Elucidating the genetic architecture controlling antioxidant status in barley

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Abstract

A core set of 138 diverse barley accessions were exposed to salt stress under field conditions at the reproductive phases, and then a genome-wide association scan (GWAS) was conducted using 19,276 single nucleotide polymorphisms (SNPs) to uncover the genetic basis of morpho-physiological and agronomic traits. A wide range of responses among the accessions to the salt stress was explored in the current study. GWAS detected 299 significantly associated SNPs with the antioxidants, K^+/Na^+ ratio, and agronomic traits. Five genomic regions harbor interesting putative candidate genes within LD ±1.2 Mbp. Exclusively, 2H harbors many candidate genes associated with antioxidant contents such as SOD, APX, GR, AsA, and GSH under salt stress. Markedly, on chromosome 7H at 153773211 bp, A:C SNP is physically located inside the gene *HORVU.MOREX.r3.7HG0676830.1* (153,772,300-153,774,057 bp) that annotated as L-gulonolactone oxidase, regulating the natural variation of SOD_S and APX_S. The allelic variation at this SNP reveals a negative selection of accessions carrying C allele which are predominantly landraces originated from Far-, Near-East carrying photoperiod sensitive alleles having lower activity of enzymatic antioxidants. The SNP-trait associations detected in the current study constitute a benchmark for developing molecular selection tools for antioxidant compound selection in barley.

Introduction

Soil salinity condition is one of the most serious environmental factors which decrease crop performance and productivity, especially under arid and semi-arid conditions. It was found that 6% of the world's soil and 20% of agricultural lands are already affected by salinity 1. According to recent reports, salinity stress costs the agricultural sector over $27.3 billion in lost revenue per year, exacerbating the food security crisis. More than 0.8 billion hectares of arable land worldwide are salt-affected 2. Parallelly, the world's human population is expected to cross 9.3 billion by 2050 1. Based on that evidence, developing salinity-tolerant crop plants is an important challenge in order to meet the expected food demand.

Soil salinization significantly decreases soil water retention and mineral absorption, resulting in salt potential, oxidative stress, nutrient deficits, and water insufficiency. Further, high sodium ion concentrations damage photosynthetic leaves, causing chlorosis and premature leaf senescence 3. Panta, et al. 4 reported that glycophytic plant crops considerably reduce average yields by 50–80% under moderate salt stress (EC 4-8 dS m^{-1}). Plant morphology, physiology, and metabolic activity are all altered as a result of salt stress 5. Therefore, understanding plant physiological and biochemical pathways is critical in breeding new barley genotypes that can grow under salt stress. Due to low sodium ions (Na^+) sequestration into vacuoles, salinity stress conditions cause severe cellular toxicity resulting in premature senescence of older leaves and toxicity symptoms (chlorosis, necrosis) in mature leaves 6,7. Also, sodium ions can disturb the integral potassium ion (K^+) in proteins, interfering with the activity of an enzyme, cellular protein, and transcription factors 8,9. Hence, the tissue K^+/Na^+ ratio is often considered as a basis of differential salt tolerance in various plant species 10. Moreover, Na^+ could be prevented from entering the cytosol by controlling Na^+ influx and/or efflux from the cytoplasm into the vacuoles. Hence, plants' ability to sustain a high K^+/Na^+ ratio is among the critical factors in response to salt stress 11. Soil salinity increases the generation of active oxygen radicles including hydrogen peroxide (H_2O_2), which could injure or kill the plant 12.

In response to external stimuli, molecular oxygen—O_2 acts as an electron acceptor, leading to the production of different types of reactive oxygen species (ROS) molecules. Singlet oxygen—$^1O_2$, hydroxyl radical—OH^-, superoxide—O_2^-, and hydrogen peroxide—H_2O_2, all seem to be highly oxidizing molecules that caused chlorophyll degradation, proteins injury, and lipids of the membrane and nucleic acids, resulting in toxicity. Therefore, to protect these

components, plants turn on the synthesis of several metabolites and proteins with a protective function 13. Many ROS detoxifying proteins, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and antioxidants, like glutathione (GSH), ascorbic acid (AsA), tocopherols, and phenolic compounds which are present in almost all subcellular compartments, play a key role to vanquish the deleterious effects of ROS 14.

Due to the high genetic diversity in terms of stress tolerance, barley is considered to be one of the most saline-tolerant important crops, and it is frequently used as a model to understand salinity adaptive responses in cereals 15,16. This adaptability to a variety of environments and abiotic stress conditions differs significantly among barley genotypes and growth developmental stages 17.

Genome-wide association studies (GWAS) are intensely used to discover and elucidate the genetic basis of complex traits that are frequently controlled by a large number of genes 18. GWAS is used to detect associations between a large number of genetic variants and traits across a large number of genotypes from natural populations based on linkage disequilibrium (LD). GWAS generally achieves higher mapping resolution than conventional QTL mapping due to the higher recombination levels among linked marker loci and the traits of interest 19. Moreover, GWAS could accurately locate polymorphisms and the underlying genetic loci responsible for phenotypic variations, allowing gene-targeted searches 18. Up to date, GWAS has been widely applied in trait-associated genetic studies in barley, for instance, single nucleotide polymorphisms (SNPs) had a significant associations with antioxidant components at the vegetative stage under stress conditions 20-22. Although GWAS has identified a set of allelic variations in controlling plant salt tolerance, there is still a lack of applicable and sufficient salt-tolerant loci or genes in crop breeding, which may be due to a loss of elite germplasm resources or optimal conditions.

Our study detected genomic regions/ putative candidate genes for a variety of morphological, physiol-biochemical and yield-related traits in response to salt stress using a core set collection of 138 worldwide spring barley accessions, genotyped using genotyping-by-sequencing (GBS). Our results discover the natural variation in response to salt stress in barley plants and the most promising genomic regions. The findings will not only provide an extensive insight into the potential salinity tolerance mechanisms that underlie salt tolerance in barley during the reproductive stages, but they would as well be used for the breeding program to develop barley genotypes with improved salinity tolerance.

Results

Molecular markers, population structure, and linkage disequilibrium (LD)

The core collection of barley accessions belongs to ten geographical regions **(Figure 1)**. There are two main clusters among the accessions with biological status and row-type whereas most of the accessions were 2-rowed and landraces were 6-rowed (**Figure** 2a and b). The accessions of the collection were originally from different geographical origins and could see a trend among the accessions that most of Northern Europe, Northern America, and South America were clustered around the center while the accessions from other origins were distributed (**Figure** 2c). These results showed a wide diversity among the landraces coming from central Asia, the Near and the Far East, North Africa, and Ethiopia compared to accessions mostly coming from Europe and the Americas (**Figure** 2).

All of the barley accessions were genotyped using GBS, which yielded 19,276 SNPs as described by Milner, et al. 23. Information on the number of markers for each chromosome, with the map length and marker density for each chromosome, are presented in **Figure** S1. The largest number of markers were detected on chromosome 2H by 3,373

SNPs, while the highest marker density was detected on chromosome 7H. Only 2,193 markers were noted on chromosome 1H with the lowest marker density among chromosomes. For the whole barley genome, the overall average values of r^2 decreased as the distance between SNPs increased as well as the average LD decay distance was approximately 2.4 Mbp (**Figure** S2).

