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RESEARCH PAPER

Drought effect on nitrate reductase and sucrose-phosphate synthase activities in wheat (*Triticum durum* L.): role of leaf internal CO_2

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Abstract

In order to study the impact of a decline of leaf internal CO_2 molar ratio on nitrate reductase (NR) and sucrose-phosphate synthase (SPS) activities, leaves of wheat (*Triticum durum*) were submitted to different treatments: slow or rapid dehydration and decline in ambient CO_2 concentration and abscisic acid (ABA) supply. In agreement with the literature, NR activity of slowly dehydrated leaves was inhibited by about 50% when net CO_2 assimilation (A_n) decreased by 45%. NR activity of stressed leaves kept 4 h in air containing 5% CO_2 or after 2 d of re-watering was only partially restored. NR activity was slightly dependent on ambient CO_2 molar ratio, declining by 30% when non-stressed leaves were kept in CO_2-free air for 4 h. The decline of NR activity after ABA supply (through the transpiration stream) and after rapid dehydration of non-stressed leaves was comparable with the decrease observed under low CO_2 treatment. Overall, these data suggest that a drought-induced decrease of the leaf internal CO_2 concentration is only part of the signal triggering the decline of NR activity. In disagreement with most of the literature, SPS activity increased during slow dehydration, being stimulated by 30% when A_n declined by 40%. SPS activity of stressed leaves kept 4 h in air containing 5% CO_2 or 2 d after re-watering was slightly increased or unchanged, respectively. By contrast to NR activity, SPS activity of well-hydrated leaves was hardly affected by low CO_2. Increased SPS activity was mimicked, in non-stressed leaves, by a rapid dehydration within 4 h and by ABA fed through the transpiration stream. In durum wheat, the increase in SPS activity could be linked to ABA-based signalling during a drought stress.

Key words: Abscisic acid, CO_2 assimilation, drought, nitrate reductase, sucrose-phosphate synthase, *Triticum durum*.

Introduction

It is well established that the photosynthetic apparatus is resistant to a mild drought treatment (Kaiser, 1987; Cornic, 2000). In some C_3 plants submitted to drought [corresponding to a decrease in leaf relative water content (RWC) of about 20–25%], the net CO_2 uptake can decrease up to 80% without an effect on leaf photosynthetic capacity measured at high CO_2 (Cornic and Fresneau, 2002). As a result, the CO_2 molar ratio within photosynthetic cells, under these conditions, is likely to be low. Metabolic changes occur in such leaves (Sharkey and Seeman, 1989) and the photosynthetic apparatus can be damaged when the dehydration of mesophyll cells proceeds (Kaiser, 1982).

Drought effects are characterized by changes in physiological and biochemical processes, which have been extensively studied, including inhibition of protein synthesis (e.g. Hsiao, 1973; Bradford and Hsiao, 1982). Many changes in gene expression (up- and down-regulation) occur in plants growing under limiting water conditions as seen by transcriptomics (Bray, 2002). Some of these changes appear to be part of a general adaptive response to desiccation. For example, some up-regulated genes have been shown to be involved in many cellular

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Abbreviations: ABA, abscisic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NR, nitrate reductase; RWC, relative water content; SPS, sucrose-phosphate synthase.

functions, such as signalling, redox homeostasis, maintenance of the cell (chaperone activities), and maintenance of the water balance. In parallel, many enzyme activities have been shown to increase or decrease in response to cell desiccation (Hsiao, 1973). In particular, activities of nitrate reductase (NR; EC 1.7.1.3) and sucrose-phosphate synthase (SPS; EC 2.4.1.14), two key enzymes of nitrogen and carbon assimilation pathways, respectively, are strongly modulated during drought.

Under water stress, leaf NR activity decreases (Hsiao, 1973; Plaut, 1974; Larsson *et al*., 1989; Ferrario-Méry *et al*., 1998; Foyer *et al*., 1998; Mahan *et al*., 1998; Garg *et al*., 2001; Correia *et al*., 2005). As shown for spinach leaves, the decline in nitrate reduction is correlated with low photosynthetic activity, i.e. when internal CO_2 concentration is reduced due to stomatal closure (Kaiser and Förster, 1989). Kaiser and Brendle-Behnisch (1991) presented evidence that photosynthesis regulates nitrate reduction by modulating NR activity. When spinach leaves are incubated in CO_2-free air under light, active NR (measured in the presence of free Mg) rapidly decreases. This activity is restored up to control levels either by restoring normal air conditions *in vivo* (Kaiser and Brendle-Behnisch, 1991) or by incubating leaf extracts with AMP *in vitro* (Kaiser and Spill, 1991). These authors showed that photosynthesis was linked to NR activity via ATP/AMP ratios. Indeed, under low CO_2, when NR activity was reduced, photorespiration (high ATP/AMP) increased while photosynthesis (low ATP/AMP) decreased, suggesting that the mechanism of NR regulation depended on the phosphorylation/dephosphorylation state of the protein (see also Huber *et al*., 1994).

NR activity under drought could also be modified via changes in NR transcript levels. Accordingly, Foyer *et al*. (1998) and Ferrario-Méry *et al*. (1998) showed that, in maize and tobacco, NR transcript levels decreased (by about 80% in maize leaves) under drought. They suggested that there was presumably a decrease in protein synthesis due to an inhibition of processes involving transcription and formation of NR native protein. The decreases observed in the quantity of NR mRNA may have been caused by a decrease in foliar nitrate levels due to a subsequent shortage of nutrient availability under drought. Indeed, Correia *et al*. (2005) have shown that the drought-induced decrease in NR activity in sunflower and white lupin leaves was linearly correlated with nitrate depletion.

