± 0.6a	14.4 ± 0.7
Cooperativa Maipún	15.5 ± 0.3	6.5 ± 0.7a	11.5 ± 0.9 b

Values are the means ± SE of four independent experiments. a indicates means statistically different between stressed and control plants. b indicates means statistically different from control and droughted treatments (ANOVA, $P \leq 0{,}05$).

(Bartoli *et al.* 2004). Taken together, these data indicate that cv CM incurs more damage during drought than BCH.

In water-replete plants, cv BCH had almost twice as much GalLDH activity and protein as cv CM (Fig. 4). Whereas drought treatment did not affect GalLDH protein or activity significantly in cv BCH, it had a marked effect on these parameters in cv CM (Fig. 4). Although drought increased the amount of GalLDH protein and activity to values similar to those observed in water-replete cv BCH, it failed to have any impact on leaf AA content (Fig. 5a). Indeed, the AA content of droughted CM leaves was still less than half the AA content of cv BCH. These results indicate that the capacity to accumulate AA in optimal and stress conditions is much lower in cv CM than cv BCH and that this is governed by factors other than the maximal extractable activity of GalLDH.

Although leaves of cv BCH leaves had twice as much AA as those of cv CM (Fig. 5a), the DHA content was similar (Fig. 5b), such that the ratio of reduced to oxidized AA was higher in cv BCH leaves. This suggests that, in addition to a higher capacity for AA synthesis, cv BCH is more competent at AA regeneration than cv CM. In agreement with this, DHAR and GR activities were higher in cv BCH than in cv CM, although MDHAR activity was similar in both cultivars (Table 4). APX activities were also higher in BCH leaves of well-watered plants (Table 4). This result confirms previous observations that the amount of AA is determined in part by the operation of regeneration systems, as demonstrated by increases in AA following the over-expression of regenerating enzymes in transgenic plants (Foyer *et al.* 1995; Chen *et al.* 2003). However, leaf DHAR, MDHAR, GR and APX activities were unchanged by drought (Table 4), suggesting that the capacity for regeneration was also unchanged by this treatment.

CONCLUSIONS

As there is very little information concerning the regulation of GalLDH protein and activity in the literature, this study set out to investigate the relationship between these parameters and AA accumulation in leaves. As a first step, a specific polyclonal antiserum was generated, which recognized GalLDH in a range of plant species. This antiserum was used subsequently to quantify GalLDH protein in wheat leaves under optimal and stress conditions. GalLDH activity and AA were also measured. Two important conclusions arose from these experiments: first, there is no

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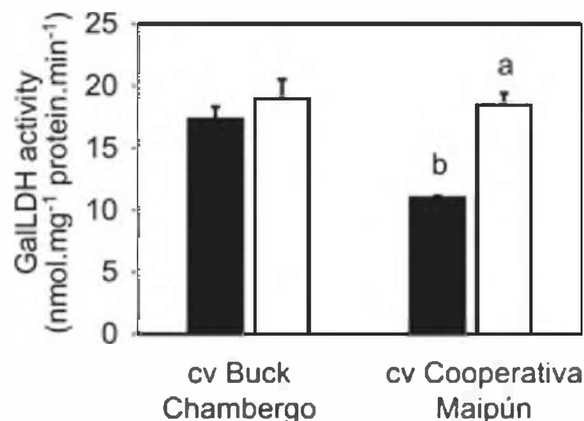

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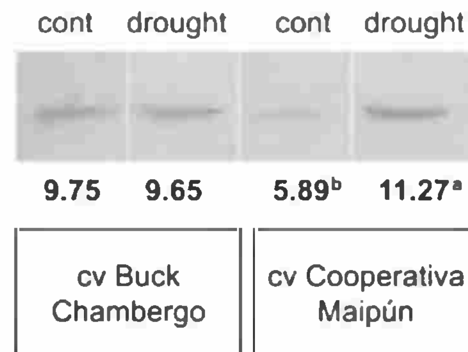

