Salt and Drought Stress Differentially Affect the Accumulation of Extracellular Proteins in Barley

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Barley (*Hordeum vulgare*) was grown for eight days in the presence of a range of salt concentrations or subjected to repeated cycles of wilting and rehydration. Changes in apoplastic protein content, protein pattern, enzymic activities and ion composition were investigated under salinity and drought. The protein content of intercellular washing fluid (IWF) increased 2.5- to 3.0-fold when the NaCl concentration in the growth medium was increased from 0 to 100 mM. The elevated protein content was the result of a general increase in most polypeptides and a pronounced increase in the abundance of specific polypeptides of apparent molecular masses of 15, 21, 22, 26, 36, 40 and 62 kDa. Conversely, the IWF protein content decreased during wilting similar as after application of colchicin, cytochalasin B or cycloheximide suggesting that inihibition of protein synthesis or vesicle transport may be the cause for the decrease in apoplastic protein content and enzyme activities in dehydrating plant tissue. The changes in apoplastic protein content were accompanied by stress-specific alterations in activities of apoplastic enzymes. The greater apoplastic protein content was the consequence of stimulated protein synthesis in the presence of NaCl, as evidenced by increased incorporation of [^{35}S]-methionine into IWF protein. The results demonstrate that the leaf apoplast is a compartment which sensitively and differentially responds to drought and salinity with consequences for plant growth.

Introduction

Saline growth conditions and drought inhibit germination efficiency and growth of most glycophytes and reduce the yield of crop species in arid and semi-arid zones of the world. Up to now physiological research has mainly focused on three topics related to salinity and drought, namely water relations, photosynthesis and the accumulation of specific metabolites such as the compatible solutes glycinebetaine and proline. These processes have been investigated in detail in respect to their contribution to the described limitations of growth and to tolerance under saline conditions (Munns, 1993).

Under salinity, the apoplastic salt concentration rises if the transport of salt into the leaf exceeds uptake by cells. The excessive accumulation of salt in the apoplastic and cytoplasmic compartments of mature tissues of hydroponically grown plants accelerates the development of damage and plant death (Munns, 1993). However, the apoplast has not adequately been investigated so far. Only a few studies have been performed related to the salt induced changes in ion composition (Oertli, 1968; Speer and Kaiser, 1991).

The apoplast is involved in many physiological processes of the cell, for instance nutrition, growth and defence. (i) The contents of the xylem-mediated transpiration stream first spread over the leaf apoplast in most species before the leaf cells specifically import nutrient elements from the apoplast (Dietz, 1996). (ii) The physico-chemical properties of the cell wall and the biochemical activities in the apoplast determine the capacity and the rate of cell expansion (Grignon and Sentenac, 1991). (iii) The apoplast is a major site where plants express proteins involved in the defence against pathogens (Jung *et al.*, 1993). Besides these and other functions recent studies have also established the

Abbreviations: IEF, isoelectric focussing; IWF, Intercellular washing fluid.

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role of the apoplast in adaptation to environmental stress (Dietz, 1996). For example, apoplastic reactions allow for the detoxification of SO_2 (Pfanz *et al.*, 1990) and ozone (Luwe and Heber, 1995) The apoplast is an important compartment for cold acclimation (Marentes *et al.*, 1993) and responds to heavy metal stress, for instance to elevated supply with Zn (Brune *et al.*, 1994). A role in aluminium resistance either by exclusion of aluminium from the apoplast and/or detoxification within the apoplast has also been reported (Horst, 1995). The examples illustrate the important functions served by the apoplast both under favourable and stressful conditions. However, a detailed analysis is not available comparing the apoplastic responses towards various stressors. Only individual genes and a few specific enzymes such as the extracellular peroxidases have been investigated in depth with regard to various stress regimes and have been employed as stress markers. Recently, a comparative investigation was performed on the effect of various heavy metals on apoplastic polypeptides (Blinda *et al.*, 1997) indicating a qualitatively similar but quantitatively distinct response towards Ni, Cd, Zn and Mo in barley. A comparative work has also been published about the effect of air pollutants such as ozone and UV B radiation in tobacco (Langebartels *et al.*, 1997). There is a requirement to extend this type of analysis of apoplastic responses to other stressors in one species, better in one cultivar, in order to work on a defined genetic background. Therefore, this investigation addresses the question of how salinity and drought compare in their effects on protein content, qualitative polypeptide composition and enzyme complement of the leaf apoplast.

