Expression of thioredoxins $f$ and $m$, and of their targets fructose-1,6-bisphosphatase and NADP-malate dehydrogenase, in pea plants grown under normal and light/temperature stress conditions

Eduardo A. Pagano¹, Ana Chueca² and Julio López-Gorgé²,³

¹ Faculty of Agronomy, Universidad de Buenos Aires, Argentina
² Department of Plant Biochemistry, Estación Experimental del Zaidín (CSIC), 18008-Granada, Spain

Received 27 September 1999; Accepted 12 March 2000

Abstract
Thioredoxins (Trxs) $f$ and $m$, as well as their targets chloroplast fructose-1,6-bisphosphatase (FBPase) and NADP⁺-malate dehydrogenase (NADP-MDH), displayed transcriptional expression in both photosynthetic and non-photosynthetic organs of pea plants (Pisum sativum L. cv. Lincoln) grown for 50 d under normal irradiance. However, whereas Trx $m$ and both target enzymes were poorly expressed in non-photosynthetic tissues, the content of the precursor form of the Trx $f$-specific mRNA was high in pea roots. In contrast, the translational expression of Trx $f$ was low in this organ. The high FBPase activity in immature seeds, and the low activity of leaves, must be related to high starch synthesis in the first, and with high sucrose formation in the second. The transcriptional expression of FBPase and NADP⁺-MDH, and to a lesser extent that of Trxs $f$ and $m$, was inhibited under low irradiance in plants grown under both normal and high temperatures. Pea plants grown at low temperature displayed a high level of mRNAs for Trxs and their targets, especially when the growth was carried out at low light. To a lesser extent, similar behaviour was observed at the protein level. Chloroplasts of mesophyll leaf cells of pea plants grown under saturating light, or under sub-saturating continuous irradiance, showed broken envelopes, distorted structural elements and disorganized starch grains, as a consequence of a photobleaching process and high starch accumulation.

Key words: Thioredoxins, FBPase, light, temperature, stress, pea plants.

Introduction
Thioredoxins (Trxs) are low molecular mass proteins (about 12 kDa) ubiquitous in bacteria, yeasts, algae, plants, and animals, where they appear involved in redox processes, but also in structural ones (Holmgren, 1985). Two chloroplast Trxs have been described in higher plants: Trx $f$, involved in the reductive activation of fructose-1,6-bisphosphatase (FBPase) and other Calvin cycle enzymes, and Trx $m$, specialized in NADP⁺-malate dehydrogenase (NADP-MDH) modulation (Jacquot et al., 1997). In addition, a third group of Trxs (Trx $h$) has been identified in the cytosol, of which up to five different isoforms have been described in Arabidopsis (Rivera-Madrid et al., 1995). It is noteworthy that, in spite of the different amino acid sequences of plant Trxs, all of them display similar size and folding (Eklund et al., 1991). In addition, they all support the -C-G-P-C- cluster which is responsible for their low redox potentials (in the range $−200$ and $−300$ mV), and that participates in the oxidation–reduction processes in which Trxs are engaged. These similarities make the existence of so many Trx isoforms very intriguing, and strengthen their involvement in additional processes.

Higher plants are subjected to the deleterious effects of partially reduced oxygen species (Foyer et al., 1994).
They are currently generated under normal growth, but their concentrations sharply increase when plants are subjected to stress conditions (Bohnert and Sheveleva, 1998). Plants have developed different mechanisms, either enzymatics (superoxide dismutases, ascorbate-peroxidase) or not (carotenoids, flavonoids, etc.), for the elimination of these toxic components. Among those of an enzymatic nature the ascorbate-peroxidase system, coupled to the glutathione cycle as an ascorbate regeneration mechanism is by far the most effective for H₂O₂ wasting (Foyer and Mullineaux, 1998; Noctor and Foyer, 1998).

However, some plants thiol-proteins have recently been described as involved in protection mechanisms. This is the case of 2-cys-peroxiredoxin BAS1, a homodimeric nuclear-coded chloroplast protein of about 28 kDa molecular mass per subunit, found in Arabidopsis and barley (Baier and Dietz, 1996). Although under investigation, it seems to be involved in the wasting of the oxygen singlet and alkyl hydroperoxides more than in H₂O₂ removal, against which peroxiredoxins display negligible activity. Because of its plastidic location, the ferredoxin-thioredoxin system could be involved in the regeneration of the reduced form of peroxiredoxin BAS1 (Baier and Dietz, 1997).

