Journal of Experimental Botany, Vol. 58, No. 11, pp. 3037–3046, 2007
doi:10.1093/jxb/erm161
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Amino acid pattern and glutamate metabolism during dehydration stress in the 'resurrection' plant *Sporobolus stapfianus*: a comparison between desiccation-sensitive and desiccation-tolerant leaves

Tommaso Martinelli[1,*], Anne Whittaker[1], Adriana Bochicchio[1], Concetta Vazzana[1], Akira Suzuki[2] and Céline Masclaux-Daubresse[2]

[1] *Dipartimento di Scienze Agronomiche e Gestione del Territorio Agroforestale, Facoltà di Agraria, Università degli Studi di Firenze, I-50144 Firenze, Italia*

[2] *Unité de Nutrition Azotée des Plantes, INRA, Route de Saint Cyr, F-78000 Versailles, France*

Received 26 February 2007; Revised 4 June 2007; Accepted 12 June 2007

Abstract

The present study analyses changes in nitrogen compounds, amino acid composition, and glutamate metabolism in the resurrection plant *Sporobolus stapfianus* during dehydration stress. Results showed that older leaves (OL) were desiccation-sensitive whereas younger leaves (YL) were desiccation-tolerant. OL lost their soluble protein more rapidly, and to a larger extent than YL. Enzymes of primary nitrogen assimilation were affected by desiccation and the decrease in the glutamine synthetase (GS, EC 6.3.1.2) and ferredoxin-dependent GOGAT (Fd-GOGAT, EC 1.4.7.1) activities was higher in OL than in YL, thus suggesting higher sensibility to dehydration. Moreover, YL showed higher total GS enzyme activity at the end of the dehydration stress and was shown to maintain high chloroplastic GS protein content during the entire stress period. Free amino acid content increased in both YL and OL between 88% and 6% relative water content. Interestingly, OL and YL did not accumulate the same amino acids. OL accumulated large amounts of proline and γ-aminobutyrate whereas YL preferentially accumulated asparagine and arginine. It is concluded (i) that modifications in the nitrogen and amino acid metabolism during dehydration stress were different depending on leaf development and (ii) that proline and γ-aminobutyrate accumulation in *S. stapfianus* leaves were not essential for the acquisition of desiccation tolerance. On the contrary, the accumulation of large amounts of asparagine and arginine in the YL during dehydration could be important and serve as essential nitrogen and carbon reservoirs useful during rehydration. In this context, the role of GS for asparagine accumulation in YL is discussed.

Key words: Arginine, asparagine, GABA, glutamine synthetase, GOGAT, proline.

Introduction

Angiosperms with mature foliage which survive desiccation and, upon rehydration, completely recover their physiological functions are known as 'resurrection' plants (Gaff, 1971). In angiosperms, desiccation tolerance is not constitutional and is acquired during water stress. The acquisition of desiccation tolerance is the result of morphological and physiological responses to water stress (Oliver *et al*., 1998). Even though the metabolic adaptations that come into play during dehydration stress are diverse, the accumulation of late embryogenesis abundant proteins and carbohydrates are common traits of taxonomically distant resurrection plants and have been hypothesized to be pivotal factors for

* To whom correspondence should be addressed. E-mail: tommaso.martinelli@unifi.it

Abbreviations: Chl, chlorophyll; Fd-GOGAT, ferredoxin-dependent glutamate synthase; GABA, γ-aminobutyrate; GDH, glutamate dehydrogenase; GS/GOGAT cycle, glutamine synthetase/glutamate synthase cycle; GS1, glutamine synthetase cytosolic isoform; GS2, glutamine synthetase chloroplastic isoform; NADH-GOGAT, NADH-dependent glutamate synthase; OL, older leaves; ROS, reactive oxygen species; *RWC*, relative water content; YL, younger leaves.

the acquisition of desiccation tolerance (Bewley and Oliver, 1992; Ingram and Bartels, 1996; Bernacchia and Furini, 2004; Rascio and La Rocca, 2005).

In plants, stress conditions are generally correlated with enhanced proteolytic activity (Davis, 1982) and increased protein turnover, leading to either acclimation to the stress condition or to senescence and subsequent cell death (Brouquisse *et al*., 2001). Resurrection plants are able to synthesize new proteins by actively modifying their metabolism even at very low Relative Water Contents (*RWC*; Kuang *et al*., 1995). Soluble and insoluble protein breakdown and amino acid accumulation during dehydration are features of many resurrection plants (Tymms and Gaff, 1978; Gaff and McGregor, 1979). Amino acid accumulation (especially proline) has been widely suggested to be directly involved as a protective mechanism in many water-stressed plants (Hare and Cress, 1997; Rhodes *et al*., 1999), but up until now the pattern of amino acid accumulation and how amino acid accumulation is regulated during dehydration stress in resurrection plants is not known.

In the leaf, amino acid pools may be affected by many factors: (i) nitrogen assimilation into amino acids (through the glutamine synthetase/glutamate synthase cycle, GS/GOGAT cycle), (ii) amino acid interconversions (through aminotransferase reactions), (iii) active protein biosynthesis/degradation, (iv) amino acid transport, and (v) catabolism or utilization of amino acids for the biosynthesis of other metabolites (Noctor *et al*., 2002).

The amino acid glutamate is known to have a central role in nitrogen metabolism. Glutamate is both the product of ammonia assimilation through the GS/GOGAT cycle (Hirel and Lea, 2001) and the preferential amino-donor for the different aminotransferase reactions for subsequent amino acid interconversions (Lea and Ireland, 1999). Glutamine synthetase (GS, EC 6.3.1.2) is present in leaves in two isoenzymic forms (GS1 and GS2) and catalyses the biosynthesis of glutamine utilizing ammonia and glutamate as substrates. GS2, together with the ferredoxin-dependent isoform of glutamate synthase (Fd-GOGAT, EC 1.4.7.1), is reported to be responsible for the chloroplastic GS/GOGAT cycle. On the other hand GS1 is considered to be operational along with the NADH-dependent isoform of GOGAT (NADH-GOGAT, EC 1.4.1.14) in the cytosol. Glutamate dehydrogenase (GDH, EC 1.4.1.2) is also directly involved in glutamate metabolism and its *in vivo* role is still under debate (Lea and Ireland, 1999; Masclaux-Daubresse *et al*., 2006; Skopelitis *et al*., 2006). Despite the central importance of the amino acid glutamate, its metabolism has never been studied in resurrection plants during dehydration stress.