Materials and Methods

Plant growth, stress treatments and extraction of intercellular washing solution

Barley (*Hordeum vulgare*, cv. Gerbel) was grown in hydroponic culture in a growth chamber at a day/night cycle of 14h/10h at temperatures of 22 °C and 18 °C, respectively. Photon fluence rate was about 200 μmol m^{-2} s^{-1}. Barley seeds were mixed with vermiculite and imbibed with water over night. The seeds were transfered on nylon nets clamped between two plastic rings and placed on nutrient solution containing 1.25 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.375 mM KH_2PO_4, 0.032 mM NaFe EDTA, 0.069 mM H_3BO_3, 0.5 μM $CuCl_2$, 14 μM $MnCl_2$, 1.5 μM Na_2MoO_4 and 1.5 μM $ZnSO_4$. The nutrient solution was supplemented with NaCl at concentrations between 10 and 100 mM as indicated. Primary leaves of 10-day old barley seedlings were used for the experiments. Drought stress was developed by repeated cycles of wilting and rehydration. The hydroponic medium was removed during the light period for 8 h or 16 h beginning with the 8th day after sowing. At that time the blades of the primary leaves were fully expanded. The hydroponic medium was added to the plants at the end of the drought period. The wilting/rehydration treatment was repeated four times. Intercellular washing fluid (IWF) was extracted after infiltration of the excised leaves with a solution containing 100 mM sorbitol and Triton X-100 (0.1% v/v) by centrifugation of the leaves at 1000×*g* for 5 min. Apoplastic protein content was determined by a modified Lowry procedure as outlined before (Brune *et al.*, 1994).

Determination of ion contents

IWF was extracted in HNO_3 (10% v/v). The extracts were analyzed with an inductively coupled plasma atomic emission spectrometer (Jobin Yvon JY70, Instruments S. A., Longjumea, France) with an argon plasma. Each measurement was performed in triplicate. The element contents of the samples were quantified by comparison with standard solutions at appropriate dilutions (Merck, Darmstadt, Germany): 10 μg/ml P and K, 2 μg/ml Mg, Na and Ca, 0.1 μg/ml Mn. The factor of ion concentration increase was calculated as $f=c_{100\ mM}(c_{0\ mM})^{-1}$ i.e. the ion concentration at 100 mM NaCl divided by the concentration in the control.

Analysis of polypetide composition by one- and two-dimensional gel electrophoresis

Following isolation of the IWF the proteins were concentrated by phenol extraction and ethanol/NH_4-acetate precipitation. For the analysis by one-dimensional sodium dodecyl sulfate (SDS) PAGE, intercellular washing fluid was diluted with identical volumes of a two-fold concentrated loading buffer to final concentrations of 2.5% SDS, 125 mM Tris-HCl pH 6.8, 10 mM dithiothreitol

(DTT) and 10% glycerol. After heating at 95 °C for 5 min, polypeptides were concentrated in a 6% stacking gel and separated in a 12.5% resolving polyacrylamide gel (Dietz and Bogorad, 1987). Polypeptides were visualized by staining with silver nitrate. Two-dimensional gel electrophoresis was carried out as described by O'Farrell (1975) with minor modifications. Polypeptides were precipitated at −20 °C after extraction into 250 µl of water-saturated phenol and addition of 100 mM NH_4 acetate in 1 ml ethanol in the presence of 1 mM DTT. The pellet obtained by centrifugation was washed with ethanol, dried briefly and resuspended in a modified lysis buffer (Dietz and Bogorad, 1987). Resuspended samples were loaded into the acidic end of the tube gels of the first dimension. Separation in the second dimension was achieved by SDS-PAGE as described above. Polypeptides were visualized by staining with silver nitrate. Molecular weight markers were α-lactalbumin (14 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa).

