PHOTOSYNTHETICA 45 (2): 182-188, 2007

Involvement of betacyanin in chilling-induced photoinhibition in leaves of *Suaeda salsa*

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Abstract

Seeds of *Suaeda salsa* were cultured in dark for 3 d and betacyanin accumulation in seedlings was promoted significantly. Then the seedlings with accumulated betacyanin (C+B) were transferred to 14/10 h light/dark and used for chilling treatment 15 d later. Photosystem 2 (PS2) photochemistry, D1 protein content, and xanthophyll cycle during the chilling-induced photoinhibition (exposed to 5 ºC at a moderate photon flux density of 500 μmol m^{-2} s^{-1} for 3 h) and the subsequent restoration were compared between the C+B seedlings and the control (C) ones. The maximal efficiency of PS2 photochemistry (F_v/F_m), the efficiency of excitation energy capture by open PS2 centres (F_v'/F_m'), and the yield of PS2 electron transport (Φ_{PS2}) of the C+B and C leaves both decreased during photoinhibition. However, smaller decreases in F_v/F_m, F_v'/F_m', and Φ_{PS2} were observed in the C+B leaves than in C ones. At the same time, the de-epoxidation state of xanthophyll cycle, indicated by (A+Z)/(V+A+Z) ratio, increased rapidly but the D1 protein content decreased considerably during the photoinhibition. The increase in rate of (A+Z)/(V+A+Z) was higher but the D1 protein turnover was slower in C+B than C leaves. After photoinhibition treatment, the plants were transferred to a dim irradiation (10 μmol m^{-2} s^{-1}) at 25 ºC for restoration. During restoration, the chlorophyll (Chl) fluorescence parameters, D1 protein content, and xanthophyll cycle components relaxed gradually, but the rate and level of restoration in the C+B leaves was greater than those in the C leaves. The addition of betacyanins to the thylakoid solution *in vitro* resulted in similar changes of F_v/F_m, D1 protein content, and (A+Z)/(V+A+Z) ratio during the chilling process. Therefore, betacyanin accumulation in *S. salsa* seedlings may result in higher resistance to photoinhibition, larger slowing down of D1 protein turnover, and enhancement of non-radiative energy dissipation associated with xanthophyll cycle, as well as in greater restoration after photoinhibition than in the control when subjected to chilling at moderate irradiance.

Additional key words: chlorophyll fluorescence; D1 protein; hydrogen peroxide (H_2O_2); photosystem 2; xanthophyll cycle.

Introduction

Betalains are water-soluble nitrogen-containing pigments, which comprise the red-violet betacyanins and the yellow betaxanthins. Betalains accumulate in flowers, fruits, and occasionally in vegetative tissues of plants in most families of the Caryophyllales and in some higher fungi. Betalain-producing plants are unable to convert flavan-3,4-diols to anthocyanidin. Instead, they convert tyrosine *via* Dopa and a series of intermediate metabolites to the red-violet betacyanin and/or yellow betaxanthin (Steglich and Strack 1990). So betalains are important chemo-taxonomical markers that have never been found jointly with anthocyanins in the same plant (Stafford 1994, Clement and Mabry 1996). In addition, betalains have been used as natural additives for food, drugs, and cosmetic products, and they are free-radical scavengers and prevent active oxygen-induced and free radical-mediated oxidation of biological molecules (Dörnenburg and Knorr 1996, Pedreño and Escribano 2001). Documented up-regulation of betalain biosynthesis under adverse environmental conditions suggests that it plays an important role in plant stress tolerance (Vogt *et al.* 1999, Ibdah *et al*. 2002).

The Chenopodiaceae C_3 halophyte *Suaeda salsa* is one of the most important halophytes in China. It is native to saline soils and grows even in the intertidal zone of the Yellow River Delta, where soil salt content is often higher than 3 % (Zhao 1998). Identification and biosynthesis regulation of betacyanins in *Suaeda salsa* have been preliminarily studied in our laboratory (Wang *et al.* 2006, 2007, Wang and Liu 2006). Betacyanin content is regulated by environmental factors such as irradiance, temperature, and salinity. Dark at germination stage is one of the most important environmental factors to induce betacyanin biosynthesis in *S. salsa* (Wang *et al.* 2006). However, no information exists about the biological roles of betacyanins in chilling-induced photoinhibition in *S. salsa*.