Concerning SPS activity, contradictory data have been reported regarding the effect of drought. Quite often, SPS maximal activity (SPS_{max}) was shown to decrease during leaf desiccation (Vassey and Sharkey, 1989; Vassey *et al*., 1991; Castrillo, 1992, Pelleschi *et al*., 1997; Foyer *et al*., 1998), or to remain constant (Quick *et al*., 1989; Zrenner and Stitt, 1991). However, more recently, Yang *et al*. (2002) and Niedzwiedz-Siegen *et al*. (2004) observed an increase in SPS_{max} activity in rice and wheat leaves, respectively, under mild drought. In some cases, increased SPS activities were observed under limiting substrate conditions (i.e. SPS_{lim}) (Quick *et al*., 1989; Zrenner and Stitt, 1991; Foyer *et al*., 1998). As for NR, the mechanism of SPS regulation depends on the phosphorylation/dephosphorylation state of the protein (Pathre *et al*., 2000).

The decrease in SPS_{max} activity observed in dehydrated leaves under normal atmospheric conditions (air with 370 ppm of CO_2) is restored to the control value when leaves are submitted to elevated CO_2 (1%) (Vassey *et al*., 1991). On the other hand, a 1 h incubation of non-stressed leaves under low CO_2 led to about a 55% decrease in SPS_{max} activity which could be totally reversed by a 20 min incubation in elevated CO_2 (5%). These authors concluded that the effect of water stress on SPS activity was due to an inhibition of photosynthesis caused by stomatal closure.

By contrast to the important decline in NR mRNA levels measured under drought conditions, the SPS mRNA pool is only slightly decreased (10–20%) in maize leaves, suggesting that this effect is relatively specific to NR transcripts (Foyer *et al*., 1998).

The decrease or increase in SPS activity during drought is generally accompanied by an increase in leaf sucrose (Zrenner and Stitt, 1991; Pelleschi *et al*., 1997; Ferrario-Méry *et al*., 1998; Yang *et al*., 2002; Niedzwiedz-Siegen *et al*., 2004; Correia *et al*., 2005) and/or hexose contents (Keller and Ludlow, 1993; Rodriguez *et al*., 1993), which could contribute to an osmoregulation under such conditions.

In some cases, metabolic changes within a stressed leaf can be the consequence of the resistance to dehydration of the photosynthetic apparatus itself. The signal could be a low CO_2 concentration in leaves due to stomatal closure under light. This appears to be true for the increase of the glycolate pathway during leaf desiccation (Cornic, 1994). Indeed, photorespiration drains electrons in excess, protecting the photosynthetic apparatus submitted to drought conditions.

The focus of this paper is to determine whether or not the low leaf internal CO_2 molar ratio, induced by stomatal closure during water stress, can modulate NR and SPS activities in *Triticum durum*. Leaf net CO_2 uptake was inhibited by (i) withholding watering of intact plants (slow drought), (ii) cutting leaves off the plant (rapid drought), or (iii) decreasing the CO_2 molar ratio around turgid leaves. Also measured, in well-watered leaves, was the effect of abscisic acid (ABA) on NR and SPS activities, because this plant hormone is known to inhibit leaf net CO_2 uptake by inducing stomatal closure. It is shown that both stomatal and non-stomatal effects of drought explain the observed modulations of NR and SPS activities when durum wheat leaves are dehydrated.

Materials and methods

Plant material, growth conditions, and drought stress treatment

Experiments were carried out on *Triticum durum* cv. Lloyd. Plants were grown under a 16 h photoperiod with 21 °C/15 °C day/night temperatures in a growth chamber in square containers (40×40×7 cm) containing vermiculite and watered every 2 d with modified Knop's nutrition medium (Feierabend and Schrader-Reichhardt, 1976). Photon flux density at the top of the plants was 250 μmol m^{-2} s^{-1}. Unless otherwise stated, experiments started 3 h after the beginning of the photoperiod, when NR and SPS activities were close to their maximum values (NR and SPS activities are under circadian control; Deng *et al*., 1990; Jones and Ort, 1997).

Drought was imposed by withholding nutrition medium after 16 d of culture. All measurements were made on the central part (6 cm at 8 cm from the stem) of the third leaf which was not mature at the beginning of the experiments (16th day of growth), then fully expanded (21st day of growth), liguled, and finally becoming senescent.

Different treatments

After withholding nutrition medium, stressed and control leaves were used to measure gas exchange, water status, and enzyme activities under normal air conditions. Eleven days after watering was stopped, a re-watering experiment was carried out, as well as incubations at a high CO_2 concentration under light (i) in an oxygen electrode to evaluate the contribution of stomatal closure to CO_2 assimilation decline under drought and (ii) in a large assimilation chamber to collect enough material for enzymatic activity measurements.

On 21-d-old third leaves, cut from well-hydrated plants and maintained in Eppendorf tubes filled with water, three types of experiments were carried out in a large assimilation chamber under light: (i) a rapid drought of 4 h obtained by removing water under 370 ppm CO_2; (ii) a 4 h incubation under different CO_2 molar ratios from 0 to 370 ppm; and (iii) a 4 h incubation under 370 ppm CO_2 in the presence of ABA fed through the transpiration stream. During these experiments, gas exchange measurements were performed and enzymatic activities were measured after each treatment.

