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Cytokinin-induced changes of nitrogen remobilization and chloroplast ultrastructure in wheat (*Triticum aestivum*)

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Summary

Nitrogen (N) remobilization in wheat (*Triticum aestivum*) plants is crucial because it determines the grain protein concentration and the baking quality of flour. In order to evaluate the influence of cytokinins on N remobilization during N starvation, we analyzed various N remobilization parameters in wheat plants that were watered with 6-benzylaminopurine (BAP) either with or without KNO_3. Besides, the effects of BAP on protein synthesis were evaluated, and the size and ultrastructure of chloroplasts of BAP-treated plants were studied. BAP supply inhibited N remobilization of plants independently of N supply as shown by the increase in protein, Rubisco, chlorophyll, sugar and starch concentrations in the older leaves, the decrease in amino acid and sugar export to the phloem, and the decrease in protein, Rubisco and chlorophyll concentrations in the younger leaves. Besides, BAP supply increased nitrate reductase activity and decreased nitrate concentration, thus suggesting an increased assimilatory capacity. The increase in protein concentration could be explained mainly by a significant decrease in protein degradation and, to a lesser extent, by an increase in protein synthesis. Finally, an increase both in the size of the chloroplast and in the plastoglobuli and starch contents in BAP-supplied plants was observed. We propose that cytokinins retain the sink activity of the older leaves by inhibiting amino acid and sugar export to the phloem and stimulating assimilate accumulation in the chloroplasts of the older leaves. Besides, BAP may increase

Abbreviations: ABA, abscisic acid; BAP, 6-benzylaminopurine; G, grana; I, protein synthesis inhibitors; iPA, isopentenyl adenosine; L3, the third leaf; L5, the fifth leaf; LS, Rubisco large subunit; N, nitrogen; NR, nitrate reductase; P, plastoglobuli; S, stroma; SI, starch inclusion; SS, Rubisco small subunit; T, thylakoid.

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protein concentration of the older leaves both by decreasing protein degradation and maintaining protein synthesis even under stress conditions.

Introduction

Senescence can be described as a nutrient remobilization process where mature leaves behave as source organs, providing both carbon and nitrogen organic molecules to the sink tissues, including young developing roots, leaves and seeds (Gan and Amasino, 1997; Roitsch and Ehness, 2000). In wheat (*Triticum aestivum*), 60–95% of the grain nitrogen (N) comes from the remobilization of N stored in roots and shoots before anthesis (Hirel et al., 2007), determining the grain protein concentration and the baking quality of flour. This remobilization is carried out by proteolytic enzymes that hydrolyze leaf proteins, releasing amino acids that may be transported to the ear (Vierstra, 1996). Degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) has been considered as a key component of the N-redistribution process since it constitutes the main plant reserve of N (Crafts-Brandner et al., 1998). However, the way in which the proteolysis and the export rate of amino acids are regulated is far from clear (Schwechheimer and Schwager, 2004; Barneix, 2007). Increased knowledge of the regulatory mechanisms controlling plant N economy is necessary for improving N use efficiency and for reducing excessive input of fertilizers, while maintaining an acceptable yield (Hirel et al., 2007).

Leaf senescence is controlled by many internal and external factors and can be accelerated or delayed by alteration of these signals (Feller and Fischer, 1994). The external cues include stresses such as extreme temperatures, drought, ozone, nutrient deficiency, pathogen infection, wounding, and shading. Among these stresses, limited N availability is one of the main factors that induce senescence in crop leaves (Gan and Amasino, 1997; Crafts-Brandner et al., 1998).

Cytokinins are a group of plant growth regulators which play a role in many aspects of plant growth and development, such as cell division, photosynthesis, senescence, chloroplast development and assimilate partitioning (reviewed by Binns, 1994). Despite the importance of cytokinins, this group of plant growth regulators is the least understood with respect to the mode of action (Balibrea Lara et al., 2004). The way by which physiological effects are evoked at the molecular level and, especially, how cytokinins regulate assimilate partitioning remains to be elucidated (Roitsch and Ehness, 2000). Transgenic tobacco (*Nicotiana tabacum*) plants expressing a cytokinin biosynthesis gene (isopentenyl transferase, *ipt*) under the control of the senescence-specific promoter *SAG12* (P_{SAG12}-*IPT*) have been used to avoid a direct interference with other aspects of normal plant development (Gan and Amasino, 1995). However, later studies have demonstrated that there are also indirect effects in these transgenic tobacco plants, and that the sink–source relation is modified, showing a preferential allocation of N to old senescing leaves and a reduced N accumulation in young leaves (Jordi et al., 2000; Cowan et al., 2005). Besides, only few reports have focused on the cytokinin-induced expression of genes that codify for enzymes such as nitrate reductase (NR), extracellular invertase and hexose transporter (Brenner et al., 2005).