Resurrection plants provide a unique model system to understand the involvement of amino acids in the protection mechanism to extreme water stress. In the resurrection plant *Sporobolus stapfianus* Gandoger (Poaceae) the majority of older leaves (OL) do not acquire desiccation tolerance during dehydration stress (to be reported in the present study). Therefore, using *S. stapfianus* as a model plant, it is possible to compare the response to desiccation stress in genetically identical desiccation-sensitive and desiccation-tolerant leaf material. The present research aims at studying: (i) the pattern of amino acid accumulation and (ii) the enzymes central to glutamate metabolism (namely GS1, GS2, Fd-GOGAT, NADH-GOGAT, and GDH) in desiccation-sensitive (older leaves, OL) and desiccation-tolerant leaves (younger leaves, YL) of *S. stapfianus* during dehydration stress.

Materials and methods

Plant material, growth conditions, leaf viability, and RWC *determinations*

Two-year-old plants of *S. stapfianus* were divided into single tillers and vegetatively propagated in three pots (60×40×15 cm, eight plants per pot) containing soil composed of 40% commercial potting mix, 35% sand, and 25% leaf mould. Plants were grown in a greenhouse for four months. Dehydration stress was imposed in the greenhouse by withholding water for 12 d. Over the experimental period, plants were exposed to summer sunlight (maximum photon flux density 900 μmol m^{-2} s^{-1}; 14 h photoperiod), maximum and minimum temperatures were on average 40 °C and 20 °C, respectively; relative air humidity during the day cycle ranged between 25% and 70%. Sampling was performed at four dehydration stages. Once the leaves started to show stress (i.e leaf rolling), sampling was performed daily. Mature leaves were harvested at 16.00 h from different plants in different pots. Only a portion of the leaf blade (8–10 cm) from the base (excluding the leaf sheath) was used for the analyses. Immediately after sampling, the leaves were frozen in liquid nitrogen and stored at –80 °C until analysis.

In *S. stapfianus*, each tiller is usually composed of four fully expanded leaves. Within each tiller, the innermost leaf is the youngest and the outermost is the oldest. During the sampling, OL and YL were collected separately. The two inner leaves (i.e. YL) and the two outer leaves (i.e. OL) were separated and grouped together. At each sampling point YL and OL from the different tillers were pooled together. Younger leaves were excluded from the sampling if not completely expanded. During the dehydration stress some of the OL senesce and turn yellow. The senescent leaves were easily recognizable even at low *RWC* and were not included in the sampling. At the end of the dehydration stress, after 48 h rehydration, leaf viability was visually evaluated using 60 tillers. Leaves were considered non-desiccation-tolerant if not able to recover turgor and Chl content.

The *RWC* was calculated according to the formula: *RWC*=(fresh weight–dry weight)/(full turgor weight–dry weight). At each sampling point during dehydration stress, four leaf samples were utilized for the determination of leaf *RWC*. Full turgor weight of the leaves was determined following a 24 h incubation in the dark, in flasks containing distilled water. Dry weight was determined after oven drying at 80 °C for 2 d.

Total nitrogen content

Frozen leaf tissue was ground and oven-dried for 2 d at 80 °C. The total nitrogen content was then measured with the Flash E1112

Automatic Elemental Analyser according to the instructions of the manufacturer (CE instruments, Wigan, UK).

Chlorophyll content

Frozen leaf tissues were ground and chlorophyll extracted in 80% acetone at 4 °C overnight. After centrifugation Chl concentration was determined according to Wellburn (1994).

Amino acid and ammonium analysis

Amino acids were determined after extraction of frozen ground leaf tissue in a 2% (w/v) solution of 5-sulphosalicylic acid (30 mg DW ml^{-1}). The composition of individual amino acids was determined by ion-exchange chromatography using the Amino Tac JLC-500/V amino acid analyser (ninhydrin coloration) according to the instructions of the manufacturer (JEOL (Europe) Croissy sur Seine, France). Total amino acid content was calculated by summing the 20 protein amino acids.

Ammonium was determined by the phenol hypochlorite colorimetric method (Berthelot reaction) using ammonium sulphate as a reference (Russell, 1944).

Enzyme assays

The entire extraction procedure was performed at 4 °C using frozen (–80 °C) leaf tissues. Fd- and NADH-GOGAT were extracted in 25 mM phosphate buffer (pH 7.5), 14 mM β-mercaptoethanol, 1 mM dithiothreitol, and 5 mM EDTA. Cold extraction buffer was added to ground leaf tissue in the proportion of 80 mg DW ml^{-1}. Enzymatic activity was measured according to Suzuki *et al*. (1994), using either methyl viologen or NADH as an electron donor.

For GS and GDH assays, frozen, and ground leaf material was extracted in a solution containing 100 mM TRIS buffer (pH 8), 1 mM dithiothreitol, 1 mM EDTA, 0.05% (v/v) Triton X-100, and 5% (v/v) glycerol. Crude extracts were desalted using 2.5 ml Sephadex G-25 columns equilibrated with extraction buffer. GS enzymatic activity was measured spectrophotometrically (Lambda 20 from Perkin Elmer) following the oxidation of NADH according to Lea *et al*. (1990). GDH aminating and deaminating activity was measured as described by Turano *et al*. (1996).

Soluble proteins were measured according to Bradford (1976) using bovine serum albumin as a standard.

Gel electrophoresis and protein blot analysis

Proteins were extracted as previously described for Fd- and NADH-GOGAT. Proteins were separated by SDS-PAGE (Laemmli, 1970). Equal amounts of protein (10 μg) were added to each lane. As a standard (ST) 10 μg protein from a mixture of different leaves (dry and fresh leaves) of *S. stapfianus* was added to the last lane of each gel. The ST was necessary to make possible the comparison between different gels. The percentage of polyacrylamide in the running gel was 8% for both the GS and Fd-GOGAT gels, respectively. Denatured proteins were electrophoretically transferred to nitrocellulose membranes. Polypeptide detection was performed using polyclonal antiserum raised against chloroplastic GS of tobacco (Becker *et al*., 1992) and Fd-GOGAT (Suzuki *et al*., 1994). After incubation with the secondary antibody, conjugated with peroxidase, immunodetection was performed. The analysis was performed twice with similar results.

Statistical analysis

Differences between two separates means were analysed by the ANOVA test. Values expressed as a percentage were transformed (arcsine $\sqrt{}$percentage) before the statistical analysis.

Results

Leaf viability

To evaluate if the acquisition of desiccation tolerance is correlated with leaf ageing, the leaves of different ages within each tiller were scored based on the ability to recover from the dehydrated status (Fig. 1). The first two inner, YL (leaves in position number one and two), sampled from 4-month-old plants (see Materials and methods), were able to recover completely from the dehydrated status and, taken together, they showed almost 100% viability after 48 h rehydration. In order to obtain completely desiccation-tolerant leaf material, leaves numbers one and two were sampled together (YL) during dehydration stress. OL (i.e. leaves in position numbers three and four) showed a lower capability to recover from the dehydrated state and, upon rewatering, only 60% of the leaves in position three and 9% of the leaves in position four were viable (Fig. 1), resulting, on average, in a reduced desiccation tolerance in OL (*c*. 35% viable leaves). Additional experiments were performed on younger plants to investigate the relationship between leaf age and leaf viability during desiccation stress. Dehydration of 2-month-old plants showed that, in this case, both YL and OL were able to rehydrate upon rewatering, thus showing that leaf viability depends on leaf age (data not shown).