Leaf gas exchange measurements

During the slow drought treatment, leaf gas exchanges (net CO_2 assimilation and water transpiration) were measured in a gas exchange system already described by Cornic and Ghashghaie (1991). Measurements were made on three leaves, still attached to the seedlings, placed in a small assimilation chamber (1.45 cm^2 leaf surface; volume 4 ml). During measurements, unless otherwise stated, conditions were as follows: leaf temperature, 21 °C; photosynthetic photon flux density, 350 μmol m^{-2} s^{-1}; ambient CO_2 molar ratio (C_a), 370 ppm; leaf vapour pressure deficit, 0.9 kPa. Net CO_2 uptake, leaf conductance for CO_2, and CO_2 molar ratio at the evaporating site of the leaf (C_i) were calculated according to Von Caemmerer and Farquhar (1981).

Measurements of CO_2-dependent O_2 evolution were also made at high C_a (5%) using a leaf disc oxygen electrode. During measurements, leaf temperature was 21 °C under a photosynthetic photon flux density value of 350 μmol photons m^{-2} s^{-1}.

Leaf photosynthetic activity was followed on 21-d-old leaves, cut from well-hydrated plants, maintained in water and placed in a large assimilation chamber (volume 200 ml) connected to the same gas exchange system. After either 4 h incubation in the presence of ABA (added to water), 4 h rapid dehydration (removing cut leaves from water), or 4 h exposure to different CO_2 molar ratios, enzymatic activities (NR and SPS) were determined in extracts made from the central part of the leaves. This allowed photosynthetic activities to be related to enzymatic activities. Four hours of treatment with different CO_2 molar ratios were shown to be long enough to obtain the maximum response, as already observed in spinach leaves by Kaiser and Brendle-Benisch (1991). Each experimental condition provided two samples of four leaves for activity measurements and this was repeated twice.

NR and SPS activity measurements

The central part of the third leaf was divided into two segments, of 4 cm and 2 cm in length for activity (NR and SPS) measurement and total protein determination, respectively. After a rapid determination of fresh weight, samples of four leaves were rapidly frozen in liquid nitrogen. In parallel, the central part of two similar leaves was used to measure RWC and leaf water potential (see below).

Leaf samples (around 70 mg) were ground in liquid nitrogen and the resulting powder dissolved in 1 ml of 50 mM HEPES-NaOH buffer, pH 7.6, containing either 0.5% BSA, 0.1% Triton X-100, 5 mM $MgCl_2$, 10 μM leupeptin, 0.5 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride], 3 mM dithiothreitol (DTT), 1% polyvinylpolypyrrolidone, and 5 mM flavine adenine dinucleotide, for NR activity measurements, or 0.6% BSA, 0.02% Triton X-100, 5 mM $MgCl_2$, 10 μM leupeptin, 0.5 mM AEBSF, and 3 mM DTT, for SPS activity measurements. In both cases, this was followed by centrifugation for 10 min at 13 000 *g*.

Measurements of total [in the presence of EDTA (ethylenediaminetetraacetic acid), NR-EDTA] and active (in the presence of Mg^{2+}, NR-Mg) NR activities were carried out on the supernatant following the method described by Foyer *et al*. (1998). SPS activity was measured under limiting (SPS_{lim}) and saturating (SPS_{max}) substrate conditions, using the method described by Pelleschi *et al*. (1997), after desalting extracts by centrifugal filtration on a Sephadex G25 gel (Amersham Biosciences).

Treatment by ABA and rapid dehydration of excised leaves

Eight leaves, cut under water, from well-hydrated 21-d-old plants with their bottom part in an Eppendorf tube, containing 1.5 ml of distilled water, were placed in the large assimilation chamber of the gas exchange system. When a steady-state of net CO_2 assimilation was reached (about 1 h after the beginning of experiment), either (i) a solution of ABA in 10% ethanol was added to water in the Eppendorf tube to give a final concentration of 5 μM or 10 μM (ethanol 0.1%), or (ii) the bottom part of the leaves was removed from water to dehydrate the cut leaves rapidly. A_n was measured continuously during the next 4 h. Each experiment (repeated twice) provided two samples that were used to measure NR and SPS activities as well as total protein content.

Incubation in 5% CO_2-enriched air

The effect of CO_2-enriched air was examined using a larger assimilation chamber (volume 475 ml) containing either eight stressed or eight control leaves attached to the seedling. In order to maintain a leaf temperature of 21 °C, leaves were kept stuck to the top of the chamber where temperature-controlled water was rapidly circulating between two transparent glass walls. To maintain a high CO_2 molar ratio inside the chamber during the 4 h treatment time, the chamber was flushed for 5 min every 30 min with CO_2-enriched air (5%) delivered by two mass flow controllers (flow rate: 100 ml min^{-1}). At the end of incubation, the central part of the leaves was immediately placed in liquid nitrogen until NR, SPS, and protein measurements were performed. The experiment was repeated three times.

Protein content determination

Leaf samples were ground in liquid nitrogen and the powder was dissolved in 1 ml of 50 mM HEPES-NaOH buffer pH 7.6 containing

3 mM DTT. After centrifugation for 10 min at 13 000 *g*, the protein concentration was measured using the Sedmak and Grossberg method (Sedmak and Grossberg, 1977) using BSA as standard protein. This allowed all enzymatic activities to be expressed relative to the soluble protein concentration.

Leaf RWC determination

RWC was measured simultaneously on two leaves from either stressed or well-watered plants taken 3 h after the beginning of illumination of the culture chamber. After weighing (fresh weight, FW), leaves were cut into three parts and placed in water in a closed Petri dish with filter paper placed on the samples. After 24 h at 4 °C, leaf pieces were weighed (turgid weight, TW). Dry weight (DW) was measured after 48 h at 70 °C. RWC was calculated as [100×(FW–DW)]/(TW–DW).

