Water Stress Enhances Expression of Genes Encoding Plastid Terminal Oxidase and Key Components of Chlororespiration and Alternative Respiration in Soybean Seedlings

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Plastid terminal oxidase (PTOX) is a plastid-localized plastoquinone (PQ) oxidase in plants. It functions as the terminal oxidase of chlororespiration, and has the potential ability to regulate the redox state of the PQ pool. Expression of the *PTOX* gene was up-regulated in soybean seedlings after exposure to water deficit stress for 6 h. Concomitantly expression of the *NDH-H* gene, encoding a component of the NADPH dehydrogenase (NDH) complex which is a key component of both chlororespiration and NDH-dependent cyclic electron transfer (CET), was also up-regulated. Transcript levels of the proton gradient regulation (*PGR5*) gene, which encodes an essential component of the PGR5-dependent CET, were not affected by water stress, while the expression of the alternative oxidase (*AOX1*) gene, which encodes a terminal oxidase of alternative respiration in mitochondria, was also up-regulated under water stress. Therefore, our results indicate that water stress induced the up-regulation of genes encoding key components of chlororespiration and alternative respiration. Transcript levels of the *AOX1* gene began to increase in response to water stress before those of *PTOX* suggesting that alternative respiration may react faster to water stress than chlororespiration.

Key words: Chlororespiration, Plastid Terminal Oxidase (PTOX), Water Stress

Introduction

Water stress is considered a major environmental factor limiting plant growth and yield all over the world, especially of those species which react sensitively to water deficit. It is well known that one of the primary physiological consequences of water stress is the inhibition of photosynthesis, since CO_2 diffusion to the chloroplasts is reduced due to stomatal closure; photosystem activity and electron transport are also directly affected by water stress (Chaves *et al*., 2003; Flexas *et al*., 2006). Nevertheless, when exposed to water stress, plants are able to activate mechanisms to harmonize photosynthetic light reactions and carbon assimilation, and thus alleviate photosynthesis inhibition. Among these mechanisms are photorespiration (Wingler *et al*., 2000), cyclic electron transfer (CET) around photosystem I (PSI) (Rumeau *et al*., 2007; Lehtimäki *et al*., 2010), and even pathways outside chloroplasts like alternative respiration (Feng *et al*., 2008). Besides these well-studied mechanisms, the physiological roles of plastid terminal oxidase (PTOX) have become a new focus in recent years (Sun and Wen, 2011).

PTOX, a plastid-localized plastoquinone (PQ) oxidase, exists widely in photosynthetic species, includ-

ing algae and higher plants (Carol and Kuntz, 2001; Kuntz, 2004). Evidence indicates that PTOX is an interfacial membrane protein with a di-iron carboxylate center in the active site (Aluru and Rodermel, 2004). It transfers electrons from reduced PQ to molecular oxygen with the formation of water, and acts as terminal oxidase of chlororespiration, a respiratory electron transport chain in the thylakoid membrane involving both non-photochemical reduction by the NADPH dehydrogenase (NDH) complex and oxidation of PQ by PTOX (Peltier and Cournac, 2002; Aluru and Rodermel, 2004). The physiological roles of PTOX have been investigated since it was isolated from plants (McDonald *et al*., 2011). There are indications that PTOX functions in carotenoid biosynthesis by transferring electrons abstracted from precursors during the desaturation process to O_2 via the PQ pool, and that it plays an important role in chloroplast biogenesis (Carol and Kuntz, 2001; Aluru *et al*., 2006). Recent studies also suggested that PTOX is beneficial for plants under various forms of environmental stress, because of its ability to regulate the redox state of the PQ pool and to protect the photosynthetic electron transport chain from over-reduction (Sun and Wen, 2011). However, there are also arguments that PTOX alone cannot act as a safety valve in protecting photosynthesis (Rosso *et al*., 2006; Okegawa *et al*., 2010). Related physiological processes, such as CET around PSI and antioxidation mechanisms, must rather be co-regulated for PTOX-dependent stress tolerance (Sun and Wen, 2011).

