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Searching for salt tolerance among wild relatives of wheat: What should we look for?

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Abstract

Common strategies for identifying new genetic sources for crop salt tolerance have had limited success. One of the problems lies in the focusing on the seedling vegetative stage, despite the fact that tolerance at the reproductive stages is different. This article highlights a new strategy for such research. The first phase of this study focused on finding closely-related genotypes that differed in a key trait related to salt tolerance. Four hundred lines of four species were subjected to 150 mM NaCl in sand culture. Four *Aegilops kotchyi* lines were selected in this phase. Two tolerant lines (Ak3390 and Ak3393) showed salinity tolerance to 150 mM NaCl both in the vegetative and the reproductive phase. The other two sensitive lines (Ak3511 and Ak4533) reached flowering but failed to produce viable seeds under salt. The four lines were compared during the second phase of the study in terms of ion accumulation and other processes leading to grain production. All lines exhibited decreasing ion content along the spike with no Na^+ reaching the stigma. No significant difference was found between the tolerant and sensitive lines in pollen production and fertilization processes. The sensitive lines did not fill the seed endosperm under salt, and the seeds produced by the salt-treated plants were not viable; while the tolerant lines produced viable seeds with solid full endosperm. It is suggested that future studies will use this two-phase selection strategy in order to identify those genotypes that could be used for the study of key processes related to salt tolerance.

Keywords: *Aegilops*; endosperm; grain filling; pollen viability.
Abbreviations: SHI- spike harvest index; TKW- thousand kernel weight.

Introduction

Wheat is considered among the three most important cereal crops, providing about one-fifth of the calories consumed by man, with over 600 million tons being harvested annually (Shewry, 2009). Global food production will have to increase by approximately 50% by 2050 to match the projected population growth (Flowers, 2004; Rengasamy, 2006). At the same time, the most suitable land is already under cultivation, implying a need for expansion into new areas to meet the above target. One of the major limitations to such expansion is soil salinity. Over 800 million hectares of land worldwide are affected by salinity (Bot et al., 2000; Munns and Tester, 2008), comprising nearly 7% of the world's total land area. A severe reduction in wheat yield at moderate salinity levels (100-150 mM) has been documented in a number of studies (e.g., Hu et al., 1997; Grieve and Poss, 2000; Sairam et al., 2002), and various research methods for screening wheat genotypes for salt tolerance have been proposed (e.g., Munns and James, 2003; Munns et al., 2006).

Improving crop salt tolerance is fast becoming one of the key aspects of future plant breeding. However, all attempts to date towards improving crop salt tolerance through conventional breeding programs have met with very limited success (Colmer et al., 2005; Shabala and Cuin, 2007). The main challenge confronting conventional plant breeders is the magnitude of genetic variation, which is very low in the gene pool of most crop species. As a result, major improvements in salt tolerance cannot be expected. However, utilizing the wild relatives of crop plants as a source of genes to confer salt tolerance can broaden the range of variation that could be used in crop improvement programs (Ashraf et al., 2008). A number of recent reviews noted that progenitors and wild relatives may provide sources of salt tolerance for crops (Colmer et al., 2005; Peleg et al., 2009; Rajendran et al., 2009; Nevo and Chen, 2010). Flowers and Colmer (2008) have already suggested that because of the importance of members of the Triticeae as crops, the halophytic relatives of wheat clearly also deserve investigation. Munns et al. (2012) successfully increased the salt tolerance of durum wheat through the introduction of a sodium exclusion gene from an ancestral wheat relative. *Aegilops* spp. were among the contributors to the wheat genome (Feldman and Millet, 2001; Shewry, 2009), and this is the most closely-related genus to *Triticum*, which has already been used in wheat improvement (Ortiz et al., 2008; Schneider et al., 2008). The study reported here was aimed at identifying a new strategy for selecting sources for salinity tolerance among *Aegilops* spp. lines. Salt tolerance traits discovered using this strategy could be used in the future for the improvement of salt tolerance of commercial wheat cultivars. Inter-specific and inter-generic crosses within the Triticeae are already routine procedures in wheat breeding. Products of inter-specific hybridization expressing superior physiological traits are available for crossing with wheat (Noori, 2005; Marais et al., 2006; Hajjar and Hodgkin, 2007; Islam et al., 2007; Fischer, 2011; Reynolds et al., 2011). Genetic transformation of wheat and utilization of transgenic wheat plants have also become a reality (Rieben et al., 2011; Harwood, 2012; Saint Pierre et al., 2012; Xia et al., 2012). There have been reports in the literature for several decades now that the vegetative and reproductive phases of cereal growth differ with respect to salinity tolerance in rice (Khatun et al., 1995; Khatun and Flowers, 1995a and references cited therein; Abdullah et al.,

2001) and wheat (Blum, 1988; Poustini and Siosemardeh, 2004). Nevertheless, much of the research to date is still being focused on the seedling stage (cf., Munns and Tester, 2008; Sirault et al., 2009; Damon et al., 2011; Chen et al., 2011; Faiyue et al., 2012; Katerji et al., 2012; Xu et al., 2012a,b). More is known about seedlings than mature plants, primarily because results can be obtained more quickly and easily with small vegetative plants than with large flowering plants. Such experiments on seedlings have been justified, since without seedling establishment there would be no plants to flower (Khatun et al., 1995). However, it was already noted that the use of germination or survival at the seedling stage as selection criteria, while rapid and simple, will not guarantee sufficient yield under salt stress (Colmer et al., 2005; Genc et al., 2007). Nonetheless, only a few controlled environment studies continued to the stage of plant maturity and yield measurement (e.g., Gul and Ahmad, 2006; Genc et al., 2007; Samineni et al., 2011). The study by Pranhan et al. (2012) is a rare example of an attempt to discern the stress effect at the grain-filling stage. Samineni et al. (2011) have suggested that future evaluation of salt tolerance should be conducted at both the vegetative and the reproductive stages. In the study reported here salt tolerance was indeed tested at both these stages of the plant's life cycle. At the vegetative stage emphasis was given to selectivity between Na^+ and K^+ in order to maintain favourable K^+/Na^+ ratios (Munns and Tester, 2008) and reduce Cl^- accumulation (Teakle and Tyerman, 2010). At the reproductive stage, pollen viability, fertilization and seed-filling processes were studied. The first phase of this study focused on finding closely- related genotypes which differed in a key trait related to salt tolerance. The second phase focused on four *A. kotchyi* genotypes which showed salt tolerance at the vegetative stage but differed in their salt tolerance at the reproductive stage, which is crucial for future crop improvement. Using this small group of genotypes we demonstrate a selection strategy that may lead to improvement of key mechanisms related to crop salt tolerance (Inbart-Pompan, 2010; Inbart-Pompan et al. 2010).