Trxs have a sufficiently low oxidation-reduction potential, about −290 mV for pea and spinach Trx f, and −300 mV for spinach and corn Trx m (Rebeille and Hatch, 1986; Hirasawa et al. 1999), for the fast dissipation of oxygen radicals or, alternatively, for an efficient restoration of the reduced forms of chloroplast scavenging compounds (Eₐ of glutathione and ascorbate are −250 and +58 mV, respectively). Indeed one unfavourable circumstance is the low concentration of Trxs within the chloroplast (100–160 μM in pea) (Scheibe, 1981), in comparison with 10–30 mM ascorbate (Ivanov, 1998) and 5 mM glutathione (Alschier, 1989). A high concentration of Trx h has been found in the phloem sap of rice plants (Ishiwatari et al., 1995), suggesting its involvement in the protection mechanisms against plant stress, although, as occurs with glutathione transfer (Herschbach et al., 1998), phloem is the path along which Trx could be exported to other plant organs.

Looking for targets of the different Trx isoforms, Verdoucq et al. have recently isolated a complex between a C35S mutant of AtTRX3, a h-type Trx from Arabidopsis thaliana, and a yeast protein which shows high homology with other proteins from bacteria, animals and Arabidopsis (Verdoucq et al., 1999). This protein displays low homology with peroxiredoxins, but shows a Trx-dependent peroxidase activity. Moreover, AtTRX3 confers H₂O₂ tolerance when introduced to Trx-depleted mutants of Saccharomyces cerevisiae (Meyer et al., 1999). Another case is that of a 32 kDa protein found in potatoes subjected to water stress (Reya et al., 1998). This chloroplast protein displays a high homology with Trxs in its carboxy-half, holds the CGPC motif of the Trx active site, and is able to reduce the insulin disulphide bridges. The authors propose, for this thiol-containing protein, a role as a defence mechanism against structural modifications of regulatory enzymes, a consequence of drought-induced ionic imbalances or an increase in active oxygen species.

The situation is even more confused with glutaredoxins, a group of about 11 kDa molecular mass proteins, which display low homology with Trxs, but parallel folding. They support a CPFC active cluster similar to that of Trxs, with which they show overlapping functions. Even though lacking endogenous dehydroascorbate reductase activity, a glutaredoxin isolated from rice aleurone seems to be involved in the oxygen scavenging system of this outer layer of the seed endosperm (Minakuchi et al., 1994).

NADP-MDH is a crucial enzyme in C₃ metabolism, where it acts as a CO₂ pump from the mesophyll cells to those of the bundle sheet tissue. By contrast, its role in C₄ plants is to act as a shuttle between the chloroplast and cytosol of photosynthetic cells in the transfer of reducing power among both compartments. Chloroplast FBPase is one of the regulatory enzymes of the CO₂ assimilation pathway and its key position in the Calvin cycle catalyses the flow of photoassimilated carbon either to starch accumulation within the chloroplast or to trioses-phosphate export to the cytosol for sucrose synthesis. This enzyme is inactive in the dark, but becomes active in the light by the reduction of an essential -S-S- bridge through the ferredoxin-thioredoxin system (Scheibe, 1990; Buchanan, 1992). A second FBPase isozyme exists in the cytosol of both photosynthetic and non-photosynthetic cells, where it appears involved in sucrose synthesis and the gluconogenic pathway. As occurs in bacteria, yeasts and mammals, it is not modulated by a light–dark switch, but by the AMP and fructose-2,6-bisphosphate concentration (Stitt et al., 1987).