Leaf water potential determination

Leaf water potential was measured using a dew point microvoltmeter (Wescor, HR-33-T) equipped with thermocouples mounted in Wescor C-52 sample chambers. Discs of 0.28 cm^2 were taken from the central part of two leaves from stressed and well-watered plants at the same time as for the water determination analysis. Leaf water potential measurements were made 1.5 h after leaf discs were installed in the chambers, thus allowing hygroscopic equilibrium to be reached.

Results

Slow drought

The net CO_2 uptake (A_n) of control leaves slightly increased until maturity and then slowly decreased, but remained high (around 10 μmol m^{-2} s^{-1}) throughout the experimental period. Net CO_2 uptake of stressed leaves significantly decreased 5 d after watering was withheld and was found to be ~55% of the control value after 11 d of drought. By this time, the CO_2 molar ratio inside the leaf (C_i) decreased 2-fold from about 250 ppm to about 125 ppm (Fig. 1A). This was accompanied by a decrease in the C_i/C_a ratio from 0.67±0.03 to 0.31±0.01 (C_a is the ambient CO_2 molar ratio) showing that stomatal closure (data not shown) was the main cause of the change in A_n (Kaiser, 1987; Cornic, 2000).

A significant decrease in RWC was seen only after 9 d in stressed plants (Fig. 1B), while leaf water potential rapidly declined, reaching a steady value of about –0.85 MPa after 7 d (Fig. 1C). During the experiment, the RWC of control leaves remained constant while leaf water potential slowly decreased during the growth period, but always remained higher than in stressed leaves.

Total NR activity (NR-EDTA) first increased and then decreased, in both control and drought-stressed leaves, thus reflecting leaf growth and ageing. However, total NR activity was always lower in drought-stressed leaves (Fig. 2A). As shown in the inset of Fig. 2A, the activation state of NR (percentage of NR-Mg in comparison with NR-EDTA activity) was not affected by drought and remained more or less constant during the growth of the third leaf (84.6±0.1%).

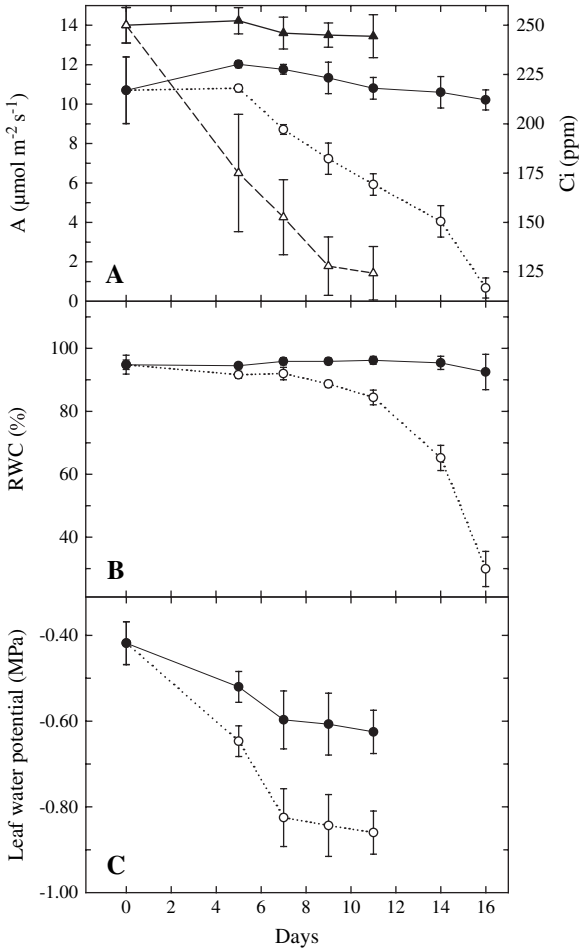

Fig. 1. Variations of net CO_2 assimilation (A_n), internal CO_2 concentration (C_i) (A), leaf relative water content (RWC) (B), and leaf water potential (C) of wheat leaves under control (closed symbols) and slow drought (open symbols) conditions. Watering was withheld at day 0 which corresponded to the 16th day of growth. Mean values are given ±SE (n=3). Conditions during assimilation measurements were: leaf temperature, 21 °C; photosynthetic photon flux density, 350 μmol m^{-2} s^{-1}; leaf vapour pressure deficit, 0.9±0.2 kPa; ambient CO_2 molar ratio (C_a), 370±5 ppm.

The maximum SPS activity (SPS_{max}) increased in leaves of both drought-stressed and control plants. Activity was always higher in stressed leaves from day 5 (Fig. 2B). The activation state of SPS (percentage of SPS_{lim} in comparison with SPS_{max} activity) was only 9.4±0.6% and it did not vary during the stress or during the growth period (inset Fig. 2B). This result is similar to that obtained by Trevanion *et al*. (2004) on leaves of two *Triticum aestivum* cultivars.

