Assessment of the salt tolerance of wheat genotypes during the germination stage based on germination ability parameters and associated SSR markers

Salah El-Hendawy, Adel Elshafei, Nasser Al-Suhaibani, Majed Alotabi, Wael Hassan, Yaser Hassan Dewir, and Kamel Abdella

Department of Plant Production, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia; Department of Agronomy, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt; Genetic Engineering and Biotechnology Division, National Research Centre, Cairo, Egypt; Department of Biology, College of Science and Humanities at Quwayyah, Shaqra University, Shaqra, Saudi Arabia; Department of Agricultural Botany, Faculty of Agriculture, Suez Canal University Ismailia, Egypt; Horticulture Department, Faculty of Agriculture, Kafrelsheikh University, Kafr El Sheikh, Egypt

ABSTRACT

Although the germination stage accounts for a very short period of a plant’s life cycle, it involves numerous mechanisms and multistage processes that potentially differ among genotypes under salt stress. Therefore, we hypothesized that the parameters controlling the water uptake pattern and α-amylase activity during the seed germination process could be helpful for assessment the salt tolerance of wheat genotypes at the early growth stage. Genotypic differences in the germination ability parameters and α-amylase activity were assessed for seven wheat cultivars under normal and salt stress conditions at the molecular marker level using 30 simple sequence repeat (SSR) markers linked to salt tolerance. Results found that genotypic variations in the water uptake pattern were appeared at the late stage of phase II under both salinity levels and more obviously during phase III. Genotypic variations were observed in the germination time (GT), which was delayed by increasing salinity levels. The α-amylase activity and GT were positively and negatively correlated, respectively, with each time of water uptake rate. Significant correlation ($r = 0.49$, $P = 0.026$) was observed between similarity coefficients of germination ability parameters and SSR data based on the Mantel test. Among the 24 SSR markers, which showed polymorphism, Cdf 9, Cdf 46, Cdf 49, Wmc 503, and Gwm 312 were associated with almost germination ability parameters ($R^2$ ranged from 0.43 to 0.95). Therefore, based on the molecular marker-phenotypic trait association at germination stage, assessment of salt tolerance in many wheat genotypes in a relatively short time could be conceivable.

Introduction

Seed germination is one of the most critical stages in the plant life cycle, as it directly determines the failure or success of the subsequent growth stages (Saritha and Prasad, 2007; El-Hendawy et al. 2011; Liu et al. 2018). Salinity is one of the major environmental stresses responsible for the inhibition of seed germination in glycophyte plants. Saline soil can inhibit seed germination by either creating osmotic stress that prevents water uptake or by specific ion toxicity that inhibits the processes of dividing and expanding cells, as well as altering the activity of some important enzymes, which finally reduces the utilization of seed reserves (Zhang et al. 2010, 2017). In general, under normal conditions, there are three phases responsible for the progress of seed germination. The water uptake in these phases follows a triphasic pattern (Bewely, 1997). Phase I represents fast water uptake by the dry seed. This is followed by a plateau phase (phase II, metabolism reactivation), which is characterized by the limited water uptake. Then comes phase III, a post-germination phase of water uptake, which is characterized by continuous water uptake until germination is complete. Based on these three phases, the inhibition of seed germination under salinity stress could be generally attributed to osmotic stress in the phase I and ionic stress in the phase II. During the phase III, both components of salt stress interact together to inhibit seed germination. Therefore, to decrease the negative effects of salinity stress on seed germination, it is important to know to what extent the genotypic variation in the water uptake pattern during these phases is associated with the salt tolerance of genotypes at the germination stage.

α-amylase is a major enzyme in cereal crops, as it plays an important role in seed germination. It is excreted into the endosperm to degrade the stored starch into metabolizable sugars that provide readily available energy and nutrients to the growing embryo, plumule, and radicle. Several studies reported that salinity stress may have little effect on the final germination percentage of most crops, but it might cause a significant delaying of the germination time. A delay of water uptake as well as a decrease in the α-amylase activities might be the main reasons for the delaying of the germination time under salinity stress (Kaneko et al. 2002; Murtaza and Asghar, 2012). Several studies reported that the α-amylase activity decreased with an increase in the concentration of NaCl, even at low concentrations. However, this decrease was more pronounced in the salt-sensitive genotypes than in the salt-tolerant ones. This reduction in the α-amylase activity causes a significant reduction in the translocation of sugars to the developing embryo. As a result of decreasing sugar concentrations, the osmotic potential of growing cells is increased.
and then water uptake is decreased, which delays seed germination (Ashraf et al. 2002; Jamil et al. 2006; Dkhil and Denden, 2010; Nizam, 2011; Hua-long et al. 2014; Zhang et al. 2017; Liu et al. 2018).

Molecular markers provide a virtually unlimited number of markers to compare individual genotypes under a wide range of environmental conditions, are not associated with crop growth stages, and provide data that can be analyzed objectively. Intriguingly, simple sequence repeats (SSRs), otherwise known as microsatellite markers, have been found to be effective for creating contrasting genetic maps, studying genetic diversity, studying genomic polymorphism in different germplasms, and assisting genotypic selection (Islam, 2004; Ott et al. 2011; Shahzad et al. 2012; Miah et al. 2013; Shirnasabian et al. 2014; Kumar et al. 2016). Therefore, SSR analysis could play an important role in identifying the major gene relating to salt tolerance, which would be helpful for plant breeders to discriminate between the levels of salt tolerance for different genotypes and to identify the salt-tolerant genotypes that can be used as new sources for future breeding programmes.

