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Effects of salicylic acid on the photosystem 2 of barley seedlings under osmotic stress

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Abstract

The effects of exogenous salicylic acid (SA) on photosystem 2 (PS 2) in barley (*Hordeum vulgare* L.) seedlings were investigated. SA pretreatment provided protection against subsequent osmotic stress. The highest protective effect of 0.25 mM SA was confirmed by determination of chlorophyll fluorescence, electrolyte leakage, malonyldialdehyde contents, PS 2 mRNAs and proteins. SA pretreatment increased reactive oxygen species (ROS), decreased net photosynthetic rate and stomatal conductance immediately, but prevented ROS accumulation during subsequent osmotic stress by activating antioxidant enzymes. Elimination of H_2O_2 during SA pretreatment inhibited almost all above mentioned SA effects. Therefore, SA pretreatment enhanced osmotic stress tolerance in barley seedlings mainly through ROS signals, rather than SA itself. The only SA-dependent and ROS-independent effect of exogenous SA on PS 2 was reduction of non-photochemical quenching.

Additional key words: chlorophyll fluorescence, electrolyte leakage, *Hordeum vulgare*, net photosynthetic rate, reactive oxygen species, stomatal conductance.

Introduction

Previous studies showed that the two photosystems, PS 1 and PS 2, particularly PS 2, were affected by osmotic stress and this led to lowered electron transport (Yuan *et al*. 2005, Liu *et al*. 2006). The steady-state-levels of PS 2 proteins D1, D2 and LHC 2 and corresponding genes *psbA*, *psbD* and *cab* mRNAs decreased dramatically under osmotic stress, which can be attributed to decreased transcription and translation rates and accelerated degradation (Yuan *et al*. 2005, 2007, Duan *et al*. 2006, Liu *et al*. 2006). Furthermore, LHC 2 could protect PS 2 core against various stresses by altering its conformation and migration between PS 2 and PS 1 (Allen 1992, 2003).

Salicylic acid (SA), a phenolic compound produced *via* the phenylpropanoid pathway (Metraux 2002), is implicated in pathogenesis-related gene expression, systemic acquired resistance, and the hypersensitive response (Shah 2003). SA is also believed to play a role in plant responses to abiotic stresses including osmotic stress, drought, salt and UV stress (Al-Hakimi *et al*. 2001, Horvath *et al*. 2007, Mahdavian *et al*. 2008).

Environmental stresses such as drought can cause lipid peroxidation, membrane deterioration, protein degradation and chlorophyll bleaching by forming potentially toxic reactive oxygen species (ROS) (Kang *et al*. 2003). Suitable SA pretreatment (usually ranges from 0.1 mM to 0.5 mM) could enhance stress tolerance and the effect is closely related to ROS metabolism. SA pretreatment immediately causes increased ROS, therefore activates antioxidant enzymes, which decreases ROS during subsequent stress (Yuan and Lin 2008). Exogenous H_2O_2 treatment also increases stress tolerance and pretreatment of ROS eliminators inhibits the protective effect of SA (Kang *et al*. 2003, Wahid *et al*. 2007), suggesting that SA in this level protects plants mainly through a ROS-dependent but SA-independent pathway (Yuan and Lin 2008).

The aim of the presented paper has been to elucidate the effects of SA on the PS 2 under osmotic stress.

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Abbreviations: DMTU - dimethylthiourea; PS 2 - photosystem 2; ROS - reactive oxygen species; RWC - relative water content; SA - salicylic acid.
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Materials and methods

Preparation of plant materials: Seeds of barley (*Hordeum vulgare* L.) were surface-sterilized with 1 % NaClO for 10 min. The germinated seeds were planted in sterilized sand and grown at 25 °C under a 12-h photoperiod and photosynthetic photon flux density (PPFD) of 100 μmol m^{-2} s^{-1}. The seedlings were cultivated with half-strength Hoagland's nutrient solution for 10 d and 0 - 1.5 mM SA was added. After SA pretreatment for 24 h, seedlings were transferred into 16 % PEG solutions with an osmotic potential of -0.6 MPa for 24, 48 and 72 h. Then the electrolyte leakage of membranes and malonyl-dialdehyde (MDA) content were measured.