Effect of CO_2 on NR and SPS activities measured in turgid leaves

Lowering the CO_2 molar ratio, during a 4 h period, around intact third leaves from 21 d well-watered plants (control leaves corresponding to day 5 in Fig. 2), caused a decrease in both A_n and NR-EDTA (Fig. 3A, open triangles) and

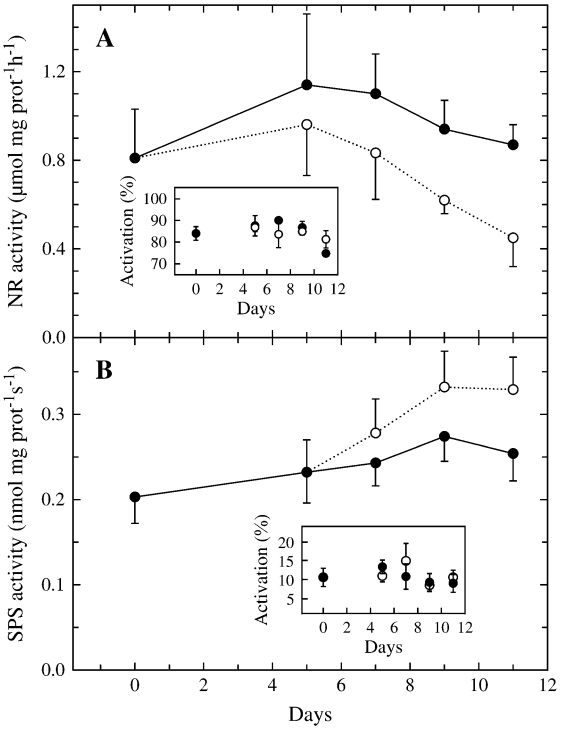

Fig. 2. Variations in total NR activity (A) and maximum SPS activity (B) during slow drought. (A) Total NR activity in the presence of EDTA (closed squares, control leaves; open squares, stressed leaves). Inset: activation percentage of control (closed circles) and stressed (open circles) leaves. (B) SPS_{max} activity (closed squares, control leaves; open squares, stressed leaves). Inset: activation percentage of control (closed circles) and stressed (open circles) leaves. Day 0 corresponds to the 16th day of growth and also to the beginning of slow drought. Mean values are given ±SE (n=3–6).

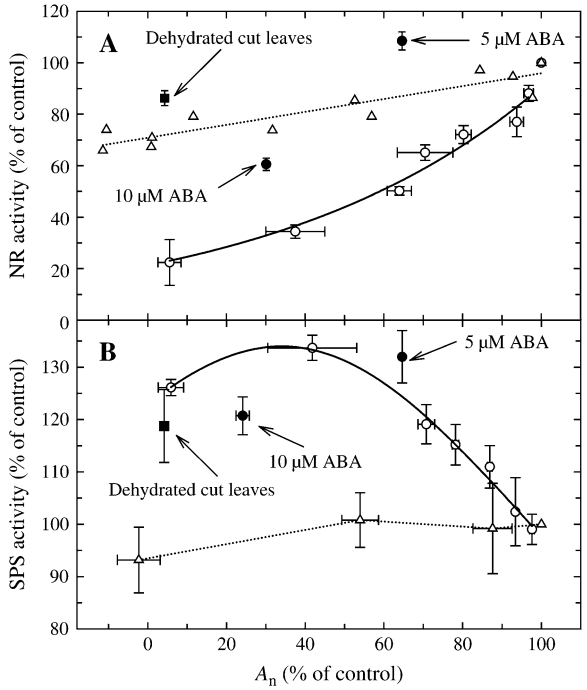

Fig. 3. NR total activity (A) and SPS maximum activity (B) as a function of net CO_2 uptake, A_n, expressed as a percentage of the control values. Changes in A_n were obtained either by submitting plants to slow drought (open circles) or by lowering the CO_2 level around well-watered leaves (open triangles). The relationships for NR and SPS maximum activities in stressed leaves are expressed as a percentage of NR and SPS maximum activities in turgid leaves of the same age (open circles). The relationships for NR and SPS activities at a given C_a are expressed as a percentage of NR and SPS activities in similar leaves (open triangles). The effects of ABA (closed circles) and a rapid dehydration (closed square) are also shown. In both cases the corresponding values of A_n at the steady-state were considered. The control leaves for the three treatments were 21 d turgid leaves maintained for 4 h in water at C_a=370 ppm under light. Conditions during measurements of CO_2 assimilation were the same as in Fig. 1. For all the measurements, mean values are given ±SE (n=2–5).

NR-Mg activities (not shown). The 100% value for CO_2 assimilation and enzyme activity was determined on well-hydrated leaves of the same age and maintained in air containing 370 ppm of CO_2. For comparison, the curve showing the effect of a slow drought (open circles) on leaf NR-EDTA activity in relation to A_n is plotted on the same figure. On turgid leaves, total NR activity measured in CO_2-free air, when A_n was negative, remained at about 70% of the value observed with a normal CO_2 molar ratio, while it was inhibited by 80% in dehydrated leaves that still exhibited a positive net CO_2 uptake. Since changes in relative rates of NR-EDTA and NR-Mg activities were always identical, only NR-EDTA data are shown in Fig. 3A.

The maximum SPS activity barely changed when A_n decreased as a result of a decline in the atmospheric CO_2 molar ratio (Fig. 3B, open triangles), representing about a 10% inhibition in a CO_2-free atmosphere. This contrasted with the increase observed during a slow drought treatment (Fig. 3B, open circles), where SPS_{max} activity reached a maximum value when A_n was inhibited by 55%. Since, in both experiments, variations in relative rates of SPS_{max} and SPS_{lim} were identical, only SPS_{max} values are given in Fig. 3B.