In this study we hypothesized that the parameters affecting the seed water uptake pattern and α-amylase activity during seed germination will differ among wheat genotypes under salt stress. Therefore, the objectives of this study were to exploit these differences to examine the possibility of effectively using these parameters as screening criteria to discriminate between salt-tolerant wheat cultivars and salt-sensitive ones at an early growth stage and to validate these screening criteria using different SSR markers linked to salt tolerance in wheat.

Materials and methods

Plant materials and experimental set-up

Five bread wheat cultivars (Triticum aestivum L.) (Drysdale, Sakha 93, Yecora Rojo, Sakha 61, and Pavon 76) and two durum wheat cultivars (Triticum durum Desf.) (Kronos and Orita) were evaluated in two different experiments. Two of the bread cultivars (Sakha 93 and Sakha 61) were tested for their salt tolerance across the different growth stages (from seedling until maturity). The results of which confirmed that Sakha 93 and Sakha 61 are salt-tolerant and salt-sensitive cultivars, respectively (El-Hendawy et al. 2005).

The first experiment was conducted in a dark growth chamber with the temperature and relative air humidity maintained at about 23°C and 80%, respectively. This experiment monitored the water uptake pattern and germination time during the seed germination process. It was conducted using a randomized complete block design with three replicates. The second experiment was conducted under the same conditions as the first, to determine the total amylase activity in the entire germinating seeds.

In the two experiments, the seven wheat cultivars were subjected to three treatments of different salinity levels (the control (no added NaCl), low salinity (60 mM NaCl), and high salinity (120 mM NaCl)). To prepare the non-saline soil and artificial saline soils, a loamy soil consisting of 48.4% sand, 31.5% silt, 20.1% clay, and 1.65% organic matter was air-dried, ground, and then sieved through a 5 mm mesh screen. Following this, the soil was thoroughly mixed by hand with either tap water for the control treatment or salt solution for the salinity treatments. The salt concentration of the artificial saline soils was calculated based on a final soil water content of 25%. Thereafter, in the first experiment, a plastic tray (50 cm in length × 31 cm in width × 4 cm in height) containing 126 cells was filled with the soil. One seed was manually dry seeded in each cell at a depth of about 2 cm. In the second experiment, plastic pots (9 cm in diameter × 7 cm in height) were filled with the soil. Fifteen seeds of each cultivar were manually dry seeded in each pot at a depth of about 2 cm. All plastic trays and pots were covered with a 1.5 mm thick plastic sheet to avoid any water loss.

Assessment of the seed water uptake pattern and germination time

To determine the amount of water uptake by the seeds, the initial weight of each individual seed was recorded before the start of the experiment. At 8, 16, 24, 32, 40, 56, 64, 72, 80, 88, 96, 104, 112, 120, 128, 136, and 144 h after sowing, the seeds were withdrawn from the soil, washed, and rubbed carefully between paper towels to remove the external water from the surface of the seeds before they were weighed. The percentage of water uptake was calculated as the difference between the wet and initial weights of the seed as a proportion of the initial weight multiplied by 100. The rate of water uptake at different times after seed sowing was calculated by subtracting the percentage of water uptake of two consecutive measurements and dividing the result by the elapsed time between the two measurements. Germination time was recorded when the radicle protrusion penetrated the seed coat.

Quantitative assay for α-amylase activity in the germinating seeds

At 48, 72, and 96 h after sowing, the fifteen seeds in each pot were removed from the soil, washed, and rubbed carefully between paper towels to remove the external water from the surface of the seeds. The seeds were ground and homogenized in 100 ml chilled distilled water. The homogeneous mixture was soaked in a cooling bath at 4°C for 10 min and then filtered through a muslin cloth. The filtrate was then centrifuged at 10,000 × g for 15 min at 3°C. The clear supernatant was used as the crude extract to assay the α-amylase activity using the dinitrosalicylic acid method established by Miller (1959).

The crude extract was heated at 70°C for 15 min to inactivate β-amylase. Then, a mixture of 1 ml of the crude extract and 1 ml of 1% starch solution was dissolved in Na-acetate buffer at pH 5.6, incubated at 40°C for 15 min, and boiled for 5 min in the presence of 0.5 ml of 3,5-dinitrosalicylic acid reagent to terminate the reaction. The assembly was incubated for 5 min in a boiling water bath and then cooled. After completing these steps, the absorbance of the reaction mixture was measured at 540 nm using a spectrophotometer (Jenway 7315 series model) with maltose as the reducing sugar standard. One unit of α-amylase activity was defined as μ moles of maltose produced per minute under the standard assay conditions.

DNA extraction and SSR analysis

Each cultivar had young leaves removed and frozen (0.5 g; derived from the shoot tips), which were then ground to a powder in a mortar with liquid nitrogen. Genomic DNA of
each cultivar was extracted using the Wizard Genomic DNA Purification Kit (PROMEGA Corporation Biotechnology, Madison, Wisconsin, USA). After extraction, the samples were treated with RNase and then maintained at a temperature of −20°C. The quality of DNA was checked by electrophoresis on 0.8% agarose gel and the concentration of DNA was determined using an Epoch Multi-Volume Spectrophotometer (Thermo scientific, USA). Then, the quantified DNA stock was diluted to a final concentration of 25 ng μl−1.