Natural variation of agronomic trait performance

Barley accessions showed excessive significant phenotypic variation for all morphological and yield-related traits with normal distribution under both treatment conditions **(Figure S3)**. The minimum, maximum, and mean of each trait for all genotypes are presented in **Table S2**. All yield traits (SL, NSS, NGS, WGS, and TKW) had a lower mean performance under salinity stress than under control conditions as expected (**Table S2 and Figure S8)**. On average, all yield traits had a reduction due to salt stress ranging from 15% (NGS) to 48% (WGS). Based on salt-tolerant indices (STI), genotypic mean values for all yield traits ranged from 52% (WGS) to 85% (NGS) **(Table S2).** The broad-sense heritability (H^2) values were ranged from 99.38 for NGS_C to 96.32 for NSS_C. In response to salt stress, the highest H^2 was detected for NGS (99.3) followed by SL (97.91) and WGS (97.06). Moreover, H^2 was calculated and ranged from 53.64 (TKW_STI) to 81.26 (SL_STI) **(Table S2)**.

Natural variation of enzymatic antioxidants

Significant natural variation was also detected for all enzymatic antioxidant activities with normal distribution in response to salinity conditions **(Figure S4)**. Under control conditions, the genotypic mean values for SOD, CAT, APX, and GR were ranged from 2.29 (SOD) to 1.10 (APX). The mean values were ranged from 5.60 (SOD) to 2.37 (APX) under salt treatment. A significant increment in SOD, CAT, APX, and GR was observed under salt stress (58%, 48%, 50%, and 65%), respectively, compared to the control condition **(Table S4 and Figure S9).** For STI, genotypic mean values ranged from 252.18. (APX) to 1986.54 (CAT). The highest value was detected for CAT_STI by 53666.67. The highest values of H^2 were observed for CAT_S by 99.74 and for GR _C by 97.93. H^2 values ranged from 77.96 (SOD_STI) to 99.74 (CAT_STI) as shown in **Table S3**.

Natural variation of proline contents, soluble protein, and non-enzymatic antioxidants

Natural variation was detected for all non-enzymatic antioxidants and compatible solutes which expressed normal distribution under salt stress (**Figure S5 and Figure S6).** Proline content (PC) accumulation remarkably increased by 47% under salt treatment (**Figure S10a)**, compared to the control condition **(Table S4)**. Soluble protein (SP) significantly decreased under salt stress by 57% when compared to the control **(Figure S10b)**. The mean salt tolerance indices for SPC and PC were 43 and 190, respectively. A significant increase in AsA, GSH, TPC, TFC, and TAC was detected under salt treatment by 65%, 49%, 32%, 66%, and 72%, respectively, in comparison to the control across over genotypes **(Table S4).** Genotypic mean values for such traits STI ranged from 154.72 (TPC) to 542 (AsA) **(Table S4).** H^2 values were ranged from 97.94 for AsA_C to 61.78 for TAC_C. Under salt treatment, the highest H^2 was detected for AsA (95.27) followed by TFC (89.13) and SP (76.3). H^2 values for STI were low for PC_STI (34.70) and reached 95.27 in AsA_STI

Natural variation of ionic Na^+ and K^+

Na^+ content showed a significant increment by 87% under salt treatment compared to the control treatment **(Table S5)**. In contrast, K^+ accumulation was decreased by 67% under salt treatment in comparison to the control across over accessions **(Table S5)**. Moreover, the K^+/ Na^+ ratio was significantly increased under salt stress by 27% when

compared to the control treatment **(Table S5)**. For STI, mean values were 768.51, 32.64, and 4.25 for Na^+, K^+, and the K^+/ Na^+ ratio, respectively. Additionally, high H^2 values were detected which ranged from 84.06 (K^+/ Na^+) to 90.87 (Na^+) **(Table S5).**

Correlations analyses

Pearson's correlations among traits based on the mean of all accessions under each treatment condition reveal a significant correlation was observed amongst all studied traits under both treatments. SOD showed positive significant correlation at p-value 0.0001 **** with CAT, APX, and GR (r = 0.55***, 0.99***, 0.86***), respectively. Similarly, a positive significant correlation was noted between SOD and all agronomic traits except NGS and WGS. In contrast, there was a negative correlation between SOD activity and non-enzymatic antioxidants such as AsA, TPC, TFC, TAC, and compatible solutes such as SP and PC. Moreover, APX showed positive correlations with SOD, CAT, GR, and TKW (r = 0.99***, 0.54***, 0.85***, 0.26), respectively **(Figure 3a)**.

Under salinity conditions, SOD activity detected positive associations with all physiological and antioxidant systems where the highest significant associations were detected for APX and GR (0.87***, 0.75***), respectively. Contrarily, SOD was negatively correlated with all agronomic traits except for NGS and WGS. Interestingly, high significant positive correlations were detected between APX activity with SOD, CAT, GR, AsA and GSH (r = 0.87***, 0.75***, 0.86***, 0.70***, 0.70***), respectively. Like SOD, APX was negatively correlated with all agronomic traits except for NGS and WGS **(Figure 3b)**.

Genetic associations and candidate genes underlying the studied traits

GWAS identified a total of 299 SNPs as significantly associated with 19 traits and passing thresholds of ($-Log_{10}$ (p) ≥ 4.0) and with R^2 ≥ 10% under both control and salinity conditions **(Table S6)**. Out of the 299 SNPs, we identified 163 SNPs ($-log_{10}$ p-value ≥ 4) that had highly significant associations with enzymatic and non-enzymatic antioxidants under both stress and non-stress conditions **(Table S6)**. For example, we observed sixteen, fifteen, fourteen, eleven, and ten SNPs associated respectively with APX_STI, CAT_STI, AsA_C, CAT_S, and SOD_S, whereas less than ten SNPs were associated with each of the remaining traits**.** Moreover, thirty-seven SNP markers were significantly associated with Na^+, K^+, and K^+/Na^+ ratio where the highest number of was detected for K^+/Na^+_S (12 SNPs), followed by K^+/Na^+_C (9 SNPs), whereas less than nine SNPs were associated with each of the remaining traits under both control and salinity treatments. Compatible solutes such as soluble protein (SP) and proline content (PC) showed 13 significant SNP markers present on chromosomes 1H, 2H, and 3H. For instance, we detected eight, four, and one SNPs highly associated, respectively, with SP_STI, SP_C, and PC_STI with ($-log_{10}$ p-value ≥ 4)**.**

For all agronomic traits, 86 SNPs ($-log_{10}$ p-value ≥ 4) were significantly detected on all chromosomes **(Table S7)**. The highest number of markers was detected for SL_S (23 SNPs), followed by NGS_C and SL_C (11 SNPs each), whereas ten SNPs were associated with NGS_S and eight SNPs for TKW_C.

Interestingly, highly significant associations were discovered on chromosomes which were found to be highly associated with the antioxidant system, K^+/Na^+ ratio, and some agronomic traits **(Table S8 and Table S9).** In this investigation, we identified highly significant SNPs associated with enzymatic and non-enzymatic antioxidants present on all chromosomes. For instance, we observed five, seven, three, and one SNPs highly associated respectively with CAT_S, CAT_STI, APX_STI, and TFC_STI with a $-log_{10}$ (p-value) > 6 (i.e. p-value $<10^{-6}$). For K^+/Na^+_C, four highly significant associations were detected on chromosomes 2H, 5H, and 7H. Moreover, we identified highly

significant SNPs associated with some agronomic traits distributed on all over chromosomes. For instance, we observed four, five, three, and four SNPs highly associated with NGS_S, NGS_C, TKW_S, and TKW_C, respectively.