Results

First stage; selecting closely related genotypes that differ in grain-filling under salt

This study commenced with a wide screening of 400 lines of wild relatives of wheat for salt tolerance. The selection focused on vegetative growth stages and the surviving 10% of these lines following increasing exposure to salt were further exposed to 150 mM NaCl salinity during two consecutive growth seasons; and the best 12 lines, based on their vegetative growth under salt treatment, were chosen for the current research. The selected lines belonged to several *Aegilops* species and wild emmer wheat, all originated from arid or coastal habitats, as listed in Table 1. The plants were grown from seed in sand culture. From the four-leaf stage (Zadok's #14) to maturity, control plants were irrigated with nutrient solution and treated plants were subjected to 150 mM NaCl irrigation. The effect of the salt treatment was assessed by comparing the performance under the two regimes (Table 2). Salt treatment shortened the time-to-flowering, and reduced vegetative and reproductive growth to varying degrees. The lines of *A. sharonensis* and *A. longissima* proved to be the most vulnerable to salt, with their growth reduced by 65-80%. *A. longissima* did not produce any seeds at all and less than 10% of the *A. sharonensis* seeds germinated. The vegetative growth of Td875 was reduced by 85% and its reproductive growth by almost 90%. Based on these results we continued working with the *A. kotschyi* lines Ak3390, Ak3393, which were considered to be tolerant to salt stress at all growth stages; and Ak3511, Ak4533, were considered to be sensitive to salt at the reproductive stage.

Ion relations and grain-filling

From the data on vegetative growth and ion content of the selected lines, shown in Fig. 1, it is evident that the effect of the salt treatment was similar in all lines. Vegetative growth as well as K^+ content was reduced, and Na^+ and Cl^- content increased, as expected. The low level of Na^+ and Cl^- content in the control plants resulted from the use of tap-water as the basis of all irrigation solutions. A more detailed analysis was performed on the upper leaves and reproductive parts (Fig. 2). The top of the main culms of control and salt-treated plants were harvested at anthesis and separated into the leaf blades of the three uppermost leaves and the part of the spike present at that stage, rachis, lemma-palea, and glumes. The results indicate that in this part of the plant the salt effect on ion content differed from that in the vegetative parts as a whole. K^+ content was lower, especially in the spike parts, and was not reduced significantly by the salt treatment. Na^+ and Cl^- content decreased in the apical direction, with the spike parts being protected from the toxic ion accumulation. This analysis revealed some differences among the tested lines, with Ak4533 having a somewhat higher content of Na^+ and Cl^- in the upper leaves and spikes. Plants were grown to maturity and their reproductive performance was assessed. As these are hulled species, only a small (10 seeds) subsample was threshed by hand for determination of Thousand Kernel Weight (TKW), while harvest index was calculated by dividing the overall spike weight by the vegetative weight of the plants, and is therefore referred to as Spike Harvest Index (SHI). Salt did not affect SHI of Ak3990 and Ak3993 and its small effect on SHI of Ak4533 was not significant, since both vegetative and reproductive growth were reduced to a similar degree (Fig. 3). However, salt treatment decreased the other measures of the yield, TKW, spike number and spike weight per plant. Again, the effect on Ak3990 and Ak3393 was smaller than on the sensitive lines Ak3511, Ak4533.

Pollen production and viability

Reduction in seed production and seed weight (Table 2, Fig. 3) could have been caused by damage to any of a number of processes and parameters related to pollination and seed set. Pollen production, release and viability were examined first. All the lines reached anthesis and had normal anthers and stigmas. Anthers also extended and dehisced normally under artificial light in the laboratory, indicating that the salt did not hamper either the lodicule swelling or the filament extension that depend on rapid water uptake. Pollen viability was first tested by germinating freshly released pollen on agar medium. This medium did not include excessive amounts of salts and its osmotic pressure was adjusted with the oligosaccharide raffinose. A 1 M raffinose concentration was found to be optimal for this purpose but pollen grain germination was never higher than 65%, and a considerable fraction (~20%) of the pollen grains always burst, both in control and salt treatments (Fig. 4). Only Ak3390 grains from control plants burst at a significantly higher proportion. Pollen from control and salt-treated plants of the tested lines germinated at about the same percentage. We also used

Table 1. The *Aegilops* spp. and *Triticum* lines screened in the trial and their salt-affected natural habitats.

Line	Location	Global coordinates	Habitat
Aegilops kotschyi Boiss.			
Ak3390	Ashalim	30°57' N 34°42' E	Desert sandy plateau
Ak3393	Ashalim	30°57' N 34°42' E	Desert sandy plateau
Ak3511	Mamshit	31°02' N 35°05' E	Desert sandy plateau
Ak4533	Nahal Hatzatz	30°55' N 34°52' E	Desert highlands
Aegilops sharonensis Eig			
As405	Mihmoret	32°24' N 34°52' E	Coastal sand
As2178	Heftsiba	32°27' N 34°54' E	Coastal sand
Aegilops longissima Schweinf. & Muschl.			
Al1669	Sede Boker	30°52' N 34°46' E	Desert highlands
Al1721	Akko	32°55' N 35°05' E	Coastal saline
Al2893	Yeruham	30°59' N 34°55' E	Desert highlands
Al2895	Yeruham	30°59' N 34°55' E	Desert highlands
Triticum turgidum L. ssp. *dicoccoides* (Korn. ex Asch. & Graeben.)			
Td1477	Hotze Shomron	32°07' N 35 16' E	Arid hills
Td875	Kochav Hashahar	31°57' N 35°21' E	Arid hills