Previous reports have shown the light-induced regulation of many nuclear-coded chloroplast proteins at a transcriptional level. This is the case for the Calvin cycle enzymes phosphoribulokinase, phosphoglycerate kinase, sedoheptulose-1,7-bisphosphatase, and FBPase (Sahrawy et al., 1990; Raines et al., 1991), as well as for the related enzyme NADP-MDH (Crétin et al., 1988). As regards Trxs, a light-induced synthesis of Trx f that was detectable after 3 h illumination of etiolated spinach plants has been found, with the highest values appearing after 50 h light, and with a half-life of 7 h (Carrasco et al., 1992). In addition to the light-induced transcriptional control of these chloroplast proteins, there is a developmental regulation responsible for the lag phase after a prolonged darkness, and for the different transcriptional expression of young and mature cells within the same organ (Raines et al., 1989, 1991). Additional up-stream DNA sequences,
Chemical and biological material

Fructose-1,6-bisphosphate, oxalacetate, NADP⁺, NADPH, glucose-6-phosphate dehydrogenase, phosphohexose isomerase, mRNA isolation kit, RNase inhibitor, digoxigenin-labelled dUTP, alkaline phosphatase-labelled antidigoxigenin, and nylon membranes, were all from Boehringer (Mannheim, Germany). The RT-PCR system was from Promega (Madison, WI, USA). Nusieve-GTG agarose is a product from FMC Bio-Product (Rockland, ME, USA). Other chemicals, including ELISA and electrophoretic reagents, were of molecular biology grade, and were obtained from Sigma (St Louis, MO, USA). Synthetic oligonucleotides were prepared in the Instituto ‘López-Neyra’ de Parasitología (Granada, Spain). Polyclonal antibodies against pea FBPase, NADP-MDH, Trx f, and Trx m were prepared in rabbits following the method outlined previously (Hermoso et al., 1987). The corresponding proteins for rabbit immunization were obtained by heterologous expression in Escherichia coli of the corresponding cDNA clones subcloned in the pET-3d expression vector, and then purified to homogeneity by conventional methods.

Plant cultures

Pea (Pisum sativum L. cv. Lincoln) seeds were germinated in moistened vermiculite contained in plastic trays, and grown in a germination chamber for 10 d under 200 μmol m⁻² s⁻¹ PAR, with a 16 h photoperiod and a day/night temperature gradient of 25/20 °C. Seedlings were then transferred to aerated Hewitt medium arranged in metacrylate pots covered with opaque cardboard to prevent algal contamination. Nutrient solution was renewed each week, and the transpired water replaced. Plants were then harvested and dissected into petioled and sessile leaves (top position), stem, root, pod, and grains. In another set of experiments seedlings were grown for 10 d under the following conditions: optimal temperature (25/20 °C day/night) and low light (100 μmol m⁻² s⁻¹ PAR), normal temperature and high light (2500 μmol m⁻² s⁻¹ PAR), high temperature (35/30 °C day/night) and low light, high temperature and high light, low temperature, low light and low temperature and high light. In order to avoid differences in their ontogenetic state, similar petioled leaflet samples from the upper position were harvested after 10 d treatment. After fresh weight determination, samples were frozen and stored at −80 °C until use.

For electron microscopy visualization plants were grown under normal temperature day/night gradient, and 2500 μmol m⁻² s⁻¹ PAR (saturating light) or continuous 500 μmol m⁻² s⁻¹ PAR (subsaturating irradiance). Samples of upper leaves of 10-d-old plants were harvested for fixation and inclusion.

mRNA and protein determinations

Total mRNA was obtained from extracts of 100 mg samples by retention on a biotin-labelled oligo-dT probe. This was fixed on streptavidin magnetic particles, which were then isolated with a magnetic device. The fixed mRNA was eluted with a low ionic strength solution. Total mRNA was determined by spectrophotometric reading at 260 nm. Semi-quantitative concentrations of mRNA corresponding to FBPase, NADP-MDH, Trx f, and Trx m were determined by reverse transcription (RT) and DNA amplification (PCR), in the presence of synthetic oligonucleotides complementary to the ends of the coding sequence for each protein, according to the manufacturer's protocol. The deoxynucleotide mixture of the kit was supplemented with 5% (w/v) of digoxigenin-labelled dUTP, which was then incorporated into the polymerase-mediated DNA synthesis. The RT-PCR conditions for FBPase and NADP-MDH were optimized in the following way: 45 min at 48 °C (RT), 3 min at 94 °C (denaturation), and 40 cycles amplification (PCR), which includes 30 s at 94 °C (denaturation), 2 min at 58 °C (annealing), and 3 min at 68 °C (polymerization), with a final step of 7 min at 68 °C. The program was modified for Trxs f and m as follows: 25 cycles amplification (PCR), and 2 min at 68 °C polymerization. Two per cent amplified DNA was electrophoresed in 1.5% agarose and, after transfer to a positively-charged nylon membrane, was developed with alkaline phosphatase-labelled antidigoxigenin. Bands were quantified by densitometry, and the results were quoted with reference to fresh weight and total mRNA. The linearity between mRNA concentration and the densitometric optical reading of the alkaline phosphatase reaction was verified with both PCR programs by using increasing amounts of isolated total mRNAs.