The aim of this work was to gain new insights into the knowledge of the role of cytokinins on reserve mobilization from older to younger leaves. Based on the studies mentioned above, we propose that cytokinins determine the source–sink relations of reserve remobilization by inhibiting assimilate exports to the phloem, and stimulating their accumulation in the chloroplasts of older leaves. To test this hypothesis, we studied assimilate partitioning in plants subjected to both N-starvation and 6-benzylaminopurine (BAP) addition, a synthetic cytokinin that has been widely used in plant physiology studies (Roitsch and Ehness, 2000).

Materials and methods

Plant material and growth conditions

Wheat (*Triticum aestivum* L. var. Pro INTA Isla Verde) seeds were germinated on moist filter paper at 25 °C in the dark. The seedlings were then transferred to plastic pots containing vermiculite and cultivated in a growth chamber at 25 °C, with a photoperiod of 16 h, and a photosynthetic photon flux density of 300 $\mu mol\,m^{-2}\,s^{-1}$ provided by General Electric® 400 W HPL lamps. Each pot was supplied with 50 mL of nutrient solution according to Hoagland and Arnon (1950) with 10 mM KNO_3.

Seventeen days after sowing (0 h), plants were watered with nutrient solution either with or without 10 mM KNO_3 and either with or without 20 μM BAP (SIGMA, USA) at a rate of 50 mL per pot per day for 2 consecutive days. For convenience, plants were designed as N+, N−, N+/BAP and N−/BAP, respectively. The concentration used for the BAP treatment was chosen from a dose–response curve (data not shown). The third leaf (last fully expanded leaf, L3) and the fifth leaf (young expanding leaf, L5) from the base of all treated plants were collected at 48 h. N+ plants were used as controls since they showed no differences with plants collected at 0 h (Criado et al., 2007). Samples were weighed, immediately frozen in liquid nitrogen, and stored at −50 °C until used for biochemical determinations.

Biochemical analysis

Leaf tissue was homogenized (2.5 mL buffer g^{-1} fresh weight) with 50 mM Tris–HCl buffer pH 7.5 containing 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged at 10,000*g* for 20 min. The supernatant was used for the determination of soluble protein (Bradford, 1976), amino acid (Yemm and Cocking, 1955), sugar (Yemm and Willis, 1954) and nitrate (Cataldo et al., 1975) concentrations, and for the determination of NR (EC 1.6.6.1) activity (Lewis et al., 1982). For amino acid and sugar analysis, aliquots of homogenate were quickly precipitated with trichloroacetic acid or boiled, respectively. Chlorophyll and starch contents were determined in the leaf homogenates prior to centrifugation, according to Arnon (1949) and McCready et al. (1950), respectively.

Collection of phloem exudates

L3 of each plant was excised, and the cut ends of five leaves were placed into 1 mL of 20 mM ethylene-diamine-tetraacetic acid (EDTA) pH 8.0 solution (Caputo and Barneix, 1997) in 10-mL glass tubes. After 15 min in the dark, this pre-incubation solution was discarded since this exudate consists primarily of xylem and cellular fluid. The leaves were rinsed, transferred to another 1 mL of the same solution, and kept for 3 h in the dark to avoid transpiration. The exudates were stored at −18 °C until amino acid and sugar determinations.

Analysis of plant growth regulators

Isopentenyl adenosine (iPA) and abscisic acid (ABA) were extracted as previously described (Criado et al., 2007) and determined by ELISA using monoclonal antibodies (Phytodetek, Agdia, Elkhart, IN, USA) following the protocols provided by the manufacturer.