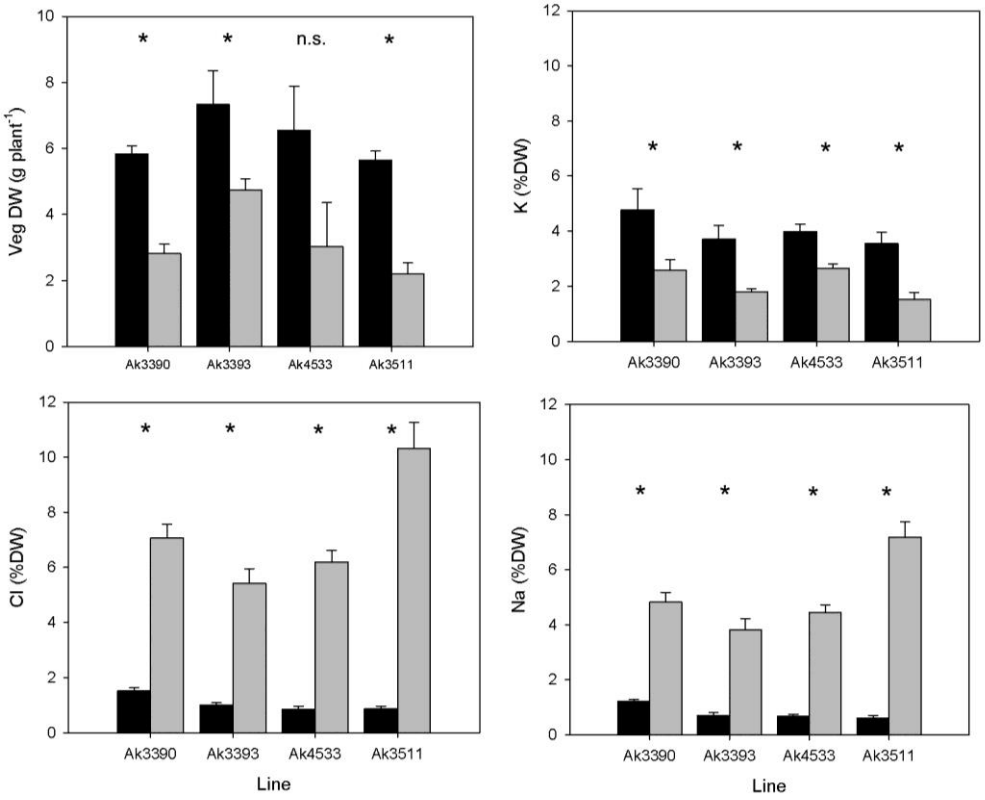

Fig 1. Growth and ion content of vegetative plant parts. Plants of selected lines were grown from seeds in sand-culture either under control [black columns] or salt (150 mM) [grey columns] irrigation and harvested at anthesis (Zadoks' scale #60). Above-ground vegetative parts (culms and leaves) were dried and analyzed for ion content. Data are average ± SE of 3 replicates for the VDW, and 5 replicates for ion content. Asterisks indicate a significant difference between control and salt-treated plants of the same line $p<0.01$, n.s. indicates no significant difference between control and salt-treated plants of the same line.

nuclei fluorescence as an indicator of pollen viability. Pollen germinated on the agarose medium was stained with propidium iodide (PI), and generative nuclei fluorescence was measured under a microscope to indicate any aberrations in nuclear structure (Fig. 5). Salt did not affect nuclear fluorescence of the Ak3390, Ak3393, and Ak4533. The reduction in fluorescence of nuclei of Ak3511 pollen was small but did not indicate a defect in the mitotic nuclei division. Due to the uncertainty of pollen germination on artificial media being representative of what happens on the stigmas, germination of freshly-released pollen on the stigmas of control and salt-treated plants was scored. Pollen of control plants was tested on stigmas of control and of salt-treated plants, and *vice versa* (Fig. 6). In all cases pollen germination on the same type plants (control/control and salt/salt) was much higher than on opposite types, indicating a complementarity between pollen and stigmas of the same type. In the more tolerant lines, Ak3390 and Ak3393, pollen of control/control was significantly higher than that of salt/salt, but the salt had no effect on pollen germination in the other tested cases.

Stigma ion content and fertilization

The complementarity between pollen and stigma might have indicated ion accumulation in the stigma itself. We attempted

Table 2. Salt effect on selected accessions of wild relatives of wheat listed in Table1. Plants were grown from seed in sand culture and subjected to 150 mM NaCl from the four leaf stage (Zadok's #14) to maturity.

Accession	Treated as percentage of control						
	Earlier flowering (d)	Vegetative DW	Spike DW	Spike no.	Spike HI	TKW	Seeds per spike
Ak3390	9	48.5	47.5	52.6	100.7	41.7	112.8
Ak3393	0	64.8	69.6	91.6	105.8	51.3	104.0
Ak3511	12	39.0	22.2	31.9	62.8	38.7	36.6
Ak4533	6	46.2	23.1	29.5	69.2	26.6	24.0
As405	16	28.9	9.0	17.3	30.6	0.0	8.8
As2178	7	35.0	15.8	25.6	50.4	0.0	3.1
Al1669	6	23.7	5.2	11.5	33.9	0.0	0.0
Al1721	3	21.6	6.3	22.1	41.2	0.0	0.0
Al2893	2	24.1	3.2	8.9	8.1	0.0	0.0
Al2895	3	22.2	0.5	2.7	1.8	0.0	0.0
Td875	10	15.8	11.3	11.1	15.8	0.0	0.0
Td1477	8	55.4	36.2	50.4	64.1	27.1	61.2

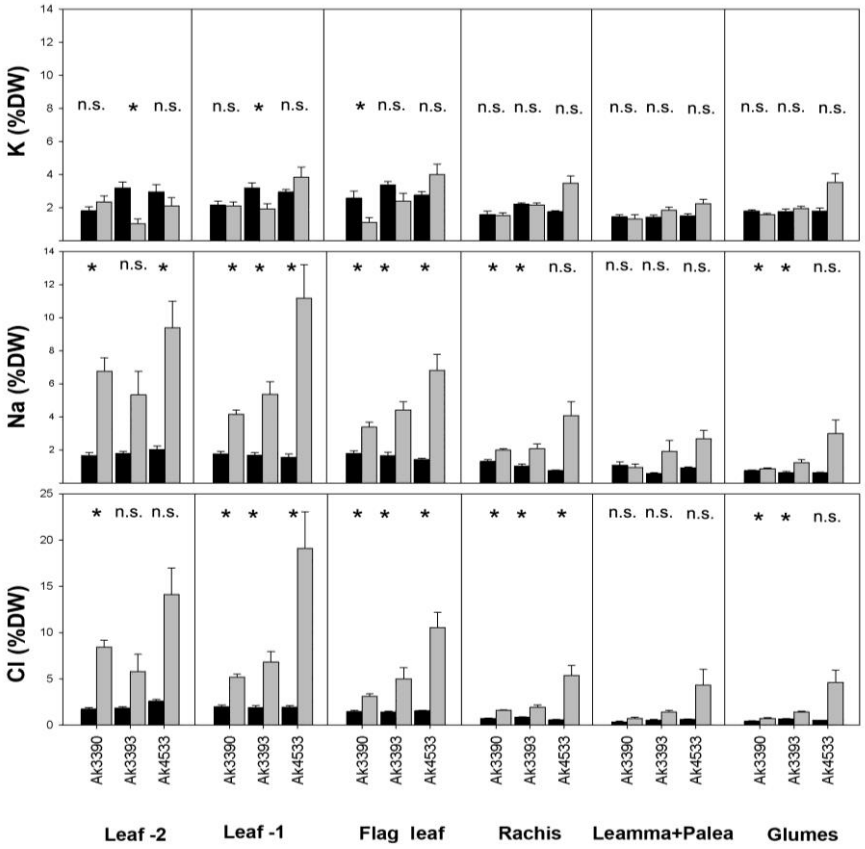

Fig 2. Ion content in upper leaves and spike parts of control [black columns] and salt-treated (120mM) [grey columns] plants. The top of the main culm in each plant, including the three upper leaves and the spike, were sampled at anthesis (Zadok's scale #60), separated into the various parts, dried, and analysed for ion content. Data are average ± SE of 5 replicates. Asterisks indicate a significant difference between control and salt-treated plants of the same line $p<0.01$, n.s. indicates no significant difference between control and salt-treated plants of the same line. Culms and spikes of Ak3511 were not included in this analysis.

to analyse the ion content of stigmas by flame photometry, but the readings obtained from the material we managed to collect were always too low. We therefore analysed stigma ion content by X-ray microanalysis. Images of the material used are presented in Fig. 7. As the thickness and geometry of the samples were not uniform, no suitable standard could be used to quantify the results. The results of the analysis (presented in Fig. 8) are normalized spectra after background subtraction at the energy range of Kα emission of elements 11-20 atomic numbers. There were no differences in the content of the essential elements – S, P, K, Ca – between the control and salt-treated plants. Cl was present in the stigmas of control plants and accumulated to a higher level in the salt-treated ones. Surprisingly, Na did not show a significant reading for either the control or the salt-treated plants. Microscopic sections of the ovules of Ak3393 (A), Ak4533 (B), and Ak3511 (C), made 24 h after anthesis, are shown in Fig. 9. As these plants are known to propagate by selfing, it may well be that fertilization took place by cleistogamy within the florets (Evers and Millart, 2002; Maeng et al., 2006). The presence of the egg cell, the polar nuclei, and the antipodal cells, indicates that fertilization took place in all the lines tested, sensitive and tolerant alike.