Received 20 April 2006, *accepted* 24 August 2006.
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Chilling induced damage of photosynthesis under moderate irradiance, which can be regarded as a kind of photoinhibition (Sonoike 1999), was investigated by following the ratio of variable to maximum chlorophyll (Chl) fluorescence (F_v/F_m). F_v/F_m is a measure of the maximal quantum yield of photosystem 2 (PS2), which is the main regulatory site of photoinhibition as well as the main target of photo-oxidative damage (Prášil *et al.* 1992, Barber 1994). Decreases in F_v/F_m can be attributed to at least two processes (Osmond 1994). The first one is a rapidly reversible down-regulation of PS2 activity and is described as dynamic photoinhibition. It involves biophysical events and is partially a zeaxanthin related process. The second process is presumably related to net degradation of the D1 protein of the PS2 reaction centre (RC) and is described as chronic photoinhibition. Both processes are interactive and their respective contributions depend on the extent of photon excess and the duration of exposure (Russell *et al.* 1995). The relative proportions can be determined by following the recovery response in dim radiation at optimal temperatures. The first stage is quickly reversible and involves epoxidation of zeaxanthin. Recovery from the second stage involves synthesis of the D1 protein. Hence, the two types of photoinhibition are visible in a biphasic recovery response. An initial fast recovery phase of maximally 1 h is followed by a slow phase of several hours (Leitsch *et al.* 1994, Thiele *et al.* 1996).

There are various strategies to minimise photodamage, including enhanced rates of photosynthetic electron transport, increased D1 turnover to prevent net photoinactivation of PS2, and increased energy dissipation in the light-harvesting antenna, *i.e.* before it reaches the RCs. The latter response includes enhanced dissipation of excitation energy *via* the xanthophyll cycle, the formation of the membrane-bound radical scavenger α-tocopherol, superoxide dismutase, antioxidants of the ascorbate-glutathione cycle, or the more availability of appropriate electron sinks for the consumption of excitation energy (Demmig-Adams and Adams 1996, Foyer 1997). However, the connection between the important second metabolite betacyanin and the photo-protection during the chilling process is not clear.

The objective of this study was to gain more insight into the relation between betacyanin accumulation and susceptibility to chilling-induced photoinhibition in *Suaeda salsa*. For this purpose we developed *S. salsa* seedlings that accumulated betacyanin (C+B) for chilling treatment. The Chl fluorescence parameters, D1 protein content, and xanthophyll cycle components during the photoinhibition and the subsequent restoration were investigated in C+B seedlings. Compared to the control (C), C+B seedling leaves exhibited less photoinhibiton following the exposure to 5 ºC and photosynthetic flux density (PFD) of 500 μmol m^{-2} s^{-1}. Not only the significant differences of Chl fluorescence parameters but also those of the D1 protein content and (A+Z)/(V+A+Z) ratio between the C+B and C leaves were observed throughout the chilling treatment and restoration. So the higher betacyanin content may play an important role in enhancing the photoprotection *via* slowed-down D1 protein turnover and increased (A+Z)/(V+A+Z) ratio in C+B seedlings of *S. salsa*.

Materials and methods

Plants and induction of betacyanin accumulation: Seeds of the *S. salsa* were collected from the Yellow River Delta. After being sterilized with 0.5 % $HgCl_2$ for 3 min, seeds were washed and germinated in plastic plates filled with sand and watered with 1/2 MS solution containing 100 mM NaCl. The seeds were placed in the growth cabinet with a 24-h dark, temperature was 25 °C, relative humidity (RH) was 60 %. After 3 d, uniform seedlings were transplanted to and cultured in a growth cabinet with a photoperiod of 14/10 h light/dark; photon flux density (PFD) was 200 μmol m^{-2} s^{-1}, the other culture conditions were the same as previously described. 15 d later, the 18-d-old dark pre-treated seedlings were used as C+B plants for chilling treatment. Seedlings obtained from the seeds cultured from the very beginning at the described growth cabinet conditions were used as control (C plants).