Total soluble protein, free amino acid, nitrogen, and chlorophyll contents

At the beginning of water stress, total soluble protein content, total free amino acid content, and total nitrogen content were not different in YL and in OL (Table 1). The

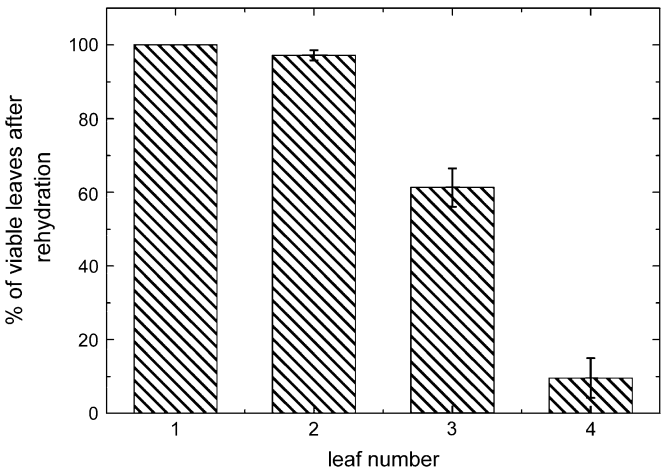

Fig. 1. Relationship between leaf age and 'resurrection' capacity. At the end of dehydration stress plants were rewatered and after 48 h rehydration leaf viability was visually evaluated. Leaves were considered non-desiccation-tolerant if not able to recover turgor and Chl content. Three groups of 20 tillers each have been analysed. Each column is the mean of the three groups ±SE.

Table 1. *Changes in chlorophyll* a *and chlorophyll* b *contents, total nitrogen, soluble protein, total amino acid, and ammonia contents in YL and OL during desiccation stress.*

The two inner leaves of each tiller have been classified as younger leaves (YL) and the outer leaves of each tiller as older leaves (OL), respectively. Small letters near the results indicate significant ($P \leqslant 0.05$) differences within columns; capital letters indicate significant differences between columns. Each point is the mean of three samples ±SE; n.a., not analysed.

RWC (%)	Chl a[a]		Chl b[b]		Total nitrogen[c]		Sol proteins[a]		Amino acids[b]		Ammonia[b]	
	YL	OL	YL	OL	YL	OL	YL	OL	YL	OL	YL	OL
97	3.5±0.1 aA	5.4±0.5 aB	1.9±0.1 aA	2.8±0.3 aB	2.0±0.2 a	1.90±0.3 a	33.0±2.2 a	36.0±4.2 a	73.5±8.9 a	64.4±10.7 a	1.4±0.6	1.1±0.1 a
88	2.3±0.2 b	3.1±0.2 b	1.4±0.1 b	1.7±0.1 b	n.a.	n.a.	26.0±0.9 bA	17.8±2.3 bB	37.3±7.2 b	33.9±6.4 b	1.0±0.1 a	1.5±0.1 a
56	2.1±0.0 b	2.3±0.5	1.2±0.0 b	1.3±0.2	1.6±0.0	1.40±0.2	30.2±1.2 b	21.7±2.1 b	53.5±5.8	55.3±4.6	0.8±0.2 a	0.4±0.3 a
30	1.4±0.0 c	1.6±0.1 c	0.9±0.0 c	1.1±0.1 c	n.a.	n.a.	30.4±2.4 bA	20.1±0.7 bB	54.8±8.4	65.9±6.3	0.8±0.4 a	0.8±0.3 a
6	1.6±0.1 c	2.1±0.2 c	1.0±0.1 b	1.5±0.2	1.5±0.1 b	1.3±0.1 b	32.4±1.0 a	26.5±2.0 a	101.9±18.4 a	101.5±15.2 a	2.6±0.7 b	3.3±0.6 b

[a] mg g^{-1} DW.
[b] µmol g^{-1} DW.
[c] % of DW.

Chl *a* and Chl *b* contents were higher in OL than in YL. Between 97% and 88% *RWC*, there was a *c*. 30% and 40% decrease in total Chl content (Chl *a*+*b*) was evident for YL and OL, respectively. However, in both YL and OL, dehydration stress caused a decrease in Chl content over the stress, finally resulting in *c*. 50% loss. In both YL and OL, the total nitrogen content decreased (*c*. 25%) during dehydration stress. A large decrease in the total soluble protein content was observed in OL and YL between 97% *RWC* and 88% *RWC*. This decrease was higher in OL than in YL (*c*. 50% versus 20%, respectively). Thereafter, the decrease was reversed in the remaining steps of dehydration, in both YL and OL. The total free amino acid contents changed in a similar manner in OL and YL and in the first step of the desiccation stress (from 97% to 88% *RWC*) decreased by *c*. 50%. Thereafter, YL and OL accumulated free amino acids during dehydration stress. Ammonium content, detected in *S. stapfianus* leaves, were very low and not different between YL and OL. However, an increase was detected in the last step (6% *RWC*) of dehydration in both YL and OL (Table 1).

Changes in glutamate metabolism enzyme activities during dehydration

Since a decrease in total nitrogen content was observed during dehydration in *S. stapfianus* whereas free amino acids and ammonia increased, the changes in the activities of the enzymes involved in nitrogen metabolism were analysed to investigate a potential switch from primary nitrogen assimilation to nitrogen recycling and mobilization.

The total GS activity (cytosolic GS1 activity plus chloroplastic GS2 activity) was similar in unstressed YL and OL (Fig. 2A). In the first step of dehydration, GS activity decreased in both YL and OL until 30% *RWC*. Afterwards, GS activity increased significantly in both YL and OL and was significantly higher in YL than in OL at 30% and 6% *RWC*. The Fd-GOGAT and NADH-GOGAT activities were similar in unstressed YL and OL (97% *RWC*). When dehydration stress started, Fd-GOGAT activity decreased in OL, but was maintained in YL until 56% *RWC* (Fig. 2B). In dehydrated leaves, the Fd-GOGAT activity was reduced to about 50%. NADH-GOGAT activity decreased earlier and to a greater extent than Fd-GOGAT in both OL and YL (Fig. 2C). However, Fd-GOGAT activity seemed to remain higher in YL than OL, whereas at 56% *RWC* NADH-GOGAT activity was less affected by dehydration in OL than in YL. It must be noted that the NADH-GOGAT activity was high in *S. stapfianus* compared with other plant species, and in the same range as Fd-GOGAT. The aminating and deaminating GDH activities were measured *in vitro* (Fig. 3). Both NADH-dependent (Fig. 3A) and NAD^+-dependent (Fig. 3B) GDH displayed higher activity in YL than in OL.