According to a preliminary experiment, 0.25 mM of SA was chosen as an optimal concentration for barley seedlings. They were randomly divided into three groups: CK - control seedlings, SA - 0.25 mM SA pretreated seedlings (SA), and SA+D seedlings pretreated with 0.25 mM SA and 0.5 mM dimethylthiourea (DMTU, a trap for H_2O_2). The pH of all solutions was adjusted to 6.0. Then the osmotic stress was applied as mentioned above. The degree of osmotic stress was characterized by RWC, which was determined as the ratio of [(fresh mass - dry mass)/(water-saturated mass - dry mass)] × 100.

Electrolyte leakage and MDA content: Electrolyte leakage was measured according to Szalai *et al*. (1996). After measuring the conductivity, the barley samples were boiled for 15 min to achieve 100 % electrolyte leakage.

The MDA content of leaves was measured according to Karabal *et al*. (2003) with some modification (Sun *et al*. 2006). Approximately 0.2 g of fresh leaves were cut into small pieces and homogenized by the addition of 5 cm^3 5 % trichloroacetic acid (TCA) in ice bath. The homogenates were transferred into a tube and centrifuged at 10 000 *g* for 10 min at 4 °C. Equal volumes of supernatant and 0.5 % thiobarbituric acid (TBA) in 20 % TCA solution were added into a new tube. This mixture was incubated at 98 °C for 40 min, then cooled to room temperature and centrifuged at 8 000 *g* for 5 min. The supernatant was subjected to analysis with a spectro-photometer. The MDA content was calculated from A_{535} - A_{600} using coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Preparation of chloroplasts and thylakoids: Intact chloroplasts were prepared immediately from fresh barley leaves according to Tripathy and Prasanna (1980). High active thylakoids were prepared according to Kuwabare and Murata (1982) with some modifications: barley leaves were mashed by triturator in K1 isolation solution (100 mM sucrose, 200 mM NaCl, 50 mM phosphate buffer, pH 7.4) for 40 s, then filtered through two layers of cheesecloth and filtrate was centrifuged at 3 000 *g* for 10 min, deposits were centrifuged at 500 *g* for 60 s after suspending with K1, then supernatant was centrifuged at 3 000 *g* for 10 min, and thylakoids were isolated after suspending with K2 isolation solution (300 mM sucrose, 500 mM NaCl, 50 mM phosphate buffer, pH 6.9).

Superoxide and H_2O_2 were detected with nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively, as described previously (Yang *et al*. 2004). Barley leaves were excised at the base with a razor blade and supplied through the cut ends with NBT (1 mg cm^{-3}) or DAB (0.5 mg cm^{-3}) solutions for 8 h. Leaves were then decolorized in boiling ethanol (95 %) for 15 min. At least five leaves were used for each treatment in these experiments.

H_2O_2 content of leaves was measured as described by Velikova *et al*. (2002). Approximately 0.5 g of fresh leaves were cut into small pieces and homogenized in ice bath with 5 ml 0.1 % (m/v) TCA. The homogenate was centrifuged at 12 000 *g* for 20 min at 4 °C. 0.5 cm^3 of the supernatant was added to 0.5 cm^3 10 mM potassium phosphate buffer (pH 7.0) and 1 cm^3 1 M KI. The absorbance of supernatant was read at 390 nm.

SDS-PAGE and Western blot analysis: Equal amounts of thylakoid membrane proteins were applied on SDS-PAGE. Stacking gel and resolving gel were 5 and 12.5 %, respectively. For analyzing the changes in polypeptide composition of thylakoid complex, gels were stained with Coomassie blue R-250.

For Western blotting, electrophoresed proteins were immediately transferred onto nitrocellulose membrane according to Liu *et al* (2006). Then antiserum to the D1, D2 (provided from Prof. Eva-Mari Aro) and LHC 2 proteins (purchased from *AgriSera Comp*., Stockholm, Sweden) were applied. The signals revealed by using secondary antibodies of alkaline phosphatase goat anti-rabbit IgG. Semi-quantitative data on the contents of proteins were obtained by densitometric scanning of the signal intensity of Western blots.

