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ORIGINAL ARTICLES

Differential Transcript Expression of Wall-loosening Candidates in Leaves of Maize Cultivars Differing in Salt Resistance

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Abstract Salt-sensitive crop plants such as maize (*Zea mays* L.) exhibit a strong and rapid growth reduction in response to NaCl stress. The unique salt-resistant maize hybrid SR03 and the salt-sensitive maize hybrid Lector provide good tools to characterize various genotypic responses to salinity in terms of shoot growth, shoot extensibility, and the expression pattern of wall-loosening candidates. The mRNA transcript levels of wall-loosening candidates such as xyloglucan endotransglucosylase (XET), endo-1,4-β-D-endoglucanase (EGase), α-expansins (EXPA), and the plasma membrane proton pump (PM-H^+-ATPase) are correlated with cell-wall extensibility and with shoot growth under NaCl stress. We have found for the salt-sensitive maize that a decrease in the relative transcript abundance of ZmXET1, ZmEXPA1, and the composite PM-H^+-ATPase mRNAs correlates with a decrease in wall extensibility and shoot growth. We suggest that this downregulation of wall-loosening candidates contributes to a reduction in extensibility and consequently in growth. In contrast, the decrease in wall extensibility is less strong in the salt-sensitive hybrid SR03. In the salt-resistant maize genotype, an upregulation of ZmXET1, ZmEXPA1 and PM-H^{+-}ATPase transcripts possibly mitigates the salinity-induced decrease in wall extensibility and thus in shoot growth.

Keywords Salinity · Growth inhibition · Wall extensibility · XET · EGase · PM-H^+-ATPases · α-expansins · Real-time quantitative RT-PCR

Introduction

Plant growth responds to salinity in two phases: a first rapid osmotic phase that decreases the growth rate of young leaves and a second ionic-toxic phase in which the senescence of mature leaves accelerates. The mechanism that downregulates the growth of young leaves under salt stress is not precisely known. Changes in the mechanical properties of the cell wall have been demonstrated to control elongation-type growth in most systems studied so far and are predicted to be involved in salinity-related growth adaptations (Cramer and Bowmann 1991; Munns 1993; Munns and Tester 2008).

The primary cell wall of plants is a complex polymeric network that must provide a rigid constraint to cell turgor and yet be sufficiently compliant to allow controlled cell expansion. The cell-wall framework is composed primarily of cellulose microfibrils embedded in matrices of hemicellulosic and pectic polymers, with additional minor components such as structural proteins (Carpita and Gibeaut 1993; Catalá and others 1997; Cosgrove 1997). According to

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current models of primary walls (Carpita and Gibeaut 1993), cellulose microfibrils interact with matrix components such as hemicelluloses and form a complex network. Xyloglucan, an extremely abundant hemicellulosic compound in primary cell walls, binds noncovalently to cellulose microfibrils and thus coats and crosslinks adjacent microfibrils. This results in an extensive xyloglucan-cellulose network that is thought to act as the major tension-bearing structure in the primary wall (Hayashi 1989; McCann and others 1990; Rose and others 2002). However, the primary cell wall is also capable of expanding, indicating that the interactions between wall polymers can be modified to make walls extensible for elongation (Wu and others 2001; Cosgrove 2005). Nevertheless, cellulose microfibrils are neither extensible nor degradable during cell elongation; they can only move apart. This explains why the network between microfibrils is crucial for determining cell-wall yielding behavior (Wu and others 2001).

Cell-wall enlargement begins with wall stress relaxation, allowing the cells physically to enlarge. According to the acid growth theory, an auxin-mediated acidification of the leaf apoplast is the major requirement for increasing wall extensibility (Hager and others 1971; Hager 2003). This theory is supported by the finding that the fungal toxin fusicoccin and, at least in part, the plant hormone auxin mediate the acidification of the apoplast below a pH of 5.0, thus stimulating cell elongation in maize and pea (Jacobs and Ray 1976; Peters and others 1998). Auxin is assumed to cause the plasma-membrane proton pump (PM-H^+-ATPase) to actively pump protons from the cytosol into the apoplast, resulting in wall-loosening and cell expansion (Moriau and others 1999). The pH of the apoplast of growing cells typically lies between 4.7 and 6 (Mühling and others 1995), which is the range in which acidification activates expansin activity (Cosgrove 2005). Expansins are a group of nonenzymatic cell-wall proteins that are thought to mediate acid-induced growth. In this context, isolated cell walls have been shown to exhibit acid growth resulting from the action of these pH-dependent wall-loosening proteins (Cho and Kende 1997). However, the precise way in which expansins mediate wall enlargement remains unclear. One interpretation is that expansins intercalate within carbohydrate matrices in the cell wall, leading to the transient loosening of noncovalent interactions, and thus enhance the ability of these matrices to move relative to each other (Cosgrove 2000, 2005). In addition to expansin, other candidate wall-loosening proteins are assumed to contribute to cell elongation. Catalá and others (1997) have summarized that xyloglucan undergoes substantial depolymerization and solubilization during auxin-induced cell elongation, suggesting that xyloglucan turnover is integral to auxin-induced cell-wall loosening. An enzymatic modification of load-bearing xyloglucan tethers, resulting in cell-wall loosening, is considered a key process necessary for cell expansion (Fry 1989). Xyloglucan-metabolizing enzymes therefore represent potentially important agents in controlling wall strength and extensibility. The basis of xyloglucan metabolism during cell elongation is not precisely known, but the involvement of endo-1,4-β-D-glucanases (EGases) and xyloglucan endotransglucosylase/hydrolase (XTH) has been suggested (Fry 1989; Nishitani 1995).

