Journal of Integrative Plant Biology http://www.blackwell-synergy.com
Formerly *Acta Botanica Sinica* 2005, **47** (10): 1211–1219 http://www.chineseplantscience.com

Possible Involvement of Anti-Oxidant Enzymes in the Cross-Tolerance of the Germination/Growth of Wheat Seeds to Salinity and Heat Stress

Yan-Bao LEI[1, 2], Song-Quan SONG[1, 2*] and Jia-Rui FU[2]

(1. *Xishuangbanna Tropical Botanical Garden*, *the Chinese Academy of Sciences*, Yunnan 666303, China;
2. *School of Life Sciences*, *Sun Yat-Sen University*, Guangzhou 510275, China)

Abstract: The germination/growth of wheat (*Triticum aestivum* L. cv. Zimai 1) seeds and changes in the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), as well as in the content of thiobarbituric acid-reactive substances (TBARS), in response to salt and heat stress, as well as cross-stress, were investigated in the present study. With increasing temperature and decreasing water potential caused by NaCl solution, the germination percentage of seeds and the fresh weight of seedlings decreased markedly, SOD activity increased, activities of APX and CAT decreased distinctly, and the TBARS content increased gradually. Seeds pretreated at 33 °C for different times displayed increased tolerance to subsequent salt stress, enhanced SOD, APX, and CAT activities, and decreased TBARS content. Seeds pretreated at –0.8 MPa NaCl for different times displayed increased tolerance to subsequent heat stress and marked increases in SOD, APX, and CAT activities, which were associated with decreased TBARS content. It is considered that the common component in the cross-tolerance of the germination and growth of wheat seeds to salinity and heat stress is the anti-oxidant enzyme system.

Key words: anti-oxidant enzymes; cross tolerance; germination; growth; heat stress; salinity stress; *Triticum aestivum* seeds.

Soil salinity is a major abiotic stress for agricultural plants worldwide. Approximately 20% of the world's cultivated land and nearly half of all irrigated land are affected by salinity (Rhoades and Loveday 1990). High or low temperature stress is also a very common type of stress that plants receive from their surroundings (Iba 2002). In many plants, gradual changes in environmental conditions could induce tolerance to the extreme conditions. An increasing number of studies has shown the existence of cross-tolerance in plants: exposure of tissue to a moderate stress induces resistance to other stresses. Water stress confers cold hardiness upon a variety of winter cereals (Cloutier and Andrews 1984) and induces chilling resistance in rice (Takahashi *et al*. 1994). Salt stress induces/increases cold hardiness in potato and spinach seedlings (Ryu *et al.* 1995). Mechanical injury increases chilling tolerance in tomato leaves (Keller and Steffen 1995). Heat stress protects against heavy metal toxicity (Bonham-Smith *et al.* 1987; Orzech and Burke 1988), increases salt resistance (Kuznetsov *et al.* 1993), induces water stress tolerance (Bonham-Smith *et al*. 1987), and reduces chilling injury in chilling-sensitive species, such as tomato fruits, mung bean hypocotyls, and cucumber cotyledons (Jennings and Saltveit 1994; Collins *et al.* 1995).

One mechanism that may be involved in the resistance to many types of stress is the activity of the anti-oxidant enzymes. These enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), as well as those of the ascorbate-glutathione cycle. High activities of these enzymes have

Received 9 Nov. 2004 Accepted 5 Jun. 2005
Supported by the KIP Pilot Project (KSCX2-SW-117) and Hundred Talents Program of the Chinese Academy of Sciences, the Natural Science Foundation of Yunnan of China (2003C0068M), and the Ministry of Sciences and Technology of China (2004DKA30430).
*Author for correspondence. Tel: +86 (0)691 871 5474; Fax: +86 (0)691 871 5070; E-mail: <sqsong@xtbg.org.cn>.

been found in response to heat, chilling, freezing, salt, drought, and wounding, as well as to oxidative stress (Foyer *et al.* 1994). The increased expression of anti-oxidant enzymes under different types of stress may play a general role in the acquisition of tolerance by plants (Sabehat *et al.* 1998).