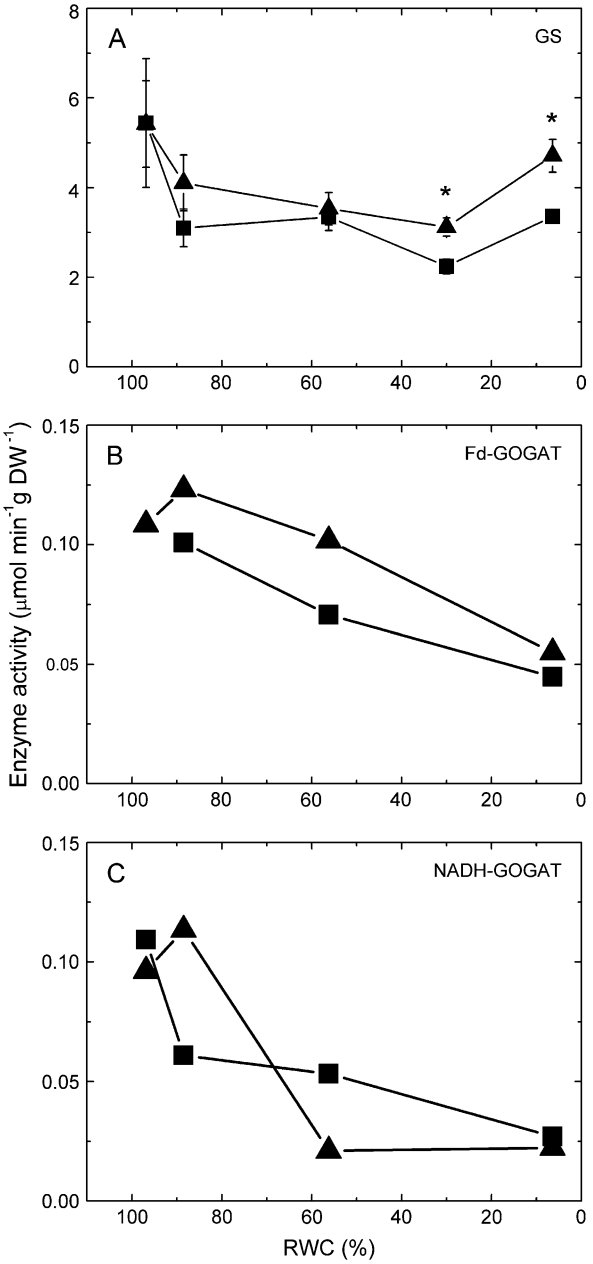

Fig. 2. GS/GOGAT cycle enzyme activity during desiccation stress in YL (black triangles) and OL (black squares). (A) Total GS activity. Each point is the mean of three samples ±SE. Error bars are shown when larger than symbols. Asterisks indicate significant ($P \leqslant 0.05$) differences between OL and YL. (B, C) Fd- and NADH-GOGAT activity during desiccation stress; each value shows the activity of three pooled samples.

Changes in enzyme protein contents during dehydration

Fd-GOGAT protein content was investigated by western blots using polyclonal antibodies raised against tobacco Fd-GOGAT (Suzuki *et al.*, 1994). Immunolocalization allowed a single 133 kDa protein to be detected, that migrated at exactly the same level as the Fd-GOGAT signal detected on the *Arabidopsis* control that was used as a standard for comparison (data not shown). Results,

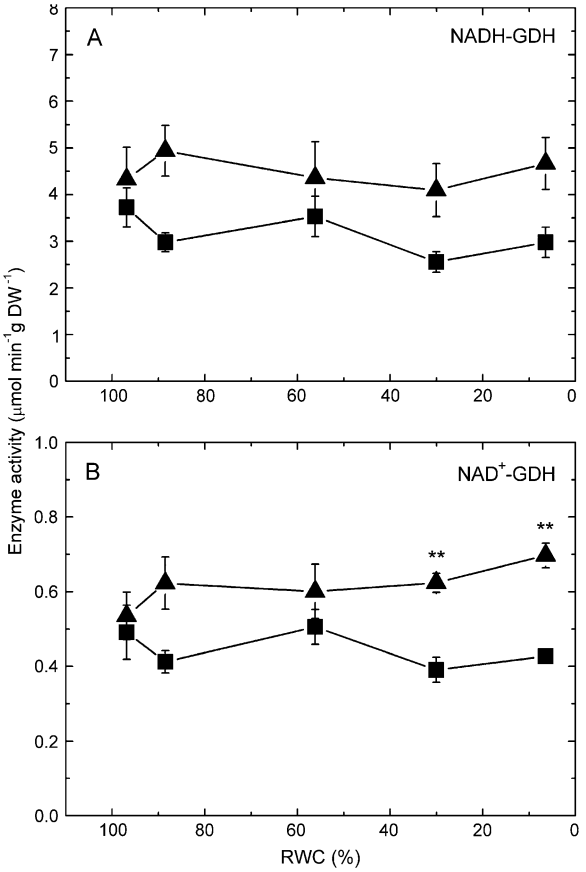

Fig. 3. GDH activity during desiccation stress in YL (black triangles) and OL (black squares). (A) NADH-dependent GDH activity (aminating activity). (B) NAD^+-dependent GDH activity (deaminating activity). Each point is the mean of three samples ±SE. Error bars are shown when larger than symbols. Asterisks indicate highly significant ($P \leqslant 0.01$) differences between OL and YL.

were consistent with the measured enzymatic activity (Fig. 4), and showed that Fd-GOGAT protein content diminished over the entire dehydration stress period in OL, but was maintained at high levels in YL until 56% *RWC*. At 6% *RWC*, Fd-GOGAT protein content was very low in both YL and OL.

To compare the changes in the cytosolic GS1 and chloroplastic GS2 contents, western blots were also carried out using polyclonal antibodies raised against the tobacco GS2 protein (Becker *et al.*, 1992). Dehydration stress led to a decrease in the GS2 protein content in OL. By contrast, in YL, GS2 protein content was maintained at the level of unstressed leaves during the desiccation stress (Fig. 4). This finding could explain why OL showed lower total GS activity at the end of desiccation stress than YL (Fig. 2A). In YL (at 56% *RWC*) GS protein content is similar to the level measured in unstressed leaves (Fig. 4). By contrast, a large decline in total GS activity was measured between 97% and 56% *RWC* (Fig. 2A). No difference in the GS1 content could be detected in the course of dehydration for both YL and OL.

