S35-028

Photosystem II regulation under cold stress in *S. tuberosum* and *S. commersonii* genotypes and their hybrids

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Keywords: Photosystem II, cold stress, potato hybrids, phosphoserine and phosphothreonine kinase activity

Introduction

Plants are exposed to a variety of environmental extremes over the course of their lifetimes. Atmospheric conditions are determining to agricultural productivity and an increased understanding of plant adaptation to adverse conditions can lead to improved crop.
Although there are many different stresses, plant responses can be very similar. Plants respond to stress with changes in the rate of protein synthesis and degradation, alterations in membrane lipid composition, and changes in redox state and in protein phosphorylation. The effects of low temperature are particularly sustained at the level of PS-II [Barber J & Andersson B 1992], the multiprotein complex of the chloroplast involved in the reactions of photosynthesis. PS-II is made up of a reaction center "core", light harvesting antennae and the oxygen-evolving complex. The core comprises the D1, D2 and Cyt b559 proteins as well as the internal antennae CP43 and CP47 and a 9 kDa protein of unknown function; this is the site of the initial photosynthetic electron transport reactions. The entire complex is immersed in a phospholipid bilayer within the thylakoid grana.
PS-II is inactivated by cold stress and the damage is localised primarily to the D1 protein. Under stress conditions D1 turnover is altered, as the result of damage to the Q_B site that binds plastoquinone [Giardi MT et al. 1996]. Low temperatures lead to a decrease in photosynthesis due to a general slowdown of metabolic processes and consequently, an increased susceptibility of PS-II to photoinhibition under physiological light. Recent studies have demonstrated the role of phosphorylation on the recovery process, following exposure to several kinds of stress. Phosphorylation is an integral part of light-mediated metabolism that aids in the protection of PS-II from photoinhibition and degradation of the D1 protein [Rintamaki E et al. 1996]. The effects of cold stress include an altered composition of glycerolipids, changes in the level of fatty acid unsaturation, the redistribution of saturated and unsaturated fatty acids, associated with changes in protein composition and the activation of ion channels. It is known that unsaturation of fatty acids in thylakoid membranes stabilizes the photosynthetic machinery against low-temperature photoinhibition by accelerating the recovery of the PS-II [Moon BY et al. 1995].
In this work we report on the cold stress adaptation of genetically different potato plants, to study the nuclear and plastid gene contribution at both physiological and molecular level.

Materials and methods

Plant material
Seeds of diploid *Solanum commersonii* (coded TP, tolerant parent) were sown, and shoot cultures were maintained as previously described [Cardi T et al. 1990]. Shoot cultures of a dihaploid *Solanum tuberosum* clone (code SP, sensitive parent) were bleached by adding the herbicide SAN 9789 to the culture medium [Puite KJ et al. 1986]. Somatic hybrids were obtained by the

electrofusion of mesophyll protoplasts of *S. commersonii* and *S. tuberosum*. The four hybrids have a nucleus derived from the fusion of the parental nuclei; of these, TH1 and TH2 have chloroplasts derived from TP, while SH1 and SH2 have chloroplasts derived from SP.

Cycle of cold stress
The plants were exposed to a cycle of cold stress designed to reproduce the conditions of a winter day. The conditions of the cold stress cycle were imposed using a refrigerated cell. The initial temperature of +20°C was shifted gradually to +4°C in the dark (A). Within 4 hours, the plants were brought to -3°C (B) and kept there for 2 hours (B'), then returned to +4°C (C) over a 4-hour period. During the subsequent 15 hours, the plants were kept at +4°C, during which time a few plants were exposed to a light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$, while the rest were kept in the dark (D). Finally, the temperature was gradually increased to +20°C (E).

Chlorophyll fluorescence and chlorophyll content
The maximum photochemical efficiency of PSII was measured as the Fv/Fm ratio after 20 min of dark adaptation using the Plant Efficiency Analyser (Hansatech) at an irradiance of 3000 µmol photons $m^{-2}s^{-1}$. The chlorophyll content was measured after extraction with 80 % (w/v) acetone.