Although there are many reports on salt and heat tolerance in plants, as well as on the relationship between these two stress tolerances and anti-oxidant enzymes, most plant materials involved in reports are seedlings or adult plants. To our knowledge, there is little information on the relationship between the cross-tolerance of seed germination/growth and anti-oxidant enzymes. Seeds are the most basic means of production in agriculture, forestry, and horticulture. It is very important to increase the germination percentage and emergence rate of seeds under a certain stress condition, as well as to increase tolerance to another stress condition via pretreatment with a sub-lethal stress factor in production practice, especially in salinity soil and extreme temperature. In the present study, wheat seeds were used to investigate germination/growth under salt and heat stress, and cross-tolerance between both stresses, as well as the relationship between these tolerances and anti-oxidant enzymes.

1 Materials and Methods

1.1 Plant materials and chemicals

Wheat (*Triticum aestivum* L. cv. Zimai 1) seeds harvested in summer 2001 (June–August), were purchased from the Qufu Seed Company (Shandong Province, China) and were kept at 15 °C until September 2002.

1,1,3,3-Tetraethoxypropane, ascorbic acid, glycerol, polyvinylpolypyrrolidone, EDTA, reduced glutathione (GSH), nitrobule tetrazolium (NBT), riboflavin, Triton X-100, Trizma base, and dithiothreitol were purchased from Sigma (St Louis, MO, USA) and other chemicals were obtained from Shanghai Sangon Biological Engineering Technology (Shanghai, China) and Shanghai Chemical Reagent (Shanghai, China).

1.2 Stress treatments and germination of seeds

For salt stress treatment, 100 seeds were allowed to germinate on filter paper in a Petri dish (9 cm in diameter) containing 10 mL NaCl solution of different water potentials for 5 d in the dark at 20 °C. The water potential of the NaCl solution is estimated by the van't Hoff equation $\psi_s = -RTC_s$ (Taiz and Zeiger 2002), where ψ_s is the solute potential, R is the gas constant (8.32 J·mol^{-1}·k^{-1}), T is the absolute temperature (in degrees Kelvin, or K), and C_s is the solute concentration of the solution, expressed as osmolality (moles of total dissolved per liter of water (mol/L).

For heat stress treatment, seeds were allowed to germinate for 5 d in a Petri dish (9 cm in diamter) containing 10 mL water at different temperatures.

For cross-tolerance treatment, seeds pretreated in distilled water at 33 °C for different times were transferred to –0.8 MPa NaCl solution to continue germination at 20 °C or seeds pretreated in –0.8 MPa NaCl solution at 20 °C for different times were transferred to 33 °C to continue germination. The total time (pretreatment time and germination time) was 5 d.

Seeds showing radicle emergence were counted as germinated. Endosperm weight is excluded in the fresh weight of seedlings.

1.3 Superoxide dismutase

Seedlings and germinated seeds after treatment were homogenized to a fine powder using a mortar and pestle under liquid nitrogen. Subsequently, proteins were extracted by grinding the powder in an extraction medium composed of 50 mmol/L potassium phosphate, pH 7.0, 1.0 mmol/L EDTA, 0.05% (v/v) Triton X-100, 2% (w/v) polyvinylpolypyrrolidone and 1 mmol/L ascorbic acid. The homogenate was centrifuged at 16 000*g* for 15 min, after which the supernatant was transferred to a new tube and stored at –20 °C.

The assay for SOD (EC 1.15.1.1) activity was based on inhibition of the photochemical reduction of NBT as modified by Schickler and Caspi (1999). The reaction mixture contained 50 mmol/L potassium phosphate, pH 7.8, 0.1 mmol/L EDTA, 13 mmol/L methionine, 75 μmol/L NBT, 16.7 μmol/L riboflavin, and enzyme source (approximately 25 μg protein). Riboflavin was added last and the reaction was performed

under one 18-W fluorescent lamp (350 μmol·m^{-2}·s^{-1}) for 15 min. An illuminated blank without protein gave the maximum reduction of NBT and the absorbance was measured at 560 nm. One unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%. The specific activity of SOD was expressed as units/mg protein.