Figure 4. Effect of drought stress on GalLDH activity and protein in leaves of two wheat cultivars which differ in ascorbate content. (a) Maximal extractable L-galactono-1,4-lactone dehydrogenase (GalLDH) activity. Black bars: well-irrigated plants; open bars: droughted plants. The values are the mean ± standard error of four independent experiments. (b) Leaf GalLDH content under well-irrigated (cont.) and droughted conditions. Samples were prepared and subjected to western analysis as described in the legend to Fig. 1. Relative protein level (arbitrary units) was determined by densitometry of western blots; the values are the mean of three independent experiments. For a given cultivar, a indicates significant differences between well-irrigated and droughted plants; b indicates significant differences between cultivars with the same treatment (ANOVA, $P \leq 0.05$). Drought provoked a pronounced decrease in leaf water conductance, such that the leaf water potential fell to values between −1.65 and −2.1 MPa in both cultivars when plants were deprived of water (data not shown). This observation indicates that both cultivars experienced a similar level of drought.

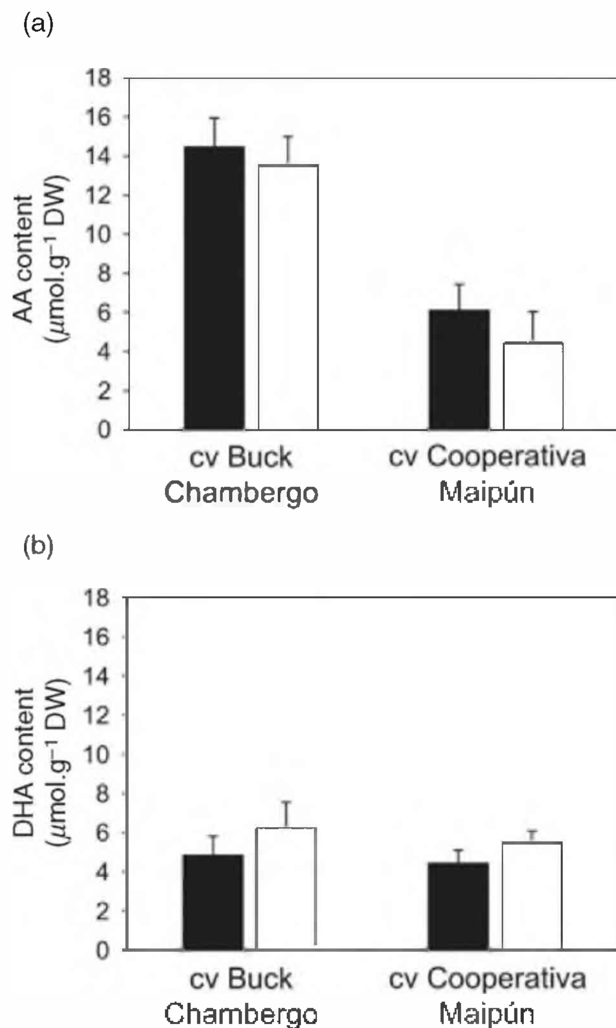

Figure 5. Effect of drought stress on AA and DHA content in wheat leaves. AA content (a) and DHA content (b) under well-irrigated (black bars) and droughted conditions (open bars). Error bars represent the standard error of the mean.

relationship between GalLDH protein and maximum extractable activity in wheat leaves or other species. Second, we found no evidence that either of these parameters are related to the level of AA in leaves.