Determination of enzyme activites

Activities of acid hydrolases were determined by liberation of *p*-nitrophenol from synthetic substrates as described by Boller and Kende, (1979). The incubation assay contained 100 mM citric acid – KOH, pH 4.6, 2 mg *p*-nitrophenol substrates and extracts (5–50 µl) in a final volume of 400 µl. After incubation at 37 °C for 60 min, the reaction was terminated by alkalinization with 1 ml of 100 mM Na_2CO_3. The amount of liberated *p*-nitrophenol was quantified spectrophotometrically at 405 nm using the molar extinction coefficient of $\varepsilon = 18.400\ M\ cm^{-1}$. Apoplastic peroxidase activity was measured using the tetramerization reaction of guaiacol. The spectrophotometric assay contained 100 mM K-phosphate buffer, pH 6.5, 2 mM guaicol, 1 mM H_2O_2 and sample. Changes in absorption were monitored at 436 nm. Tetramerization rate was calculated using the molar extinction coefficient ($\varepsilon = 2550\ M\ cm^{-1}$).

^{35}S-Met incorporation into apoplastic proteins

The primary leaves of barley grown on hydroponic medium with 0, 10 or 100 mM NaCl were excised and placed on 100 µM $CaCl_2$ solution containing [^{35}S]-methionine at an activity of 40 kBq. After different time periods of incubation, IWF was isolated and the amount of [^{35}S]-Met incorporation in proteins was determined with a scintillation counter following trichloroacetic acid-dependent precipitation of the proteins on filter discs (cf. Dietz and Bogorad, 1987). Another aliquot was prepared for gel electrophoretic separation followed by fluorography.

Results

Na^+ and protein contents in the IWF

Barley seedlings grown in the presence of increasing NaCl concentrations in the hydroponic medium during a ten day period showed elevated Na contents in the apoplastic space of leaves (Table I). At this point it should be noted that the control which in the following is denominated "0 mM NaCl" contained about 30 µM NaCl added with the micronutrients. The Na^+ content of the IWF increased less than the Na^+ concentration in the growth medium. The apoplastic concentrations of other elements were also elevated at conditions of high salt. For example K^+ increased by a factor of about 6 from 2.4 mM in the control to 14 mM in the presence of 100 mM NaCl. The factor of increase was between 3 and 4 for Ca^{2+}, P_i and Mg^{2+} (data not shown).

The leaf and apoplastic protein contents were measured. The whole leaf protein content was not affected by NaCl concentrations up to 50 mM. It decreased slightly by about 10% in plants grown at 100 mM NaCl (data not shown). On the other hand, the apoplastic protein content was strongly influenced by salinity. The protein content of the IWF was increased even at the lowest NaCl con-

Table I. Protein and Na^+ contents of intercellular washing fluid (IWF) isolated from barley primary leaves subjected to NaCl-salinity. The data are means of 12 (protein) and 6 (Na^+) independent determinations ±SD, respectively.

NaCl in the hydroponic medium [mM]	Protein contents of IWF [µg · µl^{-1}]	Na^+ contents of IWF [mM]
0	0.29 ± 0.02	0.24 ± 0.02
10	0.39 ± 0.02	1.59 ± 0.12
20	0.44 ± 0.03	2.75 ± 0.22
50	0.56 ± 0.03	4.71 ± 0.54
100	0.76 ± 0.05	7.14 ± 0.75

centration of 10 mM. This effect was further enhanced with increasing NaCl concentrations. In control plants apoplastic protein content ranged between 0.27 and 0.30 μg μl^{-1} IWF whereas the apoplastic protein content was 2.5-fold higher in the leaves stressed with 100 mM NaCl (0.76 μg μl^{-1} IWF) (Table I).

Apoplastic polypeptide pattern under salinity

Analysis by SDS-PAGE of leaf apoplastic proteins revealed the presence of at least 40 proteins in the molecular mass range of 14 to 80 kDa. Silver staining visualized dominant polypeptides with molecular masses of 67, 50 and 32 kDa; in control plants prominent polypeptides were also found at 69, 45, 24, 21 and 15 kDa (data not shown). In order to look for polypeptides responsive to NaCl, apoplastic fluids were isolated from barley seedlings grown in the presence of NaCl concentrations ranging from 10 to 100 mM and analysed for polypeptide composition by SDS PAGE. Identical amounts of IWF were loaded in each lane. Most protein bands increased in intensity as a function of the NaCl concentration in the rooting medium.