Chilling treatment and recovery experiment: C+B seedlings and C plants were placed in a pre-cooled (5 ºC) climate chamber at a PFD of 500 μmol m^{-2} s^{-1}. Humidified air containing 360 μmol mol^{-1} CO_2 was passed through the chamber during the treatment. During the 3-h chilling, leaves of seedlings were analyzed to determine xanthophyll cycle components, D1 protein and H_2O_2 contents, and Chl *a* fluorescence at 0 (the hour before treatment), 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h. Then temperature was increased at a rate of 0.5 °C per 1 min to 25 ºC and PFD was set to 10 μmol m^{-2} s^{-1}. The recovery of the Chl fluorescence parameters (F_v/F_m, F_v'/F_m', and Φ_{PS2}), H_2O_2 and D1 protein contents, and de-epoxidation state of xanthophyll cycle [(A+Z)/(V+A+Z)] were measured every 30 min for 5 h. All inhibition and recovery series were repeated three times (n = 3).

Pigments and D1 protein analysis: Betacyanin of *S. salsa* seedlings was extracted and measured according to the method of Wang *et al.* (2006). Chl content was determined by the method of Mackinney (1941). Xanthophyll cycle components were separated and quantified by HPLC based on the method of Kraay *et al.* (1992). The HPLC system consisted of a *Waters 2690* Separations Module and a *Waters 996* Photodiode Array Detector (Milford, MA, USA). A *Waters Delta-Pak* reversed-

phase column (C_{18}, 5 μm, 10 nm, 150×3.9 mm, fully end capped) was used. Pigments were identified by comparing their retention times and absorption spectra with pigment standards and quantified at 436 nm. The standard reagents of violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) were purchased from *Sigma Company*. D1 protein content was determined with ^{14}C-atrazine as described by Ji and Jiao (2001).

H_2O_2 content: After exposure to the chilling treatment and restoration, the attached leaves were collected every 30 min and rapidly frozen in liquid N_2. The frozen tissue was ground to a powder at liquid N_2 temperature, and the H_2O_2 was extracted with ice-cold 0.2 M $HClO_4$. After neutralization with KOH and centrifugation, the extract was assayed for H_2O_2 following the modified method of Okuda *et al*. (1991). The concentration of 3-methyl-2-benzothiazoline hydrazone in the reaction mixture was 750 μM.

Chl fluorescence emission was measured from attached fully developed leaves with a pulse-modulated fluorometer (*FMS2*, *Hansatech*, UK) through a port in the oxygen electrode chamber at various times during the 3-h chilling treatment and 5-h recovery. The experimental protocol of Schreiber *et al.* (1986) and nomenclature of van Kooten and Snel (1990) were used. The quantum efficiency for electron transport by PS2 was calculated as $\Phi_{PS2} = (F_m' - F)/F_m'$ (Genty *et al.* 1989), where F and F_m' are the steady-state and maximal Chl fluorescence for light-acclimated leaves, respectively. The level of thermal energy dissipation in PS2 antennae was estimated by calculating the excitation capture efficiency of PS2 using the ratio F_v'/F_m', where $F_v' = F_m' - F_0'$ is variable Chl fluorescence for light-acclimated leaves. According to Harbinson *et al.* (1989), the ratio of variable to maximal PS2 fluorescence (F_v/F_m) estimates the efficiency of excitation energy transfer to PS2 RCs, and a loss in F_v/F_m represents more photoinhibition. Measurements of F_0', minimal Chl fluorescence for light-acclimated leaves were performed after a 10-s application of feeble far-red irradiation.

***In vitro* experiment**: Thylakoids were prepared from freshly harvested C leaves as described by Jennings *et al.* (1981). The final pellet was re-suspended in 20 mM *Tricine* (pH 7.6), 0.33 M sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$. Chl content was determined using the extinction coefficients given by Mackinney (1941). Betacyanin was added in the thylakoid solution with the final solvent concentration being less than 1 % (m/v). Chilling treatments were performed in a 1-cm cuvette at a Chl concentration of 20 g m^{-3} at 5 ºC and PFD of 500 μmol m^{-2} s^{-1}. F_v/F_m, xanthophyll cycle pigment composition, and H_2O_2 and D1 protein contents were determined as previously described at 0, 1, 2, and 3 h thereafter.