Preparation of total RNA and Northern blot hybridization: Total RNA was extracted from leaves according to Yuan *et al*. (2005). For Northern blot analysis, an equal amounts of total RNA (20 μg) were separated by formaldehyde agarose gel electrophoresis, then RNA was transferred to nitrocellulose filters for subsequent probe hybridization according to Yuan *et al*. (2007). DNA fragments of the *psb*A gene (provided from Prof. N.-H. Wu), *psb*D gene (provided from Prof. L.-F. Yan), *cab* gene (provided from Dr. Y.-S. Zhu), and *rbcS* (provide from Dr. N.-H. Zhang) were used as probes for RNA blot analyses.

Gas exchange: Gas exchange was measured by an open system TPS-1 (*PP systems*, Hitchin, UK). Net photo-synthetic rate (P_N) was determined at CO_2 concentration of 360 μmol mol^{-1}, relative humidity of 80 %, irradiance of 100 μmol m^{-2} s^1 and temperature of 25 °C. Leaf stomatal conductance (g_s) was measured under the same conditions with the steady-state porometer TPS-1.

Measurements of chlorophyll fluorescence: Chlorophyll fluorescence quenching analysis was carried out at room temperature (25 °C) with a portable fluorometer (PAM-2100, *Walz*, Effetrich, Germany). The minimal fluorescence in the dark-adapted (10 min) leaves (F_o) was measured at irradiance < 0.1 μmol m^{-2} s^{-1}. The minimal fluorescence in the light-adapted leaves (F_o') was measured after turning off the actinic light, which was equivalent of the growth irradiance and far-red light for 3 s. The maximum fluorescence in dark- and light-adapted leaves (F_m and F_m') was induced by by 0.8 s pulse of saturating white light (8000 μmol m^{-2} s^{-1}). The steady-state fluorescence (F_s) was also recorded after 5 min irradiance at 100 μmol m^{-2} s^{-1} using the actinic light. Using measured fluorescence parameters, we calculated: *1*) the maximum efficiency of PS 2 photochemistry in the dark-adapted leaves (F_v/F_m), *2*) the efficiency of excitation energy capture by open PS 2 reaction centers, $F_v'/F_m' = (F_m' - F_o')/F_m'$, *3*) the non-photochemical quenching coefficient, $NPQ = (F_m - F_m')/F_m'$, and *4*) the quantum yield of PS 2 electron transport, $\Phi_{PS2} = (F_m' - F_s)/F_m'$.

Statistical analysis: Means of 3 triplicates were measured. Student's *t*-test was used for comparison among CK, SA and SA+D. Differences of treatments were considered to be statistically significant when $P < 0.05$.

Results

Determination of optimal concentration of SA applied: The rate of electrolyte leakage and MDA content has been used as an indicator of osmotic stress injuries. Barley seedlings pretreated with different concentrations of SA for 1 d were transferred into PEG solutions with an osmotic potential of -0.6 MPa for 48 h, and then the electrolyte leakage of membranes and MDA contents of leaves were measured (Fig. 1). Electrolyte leakage of cells firstly decreased as the SA concentration increased, while higher concentrations caused increased electrolyte leakage. The lowest electrolyte leakage was at 0.25 mM SA. The MDA contents demonstrated the same effect. Consequently, 0.25 mM was selected and used for following studies.

At 0 h, the electrolyte leakages of SA pretreated plants were higher than that in control seedlings, indicating SA pretreated plants were suffered form a slight stress. As the osmotic stress developed, the electrolyte leakages of CK and SA+D pretreated plants increased more than in SA, which were in accordance with the changes of ROS. The MDA contents changed similarly as electrolyte leakage (Fig. 2).

RWC in CK, SA, SA+D seedlings: When roots of barley seedlings were immerged into PEG solution with an osmotic potential of -0.6 MPa for 24, 48, and 72 h, leaf RWC decreased gradually. We considered stress after 24, 48, and 72 h as mild, moderate, and severe osmotic stress, respectively. In control seedlings, RWCs decreased by 17.1, 22.3 and 45.8 %, respectively, relative to 0 h. In SA+D pretreated seedlings, RWCs decreased by 20.2, 29.7 and 50.5 %, respectively, while, RWCs of SA pretreated seedlings decreased only by 12.3, 15.6 and 32.7 %, respectively. These results showed that the SA could defer the decline of RWC in barley leaves under osmotic stress, but the SA+D could not (Fig. 2).