For a more detailed analysis, two-dimensional polypeptide patterns were obtained from IWF, extracted from plants grown at 0, 10 or 100 mM NaCl. For these experiments protein was first quantified and then identical protein amounts were extracted and separated (Fig. 1). Based on their responsiveness to salinity the polypeptides were categorised in 4 groups.

a. Polypeptides responsive to low salt concentration: A polypeptide of about 70 kDa was responsive to low NaCl concentration (10 and 20 mM) and its intensity decreased at 50 and 100 mM NaCl.

b. Polypeptides responding to NaCl over the whole concentration range: The polypeptides of this group were responsive to low salt concentrations. Their intensity continued to increase with the NaCl concentration in the hydroponic medium up to 100 mM. Polypeptides with apparent molecular masses of 69, 62 and 52 kDa, as well as most of the polypeptides in the molecular mass range between 22 and 40 kDa belonged to this group.

c. Polypeptides responsive to high salt concentration: At least one polypeptide with an apparent molecular mass of 21 kDa responded only to high salt concentration. The spot intensity was almost unaltered at low salt concentration.

d. Unaffected polypeptides. At least two major apoplastic polypeptides with apparent molecular masses of 50 and 67 kDa were unaffected by NaCl salinity.

In summary, the expression of 7 major polypeptides of the IWF was strongly stimulated during saline conditions as compared to control IWF. The relative molecular masses of these polypeptides were 15, 21, 22, 26, 36, 40 and 62 kDa.

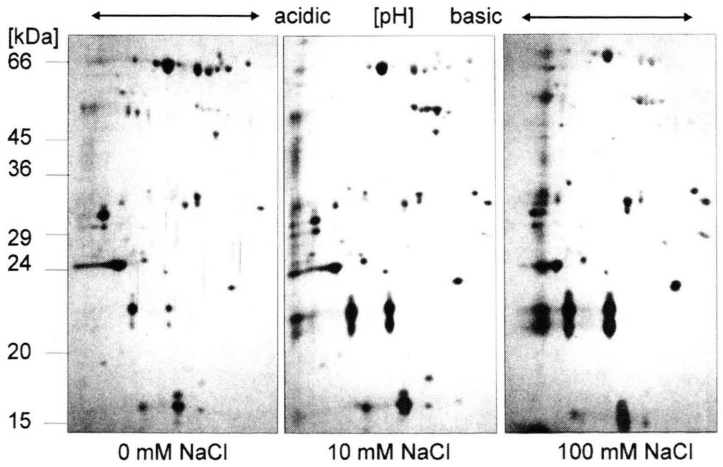

Fig. 1. Two-dimensional separations of IWF isolated from primary leaves of 10 d old barley grown in the presence of 0, 10 or 100 mM NaCl. Similar amounts of proteins were extracted, loaded on the tube gels of the first dimension and stained with silver nitrate following their separation in the second dimension.

Effect of drought-cycling on apoplastic proteins

Wilting of the plants was initiated by withdrawal of the medium for 8 h or 16 h per day followed by rehydration for 16 h or 8 h, respectively. The drought cycling was repeated four times. IWF was isolated after the fourth rehydration period. Each cycle resulted in a loss of water content by about 30%. Nevertheless, the initial quantum yield as determined as modulated chlorophyll fluorescence transients was not different between the drought-treated and the control plants after the rehydration period (data not shown).

The IWF protein content of the repeatedly wilted plants was reduced from 0.29±0.03 in the untreated controls to 0.26±0.01 in plants wilted for 8 h per cycle and to 0.22±0.02 in the plants wilted for 16 h per cycle (Table II). IWF was isolated from plants wilted in four cycles of 8 h and compared by SDS PAGE with IWF of control plants (Fig. 2). The silver stained one-dimensional electropherogram revealed polypeptides which were unaffected by drought for instance at 15, 21, 24, 50 and 67 kDa. Other polypeptides showed a strong decrease in band intensity, for example at 22 and 31–33 kDa.