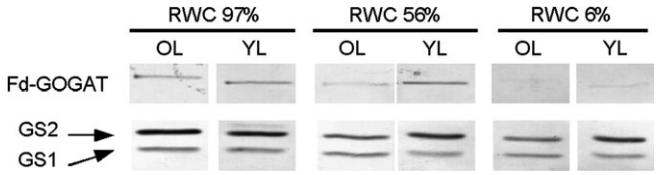

Fig. 4. Comparison of Fd-GOGAT, GS2, and GS1 protein contents in OL and YL at 97% *RWC*, 56% *RWC*, and 6% *RWC* dehydration steps. Singles bands for Fd-GOGAT, GS1, and GS2 proteins were detected after western blotting and using polyclonal antibodies raised against tobacco Fd-GOGAT and GS2. An equal amount of protein (10 μg) was loaded per lane.

Individual amino acid contents

Glutamate (Glu), glutamine (Gln), aspartate (Asp), asparagine (Asn), and alanine (Ala) together constituted *c*. 80% of the total free amino acid pool found in both unstressed OL and YL (data not shown). Three main groups of amino acids could be roughly defined: (i) amino acids with similar levels in YL and OL and unchanged during dehydration, (ii) amino acids with similar levels in YL and OL and changed during dehydration, and (iii) amino acids with dissimilar levels in YL and OL and changed during dehydration.

The only amino acid, which did not change significantly during dehydration in both YL and OL was Gln (data not shown). Gln was surprisingly low in stressed and unstressed YL and OL of *S. stapfianus* (*c*. 2%).

The curves for leucine (Leu), isoleucine (Ile), tyrosine (Tyr), tryptophan (Trp), phenylalanine (Phe), and Glu were similar in YL and OL and showed a bell shape trend (Fig. 5). Between 97% and 56% *RWC*, these amino acids increased in both YL and OL. Afterwards, between 56% and 30% *RWC*, their amount remained stable then decreased from 30% to 6% *RWC*. In the last part of the dehydration stress, the decrease of Leu, Ile, Tyr, and Trp was more pronounced in YL (Fig. 5A, B, C, D). Phe and Glu amounts did not significantly differ between YL and OL (Fig. 5E, F) except for Phe content that was higher at 56% *RWC* in OL than YL.

The amino acids that accumulated to a different extent in YL and OL, and during dehydration were: proline (Pro), Asn, Asp, arginine (Arg), valine (Val) and γ-aminobutyrate (GABA) (Fig. 6). Pro content clearly rose in both YL and OL from the beginning of the dehydration stress. Pro content increased more rapidly in OL than in YL reaching 20% of the total amino acid content at 56% *RWC* (Fig. 6A). One of the most striking results is that dehydrated YL and OL strongly and significantly differed in their Pro content. Below 30% *RWC*, a drop in Pro content was observed in YL, whereas Pro continued to accumulate in OL. At the end of dehydration, Pro represented up to *c*. 30% of the total amino acid content in OL whereas it was almost undetectable in dried YL (Fig. 6A).

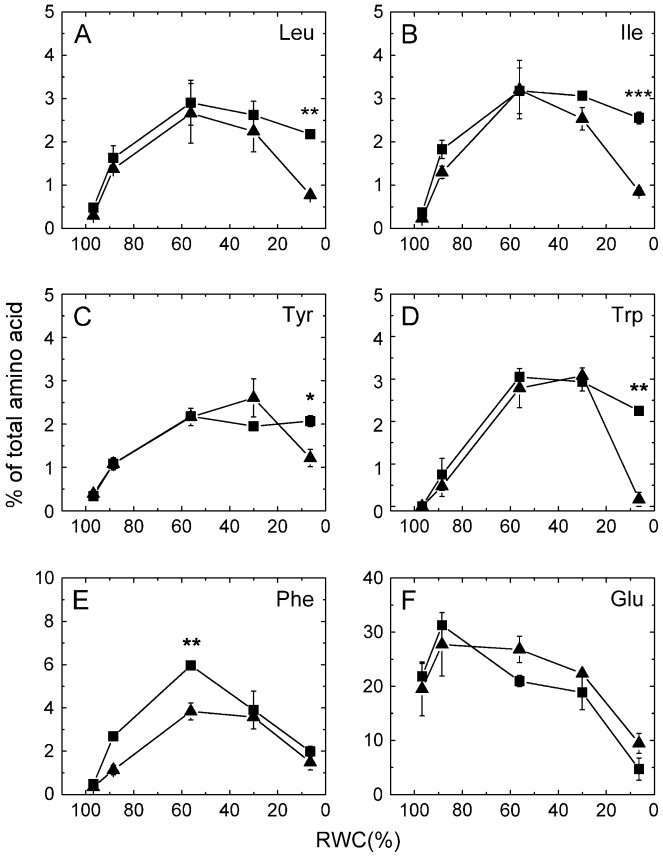

Fig. 5. Amino acid content during desiccation stress in YL (black triangles) and OL (black squares). (A) Leu, (B) Ile, (C) Tyr, (D) Trp, (E) Phe, and (F) Glu. Values are expressed in percentage of total leaf amino acids. Each point is the mean of three samples ±SE. Error bars are shown when larger than symbols. Asterisks indicate significant differences between OL and YL: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

By contrast with Pro, Asn accumulated in YL at the end of dehydration stress (Fig. 6B). It must be noted that, together with Glu, Asn is the most abundant amino acid found in *S. stapfianus* leaves. Asn represented 40% and 33% of the total amino acids contained in unstressed YL and OL, respectively. During the first step of dehydration, Asn content dropped dramatically in both YL and OL. However, whereas Asn content in OL was maintained at low levels throughout the dehydration stress, Asn increased dramatically in YL between 30% and 6% *RWC*. In dehydrated YL, Asn content was not different from unstressed leaves (around 40% of total amino acids). As a result, a significant difference in Asn content was observed between YL and OL at 6% *RWC* (Fig. 6B).

Asp content decreased slightly in YL throughout the dehydration stress. During dehydration stress it represented *c*. 13% and 11% of total amino acids in YL and OL, respectively. Asp was weakly but significantly more abundant in dehydrated YL than in OL (Fig. 6C). Arg increased to a larger extent in YL than in OL. Arg