Twenty-four different specific SSR markers linked to salt tolerance in wheat were used (Table 3). These microsatellite primers have been previously reported in several studies as being linked with salt tolerance (Lindsay et al. 2004; Byrt et al. 2007; Shahzad, 2007; Huang et al. 2008; Lindsay et al. 2008; Ahmed, 2010; James et al. 2011). The polymerase chain reaction (PCR) mixture consisted of 20–50 ng of genomic DNA, 1 × PCR buffer, 1.5 mM MgCl2, 0.1 mM dNTP, 0.5 μM primer, and 1 U Taq polymerase in a volume of 0.025 cm3. The programme of PCR for the SSR analysis included an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 51–61°C (depending on the individual SSR primers) for 1 min, an extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Amplification products were electrophoretically resolved on 3% (m/v) agarose gels, containing 0.1 μg cm−3 ethidium bromide, and photographed on a UV trans-illuminator.

**Data handling and cluster analysis**

SSR data was scored based on the presence or absence of the amplified products for each primer, after excluding the un prosecutable bands. If a product is present in all seven wheat cultivars, it will be designated as ‘1’, if absent, it will be designated as ‘0’. The genetic similarities (GS) were calculated, according to Nei and Li (1979) as follows: GS = 2Nij/(Ni + Nj). Where, Nij is the number of bands present in both i and j cultivars, and Ni and Nj are the number of bands present in i and j cultivars, respectively. Based on the similarity matrix, a dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA). Phylogenetic relationships were estimated from the nucleotide sequences using the method of maximum parsimony (fast heuristic search algorithm, PAUP software, version 4.0b10 [Sinauer Associates, Inc., Sunderland, MA, USA]) paired with a bootstrap resampling method (1000 replications). To investigate the discriminatory power of each SSR primer, the polymorphic information content (PIC) was calculated (according to Smith et al. 2000) as follows: PIC = 1−Σd2. Where, fi is the frequency of the ith allele in the set of seven wheat cultivars. Discrimination power (Dp) for each primer was calculated by dividing the number of amplified polymorphic alleles by the total number of polymorphic alleles obtained (Khierallah et al. 2011).

**Statistical analysis**

Data of the water uptake pattern and α-amylase activity were subjected to two-way analysis of variance (ANOVA). A Pearson’s correlation coefficient matrix was used to determine the relationship between the germination ability parameters at different times after seed sowing for the low and high salinity treatments (n = 21). The difference between the mean values of germination ability parameters and α-amylase activity were compared using Duncan’s test at the 0.05 probability level. The ANOVA analysis and comparison between mean values were done using CoStat 6.311 for Windows (CoHort software, Berkeley, CA 94701). Data (mean ± standard error) were done graphically using SigmaPlot 12.0 for windows.

The clusters were constructed using PAST 3.22 software. Euclidian and unweighted pair group method arithmetic average (UPGMA) was used to group cultivars based on their all germination ability parameters or SSR data. The relationships between the data of Euclidean distance matrix based on germination ability parameters and the distance matrices of genetic based on SSR data were tested according to Mantel test (Mantel, 1967).

**Results**

**Patterns of water uptake at different times after seed sowing**

Figure 1 shows the pattern of water uptake for the seven wheat cultivars subjected to three salinity treatments over a 144 h period after sowing. Interestingly, the triphasic pattern of water uptake was identical and occurred in all three salinity levels but phase II was delayed as the level of salinity increased. Phase I of water uptake was complete by 48 h after sowing in all treatments; whereas, phase II was delayed by 16 and 24 h in 60 and 120 mM NaCl, respectively, in comparison to the control treatment. There were no distinct differences in the water uptake among the cultivars in phase I. These differences appeared at the late stage of phase II under 60 and 120 mM NaCl and more obviously during phase III under all treatments (Figure 1). In all treatments, Drysdale and the salt-tolerant check cultivar Sakha 93 both had a significantly higher level of water uptake than Pavon 76, Orita, and the salt-sensitive check cultivar Sakha 61.

**Rate of water uptake**

Both the salinity level and cultivar significantly affected the water uptake rate per hour over different periods after seed sowing (0–8, 8–48, 48–72, 72–96, 96–120, and 120–144 h after seed sowing). The interaction between the salinity level and cultivar also had a significant effect on the water uptake rate per hour over all the periods, except for the period between 0 and 8 h after seed sowing (Table 1). The highest rates of water uptake per hour occurred between 0 and 8, 96 and 120, and 120 and 144 h after seed sowing (Figure 2). Rate of water uptake per hour significantly decreased as the levels of NaCl increased. However, this reduction depended on which cultivar was being tested (Figure 2). A minimum reduction in the water uptake rate per hour under 60 and 120 mM NaCl was recorded in the three bread cultivars (Drysdale, Sakha 93, and Yecora Rojo) and the durum cultivar Kronos. This reduction was more pronounced for the other bread (Sakha 61 and Pavon 76) and durum (Orita) cultivars (Figure 2).

**Germination percentage and time**

The final germination percentage reached 100% for all cultivars under the salinity treatments, as with the control. The
greatest difference between the cultivars was observed in the germination time (Figure 3). The germination time of Drysdale, Sakha 93, Yecora Rojo, Sakha 61, Pavon 76, Kronos, and Orita was slowed by 8, 8, 16, 24, 24, 16, and 48 h under 60 mM NaCl, and by 24, 24, 32, 56, 48, 40, and 48 h under 120 mM NaCl, respectively, compared with the control treatment (Figure 3). Under the high salinity treatment, the germination time was slowed by 16 h for Drysdale, Sakha 93, and Yecora Rojo, 24 h for Pavon 76 and Kronos, and 32 h for Sakha 61, compared with the low salinity treatment; whereas, the seeds of Orita reached 100% germination at the same time (104 h after sowing) under both salinity treatments (Figure 4).