This study revealed five genomic regions based on marker-trait associations (MTAs), which harbors 193 potential candidate genes which are distributed on chromosomes 1H, 2H, 4H, 6H, and 7H **(Table S10)**. Of these, 23 potential candidate genes were found to control all enzymatic and non-enzymatic antioxidant components under salt stress **(Table 1)**.

On chromosome 1H, two candidate genes namely Pentatricopeptide repeat (PPR) superfamily protein that control the variation of Na^+_C, K^+_C, Na^+_STI, TFC_C, and TFC_STI, at position (669,733,8-669,734,9 bp) and FBD-associated F-box protein that control the variation of APX_STI and SOD_STI at position (500,0276,4-512,105,27 bp). Exclusively, chromosome 2H harbors several candidate genes that are found to be associated with most enzymatic and non-enzymatic antioxidant components such as SOD, APX, GR, AsA, and GSH under salt stress **(Figure 4)**. The most prominent candidate gene is *HORVU.MOREX.r3.2HG0181470.1* at position (557,725,076-557,730,772 bp) annotated as Beta-glucosidase, putative. The third genomic region is located on chromosome 4H,chr4H:13826536:A:G SNP (138,265,36 bp) and chr4H:569747682:G:A SNP (569,747,682 bp) inside the *HORVU.MOREX.r3.4HG0336230.1* (138,232,77-138,249,82 bp) annotated as Glycosyltransferase for SL_STI, and gene *HORVU.MOREX.r3.4HG0405320.1* (569,744,191-569,748,338 bp) annotated 3-ketoacyl-CoA synthase that control the variation of K^+/Na^+_S, K^+/Na^+_STI, and K^+_STI **(Figure 5, Table S8 and S9)**. Interestingly, the fourth genomic region was detected on 6H, chr6H:451682318:A:T SNP near the gene *HORVU.MOREX.r3.6HG0605480.1* (451,676,227- 451,677,811 bp) annotated as PLATZ transcription factor, regulating the variation of CAT_S, APX_S, GR_S, AsA_S, and GSH_S related to antioxidant components **(Figure 4)**. Ultimately, the the genomic region at 7H was found to be associated with SOD_S and APX_S at 153,773,211 bp chr7H:153773211:A:C SNP inside the gene *HORVU.MOREX.r3.7HG0676830.1* (153,772,300- 153,774,057 bp) that encodes L-gulonolactone oxidase, regulating the variation of SOD_S and APX_S related to enzymatic antioxidant components. The allelic variation at chr7H:153773211:A:C SNP inside the L-gulonolactone oxidase gene demonstrates a negative selection of accessions carrying C allele. This allele appears in a cultivar with lower activity of enzymatic antioxidants e.g. superoxide dismutase and ascorbate peroxidase under salinity conditions. **(Figure 6)**.

Discussion

Salinity, in the form of NaCl, is considered to be the most serious abiotic stress worldwide as it severely constrains cereal crop yield and production. Nevertheless, the response of different plant species and accessions to salt stress may vary significantly because its genotype- and concentration-dependent. Therefore, we screened new diverse barley accessions for morphological, physiological, and antioxidant responses to salt stress in order to detect the major physiological, biochemical, and antioxidative genetic factors and mechanisms. In our study, significant variations were observed in a set core of barley accessions for all of the studied traits under salinity conditions. The high level of variation found in this study would be used to enhance barley salt tolerance in breeding programs. Moreover, higher heritability values were detected for enzymatic and non-enzymatic antioxidants under salinity conditions. Similarly, Thabet, et al. 21 detected higher heritability values for antioxidants components under salinity stress during the vegetative developmental phase. Under salinity treatment, all morphological traits showed a significant reduction when compared to the control, suggesting that salt stress might cause a decline in the plant growth and performance of the whole studied panel. Thabet, et al. 21 also detected a significant reduction of all agronomic traits including SL, NSS, NGS, WGS, and TKW.

Maintaining the osmotic adaptation in response to salinity stress, compatible organic solutes such as soluble sugars, protein, and proline contents were accumulated in plant cells and helped plants to tolerate stress. Proline accumulated due to salinity plays an important role in quenching the deleterious effects of reactive oxygen species (ROS) molecules, maintaining the membrane integrity, and adjusting osmosis 24. Our study detected a significant increase in proline contents (PC) for all barley accessions in response to salinity stress compared to the control condition. It showed that tolerant genotypes produced more ROS, which was detoxified by an increase in proline content. In contrast, soluble protein content (SP) was significantly decreased under salinity conditions in comparison to control. Our results are in agreement with Doganlar, et al. 25, who demonstrated a considerable decline in total protein content as a result of specific stress protein synthesis produced under salinity stress. Soluble protein content decreased as salinity increased, owing to a disruption in the protein synthesis mechanism or increased proteolytic activity, which eventually induced programmed cell death 26. Therefore, plants may accumulate small molecular mass proteins in response to salt stress, which may be a source of storage nitrogen and may also involve in osmotic adjustment 27,28. Similarly, an increment in the content of proline and a decline in the content of protein were also detected under salinity stress 29.

Non-enzymatic antioxidant componentssuch as : AsA, GSH, TAC, TPC, and TFC are playing a principal role to reduce oxidative damage as well as regulate the cellular ROS homeostasis in different plant species 30. Our investigation detected that the contents of AsA, GSH, TAC, TPC, and TFC were higher under salt treatment in comparison to the control treatment. It is indicated that, those non-enzymatic antioxidants played critical roles to reduce oxidative damage as well as regulate the cellular ROS homeostasis in response to salinity conditions. Moreover, a significant increase in ascorbic acid contents signified the major contribution of the ascorbate–glutathione cycle in quenching the ROS molecules. Taken together, Hernández, et al. 31 reported that the ascorbate–glutathione cycle activity may be higher under salt stress in the studied panel. Under salinity stress, tolerant plants have developed an enhanced enzymatic antioxidant defense system to combat the outcomes of ROS molecules. All barley accessions detected significantly increased SOD, CAT, APX, and GR activities under salt treatment. Our results are in agreement with Thabet, et al. 21 who observed high enzymatic antioxidant activities in response to salinity conditions. SOD contributed to the dismutation of $O_2^{\bullet-}$ to O_2 and finally to H_2O_2 after that, a variety of peroxidases used various reducing agents to decompose H_2O_2 into H_2O 32. In parallel, CAT decomposes H_2O_2 produced in the peroxisome as a result of photorespiration 33. Altogether, it is evident that the higher activities of POD, CAT, and APX cope with the activity of SOD to counteract the negative effects of $O_2^{\bullet-}$ and H_2O_2 molecules, indicating that the activities of these enzymes showed a strong correlation with salinity stress tolerance in wheat and barley 34,35.

Interestingly, the higher GR activity might be elevated in the concentrations of $NADP^+$ to gain electrons from the photosynthetic electron transport chain via reduction of ROS 36. Altogether, crop barley genotypes may exhibit a more active AsA-GSH cycle under salinity conditions. Therefore, crop plants may induce high levels of enzymatic and non-enzymatic antioxidants that increased tolerance to salt-induced oxidative damage 14.