It has been proposed that EGases act primarily on the β-1,4-linked glucan backbone of xyloglucan in the plant cell wall (Hayashi 1989; Catalá and others 1997). The enzyme is thought to digest the noncrystalline regions of cellulose microfibrils and to release trapped xyloglucans, whereby this cleavage of load-bearing xyloglucan allows cell growth (Cosgrove 2005). Moreover, increased EGase enzyme activity is associated with auxin-stimulated cell enlargement. The enzyme xyloglucan endotransglucosylase/hydrolase (XTH) is involved in the modification of cell-wall structure by acting on the xyloglucans attached to cellulose microfibrils. One action of XTH proteins is referred to enzymologically as xyloglucan endotransglucosylase (XET) activity (Rose and others 2002; Vissenberg and others 2005; Genovesi and others 2008). XETs cleave and rejoin xyloglucan chains or suitable xyloglucan-derived oligosaccharides and are therefore candidates for wall-loosening. XET-mediated transglucosylation between two potentially load-bearing xyloglucan molecules is thought to allow incremental slippage of adjacent microfibrils by facilitating hydrostatic pressure of the protoplasm against this weakened wall. This results in a "creep" of cellulose microfibrils (Nishitani 1995; Catalá and others 1997; Vissenberg and others 2005).

Because salinity inhibits cell elongation in young leaves and because proteins such as xyloglucan endotransglucosylases, endo-1,4-β-D-glucanases, PM-H^+-ATPases, and expansins are believed to play key roles in cell-wall extension, the transcript expression patterns of these wall-loosening candidates have been correlated with the wall extensibility and growth of maize shoots under conditions of salt stress.

Materials and Methods

Plant Cultivation

The salt-sensitive maize hybrid Lector and the highly salt-resistant maize hybrid SR03 (*Zea mays* L.; Schubert and others 2009) were grown in greenhouses under hydroponic culture conditions. The experimental setup consisted of three independent replicates of the salt-treated plants (100 mM NaCl) and the corresponding control plants (1 mM NaCl). Each biological replicate was run in triplicate in a completely randomized design. The roots of seedlings were embedded in

1 mM $CaSO_4$ in an aerated solution at 25°C for 1 day and placed between filter papers moistened with 1 mM $CaSO_4$ for a period of 3 days. Subsequently, the seedlings were transferred to 4.5-L plastic pots (3 plants per pot) containing one-quarter-strength nutrient solution. After 2 days of cultivation, the concentration of nutrients was increased to half-strength and, after 4 days of cultivation, to full-strength. The nutrient solution had the following composition: 2.5 mM $Ca(NO_3)_2$, 1.0 mM K_2SO_4, 0.2 mM KH_2PO_4, 0.6 mM $MgSO_4$, 5.0 mM $CaCl_2$, 1.0 μM H_3BO_4, 2.0 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.005 μM $(NH_4)_6Mo_7O_{24}$, and 200 μM Fe-EDTA. The solution was changed every second day to avoid nutrient depletion. NaCl treatment was started 2 days after the full-nutrient concentration had been reached and was increased stepwise by 25-mM increments every second day. Temperature was kept constant at 26°C for the light period and at 18°C for the dark period; relative humidity was set to about 70%.

In this study, only young expanding shoot material that had developed entirely under the full influence of the 8-day 100-mM NaCl treatment was harvested. Any remaining older shoot material that had started to develop before the full-strength NaCl concentration had been reached was not taken into account. After the measurement of fresh weight, leaf material was immediately frozen in liquid nitrogen. Tissues used for extensibility measurements were then stored at −20°C. For RNA analysis, leaves were ground in liquid nitrogen and stored at −80°C.