Rubisco content and expression

Protein extracts were analyzed by 15% SDS-PAGE according to Laemmli (1970). The same amount of extract corresponding to 3 mg of leaf fresh weight was loaded in each lane. For visualization of Rubisco, gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250. The mRNA level of the Rubisco small subunit (SS) was determined using the semiquantitative RT-PCR method. Total RNA was extracted from 500 mg leaf by using Concert TM Plant RNA reagent according to the manufacturer's instructions (Invitrogen, USA). An aliquot of 1 μg DNAse-treated RNA was used for cDNA synthesis using SuperScript® II Reverse Transcriptase and Oligo (dT)12–18 Primer also as indicated by the manufacturer (Invitrogen, USA). The primers sequences used for PCR amplification were sense: 5′-ACCCTTCCAGGGTCTCAAGT-3′ and antisense: 5′-TTGTCCAGTATCGACCATCG-3′ for wheat SS (AB042068), and sense: 5′-TCCGTCTTCCCCTCCAGGAC-3′ and antisense: 5′-GCAGGGCCTCCAGCTCCTTA-3′, for wheat elongation factor 1−α (TEF1, M90077). The SS and TEF1 PCR products were electrophoresed on 1.5% agarose gel and visualized by staining with ethidium bromide. Images were recorded using Kodak Digital Science TM Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, Rochester, NY, USA) associated with Kodak 1D Image Analysis Software. The integrated density values of the bands from protein and PCR products were analyzed using Image J. software, free available at http://rsb.info.nih.gov/ij/index.html.

Inhibitors of protein synthesis

The third leaves of 17-day-old plants were detached and placed in 50-mL glass tubes with BAP or/and inhibitors of protein synthesis and incubated in the growth chamber under the same conditions. Control leaves were incubated with distilled water. The solutions were changed at 24 h and the leaves were sampled at 48 h. The solutions used were: 5 μM BAP, 0.1 μM puromycin (SIGMA, USA), 10 μM fusidic acid (SIGMA, USA), and 1 mM chloramphenicol (SIGMA, USA). Puromycin and fusidic acid are potent inhibitors of protein synthesis of both prokaryotic and eukaryotic systems (Carpenter and Cherry, 1966;

Goswami et al., 1973). Although chloramphenicol is a specific inhibitor of protein synthesis of prokaryotic systems, there is good evidence that chloramphenicol inhibits protein synthesis in higher plants, probably by inhibiting synthesis of chloroplast-encoded proteins (Margulies and Brubaker, 1970).

Light microscopy

The central area of L3 of N+ and N+/BAP plants was fixed in FAA (50% ethanol+5% formaldehyde+10% glacial acetic acid, in water) for 48 h, dehydrated in an ethanol series, and embedded in paraffin. Then, the segments were transversally sectioned at 8–10 μm with a Minot-type rotary microtome and stained with safranin-fast green (Johansen, 1940). Chloroplasts were visualized with a Nikon Eclipse 50i microscope and images were taken with a colour digital camera (Nikon Coolpix S10). Finally, the area of chloroplasts was measured on digital micrographs by using UTHSCSA Image Tool software, freely available at http://ddsdx.uthscsa.edu/dig/itdesc.html.

Electron microscopy

Segments obtained as described above were fixed in 3% (v/v) glutaraldehyde in 20 mM phosphate buffer pH 7 at 20 °C for 2 h, and washed with the buffer for 1 h and postfixed in 1% (w/v) aqueous OsO_4 at 20 °C for 2 h. Samples were then washed with the same buffer for 1 h, dehydrated through a graded acetone series at 20 °C, and embedded in low viscosity Spurr's resin (Spurr, 1969). Ultrathin sections were cut using a glass knife and a Sorvall MT 2-B ultracut microtome, and transverse ultrathin sections were collected on formvar-coated slot copper grids and double stained with uranyl acetate and lead citrate (Reynolds, 1963). Sections were examined using a Jeol JEM 1200 EX II transmission electron microscope at an accelerating voltage of 80 kV.

Statistical treatment

Data were analyzed with a two-way ANOVA, except for the chloroplast size which was analyzed with a one-way ANOVA. The software used was Statistica (R). All experiments consisted of four replicates per treatment, where each replicate was a pot containing five plants. Each experiment was repeated at least twice and data from each experiment were analyzed separately. The results from the different experiments were very similar and thus only one is reported in this paper.

Results

Nutrient remobilization

After 48 h of N starvation, protein, chlorophyll, amino acid and starch concentrations in L3 decreased as compared to control plants (N+ plants), whereas sugar did not vary significantly (Table 1). Conversely, the supply of BAP to both N+ and N− plants significantly increased protein, chlorophyll, starch and sugar concentrations. No significant interactions were detected between treatments for each parameter (Table 1).

On the other hand, in expanding leaves (L5), a decrease in protein and chlorophyll, but not in amino acid, starch and sugar concentrations, was observed in N− plants. The supply of BAP to N+ plants decreased protein and chlorophyll concentrations, while the supply of BAP to N− plants had no effect (Table 1).