With respect to the first conclusion, an explanation based on post-translational regulation can be proposed. GalLDH has at least two redox-active components which could be subject to regulation: the maximum extractable activity could reflect the activation state of GalLDH at the time of extraction and therefore would not necessarily be proportional to the amount of protein. It has been shown that GalLDH is inhibited by the thiol-reactive reagents *p*-chloromercuribenzoate, *N*-ethylmaleimide and monoiodoacetic acid (Mapson & Breslow 1958; Ôba *et al.* 1995; Østergaard *et al.* 1997) indicating that it requires a sulfhydryl group(s) for its activity. Multiple sequence alignments show the presence of five conserved cysteine residues in GalLDH, two of which are also conserved in gulonolactone oxidase (data not shown). These residues could be involved in catalysis, disulphide bonding or other forms of redox regulation. Furthermore, GalLDH has been suggested to contain a non-covalently bound flavin prosthetic group, based on the effect of flavoenzyme inhibitors (Mapson & Breslow 1958; Ôba *et al.* 1995), spectral properties of the purified enzyme (Imai *et al.* 1998) and the identification of a putative FAD binding site in the protein sequences of GalLDH from different species. Redox regulation could therefore be mediated by the redox state of FAD, via effectors other than the substrates. This regulation could affect the tightness of the association between FAD and the GalLDH protein. Although the same isolation procedure was used for each species, it is possible that differential extraction of FAD and GalLDH protein occurred, which again could influence the enzyme activity. Interestingly, although cauliflower GalLDH (accession number: O47881) contains a FAD binding motif, Østergaard *et al.* (1997) observed neither acriflavine inhibition nor a flavin absorption spectrum for pure cauliflower GalLDH, in contrast to the data for sweet potato (Ôba *et al.* 1995; Imai *et al.* 1998). The differing K_m values reported for sweet potato and cauliflower GalLDH strongly suggest that the presence of FAD substantially increases the affinity of the enzyme for its substrate (Ôba *et al.* 1995; Østergaard *et al.* 1997). However, further experimentation is necessary to support this hypothesis.

With respect to the second conclusion, the fact that GalLDH protein can be induced by drought in a cultivar-specific manner, without affecting AA content in wheat

Table 4. Activities of enzymes involved in ascorbic acid metabolism in the leaves of two wheat cultivars differing in their ascorbate content

		Enzyme activities (nmol mg prot^{-1} min^{-1})			
		MDHAR	DHAR	GR	APX
cv. Buck Chambergo	Well-irrigated	48.5 ± 3	121 ± 6	67.3 ± 4	729 ± 51
	Droughted	42.2 ± 4	116 ± 10	66.6 ± 12	566 ± 64
cv. Cooperativa Maipún	Well-irrigated	41.5 ± 3	77 ± 12[b]	49.0 ± 3[b]	544 ± 40[b]
	Droughted	46.0 ± 4	50 ± 22[b]	61.2 ± 4	552 ± 47

MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; APX, ascorbate peroxidase. The values are mean ± standard error of five independent experiments. b indicates significant differences between cultivars with the same treatment (ANOVA, $P \leq 0.05$).

illustrates the importance of AA regeneration as well as enzyme regulation. The cultivar with the higher total AA also had the highest level of DHAR activity, which no doubt contributes to its higher maintenance of AA, in agreement with a study in which maize and tobacco plants constitutively expressing DHAR had higher levels of leaf AA (Chen *et al*. 2003). We cannot rule out the possibility that cv CM synthesizes AA at a higher rate due to increased GalLDH under drought stress, but that the oxidized AA is degraded faster in this cultivar due to lower DHAR compared to BCH, such that the net result is no change to the AA pool compared to control conditions. In contrast to this, Chen & Gallie (2004) argued that suppressing DHAR in tobacco conferred increased drought tolerance, because tobacco guard cells with an increase in AA redox state were less responsive to H_2O_2 or abscisic acid signalling. Plants with greater AA and DHAR exhibited higher water loss under drought conditions. However, our AA measurements and stomatal conductance data suggest that this is not the case in wheat (Table 3 and Fig. 5). Clearly, much remains to be resolved regarding AA homeostasis in different plant species. The conclusions from the present study, together with our report on Arabidopsis (Millar *et al*. 2003) lay a foundation for future work on GalLDH regulation and illustrate the importance of inter-pathway redox regulation, where the mitochondrial electron transport chain and/or associated redox regulators control the synthesis of AA.

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