In this study, all barley accessions showed a significant increase in Na^+ accumulation and a significant decrease in K^+ accumulation. Hence, all barley genotypes can maintain a high solute concentration while also absorbing too much water and, as a result, adjusting osmosis. The detrimental effects of Na^+ are associated with the Na^+ accumulation in leaf tissues and results in necrosis of mature leaves, and reducing the photosynthetic capacity to a level that cannot sustain growth 3. High Na^+ ion concentrations may inhibit nutrient uptake because it interferes with K^+-selective ion channels in the root plasma membrane 37. Due to the K^+ requirement for tRNA binding to ribosomes, osmotic damage and a decrease in K^+ ion intake can disrupt protein synthesis 38. It was postulated that a higher

ionic K^+/Na^+ ratio plays a key role in plant tolerance to salinity stress 10. Furthermore, Na^+ should be prevented from reaching the cytosol by restricting Na^+ influx and/or efflux from the cytoplasm into the vacuoles. Hence, the ability to sustain a high K^+/Na^+ ratio is one of the critical factors in plant responses to salinity stress 11.

GWAS identified five genomic regions harboring significant association SNPs with 19 traits, many of which were clustered into. These genomic regions harbor 193 candidate genes and are distributed on chromosomes 1H, 2H, 4H, 6H, and 7H. Markedly, 23 potential candidate genes were found to be potentially involved in controlling all enzymatic and non-enzymatic antioxidant components under salt stress.

On chromosome 1H, two candidate genes control the variation of Na^+_C, K^+_C, Na^+_STI, TFC_C, and TFC_STI, and FBD-associated F-box protein that control the variation of APX_STI and SOD_STI. The first gene *HORVU.MOREX.r3.1HG0003310.1* at position (678,704,1- 678,880,8 bp) encodes pentatricopeptide repeat (PPR) superfamily protein that known to have a wide range of physiological and developmental processes, such as photosynthesis, respiration, seed development, and embryogenesis 39,40. In Arabidopsis, PPR40 had an important role in salinity tolerance 40. Mitochondrial mutants ppr40 showed hypersensitivity towards these adverse conditions during early growth stages 41. Also, the authors reported that upregulated expression of mitochondrial PPR40 gene conferred salinity tolerance in Arabidopsis through enhancing the mitochondrial electron transport's stability and reducing oxidative damage that could also be the role of our candidate gene.

The second gene *HORVU.MOREX.r3.1HG0016850.1* encodes F-box family protein at position (505,936,57- 505,957,00 bp) which is recognized by a conserved 40-50-amino acid F-box motif as well as reported to play critical role in abiotic stress tolerance through the ubiquitin pathway 42,43. Zhou, et al. 44 suggested that *TaFBA1* gene is involved in oxidative stress tolerance in wheat. In transgenic plants, enhanced oxidative stress responses was attributed to higher enzymatic antioxidant activities enzymes such as SOD, CAT, APX, and POD compared to WT, which may be related to the overexpression of *TaFBA1*gene 44. Together with our candidate gene, it is critical to comprehend the roles of F-box proteins in barley tolerance to various abiotic stress conditions.

Exclusively, chromosome 2H harbors several potential candidate genes which involved in most of the enzymatic and non-enzymatic antioxidant components in response to salt stress. The most prominent candidate gene is *HORVU.MOREX.r3.2HG0181470.1* at position (557,725,076-557,730,772 bp) annotated as Beta-glucosidase, putative. Xu, et al. 45 reported that several β-glucosidase proteins synthesise ABA from glucose-conjugated abscisic acid (ABA-GE), as well as the multiple ABA production pathways work together to enhance ABA levels. In Arabidopsis, a vacuole-glucosidase homolog1 (BG1) with glycosylated abscisic acid (Glc-ABA) hydrolyzing activity is important in osmotic stress responses. Therefore, we suggest that β-glucosidase proteins play a key role in altering the antioxidant pool by inducing high levels of enzymatic and non-enzymatic antioxidant components that increased tolerance to salt-induced oxidative damage.

The third genomic region is located on chromosome 4H, chr4H:13826536:A:G SNP (138,265,36 bp) inside the *HORVU.MOREX.r3.4HG0336230.1* (138,232,77-138,249,82 bp) annotated as Glycosyltransferase belongs to the family 1 UDP glycosyltransferases (UGTs) and chr4H:569747682:G:A SNP (569,747,682 bp) with a candidate gene *HORVU.MOREX.r3.4HG0405320.1* (569,744,191-569,748,338 bp) annotated as 3-ketoacyl-CoA synthase that control the variation of K^+/Na^+_S, K^+/Na^+_STI, and K^+_STI. Lairson, et al. 46 detected that endogenous cytokinin levels are linked to small molecular modification even by UGTs family, which transports sugar substituents to small acceptor molecules. Hou, et al. 47 identified the *Arabidopsis* UGT76C1 and UGT76C2 which finely tune the glycosylation of cytokinins and regulate the cytokinin homeostasis in several plant species. Furthermore, Li, et al. 48 demonstrated that *AtUGT76C2*, Arabidopsis cytokinin glycosyltransferase is implicated in the adaptation to osmotic and drought

stress. The second gene in the identified genomic region is *HORVU.MOREX.r3.4HG0405320.1* that annotated as 3-ketoacyl-CoA synthase, regulating the variation of K^+/Na^+_S, K^+/Na^+_STI, and K^+_STI. 3-ketoacyl-CoA synthase and is considered to be the key enzyme in the fatty acid elongation process 49. In *Arabidopsis thaliana*, upregulation of -Ketoacyl-CoA Synthase from Vitis vinifera L. enhances salinity stress responses. The enhanced salt stress was the consequence of various mechanisms cooperating together, along with the regulatory oversight of ion transporters, the accumulation of osmolytes, and the maintenance of membrane integrity 49. These genomic regions associated with the ionic contents of Na^+, K^+ and Na^+, K^+ ratio found in this study might be a target in future studies focused on improving salt-tolerant barley genotypes.

Interestingly, the fourth genomic region at 6H, chr6H:451682318:A:T SNP near the gene *HORVU.MOREX.r3.6HG0605480.1* (451,676,227- 451,677,811 bp) annotated as PLATZ (plant AT-rich protein and zinc-binding protein) transcription factor, regulating the variation of CAT_S, APX_S, GR_S, AsA_S, and GSH_S related to antioxidant components. The PLATZ transcription factor family is a class of plant-specific zinc-dependent DNA-binding proteins. PLATZ plays a critical role in the development of seed endosperm during the early developmental crop stages. PLATZ proteins are involved in the response to environmental stress. *GmPLATZ1 (Glycine max PLATZ1)* transcription levels in soybean were increased by ABA and drought stress 50. Upregulation of *GmPLATZ1* in transgenic Arabidopsis exhibited slowed in germination rate under osmotic and ABA stress 51. Altogether, this gene was linked to enzymatic antioxidants, suggesting that it participates in the salt tolerance pathway via the antioxidant defence system, that may have enhanced plant growth and performance. The last genomic region at 7H was found to be associated with SOD_S and APX_S at 153,773,211 bp chr7H:153773211:A:C SNP inside the gene *HORVU.MOREX.r3.7HG0676830.1* (153,772,300- 153,774,057 bp) that encodes L-gulonolactone oxidase. The allelic variation at chr7H:153773211:A:C SNP inside the L-gulonolactone oxidase gene reveals a negative selection of accessions carrying C allele. This allele is found in landraces from the Far and Near East that have lower enzymatic antioxidant activity e.g. SOD and APX in response to salinity. L-gulonolactone oxidase (GLOase) is one of the key enzymes that catalyzes the transformation of L-Gulonolactone to AsA 52. Relative expression of *GLOase* showed a significant increase in treated *Aeluropus littorali* shoots in response to salt stress 53. This enhancement may be correlated with the reduction in K^+ and water content, and the increase in Na^+ concentration that induced oxidative stress that results in increasing ROS molecules. Therefore, Na^+ should be prevented from reaching the cytosol by restricting Na^+ influx and/or efflux from the cytoplasm into the vacuoles. Hence, one of the critical factors in plant tolerance to salt stress is the ability to maintain a high K^+/Na^+ ratio. Altogether, GLOase actively participated in multiple stress responses by influencing the activity of ROS enzymes. Markedly, A:C SNP was associated with important enzymatic antioxidant components, such as SOD_S and APX_S, and thus, could be a candidate genomic region for helping to improve barley tolerance to salt stress.