Changes in α-amylase activity in the germinating seeds

The α-amylase activity during different periods of germination was remarkably affected by the salinity level, cultivar, and their interaction (Table 1). In general, the activity of this enzyme was significantly reduced by increasing the level of salinity. Compared with the control treatment, enzyme activity reduced by 18.7, 17.0, and 27.9% under 60 mM NaCl and by 34.0, 29.9, and 41.3% under 120 mM NaCl after 48, 72, and 96 h of seed sowing, respectively (Figure 4). However, this reduction depended upon the cultivar being tested. The maximum reduction in α-amylase activity under

Figure 1. Increase in the fresh weight of the seed (%) of seven wheat cultivars at different times after sowing under different salinity treatments.
both salinity treatments during all three phases of germination was recorded for Orita, followed by the salt-sensitive check cultivar (Sakha 61) and Pavon 76. The minimum reduction was found for Drysdale, followed by the salt-tolerant check cultivar (Sakha 93), Yecora Rojo, and Kronos (Figure 3). For instance, the activity of α-amylase in germinating seeds of Orita was reduced by 45.5, 46.0, and 53.2% under 60 mM NaCl and by 56.2, 54.9, and 62.6% under 120 mM NaCl after 48, 72, and 96 h of seed sowing, respectively, compared with the control treatment. However, α-amylase activity reduced in germinating seeds of Drysdale by 4.5, 2.8, and 13.6% under 60 mM NaCl and 18.1, 19.8, and 22.6% under 120 mM NaCl after 48, 72, and 96 h of seed sowing, respectively, compared with the control treatment (Figure 4).

**Pearson correlation matrix**

The relationships among all the parameters associated with the germination ability were evaluated for the salinity treatments \((n = 21)\) (Table 2). Results of the correlation matrix showed that the water uptake rate had a strong positive \((r \text{ ranged from 0.82 to 0.99)}\) and negative \((r \text{ ranged from } -0.79 \text{ to } -0.97)\) correlation with α-amylase activity at different period of germinating seeds and germination time, respectively. However, there was an exception for the water uptake rate between 0 and 8 h for the two salinity treatments and between

![Figure 2](image-url)
8 and 48 h for the high salinity treatment, which showed no correlation with the other parameters. For the low salinity treatment, the α-amylase activity at 72 h after sowing and germination time also did not show a significant correlation with the water uptake rate between 8 and 48 h (Table 2).

Genetic diversity of germination ability parameters

Figure 5 shows the Hierarchical cluster based on germination ability parameters for the seven cultivars. The seven genotypes were grouped into three main clusters. The first cluster includes only a durum wheat cultivar (Orita). The second cluster includes four cultivars and consists of two subgroups. Yecora Rojo and Kronos were grouped together in first subgroup, while the salt-sensitive check cultivar Sakha 61 with Pavon 76 were grouped in the second subgroup. The third cluster includes the salt-tolerant check cultivar Sakha 93 with Drysdale.

Genetic diversity of SSR molecular markers

Out of 30 different SSR primers tested, only 24 SSR primer pairs generated polymorphisms among the seven wheat genotypes (Table 3). Cluster analysis, using SSR data of the 45 scored bands, grouped the seven genotypes into two main clusters with the similarity coefficient ranging from 0.13 to 0.75 (Figure 6). The highest genetic similarity (0.75) was found between Yecora Rojo and Drysdale, while the lowest (0.13) was found between Orita and the salt-tolerant check cultivar (Sakha 93) (Figure 6). The first cluster, supported by a bootstrap value of 80%, includes two cultivars (Pavon
The second cluster, supported by a bootstrap value of 77%, includes five cultivars and consists of two subgroups. The first subgroup, supported by a bootstrap value of 50%, includes four cultivars (Kronos, Sakha 93, Yecora Rojo, and Drysdale). The second subgroup included only the salt-sensitive check cultivar Sakha 61 (Figure 6).
Levels of genetic information generated by SSR primers

The 24 SSR primers covered the three genomes (A, B, and D) and detected 42 polymorphic alleles among the seven wheat cultivars (Table 3). The number of amplified alleles per primer ranged from one allele (Cfd 9) to four alleles (Wmc 405) with an average number of 1.89 alleles. The highest amplicon size belongs to Gwm 410 (500 bp) and the lowest to Barc 182 (110 bp). The polymorphic information content (PIC) varied greatly between SSR primers and their value