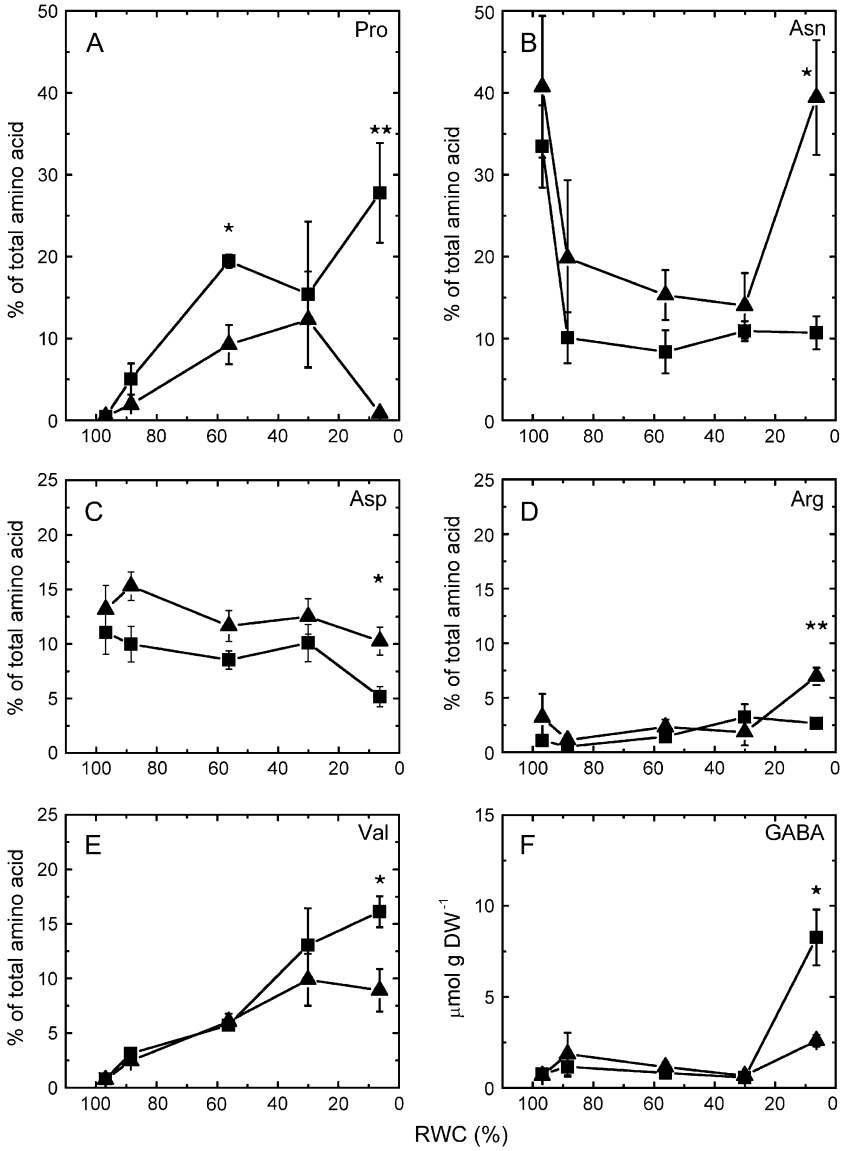

Fig. 6. Amino acid content during desiccation stress in the YL (black triangles) and OL (black squares). (A) Pro, (B) Asn, (C) Asp, (D) Arg, (E) Val, and (F) GABA. Values are expressed in percentage of total leaf amino acids. GABA is expressed in µmol g^{-1} DW because it has not been included in the calculation of total amino acid content. Each point is the mean of three samples ±SE. Error bars are shown when larger than symbols. Asterisks indicate significant differences between OL and YL: * $P \leqslant 0.05$, ** $P \leqslant 0.01$.

increased sharply in YL towards the end of the dehydration stress, and at 6% *RWC* represented 7% of total amino acids (Fig. 6D). Val and GABA increased in both YL and OL with dehydration, but to a much larger extent in OL than in YL (Fig. 6E, F). Like Pro, GABA has been proposed to be important in the stress protection mechanism (Rhodes *et al*., 1999). This non-proteic amino acid accumulated at the final step of dehydration stress between 30% and 6% *RWC*. A *c*. 15-fold and a *c*. 4-fold increase of GABA took place in OL and in YL, respectively. As a result, at the end of dehydration stress GABA content in OL was *c*. three times higher that in YL. Ser and Gly decreased during water stress. If compared with YL, higher values of both amino acids have been observed in OL at 88% *RWC* (data not shown). The content of citrulline, cysteine, methionine, histidine, lysine, ornithine, and hydrolysine was below 1% of the total amino acid content during dehydration stress in both OL and YL, and did not show a clear trend (data not shown).

Discussion

The identification, in *S. stapfianus,* of leaves that are desiccation sensitive and of leaves that are desiccation tolerant made possible the physiological comparison of desiccation-tolerant and desiccation-sensitive leaf material

for the discovery of specific physiological markers. The differential tolerance of *S. stapfianus* leaves depended on leaf age, and as is previously described in the resurrection grass *Eragrostis nindensis* (Vander Willigen *et al*., 2003), YL and OL proved to be desiccation tolerant and sensitive, respectively.

Since desiccation tolerance is related to leaf age, the question of the role of the developmental and physiological status of the leaves at the beginning of water stress was assessed. Interestingly, in hydrated tissues (97% *RWC*), the analyses to determine total amino acid, soluble protein, metabolite, and enzyme contents showed similar results in YL and in OL (with the exception of Chl *a* and *b* contents that were lower in YL). This finding suggested that the physiological differences that may explain the differential tolerance behaviour of OL and YL actually arose during desiccation stress.

At the beginning of desiccation stress, soluble protein content and total free amino acid content decreased in both YL and OL, thus suggesting that, in the first half of dehydration, leaf nitrogen was translocated to other parts of the plant (i.e. roots). This translocation hypothesis was supported by the large decrease in Asn between 97% and 88% *RWC*. Previous reports proposed that Asn was utilized for nitrogen transfer to the roots thus ensuring a metabolic safeguard in stress conditions (Chaffei *et al*., 2004; Olea *et al*., 2004). Then below 88% *RWC*, the total free amino acid pool started to increase in *S. stapfianus*.

Since both Chl and Fd-GOGAT contents decreased in YL and OL, it was likely that a senescence-like process was induced during desiccation stress (Masclaux *et al*., 2000). In parallel, the persistence of GDH activities and GS1 content suggested that these enzymes could have a more important role during dehydration and mobilize nitrogen as is the case during senescence. Indeed, insoluble protein degradation (Gaff and McGregor, 1979) and partial thylakoid disorganization (Dalla Vecchia *et al*., 1998) have been previously observed in *S. stapfianus* during dehydration stress. Together with the increase of polyubiquitin transcripts (Oliver *et al*., 1998) and the loss of Rubisco content throughout the dehydration stress (data not shown), this suggests that amino acid accumulation may have been generated from the chloroplastic protein degradation. Since during dehydration stress, the extent of total amino acid accumulation was the same in YL and OL, it is likely that the dehydration is paralleled by nitrogen recycling in both YL and OL.