Soybean (*Glycine max* L.) is an important economic crop worldwide, whose growth is highly affected by the water supply (Liu *et al*., 2013), especially at the seedling stage. In the present study, we investigated the expression of the *PTOX* gene in soybean seedlings under water stress to obtain evidence for the role of PTOX in stress responses. The expression of key genes in some related physiological processes, *e. g.* CET and alternative respiration, was also determined, in order to clarify the relationship between PTOX and these processes.

Materials and Methods

Plant growth and treatments

Surface-sterilized seeds of soybean [*Glycine max* (L.) Merr.] cv. Gongxuan 1, provided by the Key Laboratory of Crop Eco-physiology and Farming System in Southwest China (Ministry of Agriculture), Sichuan Agricultural University, Chengdu, China were germinated and grown in pots in a greenhouse (120 μmol photons m^{-2} s^{-1}, 14-h light/10-h dark cycle, 25 °C). For water stress treatment, the roots of two-week-old seedlings were immersed in polyethylene glycol (PEG-6000) solutions with an osmotic potential of −0.2 MPa for 3, 6, 9, and 12 h, respectively. Seedlings receiving no stress treatment were used as control. In order to study the different responses under light and dark conditions, control and stressed seedlings were both grown under continuous light or in total darkness during the respective treatments.

RNA extraction and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from liquid nitrogen-powdered leaf tissue by extraction buffer [20 mM Tris-HCl, pH 8.0, 1 % (w/v) sodium dodecyl sulfate (SDS), 200 mM NaCl, 5 mM EDTA] and phenol/chloroform (1:1, v/v) according to Lei *et al*. (2010). RNA concentrations were determined spectrophotometrically.

For semi-quantitative RT-PCR analysis, first-strand cDNA was synthesized from 1 μg of total RNA, us-

Table I. Gene-specific primers used in RT-PCR analysis.

Gene	Primers	
PTOX	forward	5′-CTTGATACTCTGTACCATGA-3′
	reverse	5′-AATTCATCTCCTTGGACC-3′
NDH-H	forward	5′-ATGAATATCTCAACTACAAGA-3′
	reverse	5′-TCAACGATCAACTTCTTCCA-3′
PGR5	forward	5′-TGCTGGCCAAGTCAGTGCC-3′
	reverse	5′-GCCATTCTCCATTCTTCTTGGCCAACC-3′
AOX1	forward	5′-GAAGCACCATGCTCCAAC-3′
	reverse	5′-CCCTTGATAGTGAATGTCC-3′

ing M-MLV reverse transcriptase (TaKaRa Biotech., Dalian, China) with universal oligo(dT)$_{16}$ primer. The resulting cDNA was used for PCR in a MyCycler gradient PCR amplifier (Bio-Rad, Richmond, CA, USA) with gene-specific primers for *PTOX*, NDH-H subunit (*NDH-H*), proton gradient regulation (*PGR5*), and alternative oxidase (*AOX1*), which are presented in Table I.

Measurement of relative water content (RWC)

RWC of leaves was calculated according to $(\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW}) \cdot 100\,\%$, where FW means fresh weight, TW means turgid weight, and DW means dry weight, as described by Cruz de Carvalho *et al*. (2011).

Determination of photosynthetic pigments

Chlorophylls and carotenoids were extracted from fresh leaves with 80 % acetone. The absorbance of the extract was read at 663, 646, and 470 nm. Contents of these pigments were calculated according to Lichtenthaler and Wellburn (1983).

Lipid peroxidation analysis

Lipid peroxidation was assessed by the malondialdehyde (MDA) content. Fresh leaves were homogenized with 5 % (w/v) trichloroacetic acid in an ice bath and centrifuged at $3000 \times g$ for 10 min at 4 °C. Then equal amounts of supernatant and 0.67 % (w/v) thiobarbituric acid were mixed, and the mixture was incubated in a boiling water bath for 15 min. After cooling to room temperature, the mixture was centrifuged at $4000 \times g$ for 10 min. Absorbance of the supernatant was measured at 600, 532, and 450 nm. Concentration of MDA was calculated according to Du *et al*. (2011).