Results

Betacyanin accumulation induced by dark pre-treatment: Our previous data (Wang and Liu 2006) indicated the dark treatment significantly promoted betacyanin accumulation in cotyledons of *S. salsa* seedlings. In order to induce betacyanin accumulation, seeds of *S. salsa* were grown in dark for 3 d before being transferred to the 14/10 h light/dark photoperiod. Fifteen days after the transferring, leaves were unfolded fully and suitable for Chl fluorescence analysis. Betacyanin content in the leaves of dark pre-treated seedlings was much higher than that in the C seedlings (grown without the pre-darkening), but the difference of the Chl *a*+*b* content between the two groups was not significant (Fig. 1). The phenotype of the C+B seedlings was also different from the control (Fig. 2). Hence C+B seedlings were used for chilling treatment to examine the role of betacyanin in chilling-induced photoinhibition.

Photoinhibition of PS2 during the chilling treatment and recovery: The Chl fluorescence parameter F_v/F_m represents the maximum quantum yield of PS2 photochemistry. Before chilling, the F_v/F_m ratios of the C+B and C leaves were very similar (Fig. 3*A*). F_v/F_m decreased when plants were subjected to chilling-induced photoinhibition, but the C+B leaves showed a smaller decrease in F_v/F_m than the C leaves. After 3-h chilling, the F_v/F_m of C leaves decreased by 63.4 %, while that in C+B leaves decreased only by 43.2 %. F_v/F_m in both the C+B and C leaves increased during restoration under dim irradiation at 25 ºC after chilling, but none of them could restore it to the initial levels. After 5-h restoration, F_v/F_m of the C+B leaves was restored to 90.2 % of their initial values, while those of the C ones only to 77.5 %.

The efficiencies of excitation energy capture by open PS2 centres (F_v'/F_m') of the C+B and C leaves were similar prior to chilling treatment (Fig. 3*B*) but they decreased when subjected to chilling combined with moderate PFD. The rate of decrease in F_v'/F_m' in the C+B leaves was lower than that in the C leaves. F_v'/F_m' of the C+B leaves decreased by 37.5 % after the 3-h treatment, while that in the C leaves by 56.3 %. F_v'/F_m' kept

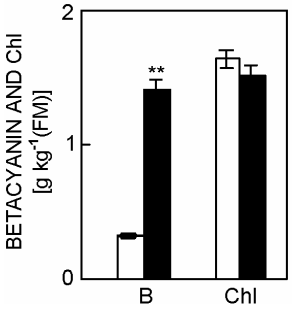

Fig. 1. The betacyanin (B) and chlorophyll (Chl) contents in the dark-pre-treated leaves of *Suaeda salsa* seedlings (■) and the control (□). Means ± SE ($n = 5$). Significance level of difference between the dark pre-treatment and control: $^{**}p<0.01$.

recovering when transferred to dim irradiation at 25 ºC. The rate of restoration in the C+B leaves was greater than that in the C leaves.

The quantum yield of PS2 electron transport (Φ_{PS2}) showed similar changes to the F_v/F_m and F_v'/F_m' (Fig. 3*C*). Φ_{PS2} in the C+B leaves was higher than that of the C ones. Φ_{PS2} in the C leaves decreased more markedly during photoinhibition and restored more slowly than that of the C+B leaves.

D1 protein content, xanthophyll cycle components, and H_2O_2 contents in leaves during the chilling treatment and recovery: D1 protein content in photosynthetic apparatus changed in a trend similar to that of PS2 photochemical efficiency (F_v/F_m). As shown in Fig. 3*D*, D1 protein in both the C+B and C leaves decreased markedly with time during the chilling treatment but increased with time during restoration under dim irradiation at 25 ºC. However, the D1 protein content in C+B leaves showed a slower decrease during photoinhibition and a faster increase during restoration than that of the control.