Although the total amino acid concentration did not differ between YL and OL, individual amino acids differed and might have been individually important for the acquisition of desiccation tolerance. Pro has been widely proposed as an important amino acid involved in drought stress protection (Hare and Cress, 1997; Rhodes *et al*., 1999). In dehydrated leaves of *S. stapfianus*, Pro accumulation was only detected in the desiccation-sensitive OL and not in the desiccation-tolerant YL. Previously a similar age-related trend in Pro accumulation has also been observed in wheat during salt stress (Colmer *et al*., 1995). Hoekstra *et al*. (2001) have argued that Pro can be considered as a protectant only when the leaf water content is maintained over 0.3 g H_2O g^{-1} DW. Interestingly in *S. stapfianus*, the fully desiccation-tolerant YL accumulated Pro until *c*. 0.5 g H_2O g^{-1} DW (30% *RWC*), and did not contain Pro at 0.1 g H_2O g^{-1} DW (6% *RWC*). This study clearly shows that Pro was no longer required as a protectant in dehydrated desiccation-tolerant YL.

Several reports emphasize the role of GABA as a protectant against reactive oxygen species (ROS) in response to environmental stresses in plants (Rhodes *et al*., 1999). In *S. stapfianus* GABA increased during the last step of dehydration (6% *RWC*) in YL and to a greater extent in the desiccation-sensitive OL. GABA increase in OL suggested that the ROS level that can control the GABA shunt activation (Fait *et al*., 2005) was higher in desiccation-sensitive OL. Finally, the accumulation of high concentration of GABA has no function in the acquisition of desiccation tolerance in *S. stapfianus*.

By contrast with OL, YL accumulated Arg and Asn in dehydrated leaves. Each Arg molecule contains four nitrogen atoms and acts as a nitrogen reservoir. In seeds, Arg can constitute 10–40% of the total nitrogen (Van Etten *et al*., 1967), which is later remobilized during seed germination. In dehydrated YL of *S. stapfianus,* Arg could also be a nitrogen reservoir usable during leaf rehydration. Similarly, Asn, being composed of two nitrogen and four carbon atoms, is an 'economical' way of storing nitrogen. In *S. stapfianus,* given that a quantitatively high amount of sucrose is accumulated during dehydration (Whittaker *et al*., 2001), Asn may also represent a suitable compound for the storage of nitrogen during the stress. Asn build-up has indeed been observed in several water-stressed plants (Stewart and Larher, 1980; Sieciechowicz *et al*., 1988) and has been proposed as a transient nitrogen storage metabolite in sugar-starved maize roots (Brouquisse *et al*., 1992), seeds during dehydration and germination (Canas *et al*., 2006; Fait *et al*., 2006) and, together with Arg, in *Coleus blumei* Benth. sink leaves during salinity stress (Gilbert *et al*., 1998). In dry YL, *c*. 50% of amino acids are composed of Asn and Arg. Since sucrose is not immediately remobilized upon rewatering (Whittaker *et al*., 2004) it is proposed that Arg and Asn may be important as both carbon and nitrogen sources during the early stages of YL rehydration before the recovery of photosynthetic activity.

By contrast, dehydrated OL accumulate a wider pattern of amino acids. This is probably due to slower amino acid interconversions during the second half of dehydration that finally result in higher levels of Leu, Ile, Tyr, Trp, and Val in dehydrated OL.

The present report shows that Glu might have been channeled into two different pathways leading to Pro and

GABA biosynthesis in OL and Arg and Asn accumulation in YL. It was observed that both Fd-GOGAT and NADH-GOGAT activities decreased with stress in both YL and OL. The desiccation-tolerant YL showed high total GS activity (if compared with unstressed leaves), higher GS2 protein content and higher GDH deaminating activity. It can then be hypothetized that ammonia supply from the GDH deaminating activity could have participated in the Gln biosynthesis needed for Asn accumulation in YL.

The principal findings of this study include the large accumulation of Asn and Arg in the desiccation-tolerant YL of *S. stapfianus*, and the accumulation of Pro and GABA in the desiccation-sensitive OL. The possible roles of Asn during rehydration have to be further investigated, as well as the difference in the control of Asn, Pro, Arg, and GABA biosynthetic pathways in OL and YL. *S. stapfianus* will provide a suitable model for these further studies.

Acknowledgements

We are grateful to Stéphanie Boutet (INRA, Versailles, France) for technical help with the amino acid analyser, to Dr Bertrand Hirel for providing GS antibodies (INRA, Versailles, France) and to Adriano Baglio (DISAT, Florence, Italy) for technical help with the elemental analyser. The Marie Curie European project (agreement MCF00049) provided the financial support that made possible the three month visit of Dr Tommaso Martinelli to the Laboratory of Plant Nitrogen Nutrition in INRA, Versailles.

References

Becker TW, Caboche M, Carrayol E, Hirel B. 1992. Nucleotide sequence of tobacco cDNA encoding plastidic glutamine synthetase and light inducibility, organ specificity and diurnal rhythmicity in the expression of the corresponding genes of tobacco and tomato. *Plant Molecular Biology* **19,** 367–379.

Bernacchia G, Furini A. 2004. Biochemical and molecular responses to water stress in resurrection plants. *Physilogia Plantarum* **121,** 175–181.

Bewley JD, Oliver MJ. 1992. Desiccation tolerance in vegetative plant tissue and seeds: protein synthesis in relation to desiccation and a potential role for protection and repair mechanisms. In: Somero GN, Osmond GB, Bolis CL, eds. *Water and life: a comparative analysis of water relationship at the organismic, cellular and molecular levels*. New York: Springer, 141–160.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Brouquisse R, James F, Pradet A, Raymond P. 1992. Asparagine metabolism and nitrogen distribution during protein degradation in sugar-starved maize root tips. *Planta* **188,** 384–395.

Brouquisse R, Masclaux C, Feller U, Raymond P. 2001. Protein hydrolysis and nirtrogen remobilization in plant life and senescence. In: Lea PJ, Morot-Gaudry JF, eds. *Plant nitrogen*. Berlin: Springer, 275–293.

Canas RA, de la Torre F, Canovas FM, Canton FR. 2006. High levels of asparagine synthetase in hypocotyls of pine seedlings suggest a role of the enzyme in re-allocation of seed-stored nitrogen. *Planta* **224,** 83–95.

Chaffei C, Pageau K, Suzuki A, Gouia H, Ghorbel MH, Masclaux-Daubresse C. 2004. Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. *Plant and Cell Physiology* **45,** 1681–1693.

Colmer TD, Epstein E, Dvorak J. 1995. Differential solute regulation in leaf blades of various ages in salt-sensitive wheat and salt-tolerant wheat×*Lophopyrum elongatum* (Host) A. love amphiploid. *Plant Physiology* **108,** 1715–1724.

Dalla Vecchia F, El Asmar T, Calamassi R, Rascio N, Vazzana C. 1998. Morphological and ultrastructural aspects of dehydration and rehydration in leaves of *Sporobolus stapfianus*. *Plant Growth Regulation* **24,** 219–228.

Davis DD. 1982. Physiological aspects of protein turnover. In: Boulter D, Parthier B, eds. *Nucleic acids and proteins in plants. Structure, biochemistry and physiology of proteins.* Vol. I. *Encyclopedia of plant physiology*. Berlin: Springer-Verlag, 189–228.