ROS contents: During 24 h pretreatment, H_2O_2 content in SA-treated seedlings increased significantly; while that of SA+D was a little lower than that in CK. As the osmotic stress developed, H_2O_2 content increased in all treatments, but after 72 h, the increase was highest in SA+D treatment and the lowest in the SA-treated seedlings.

Similarly to total cellular H_2O_2, H_2O_2 content of chloroplasts increased significantly during 24 h of SA pretreatments. As the osmotic stress developed, SA deferred the increase of H_2O_2. Chloroplast H_2O_2 of SA+D increased slowly, while the total cellular H_2O_2 of SA+D increased rapidly and was almost the same as in the control seedlings.

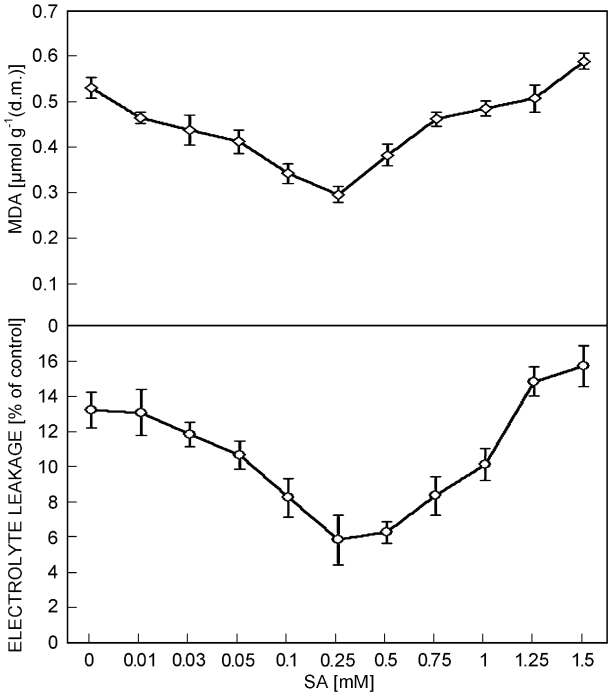

Fig. 1. Effects of SA concentrations on electrolyte leakage and MDA content of barley seedling membranes after 1 d of SA pretreatment and 2 d of osmotic stress. *Bars* represent standard deviations of 5 independent replicates.

Little DAB and NBT staining was observed in control leaves, except in some epidermal cells. Leaves treated with 0.25 mM SA for 1 d showed significant accumulations of H_2O_2 and superoxide in the vascular tissue (data

not shown). After 3 d of osmotic stress, leaves of both control and SA+D-pretreated plants showed significant staining. Different from the DAB staining, the NBT staining in SA+D at 0 h was more marked than that in SA, showing that DMTU eliminated H_2O_2 but not superoxide (data not shown).

Gas exchange: After 24 h of SA pretreatments, g_s and P_N were significantly lower than in CK or SA+D. As the osmotic stress developed, less decrease of stomatal conductance and CO_2 assimilation rate was observed in SA treated plants, and the effect of stomatal closure induced by SA pretreatment could not be observed when H_2O_2 was eliminated in SA+D pretreated plants (Fig. 3). Transpiration rate changed similarly as stomatal conductance (data not shown).

Chlorophyll fluorescence parameters: F_v/F_m, F_v'/F_m' and Φ_{PS2} declined gradually and NPQ increased gradually in all plants as the osmotic stress developed. 24 h of SA pretreatment did not affect F_v/F_m and Φ_{PS2}, but they decreased more slowly during osmotic stress in SA and SA+D than in CK seedlings. SA pretreatment significantly promoted F_v'/F_m', and reduced NPQ correspondingly (Fig. 3). Elimination of H_2O_2 during pretreatment only partly reversed the effects of SA to F_v'/F_m' and NPQ, suggesting that they may be mediated through a SA-dependent and ROS-independent pathway.

PS 2 polypeptides and mRNAs: Steady state contents of thylakoid polypeptides in CK seedlings declined gradually as osmotic stress developed, including peripheric antenna complex LHC2, core antennae CP43 and CP47, oxygen-enhancer protein OEE1, and PS1 complex CF1 (Fig. 4*A*). In contrast, these polypeptides in SA and SA+D pretreated seedlings showed only a slight decrease, indicating that both SA and SA+D pretreatment provided a good protection on these proteins under osmotic stress.