The (A+Z)/(V+A+Z) ratio, expressed as de-epoxidation state of V, in both the C+B and C leaves, increased during photoinhibition and decreased markedly during restoration (Fig. 3*E*). Betacyanin accumulation in leaves resulted in a statistically significant change in the pigment composition compared to the control. The dynamics of the xanthophyll cycle differed between the attached C+B and C leaves during the chilling treatment and restoration. The C+B leaves exhibited a larger increase during the photoinhibition but greater decrease of (A+Z)/(V+A+Z) during recovery compared to the control. So the lesser

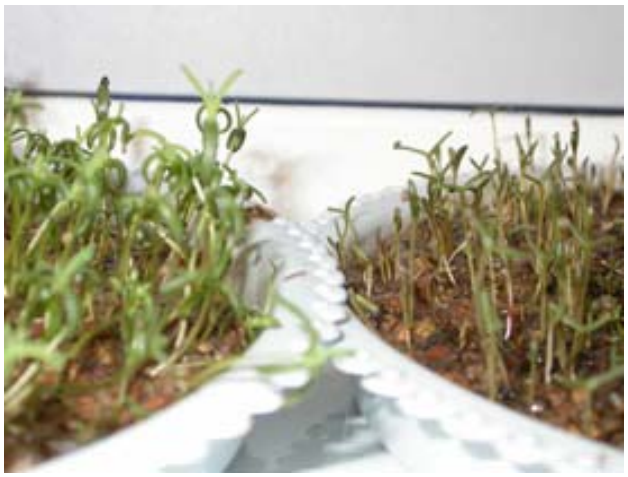

Fig. 2. The phenotypes of the dark-pre-treated *Suaeda salsa* seedlings (*right*) and the control plants (*left*).

photoinhibition in C+B leaves may not only result from the slowed-down D1 protein turnover but also from enhanced energy dissipation mediated by de-epoxidational xanthophyll cycle components.

For both C+B and C leaves, the total leaf H_2O_2 contents were much greater after chilling treatment than before. The leaf H_2O_2 content increased quickly upon irradiation at 5 ºC, reaching the highest value in 30 min and then declining with time. After 5 h of restoration, H_2O_2 content was similar to that found just before the irradiation. However, the H_2O_2 content in C+B seedlings was much lower than in the C leaves during photoinhibition (Fig. 3*F*). Thus the C+B leaves suffered from lesser

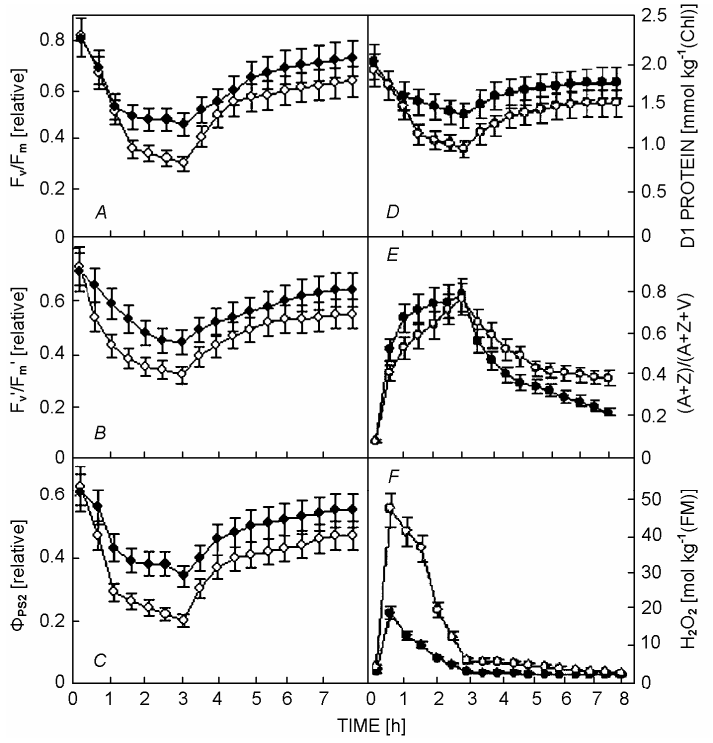

Fig. 3. F_v/F_m (*A*), F_v'/F_m' (*B*), Φ_{PS2} (*C*), D1 protein (*D*) and H_2O_2 (*F*) contents, and (A+Z)/(A+Z+V) ratio (*E*) in *Suaeda salsa* C+B (●) and control, C (○) leaves during the chilling-induced photoinhibition and restoration. Means ± SE (n = 5).