Isolation of thylakoids and PS-II particles
Thylakoids were prepared from control and stressed plants as previously described [Giardi MT et al. 1996]. PS-II enriched particles were prepared after solubilization with Triton X-100 using a chlorophyll to detergent ratio of 15 [Bertold DA et al 1981].

Radioactive incorporation and the detection of labelled proteins
For pulse experiments, small leaf disks (3 mm diameter) were incubated in a solution containing [^{35}S] methionine as previously described [Geiken B et al. 1998]. They were subsequently immersed in buffer containing 1 mM cold methionine. Thylakoid proteins were analyzed using SDS-PAGE. For autoradiography, the gels were stained with Coomassie Blue, dried and exposed to X-ray film (Kodak-Safety light) after a 20 min treatment with amplifier (Amplify, Amersham).

SDS-PAG, immunoblot and lipid analyses
The protein patterns of isolated thylakoids were analyzed by SDS-PAGE using denaturing 12-17 % polyacrylamide gradient gels. Detection of the PSII proteins by immunoblot analyses was performed using specific polyclonal antibodies kindly provided by Dr. Barbato R. (Padova UNI, Italy). PSII particles, equivalent to 60 µg chlorophyll, were extracted with $CHCl_3/CH_3OH/H_2O$ (65:25:4) to isolate total lipids. Phospholipid analysis was performed on thin-layer chromatography plates (Merck Silica Gel 60) using monogalactosyldiacylglycerol (MGDG) and digalactosylidiacylglycerol (DGDG) (Sigma) as reference standards and the above solvent as the eluent.

^{31}P-NMR spectra
Samples from PSII preparations equivalent to 6 mg chlorophyll were extracted with $CHCl_3/CH_3OH/H_2O$ (65:25:4) to isolate total lipids from the protein fraction. The protein fraction was incubated in 10 ml HCl (6N) at 110°C for 2 h, to hydrolyse the proteins. The samples were neutralised with NaOH and lyophilised. The resulting residue was dissolved in 1 ml of 1 M NH_4OH. Since the position of the bands strongly depends on ionic strength, conductivity and pH were controlled.
The spectrometer was a Bruker AMX 600 operating on ^{31}P at 242.88 MHz. All spectra were proton broad-band decoupled with a time domain of 16 K, a relaxation delay of 1 sec, scan numbers 18000, an acquisition time of 0.2 sec, and a 90º pulse of 13.5 µs. Prior to Fourier transformation, an exponential multiplication with a line broadening of 16 Hz was applied. All spectra were referenced

with respect to an external standard of 85% H_3P0_4 located in a capillary inside the sample tube. In order to obtain reproducible intensities, the probe was returned before each experiment. All spectra run at 278 K ± 0.2 had the same number of scans, receiver gain and approximately the same 90º flip angle.

Results and Discussion

Photosynthetic activity during the cold stress cycle

The focus of our study was the regulation of metabolic processes in response to cold stress. First, we were interested in determining the relative contributions of the plastid and nuclear genomes to plant metabolic processes. To this end we used clones of potato with genetic differences that would permit the determination of the involvement of the nucleus and the chloroplast in stress induced metabolic modifications. In the hybrids, the chloroplasts were derived directly from one of the parents. On the other hand, they inherited all nuclear chromosomes from both parents. Hence, the nuclear genomes are supposed to be highly isogenic. The plants were initially grown under normal temperature conditions and then successively subjected to a cycle of cold stress. We looked at chlorophyll fluorescence emission in order to obtain information on the general physiological status of the plants. Fluorescence measurements were taken for all genotypes during the cycle of cold stress and compared to control plants kept at +25°C. The fluorescence values were similar for the controls of all genotypes and variation in fluorescence levels is not detected before the D point of the cycle of cold stress. As expected, the photosynthetic activity at D point of SP was more severely impacted, with light acting synergistically on the stress response (about 40% of control). This synergism is evident in the behaviour of the hybrids, in particular, the lowest Fv/Fm values (about 25% of relative controls) were detected in the two hybrids (SH1 e SH2) whose chloroplasts were derived from the sensitive parent, the other two hybrids behaved in a similar manner to SP. In the dark, all of the hybrids were equally resistant, independent of the chloroplast genome. In the dark TP showed no detectable decrease in response to cold, while in the light is partially resistant (60% of the control). This indicates that resistance in the dark is principally nuclear in origin, while the chloroplast may contribute more to cold tolerance than the nucleus in the light.