De novo synthesis of apoplastic proteins

Incorporation of [^{35}S]-methionine into apoplastic proteins of intact leaves was used as a measure for *de novo* protein synthesis. Primary leaves were cut from control plants and from plants treated with 100 mM NaCl and supplied with radiolabeled methionine via the petiole. IWF was isolated as a function of time and analyzed for incorporation of radiolabel in the protein fraction (Table III). The initial incorporation was similar in control and high salt leaves. After two hours, 0.26–0.27% of the total radioactivity was associated with the apoplastic protein fraction both in control and high salt leaves. The incorporation of radiolabel did not further increase with time in control plants. Conversely, incorporation into apoplastic proteins of salt-treated plants increased up to 6 hours. The pattern of radiolabeled polypeptides was investigated by SDS-PAGE after 2, 4 and 6 h of incubation (Fig. 3). The labeling pattern of control plants did not change with incubation time greater than 2h except the decrease in intensity of a 67 kDa band and the increase in a 50 kDa band. After 2h of incubation, the salt-treated sample showed a similar or even weaker incorporation into apoplastic proteins than the control in the size range between 14 and 67 kDa. However, the most conspicuous difference was the strong labeling of a 69 kDa band. During the following four hours, the radiolabeling intensity also increased in bands of apparent molecular masses of 14, 15, 21, 22, 50 and 52 kDa. The 69 kDa remained strongly labeled.

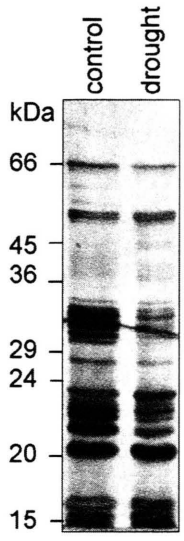

Fig. 2. SDS-PAGE of apoplastic proteins of barley seedlings grown under control conditions or subjected to drought cycling of 4 times 8h. Identical amounts of IWF (30 µl) were loaded in each lane of the 12.5% PAGE and stained with silver at the end of the separation.

Table II. Apoplasmic protein contents of barley primary leaves as affected by drought or by a 24 h treatment with colchicine, cytochalasin B and cycloheximide (effector concentration 10 µM each). The control leaves were supplied with 0.1 mM $CaCl_2$. For details see text and Materials and Methods. The data are means of 6 measurements ±SD.

Treatment	Protein contents of IWF [$\mu g_* \mu l^{-1}$]
Control	0.29 ± 0.03
Colchicine	0.22 ± 0.03
Cytochalasin B	0.21 ± 0.04
Cycloheximide	0.17 ± 0.03
8 h drought, 4 cycles	0.26 ± 0.01
16 h drought, 4 cycles	0.22 ± 0.02

Table III. Incorporation of [^{35}S]-methionine into IWF proteins of control plants and plants stressed with 100 mM NaCl as a function of time. The radiolabeled methionine was fed to the cut leaves of 10 d old plants via the petiole. The IWF was isolated 2, 4, 6 and 8 h after the beginning of the incubation. Incorporation in leaves of barley seedlings treated with 10 mM NaCl was 32±3 Bq after 6 h. The data are mean values of 6 determinations ± SD in two experiments.

Time of incubation [h]	Bq		Incorporation [% of total]	
	NaCl concentration [mM]		NaCl concentration [mM]	
	0	100	0	100
2	28±5	29±3	0.27	0.26
4	27±5	42±5	0.26	0.34
6	28±2	51±10	0.27	0.41
8	28±2	49±7	0.26	0.39

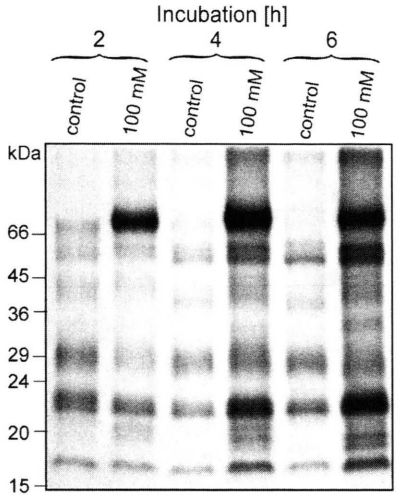

Fig. 3. Pattern of newly synthesized apoplastic proteins in leaves of control plants and plants treated with 100 mM NaCl. ^{35}S-met was fed to cut leaves via the petiole. IWF was prepared after 24 h following the incubation with the label and analyzed by fluorography of the 12.5% SDS PAG electropherogram.