Measurement of electrolyte leakage

Electrolyte leakage was measured according to Sun *et al*. (2006). Fresh leaves were placed in deionized water at room temperature. After 45 min, the electrical conductivity was measured, and then the sample was incubated in a boiling water bath for 10 min to achieve 100 % electrolyte leakage. The result was calculated as the ratio of electrical conductivity before and after boiling.

Measurement of hydrogen peroxide (H_2O_2) content

H_2O_2 content was measured according to Sun *et al*. (2009). Fresh leaves were homogenized with 0.1 % (w/v) trichloroacetic acid in an ice bath and centrifuged at $12{,}000 \times g$ for 20 min at 4 °C. Then the supernatant was mixed with 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI. The absorbance of the supernatant was read at 390 nm. The content of H_2O_2 was determined by comparison with a standard curve.

Enzyme assays

NADP-malate dehydrogenase (NADP-MDH) and NAD-malate dehydrogenase (NAD-MDH) were extracted from liquid nitrogen-powdered leaf tissue by 25 mM HEPES-KOH buffer (pH 7.5) as described by Zhang *et al*. (2010). NADP-MDH was assayed with 25 mM Tricine-KOH buffer (pH 8.3) containing 150 mM KCl, 1 mM Na_2EDTA, 5 mM dithiothreitol, 0.2 mM NADPH, and 2 mM oxaloacetate (OAA). NAD-MDH was assayed with 50 mM TES-HCl buffer (pH 7.2) containing 10 mM $MgCl_2$, 0.02 % (v/v) Triton X-100, 0.2 mM NADH, and 2 mM OAA. Activities of these two enzymes were measured spectrophotometrically at 340 nm according to Zhang *et al*. (2010).

Statistical analysis

The results are the means of three independent measurements and were statistically evaluated using the standard deviation and *t*-test methods. The difference was considered to be statistically significant when $p < 0.05$.

Results

Expression of the PTOX gene under water stress

Levels of soybean *PTOX* transcripts were determined by semi-quantitative RT-PCR analysis to evaluate the expression of *PTOX* under water stress. Figure 1a shows that, when seedlings were exposed to stress under light condition, the level of *PTOX* transcripts did not change in the first few hours (0 – 3 h). However, a persistent increase was detected after 6 h of water stress, in contrast to the control which displayed no change in the *PTOX* transcript level during the entire treatment period of 12 h (Fig. 1b). For the seedlings in the dark, only weak signals could be detected by RT-PCR, regardless of whether they were

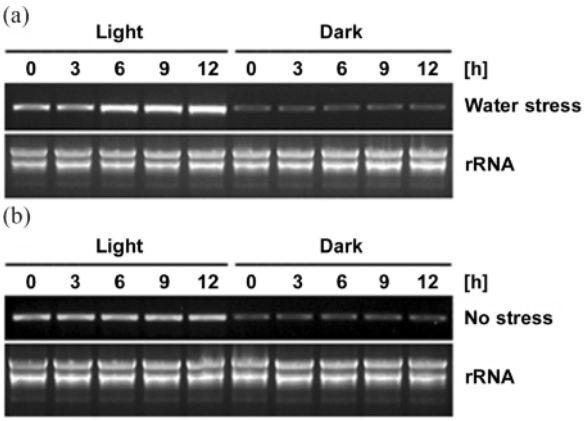

Fig. 1. Semi-quantitative RT-PCR analysis of *PTOX* transcripts in (a) water-stressed and (b) non-stressed soybean seedlings under light or dark conditions. rRNA is shown as a loading control and indicator of RNA intactness.

water-stressed or not, and these low transcript levels did not change during the entire treatment period of 12 h (Figs. 1a, 1b). These results suggest a role for PTOX in response to water stress in soybean seedlings in the light, but not in the dark. Therefore, for the following experiments all seedlings were kept in the light.