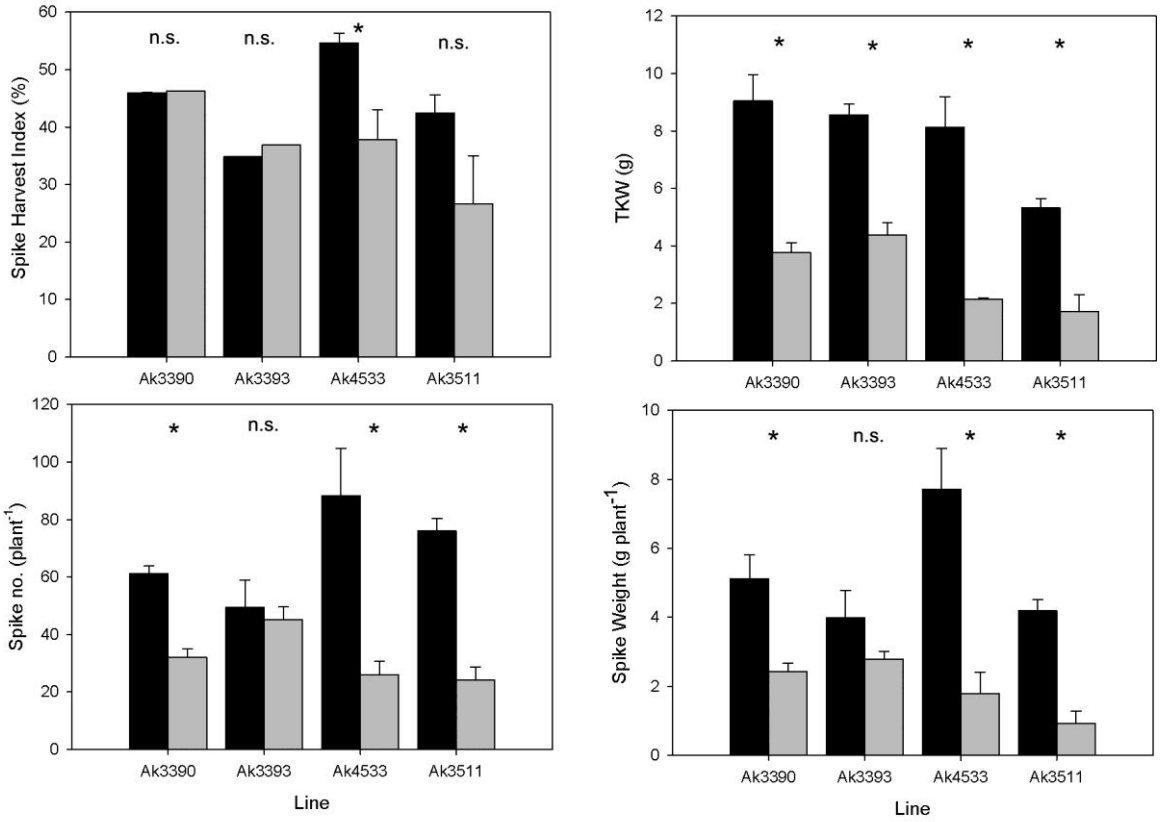

Fig 3. Yield components. Spike Harvest Index (SHI), spike number and spike weight per plant and TKW of control [black columns], and salt-treated (150 mM) [grey columns] plants of selected lines. Data are average ± SE of 3 replicates. Asterisks indicate a significant difference between control and salt-treated plants of the same line $p<0.01$, n.s. indicates no significant difference between control and salt-treated plants of the same line.

Grain-filling

All the seeds showed a developing embryo, indicating that fertilization took place in all instances. However, endosperm development was normal only in the tolerant lines Ak3390 and Ak3393, but was inhibited in the salt-treated sensitive lines Ak3511and Ak4533. Stereo-microscope images of the mature seeds (Fig. 10) also show that while endosperm was normal in all lines under control conditions, only the tolerant Ak3390 and Ak3393 had a fully developed endosperm under salt treatment, while in the sensitive lines the endosperm was missing. These results are supported by the TKW data (Fig. 11) which show a decrease in seed weight under salt only of the sensitive lines Ak3511, Ak4533; and by germination score of these seeds (Fig. 12), which showed that those of control plants of all lines germinated well, but among the seeds produced under salt treatment only those of Ak3390 and Ak3393 germinated, while those of the sensitive lines Ak3511and Ak4533 did not.

Discussion

The first phase of this study was aimed at selecting lines that had survived the salt stress at the vegetative stage in order to study salt tolerance effect on the reproductive stage. We later focused on a smaller group of lines which had exhibited both vegetative growth and flowering under saline conditions. Among these, there were four lines of *Aegilops kotchyi*, a tetraploid species containing the UUSS genomes (Ortiz et al., 2008). Based on the performance of these lines in the first phase, they were designated as either "sensitive" lines (Ak3511, Ak3455) that had normally looking spikes but did not produce viable seeds under salt stress, and "tolerant" lines (Ak3390, Ak3393) that did produce viable seeds under such conditions (Table 2, Fig. 12). These lines of different salt tolerance at the reproductive stage were compared in order to identify the key factors responsible for this difference, following a strategy advocated by Senadheera et al. (2009).