Increased IWF Protein contents is accompanied by elevated enzymic activities

The changes in the apoplastic protein content brought about by NaCl and drought stress were further investigated with regard to specific enzyme activities known to be expressed in the extracellular space. Saline growth conditions stimulated the activities of α-mannosidase by up to 455%, peroxidase by 343%, β-galactosidase by almost 200% and acid phosphatase by about 90%. Conversely, phosphodiesterase, α-galactosidase and N-acetylglucosaminidase activities were not or only little affected by growth on NaCl-enriched media (Table IVA). The changes in enzyme activities under salinity were compared with the activities following a repeated drought treatment (Fig. IVB). With the exception of β-galactosidase and N-acetylglucosaminidase, all activities decreased as a consequence of the drought treatment. The decrease was significant in the cases of peroxidase and phosphodiesterase activity after for drought periods of 8 h and further enhanced in the 16 h drought treatment. Only about half of the control activity was measured for the peroxidase, α-mannosidase, phosphodiesterase and α-galactosidase.

Influence of colchicine, cytochalasin B and cycloheximide on IWF-protein content

An experiment was performed in order to determine the effect of inhibitors of cytoskeleton formation and protein synthesis on apoplastic protein contents. Colchicine inhibits the polymerisation of tubulins, cytochalasin B blocks the formation of microfilaments and cycloheximide binds to 80S ribosomes and inhibits protein synthesis. The three treatments caused a decrease in apoplastic protein contents similar to the severe drought regime.

Discussion

Responsiveness of the apoplast is maintained under salinity

Hordeum vulgare cv. GERBEL is a barley cultivar being moderately salt tolerant. This cultivar is able to maintain its tissue turgor over the whole range of NaCl concentrations that were used in the experiments. Conversely, turgor was lost dur-

Table IV. Activity of acidic hydrolases and peroxidases in the IWF of barley primary leaves subjected to NaCl salinity (A) or drought stress (B). The data are means of 12 determinations ± SD. The numbers in brackets give the percent increase as compared to the control. It should be noted that the ratio of intercellular gas space to fresh weight is fairly constant in barley leaves under most conditions and consequently also the ratio of IWF per g fresh weight (Dietz, 1996; Blinda *et al.*, 1997). In barley 300 µl IWF are extracted from 1 g fully infiltrated primary leaves. Therefore, enzyme activity was related to volume units IWF in order to allow for a comparison of enzyme activity allocated to the apoplast in dependence of the salt concentration in the hydroponic medium.

(A)

	Salt concentration in the medium [mM]				
	0	10	20	50	100
Enzyme	Enzyme activity [nmol*ml $IWF^{-1}min^{-1}$]				
Peroxidase	409 ± 30 (0)	645 ± 41 (58)	702 ± 62 (72)	1283 ± 312 (214)	1807 ± 312 (343)
Phosphatase	383 ± 35 (0)	424 ± 42 (11)	467 ± 64 (22)	503 ± 53 (31)	725 ± 89 (89)
β-Galactosidase	118 ± 9 (0)	183 ± 17 (55)	209 ± 30 (77)	266 ± 39 (125)	347 ± 31 (194)
α-Mannosidase	33.5 ± 4.6 (0)	69.4 ± 8.2 (106)	82.8 ± 11.8 (147)	127 ± 14 (279)	186 ± 22 (455)
Phosphodiesterase	8.37 ± 0.67 (0)	8.42 ± 1.92 (1)	8.54 ± 0.82 (2)	8.95 ± 2.70 (7)	9.52 ± 3.60 (14)
α-Galactosidase	1.89 ± 0.42 (0)	1.97 ± 0.48 (4)	2.07 ± 0.61 (10)	2.39 ± 0.78 (26)	2.58 ± 0.78 (37)
N-Acetylglucoseaminidase	1.62 ± 0.36 (0)	1.55 ± 0.28 (−4)	1.60 ± 0.15 (−1)	1.64 ± 0.35 (1)	1.73 ± 0.43 (7)

(B)