Table 3. Details of 24 genomic-SSR makers including their annealing temperature (TA), location on the wheat genome, amplicon size, number of alleles, number of polymorphic alleles detected, polymorphism information content (PIC), and discrimination power (Dp) for seven wheat cultivars

| Primers | Chromosome location | TA °C | Amplicon size range (bp) | Number of alleles | Polymorphic allele | PIC value | Dp |
|---------|---------------------|-------|--------------------------|-------------------|-------------------|-----------|----|
| 1       | Barc124 2A, 2D      | 52    | 250                      | 1                 | 1                 | 0         | 0.024 |
| 2       | Barc182 7B          | 58    | 110                      | 1                 | 0                 | 0         | 0    |
| 3       | Cfd1 6A,6B,6D       | 60    | 240–270                  | 2                 | 1                 | 0.51      | 0.024 |
| 4       | Cfd3 3D             | 60    | 210                      | 1                 | 1                 | 0         | 0.024 |
| 5       | Cfd9 5D             | 60    | 200                      | 1                 | 1                 | 0         | 0.024 |
| 6       | Cfd46 7D            | 60    | 210                      | 2                 | 2                 | 0.50      | 0.048 |
| 7       | Cfd49 6D            | 60    | 175–225                  | 3                 | 3                 | 0.25      | 0.071 |
| 8       | Cfd183 5D           | 60    | 200                      | 1                 | 1                 | 0         | 0.024 |
| 9       | Gwm174 5D           | 55    | 210                      | 1                 | 1                 | 0         | 0.024 |
| 10      | Gwm205 5A,5D        | 60    | 130–280                  | 3                 | 3                 | 0.41      | 0.071 |
| 11      | Gwm249 2A           | 55    | 140–200                  | 2                 | 2                 | 0.50      | 0.048 |
| 12      | Gwm291 5D           | 60    | 180                      | 1                 | 1                 | 0         | 0.024 |
| 13      | Gwm312 2A           | 60    | 199–220                  | 2                 | 2                 | 0.48      | 0.048 |
| 14      | Gwm350 4A,7A,7D     | 55    | 135                      | 1                 | 1                 | 0         | 0.024 |
| 15      | Gwm410 2B           | 55    | 290–500                  | 3                 | 3                 | 0.64      | 0.071 |
| 16      | Wmc11 3A            | 61    | 100–270                  | 3                 | 3                 | 0.33      | 0.048 |
| 17      | Wmc18 2D            | 61    | 160–280                  | 2                 | 2                 | 0.33      | 0.048 |
| 18      | Wmc154 2B           | 61    | 110–170                  | 2                 | 2                 | 0.44      | 0.048 |
| 19      | Wmc169 3A,5A        | 61    | 125–140                  | 2                 | 2                 | 0.38      | 0.048 |
| 20      | Wmc170 2A,2D        | 61    | 220                      | 1                 | 1                 | 0         | 0.024 |
| 21      | Wmc405 7A,5B,1D     | 61    | 120–245                  | 4                 | 4                 | 0.66      | 0.095 |
| 22      | Wmc432 1D           | 51    | 195–220                  | 2                 | 2                 | 0.48      | 0.048 |
| 23      | Wmc503 2D           | 61    | 180–330                  | 2                 | 2                 | 0.45      | 0.048 |
| 24      | Wmc661 2B           | 61    | 210–236                  | 2                 | 2                 | 0.48      | 0.048 |
| Total   | —                   | —     | —                        | 45                | 42                | 6.82      | 0.99 |
| Average | —                   | —     | —                        | 1.89              | 1.75              | 0.28      | 0.042 |
| Ranged  | —                   | —     | —                        | 1–4               | 0–4               | 0–0.66    | 0–0.095 |
| Maximum | —                   | —     | —                        | 4                 | 4                 | 0.66      | 0.095 |
| Minimum | —                   | —     | —                        | 1                 | 0                 | 0.0       | 0     |

Figure 5. Hierarchical cluster of seven wheat cultivars based on their germination ability parameters.
varied from 0.0 to 0.66. The lowest PIC values belonged to 9 SSR primers (Barc 124 and 182; Cfd 9, 18, and 183; Gwm 174, 291, and 350; and Wmc 170), while Wmc 405 showed the highest PIC value (0.66). The discrimination power (DP) ranged between 0.0 (Barc 182) and 0.095 (Wmc 405) (Table 3).

**Correlation between different germination ability parameters and genetic diversities**

The Mantel test showed significant correlation ($r = 0.49$, $P = 0.026$) between the matrix based on germination ability parameters and those derived from the molecular data using SSR markers. Thus, clustering of cultivars based on germination parameters was associated with those derived from the SSR data analysis.

Therefore, the data for the 24 SSR markers, which showed polymorphism, was used to identify the markers that are associated with the different parameters of germination ability (water uptake rate per hour over different periods after seed sowing, α-amylase activity at different periods of germination, and germination time) under 60 and 120 mM NaCl. The Cfd 9, Cfd 46, Cfd 49, and Wmc 305 SSR markers are associated with all germination ability parameters ($R^2$ ranged from 0.44 to 0.95), except for the water uptake rate between 48 and 72 h under both salinity treatments and between 72 and 96 h under the high salinity treatment (Table 4). The marker Gwm 312 was associated with α-amylase activity at 48 and 96 h under 60 mM NaCl and at 72 h under 120 mM NaCl ($R^2$ ranged from 0.44 to 0.57). Additionally, Gwm 312 was associated with the water uptake rate ($R^2$ ranged from 0.43 to 0.89), except for between 72 and 96 h under the two salinity treatments and between 8 and 48 h under the high salinity treatment (Table 4). Cfd 1 was associated with the germination time and water uptake rate between 120 and 144 h under both salinity treatments. Furthermore, Cfd 1 was associated with the α-amylase activity at 48 h and the water uptake rate between 48 and 72 h and between 96 and 120 h under 60 mM NaCl. Six of the SSR markers (Wmc 154, Wmc 169, Wmc 170, Wmc 432, Wmc 661, and Gwm 410) were significantly associated with the water uptake rate between 72 and 96 h under 60 mM NaCl ($R^2 = 0.42$) and between 8 and 48 h under 120 mM NaCl ($R^2 = 0.62$) (Table 4).