To check the Trx f-coding ability of the DNA produced by RT-PCR of corresponding mRNA, samples of the amplified DNA were purified by electrophoresis in Nusieve GTG agarose, recovered from the gel, and re-amplified by PCR as above. After a new electrophoresis under the same conditions, the DNA fragment was sequenced using the fmol sequencing system and fluorescent primers.

Parallel 100 mg samples were extracted by crushing in a liquid N₂-chilled mortar with 0.4 ml of 25 mM TRIS-HCl (pH 7.5), 5 mM MgCl₂, 5 mM 2-mercaptoethanol, and 0.5 mM EDTA-Na₂. After 12 000 g centrifugation for 15 min, the content of Trxs f and m, FBPase, and NADP-MDH were determined in the supernatant by ELISA with antibodies raised in rabbits against the corresponding purified proteins. Total protein was measured according to Bradford (Bradford, 1976), and chlorophyll by the Arnon (Arnon, 1949) method.
Trx-dependent FBPase activity was determined by the two-step procedure of Hertig and Wołosiuń (Hertig and Wołosiuń, 1980). Ten μmol of TRIS-HCl (pH 7.9), 0.25 μmol of DTT, 0.04 μmol of fructose-1,6-bisphosphate, 0.005 μmol of CaCl₂, and the sample extract, were preincubated for 15 min at 20 °C in a final volume of 0.1 ml. The catalytic step was performed by the addition of 0.9 ml solution containing 50 μmol of TRIS-HCl (pH 7.9), 1 μmol of MgCl₂, 0.36 μmol of fructose-1,6-bisphosphate, 0.02 μmol of EGTA, 0.3 μmol of NADP⁰, 0.7 units of glucose-6-phosphate dehydrogenase, and 1.4 units of phosphoglucone isomerase. FBPase activity was assayed by the two-step procedure of Fickenscher and Scheibe (Fickenscher and Scheibe, 1983). Ten μmol of TRIS-HCl, 2.5 μmol DTT, 50 μmol NaCl, and the sample extract, were preincubated at 25 °C (pH 8.0) for 15 min, in a final volume of 0.1 ml. The catalytic step was performed by addition of 0.9 ml solution (pH 8.0) containing 90 μmol of TRIS-HCl, 0.78 μmol of fructose-1,6-bisphosphate, 0.005 μmol of EGTA, 0.3 μmol of NADP⁺, 0.50 units of glucose-6-phosphate dehydrogenase, and 0.35 units of phosphohexose isomerase, in a final volume of 1 ml. Trx-dependent NADP-MDH activity was assayed by the two-step procedure of Fickenscher and Scheibe (Fickenscher and Scheibe, 1983). Ten μmol of TRIS-HCl, 2.5 μmol DTT, 50 μmol NaCl, and the sample extract, were preincubated for 15 min at 20 °C (pH 7.4) for 15 min, in a final volume of 0.1 ml. The catalytic step was performed by addition of 0.9 ml solution (pH 8.0) containing 90 μmol of TRIS-HCl, 10 μmol MgCl₂, 1 μmol oxalacetate, and 0.2 μmol NADPH. In all cases activity was measured through the first 1 min recording of the absorbance increase (FBPase) or decrease (NADP-MDH) at 340 nm. One enzyme unit is the enzyme activity which produces the appearance (FBPase) or disappearance (NADP-MDH) of 1 μmol of NADPH per min under the experimental conditions. Results were quoted with reference to fresh weight and total protein.

Ultrastructural analysis

Three mm wide strips from the central part of the upper leaves were longitudinally cut into 1 mm section pieces. These were fixed overnight at 4 °C with 4% (v/v) p-formaldehyde and 0.1% (v/v) glutaraldehyde in 0.1 M Na-cacodylate (pH 7.2), and washed three times for 30 min each in the same buffer. Samples were then dehydrated by successive treatments with increased concentrations of ethanol at 4 °C. Finally, they were included in Unicryl by 3 d polymerization at −25 °C under UV light. Ultrathin sections obtained with a Reichert-Jung ultramicrotome were observed in a Zeiss EM10C transmission electron microscope under 60 kV.