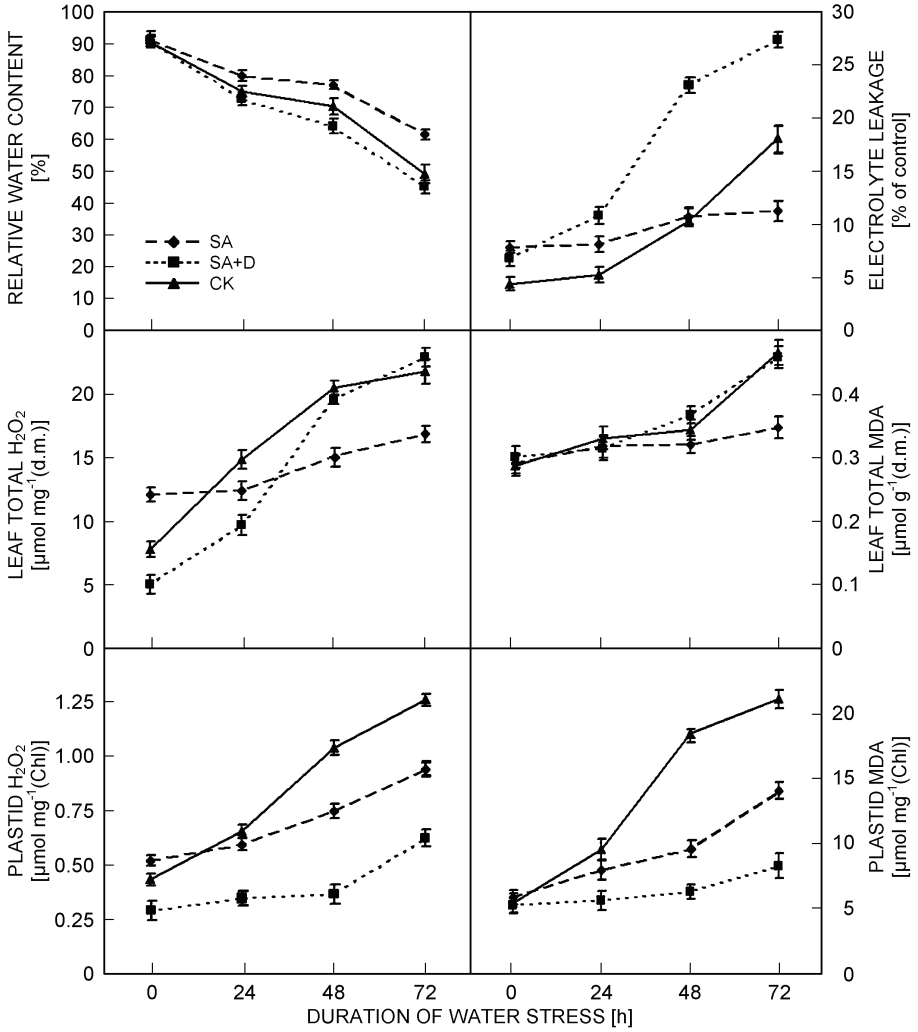

Fig. 2. Effects of exogenous SA, SA+D pretreatments on leaf relative water content (RWC), electrolyte leakage, lipid peroxidation (MDA content) and H_2O_2 content, plastid lipid peroxidation and plastid H_2O_2 content in barley seedlings under PEG osmotic stress. *Bars* represent standard deviations of 5 independent replicates. CK, SA and SA+D represent control seedlings, SA-pretreated seedlings, and SA + DMTU-pretreated seedlings, respectively.