Table 1. The effects of betacyanin addition to thylakoid solution on F_v/F_m, (A+Z)/(V+A+Z), and D1 protein [mmol kg^{-1}(Chl)] and H_2O_2 [mol kg^{-1}(FM)] contents in *Suaeda salsa* leaves that accumulated betacyanin (C+B) and control leaves (C) during the chilling-induced photoinhibition *in vitro*. Means ± SE (n = 5)

	Chilling time [h]							
	0		1		2		3	
	C	C+B	C	C+B	C	C+B	C	C+B
F_v/F_m	0.73	0.72	0.34	0.46	0.30	0.42	0.28	0.41
(A+Z)/(V+A+Z)	0.15	0.17	0.47	0.63	0.59	0.72	0.74	0.73
D1	2.20	2.17	0.99	1.23	0.81	1.12	0.80	1.10
H_2O_2	9.40	10.3	61.20	34.23	43.20	27.90	28.30	15.70

oxidative stress during the chilling treatment and recovery.

Role of betacyanin during photoinhibition in *vitro*: In order to detect the direct role of betacyanin in chilling-induced photoinhibition, betacyanin was added to the thylakoid solution before chilling treatment. F_v/F_m, xanthophyll cycle pigment composition, and H_2O_2 and D1 protein contents were determined during the chilling-induced photoinhibition. The changes in all these characteristics were consistent with those found *in vivo* (Table 1). In contrast to the control, thylakoid solution with betacyanin addition showed less photoinhibition, slower D1 protein turnover, and greater non-radiative energy dissipation associated with xanthophyll cycle, as well as smaller increase of H_2O_2 content during the chilling treatment. So the betacyanin had direct role in alleviating chilling-induced photoinhibition *via* slowed-down D1 protein turnover and increased (A+Z)/(V+A+Z) ratio.

Discussion

During the 3-h exposure to 5 °C under a PFD of 500 μmol m^{-2} s^{-1}, both the leaves of C+B and C plants showed a similar substantial photoinhibition, which approached steady state levels after a rapid decline in F_v/F_m. After return to optimal temperature (25 °C) and dim irradiance, F_v/F_m values increased with biphasic kinetics. A fast but incomplete recovery of photoinhibition occurred first and was followed by slow recovery kinetics. In addition, the extent of photoinhibition was discernibly lower but that of restoration was higher in C+B leaves than in C leaves (Fig. 3).

Difference in relative susceptibility to photoinhibition must be based on difference in protective strategies. Aro *et al.* (1993) and Park *et al.* (1995) showed that stimulating D1 turnover is the dominant strategy for protection of PS2 and is most effective in sustaining PS2 function upon exposure to excess photons. However, Demmig-Adams and Adams (1992) attribute a major role to limitations of xanthophyll cycle activity and assign only a minor role to D1 turnover. The (A+Z)/(V+A+Z) increased but D1 protein turnover decreased significantly both in the C+B and C leaves (Fig. 3), which indicated the two factors really play an important role in photoprotection during chilling-induced photoinhibition. However, significant differences were observed not only in the D1 protein turnover but also in the (A+Z)/(V+A+Z) ratio increase between the C+B and C leaves during photoinhibition. Hence the lesser photoinhibition in C+B leaves is probably due to the slower D1 protein turnover and greater increase of (A+Z)/(V+A+Z).