	Drought treatment		
	0 h	8 h	16 h
Enzyme	Enzyme activity [nmol*ml $IWF^{-1}min^{-1}$]		
Peroxidase	12410 ± 1250 (0)	8136 ± 1130 (−34)	5166 ± 870 (−58)
Phosphatase	1182 ± 78 (0)	1209 ± 103 (2)	776 ± 65 (−34)
β-Galactosidase	253 ± 42 (0)	297 ± 37 (17)	210 ± 27 (−17)
α-Mannosidase	60.6 ± 6.6 (0)	48.9 ± 4.4 (−19)	34.1 ± 6.9 (−44)
Phosphodiesterase	18.0 ± 2.80 (0)	9.93 ± 1.26 (−45)	8.70 ± 1.54 (−52)
α-Galactosidase	13.1 ± 1.10 (0)	10.4 ± 1.2 (−20)	6.54 ± 0.88 (−50)
N-Acetylglucoseaminidase	5.37 ± 1.11 (0)	4.87 ± 0.65 (−9)	5.92 ± 0.48 (10)

ing the severe wilting treatments. Both stressors affected the apoplastic protein contents and enzyme activities. The new finding was that both stressors induce a rather distinct pattern of apoplastic changes with probable consequences for growth and survival of the plants.

Apoplastic concentrations of Na^+ and Cl^- increase when plants are grown in the presence of

elevated salt concentrations (Oertli, 1968; Flowers *et al.*, 1991; Speer and Kaiser, 1994; Table I of this report). As a consequence the osmotic gradients and driving forces for cell expansion decrease. Altered cell wall elasticity and physico-chemistry are considered to be important components of the adaptive response of plants to salt and drought (Bolanos and Longstreth, 1984; Iraki *et al.*, 1989). Speer and Kaiser (1991) compared the performance of the glycophytic species pea with that of the halotolerant species spinach under saline growth conditions. The Na^+ and Cl^- concentration increased in the leaf apoplast of pea but not in spinach with the time of exposure to NaCl. Therefore, Speer and Kaiser (1991) as earlier Oertli (1968), and later Munns (1993) related apoplastic salt accumulation with damage and low apoplastic salt concentrations with tolerance. In barley apoplastic NaCl concentrations increased with the salt concentration in the growth medium. Maximum apoplastic Na^+-concentrations remained below 10 mM similar to the low concentration observed in spinach leaves (Speer and Kaiser, 1991). Accordingly, the barley seedlings did not exhibit any symptoms of salt damage. Only the growth of the shoots and particularly of the roots was inhibited.

The investigation demonstrates that most polypeptides contained in IWF were increased in abundance under saline growth conditions, and that many apoplastic enzyme activities were stimulated. For example, the activity of the β-galactosidase (EC 3.2.1.23) responded to supplementation of the growth medium with low dosis of NaCl. In the presence of 10 mM NaCl, β-galactosidase activity was already increased by 55%. Under this condition, the apoplastic Na-concentration was still low. β-Galactosidase is involved in the breakdown of pectic polymers of galactose during cell growth (Konno *et al.*, 1986) and in the breakage of bonds between cell wall polysaccharides during cell wall loosening (Murray and Bandurski, 1975). A high activity of β-galactosidases may facilitate cell growth. Sawicka and Kacperska (1995) identified a polypeptide of molecular mass close to 65 kDa as apoplastic β-galactosidase in winter rape leaves. An increase in a polypeptide with an apparent molecular mass of 65 kDa was also observed in this study.

Apoplastic enzymes such as galactosidases, glucosidases, peroxidases and xyloglucan endo-transglycosylases have been shown *in vitro* to be highly salt-insensitive up to concentrations of 500 mM NaCl (Thiyagarajah *et al.*, 1996). Therefore, the increase in the enzyme activities is less likely a measure to compensate for salt-dependent inhibition of enzymic activities but is a mechanism to increase the activities *in vivo*.