N_{creep} Measurement

Measurement of cell-wall extensibility was carried out as described previously (Büntemeyer and others 1998). Briefly, frozen-thawed basal sections of leaves were clamped between the tips of an extensiometer and incubated in a 10-mM K-phosphate buffer, pH 7.2. Extension was measured by using a positional angular transducer (TWK Instruments, Düsseldorf, Germany), which could be equilibrated with a counterweight. Further weights could be placed on the counterweight axis to induce extension. For inducing creep, we used a weight of 20 g. Time courses of creep were found to be excellently represented by a logarithmic function of the type

$$L = L_0 + N_{\mathrm{Creep}} * \log t$$

with t being the time that had elapsed since the application of the weight. N_{creep} is a measure of the slope of the cell-wall creep; this has previously been shown to represent growth-relevant changes in the cell-wall rheology of maize root cell walls (Büntemeyer and others 1998). L_0 reflects the initial change in length once the weight had been added. The extensiometer software and the data regression software were developed in house by HL.

Primer Design for Polymerase Chain Reaction

For analyzing the mRNA transcript level of wall-loosening candidates, group-specific, that is, degenerated, and isoform-specific polymerase chain reaction (PCR) primer pairs were designed (Table 1). Primer pairs for amplifying *Zea mays* xyloglucan endotransglucosylase homolog 1 (*ZmXET1*) and *Zea mays* ubiquitin-conjugating enzyme (*ZmUBC*; used as reference gene for real-time quantitative reverse transcription [qRT]-PCR) were designed by means of Primer3Plus and Primer-BLAST software (http://bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; http://www.ncbi.nlm.nih.gov/). Primer pairs for analyzing three *Zea mays* α-expansin-isoforms (*ZmEXPA1*, *ZmEXPA3*, and *ZmEXPA4*; Table 1) were designed on the basis of nucleotide alignments (ClustalW, http://www.clustal.org). To measure the transcript level of four composite *Zea mays* membrane-H^+-ATPases (*ZmMHA1*, *ZmMHA2*, *ZmMHA3*, and *ZmMHA4*), we created a degenerated, that is, group-specific, primer that was designed by first generating a multiple alignment and then manually identifying the most conservative regions for primer design. Henceforth, this degenerated primer pair is referred to as primer pair *ZmMHA_fam* (Table 1). A degenerated, that is, group-specific, primer pair for *Zea mays* endoglucanases was designed on the basis of three sequences available at the NCBI GenBank (http://www.ncbi.nlm.nih.gov; *Zea mays* endoglucanase 1 LOC100285069, *Zea mays* endoglucanase 1 LOC100285091, and *Zea mays* endoglucanase cl6880_1; see Table 1 for GenBank accession numbers). This group-specific primer pair (*ZmEGases*; Table 1) allows the quantitation of the composite transcript level and was designed as described for *ZmMHA_fam.* All primer pairs were purchased from Eurofins MWG Operon (Ebersberg, Germany). For the prevention of false priming sites, all primer pairs were checked *in silico* by BLASTN against a *Zea mays* nucleotide collection (nr/nt) and *Zea mays* reference mRNA sequences (refseq_rna). To avoid primer-dimer formation and the formation of hairpin structures, primers were evaluated *in silico* by using Primer-BLAST and Primer3Plus.

For demonstrating the specificity of the degenerated primer/template interactions, and for proving that no cross-amplifications bias the measurements, the real-time qRT-PCR products were sequenced (MWG Eurofins, Munich, Germany). DNA sequencing of the amplicons that were generated with the degenerated primer pairs (*ZmMHA_fam* or *ZmEGases*) revealed correct products. No undesired PCR products were generated (Supplementary Table 1). Moreover, dissociation curve analysis (data not shown) gave no suggestion of erroneous amplification of templates during real-time qRT-PCR. Furthermore, after real-time qRT-PCR measurements, agarose gels were run to ensure that only a PCR product with the correct length was amplified (Supplementary Fig. 1).

Table 1 Real-time quantitative RT-PCR primer pairs for maize (*Zea mays* L.)