**Validation of wheat cultivars using Gwm 312 SSR markers**

Agarose gel electrophoresis of SSR fragments generated by primer Gwm 312 (as an example) for seven wheat cultivars is shown in Figure 7. The Gwm 312 marker was used to check the presence or absence of Nax1 alleles in the diploid accessions (Nax1, Na+ exclusion trait). This primer recorded a specific band at 199 bp for Drysdale, Sakha 93, Yecora Rojo, and Kronos, while it was absent in Sakha 61, Orita, and Pavon 76 (Figure 7).

**Discussion**

Francois et al. (1986) reported that both bread and durum wheat are more sensitive to salt stress during germination than after the three-leaf stage of growth. Therefore, if the evaluation of genotypes for salt tolerance has been done during the seed germination process, the tolerant genotypes can be identified at the early growth stage (Feyzi, 2003; Aflaki et al. 2017). The water uptake pattern and the degradation of the stocks of stored starch and proteins into soluble sugars to provide energy and nutrients for germinating seeds are considered the main sequential steps of the germination

**Figure 6. Dendrogram based on the similarity coefficient of seven wheat cultivars based on the allelic data of 24 SSRs linked to salt tolerant genes.**
process (Almansouri et al. 2001; Yang et al. 2007; Zhang et al. 2017). Thus, we hypothesized that the water uptake pattern and \( \alpha \)-amylase activity during seed germination may provide useful information about the mechanisms of salt tolerance in wheat at an early growth stage. Once the mechanisms behind salt tolerance are understood, the ability to assess the salt tolerance of a large number of wheat genotypes in a relatively short time will be conceivable.

The results of this study indicate that the triphasic pattern of water uptake is identical, as demonstrated with three different salinity levels, with no distinct differences among the cultivars in phase I under either the control or salinity treatments. The significant differences in water uptake among tested cultivars appeared at the late stage of phase II under the salinity treatments and during phase III under all treatments (Figure 1). The \( \alpha \)-amylase activity at 48, 72, and 96 h after seed sowing significantly decreased with increasing salinity levels. However, Sakha 93 (a salt-tolerant cultivar) and Drysdale (a drought-tolerant cultivar) showed a lower reduction in the \( \alpha \)-amylase activity compared with Sakha 61 (a salt-sensitive cultivar) and Orita (a durum wheat cultivar) under 60 and 120 mM NaCl (Figure 4). Furthermore, we found a strong positive correlation between the \( \alpha \)-amylase activity at the three times after seed sowing and the rate of water uptake per hour between 48 and 72 h, 72 and 96 h, 96 and 120 h, and 120 and 144 h under 60 mM NaCl (r ranged from 0.75 to 0.96) and 120 mM NaCl (r ranged from 0.83 to 0.96) (Table 2). The significant genotypic differences in the regulation of the water uptake rate, especially at the late stage of phase II and during phase III, as well as in the level of \( \alpha \)-amylase activity indicates that these criteria may play a significant role in salt-tolerant wheat cultivars during the germination process. Due to the first response of seeds in saline

Table 4. SSR markers which were significantly associated with the different germination ability parameters. GT indicates germination time.

| Par. Time | Primers | R² | Par. Time | Primers | R² |
|----------|---------|----|----------|---------|----|
| 48 h     | Cfd 9   | 0.72** | 8-48 h   | Cfd 9   | 0.62** |
|          | Cfd 46  | 0.72** |          | Cfd 46  | 0.62** |
|          | Cfd 49  | 0.72** |          | Cfd 49  | 0.62** |
|          | Wmc 503 | 0.62** |          | Wmc 503 | 0.62** |
|          | Gwm 312 | 0.57*  |          | Gwm 312 | 0.89*** |
|          | 312     |       |          | 312     |       |
| 72 h     | Cfd 9   | 0.71** | 48-72 h  | Cfd 1   | 0.56*  |
|          | Cfd 46  | 0.71** |          | Cfd 46  | 0.64** |
|          | Cfd 49  | 0.71** |          | Cfd 49  | 0.43*  |
|          | Wmc 71**| 0.71** |          | Gwm 312 | 0.95*** |
|          | 405     |       |          | 405     |       |
| 96 h     | Cfd 9   | 0.56*  | 72-96 h  | Cfd 46  | 0.95*** |
|          | Cfd 46  | 0.56*  |          | Gwm 312 | 0.42*  |
|          | Cfd 49  | 0.56*  |          | Wmc 170 | 0.42*  |
|          | Wmc 503 | 0.56*  |          | Wmc 169 | 0.42*  |
|          | Gwm 170 | 0.44*  |          | Gwm 410 | 0.73** |
|          | 312     |       |          | 312     |       |
| 48 h     | Cfd 9   | 0.64** | 96-120 h | Cfd 9   | 0.53*  |
|          | Cfd 46  | 0.64** |          | Cfd 49  | 0.53*  |
|          | Cfd 49  | 0.64** |          | Wmc 503 | 0.53*  |
|          | Wmc 1   | 0.66** |          | Wmc 46  | 0.53*  |
|          | Cfd 46  | 0.71** |          | Cfd 49  | 0.53*  |
|          | Cfd 49  | 0.71** |          | Wmc 312 | 0.60** |
|          | Wmc 503 | 0.52*  | 120-144 h| Cfd 9   | 0.46*  |
|          | Gwm 312 | 0.52*  |          | Cfd 9   | 0.52*  |
|          | 312     |       |          | Cfd 1   | 0.52*  |
| 96 h     | Cfd 9   | 0.44*  | 120-144 h| Cfd 46  | 0.52*  |
|          | Cfd 46  | 0.44*  |          | Cfd 49  | 0.52*  |
|          | Cfd 49  | 0.44*  |          | Wmc 503 | 0.52*  |
|          | Wmc 1   | 0.44*  |          | Wmc 46  | 0.52*  |
|          | Cfd 46  | 0.44*  |          | Cfd 49  | 0.52*  |
|          | Cfd 49  | 0.44*  |          | Wmc 503 | 0.52*  |
|          | Gwm 312 | 0.46*  |          | Gwm 18  | 0.46*  |
|          | 410     |       |          | 410     |       |