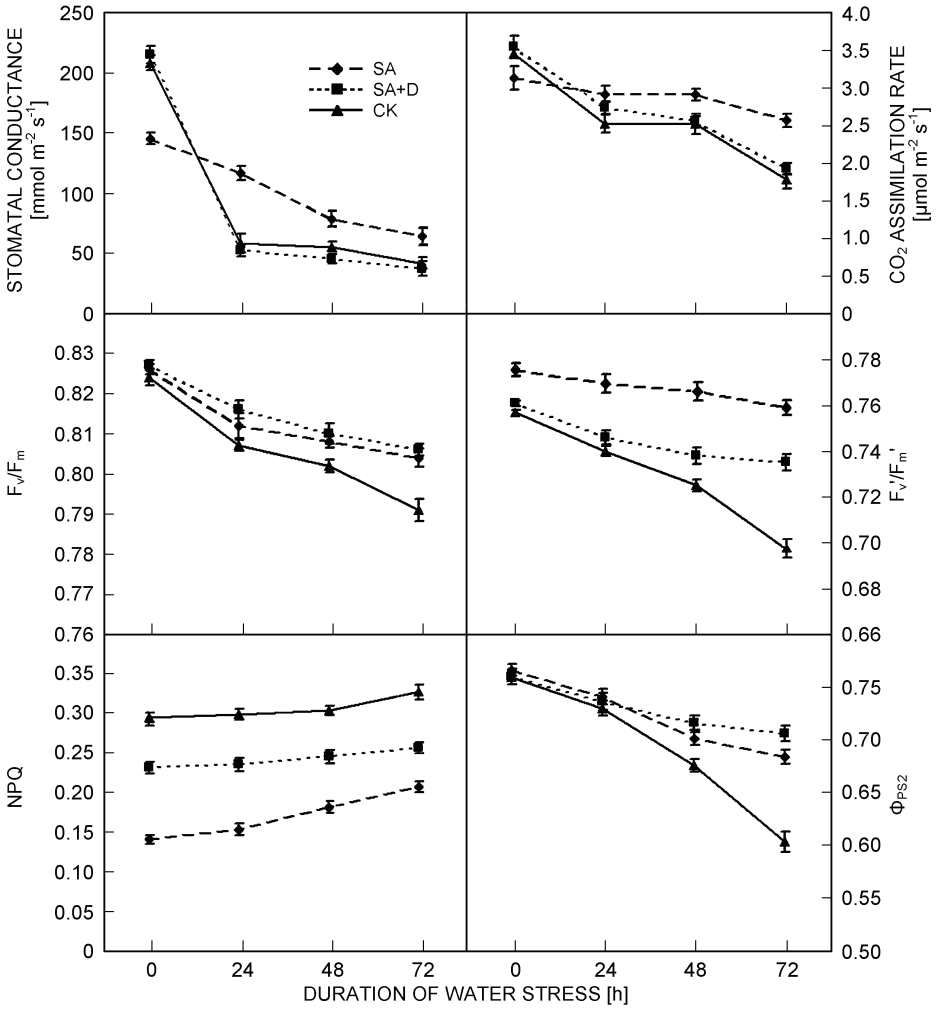

Fig. 3. Changes of gas exchange (indicated by CO_2 assimilation rate and leaf stomatal conductance) and PS 2 photochemical activities (indicated by F_v/F_m, F_v'/F_m', NPQ and Φ_{PS2}) under osmotic stress. *Bars* represent standard deviations of 5 independent replicates. CK, SA and SA+D represent control seedlings, SA-pretreated seedlings, and SA + DMTU-pretreated seedlings, respectively.

Western blot confirmed the changes of PS 2 polypeptides. Progressive declines in D1, D2, LHC 2 proteins were found in CK. In contrast, the contents decreased very slightly in SA and SA+D (Fig. 4*B*).

Northern blot analysis indicated that the transcripts of nuclear-coded genes *cab* and *rbcS* changed different from plastid-coded genes *psbA* and *psbD*. The decreases of *cab* and *rbcS* mRNAs in SA+D barley seedlings were more serious than those in SA and CK, which were in accordance with the changes of total cellular ROS. Compared with CK, the transcripts of *psbA* and *psbD* in SA and SA+D decreased only slightly, which were in accordance with the changes of ROS in plastid (Fig. 5). In SA+D seedlings, *cab* mRNA decreased largely after 72 h of osmotic stress, while its protein maintained stable in plastids.