Primer name	GenBank accession No.	Sequence of forward (f) and reverse (r) primers (5′–3′)		Annealing temp (°C)	Product size (bp)
ZmXET1	NM_001111897.1	f	CTACCAGGACGTGGACATCA	63.3	208
		r	ACCCTGCGACGAAAGATAGA		
ZmEGases	NM_001157986	f	SMGSVVGCAGRTGGACTAC	60.0	200
	NM_001157964	r	CGRYGAGCWSGTTGGGRTT		
	NM_001158465				
ZmMHA_fam	NM_001111890	f	AGCCAGGCYCTKATCTTCGT	64.0	195
	NM_001112000				
	AJ441084	r	SACGATGYTGTASAGCCAGA		
	AJ539534				
ZmEXPA1	AAK56119	f	ATGGCGGCAGCTGCTAGTG	65.9	100
		r	CGCTCTGCCACGAGCCGTA		
ZmEXPA3	AAK56121	f	CGCCACCTTCTATGGCGGTA	633	171
		r	GCCTGGTAGTCGCAGGAGAT		
ZmEXPA4	AAK56122	f	GTGCCGTGCCGCAAGTCC	65.5	126
		r	GCTGGCCGCCTTGACGCT		
ZmUBC	AF034946.1	f	GTCCTGCTCTCCATCTGCTC	52.0–65.0	113
		r	CGGGCCGTCGACTCGTACTT		

XET = xyloglucan endotransglucosylase; EGases = endoglucanases; MHA = plasma membrane (PM) proton pump (H^+)-ATPase; EXPA = α-expansins; UBC = ubiquitin-conjugating enzyme

The letter code Y, K, S, R, or M within the primer sequences represents abbreviations for nucleotide combinations following IUPAC recommendations (Nomenclature Committee of NC-IUB and IUPAC-IUB Joint Commission on Biochemical Nomenclature)

No adequate primer pairs were found for the α-expansin isoforms *ZmEXP2* and *ZmEXP5*.

RNA Extraction and cDNA Synthesis

Aliquots of 100 mg ground shoot material were used for RNA extraction. Total maize RNA was isolated according to a modified method of Cox and Goldberg (1988). The quality and quantity of RNA was checked by OD_{260}. Poly(A)$^+$ RNA purification was carried out with oligo(dT)$_{25}$-coupled paramagnetic particles (Dynabeads® mRNA Purification Kit, Invitrogen GmbH, Karlsruhe, Germany) by using 75 µg total RNA according the instructions of the manufacturer. Highly purified maize poly(A)$^+$ RNA (3 µg) was reverse-transcribed in a 10-µl cDNA reaction with a first-strand cDNA synthesis system following the manufacturer's instructions (SuperScript® VILO cDNA synthesis kit, Invitrogen). Single-stranded cDNA was diluted to a concentration dependent on the level of expression of the studied gene; cDNA was aliquoted to avoid discrepancy in the data attributable to the repetition of freezing-thawing cycles.

Real-time Quantitative RT-PCR

The SYBR® Green-based real-time qRT-PCR technique was performed on an Applied Biosystems 7300 real-time PCR system. For each reaction, 2 µl diluted single-stranded cDNA was used in a total volume of 20 µl (0.8 pM each forward and reverse primer, 0.82 mM dNTP mix, 0.1 × SYBR Green, 1 × ROX, 0.6 U *Taq* DNA polymerase; Invitek, Berlin, Germany). After an initial denaturation step (92°C, 2 min), real-time qRT-PCR was carried out over 40 cycles [denaturation: 92°C, 30 s; amplification and quantification: 40 s (for primer-pair-specific temperatures, see Table 1), elongation: 72°C, 20 s]. To check the specificity of the annealing of the oligonucleotides, dissociation kinetics were performed by the real-time PCR system at the end of the experiment (60–95°C, continuous fluorescence measurement). The comparative C_t (threshold cycles) method of relative quantification was used to analyze the real-time qRT-PCR data. With this method, the C_t values were normalized by comparison with the endogenous reference gene, that is, the ubiquitin-conjugating enzyme. The normalized C_t values were then used to compare NaCl-treated plants and corresponding controls. Data were expressed as the relative change in transcript expression (+100 relative expression = 2-fold upregulation; −100 relative expression = 2-fold downregulation). Threshold cycles were calculated by the internal software of the real-time PCR system and were the means of three biological replicates of each run in triplicate. The sizes of amplified products were confirmed by gel electrophoresis.

Negative controls with no templates were carried out concurrently.

Results

Shoot Growth and Wall Extensibility

All measurements were based on expanding leaf material derived from plants grown entirely under the influence of an 8-day 100-mM NaCl treatment. In response to the salt treatment, a significant decrease in shoot fresh weight of about 60% was measured for the salt-sensitive cultivar Lector when compared with control plants (Fig. 1). In contrast, only a slight decrease in shoot fresh weight was measured for the salt-resistant SR03. This was not significant. Creep activity (N_{creep}) was used as a rheological parameter to monitor wall extensibility in shoots (Table 2). Under conditions of salt stress, N_{creep} decreased in both genotypes. The percentage of N_{creep}(100 mM NaCl) on N_{creep}(control) was about 80% for Lector and 90% for SR03. Thus, in response to salt treatment, N_{creep} decreased about 20% in the salt-sensitive hybrid Lector and about 10% in the salt-resistant hybrid SR03.