***, **, * indicates significance at 0.001, 0.01 and 0.05 P levels, respectively.

Figure 7. Agarose gel electrophoresis of SSR fragments generated by the primer Gwm 312 of seven wheat cultivars.

The results of this study indicate that the triphasic pattern of water uptake is identical, as demonstrated with three different salinity levels, with no distinct differences among the cultivars in phase I under either the control or salinity treatments. The significant differences in water uptake among tested cultivars appeared at the late stage of phase II under the salinity treatments and during phase III under all treatments (Figure 1). The \( \alpha \)-amylase activity at 48, 72, and 96 h after seed sowing significantly decreased with increasing salinity levels. However, Sakha 93 (a salt-tolerant cultivar) and Drysdale (a drought-tolerant cultivar) showed a lower reduction in the \( \alpha \)-amylase activity compared with Sakha 61 (a salt-sensitive cultivar) and Orita (a durum wheat cultivar) under 60 and 120 mM NaCl (Figure 4). Furthermore, we found a strong positive correlation between the \( \alpha \)-amylase activity at the three times after seed sowing and the rate of water uptake per hour between 48 and 72 h, 72 and 96 h, 96 and 120 h, and 120 and 144 h under 60 mM NaCl (r ranged from 0.75 to 0.96) and 120 mM NaCl (r ranged from 0.83 to 0.96) (Table 2). The significant genotypic differences in the regulation of the water uptake rate, especially at the late stage of phase II and during phase III, as well as in the level of \( \alpha \)-amylase activity indicates that these criteria may play a significant role in salt-tolerant wheat cultivars during the germination process. Due to the first response of seeds in saline

Figure 7. Agarose gel electrophoresis of SSR fragments generated by the primer Gwm 312 of seven wheat cultivars.
soils being osmotic stress, which can be of sufficient magnitude to disrupt the water uptake by seeds, α-amylase needs to break down reserve starches into sugars to regulate the water uptake by seeds. Increasing the soluble sugar concentration is useful to attempt to osmotically adjust the germinating tissues. A higher soluble sugar concentration also reduces the loss of turgidity for these tissues, this helps seeds to take water up from saline soils rapidly and sustain the energy required during the germination process (Almansouri et al., 2001; Ashraf et al., 2002; Singh, 2004; Dkhil and Denden, 2010).

Several studies have reported that the mobilization of stored starch and proteins on a large-scale mainly occurred during the late stage of phase II (Ostergaard et al., 2004; Yang et al., 2007; Liu et al., 2015, 2016). In addition, Yang et al. (2007) found that the expression of some defense proteins such as the saltTgene product (111) also increased during phase II to protect the germinating seeds from possible stresses. This may explain why the genotypic differences and increase in seed fresh weight were significant at the late stage of phase II and during phase III under the salinity treatments (Figure 1). Consequently, it seems that the salt tolerance of wheat genotypes during the germination process is maintained through the regulation of water uptake and activity of α-amylase, which in turn stimulates the hydrolysis, degradation, and mobilization of seed storage reserves to provide energy for germination and for active growth of the radicle and plumule after germination.

The genotypic differences in the germination time (Figure 3), as well as the strong negative correlation of germination time with the water uptake rate and α-amylase activity under the salinity treatments (Table 2), confirmed that the regulation of water uptake and activity of α-amylase may play a key role in the salt tolerance mechanisms of wheat genotypes by helping the seed to germinate faster. Although there were significant genotypic differences in the germination time under the salinity treatments, the final germination percentage reached 100% for all cultivars under the salinity treatments (Figure 3). This result implies that the increase in the germination time without a decrease in the germination percentage in salt-sensitive wheat cultivars, such as Sakha 61 and Orita, under salinity stress (Figure 3) may be caused by the germinating seeds allowing Na+ and Cl⁻ to enter the cells and using them as an osmoticum to facilitate water uptake, thus allowing germination. Seeds may have the ability to avoid the toxic effect of Na⁺ within the germinating tissues by binding it to starch granules (Kanai et al., 2007; Zhang et al., 2010). This binding would inhibit the rate of starch degradation by α-amylase and thus decrease the energy required for dividing and expanding the cells of germinating tissues. However, the exclusion of toxic ions from the cells would increase the rate of starch remobilization by preventing the negative effects of toxic ions on the α-amylase activity, which enables the seeds to uptake water rapidly and germinate faster. These results imply that a delay in germination could be a more useful measure for evaluating the salt tolerance of wheat genotypes than the germination percentage at an early growth stage.