Results and discussion

The total mRNA content among the different organs was in the same order of magnitude when expressed on a fresh weight basis. They were in the range 0.55–0.78 μg mg⁻¹ FW. The only exception was that of immature grains, which displayed a higher level of transcripts (1.12 μg mg⁻¹ FW). Trx f and m, and their targets FBPase and NADP-MDH, displayed transcriptional expression in both photosynthetic and non-photosynthetic organs of 50-d-old pea plants normally illuminated (16 h photoperiod). However, the content of specific mRNAs for FBPase and NADP-MDH was very low in non-photosynthetic organs (root and grain), and the same occurs for Trx m in roots (Fig. 1). On the contrary, Trx f was strongly expressed in non-photosynthetic organs. The high expression of Trx m in the grain, and not in the roots, could be related to the photosynthetic nature of the young green seeds. When the RT-PCR was performed in the presence of synthetic oligonucleotides which were complementary to the 3'-end of the mature proteins and the 5'-end of the precursor proteins, mRNA expression displayed a similar pattern with respect to organ specificity. In order to check the Trx f-coding nature of the RT-PCR product originated from root Trx f-mRNA, a sample of the amplified DNA was purified by agarose electrophoresis, recovered from the gel and sequenced. The homology was 100% with respect to the published coding sequence of pea Trx f (Lepiniec et al., 1992), which unambiguously showed the Trx f-coding nature of the root mRNA transcript.

In spite of the close relation of photosynthetic FBPase with green tissues, there are reports concerned with the expression of this enzyme in the proplastids of non-photosynthetic cells, where it plays a role in starch synthesis (Emes and Neuhaus, 1997). At first it seems implausible that chloroplastic FBPase plays a physiological role in non-photosynthetic tissues, since this enzyme must be light-activated by the ferredoxin-Trx system via
the photosynthetic electron transport (Scheibe, 1990; Buchanan, 1992). However, the regulation of the enzyme is so complex, that some activity may occur even in the absence of reductant. While these results for pea roots showed a low transcriptional expression of chloroplast FBPase, they also showed a high Trx f-related mRNA content. However, when Trx f expression was analysed at the translational level by Western blotting, the protein was fully expressed in the photosynthetic organs, including green immature grains, and poorly expressed in roots (Fig. 2). This seems to indicate a potential role for Trx f in some processes other than FBPase modulation. This potential was masked in roots under normal growth, although the high content of the corresponding mRNA could permit an efficient expression in stressing situations via a post-transcriptional regulatory mode.

Low translational expression in roots, if any does exist, was also observed for Trx m and for FBPase and NADP-MDH targets, when the enzymes were determined both as proteins (ELISA) and as functions of enzyme activities (Fig. 3). It is noteworthy that the content of NADP-MDH, as well as of Trx f and m, was high in the green immature seeds (Fig. 3b–d). This was not the case for FBPase protein. However, the high specific activity of this enzyme, in addition to the high content of its modulator Trx f, increased the FBPase activity in seeds much more than in leaves (Fig. 3g). The comparatively low FBPase activity of leaves in the fruit-bearing plant must be related to the export of triose-phosphates to the cytosol for sucrose synthesis. On the contrary, the sink nature of seeds made an increased FBPase activity necessary for starch formation. In any case, the active metabolism of both organs, leaves and seeds can be seen in their comparatively high protein content with respect to that of other plant organs (Fig. 3e). Trxs f and m, and a putative h-type Trx named Trx c, by the authors, have been found in seeds as well as in etiolated and green seedlings of barley (Crawford et al., 1981). Similarly, a Trx active against NADP-MDH has been described in spinach roots (Jacquot et al., 1978). There was not, at that time, an exact idea of the different types of Trxs existing in the plant kingdom.