Rapid dehydration of cut leaves

The following experiments were performed on leaves with their bottom part cut under water. A rapid dehydration treatment was given when A_n had reached a steady-state level under control conditions. Controls were cut leaves with their bottom part kept in water. Rapid dehydration was obtained by removing cut leaves from water. This led to a decrease of A_n to almost zero within 30–60 min. After 4 h without water, the mean RWC of leaves was 79.1±2.1%. NR-EDTA activity (as well as NR-Mg activity) was comparable with that measured on turgid leaves when A_n was inhibited by 10% (Fig. 3A, closed square) and much higher than that observed in a slowly dehydrated plant with the same rate of leaf net CO_2 uptake. The activation state of NR was about 84% (data not shown), and similar to that measured during the slow drought treatment.

By contrast, during rapid dehydration, when A_n was close to zero, SPS_{max} activity (Fig. 3B, closed square)

increased to a level approaching that measured in the slow dehydration experiment (Fig. 3B, open circles). The same pattern was observed for SPS_{lim}.

Effect of exogenous ABA

The effect of ABA was examined using cut leaves with their bottom part cut under water. After A_n had reached a steady state in the measuring conditions, ABA was added to water in order to obtain the desired concentration. It was then taken up by the transpiration stream and led to a rapid A_n decrease, reaching a new steady-state value within 45–60 min, depending on the ABA concentration (data not shown). In this series of experiments, the control was cut leaves kept in water during the same time in the assimilation chamber, but fed with water containing 0.1% ethanol. The NR activation state was not modified by addition of ABA (data not shown). When compared on the same rate of net CO_2 uptake, total NR activity values obtained for both 5 μM and 10 μM ABA were close to the value obtained by changing the CO_2 molar ratio around well-hydrated leaves (Fig. 3A, closed circles).

By contrast, the addition of ABA led to maximum SPS activities that were similar to the relationship obtained during the slow drought treatment (Fig. 3B, closed circles) and the activation state remained unchanged (data not shown).

Effect of re-watering after a slow drought

As shown in Table 1, re-watering of plants after 11 d of drought, when A_n had been inhibited by 47%, slowly removed the drought-induced inhibition. A parallel increase in RWC and A_n was observed and control values were reached after 48 h. At the same time, the C_i values of re-hydrated leaves were similar to those of control leaves of the same age and the C_i/C_a ratio was restored to control values (0.66±0.01).

NR-EDTA activity increased after re-hydration but it did not reach the control value (only 88% of control value) even after 48 h, although leaves were already well re-hydrated. NR-Mg activity followed a similar trend (therefore no change in the activation state was observed). By contrast, SPS_{max} activity remained high and was not altered by plant re-hydration (Table 1); the same was the case for SPS_{lim}.

Effect of high CO_2 treatment after slow drought

When stressed leaves were placed in the oxygen electrode in the presence of 5% CO_2, CO_2-dependent O_2 evolution increased by about 20% (Table 2) but it did not reach the control rate. This indicated that either the photosynthetic capacity of stressed leaves had decreased or that a 5% CO_2 molar ratio was not sufficient to alleviate the inhibitory effect of drought on leaf net CO_2 uptake (see Cornic *et al*., 1989).

After 4 h in 5% CO_2, NR-EDTA and NR-Mg activities (not shown) of stressed leaves increased by 20% within 4 h, but they did not attain the control levels, while SPS_{max} and SPS_{lim} (not shown) increased further (Table 2).

Table 1. *Effects of plant re-watering, after a slow drought of 11 d, on leaf relative water content (RWC), net CO_2 uptake (A_n), and on total NR and SPS_{max} activities*

Measurements were made on third leaves of control and stressed plants of the same age. Mean values are expressed as a percentage of measurements carried out on well-watered plants (±SE, n=3).

	Hours after re-watering		
	0	24	48
RWC (% control)	85.8±5.0	92.4±4.3	99.6±0.2
A_n (% control)	61.6±8.9	85.7±12.6	98±1.7
NR (% control)	55.6±5.1	79.9±9.9	88.1±4.6
SPS (% control)	131.2±5.7	130.8±8.2	132.3±9.8

Table 2. *Effects of high CO_2 (5%) on net CO_2 uptake (A_n) and total NR and SPS_{max} activities*

Measurements were made on third leaves from dehydrated plants (11 d without watering) and control third leaves of the same age. Assimilation was determined as CO_2-dependent oxygen evolution of leaf discs in an oxygen electrode while enzymatic activity measurements were carried out after incubation at high CO_2 of similar third leaves still attached to the plant and placed in an assimilation chamber under light. Mean values are expressed as a percentage of measurements done on leaves from well-watered plants submitted to 5% CO_2 treatment (±SE, n=3).

	Hours of incubation in the presence of 5% CO_2-enriched air	
	0	4
A_n (% control)	69.7±3.0	85.8±5.0
NR (% control)	47.8±0.8	68.2±0.5
SPS (% control)	131.2±5.7	148.5±10.5

Discussion

As expected, withholding watering from the *Triticum durum* plants caused a rapid decline in net CO_2 uptake accompanied by a decrease in leaf water potential within 1 week. By contrast, leaf RWC decreased significantly, but only 9 d after watering was withheld, suggesting that there was an osmotic adjustment allowing the maintenance of leaf water content during the first week. These results are in agreement with those obtained on two cultivars of durum wheat by Kameli and Lösel (1993). The decrease in the water potential of control leaves was probably due to the ageing of the third leaf which was immature at the beginning of the experiment, but became fully expanded and eventually senesced after the growth of the 4th and the 5th leaves, before the end of the experiment.