Effects of water stress on RWC and contents of photosynthetic pigments

For a quantitative assessment of the stress effects, the RWC and contents of photosynthetic pigments of the seedlings were determined. In non-stressed seedlings, the RWC always exceeded 90 %. After exposure to water stress, the RWC decreased continuously during the entire treatment period. After 12 h of stress, the RWC had dropped to a level below 80 % (Fig. 2a). Contents of photosynthetic pigments, *i.e.* chlorophylls and carotenoids, also decreased markedly during this period (Figs. 2b, 2c). After 12 h of water stress, the contents of these pigments had decreased by about 50 % in water-stressed seedlings compared to non-stressed seedlings, implying that the water stress had significant negative effects on the physiological processes of the seedlings.

Effects of water stress on oxidative damage

In order to investigate the oxidative damage caused by water stress, MDA content and electrolyte leakage of the seedlings were studied. In the absence of stress, both MDA content and electrolyte leakage remained at

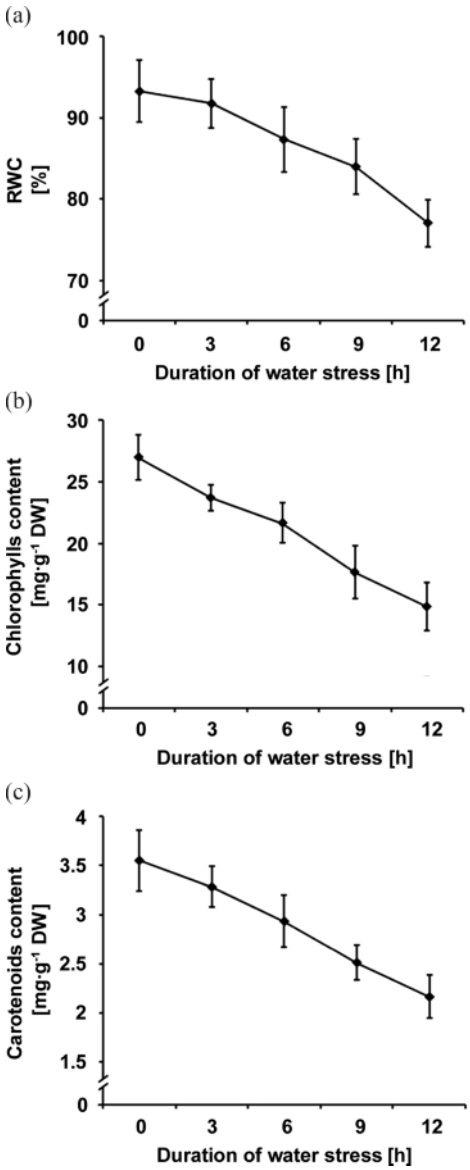

Fig. 2. (a) RWC, (b) chlorophyll content, and (c) total carotenoid content of soybean seedlings under water stress. Bars represent standard deviations of three independent measurements.

a low level. However, after exposure to water stress, the MDA content increased rapidly and was enhanced about three-fold after 12 h compared to seedlings without stress (Fig. 3a). Electrolyte leakage increased almost two-fold in parallel (Fig. 3b).