Fait A, Yellin A, Fromm H. 2005. GABA shunt deficiencies and accumulation of reactive oxygen intermediates: insight form *Arabidopsis* mutants. *FEBS Letters* **579,** 415–420.

Fait A, Angelovici R, Less H, Ohad I, Urbanczyk-Wochniak E, Fernie AR, Galili G. 2006. *Arabidopsis* seed development and germination is associated with temporally distinct metabolic switches. *Plant Physiology* **142,** 839–854.

Gaff DF. 1971. Desiccation tolerant plants in Southern Africa. *Science* **174,** 1033–1034.

Gaff DF, McGregor GR. 1979. The effect of dehydration and rehydration on the nitrogen content of various fractions from resurrection plants. *Biologia Plantarum* **22,** 92–99.

Gilbert GA, Gadush MV, Wilson C, Madore MA. 1998. Amino acid accumulation in sink and source tissues of *Coleus blumei* Benth. during salinity stress. *Journal of Experimental Botany* **49,** 107–114.

Hare PD, Cress WA. 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regulation* **21,** 79–102.

Hirel B, Lea PJ. 2001. Ammonia assimilation. In: Lea PJ, Morot-Gaudry JF, eds. *Plant nitrogen*. Berlin: Springer, 79–99.

Hoekstra FA, Golovina EA, Buitink J. 2001. Mechanisms of plant desiccation tolerance. *Trends in Plant Science* **6,** 431–438.

Ingram J, Bartels D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology* **47,** 377–403.

Kuang J, Gaff DF, Gianello RD, Blomstedt CK, Neale AD, Hamill JD. 1995. Changes in *in vivo* protein complements in drying leaves of the desiccation-tolerant grass *Sporobolus stapfianus* and the desiccation-sensitive grass *Sporobolus pyramidalis*. *Australian Journal of Plant Physiology* **22,** 1027–1034.

Laemmli UK. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lea PJ, Blackwell RD, Chen FL, Hecht U. 1990. Enzymes of ammonium assimilation. In: Dey PM, Harborne JB, eds. *Methods in plant biochemistry*. London: Academic Press, 257–276.

Lea PJ, Ireland RJ. 1999. Nitrogen metabolism in higher plants. In: Singh BK, ed. *Plant amino acids: biochemistry and biotechnology*. New York: Marcel Dekker, 1–47.

Masclaux C, Valadier MH, Brugière N, Morot-Gaudry JF, Hirel B. 2000. Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. *Planta* **211,** 510–518.

Masclaux-Daubresse C, Reisdorf-Cren M, Pageau K, Lelandais M, Grandjean O, Kronenberger J, Valadier MH, Feraud M, Jouglet T, Suzuki A. 2006. Glutamine synthetase–glutamate synthase pathway and glutamate dehydrogenase play distinct roles for sink–source nitrogen cycle in tobacco (*Nicotiana tabacum* L.). *Plant Physiology* **140,** 444–456.

Noctor G, Novitskaya L, Lea PJ, Foyer CH. 2002. Co-ordination of leaf minor amino acids contents in crop species: significance and interpretation. *Journal of Experimental Botany* **53,** 939–945.

Olea F, Perez-Garcia A, Canton FR, Rivera ME, Canas R, Avila C, Cazorla FM, Canovas FM, de Vicente A. 2004. Up-regulation and localization of asparagine synthetase in tomato leaves infected by the bacterial pathogen *Pseudomonas syringae*. *Plant and Cell Physiology* **45,** 770–780.

Oliver MJ, Wood AJ, O'Mahony P. 1998. 'To dryness and beyond': preparation for the dried state and rehydration in vegetative desiccation-tolerant plants. *Plant Growth Regulation* **24,** 193–201.

Rascio N, La Rocca N. 2005. Resurrection plants: the puzzle of surviving extreme vegetative desiccation. *Critical Review in Plant Science* **24,** 209–225.

Rhodes D, Verslues PE, Sharp RE. 1999. Role of amino acids in abiotic stress resistance. In: Singh BK, ed. *Plant amino acids: biochemistry and biotechnology*. New York: Marcel Dekker, 319–356.

Russell JA. 1944. The colorimetric estimation of small amounts of ammonia by the phenol–hypochlorite reaction. *Journal of Biological Chemistry* **156,** 457–461.

Sieciechowicz KA, Joy KW, Ireland RJ. 1988. The metabolism of asparagine in plants. *Phytochemistry* **27,** 663–671.

Stewart GR, Larher F. 1980. Accumulation of amino acids and related compounds in relation to environmental stress. In: Miflin BJ, ed. *The biochemistry of plants,* Vol. V. New York: Academic Press, 609–635.

Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Delis ID, Yakoumakis DI, Kouvarakis A, Papadakis AK, Stephanou EG, Roubelakis-Angelakis KA. 2006. Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenase to form glutamate for proline synthesis in tobacco and grapevine. *The Plant Cell* **18,** 2767–2781.

Suzuki A, Vergnet C, Morot-Gaudry JF, Zehnacker C, Grosclaude J. 1994. Immunological characterization of ferredoxin and methyl viologen interaction domains of glutamate synthetase using monoclonal antibodies. *Plant Physiology and Biochemistry* **32,** 619–626.

Turano JF, Dashner R, Upadhayaya A, Caldwell CR. 1996. Purification of mitocondrial glutamate dehydrogenase from dark-grown soybean seedlings. *Plant Physiology* **112,** 1357–1364.

Tymms MJ, Gaff DF. 1978. Proline accumulation during water stress in resurrection plants. *Journal of Experimental Botany* **30,** 165–168.

Van Etten CH, Kwolek WF, Peter JE, Barclay AS. 1967. Plant seeds as protein source for food and feed. Evaluation based on amino acid composition of 379 species. *Journal of Agricultural and Food Chemistry* **15,** 1077–1089.

Vander Willigen C, Pammenter NW, Jaffer AM, Mundree SG, Farrant JM. 2003. An ultrastructural study using anhydrous fixation of *Eragrostis nindensis*, a resurrection grass with both desiccation-tolerant and desiccation-sensitive tissues. *Functional Plant Biology* **30,** 281–290.

Wellburn AR. 1994. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology* **144,** 307–313.

Whittaker A, Bochicchio A, Vazzana C, Lindsey G, Farrant J. 2001. Changes in leaf hexokinase activity and metabolite levels in response to drying in the desiccation-tolerant species *Sporobolus stapfianus* and *Xerophyta viscosa*. *Journal of Experimental Botany* **52,** 961–969.

Whittaker A, Martinelli T, Bochicchio A, Vazzana C, Farrant J. 2004. Comparison of sucrose metabolism during the rehydration of desiccation-tolerant and desiccation-sensitive leaf material of *Sporobolus stapfianus*. *Physiologia Plantarum* **122,** 11–24.