Conclusions

In conclusion, our study provided detailed mechanistic insights into the genetic basis of salinity tolerance-related traits in barely under salt stress conditions. Highly reliable genomic regions were detected for multiple of the salinity tolerance-related traits, such as antioxidant components (SOD, APX, GR, AsA, and GSH), K^+ content, and K^+/ Na^+ ratio. Potential candidate genes were also identified, such as *HORVU.MOREX. r3.2HG0181470.1* and *HORVU.MOREX. r3.7HG0676830.1* that annotated as Beta-glucosidase and L-gulonolactone oxidase, respectively. Novel genomic regions and the linked genes which were discovered in this study, could be the potential candidates for developing barely salt-tolerant breeding lines. These findings maximize our knowledge of understanding the genetic factors underlying morpho-physiological and grain yield traits in barley in response to salinity that can be implemented through breeding for crop improvement.

Methods

Germplasm and genotyping

A core set collection consisting of 138 worldwide spring barley accessions was selected out of 22,621 wild and domesticated barley accessions hosted in the German *ex-situ* IPK-Gatersleben GeneBank as highly diverse accessions (**Figure** 1). The collection is originally from different geographical origins include 54 cultivars and 84 landraces. The core set/panel was split into 63 two-rowed and 75 six-rowed. All the accessions were genotyped using GBS that yielded 19,276 SNPs as reported in 23. This collection was tested for population structure that is considered in the GWAS analysis (**Figure** 2). Principal component analysis was used to check the structure within the population and plotted based on geographical regions, row-type and biological status. The detailed information of barley accessions is provided in **Table** S1.

Experimental setup

A pot experiment was conducted under field conditions during the two growing seasons (2019/2020-2020/2021) at the Experimental Station of Fayoum University (29°11'20.36"N latitude, 30°10'06.45"E longitude). A diverse set of 138 barley accessions was grown under both control and salt treatment. Four seeds were sown per each accession in plastic pots (22 cm × 60 cm × 40 cm) filled with sandy loam soil. Each accession was replicated three times of each treatment per year using a randomized complete block design (RCBD). Each pot received 5 g (17:11:10/N: P: K) fertilizer and was manually irrigated as needed. At 26 DAS, 200 mM NaCl and distilled water were imposed in respective salt and control pots, respectively. Salinity treatments were continued up to 55 DAS, after which irrigation was continued till harvest by distilled water (**Figure** 7). Five phenotypic traits were measured such as spike length (SL) (The distance between the spike's base and tip (excluding awns) in cm.), number of spikelets per spike (NSS) was measured as the actual number of spikelets developed per spike, and the number of grains per spike (NGS) was measured as the actual the actual number of grains developed per spike. For the weight of grains per spike (WGS) in gram (g), and thousand kernel weight (TKW), 1000 seeds were randomly taken from each plot in gram (g). Additionally, salt tolerance indices (STI) for all traits were calculated according to the following equation: STI = trait value under salinity/ trait value under control.

Estimation of Osmolytes

Proline content (PC) was determined and extracted as described in methods 54. Briefly, 400 ml of the mixture (1.25 g ninhydrin, 20 mL phosphoric acid, and 30 mL glacial acetic acid) was added to a 200 mL supernatant aliquot and heated for 1 h at 100°C. The mixture was then allowed to cool, and 4 mL of toluene was added. At 520 nm, the absorbance was taken and the results were recorded as mmol g^{-1} DW. Soluble protein (SP) was determined by using dye-binding method and bovine serum albumin as a standard. Then, the absorbance was measured spectrophotometrically at 595 nm.

Determination of Antioxidant Enzyme Activities

Enzyme extracts were prepared from barley leaf samples according to the procedure reported by . The photochemical reduction of nitro blue tetrazolium (NBT) through inhibition of enzyme activity was assessed to determine superoxide dismutase (EC 1.15.1.1). The reaction mixture was prepared by mixing 0.1 mL enzyme extract with methionine (13.33 mM), 50 mM buffer (phosphate, PH 7.8), sodium carbonate (50 mM), NBT (75 Mm), 0.1 mL riboflavin (2mM) and EDTA (0.1 mM), then exposed to light for 15 min. The absorbance was read spectrophotometrically at 560 nm.

Catalase activity (CAT, E.C. 1.11.1.6) was calculated using the decomposed H_2O_2 measurement 55. Exactly, 50 µL of the enzyme extract was mixed with 0.5 mL H_2O_2 (75 mM), and 1.5 mL buffer (phosphate 0.1 M, pH 7). An absorbance decrease was observed at 240 nm for 1 min. Catalase activity was estimated from the amount of decomposed H_2O_2. CAT activity (1 unit) is described as the amount of enzyme needed to catalyse the oxidation of 1 µmol of H_2O_2 min^{-1}.

The activity of ascorbate peroxidase (APX, E.C. 1.11.1.11) was determined by calculating the decrease in optical density caused by ascorbate at 290 nm 56. Exactly, 0.1 ml of the enzyme extract was mixed with 50 mM buffer (potassium phosphate, pH 7.0), 0.1 mL H_2O_2 (0.1 mM), EDTA (0.1 mM), ascorbate, and water (0.5 mM). APX activity (1 unit) is described as the amount of enzyme required to convert 1 µmol of ascorbate min^{-1} at 25˚C.

Glutathione reductase activity (GR, E.C. 1.6.4.2) was performed by estimating NADPH oxidation at 340 nm . Exactly, 37 µl of the enzyme extract was mixed with 50 mM Tris-HCl buffer (pH 7.5), $MgCl_2$ (3 mM), GSSG (0.5 mM), and NADPH (0.2 mM), then GSSG was added to start the reaction. GR activity (1 unit) is described as the amount of enzyme needed to catalyze the oxidation of 1 µmol of NADPH min^{-1}.

The total antioxidant capacity (TAC) was estimated according to Prieto, et al. 57. Briefly, barley extract of 10 µL was mixed to 1 mL of 250 µM DPPH solution and 4 mL distilled water, then the extract was placed in the dark for 30 min. The absorbance was measured at 517 nm. The reference standard was Trolox, and also the results were recorded as Trolox equivalent mg g^{-1} DW. The inhibition (%) of free radical DPPH was calculated using the equation as follows:

Antioxidant activity (I%) = [(A0- Ae)/A0] × 100, where A0 is the absorbance of the blank sample and Ae is the absorbance of the tested sample.