1.4 Ascorbate poxidase and catalase

Powder (approximately 300 mg) from the homogenization described above was extracted by grinding in 5 mL of 50 mmol/L Tris-HCl, pH 7.0, containing 20% (v/v) glycerol, 1 mmol/L ascorbic acid, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 1 mmol/L GSH, 5 mmol/L $MgCl_2$, and 1% (w/v) polyvinylpolypyrrolidone. After two steps of centrifugation (6 min at 12 000*g* and 16 min at 26 900*g*), the supernatant was stored at –20 °C until measurement of the enzyme activities of APX and CAT.

Ascorbate peroxidase (EC 1.11.1.7) was assayed as the decrease in absorbance at 290 nm (2.8 L·mmol^{-1}·cm^{-1}) due to ascorbic acid oxidation according to the method of Nakano and Asada (1981). The reaction mixture contained 50 mmol/L potassium phosphate, pH 7.0, 1 mmol/L sodium ascorbate, 2.5 mmol/L H_2O_2, and enzyme source (approximately 50 μg protein) in a final volume of 1 mL at 25 °C.

Catalase (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm (0.04 L·mmol^{-1}·cm^{-1}), as described by Aebi (1983), in 50 mmol/L potassium phosphate, pH 7.0, containing 10 mmol/L H_2O_2 and enzyme source (approximately 50 μg protein) in a final volume of 1 mL at 25 °C.

1.5 Thiobarbituric acid-reactive substances

Lipid peroxidation was determined as the concentration of TBARS, equated with malondialdehyde (MDA), as described originally by Heath and Packer (1986) but modified by Hendry *et al*. (1993), where the products were quantified from the second derivative spectrum against standards prepared from 1,1,3,3-tetraethoxypropane. The TBARS content (MDA) is expressed as nmol/g dry weight (DW).

1.6 Protein assay

Protein was measured according to the procedure of Bradford (1976), using bovine serum albumin as the standard.

2 Results

2.1 Effect of salt stress on seed germination and the activities of anti-oxidant enzymes

The germination percentage of wheat seeds decreased in response to water stress caused by NaCl. The water potential at which seed germination decreased to 50% (ψ_{50}) was approximately –0.87 MPa (Fig. 1a). Seed vigor, as measured by seedling fresh weight after 5 d of germination of 100 seeds, was measured both as the rate of seed germination and as the subsequent seedling growth and decreased with decreasing water potential (Fig. 1a). It is noted that the germination percentage of seeds varied over the range of water potentials and temperatures; that is, there were ungerminated seeds, seeds with protruded radicles, and seedling in samples used for the determination of enzyme activities and TBARS.

Compared with control (water potential is 0), SOD activities were increased by –0.05 to –0.80 MPa NaCl and decreased by water potential lower than –0.8 MPa NaCl. The activity of APX was increased by water potentials of –0.05 to –0.10 NaCl and that of CAT was increased only by –0.05 MPa NaCl, whereas the activities of APX and CAT decreased with increasing salt stress. The highest activities of SOD, APX, and CAT were seen for –0.20, –0.05, and –0.05 MPa NaCl, respectively (Fig. 1b, c). The TBARS content, an indicator of cellular damage, increased in solutions of –0.05 to –0.40 MPa NaCl and decreased when the water potential was lower than –0.8 MPa NaCl (Fig. 1c).

2.2 Effect of temperature on seed germination and the activities of anti-oxidant enzymes

The optimum temperature for the germination of wheat seeds was 20–25 °C; the germination percentage decreased at lower or higher temperatures. The optimum growth temperature, as measured by seedling fresh weight, was observed at 30 °C (Fig. 2a).