The export of amino acids to the phloem from L3 in N-deprived plants significantly decreased 40% as compared to the control plants, whereas the sugar export was not affected (Table 2). The supply of BAP decreased both the export of amino acids and sugars to the phloem in N+ and N− plants (Table 2). An additive effect for the amino acid export was observed.

Assimilatory capacity

When NR activity and nitrate concentration were determined in both L3 and L5, significant effects of both N and BAP treatments – but no interaction effects between them – were found. NR activity decreased with the starvation of N, and increased with the supply of BAP (Table 3). Nitrate concentration also decreased in N− plants, but decreased even more when BAP was added to both N+ and N− plants in both L3 and L5 (Table 3).

Plant growth regulators

iPA concentration decreased in both L3 and L5 of N− plants after 48 h of treatment as compared to N+ plants (Table 4). It was interesting to note that when plants were supplied with BAP, a dramatic increase in iPA concentration in both L3 and L5 was observed. No interaction effect was detected between N and BAP treatments (Table 4). ABA concentration remained constant in all the treat-

Table 1. Content of protein, chlorophyll, amino acids, sugars and starch in L3 and L5 of N+, N−, N+/BAP and N−/BAP plants after 48 h of treatment.

	L3					L5				
	N+	N−	N+/BAP	N−/BAP	ANOVA summary	N+	N−	N+/BAP	N−/BAP	ANOVA summary
Protein ($mg\,g^{-1}$ FW)	18.0 ± 0.3	16.6 ± 0.2	20.9 ± 0.2	19.1 ± 0.2	N p:0.025 BAP $p<0.001$ NxBAP p:0.481	20.2 ± 0.5	17.3 ± 0.2	17.3 ± 0.4	17.1 ± 0.4	N p:0.136 BAP p:0.038 NxBAP p:0.033
Chlorophyll ($mg\,g^{-1}$ FW)	1.50 ± 0.04	1.31 ± 0.04	1.74 ± 0.02	1.63 ± 0.02	N p:0.04 BAP p:0.001 NxBAP p:0.596	1.65 ± 0.01	1.25 ± 0.03	1.14 ± 0.04	1.28 ± 0.01	N p:0.138 BAP p:0.016 NxBAP p:0.010
Amino acids ($\mu mol\,g^{-1}$ FW)	16.2 ± 0.2	12.7 ± 0.5	14.7 ± 0.2	14.3 ± 0.3	N p:0.031 BAP p:0.044 NxBAP p:0.490	19.9 ± 0.3	18.6 ± 0.4	20.4 ± 0.4	21.1 ± 0.5	N p:0.602 BAP p:0.081 NxBAP p:0.090
Sugars ($\mu mol\,g^{-1}$ FW)	15.5 ± 0.1	14.9 ± 0.6	23.7 ± 1.4	20.5 ± 1.4	N p:0.183 BAP p:0.013 NxBAP p:0.291	24.0 ± 1.5	22.2 ± 1.0	23.4 ± 0.8	24.5 ± 1.0	N p:0.951 BAP p:0.606 NxBAP p:0.473
Starch ($mg\,hexose\,g^{-1}$ FW)	31.1 ± 0.9	23.1 ± 0.6	40.8 ± 0.8	33.5 ± 0.6	N $p<0.001$ BAP $p<0.001$ NxBAP p:0.855	47.4 ± 0.8	46.3 ± 1.2	45.6 ± 1.4	48.2 ± 1.4	N p:0.773 BAP p:0.986 NxBAP p:0.500

Values are means ± SE. Values of $p<0.05$ are considered statistically significant.

Table 2. Content of amino acids and sugars in 1 mL EDTA solution containing the phloem exudates collected for 3 h from five expanded L3 of N+, N−, N+/BAP and N−/BAP plants after 48 h of treatment.

	N+	N−	N+/BAP	N−/BAP	ANOVA summary
Amino acids (nmol mL^{-1})	162 ± 4	94 ± 3	101 ± 4	82 ± 2	N $p<0.001$; BAP $p<0.001$ NxBAP p:0.002
Sugars (nmol mL^{-1})	246 ± 13	248 ± 21	139 ± 13	120 ± 10	N p:0.766; BAP p:0.002 NxBAP p:0.733

Values are means ± SE. Values of $p<0.05$ are considered statistically significant.

ments studied in this work, with ABA content being always higher in the young leaves (Table 4).