Low temperatures combined with moderate to high PFD cause a depression of photosynthesis and PS2 efficiency, because the dark reaction of photosynthesis is retarded more than energy absorption and electron flow, and the balance between energy absorption and utilization is disturbed (Huner *et al.* 1993, Wise 1995). These conditions promote oxidative damage mainly to PS2 which leads to the inactivation of photosynthetic electron transport and which is followed by the proteolytic degradation of the 32 kDa RC protein D1 (Virgin *et al.* 1988, Prášil *et al.* 1992), mediated by proteases DegP2 and Ftsh (Lindahl *et al.* 2000, Haussühl *et al.* 2001). This latter process might initiate the disassembly of damaged RC complexes as part of a repair mechanism. Both the inactivation of the PS2 mediated electron transport chain and the protein degradation can be induced by two different mechanisms involving the acceptor and the donor side of the photosystem (Virgin *et al.* 1988, Aro *et al.* 1993). When photoinhibition is determined by damage at the oxidising side of PS2, it is almost oxygen independent and probably related to the accumulation of highly oxidising species such as P^+_{680}, Tyr^+_{161}, and/or the accessory Chl cation (Chl^+_Z). In this case the D1 protein is specifically cleaved and two fragments, of apparent molecular masses 24 and 9 kDa, were detected, corresponding respectively to the C- and N-termini of the protein (Barbato *et al.* 1991, Shipton and Barber 1992). On the other hand, fragments of 23 and 10 kDa were detected during acceptor side induced photoinhibition corresponding to the N- and C-terminus of the protein, respectively (Aro *et al.* 1990). Degradation of D1 during acceptor side photoinhibition is dependent on the

presence of oxygen, and is accompanied by singlet oxygen ($^1O_2$) production (Macpherson *et al.* 1993, Hideg *et al.* 1994). Therefore, we suggest that the D1 degradation may be triggered by an intermediate, activated by the interaction with $^1O_2$.

The photosynthetic electron transport system has the potential to generate reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical (O_2^-), and H_2O_2. $^1O_2$ is generated in photosynthetic systems by interaction of the excited triplet state of Chl and molecular oxygen (Kramer and Mathis 1980). The triplet state in Chl-protein complexes is kept very low at normal conditions. The population of a triplet state in the RC of PS2 originating from the charged separated state (P^+_{680} $Pheo^-$), the recombination triplet, has been demonstrated in conditions in which photosynthetic electron transport chain becomes over-reduced and the primary quinone acceptor is markedly reduced and protonated. When the acceptor side of PS2 is 'over-reduced', the P680 recombination triplet is formed and it is this unquenched triplet which leads to the degradation of closely located proteins, *via* $^1O_2$ formation (Vass *et al.* 1996). So the improved ROS scavenging can positively affect photoprotection.

Betacyanins in plants do not participate in primary photosynthetic reactions in chloroplasts and are usually localized in vacuoles of the cells in or just below the epidermis (Clement and Mabry 1996, Stingtzing and Carle 2004). Although some evidences suggest that betacyanins are able to provide protection against photoinhibition induced by high "white" and UV-A irradiances (Vogt *et al.* 1999), little attention has been paid to their involvement in the defence against photo-damage caused by low temperature. The antioxidant properties of betacyanins have also been reported in some systems (Butera *et al.* 2002, Cai *et al.* 2003, Sepúlveda-Jiménez *et al.* 2004, 2005). The production of betacyanins in *S. salsa* in response to H_2O_2 treatments suggests that the pigment may function as a ROS scavenger, limiting the oxidative stress caused by environmental stressors (Wang *et al.* 2006a). Irradiation at low temperature causes a rapid increase in H_2O_2 content (Fig. 3*F*). Both the C+B and C leaves were able to dampen the rise in leaf H_2O_2 that occurred once irradiation commenced at 5 ºC but before photoinhibition (F_v/F_m) reached a steady state. But the H_2O_2 content in C+B leaves was much lower than in the C leaves during the chilling process; this may not only lead to a slower D1 protein turnover but also to a greater increase of (A+Z)/(V+A+Z), thus less PS2 photoinhibition. The results of our experiment *in vitro* were in agreement with those found *in vivo* (Table 1). So we concluded that betacyanin accumulation in *S. salsa* leaves positively affected photoprotection during chilling treatment. But the details of the protection mechanism need to be studied further.

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