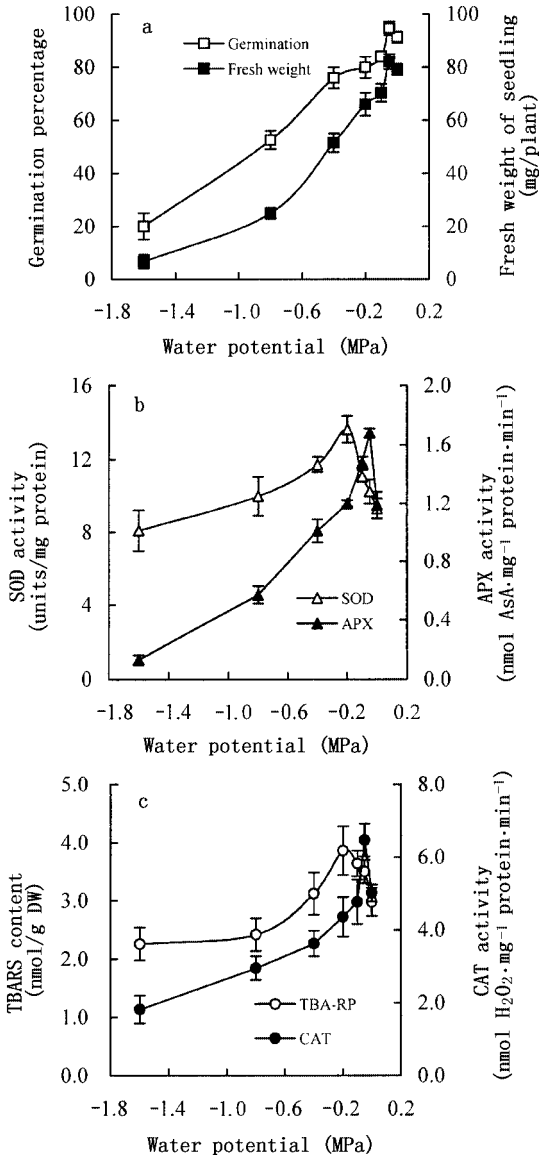

Fig. 1. Changes in (**a**) seed germination and the fresh weight of seedlings, (**b**) superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities, and (**c**) catalase (CAT) activity and thiobarbituric acid-reactive substances (TBARS) content under salt stress. Seeds were germinated at 20 °C for 5 d in NaCl solutions at the water potentials indicated. Seeds showing radicle emergence were counted as germinated. The fresh weight of seedlings produced by germinating seeds did not include the endosperm. For germination and the fresh weight of seedlings, all values are the mean ± *SD* of three replicates; for enzyme activity and TBARS, all values are the mean ± *SD* of five replicates.

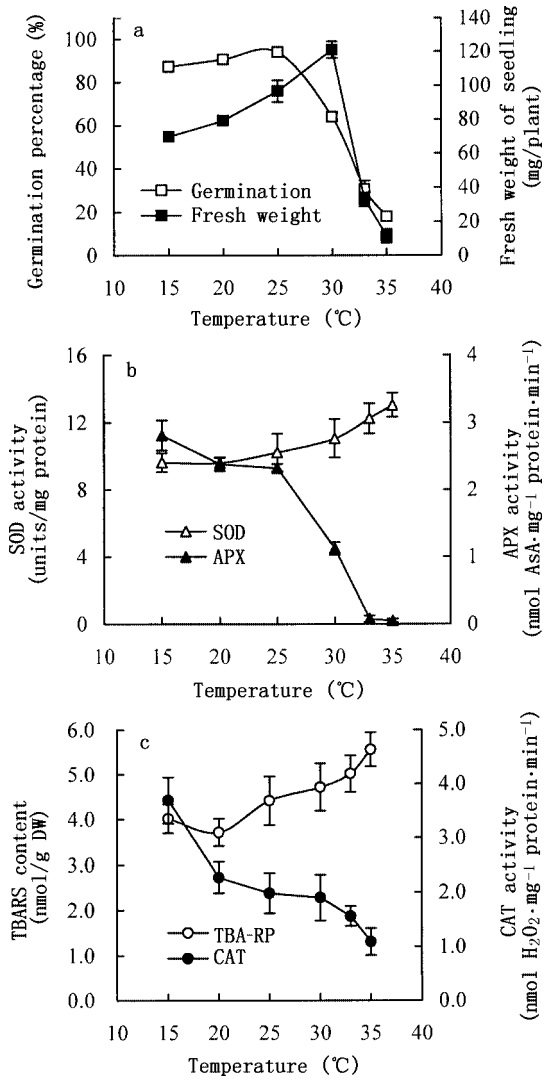

Fig. 2. Changes in (**a**) seed germination and the fresh weight of seedlings, (**b**) superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities, and (**c**) catalase (CAT) activity and thiobarbituric acid-reactive substances (TBARS) content under temperature stress. Seeds were germinated for 5 d at the temperatures indicated. For germination and the fresh weight of seedlings, all values are the mean ± *SD* of three replicates; for enzyme activity and TBARS, all values are the mean ± *SD* of five replicates.