Rubisco content and expression

When the extracts of L3 in N− plants were analyzed by SDS-PAGE, a decrease in the Rubisco large subunit (LS) was observed (Figure 1A). On the contrary, the LS content significantly increased in the plants supplied with BAP, reaching 132% and 118% in N+/BAP and N−/BAP plants, respectively (Figure 1A). In L5, both N starvation and BAP supply caused a decrease in Rubisco content, being the decrease even higher with the supply of BAP (Figure 1A).

On the other hand, the expression of the SS decreased in N− plants as compared to controls. This decrease was reversed by the supply of BAP (Figure 1B). It is of interest to note that the supply of BAP had no effect on the expression of the SS in N+ plants (Figure 1B).

Inhibitors of protein synthesis

Since an increase in protein (Table 1) and Rubisco (Figure 1A) concentrations, but not in Rubisco expression (Figure 1B), was noticed in the L3 of N+/BAP plants, detached L3 of N+ plants were treated for 48 h with different protein synthesis inhibitors (I), after which a slight but significant decrease in protein concentration was observed (Figure 2A). This effect was reversed with the addition of BAP (Figure 2A). Amino acid concentration increased after treatments with the inhibitors of protein synthesis. This increase was also reversed by the addition of BAP (Figure 2B).

Morphology and size chloroplast

Leaf sections of BAP-supplied plants observed with light microscopy exhibited a significant increase in chloroplast size as compared to control plants (Figure 3). On the other hand, when we analyzed the leaf ultrathin sections of treated plants by transmission electron microscopy, we observed considerable chloroplast ultrastructure alterations. Chloroplasts were swollen and presented numerous plastoglobuli (P) and large starch contents. As compared with controls, the grana (G) system was always restricted to the periphery of the chloroplasts (Figure 4).

Discussion

Little information is known about the cytokinin role during leaf transition from sink to source function. Our studies provide knowledge of the metabolic changes occurring during the leaf transition process in the whole plant by the addition of the synthetic hormone BAP. We found that BAP increased protein, chlorophyll, Rubisco, sugar and starch concentrations in L3 (Table 1 and Figure 1) and decreased protein and chlorophyll concentrations in L5 of N+ plants (Table 1). These results indicate that BAP compromises the reserve remobilization by maintaining the older leaves as sink organs and depriving the younger leaves of N. In agreement with this, it has been previously observed that P_{SAG12}-*IPT* plants which increase their cytokinin levels, show a preferential allocation of N to old senescing leaves and a reduced N accumulation in young leaves (Jordi et al., 2000; Cowan et al., 2005).

The cytokinins mode of action involves the inhibition of amino acid and sugar exports to the phloem (Table 2). In that respect, Roitsch and Ehness (2000) found that the extracellular invertase and hexose transporters – two functionally linked enzymes of an apoplastic phloem unloading pathway – are co-induced by cytokinins, and suggested that the stimulated sucrose cleavage in the apoplast should both induce transport to the cytokinin-treated area and inhibit export from

Table 3. NR activity and nitrate content in L3 and L5 of N+, N−, N+/BAP and N−/BAP plants after 48 h of treatment.

	L3					L5				
	N+	N−	N+/BAP	N−/BAP	ANOVA summary	N+	N−	N+/BAP	N−/BAP	ANOVA summary
NR activity (pmol NO_2^- h^{-1} g^{-1} FW)	657 ± 14	188 ± 4	874 ± 27	510 ± 29	N $p<0.001$ BAP $p<0.001$ NxBAP p:0.239	167 ± 3	113 ± 2	313 ± 1	254 ± 4	N $p<0.001$ BAP $p<0.001$ NxBAP p:0.708
NO_3^- (mg g^{-1} FW)	6.30 ± 0.03	4.78 ± 0.08	4.35 ± 0.02	3.97 ± 0.04	N $p<0.001$ BAP $p<0.001$ NxBAP p:0.003	1.12 ± 0.04	0.73 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	N $p<0.001$ BAP $p<0.001$ NxBAP p:0.002

Values are means ± SE. Values of $p<0.05$ are considered statistically significant.

Table 4. Content of iPA and ABA in L3 and L5 of N+, N−, N+/BAP and N−/BAP plants after 48 h of treatment.