Determination of non-enzymatic antioxidants

The contents of ascorbic acid and glutathione were determined and extracted using the methods described in De Kok, et al. 58. The content of total polyphenol (TPC) was estimated and extracted according to 59. Briefly,100 µL of leaf extract sample was added to 50 µL of 2N Folin-Ciocalteau reagent. After 5 min, 400 µL of 2N Na_2CO_3 and 1 mL of water were added and incubated for 90 minutes at room temperature and then removed to a microplate. The absorbance was measured at 740 nm, and the results were expressed as gallic acid (GAE) equivalent µg g^{-1} of DW.

Total flavonoids were measured and extracted using the method described in Zou, et al. 60. Briefly, 400 µL of barley extracts were mixed with 60 µL of 5% $NaNO_2$, 500 µL of distilled water, and 140 µL of10% $AlCl_3$. After 10 min, 400 µL of 1 mM NaOH was added, and incubated for 10 min at room temperature, and then removed to a microplate. The absorbance was measured at 500 nm and the results were expressed as rutin (RE) equivalent µg g^{-1} of DW.

Estimation of ionic Na^+ and K^+ Content

The ionic Na^+, K^+, and K^+/Na^+ ratios were estimated from the dried leaves using the nitric-perchloric acid digestion method according to Westerman 61. The absorbance was measured at 766.5 nm and 589.0 nm for K and Na, respectively.

Data analyses

Analysis of variance and broad-sense heritability (H^2) were performed to compare both salt and control treatments at $p < 0.05$ for all studied traits using R software. R-studio was used to calculate a comparison between treatments at $p < 0.05$ including boxplots for all the studied traits . Using the lme4 package, the mean value for the measured traits

was computed as Best Linear Unbiased Estimates (BLUEs) . Pearson correlation analysis at $p < 0.05$ was used to calculate the correlation matrix among the traits for each treatment .

$$H^2 = \frac{\sigma^2 G}{\sigma^2 G + \frac{\sigma^2 e}{r}}$$

Where, σ^2G is the variance of genotypes (accessions); σ^2e is the variance of error, r is the number of replicates.

Multi-locus genome-wide association scan (GWAS)

Multi-locus GWAS analysis was performed by using Genomic Association and Prediction Integrated Tool (GAPIT) in R software 62using 19,276 SNPs and BLUE values of studied traits of the accessions. GAPIT statistical model used in GWAS analysis is known as the Fixed and random model Circulating Probability Unification (FarmCPU) model. The FarmCPU model was also used by combining multiple markers as covariates in a fixed-effect model and optimizing the associated covariate markers in a random effect model separately, allowing us to control false-positive associations through attempting to prevent model overfitting . Moreover, a threshold P-value 0.00001 equal to -log10 (P) ≥ 4. The phenotypic variance explained by significant makers (R^2) ≥ 10% was used to determine the significance of marker-trait associations (MTAs) that were associated with more than two traits.

Linkage disequilibrium (LD) between pairs of polymorphic SNPs on each chromosome was calculated by genome-wide pairwise estimates as a squared correlation (r^2). The LD decay estimated as r^2 versus the distance between pairs of polymorphic SNPs was presented as Mbp.

The most significant SNPs were used to describe the relatively close potential candidate gene inside the LD interval based on their physical position using the barley database BARLEX 63. BARLEX is a web-based platform which includes gene annotations, gene ontology (GO), and even a summary of a barley genomic space and gene annotations. The annotation of the high-confidence (HC) candidate gene was detected using Morex v3 63.

Abbreviations

Ascorbate peroxidase
APX
Ascorbic acid
AsA
Best Linear Unbiased estimators
BLUEs
Catalase
CAT
Chromosome
Chr
Control
C
Genome Wide Association Study
GWAS
Genotyping-by-Sequencing
GBS

Glutathione reductase
GR
Glutathione
GSH
Heritability
H^2
K+/Na+
Potassium sodium ratio
K+
Potassium
Linkage Desequilibrium
LD
Na+
Sodium
Number of grains per spike
NGS
Number of spikelet per spike
NSS
Proline content
PC
Salt conditions
S
Salt tolerant indices
STI
Single Nucleotide Polimorphism
SNP
Soluble protein
SP
Spike Length
SL
Superoxide dismutase
SOD
Thousand kernel weight
TKW
Total antioxidant capacity
TAC
Total flavonoid content
TFC
Total polyphenols
TPC
Weight of grains per spike
WGS

Declarations

Author Contributions

SGT and AMA designed the experiment; SGT performed the experiments; AMA and SGT analyzed data; SGT wrote the first draft of paper; DZA, AB, HBP, and AMA; Wrote and edit the paper. SGT, DZA, AB, HBP, and AMA conceived the idea and participated in the interpretation of results. All authors read and approved the final manuscript.

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Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article

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Ethics declarations

Competing interests

The authors declare that they have no competing interests.

Ethical statement

Experimental research and field studies on plants (either cultivated or wild), including the collection of plant material,complies with relevant institutional, national, and international guidelines and legislation.

Data availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request. Passport data for all accessions are reported in Supplementary Table 1. Phenotypic data used for GWAS are reported in Supplementary Table 11. Further information regarding the GenBank accessions including genotypic information was published by Milner, et al. 23 and had been deposited and available at https://doi.org/10.5447/IPK/2018/9. All accessions used in the current study are publicly available from IPK Gatersleben Genebank.

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Tables

Table 1. The list of candidate genes based on the linkage disequilibrium of multi-traits associated marker.

Genomic region	Gene	Chromosome	Start	End	Annotation
1	*HORVU.MOREX.r3.1HG0003310.1*	1H	6787041	6788808	Pentatricopeptide repeat-containing protein
1	*HORVU.MOREX.r3.1HG0016850.1*	1H	50593657	50595700	FBD-associated F-box protein
2	*HORVU.MOREX.r3.2HG0181470.1*	2H	5.58E+08	5.58E+08	Beta-glucosidase, putative
2	*HORVU.MOREX.r3.2HG0181510.1*	2H	5.58E+08	5.58E+08	Beta-glucosidase
3	*HORVU.MOREX.r3.4HG0336230.1*	4H	13823277	13824982	Glycosyltransferase
3	*HORVU.MOREX.r3.4HG0336280.1*	4H	13895773	13899763	Beta-glucosidase
3	*HORVU.MOREX.r3.4HG0405270.1*	4H	5.7E+08	5.7E+08	Pollen allergen-like protein
3	*HORVU.MOREX.r3.4HG0405310.1*	4H	5.7E+08	5.7E+08	Protein ROOT PRIMORDIUM DEFECTIVE 1
3	*HORVU.MOREX.r3.4HG0405320.1*	4H	5.7E+08	5.7E+08	3-ketoacyl-CoA synthase
4	*HORVU.MOREX.r3.6HG0553500.1*	6H	36654394	36658229	Calmodulin-binding protein-like
4	*HORVU.MOREX.r3.6HG0553520.1*	6H	36686263	36686911	GRF zinc finger family protein
4	*HORVU.MOREX.r3.6HG0597620.1*	6H	3.87E+08	3.87E+08	Trafficking protein particle complex subunit 12
4	*HORVU.MOREX.r3.6HG0597630.1*	6H	3.87E+08	3.87E+08	Nitrilase
4	*HORVU.MOREX.r3.6HG0597650.1*	6H	3.87E+08	3.87E+08	EF-hand calcium-binding domain-containing protein 5
4	*HORVU.MOREX.r3.6HG0597790.1*	6H	3.88E+08	3.88E+08	GRAM domain-containing protein / ABA-responsive
4	*HORVU.MOREX.r3.6HG0597850.1*	6H	3.88E+08	3.88E+08	GRF zinc finger / zinc knuckle protein
4	*HORVU.MOREX.r3.6HG0597860.1*	6H	3.88E+08	3.88E+08	Endosomal targeting BRO1-like domain-containing protein
4	*HORVU.MOREX.r3.6HG0597870.1*	6H	3.88E+08	3.88E+08	Alcohol dehydrogenase
4	*HORVU.MOREX.r3.6HG0605450.1*	6H	4.51E+08	4.51E+08	Major facilitator superfamily sugar transporter-like protein