With increasing temperature, the SOD activity of seedlings and seeds increased progressively, whereas the activities of APX and CAT decreased markedly. The

activities of APX and CAT decreased by 98% and 71%, respectively, at 35 °C compared with activity determined at 15 °C. The TBARS content increased gradually, but a lower content was observed at 20 °C (Fig. 2b, c).

2.3 Cross-tolerance reaction of seed germination/growth and the activities of anti-oxidant enzymes

After pretreatment in distilled water at 33 °C for 0, 1, 2, 4, 8, and 16 h, seeds were transferred to –0.8 MPa NaCl solution to continue germination at 20 °C. Table 1 shows that seeds pretreated at 33 °C for different times had increased tolerance to subsequent salt stress (–0.8 MPa NaCl). The germination percentage of seeds and seedling fresh weight increased markedly following pretreatment in distilled water at 33 °C. The optimum pretreatment time at 33 °C was approximately 4 h.

After pretreatment at 33 °C and subsequent germination in –0.8 MPa NaCl for 5 d, the SOD, APX and CAT activities of seedling and seeds increased markedly at 1, 2, and 4 h of pretreatment, then decreased slightly after 8 and 16 h, but the activities were still higher than those of control. The TBARS content decreased with pretreatment time to a minimum at 4 h. After 4 h pretreatment, the SOD, APX and CAT activities increased by 27%, 159%, and 98%, respectively, whereas the TBARS decreased by 49% compared with control (Table 2).

Seeds pretreated in –0.8 MPa NaCl solution at 20 °C for 8, 16, 24, and 32 h also displayed increased tolerance to subsequent heat stress (33 °C). The germination percentage and fresh weight of seedlings increased markedly and the optimum pretreatment time

Table 1 Germination/growth of *Triticum aestivum* seeds in response to –0.8 MPa NaCl after pretreatment at 33 °C

Pretreatment time at 33 °C (h)+ germination time in –0.8 MPa at 20 ºC (h)	Germination percentage (%)	Fresh weight of seedlings (mg/plant)
120+0	30.5 ± 4.3	32.8 ± 4.2
0+120	58 ± 2	20.9 ± 3.6
1+119	70.7 ± 3.1	31.3 ± 4.7
2+118	82.7 ± 2.3	32.6 ± 2.4
4+116	87.3 ± 3.5	38.1 ± 2.8
8+112	68 ± 4	25.2 ± 1.8
16+104	62 ± 2	17.5 ± 2.6

All values are the mean±SD of three replicates. Seeds showing radicle emergence were counted as germinated. The fresh weight of seedlings produced by germinating seeds did not include the endosperm.

Table 2 Changes in the activities of superoxide dismutase, ascorbate peroxidase, and catalase and the thiobarbituric acid-reactive substances content of seedlings and seeds of *Triticum aestivum* in response to –0.8 PMa NaCl after pretreatment at 33 °C

Pretreatment time at 33 °C (h)+germination time in –0.8 MPa at 20 °C (h)	SOD activity (units/mg protein)	APX activity (nmol AsA·mg^{-1} protein ·min^{-1})	CAT activity (nmol H_2O_2·mg^{-1} protein·min^{-1})	TBARS content (nmol/g DW)
120+0	12.2 ± 0.9	0.08 ± 0.05	1.56 ± 0.18	5.03 ± 0.38
0+120	9.6 ± 0.6	0.61 ± 0.03	2.31 ± 0.21	4.64 ± 0.39
1+119	10.4 ± 0.7	0.87 ± 0.04	2.90 ± 0.29	4.28 ± 0.28
2+118	11.5 ± 0.8	1.22 ± 0.01	3.06 ± 0.42	3.66 ± 0.44
4+116	12.2 ± 0.5	1.58 ± 0.13	4.58 ± 0.23	2.37 ± 0.60
8+112	11.3 ± 0.9	1.19 ± 0.09	3.40 ± 0.3	2.87 ± 0.54
16+104	10.6 ± 0.6	0.82 ± 0.15	2.32 ± 0.39	2.94 ± 0.46

All values are the mean ± *SD* of five replicates. APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

in –0.8 MPa NaCl solution was approximately 24 h (Table 3).