Relative Transcript Expression

The effect of salt treatment on the relative transcript abundance of wall-loosening candidates was studied on the basis of purified poly(A)$^+$ RNA by using the real-time qRT-PCR technique. In comparison with various reference genes, the ubiquitin-conjugating enzyme was validated as the most stable reference gene, being particularly useful for the analysis of genes in salt-treated samples (Hong and others 2008). Because the report of Hong and others (2008) was based also on a monocot grass species (*Brachypodium distachyon*), we used ubiquitin-conjugating enzyme as the reference gene. In response to the 8-day 100-mM NaCl treatment, *ZmXET1* was downregulated in the salt-sensitive hybrid Lector but upregulated in the salt-resistant hybrid SR03 (Fig. 2a). The composite transcript abundance of *ZmEGases* was downregulated in both genotypes, whereas this downregulation was not noticeably different between the two genotypes (Fig. 2b). *ZmEXPA1* was downregulated

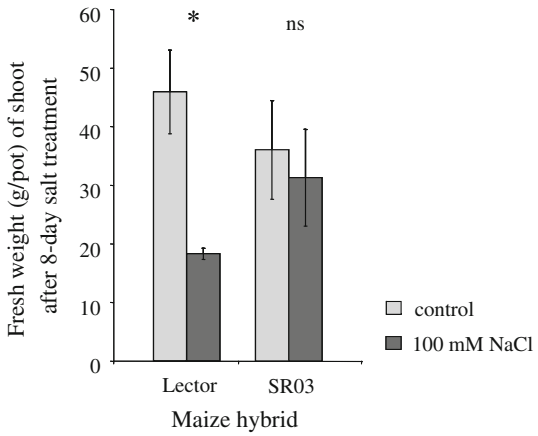

Fig. 1 Effect of salt treatment on shoot growth. Biomass reduction of expanding maize shoots (*Zea mays* L.) under conditions of salinity. Plants were treated with 100 mM NaCl for 8 days. Only expanding leaves grown entirely under the influence of the 8-day NaCl treatment and corresponding controls were studied. The remaining older shoot material was not considered. Control (*light gray*); 100 mM NaCl (*dark gray*). Data are means of three biological replicates ± SE. Each measurement was carried out in triplicate. Asterisk indicates significant differences between treatments ($p \leq 0.05\%$; ns, not significant)

Table 2 Effect of 8-day 100-mM NaCl treatment on N_{creep} activity

Hybrid	Treatment	N_{creep}			
		Mean	*n*	SE	% N_{creep}(100 mM NaCl) on N_{creep}(control)
Lector	Control	3.91	30	0.77	80.8
	100 mM NaCl	3.16	29	0.43	
SR03	Control	2.55	36	0.33	90.1
	100 mM NaCl	2.30	34	0.19	

SE standard error of the mean, *n* number of replicates

Maize genotypes: Lector, salt-sensitive; SR03, salt-resistant

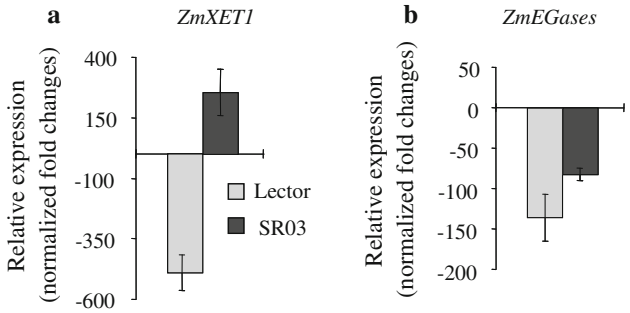

Fig. 2 Effect of salt treatment on relative XET1 and EGases transcript abundance as measured by SYBR Green-based real-time quantitative RT-PCR. Relative expression changes in response to an 8-day 100-mM NaCl treatment of (**a**) xyloglucan endotransglucosylase homolog 1 (*ZmXET1*) and (**b**) endoglucanases (*ZmEGases*). Isoforms of three endoglucanases were detected simultaneously by using a degenerated, that is, family-specific, primer pair (see Materials and Methods section). The transcript for ubiquitin-conjugating enzyme was used as the endogenous control in an Applied Biosystems 7300 real-time PCR system. Data show the effect of salt treatment on the relative transcript abundance, with the control as the calibrator sample (+100 relative expression = 2-fold upregulation, −100 relative expression = 2-fold downregulation). Salt-sensitive Lector (*light gray*); salt-resistant SR03 (*dark gray*). Expression data correspond to means of three biological replicates, each being run in triplicate, ±SE