The sensitive as well as the tolerant lines produced tillers and normal spikes under salt treatment. On average, anthesis took place a few days earlier under salt, as compared to non-saline conditions (Table 2). As can be seen in Fig. 1, the size of the vegetative parts of the sensitive and tolerant lines was reduced by the salt treatment to a similar degree. These lines also did not differ in the common criteria which are mentioned in the literature as crucial for salt tolerance: namely, low Na^+ and Cl^- uptake together with maintaining high K^+ content (Colmer et al., 2005; Mahajan and Tuteja, 2005; Chen et al., 2007; Cuin et al., 2008; Flowers and Colmer, 2008; Munns and Tester, 2008; Munns et al., 2006; Teakle and Tyerman, 2010). This may not be surprising since the relevance of overall tissue ion content to salt tolerance have already been questioned before (Kronzucker et al., 2006; Shabala and Cuin, 2007; Cuin et al., 2008; Shabala et al., 2010). As there were no distinct differences between the tolerant and sensitive lines with respect to the salt effect on their vegetative growth and ion content, we concentrated further on the factors that may have affected seed production and viability. Ion content in the upper three leaves, which are known to make the largest contribution to grain-filling (cf.,

Howarth et al., 2008), and in parts of the spike was measured. Na^+ and Cl^- content decreased going upward from the culm to the flag leaf and into the spike, similar to the trend seen in rice (Khatun and Flowers, 1995a; Khatun et al., 1995). Na^+ and Cl^- content in the upper leaves and spike parts of the sensitive lines were higher than in the tolerant lines. Ion content in the upper leaves was found by El-Hendawy et al. (2009) to be a reliable criterion for selecting wheat genotypes for salt tolerance. Unlike the findings in rice, in our plants the K^+ gradient had the same tendency as those of Na^+ and Cl^- but the differences among the tested plant parts were very small under both control and salt conditions. In tomato, Ghanem et al. (2009) found that salt treatment reduced pollen viability, and caused Na^+ ion entry into the flower stigmas. In rice, the change in the ionic concentrations in pollen and stigmas was much greater than that in the younger leaves, and in particular much greater than that in the lemmas and paleas (Khatun and Flowers, 1995a). In contrast, in the *A. kotchyi* plants tested here the lemma, palea, glumes and stigmas were protected from ion entry (Figs. 2, 8). This can be explained by the special thick-walled cells, which create a discontinuity of the xylem at the base of the spikelet in wheat and related grass species (Zee and O'Brien, 1970; O'Brienet al., 1985; Fisher and Cash-Clark, 2000). In these species this discontinuity prevents passive mass flow of the ions in the xylem sap into the florets. It was already noted by Khatun and Flowers (1995a,b) that although of crucial importance to yield, the tolerance of pollen to saline conditions has received little attention, except for a few reports indicating the adverse effects of salinity on the viability and artificial germination of pollen grains of petunia (Reddy and Goss, 1971), wheat (Abdullah et al., 1978), rice (Ota et al., 1956; Pearson and Ayers, 1960 cited by Khatun and Flowers, 1995a), and chickpeas (Dhingra and Varghese, 1993). The assumption that pollen grains germinating on a stigma of a salt-treated plant will be exposed to high salt content led scientists to perform *in-vitro* pollen germination tests by adding single salts to the nutrient solution. This caused a significant decrease in pollen germination (Reddy and Goss, 1971; Khatun and Flowers, 1995a). We obtained similar results (not shown), but after finding that the *A. kotchyi* stigma did not accumulate salts at all, this line of tests was not pursued any further, and pollen viability was tested by germination on the stigma, as discussed below. Anther development and pollen release may also be affected by the salt treatment. Even though in the *A. kotchyi* florets pollination takes place before the flowers open, we found that lodicule expansion, mediated by water uptake, filament extension and anther dehiscence (Heslop-Harrison and Heslop-Harrison, 1996), were normal in both control and salt-treated plants of all the tested lines. There were no significant differences between tolerant and sensitive lines in pollen germination on an artificial medium (Fig. 4). The tolerant Ak3390 was the only line that showed different pollen grain bursting between control and salt-treated plants, but this was considered unimportant as this line produced viable seeds under salt treatment. Little attention has been given to the effects of salinity on stigma receptivity (Khatun and Flowers,1995a,b; Khatun et al., 1995). In a study undertaken to determine the relative importance of the effects of salinity on pollen viability and stigma receptivity for seed-setting in rice, receptivity of the stigma was assessed on the basis of the germination of pollen on the stigmas of salt-treated plants. The results showed that when pollen from control plants was placed on the stigmas of salt-grown ones, germination was reduced (Khatun et al., 1995a). The results of the present study (Fig. 6) indicate that the above

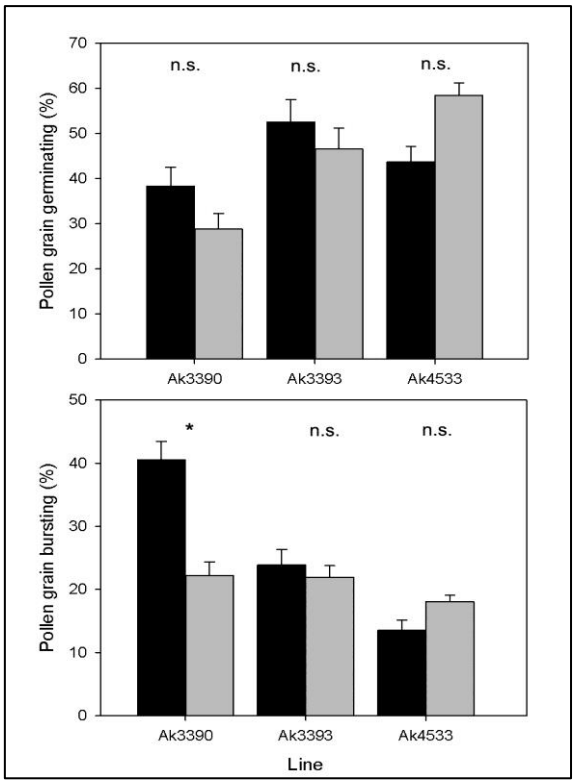

Fig 4. Pollen grain viability. Pollen grains collected from control [black columns], and salt-treated (120 mM) [grey columns] plants of selected lines, were incubated for 20 min on an agarose medium containing 1 M Raffinose. Percentage of germinating and bursting grains was determined by light microscopy. Data are average ± SE of 5 replicates. Pollen of all lines was viable. Asterisks indicate a significant difference between control and salt-treated plants of the same line $p<0.01$, n.s. indicates no significant difference between control and salt-treated plants of the same line. Pollen of Ak3511 was not included in this analysis.

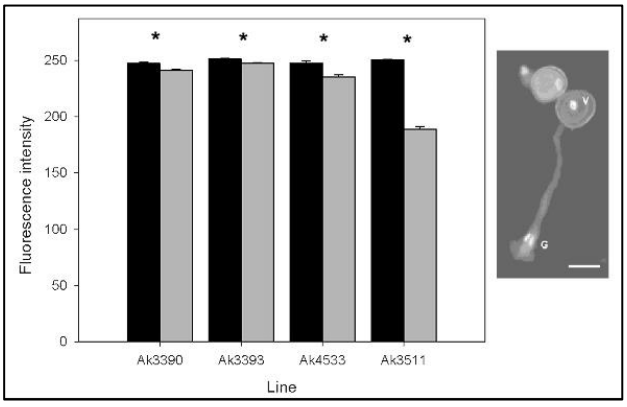

Fig 5. Fluorescence of pollen nuclei. Pollen grains of control [black columns], and salt-treated (120 mM) [grey columns] plants were germinated on agarose medium and stained with propidium iodide (PI). Fluorescence intensity was measured under a fluorescence microscope. Data in arbitrary units are average ± SE of 30 generative nuclei. Asterisks indicate a significant difference between control and salt-treated plants of the same line $p<0.01$, n.s. indicates no significant difference between control and salt-treated plants of the same line. Fluorescence image [right panel] where the vegetative (V) and generative (G) nuclei are clearly observed. Bar= 20 μm