Similarly, after pretreatment in –0.8 MPa NaCl solution and subsequent germination at 33 °C for 5 d, the SOD, APX and CAT activities of seedling and seeds increased markedly and the TBARS content decreased with increasing pretreatment time. The optimum pretreatment time in –0.8 MPa NaCl solution was approximately 24 h (Table 4).

3 Discussion

Salt stress afflicts agriculture in many parts of the world, particularly where irrigation is used. Metabolic stress caused by NaCl may result in decreased plant growth (Dodd and Donavan 1999; Ephron *et al*. 1999). The germination of wheat seeds and the fresh weight of seedlings decreased markedly in response to decreasing water potential caused by NaCl and ψ_{50} was approximately –0.87 MPa (Fig. 1a). These results are similar to those of Dionisio-Sese and Tobita (1998), who found that salt-sensitive rice varieties during salt stress exhibited a decrease in growth rate and SOD activity and an increase in electrolyte leakage and MDA content. Zhu (2001) considered that salt stress may inhibit cell division and expansion directly. However, the connection between stress signaling and the control of cell division and expansion needs to be better understood. The slight increase in germination and growth in –0.05 MPa NaCl (Fig. 1a) could be attributed to osmoconditioning caused by very low concentrations of NaCl.

High salt stress disrupts homeostasis in water potential and ion distribution. This distribution of homeostasis occurs at both the cellular and whole-plant levels. Marked changes in ion and water homeostasis lead to damage of large molecules, such as DNA and protein, growth arrest, and even death. An important cause of damage may be reactive oxygen species (ROS) generated as a result of salt stress (Zhu 2001, 2002). SOD was increased by –0.05 to –0.80 MPa NaCl solutions,

Table 3 Germination/growth of *Triticum aestivum* seeds in response to heat stress after pretreatment with –0.8 MPa NaCl solution

Pretreatment time in –0.8 MPa at 20 °C (h)+ germination time in water at 33°C (h)	Germination percentage (%)	Fresh weight of seedlings (mg/plant)
0+120	25.3 ± 3.1	38.1 ± 2.6
8+112	43 ± 4	41.5 ± 2.5
16+104	46 ± 2	50.3 ± 3.6
24+96	62.7 ± 3.2	56.0 ± 4.0
32+88	43 ± 4	43.1 ± 2.9

All values are the mean ± *SD* of three replicates. Seeds showing radicle emergence were counted as germinated. The fresh weight of seedlings produced by germinating seeds did not include the endosperm.

Table 4 Changes in the activities of superoxide dismutase, ascorbate peroxidase, and catalase and the thiobarbituric acid-reactive substances content of seedlings and seeds of *Triticum aestivum* in response to 33 °C after pretreatment in –0.8 PMa NaCl solution

Pretreatment time in –0.8 MPa at 20 °C (h)+germination time in water at 33 °C (h)	SOD activity (units/mg protein)	APX activity (nmol AsA·mg^{-1} protein·min^{-1})	CAT activity (nmol H_2O_2·mg^{-1} protein·min^{-1})	TBARS (nmol/g DW)
0+120	11.01 ± 0.90	0.08 ± 0.05	1.56 ± 0.18	5.03 ± 0.38
8+112	11.92 ± 1.17	1.16 ± 0.06	2.15 ± 0.23	4.61 ± 0.47
16+104	12.40 ± 0.90	1.52 ± 0.08	2.79 ± 0.23	3.79 ± 0.67
24+96	13.60 ± 1.01	2.15 ± 0.08	3.25 ± 0.26	3.30 ± 0.38
32+88	10.21 ± 0.78	1.33 ± 0.06	2.56 ± 0.21	3.48 ± 0.48

All values are the mean ± *SD* of five replicates. APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

but were decreased by solutions with a water potential lower than –0.80 MPa NaCl. The activities of APX and CAT were increased by solutions of –0.05 to –0.10 and –0.05 MPa NaCl, respectively, but then decreased with increasing salt stress (decreasing water potential). The TBARS content increased in solutions of –0.05 to –0.4 MPa NaCl (Fig. 1b,c). These results indicate that there is a close relationship between anti-oxidant enzymes and salt stress.