4	*HORVU.MOREX.r3.6HG0605480.1*	6H	4.52E+08	4.52E+08	PLATZ transcription factor
5	*HORVU.MOREX.r3.7HG0650870.1*	7H	31456817	31461525	Xyloglucan alpha-1,6-xylosyltransferase
5	*HORVU.MOREX.r3.7HG0676830.1*	7H	1.54E+08	1.54E+08	L-gulonolactone oxidase
5	*HORVU.MOREX.r3.7HG0687600.1*	7H	2.38E+08	2.38E+08	Beta-galactosidase

Figures

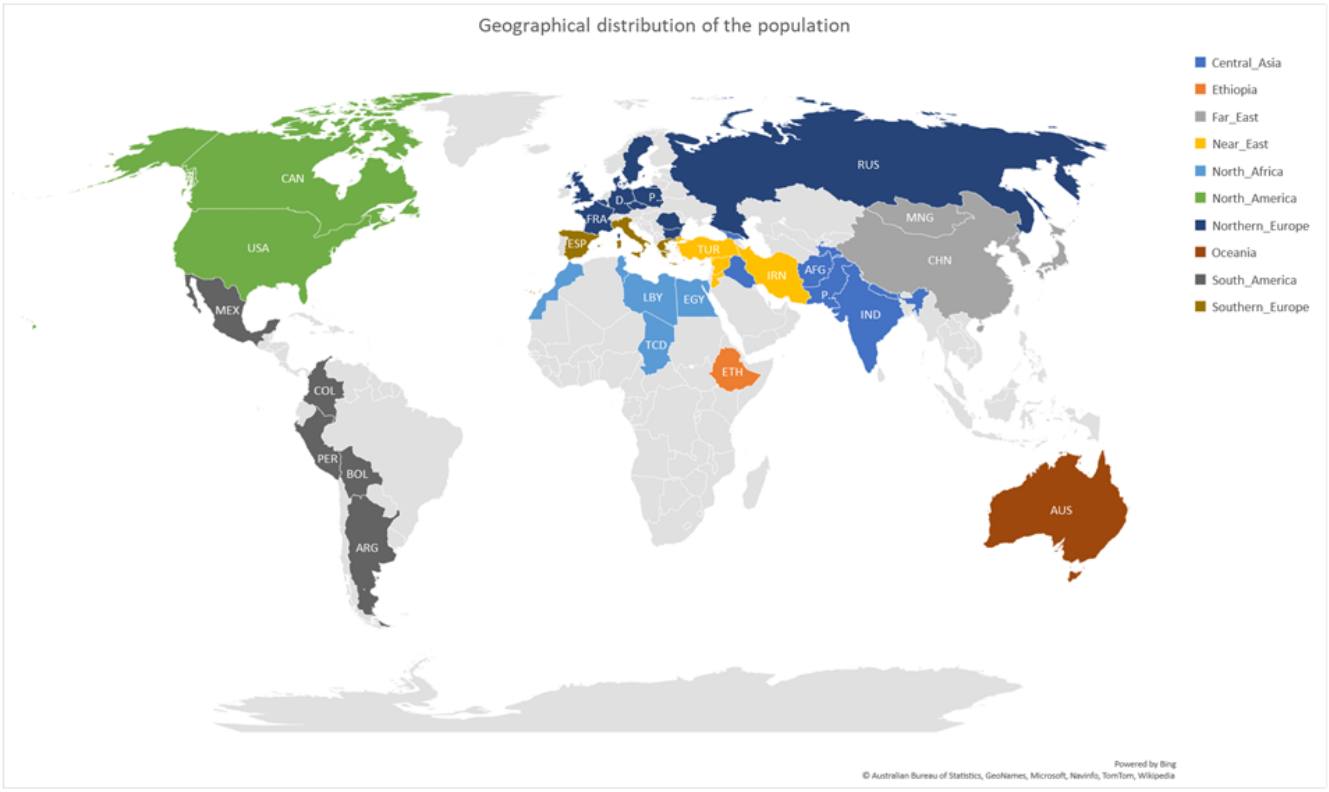

Figure 1

Geographical distribution of barley population.