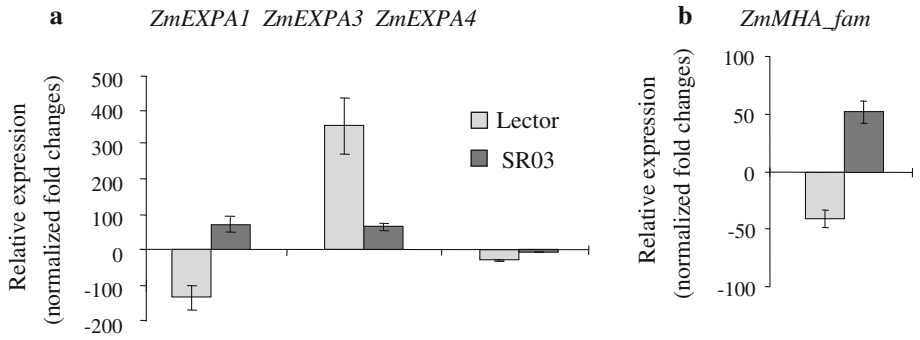

Fig. 3 Effect of salt treatment on the relative transcript abundance of EXPA1, EXPA2, EXPA3, and MHA1-4 as measured by SYBR Green-based real-time quantitative RT-PCR. Relative expression changes in response to an 8-day 100-mM NaCl treatment of (**a**) α-expansin-isoforms (*ZmEXPA1*, *ZmEXPA3*, and *ZmEXPA4*) and (**b**) four plasma membrane H^+-ATPase isoforms (*ZmMHA1*, *ZmMHA2*, *ZmMHA3*, and *ZmMHA4*). Isoforms of four plasma membrane H^+-ATPases were detected simultaneously by using a degenerated, that is, family-specific, primer pair *ZmMHA_fam* (see Materials and Methods section). The transcript for ubiquitin-conjugating enzyme was used as the endogenous control in an Applied Biosystems 7300 real-time PCR system. Data show the effect of salt treatment on the relative transcript abundance with the control as the calibrator sample (+100 relative expression = 2-fold upregulation, −100 relative expression = 2-fold downregulation). Salt-sensitive Lector (*light gray*); salt-resistant SR03 (*dark gray*). Expression data correspond to means of three biological replicates, each being run in triplicate, ±SE

in the salt-sensitive hybrid Lector but upregulated in the salt-resistant hybrid SR03 (Fig. 3a). The isoform *ZmEXPA3* was upregulated in both genotypes, whereas *ZmEXPA4* was not affected under conditions of salt stress (Fig. 3a). The composite relative transcript expression of all four PM-H^+-ATPase isoforms was downregulated in the salt-sensitive hybrid Lector but upregulated in the salt-resistant hybrid SR03 (Fig. 3b).

Discussion

This study was based on the use of young expanding leaf material (*Zea mays* L.) grown entirely under the influence of exogenously added 100 mM NaCl. For this reason, the tissue adequately reveals the effect of the 8-day salt treatment on growth. The salt-sensitive maize hybrid Lector showed a significant shoot growth reduction of 60%, whereas the highly salt-resistant SR03 hybrid exhibited no significant growth reduction under conditions of salinity (Fig. 1). These findings are in line with previously reported results describing the effect of salinity on shoot growth for these two genotypes (Geilfus and others 2010). The ability of the salt-resistant hybrid SR03 to maintain growth under condition of salt stress is remarkable, because salinity has been widely described in the literature as inhibiting the growth of young leaves via an inhibition of cell division and cell elongation (Mühling and Läuchli 2002; Munns and Tester 2008). SR03 maize was originally developed and characterized as being highly salt-resistant; it shows no harvest deficits under 10 dS m^{-1} soil salinity (Schubert and others 2009), whereas a soil conductivity of 6 dS m^{-1} usually leads to a 50% reduced harvest of almost all recent maize genotypes (Doorenbos and Kassam 1976).

Taking into account that the mechanisms that downregulate leaf growth under salt stress are not precisely known, the unique, highly salt-resistant maize genotype SR03 and the salt-sensitive genotype Lector provide good tools to characterize differences in wall extensibility and growth and, thus, differences in the expression patterns of wall-loosening candidates.