Discussion

Compounds which are able to alleviate the damaging effects of certain stresses may be of great importance from both theoretical and practical points of view (Janda *et al*. 1999). In the present study, we showed that application of exogenous SA at concentration 0.25 mM decreased markedly the electrolyte leakage of membranes and MDA contents of leaves, suggesting that SA enhanced osmotic stress tolerance of barley. In contrast, when SA concentration higher than 1.0 mM or lower than 0.1 mM were applied, more electrolyte leakage of membranes and higher MDA contents were observed, indicating that they aggravated osmotic stress injury and SA itself could be a sort of stress (Kang *et al*. 2003, Horvath *et al*. 2007, Yuan and Lin 2008). Effects of SA on plants were dependent on the concentration used for pretreatment.

Previous studies and our experiments showed that high ROS concentration was toxic for plants, but at low concentration, it plays a role in signal transductions in both

plants and animals (Foyer and Noctor 2005, Van Breusegem and Dat 2006, Xu *et al*. 2007). Through an increase in cytosolic calcium, they induce the protective mechanism (Kaeano *et al*. 2000). When plants were pretreated with 0.25 mM SA for 1 d, ROS content increased remarkably, because SA application enhanced H_2O_2 production probably by inhibition of catalase (CAT) and ascorbate peroxidase (APX) activity (Janda *et al*. 1999, Huang *et al*. 2004). Thus ROS act as a secondary signal to enhance antioxidative enzymes. Abiotic stresses including osmotic stress can also induce ethylene and NO, which may also participate in SA-ROS pathway leading to activation of antioxidative enzymes (Fujita *et al*. 2006). Elimination of H_2O_2 during pretreatment largely inhibited the protective effect of SA. It could be concluded that SA in this concentration protects plants mainly through a ROS-dependent pathway.

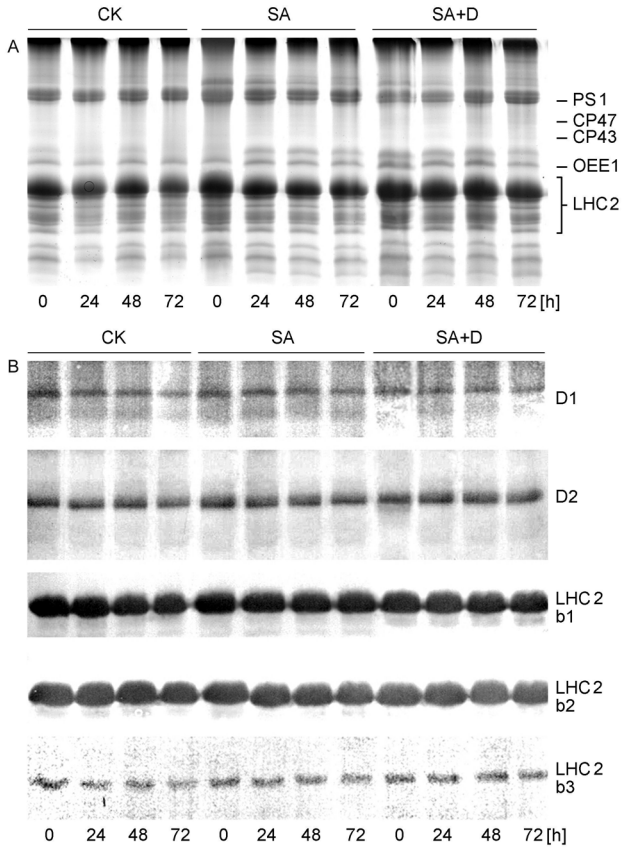

Fig. 4. SDS-PAGE (*A*) of thylakoid polypeptides isolated from leaves stressed for 0, 24, 48, and 72 h. Equal amounts of thylakoid membrane proteins were loaded. The gel was stained with Coomassie Blue R-250. Western blot analysis (*B*) of steady state levels of the D1, D2, LHC 2b1 LHC 2b2 and LHC 2b3 proteins in PS 2 during osmotic stress. Equal amounts of thylakoids from different stressed levels were separated by a SDS-PAGE and electron-transferred to nitrocellulose membranes. The resulting blots were then allowed to immunoreact with the antibodies against D1, D2, LHC 2b1 LHC 2b2, and LHC 2b3. CK, SA and SA+D represent control seedlings, SA-pretreated seedlings, and SA + DMTU-pretreated seedlings, respectively.