Generally, plant gene expression changes in response to salinity (Hurkman and Tanaka, 1987; Ramanjulu *et al.*, 1994). The stress induced adjustment in polypeptide composition plays an important role in adaptation to stress for instance by stimulation of synthetic pathways of compatible solutes (Cushman *et al.*, 1990). The changes in apoplastic protein composition described in this work may either be an active response to facilitate survival of the plants under salt stress conditions or the symptom of a general stress response. In this context two characteristics of the response must be discussed, the dose dependency and the specificity of the response. (1) As shown and pointed out above, the dose response revealed a high sensitivity of the system towards NaCl. Already at the concentration of 10 mM a significant increase in apoplastic proteins and activities was observed. It has to be concluded that the increase in apoplastic protein concentration is not the result of acute stress. (2) Brune *et al.* (1994) and Blinda *et al.* (1997) investigated the effect of various heavy metals on apoplastic proteins in barley. They also observed the accumulation of apoplastic polypeptides under heavy metal stress. A comparison of their studies (Figs. 1 and 2) which also included enzyme determinations, as well as one- and two-dimensional PAGE, with our results shows similarities in qualitative response. A set of polypeptides responded both to heavy metals and NaCl suggesting an underlying common principle of signaling. The increased protein contents of the IWFs seems to be the consequence of a stimulated synthesis of apoplastic proteins (Fig. 3) most likely on the level of the genes. Blinda *et al.* (1997) demonstrated recently that neither an altered extractability of the leaf cell wall nor a decreased protein turnover caused the accumulation of apoplastic proteins under heavy metal stress. Instead elevated mRNA levels and stimulated rates of protein synthesis were shown to account for the increase in apoplastic protein content and enzyme activities.

Hydrolytic enzymes such as the α-mannosidase are typical constituents of extraplasmic compartments of plant cells (Holden and Rohringer, 1985). α-mannosidase is involved in glycosylation and deglycosylation of proteins, and thereby in protein routing and protection against hydrolytic degradation (Faye *et al.*, 1988). A NaCl-dependent stimulation of the apoplastic α-mannosidase activity was observed in the present study. It is suggested that the increased α-mannosidase activity just indicates the stimulated vesicular transport from the cytoplasm to the apoplast by exocytosis. Salt stress is known to activitate extracellular phosphatases (Stephan *et al.*, 1994, and this report). Phosphatases are involved in the acquisition and recycling of Pi. However, the precise physiological functions of extracellular acid phosphatase activity remains to be elucidated. Elevated apoplastic peroxidase activity was registered during NaCl stress. In parallel, the intensity of protein bands at approximately 30 kDa increased which tentatively had been identified by Brune *et al.* (1994) as peroxidases. The increased activity of apoplastic peroxidase activity has also been reported in response to Zn stress (Brune *et al.*, 1994), SO_2 fumigation (Pfanz *et al.*, 1990) and upon infection with mildew (Kerby and Sommerville, 1992). The effect of increased peroxidase activity on plant growth is still under debate. Many authors correlated peroxidase activity with lignin deposition and cross linking of cell wall constitutents (Sancho *et al.*, 1996) resulting in an inhibition of cell expansion and an increased yield threshold limit for leaf growth (Cramer and Bowman, 1991). Conversely, Swoap *et al.* (1993) challenged the concept of a peroxidase-dependent inhibition of organ growth since they did not observe an inverse relation between peroxidase activity and tissue expansion. Taken together the results clearly demonstrate the maintenance of high responsiveness of the leaf apoplast under salinity despite the fact that the function of the observed changes in apoplastic protein composition and enzyme activities are not yet understood.

Responsiveness of the apoplast is lost under drought stress

The drought cycling regime employed in this work had pronounced effects on apoplastic proteins and enzymes largely opposite to salinity. Drought cycling was chosen to mimic natural conditions of daily wilting in the light followed by recovery of turgor during the night. The plants recovered completely during the period of rehydration. Nevertheless, the apoplastic proteins and enzyme activities declined strongly. Similar effects were observed when excised leaves were supplied with colchicin, cytochalasin 'B' or cycloheximide for a 24 h period. This experiment suggested that the cytoskeleton may be involved in routing proteins from the cytoplasm to the apoplast. Furtheron, the inhibitor study demonstrated that apoplastic proteins are subjected to significant turnover. Following partial or complete inhibition of exocytotic delivery of newly synthesized proteins to the apoplast, a large set of polypeptides decreased in intensity within a 24 h time period. Two possible causes are suggested to explain the disapperance of apoplastic proteins. Soluble proteins may either be linked to the cell wall or they may be degraded by proteolytic processes. Covarrubias *et al.* (1995) reported recently the appearance of basic cell wall proteins in bean seedlings subjected to water deficit. The accumulation only occured in hypocotyls and roots of bean seedlings and not in epicotyls including the leaves. Therefore, our study is not related to the observations of Covarrubias *et al.* (1995). The loss of responsiveness of the apoplast under drought stress may have far-reaching consequences for survival for instance when wilted plants are simultaneously affected by other stress factors.

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