Cell-wall Extensibility of Maize Shoots in Response to Salinity

Under salinity, rheological changes are predicted to occur in cell-wall properties, although their nature remains unknown (Cramer and Bowmann 1991; Munns and Tester 2008). In the current study, the salinity-induced rheological properties of the wall have been monitored by measuring creep activity (N_{creep}). N_{creep} has been shown to follow changes in the volumetric extensibility parameter from the Lockhart equation, which cannot be directly determined. Thus, creep activity is an adequate parameter for measuring growth-relevant processes in the cell wall of maize (Büntemeyer and others 1998). In response to salt treatment, creep activity decreases more strongly in the shoot of the salt-sensitive hybrid Lector as compared with the salt-resistant hybrid SR03 (Table 2). Because creep activity reflects changes in the volumetric extensibility (Büntemeyer and others 1998), these results show that the decrease in wall extensibility is more pronounced for the salt-sensitive hybrid Lector than for the salt-resistant SR03. This probably contributes to the remarkable genotypic differences in terms of shoot growth, with reduction being greater in the salt-sensitive Lector (Fig. 1).

These detected changes in wall extensibility strongly suggest an effect of salinity on elements that alter the rheological properties of the cell wall. In favor of this assumption, we have detected a genotype-specific stress response in terms of *ZmXET1* transcript expression (Fig. 2a).

Genotype-specific XET1 and EGases Transcript Abundances in Response to Salinity

Genovesi and others (2008) have suggested that ZmXET1 is bound to the cell wall, thereby encouraging the idea that ZmXET action affects the physical properties of the cell wall. Moreover, Saab and Sachs (1995) have assumed an involvement of ZmXET in cell-wall metabolism during processes leading to structural wall modifications. Furthermore, XET is proposed to play a role in wall-loosening during cell expansion, thereby allowing molecular grafting between polysaccharide chains that crosslink cellulose microfibrils (Fry and others 1992; Nishitani and Tominaga 1992). A decrease of *ZmXET1* mRNA as detected in the salt-sensitive Lector (Fig. 2a) probably contributes to the lower abundance of this enzyme in the cell wall of salinity-affected shoots. On the other hand, an increase of *ZmXET1* mRNA in the salt-resistant SR03 (Fig. 2a) indicates the higher abundance of this protein under salinity. This higher abundance probably improves wall extensibility by facilitating the creep of cellulose microfibrils as described by Vissenberg and others (2005). In agreement with this assumption, creep activity is strongly reduced when *ZmXET1* is downregulated (Table 2), whereas creep activity is only weakly reduced when *ZmXET1* is upregulated. This is supported by the findings that XET activity is correlated with an increasing growth rate (Nishitani and Tominaga 1992; Fry and others 1992). Thus, an increase in *XET* transcript abundance seems to counteract the salinity-induced decrease in creep activity in salt-resistant plants.

In addition to the involvement of XETs, a contribution of EGases has been assumed in xyloglucan metabolism during cell elongation (Catalá and others 1997). EGases are thought to cause the release of xyloglucans trapped in cellulose microfibrils, resulting in increased wall extensibility and cell growth (Cosgrove 2005). In this study, the composite transcript abundance of *ZmEGases* is equally downregulated in both genotypes (Fig. 2b); thus, EGases are unlikely to contribute to the genotypic differences in creep activity described in response to salinity (Table 2).

α-Expansin and Total PM-H^+-ATPase Transcript Abundance and Apoplastic pH

XETs are considered to be directly involved in xyloglucan metabolism during cell elongation, whereas expansin proteins are thought to act at the interface between cellulose microfibrils and hemicelluloses, making them obvious candidates for mediating cell-wall enlargement in growing cells (Cosgrove 2000). Expansins are grouped into α- and β-expansins. The β-expansins appear to have specialized roles in the loosening of the cell walls of grasses, whereas the α-expansins have been found to promote cell-wall growth in many species, including monocots and dicots (Cho and Kende 1997; Brummell and others 1999).

After an 8-day 100-mM salt treatment, the reduced β-expansin transcript and protein expression correlated positively with reduced shoot growth in the salt-sensitive hybrid Lector. On the other hand, an unaffected abundance of growth-mediating β-expansin protein in the salt-resistant hybrid SR03 was related to the maintenance of shoot growth under salinity (Geilfus and others 2010). In the present study, a genotype-specific stress response in terms of *ZmEXPA1* transcript expression has been detected in the shoots of both cultivars. A decrease of *ZmEXPA1* transcripts in the salt-sensitive Lector (Fig. 3a) indicates a decrease in ZmEXPA1 protein abundance in the walls of the salinity-affected leaves. Consequently, fewer ZmEXPA1 proteins seem to act as softening factors on the wall, thus mitigating wall-loosening processes in the salt-sensitive hybrid Lector. This is supported by the reduced creep activity (Table 2), reflecting a decrease in wall extensibility. On the other hand, the increased *ZmEXPA1* expression in the wall of the salt-resistant cultivar SR03 can be suggested to counteract these decreases in creep activity, explaining the less pronounced creep reduction in the salt-resistant cultivar under salinity. In favor of this assumption, Veselov and others (2007) have proposed a rapid increase of *ZmEXPA1* expression as a mechanism contributing to rapid cell wall-loosening and the maintenance of elongation-type growth in maize leaves under conditions of salinity-induced water deficit. In addition, *ZmEXPA1* has been reported to increase leaf extensibility and growth resumption after osmotic stress (Sabirzhanova and others 2005). Moreover, *ZmEXPA1* was expressed specifically in the growing region in maize roots at low water potentials (Wu and others 2001).