In SA+D seedlings, plastid H_2O_2 was maintained stable at a low level, while cytoplasmic H_2O_2 increased dramatically after prolonged osmotic stress. The reason might be that SA induced ROS mainly in cytoplasm and DMTU eliminated ROS and offset the effects of SA also mainly in cytoplasm. Meanwhile DMTU was exhausted in cytoplasm and could not maintain its activity during subsequent osmotic stress. On the other hand, some DMTU left in plastids and acted continually during stress. In order to confirm our assumption, simple DMTU pretreatment was applied, and the results showed that both plastid ROS and cytoplasmic ROS were rather low during 4 d (data not shown).

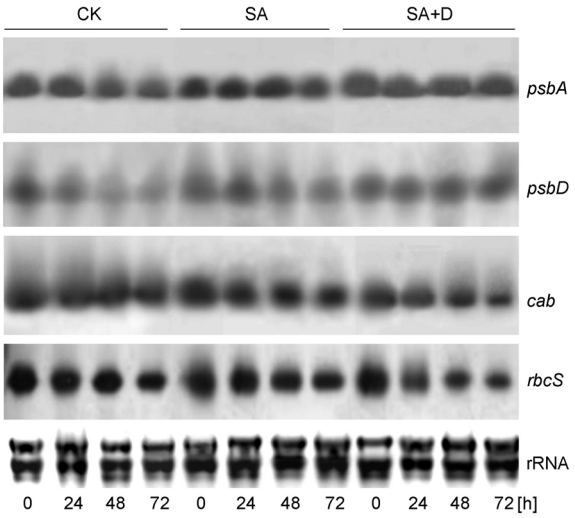

Fig. 5. Northern blot analysis of chloroplast gene *psbA*, *psbD*, and nuclear gene *cab, rbcS* transcripts. Total RNA was prepared from stressed (24 to 72 h) and non-stressed (0 h) leaves. Following blotting, *psb*A, *psb*D, *cab* and *rbcS* transcripts were detected by hybridization with specific DNA probes. The amounts of mRNAs were normalized to rRNA for each treatment. CK, SA and SA+D represent control seedlings, SA-pretreated seedlings, and SA + DMTU-pretreated seedlings, respectively.

Through Western blotting and Northern blotting, we confirmed that D1, D2 and LHC 2 proteins and their transcript levels decreased more quickly under osmotic stress in CK than in SA and SA+D, indicating the protective role of SA to PS 2 under osmotic stress. The protective effects of SA were correlated closely to the ROS content in plants. When ROS content in plastid was relatively low, the PS 2 proteins, plastid mRNAs and PS 2 photochemical activities kept stable, no matter how severe stress the plants suffered. On the other hand, electrolyte leakage and cytosol mRNAs were closely correlated with the cytosol ROS content.

In SA+D seedlings, P_N was rather low after 72 h of osmotic stress, although the PS proteins were not largely decreased at that time. The decreased g_s (over 80 %) was the reason for the declined P_N. Previous studies indicated that SA pretreatment decreased g_s and P_N (Janda *et al*. 1999). This effect is confirmed by our experiments.

Moreover, we showed that this effect could be inhibited through elimination of H_2O_2. Therefore, it can be regarded as a stress/ROS response, rather than as a direct effect of SA. H_2O_2 treatment alone also induces stomatal closure (Wahid *et al*. 2007).

After 1-d pretreatment with 0.25 mM SA, F_v'/F_m' (efficiency of excitation energy capture by opened reaction centres) largely increased. Meanwhile, NPQs in SA pretreated seedlings were remarkably lower than those in CK. In other words, pretreatment with SA could reduce the heat dissipation of photosystems. The values of F_v'/F_m' and NPQ in SA+D seedlings distributed between SA and CK, indicated that modulating effect of SA for heat dissipation of PS 2 was partly dependent on ROS.

As a whole, SA pretreatment enhanced osmotic stress tolerance of barley seedlings, and the effects of SA were related closely to its concentration. Osmotic stress tolerance enhanced by SA pretreatment was associated with ROS. The decrease in contents of PS 2 major proteins D1, D2 and LHC 2 and the transcripts of corresponding genes *psbA, psbD* and *cab* were alleviated by SA pretreatment under osmotic stress. We found that enhanced osmotic stress tolerance in barley seedlings by SA pretreatment was mainly mediated with ROS, rather than the SA itself.

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