We also determined the level of H_2O_2 as an indicator of the accumulation of reactive oxygen species

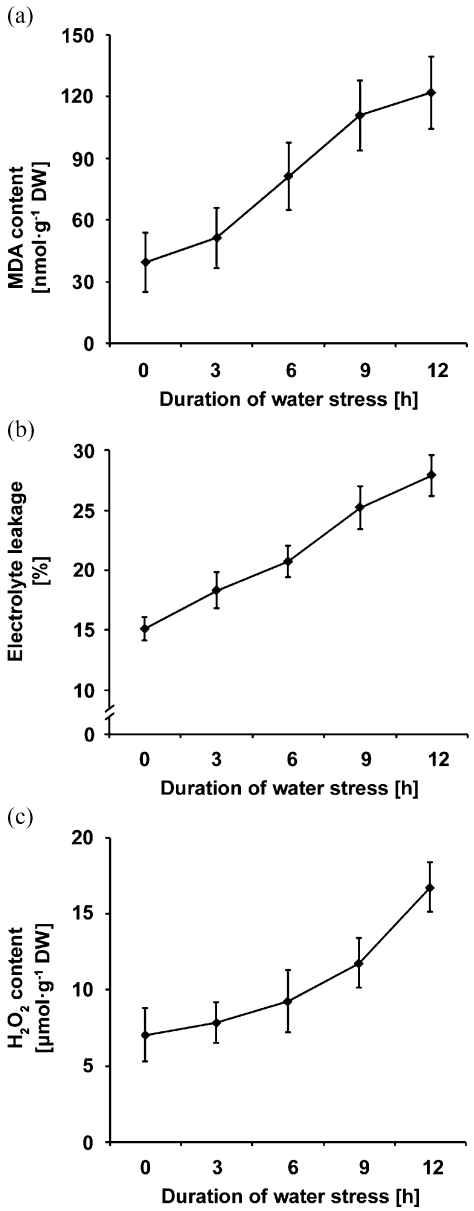

Fig. 3. (a) MDA content, (b) electrolyte leakage, and (c) H_2O_2 content of soybean seedlings under water stress. Bars represent standard deviations of three independent measurements.

(ROS) in the seedlings under water stress. H_2O_2 accumulated to a level more than two-fold after 12 h of water stress compared to non-stressed seedlings (Fig. 3c). This increase is in accord with the changes seen for the MDA content and electrolyte leakage, and confirms that water stress leads to oxidative stress.

Expression of key genes in CET and alternative respiration under water stress

To study the co-regulation of processes related to PTOX, responses of essential components of CET to water stress were determined. At least two CET pathways around PSI have been described, which are referred to as the NDH- and PGR5-dependent route, respectively (Johnson, 2011). We measured the transcript levels of the *NDH-H* and *PGR5* genes, which are known to code for the respective key components of these two electron transfer routes. The two genes responded differentially to water stress. Expression of *NDH-H* was up-regulated when the seedlings were exposed to water stress, and increased persistently during the entire treatment period, notably after 6 h of water stress (Fig. 4). On the other hand, the expression level of *PGR5* displayed no obvious change even after 12 h of water stress (Fig. 4).

We also considered the involvement of mitochondrial alternative respiration in the response to water stress, which has a close relationship with photosynthesis during environmental changes (Vanlerberghe, 2013; Zhang *et al.*, 2010). Alternative respiration represents a respiratory electron transport chain that branches from the ubiquinone pool to the terminal oxidase called AOX, which shares high homology with PTOX (McDonald *et al.*, 2011). This pathway is considered an important process in the dissipation of chloroplast-reducing equivalents outside chloroplasts, especially under environmental stress (Vanlerberghe, 2013; Zhang *et al.*, 2010). Although there are three different AOX, named AOX1, AOX2a, and AOX2b, in soybean, AOX1 is regarded as the main component in the stress response (Considine *et al.*, 2002). The expression level of *AOX1* gene increased persistently during the entire period of water stress (Fig. 4). Unlike its homologous *PTOX* gene, up-regulation of the *AOX1*

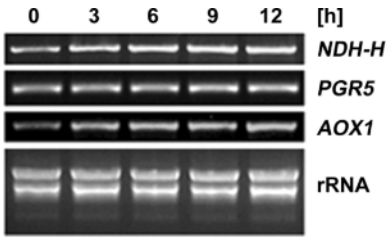

Fig. 4. Semi-quantitative RT-PCR analysis of the expression of the *NDH-H*, *PGR5*, and *AOX1* genes in soybean seedlings under water stress. rRNA is shown as a loading control and indicator of RNA intactness.

was detected already after 3 h of water stress and thus preceded the up-regulation of *PTOX* (Fig. 4).