As stated by Falk et al. (Falk et al., 1996) stress response is a special type of acclimation characterized by transient biochemical adjustments induced by short-term changes in environmental factors which may lead to plant damage and, finally, to plant senescence and death. Plant capacity for acclimation is genetically controlled, and depends on the plant plasticity in the regulation and expression of specific genes. In this sense, it is a species-(or variety-) dependent process. Although primary photochemical events are scarcely sensitive to a broad range of temperatures (Mathis and Rutherford, 1987), subsequent processes concerned with carbon assimilation are. This can be seen in the 75% drop of the light-saturated rate of CO₂ assimilation in spinach and tomato plants when the growth conditions were modified from 30°C to 4°C. It was assumed that this decrease was due to constraints of the thermodynamic rate constants (Sassenrath and Ort, 1990). The higher sensitivity of the dark reactions to low temperature favours light excess under these conditions, leading to photoinhibition as a consequence of damage in the photochemical reaction centres and metabolic imbalances (Huner et al., 1998) associated with the generation of active oxygen species.

As expected, the transcriptional expression of FBPase and NADP-MDH appeared inhibited in pea plants maintained for 10 d under low irradiance (16 h photoperiod...
at 100 μmol m$^{-2}$ s$^{-1}$ PAR), both in plants grown under normal (25/20 °C day/night) or under high (35/30 °C day/night) temperature conditions (Fig. 4a, b). By contrast, when plants were grown at low temperature (15/10 °C day/night), the level of the specific mRNAs sharply increased, with the highest values obtained under low irradiance conditions. Berry and Björkman found that acclimation of plants to low growth temperatures was accompanied by an increase in carbon metabolism (Berry and Björkman, 1980). The authors stated that this was a consequence of a higher carboxylation rate, but could also be due to decreased photorespiration. Moreover, Gray et al. reported that spinach, a cold-tolerant plant, became resistant to photoinhibition when grown at low temperature (Gray et al., 1994), whereas Martino-Catt and Ort reported a photosynthesis inhibition in tomato, a cold-sensitive plant, when grown at low temperatures in the dark, probably as a consequence of a low temperature-induced disturbance of the circadian control of transcription of certain regulatory chloroplast proteins (Martino-Catt and Ort, 1992). The behaviour of the specific mRNAs for Trxs $f$ and $m$ was not as diverse as those of their target enzymes. A positive effect of light and low temperature was observed in the former, but not as sharply as in the latter (Fig. 4c, d).

When the effects of irradiance and temperature on the expression of Trxs $f$ and $m$, and on their FBPase and NADP-MDH targets, were analysed at protein level, significant differences were rare, either when the protein content was calculated on a leaf fresh weight (Fig. 5a–d) or on a total protein basis (data not shown). The only fact that perhaps merits emphasis was, as with the mRNA level, the positive response of the plant to low temperatures under low light conditions, when results were expressed in relation to total proteins (data not shown). This tendency was not observed in FBPase and NADP-MDH activities (Fig. 5f–h), the former surprisingly displaying the highest values in plants grown under high

![Fig. 4. RT-PCR determination of specific mRNAs for FBPase (a), NADP-MDH (b), Trx $f$ (c), and Trx $m$ (d) in upper petioled leaflets of pea plants grown for 10 d under normal temperature (25/20 °C day/night) and low light (100 μmol m$^{-2}$ s$^{-1}$ PAR) (NTLL), normal temperature and high light (2500 μmol m$^{-2}$ s$^{-1}$ PAR) (NTHL), high temperature (35/30 °C day/night) and low light (HTLL), high temperature and high light (HTHL), low temperature (15/10 °C day/night) and low light (LTLL), and low temperature and high light (LTHL). Bars represent percentages in relation to the highest value (on fresh weight basis), and are the means (± SE) of two measures on each of two independent experiments. Top insets are alkaline phosphatase stains of one of the agarose electrophoresis transfers on nylon membranes of digoxigenin-labelled PCR-amplified specific DNAs.]

![Fig. 5. FBPase (a), NADP-MDH (b), Trx $f$ (c), Trx $m$ (d), and total protein (e), as well as FBPase (f, g) and NADP-MDH (h) activities of upper petioled leaflets of pea plants grown for 10 d under normal temperature (25/20 °C day/night gradient) and low light (100 μmol m$^{-2}$ s$^{-1}$ PAR) (NTLL), normal temperature and high light (2500 μmol m$^{-2}$ s$^{-1}$ PAR) (NTHL), high temperature (35/30 °C day/night gradient) and low light (HTLL), high temperature and high light (HTHL), low temperature (15/10 °C day/night gradient) and low light (LTLL), and low temperature and high light (LTHL). (a–d) were measured by ELISA with the corresponding specific antibodies, and (e) by Bradford. FBPase activity was determined at pH 8.8 under non-reducing conditions (f) or at pH 7.9 in the presence of DTT (g), whereas DTT-dependent NADP-MDH activity (h) was measured at pH 8.0, as stated in the Materials and methods. Bars represent percentages in relation to the highest value (on a fresh weight basis), and are the means (± SE) of two measures on each of two independent experiments.]