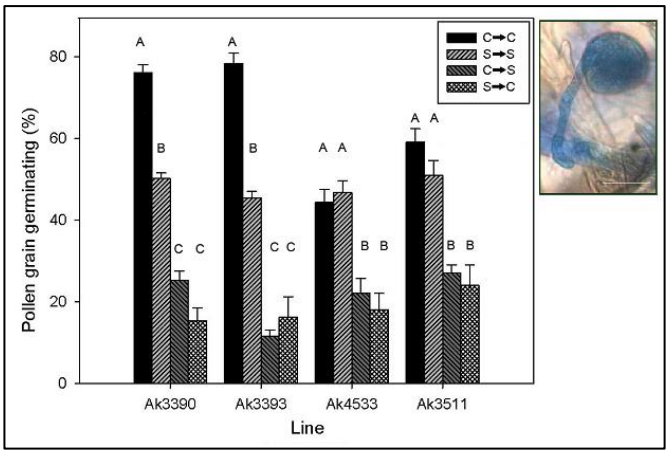

Fig 6. Pollen matches to stigmatas. Pollen grains from spikes at anthesis of control [C], and salt-treated (120 mM) [S] plants were transferred in the lab to receptive stigmas of the same and other treatment plants (see legend insert). Pollen grain germination was determined by light microscopy after staining with 0.5% Aniline blue [Right panel] (Bar= 20 µm). Data are average ± SE of 5 replicates. Within each line, different letters indicate a significant difference $p< 0.05$. Pollen of all lines germinated better on stigmas of same-treatment plants.

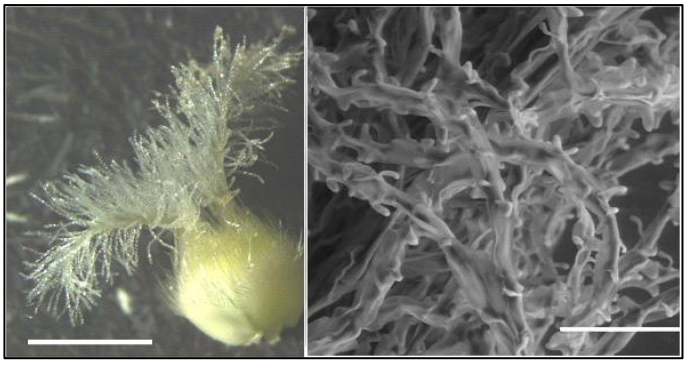

Fig 7. Stereo-microscope [left] and scanning electron [right] images of a stigma prepared for X-Ray microanalysis. Left panel: Fresh ovary and stigma isolated from a flower of *Aegilops kotchyi* which opened in the lab under artificial light, viewed under a stereo-microscope. Bar 1mm. Right panel: Scanning electron image of a stigma fixed on an aluminum disk sample holder with conductive glue, and dried under warm air. Samples were sputter-coated with gold and viewed under low vacuum in an ESEM operated at 20 keV. Bar 100 µm.

mentioned test might lead to erroneously low pollen germination values. Germination of pollen grains from salt-treated plants was better on the stigmas of a salt-treated plant than on a control and *vice versa*. This may indicate some kind of osmotic compatibility between pollen and stigmas of same-treatment plants, but cannot be represented by salt concentrations similar to those used in the irrigation solution. In tomato, Ghanem et al. (2009) found that Na^+ accumulated in style, ovaries, and anther intermediate layers but not in the tapetum or the pollen grains. In rice, both decreased pollen viability and reduced stigma receptivity were found to take part in the reduction of seed-set as a result of salt stress (Abdullah et al., 2001; Khan and Abdullah, 2003). The results presented in Fig. 5 indicate that both the sensitive and tolerant lines tested transmitted intact generative nuclei down the pollen tubes. There were no indications of major changes in nuclear content as a result of the salt treatment, while Ak3511 showed ca. 22% reduction in nuclear content, the other sensitive line, Ak4533, was not different enough from the tolerant lines to indicate a defect in the mitotic divisions. The endosperm is the most important part of the cereal kernel for exploitation by humans (Evers and Millart, 2002; Sabelli and Larkins, 2009), and its presence is crucial not only for seed viability but also for its commercial value. The results presented in Fig. 9 show that proper fertilization and formation of an embryo and antipodal cells took place in the sensitive as well as in the tolerant lines. The main difference lay in the later processes of endosperm-filling (Figs. 10-11) and, as a result, in seed viability (Fig. 12). Conventional thinking explains the poor grain-filling as the consequence of carbon limitation. Abdullah et al. (2001) concluded that, in rice, salt stress results in limited carbohydrate translocation to the developing grains and inhibition of specific activity of starch synthetase, which together bring about a reduction in seed set. Similar results were reported by Khan and Abdullah (2003). Recent studies, however, have shown that carbohydrate supply is not the major constraint of wheat yield (Reynolds et al., 2009). The low activities of key enzymes in carbon metabolism may contribute to the poor grain-filling (Weichert et al., 2010; Yang and Zhang, 2010; Li et al., 2012). A number of recent studies have targeted the genes that are involved in key processes related to the development of wheat grains: starch accumulation (Barnabas et al., 2008; Guzmán et al., 2012; Kovalchuk et al., 2012), and ion transport (Waters et al., 2009). Other recent works have identified large groups of genes involved in this process (e.g, Ge et al., 2012; Pellny et al., 2012). We therefore suggest that salt effect on grain-filling may also be a result of a specific inhibition of the genes responsible for carbohydrate mobilization, transport or polymerisation within the endosperm.

Materials and Methods

Plant material and experimental setup

Seeds were obtained from The Lieberman Germplasm Bank, The Institute for Cereal Crops Improvement, Tel Aviv University. All the trials were carried out in sand culture outdoors on the Tel-Aviv University campus, during six consecutive seasons. Seeds were planted in 10 L buckets filled with washed coarse sand in the autumn (October-December). A wide screening of 400 lines of wild relatives of wheat from this gene bank was carried out by exposing them to increasing salt concentrations until 90% had died due to salt stress. The selection focused on vegetative growth stages and identified the 10% of these lines that survived. These 40 lines were then exposed to 150mM NaCl salinity during two consecutive seasons and the best 12 lines were chosen for the current research, based on their size at anthesis.The tested lines belonged to the following species (Table 1): (1) *Aegilops bicornis* (Forssk.) Jaub. & Spach, from the Mediterranean coast, the northern Negev and Sinai, (2) *A. kotschyi* Boiss., from the northern and western Negev and Judean mountains; (3) *A. longissima* Schweinf. & Muschl., from the Galilee coast and northern Negev, (4) *A. sharonensis* Eig, from the Mediterranean coastal plain; and (5) *Triticum turgidum* L. ssp. *dicoccoides* (Korn. ex Asch. & Graeben.) Thell., from the Judean Desert and Samarian