Effects of water stress on the activities of NADP-MDH and NAD-MDH

NADP-MDH and NAD-MDH are key enzymes of the malate (Mal)/OAA shuttle, which can transport reducing equivalents from chloroplasts to other cellular compartments, where they are dissipated via pathways such as alternative respiration (Noguchi and Yoshida, 2008; Scheibe *et al*., 2005). Activities of NADP-MDH and NAD-MDH increased during water stress. After 3 h of stress, both activities were stimulated almost two-fold compared to control seedlings, and the increase continued in the subsequent period (Figs. 5a, 5b). Therefore, we inferred that the Mal/OAA shuttle was enhanced to export excess chloroplast-reducing equivalents after water stress. These results were in accord with those obtained for *AOX1* gene expression.

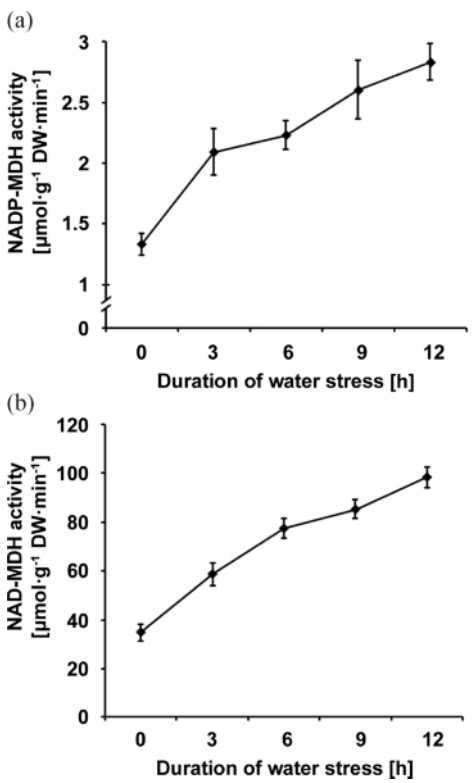

Fig. 5. Activities of (a) NADP-MDH and (b) NAD-MDH in soybean seedlings under water stress. Bars represent standard deviations of three independent measurements.

Discussion

Photosynthetic products are essential for plant growth and yield. However, photosynthesis is affected by environmental changes, and water deficit stress is a well known detrimental factor. Water stress has various effects on photosynthesis, such as reduced carbon assimilation due to limited CO_2 diffusion as a result of stomatal closure. In this case, disharmony between photosynthetic light reactions and carbon assimilation is inevitable, and the excess light energy may lead to oxidative damage to plants (Chaves *et al*., 2003; Flexas *et al*., 2006). In our study, the RWC and the contents of photosynthetic pigments decreased in the leaves of water-stressed soybean seedlings (Fig. 2). At the same time, the H_2O_2 level increased and oxidative damage was reflected by an increase in the MDA content and electrolyte leakage (Fig. 3).

Plants have developed strategies to alleviate the consequences of photosynthesis inhibition, of which PTOX has been considered a potential one (Sun and Wen, 2011). Under normal growth conditions, PTOX is only a minor component of thylakoid membranes (Lennon *et al*., 2003). However, during water stress, the PTOX level and activity were found to increase in many plant species (Simkin *et al*., 2008; Ibáñez *et al*., 2010; Muñoz and Quiles, 2013; Paredes and Quiles, 2013). In our study, expression of the *PTOX* gene was also found to be up-regulated in water-stressed soybean seedlings, in contrast to the steady low expression level in non-stressed seedlings (Fig. 1). Therefore, a physiological role of soybean PTOX in the response to water stress is likely. *PTOX* expression was up-regulated in response to water stress only in light condition, while the *PTOX* transcript levels remained at a steady low level during the whole stress period in the dark (Fig. 1). So we can assume that the physiological function of PTOX under water stress is to reduce light stress which results from reduced carbon assimilation.