Clustering pattern of seven cultivars based on all germination ability parameters was associated with those derived from SSR analysis according to the test of Mantel (r = 0.49, P = 0.026). In addition, cluster analysis based on germination ability parameters or SSR data revealed grouping of the salt-tolerant check cultivar Sakha 93 and salt-sensitive check cultivar Sakha 61 largely in separate main cluster or sub-clusters. This result indicates that the both germination ability parameters and SSR analysis of DNA polymorphism successfully assessment of salt tolerance of tested cultivars at the germination stage. Similarly, there are some reports which have revealed that clustering of genotypes based on different agromonic, morphological and/or physiological parameters provide a remarkable association with the analysis based on SSR marker data (Taamalli et al., 2006; D’Imperio et al., 2011; Ahmad et al., 2013; Amombo et al., 2018; Tahjibi-Ul-Arif et al., 2018). However, other studies have reported that clustering of genotypes based on phenotypic parameters is not associated with the DNA analysis and the molecular diversity provides a remarkably higher genetic variation among genotypes than the phenotypic parameters (Beyene et al., 2005; Tahernezhad et al., 2010; Al-faiî et al., 2013; Ali et al., 2014). The reason for these differences is probably the different numbers of genotypes tested as well as the geographical regions from which these genotypes originated, as this could play an important role in swapping genotypes from one cluster to another among different evaluation methods.

A molecular marker-phenotypic trait association proved to be a useful tool for the selection and breeding of salt-tolerant genotypes in future crop breeding programs. Here, we evaluated 24 SSR primers and studied their ability to discriminate between the salt tolerance of cultivars through identifying allele markers that are associated with each parameter of germination ability. In this study, a total of 45 alleles were detected with an average of 1.89 alleles and a PIC value of 0.28 per primer (Table 3). Furthermore, the genotypic similarity coefficients for SSR primers ranged from 0.13 to 0.75 between the tested cultivars (Figure 6). The average values of alleles and PIC per primer, in this study, are comparable with those obtained in wheat genotypes with different levels of salt tolerance using different SSR primers by Almanza-Pinzon et al. (2003), Singh et al. (2006), Ahmad et al. (2013), and Singh et al. (2018). These results indicate that the tested primers are highly informative and capable of discriminating between the levels of salt tolerance between cultivars.

Interestingly, Cdf 9, Cdf 46, Cdf 49, and Wmc 503 primers, with an average of alleles and PIC of 2.0 and 0.30 per primer, respectively, were associated with all germination abilities under 60 and 120 mM NaCl (R² ranged from 0.44 to 0.95; Table 4), with a few exceptions. This indicates that the parameters for the regulation of water uptake rate and α-amylase activity at different times after seed sowing, as well as the germination time under salinity stress, could be considered as important indicators for characterizing the genetic diversity of salt tolerance in wheat. This could provide sufficient information to understand the salt tolerance of wheat genotypes at an early growth stage. Furthermore, the Gwm 312 primer, which was reported by Byrt (2008) as an indicator for Na⁺ exclusion (Nax1), was significantly correlated with the α-amylase activity and water uptake rate between different times after seed sowing under both salinity levels (R² ranged from 0.43 to 0.89; Table 4). The specific band of this primer (199 bp) was absent in salt-sensitive cultivars and present in salt-tolerant cultivars (Figure 7). These results confirm that the exclusion of toxic ions (Na⁺ and/or Cl⁻) from the cells of germinating seeds, could be the causal
factor in avoiding the negative effects of these ions on the activity of enzymes related to the degradation of starch into sugars. These soluble sugars are not only necessary as the main substrate to generate the energy required for developing the radicle and plunule but are also required to generate additional osmotic potential in germinating tissues which enables the seeds to uptaked water rapidly and to germinate faster (Ashraf et al. 2002; Zhang et al. 2017; Liu et al. 2018). Allowing the toxic ions to enter the cells of seeds and binding them to starch would decrease the nutrients and energy required for the maintenance of the germination process. Thus, as the exclusion of toxic ions is considered an important physiological screening criterion for evaluating the salt tolerance in many crops at different vegetative growth stages (El-Hendawy et al. 2017; Amombo et al. 2018), it could also be considered an important mechanism during the germination stage to avoid the inhibition of α-amylase activity and regulate water uptake through the accumulation of organic solutes.

Conclusion

The study verified that the delaying of the germination time under salt stress was not the result of a reduction in water uptake, which was confirmed by the germination percentage reaching 100% even under high salinity levels. But instead, it is related to the water uptake regulation and α-amylase activity during the seed germination process. Therefore, both parameters can be considered as effective screening criteria for evaluating the salt tolerance of wheat genotypes in a relatively short period of their life cycle. Significant associations between cluster based on germination ability parameters and those derived from SSR markers according to Mantel analysis confirm the effectiveness of these parameters as screening criteria in the early stages of plant establishment as well as the potential utility of these markers in mapping of salinity associated germination ability parameters. Moreover, a significant association between the Gwm 312 primer, which is an indicator for Na+ exclusion and present in salt-tolerant check cultivar Sakha 93 and absent in salt-sensitive check cultivar Sakha 61, and germination ability parameters indicates that the regulation of the uptake of toxic ions during the seed germination process can aid in understanding why the water uptake regulation and α-amylase activity play important roles in the mechanisms of salt tolerance during the germination stage.

Acknowledgments

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through Research Group No. (RG-1440-024), and the Researchers Support & Services Unit (RSSU) for their technical support.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Deanship of Scientific Research at King Saud University through Research Group No. [grant number RG-1440-024].

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ORCID

Salah El-Hendawy http://orcid.org/0000-0002-8104-3410
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