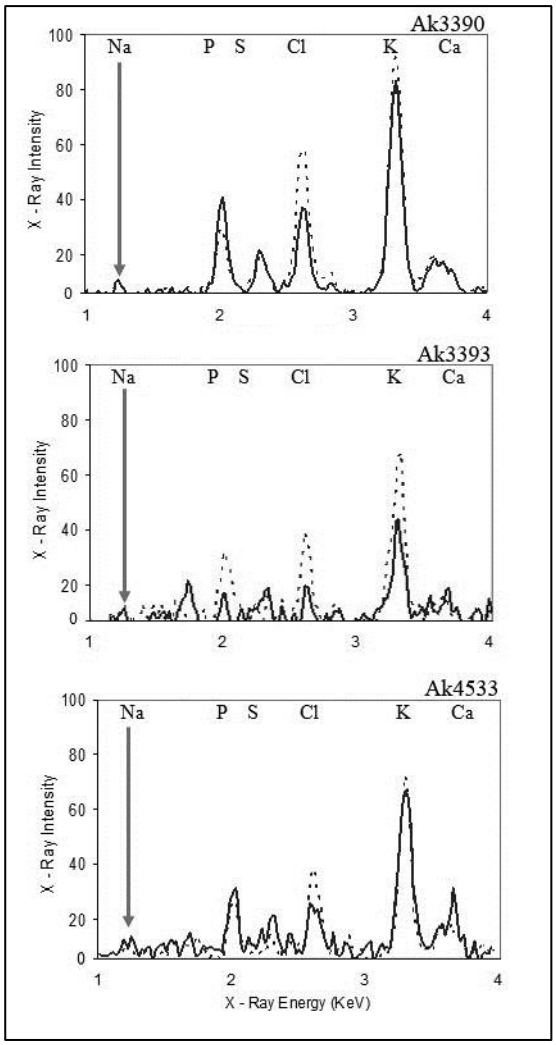

Fig 8. Ion content of the stigma. Results of X-Ray microanalysis of stigmas of control [solid lines] and salt-treated (120 mM) [dotted lines] plants of selected lines. Spectra were normalized with respect to X-ray intensity at the 4.5 – 4.7 keV range, where no X-ray emission peaks were present; and background readings, taken on the holder next to the sample, were subtracted. Kα peaks of the major ions are marked. No significant reading for Na was measured in any stigma analyzed. Four stigmas of each line/treatment were analyzed with the same results. Stigmas of Ak3511 were not included in this analysis.

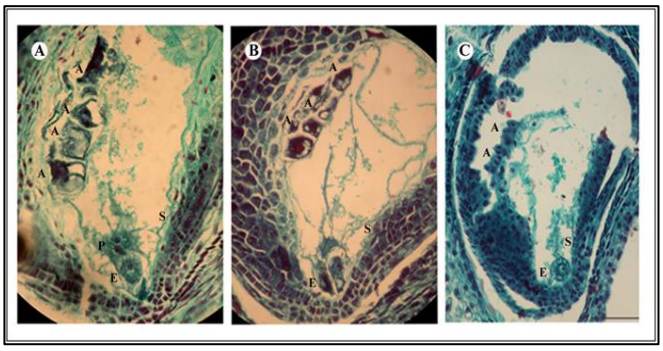

Fig 9. Microscopic sections through ovules of Ak3390 [A], Ak4533 [B], and Ak3511 [C], 24 h after anthesis. All include an embryo (E), an embryo sac (S), and antipodal cells (A), Bar=100 µm applies to all three figures.

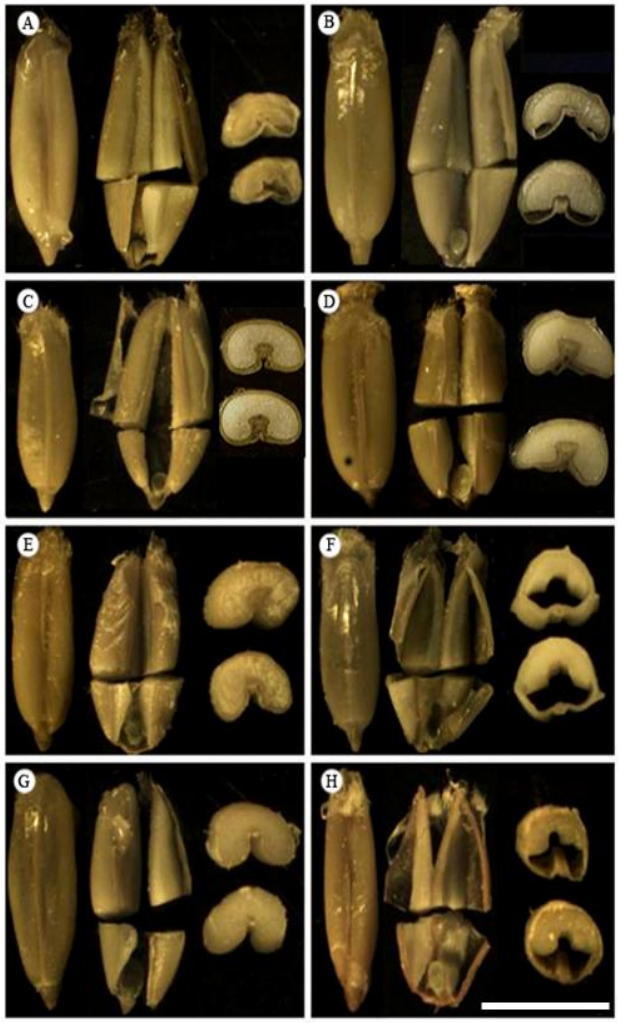

Fig 10. Seed sections. Stereo-microscope images of whole, and longitudinally- and horizontally-broken seeds, showing the endosperm of control (A,C,E,G) and salt-treated (120 mM) [B,D,F,H] plants of Ak3390 [A,B], Ak3393 [C,D], Ak4533 [E,F], and Ak3511 [G,H]. Bar=5mm applies to all panels.

mountains. In the following trials four seeds were planted in each bucket, and plants were thinned out gradually during sampling along the growth season at the following developmental stages, identified on the Zadoks' scale (Zadoks et al., 1974): #14-Four leaves unfolded, #47-Flag leaf sheath opening; #60-Beginning of anthesis; and at the end of the season when plants were completely dry. During early growth all the plants were irrigated every three days with nutrient solution containing 0.75 g L^{-1} fertilizer (20:20:20, Haifa Fertilizers, Haifa, Israel; containing 20% N compounds, 20% P_2O_5, and 20% K_2O and microelements). After the first sampling at Zadoks #14 the plants were divided into control (fertilizer irrigation) and salt-treated (NaCl + fertilizer irrigation) blocks. Salt concentration was raised gradually at 25 mM every three days until a final concentration between 120 – 150 mM, depending on the experiment; see Results for details.