temperature and low light, when the activity was measured under reducing conditions (preincubation at pH 7.9 in the presence of DTT). These results conflict with those of Stitt et al., who found a high starch content in spinach and wheat chloroplasts of plants grown under low temperature (Stitt et al., 1987). This could be due to a higher FBPase activity, but also to an effect of temperature on the ADP-glucose pyrophosphorylase, one of the key points of starch synthesis regulation; an additional explanation could be a less available P\(_i\) in the stroma, a fact associated with plants grown at low temperatures (Stitt et al., 1987). In this sense it may be reasonable to think that low temperatures could act at the P\(_i\) translocator level rather than on chloroplast bisphosphatases.

It should be noted on the basis of these results that there was an increase in the transcriptional capability of plants under low temperature stress. This fact could be considered specific to the proteins under consideration, since the increase remained when results were expressed both on a fresh weight and on a total mRNA basis. In spite of the photobleaching effect of continuous light on chlorophyll content (Table 1), high light appeared to be a positive factor on mRNA transcription. Figure 6a shows mesophyll leaf cells of pea plants grown under normal conditions. It is noteworthy that starch grains are large and refringent, probably because samples were prepared from the highly metabolic upper leaves after 10 h light. In contrast, when plants were continuously illuminated under 500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR, cells also appeared with great starch grains, but with broken envelopes and disorganized structures (Fig. 6b). The same occurs in plants grown under saturating light (2500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR) which displayed distorted structural elements and disorganized starch grains, probably as a consequence of high starch accumulation followed by a photobleaching process (Fig. 6c). However, it was noteworthy that the effects of low temperature and low illumination were synergistic, and induced the highest transcriptional response of the plant. The lack of a clear response at a protein level seems to show translation as the rate-limiting step, being necessary for protein expression under stress conditions stronger than those used in the present experiments.

**Table 1. Chlorophyll content of leaves from 50-d-old pea plants grown under different stress conditions**

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Chlorophyll (mg g(^{-1}) FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTLL 25/20°C day/night gradient and 16 h photoperiod</td>
<td>2.71 ± 0.16</td>
</tr>
<tr>
<td>NTHL 25/20°C day/night gradient and 16 h photoperiod</td>
<td>1.40 ± 0.18</td>
</tr>
<tr>
<td>HTLL 25/20°C day/night gradient and 16 h photoperiod</td>
<td>3.14 ± 0.11</td>
</tr>
<tr>
<td>HTHL 15/10°C day/night gradient and 16 h photoperiod</td>
<td>1.23 ± 0.08</td>
</tr>
<tr>
<td>LTLL 2500 (\mu)mol m(^{-2}) s(^{-1}) PAR</td>
<td>2.01 ± 0.09</td>
</tr>
<tr>
<td>LTHL 15/10°C day/night gradient and 16 h photoperiod 2500 (\mu)mol m(^{-2}) s(^{-1}) PAR</td>
<td>0.94 ± 0.14</td>
</tr>
</tbody>
</table>

**Fig. 6.** Electron micrograph of ultrathin sections of upper-leaf mesophyll from 10-d-old pea plants grown under normal conditions (200 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR, 25/20°C day/night gradient) (A), and under light stress: continuous 500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR (B), and 16 h photoperiod of 2500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR (C). Thin sections were embedded in Unicryl resin, and observed at ×8000 magnification. Bar represents 1 µm.

**Acknowledgements**

This work was supported by grant PB95–0081 from Dirección General de Investigación Científica y Técnica (Spain), and by grant No. 1067 from Junta de Andalucía. We are grateful to Professor Renate Scheibe (University of Osnabrück, Germany) for providing a cDNA clone encoding pea NADP-malate dehydrogenase, and to Dr Isabel Rodríguez García by the electron micrographs. EA Pagano was the recipient of a C Mutis (AECI) fellowship. We thank Mrs Francisca Castro and Mr Narciso Algaba for their skillful technical assistance.
References