PTOX has been suggested to protect the photosynthetic machinery by preventing the over-reduction of the PQ pool in chloroplasts (Aluru and Rodermel, 2004). As an electron sink alternative to PSI, induced PTOX is thought to effectively transfer excess electrons to O_2 with the formation of H_2O, and thus maintain the relative redox balance of the photosynthetic electron transfer chain (McDonald *et al*., 2011). However, evidence was presented that PTOX cannot act as a safety valve in protecting photosynthesis. For exam-

ple, Rosso *et al*. (2006) showed that the expression of PTOX in *Arabidopsis* is modulated only minimally by stress conditions or stress-related hormones. So PTOX may not act as a universal safety valve in all plants, but can be regarded as a beneficial strategy for certain plants which have a stress-inducible PTOX (Sun and Wen, 2011). Moreover, some studies indicated that a high level of PTOX alone might not be sufficient to improve the stress tolerance of plants. Over-expression of PTOX even promoted oxidative damage to tobacco under stress (Joët *et al*., 2002; Heyno *et al*., 2009; Ahmad *et al*., 2012). Thus, it was inferred that PTOX-related stress tolerance relies not only on the induction of PTOX but also on the necessary cooperation between PTOX and other stress responses (Sun and Wen, 2011). As the terminal oxidase of chlororespiration, PTOX usually performs its function with the NDH complex, another essential component of chlororespiration. Induction of the NDH complex and PTOX were observed under drought and other stress conditions (Ibáñez *et al*., 2010; Paredes and Quiles, 2013). In the present study, increased expression of the *NDH-H* gene was also detected in soybean seedlings under water stress (Fig. 4), just like *PTOX*. Therefore, enhanced chlororespiration involving an increased level of the NDH complex and PTOX could be proposed as a strategy of soybean to resist water stress.

In addition to its function in chlororespiration, the NDH complex has also been described as a key component of NDH-dependent CET around PSI (Rumeau *et al*., 2007). Due to the increased expression of *NDH-H*, enhanced CET could be considered a way to overcome the detrimental effects of water stress on photosynthesis in soybean seedlings. We also determined the transcript levels of *PGR5*, an essential component of the other CET pathway besides the NDH-dependent route (Johnson, 2011), but, contrary to those of *NDH-H*, they did not change in response to water stress (Fig. 4). Therefore, NDH-dependent CET and/or chlororespiration, rather than the PGR5 pathway, appear to be involved in the soybean seedlings' response to water stress.

The transcript levels of *AOX1*, the stress-responsive *AOX* gene of soybean (Considine *et al*., 2002), increased remarkably under water stress, suggesting an enhanced alternative respiration (Fig. 4). The response of *AOX1* was notably faster than that of *PTOX* (Figs. 1 and 4). Alternative respiration with its mitochondrial terminal oxidase AOX is known to dissipate reducing equivalents outside chloroplasts (Vanlerberghe, 2013; Zhang *et al*., 2010). Enhanced activities of NADP-MDH and NAD-MDH under water stress support the involvement of the Mal/OAA shuttle (Fig. 5) in the dissipation of excess reducing equivalents outside the chloroplasts.

Taken together, our present study has revealed that water stress induces up-regulation of the expression of the *PTOX*, *NDH-H*, and *AOX1* genes in soybean seedlings. This suggests that chlororespiration and NDH-dependent CET could be involved in the soybean seedlings' strategy to protect the photosynthetic machinery under water stress. Based on the kinetics of the respective increases in the transcript levels, we propose that in the initial phase of water stress alternative respiration, together with an enhanced Mal/OAA shuttle, assumes the predominant protective function, while chlororespiration becomes active at a later stage.

Acknowledgement

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