The upregulation of *ZmEXPA3* transcripts in Lector (Fig. 3a) is obviously not able to compensate for the decrease in salinity-induced growth reduction. Thus, this expression pattern is not consistent with a role in wall-loosening for shoot cell elongation under conditions of salinity, indicating the different physiological function of this isoform. For instance, in *Zinnia elegans*, *ZeEXPA3* was xylem cell-specific and possibly involved in the intrusive growth of the primary walls of differentiating xylem cells (Im and others 2000). In *Cicer arietinum*, *CaEXPA3* transcripts are related to radicle development (Sánchez and others 2004). The expression of the α-expansin isoform

ZmEXPA4 is not affected by salinity, in either Lector or SR03 (Fig. 3a), indicating that this isoform does not participate in salinity-induced differences in terms of shoot expansion growth. Muller and others (2007) have demonstrated the high correlation between *ZmEXPA4* transcript expression and the relative widening rate of the maize leaf, whereas a poor correlation has been observed between *ZmEXPA4* and the relative leaf elongation rate. In deepwater rice internodes, the transcript levels of *OsEXPA4* were induced by submergence (Cho and Kende 1997).

Expansin protein isoforms have an acidic pH optimum (Cosgrove 2000). This observation gains in importance because Pitann and others (2009) have reported apoplastic alkalinization in the shoots of a salt-sensitive maize genotype. Moreover, Zörb and others (2005) have demonstrated that salt stress decreases PM-H^+-ATPase pumping activity in a salt-sensitive maize hybrid, whereas hydrolytic activity is not affected. Apoplastic alkalization has been suggested to inactivate expansin activity, causing growth reduction under salinity (Pitann and others 2009). In the present study, a decrease in the transcript expression of the four PM-H^+-ATPase isoforms has been detected in the salt-sensitive hybrid Lector (Fig. 3b). This decrease probably contributes to the lower abundance of this enzyme in the plasma membrane of the sensitive genotype, possibly explaining the alkalization of the apoplast as detected by Pitann and others (2009). In contrast, Pitann and others (2009) have reported that the apoplastic pH is not affected in the salt-resistant SR03. In the current study, the transcript expression of the four PM-H^+-ATPases was determined to be upregulated in the salt-resistant hybrid SR03 (Fig. 3b). A higher PM-H^+-ATPases protein abundance might impede apoplastic alkalization by pumping protons from the cytosol into the apoplast. In both genotypes, the total mRNA of the composite PM-H^+-ATPases transcript correlates positively with changes in the apoplastic pH under salinity. In salt-resistant SR03, an upregulation of PM-H^+-ATPase transcripts possibly contributes to the maintenance of the apoplastic pH within a range in which the pH activates expansin activity, thus promoting wall-loosening and cell expansion.

Conclusion

In conclusion, salinity has been shown to change the transcript expression of wall-loosening candidates such as ZmXET1, ZmEGases, α-expansins, and PM-H^+-ATPases in the shoot of maize. For the first time, genotypic differences in the expression patterns of *ZmXET1*, *ZmEXPA1*, and the composite PM-H^+-ATPase transcripts have been demonstrated between a salt-sensitive and a highly salt-resistant maize hybrid in response to salinity, together with genotypic differences in wall extensibility and growth. A decrease in the relative transcript expression of *ZmXET1*, *ZmEXPA1*, and the composite PM-H^+-ATPase mRNAs has been detected in the salt-sensitive hybrid Lector. This downregulation of wall-loosening candidates correlates positively with a decreased wall extensibility and with decreased shoot growth. In contrast, the decrease in wall extensibility is less strong in the salt-sensitive hybrid SR03. An upregulation of *ZmXET1*, *ZmEXPA1*, and PM-H^{+-}ATPase transcripts possibly mitigates the salinity-induced decrease in wall extensibility and thus the decrease in shoot growth in the salt-resistant maize genotype.

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