	L3					L5				
	N+	N−	N+/BAP	N−/BAP	ANOVA summary	N+	N−	N+/BAP	N−/BAP	ANOVA summary
iPA (pmol g^{-1} FW)	15.8 ± 1.3	1.5 ± 0.2	322 ± 23	200 ± 10	N p:0.017 BAP $p<0.001$ NxBAP p:0.058	61.7 ± 5.8	15.9 ± 1.3	441 ± 66	153 ± 15	N p:0.024 BAP p:0.001 NxBAP p:0.086
ABA (pmol g^{-1} FW)	17.3 ± 2.3	20.6 ± 3.4	25.4 ± 1.6	22.9 ± 1.2	N p:0.919 BAP p:0.221 NxBAP p:0.484	81.5 ± 14.7	97.7 ± 6.3	100.3 ± 17.1	94.9 ± 15.7	N p:0.831 BAP p:0.750 NxBAP p:0.669

Values are means ± SE. Values of $p<0.05$ are considered statistically significant.

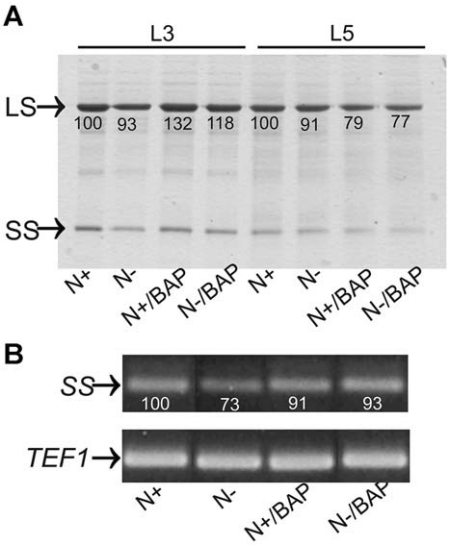

Figure 1. (A) Protein profiles of L3 and L5 of N+, N−, N+/BAP and N−/BAP plants in 15% SDS-PAGE. The gel was stained with R 250. (B) The *top panel* represents the accumulation of SS transcripts in L3 of N+, N−, N+/BAP and N−/BAP plants. TEF1-stained gel was used to assess the RNA as described in Materials and methods (*bottom panel*). Numbers in each lane represent the average density of the band as a percentage of the N+ plants band. The data were derived from at least three independent experiments.

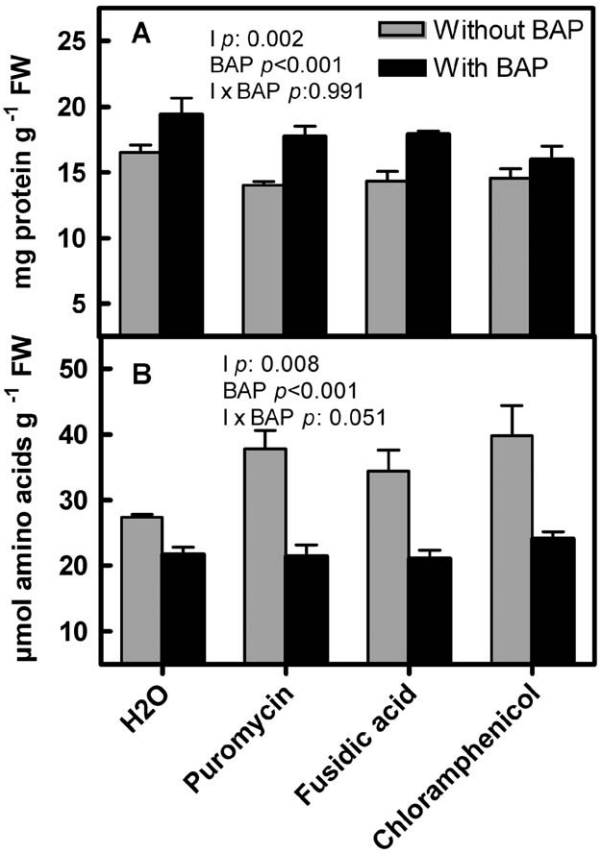

Figure 2. Contents of proteins (A) and amino acids (B) in detached leaves after 48 h of incubation with water, puromycin, fusidic acid and chloramphenicol either with or without BAP. Values are means ± SE ($n = 4$). The *p*-values indicate the significance of the I, BAP, and BAP × I interaction effects, all calculated from a two-way ANOVA.

these areas. Then, Balibrea Lara et al. (2004) demonstrated that the extracellular invertase is an essential component of the molecular mechanism of delay of senescence by cytokinins. In relation to the influence of cytokinins on the export of amino acids to the phloem, in a recent work, we found that the leaf cytosolic glutamine synthetase (GS1) might act as a key enzyme in the cytokinin-mediated amino acid allocation via phloem between source and sink tissues (Caputo et al., 2009).