Modulation of NR activity by drought

As expected, both NR activities (NR-EDTA and NR-Mg) decreased during the slow drought treatment of durum

wheat leaves. Indeed, it has already been shown that both activities were inhibited by drought in maize (Foyer *et al*., 1998), in *Nicotiana plumbaginifolia* (Ferrario-Méry *et al*., 1998), and in white lupin and sunflower (Correia *et al*., 2005) leaves. It was also reported that NR-EDTA activity declined during a drought in wheat (Plaut, 1974), in *T. aestivum* (Larsson *et al*., 1989), and in a dessication-tolerant moss, *Tortula ruralis* (Mahan *et al*., 1998). The inhibition observed in the present work was probably not directly due to a decrease in leaf water content since after 5 d both activities were inhibited by 20% without any alteration of leaf RWC when compared with the control plants. In addition, after 1 week an inhibition of 35% was reached when there was only a 5% decrease in leaf RWC. Moreover, as leaf water potential did not vary significantly after 7 d, it can also be concluded that the decrease of NR activity was not directly related to the chemical potential of water.

According to the literature, the decrease in NR activity is linked to the decline in the rate of photosynthesis due to stomatal closure (Keiser and Brendel-Benisch, 1991), rather than to water stress itself (Sharkey, 1990). This implies that the leaf internal CO_2 concentration (C_i) plays a role in NR activity. Indeed, it was observed, in durum wheat, that C_i values were already quite low in dehydrated plants on the 5th day, suggesting that C_i decline accounted for the decrease of NR activities. Two days after re-watering, when the C_i values had returned to the control values, NR activities increased but remained lower than in control leaves, which is by contrast to the total recovery observed in tobacco (Férario-Méry *et al*., 1998), sunflower, and lupin (Correia *et al*., 2005). In addition, leaf NR activities after 11 d of watering deprivation were not entirely alleviated by 5% CO_2. Moreover, in the presence of a strong inhibition of A_n (Fig. 3A), NR activities were inhibited by 80% after the slow drought treatment while they only decreased by about 30% after 4 h in CO_2-free air. It can thus be concluded that, during a slow drought, only part of the modulation of NR activity is due to a change in the leaf CO_2 molar ratio.

Under light conditions, a rapid dehydration obtained by cutting leaves caused a rapid and complete inhibition of A_n and a decline of both NR activities of the same order of magnitude as that produced by low CO_2. Although calculations of C_i in rapidly dehydrating leaves were probably inaccurate (Cornic and Masacci, 1996), it is possible that the decrease in C_i observed (data not shown) could have brought about the decrease in NR activities. Furthermore, regulation of NR by stomatal closure under light conditions was also shown by the effect of 10 μM ABA fed through the transpiration stream of cut leaves. However, the 5 μM ABA treatment, that caused a 20% inhibition of A_n, hardly affected NR activities, suggesting that the observed C_i decrease, in this case, was not large enough to produce a significant down-regulation of the different enzyme activities.

It should be noted that Kaiser and Förster (1989), as well as Kaiser and Brendle-Benisch (1991), obtained a rapid and strong inhibition (80–90%) of active spinach leaf NR, by CO_2-free air, without affecting total NR activity (Kaiser and Brendle-Benisch, 1991). Obviously, total NR activity behaves differently in *T. durum* leaves submitted to CO_2-free air.

It is worth noting that the decrease in NR activities of well-hydrated plants brought about by CO_2-free air (~30%, Fig. 3A) was within the range of stimulation (~20%, Table 2) caused to enzyme activities measured on leaves dehydrated for 11 d and then maintained for 4 h at a high atmospheric CO_2. This suggests that some regulation of NR activities by changes in C_i did occur in hydrated leaves and this was not altered by drought.

The mechanism of NR modulation by C_i was also investigated by Kaiser and Spill (1991); the effect of low CO_2 on NR activities was restored by adding AMP to leaf extracts. They suggested that low CO_2 modulates NR activity via the ATP/AMP ratio, which is linked to the phosphorylation/dephosphorylation state of protein. However, this seems unlikely in durum wheat leaves because the NR activation state was unchanged during all experimental conditions.

Foyer *et al*. (1998) and Ferrario-Méry *et al*. (1998) reported a decrease in the NR transcript levels in maize and tobacco leaves during a drought, suggesting that there is a decline in protein synthesis. Owing to the high rate of NR turnover (Zielke and Filner, 1971; Li and Oaks, 1993), the rate of NR proteolysis could become progressively higher than the rate of NR synthesis. This could occur in *T. durum* leaves during a slow drought.

In agreement with the maize (Foyer *et al*., 1998) and tobacco (Ferrario-Méry *et al*., 1998) results, a linear relationship between the rate of net CO_2 uptake by leaves and NR activity was also found in durum wheat cv. Lloyd during dehydration and re-hydration (Fig. 4), showing that a tight relationship exists between carbon and nitrogen assimilation.

Modulation of SPS activity by drought

Slow drought induced an increase in SPS_{max} and SPS_{lim} activities in durum wheat, being close to their maximum when photosynthesis was inhibited by ~50%, corresponding to an RWC of 85%. As for NR, this change in activities was probably not due to a modified RWC, which decreased by only 10%, or to the decline in leaf water potential, which remained constant while SPS activities were still increasing.