The reason why TBARS decreased at and below –0.8 MPa NaCl (Fig. 1c) may be that the germination percentage of seeds was lower and that the ungerminated seeds had a lower sensitivity to salt stress. The protein content of seeds was higher than that of seedlings (data not shown). Most of the transgenic improvements in plant salt tolerance reported to date have been achieved through this detoxification strategy. This is obvious in the case of transgenic plants overexpressing enzymes involved in oxidative protection, such as glutathione peroxidase, SOD, APX, and glutathione reductase (Allen 1997; Roxas *et al*. 1997). Support for the importance of oxidative protection in salt tolerance also comes from the characterization of the *Arabidopsis* mutant *pat1*, which has a mutation in a putative negative regulator of the oxidative stress response. The *pst1* mutant plants are more resistant to high salt concentrations and this is correlated with an increased capacity to tolerate oxidative stress (Tsugane *et al.* 1999).

In the present study, the optimum temperatures for wheat seed germination and seedling growth were found to be approximately 20–25 and 30 °C, respectively (Fig. 2a). In the range 15–35 °C, with increasing temperature, the SOD activity of seedlings and seeds increased progressively, whereas the activities of APX and CAT decreased markedly, and the TBARS content increased gradually (Fig. 2b, c). These results show that the germination and growth of wheat seeds in response to heat stress are related to anti-oxidant enzyme activity and TBARS content. It has been reported that the activity of anti-oxidant enzymes in plants decreased and the TBARS content increases in response to low (Doulis *et al*. 1997; Lee and Lee 2000) or high (Sairam *et al*. 2000; Iba 2002) temperatures.

Seeds pretreated at 33 °C for different times displayed increased tolerance to subsequent salt stress (Table 1). At the same time, the activities of SOD, APX, and CAT in seedling and seeds increased and the TBARS content decreased (Table 2). In addition, seeds pretreated in –0.8 MPa NaCl for different times also displayed increased tolerance to subsequent temperature stress (Table 3) and the activities of SOD, APX and CAT of seedlings and seeds were increased markedly, whereas the TBARS content decreased (Table 4). These results show that the germination and seedling growth of wheat seeds display cross-tolerance to salt and heat stresses. Pastori and Foyer (2002) suggested that the phenomenon whereby plants make use of common pathways and components in the stress-response relationship is known as cross-tolerance and that cross-tolerance of plants allows plants to adapt/acclimate to a range of different stresses after exposure to one specific stress. A common component in the cross-tolerance of the germination and growth of wheat seeds appears to be anti-oxidant enzymes (Tables 2, 4).

Many mechanisms explaining the phenomenon of cross-tolerance have suggested the involvement of specific proteins. These proteins are induced by one type of stress and are involved in the protection against other types of stress. Several cold-regulated proteins are homologous and have similar properties to those of drought- and Abscisic acid (ABA)-induced proteins, such as late embryogenic abundant proteins, ABA-responsive proteins, and dehydrins (Neven *et al*. 1993). The induction of chilling resistance by water stress involves the activation of drought-regulated genes (Takahashi *et al*. 1994) and salt stress stimulates cold hardiness by the activation of cold- and ABA-responsive genes (Ryu *et al*. 1995). Moderate heat shock induces the expression of heat shock proteins (HSP) in tomato and protects the fruits against chilling injury. These HSP may be involved in the protection of the fruits against damage by both high and low temperatures (Sabehat *et al.* 1996). More recent engineering with the regulatory protein NPK1, a mitogen-activated

protein kinase, is another good example. This protein kinase appears to mediate the oxidative stress response (Kovtun *et al*. 2000). The relationship between the cross-tolerance of the germination and growth of wheat seeds and specific proteins needs further investigation.

Acknowledgements The authors are grateful to Professor Ian Max Møller (Plant Research Department, Risø National Laboratory, Roskilde, Denmark) for reviewing this paper.

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(Managing editor: Ping HE)