Since NR is the first enzyme of the nitrate assimilation pathway, the increased NR activity observed in the BAP-treated plants (Table 3) indicates that BAP also modifies the N assimilatory capacity of plants. NR is transcriptionally induced by nitrate and is repressed by glutamate, cysteine, asparagines and malate (Masclaux et al., 2001; Stitt et al., 2002). Our finding strongly supports that NR is also induced by cytokinins. Besides, we found that BAP decrease the nitrate concentration in both N+/BAP and N−/BAP plants (Table 3). This decrease could be attributed to the increased assimilatory activity.

Physiological studies have demonstrated that an exogenous application of cytokinin causes a delay in senescence, and that the level of endogenous cytokinins decreases in most senescing tissues (Gan and Amasino, 1997; Buchanan-Wollaston et al., 2003; Criado et al., 2007). Indeed, in the present experiments, iPA concentration decreased in both L3 and L5 N-deprived plants (Table 4). It is worth noticing that when plants were supplied with BAP a dramatic increase in the natural cytokinin iPA was noticed (Table 4), thus suggesting that the mode of action of the synthetic hormone is through an increase in iPA concentration. Furthermore, Kamínek et al. (1997) have reported that BAP in wheat germ competes with iPA as substrate of cytokinin oxidase, decreasing iPA degradation. In the present experiments, neither N starvation nor BAP supply modified ABA concentration (Table 4). Since ABA is a stress-induced plant growth regulator (Anderson et al., 2004; Hu et al., 2005), these results indicate that the dramatic increase in iPA may not cause stress in plants. The results are consistent with our previous report in which we showed that ABA

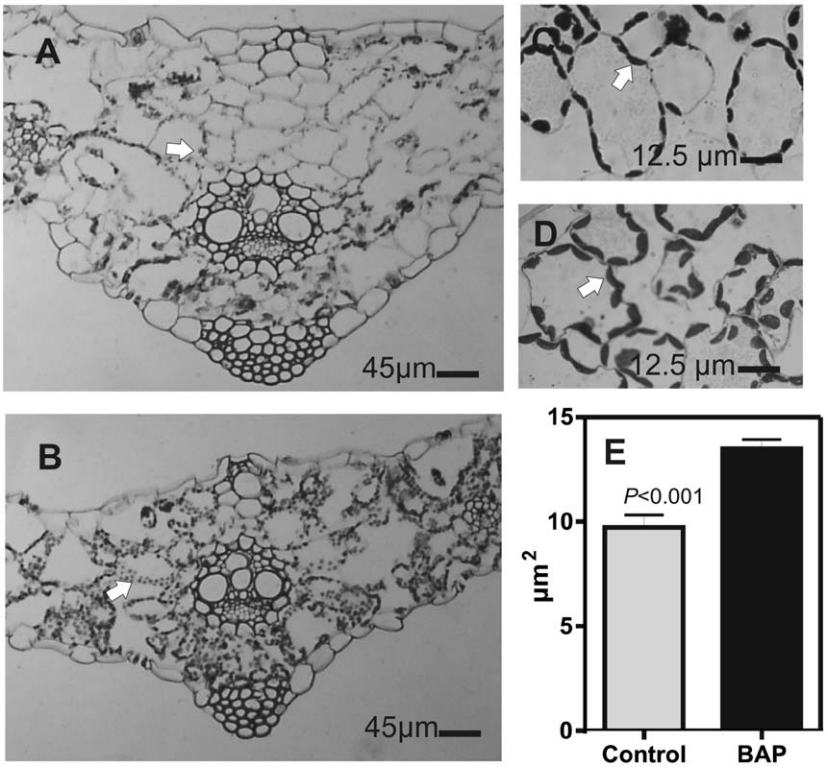

Figure 3. Transversal sections of L3 of N+ (A) and N+/BAP (B) plants. Detail of a mesophyll cell of N+ (C) and N+/BAP (D) plants. The arrows indicate characteristic chloroplasts. (E) Size of chloroplasts in N+ and N+/BAP plants. Values are means ± SE of five independent leaf samples. Sixteen chloroplasts per sample were sized.

increases only after 72 h of N starvation in wheat plants (Criado et al., 2007).