The present observations are in agreement with data reported by Yang *et al*. (2002) in rice and by Niedzwiedz-Siegien *et al*. (2004) in *Triticum aestivum* leaves submitted to mild drought. By contrast, many other reports have described either a decrease in SPS_{max} (in

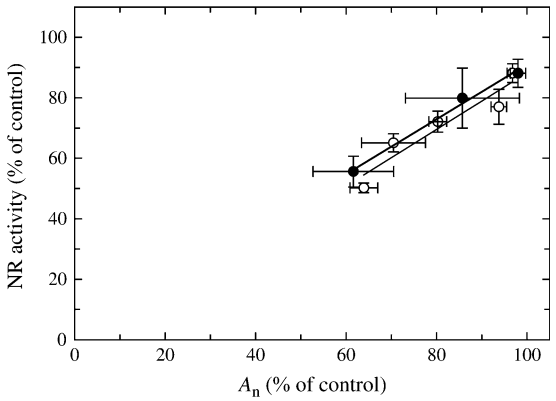

Fig. 4. Relationship between NR total activity during a drought (open circles) and during a 48 h re-watering period (closed circles) as a function of A_n expressed as a percentage of control leaves (turgid, third leaves of the same age). Mean values are given ±SE (n=3–5).

Phaseolus vulgaris: Vassey and Sharkey, 1989; Vassey *et al*., 1991; Castrillo, 1992; in *Zea mays*: Pelleschi *et al*., 1997), or a constant activity (in spinach: Quick *et al*., 1989; Zrenner and Stitt, 1991) during leaf drought stress.

By contrast to NR, the increase in SPS activities observed was not correlated with changes in C_i, since lowering A_n by decreasing the CO_2 molar ratio around well-watered leaves, from 370 ppm to a value lower than that of the CO_2 compensation point, barely affected the SPS activity. This observation is also in disagreement with the results of Vassey *et al*. (1991), showing that the SPS_{max} activity was sensitive to the atmospheric CO_2 molar ratio. Indeed, these authors showed that SPS_{max} activity, which was inhibited by 40–60% in leaves maintained under light at a CO_2 molar ratio close to the CO_2 compensation point, was restored to a control level after low CO_2-treated leaves were left for 20 min in an atmosphere containing a high CO_2 molar ratio (1%).

It is not easy to explain why drought appears to affect SPS activities differently according to plant species. However, in *T. durum* cv. Lloyd this increase was probably triggered in some way by ABA synthesized during the drought, since exogenous ABA fed to cut leaves increased SPS activities to a level similar to those brought about by a mild water deficit. Moreover, Yang *et al*. (2002) found that ABA concentrations in rice leaves during drought were positively correlated to the maximum SPS activity and to the remobilization of pre-stored carbon. In addition, Trouverie *et al*. (2003) have shown that the activity of another sugar metabolism enzyme, an acid invertase, was correlated with xylem sap ABA concentrations in water-stressed maize leaves and increased in excised maize leaves supplied with ABA.

The modulation of SPS activity could be linked to changes in the carbohydrate pool size brought about by a decreasing photosynthesis rate during drought. Wheat, which belongs to the Poaceae family, contains monosaccharides, sucrose, and fructans (Pollock and Cairns, 1991). The fructan pool is highly increased during a drought (Keresepi *et al*., 2002) and, as suggested by Goggin and Setter (2004) and Keresepi *et al*. (2002), one function of these fructans could be the protection against dehydration as osmoprotectants. Thus, it can be speculated that the increase in SPS activity during the first steps of the drought process is related to an increase in soluble sugar content in *T. durum* (including fructans). In support of this hypothesis, it was observed that 7 d after watering was withheld the total soluble sugar (sucrose+glucose+fructose) content was 25% higher in comparison with control leaves (preliminary unpublished data). The fact that a rapid osmotic adjustment of the leaves of dehydrating plants was observed is also in agreement with this hypothesis.

By contrast to what was observed for NR activity, SPS activities did not change after plants were re-watered, indicating that the increase caused by stopping plant watering was not transiently induced and, as noted above, not directly dependent on leaf water status. This is by contrast to the results of Vassey and Sharkey (1989) and Zrenner and Stitt (1991) who found a total recovery after 2 d of re-hydration, after a drought-induced decrease in SPS_{max}.

Interestingly, in *T. durum* cv. Lloyd, although SPS activities extracted from control leaves were insensitive to a decrease in the atmospheric CO_2 molar ratio, these activities were somewhat sensitive to CO_2 and showed activation after stressed leaves were maintained at high CO_2 for 4 h.

Conclusion

Overall, the results obtained on *Triticum durum* 'cv. Lloyd' leaves show that the effect of drought on NR and SPS activities is slightly different from that already reported on other plants such as spinach, bean, and maize. Therefore, the response of these enzymes to water stress is obviously different.

As in other plants, NR activity of durum wheat was down-regulated by a decrease in the CO_2 molar ratio. However, such a decrease in C_i during drought stress cannot completely explain the inhibition of NR activities under this condition. By contrast to other plants, re-watering did not totally restore NR activities in durum wheat. It is concluded that a drought-induced decrease of the leaf internal CO_2 concentration is only part of the signal triggering the decrease in NR activity. Some non-stomatal effects such as decreases in mRNA levels or in nitrate supply could also be involved.

In durum wheat, SPS_{max} activity increased during a drought treatment; this is a similar response to that of other plants in the Poaceae family (i.e. rice and *T. aestivum*),

while other species (e.g. spinach, maize, and sunflower) show the opposite response. Although, SPS activity could be up-regulated by a high CO_2 molar ratio in dehydrated leaves, it appears that this enzyme is not sensitive to a decrease in CO_2 molar ratio in control leaves. In wheat, SPS up-regulation by ABA, produced during a mild drought, could be linked to a more general response, where an increase in soluble sugar content (osmotic adjustment) would prevent leaf dehydration.

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