Protein turnover

The rate of protein synthesis and degradation varies according to the physiological conditions of the plant. Protein turnover generally increases under stress conditions, leading to the replacement of damaged proteins [Mattoo AK et al. 1999]. The capacity to increase turnover reflects their level of tolerance to abiotic stresses [Giardi MT et al. 1997]. In order to establish the different D1 protein turnover characteristic of the genotypes, we performed a series of experiments using the incorporation of radioactivity into potato leaf disks, under normal temperature conditions. In these experiments, although the synthesis of the D1 protein was similar in the two parental genotypes, the degradation rate was higher in SP. The consequent decrease in D1 protein content could explain the increased sensitivity of SP to the combination of cold and light stresses, as an integral part of the process of photoinhibition. All the hybrid genotypes had a significantly lower turnover rate with respect to the parents. This suggests that mainly the nuclear genome regulates D1 protein synthesis and degradation, even though the gene is localised to the plastid.

PS-II protein and lipid content

Electrophoretic analysis and immunoblotting illustrated a significant decrease in the PS-II proteins D1 and CP43 in the sensitive parental genotype. The protein amount correspond to 40% of D1 with respect to the control in both dark and light experiments, and 80% in dark, 70% in light of CP43, to points D of the cycle of cold stress. The accumulation of these PS-II proteins is not significantly affected in the tolerant parent. The accumulation of the CP47 and D2 proteins is not altered.

Interestingly, decreased photosynthetic activity measured as fluorescence activity and increased protein degradation exhibit two distinct patterns.
The relative abundance of the two principal classes of lipids, MGDG and DGDG, differs among the potato genotypes. We used chromatography techniques (TLC) to identify and quantify these lipids. Their ratio influences the aggregation status of PS-II and may play a role in PS-II protein degradation. The tolerant genotype has a lower content of DGDG than other genotypes (MGDG/DGDG=1.9), while the highest amount is found in the sensitive parent (MGDG/DGDG=1.4). A decrease in MGDG has been observed under cold stress and the relative amounts of the two classes of lipids vary in TP with respect to the control, resembling the control amounts of SP. The lipid content of the hybrids, approximating that of SP (MGDG/DGDG=1.5), could be the cause of the loss of tolerance in response to the stresses of cold and light. The lipid content is known to be regulated by nuclear genes, in fact hybrids show the same lipid composition.

Phosphoserine and phosphothreonine kinase activity
Nuclear magnetic resonance was used to study the involvement of phosphorylation in protection against stress. The two peaks detected in the spectra of hydrolysed PS-II proteins, one at 3.7 and the other at 4.5 ppm, were attributed to phosphothreonine and phosphoserine, respectively. The phosphoserine content was three times higher in TP with respect to SP, while the hybrids had intermediate values and an elevated phosphothreonine content (from two to five times more than in the parents). The phosphorylation status of the PS-II core proteins has been implicated in the regulation of core protein turnover. In our experiments, while phosphothreonine appears to be involved in decreasing the rate of protein synthesis and degradation, phosphoserine could be involved in increasing the turnover rate. Accordingly, the P-Ser/P-Thr ratio varies significantly in TP and SP. The high P-Ser content in TP explains the high rate of D1 protein turnover. It is interesting to note the elevated quantity of P-Thr in the hybrids, which is not detected in either the parental genotypes or in various other plant species.

In conclusion, mainly nuclear factors appear to influence tolerance in the dark, while both nuclear and plastid factors seem to be influential in the light. Both protein phosphorylation and lipid modifications seem to be involved in plant response to cold stress.

Acknowledgements

We thank F. Fiorentino for his technical contributions and Dr A.L. Segre for helpful discussions of NMR spectra. This work was supported in part by MIPAF and the CNR project for biotechnology.

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