The increase in total protein concentration after BAP supply (Table 1 and Figure 1) may be a consequence either of the redirection of the not-exported amino acids to the synthesis of protein or of an inhibition of protein degradation. In the present work, we observed that the mRNA levels of the SS were kept high by the addition of BAP in N− plants, thus suggesting a continued synthesis of the enzyme. We also demonstrated, using I, that BAP reversed the effect of these inhibitors on protein and amino acid concentrations (Figure 2). Taken together, these results indicate that BAP is acting by inhibiting protein degradation and increasing the synthesis of proteins in N-stressed plants. Similarly, Takegami (1975) demonstrated that BAP promotes synthesis and inhibits degradation of proteins in tobacco leaf disk. Also, Ananieva and Ananiev (1999) showed that BAP stimulates protein and RNA synthesis in cotyledons of zucchini.

BAP produced an increase in the size of chloroplasts, mainly through the swelling of the stroma (S), thus producing starch grains larger than those of control plants (Figure 4). What remains to be determined is if these alterations are a specific effect of BAP itself, or a consequence of the increased accumulation of Rubisco and starch. Unfortunately, only fragmented information about cytokinin effects on chloroplast development has been published. It has been shown that when half of the sugar-beet leaf is sprayed with BAP, a changeover from the source to the sink function and an increase in the levels of starch and lipids in the chloroplast are observed (Paramonova et al., 2002). Also, Zavaleta-Mancera et al. (1999) have shown that BAP accelerates the redevelopment of G and S in regreening plastids, with an increase in chloroplast size and P and starch contents as compared with the senescent leaves.

In summary, cytokinins retain the sink activity of the older leaves by inhibiting amino acid and sugar export to the phloem, redirecting the incoming nitrate into these leaves, and increasing both protein and sugar concentrations. As a consequence, despite their increased assimilatory capacity, the young expanding leaves become N starved with decreased protein concentration. Besides, BAP may increase protein concentration of the older leaves by both maintaining protein synthesis even under stress conditions, and decreasing protein degradation.

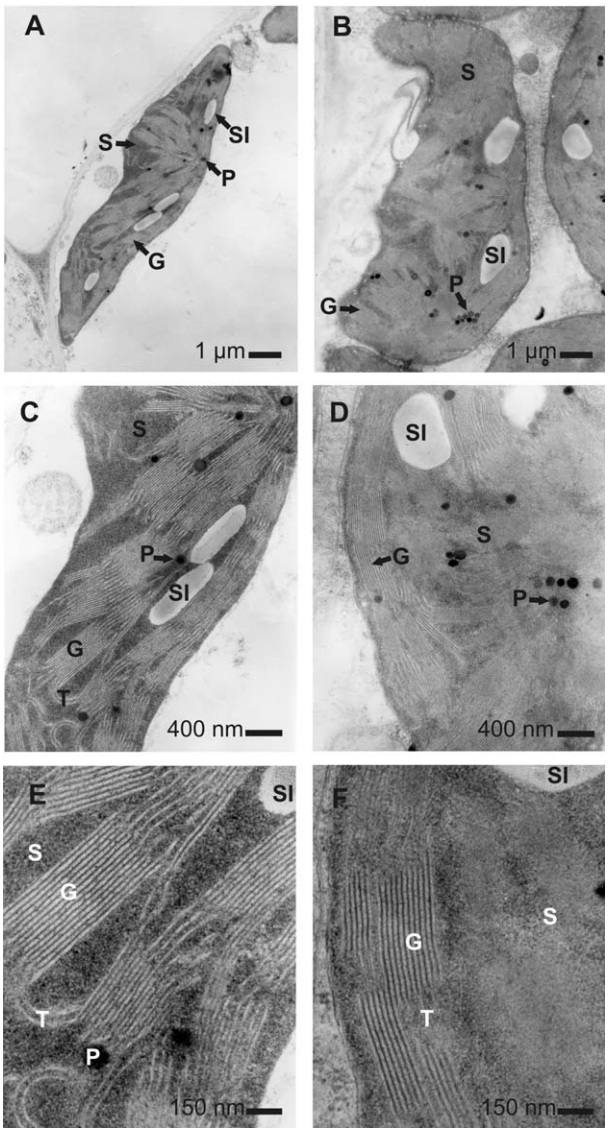

Figure 4. Ultrastructure of chloroplasts from leaves of N+ plants (A, C, E) and N+/BAP plants (B, D, F).

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