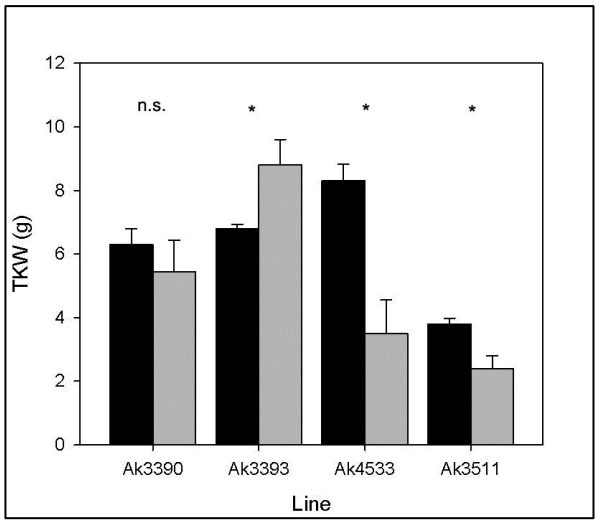

Fig 11. TKW of control [black columns], and salt-treated (120 mM) [grey columns] plants of selected lines. Data are averages ± SE of 5 replicates of 10 seeds each. Asterisks indicate a significant difference between control and salt-treated plants of the same line $p<0.01$, n.s. indicates no significant difference between control and salt-treated plants of the same line.

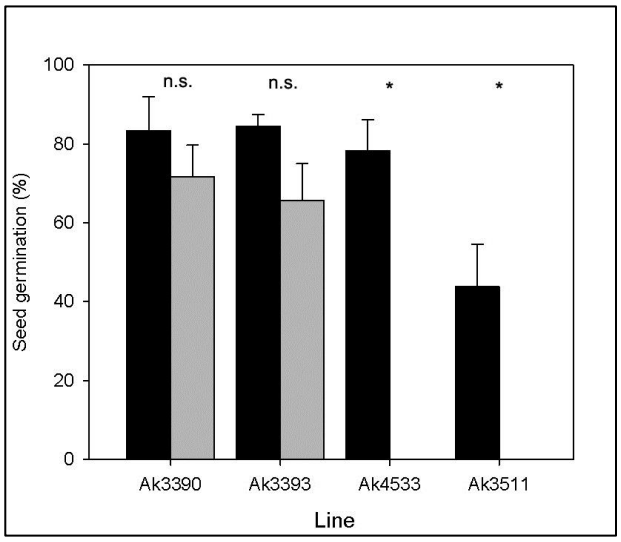

Fig *12*. Seed germination in distilled water. Percentage germination of seeds of control [black columns] and salt-treated (120 mM) [grey columns] of selected lines. The effect of salt treatment of the mother plant on germination of seeds of the tolerant lines Ak3390, Ak3393 was not significant. Seeds of salt-treated plants of the sensitive lines did not germinate at all. Data are average ± SE of 5 replicates of 8 seeds each. Asterisks indicate a significant difference between control and salt-treated plants of the same line $p<0.01$, n.s. indicates no significant difference between control and salt-treated plants of the same line.

Measuring harvest parameters and ion content

Plants were sampled by cutting at ground level. The harvested material was dried in an oven at 80°C for at least 48 h. On the last two sampling dates the samples were divided into straw and spikes that were weighed separately. As grains of these wild species are hulled and not easily threshed, spike weight was used as a measure of total yield, and spike harvest index (SHI) was calculated as the ratio between spike and straw weight of every plant. Seeds from 1 g of air-dried spikes were hulled and used for determining seed yield per spike, thousand kernels weight (TKW), and seed viability. Seed viability was determined by plating eight seeds in a 90 mm Petri dish over Whatman filter paper soaked with distilled water, incubating for four days at 4°C and another four days at 25°C, and counting the germinated seeds by radicle emergence. For determination of ion content, 100 mg samples of the dry plant material were ashed at 500°C and the ashes were dissolved in 2 mL 2N HNO_3. The acid extract was diluted in distilled water and analyzed by flame photometry (Model 410, Sherwood Scientific, UK) for Na^+ and K^+, and by coulometric chloride titration (MKII Chloride Analyzer 926, Sherwood Scientific, UK) for Cl^-. Ion content of fresh stigmas collected from flower that had opened in the lab, under artificial light, was determined in an environmental scanning electron microscope (ESEM) (FEI, Quanta 200FEG) operated in low vacuum at 20 keV, equipped with an energy dispersive X-ray spectrometer (EDS), (Oxford Instruments). Individual stigmas were isolated from mature flowers of control and salt-treated plants, fixed to an aluminum sample holder with conductive glue, and dried in warm air. Prior to analysis the samples were sputter coated with gold. As sample geometry and thickness were irregular, all X-ray spectra were normalized by intensity at the 4.5 – 4.7 keV range, where no peak was observed. X-ray background intensity was recorded on the sample holder next to each sample, and subtracted from all channels.

Testing pollen viability and fertilization

Pollen was collected in the lab from spikes brought in just before anthesis. The upper third of the glumes of each spikelet was cut under a binocular. The spikes were placed on the lab bench under white light until the filaments extended and the anthers opened. The pollen grains were dispersed in a 35 mm Petri dish on a solid pollen germination substrate, modified after Cheng and McComb (1992) (Agarose Low EEO 0.05-0.1 0.7%, H_3BO_3 100 mg L^{-1}, $CaCL_2.2H_2O$ 300 mg L^{-1} and Raffinose 1M), and allowed to germinate at room temperature for 20 min. The percentage of germinating pollen grains according to the length of germination tubes was determined under a light microscope (Nikon ECLIPSE E600). For fluorescence measurements, germinating pollen grains were stained with propidium iodide (PI) according to Eilam et al. (2009) and examined under a Zeiss epi-fluorescence microscope with filter set (510-560 FT 580, LP 590). Images were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/index.html).

In-vivo pollen germination was tested by transferring pollen grains from the just opened anthers to the stigmas of emasculated flowers opened in the lab under white light. After 1 h stigmas were stained with Aniline blue 0.5% and examined under a light microscope. Germinating pollen grains were counted under the light microscope. For light microscopy of flower ovaries and developing seeds, spikelets were collected 1, 3, 6, 12, 16, and 18 days after anthesis, fixed in FAA (Formalin: Glacial Acetic acid: Ethyl Alcohol, 5:5:90), dehydrated by the T.B.A. (Tertiary Buthyl Alcohol) method, embedded in paraffin, sectioned at 8 μm by a rotary microtome, and stained with Safranin-Fast green (Johansen 1940). The t-tests, two-way ANOVA, and Tukey's multiple comparison tests were done at $p<0.05$ using JMP (SAS Institute Inc.) and SPSS (IBM).

Conclusion

This study demonstrated a novel two-phase strategy for discovering sources for salt tolerance. The first phase selected closely-related genotypes that are similarly affected by the salt in their vegetative phase, and differ only in their ability to fill the grains under salt. The second phase examined the salt effect on all the processes involved in grain production and filling; flowering, pollen production, release and viability, fertilization, and endosperm filing. It was shown that tolerant and sensitive lines differed in the last process only. It is suggested that future studies should follow the same strategy in order to identify the mechanisms behind the salt effect on the yield of wheat and other cereals. Due to the high synteny among the genomes of these grasses, it may be possible in future breeding programs to target the specific genes involved.

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