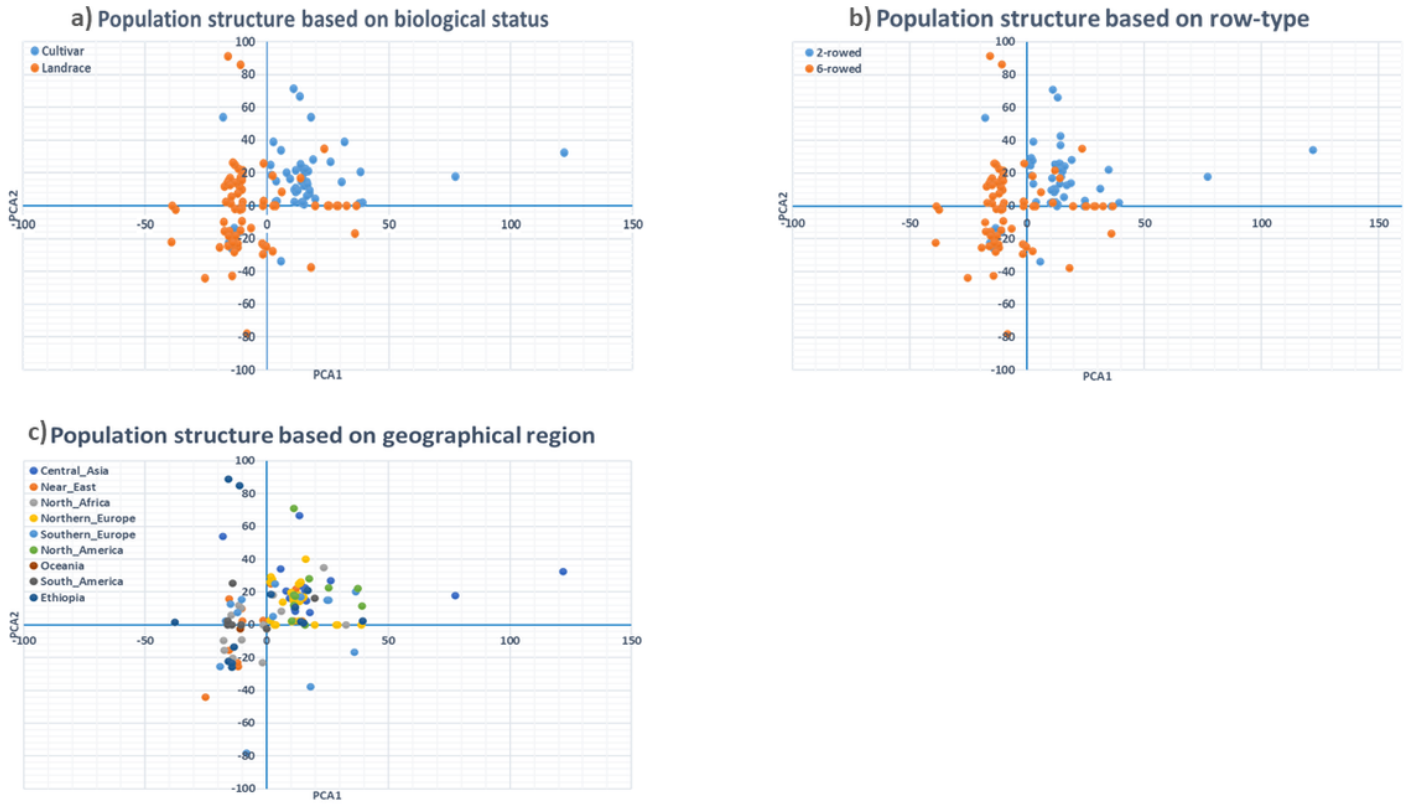

Figure 2

Population structure based on a) biological status, b) row-type, c) growth habit, and d) geographical region.

Figure 3

Correlations of the studied traits in barley accessions under both (a) control and (b) salinity treatment. The degree of significance is indicated as *p, 0.05; **p, 0.01; ***p, 0.001; ****p, 0.0001.

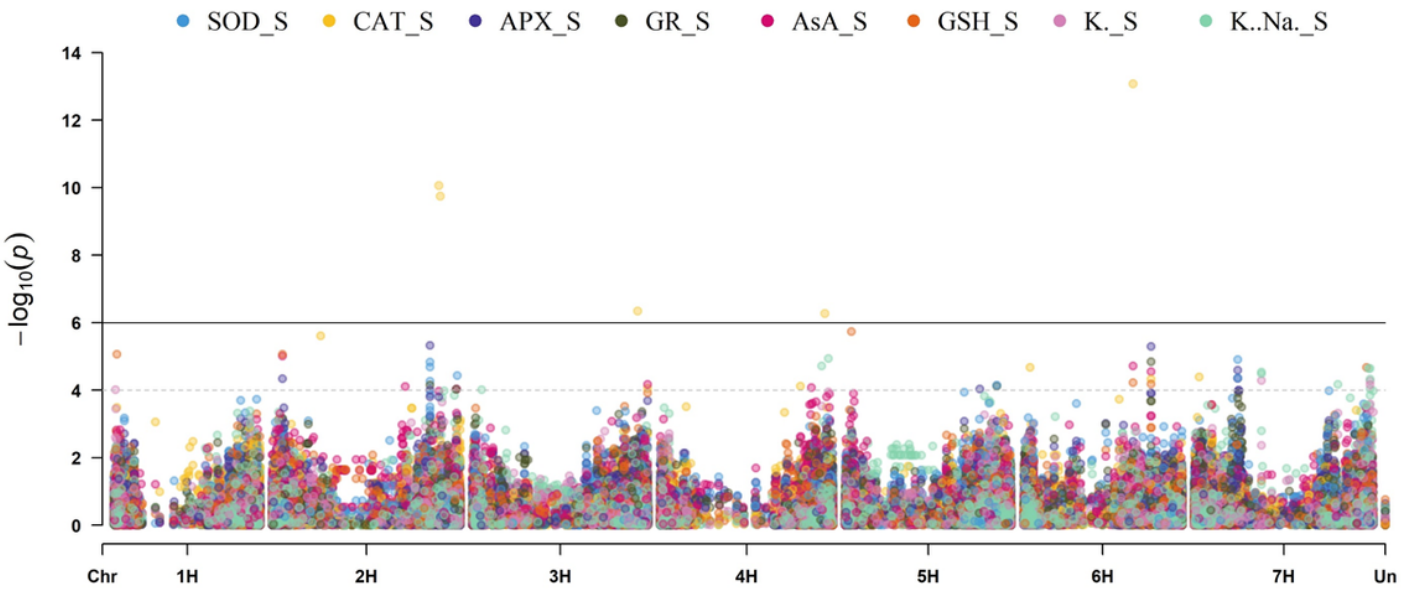

Figure 4

Combination of association studies regarding salt stress (SOD, CAT, APX, GR, AsA, GSH, K, K/Na ratio).

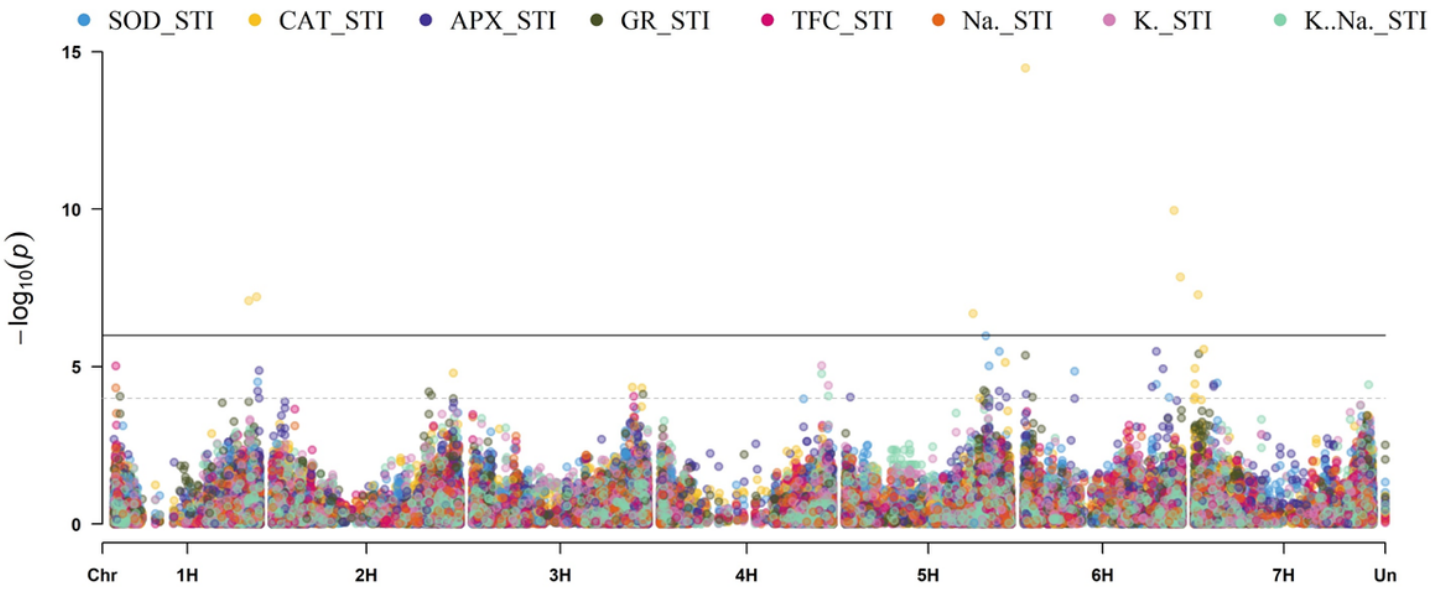

Figure 5

Combination of association studies regarding salt tolerance indices (SOD, CAT, APX, GR, TFS, Na, K, K/Na ratio).

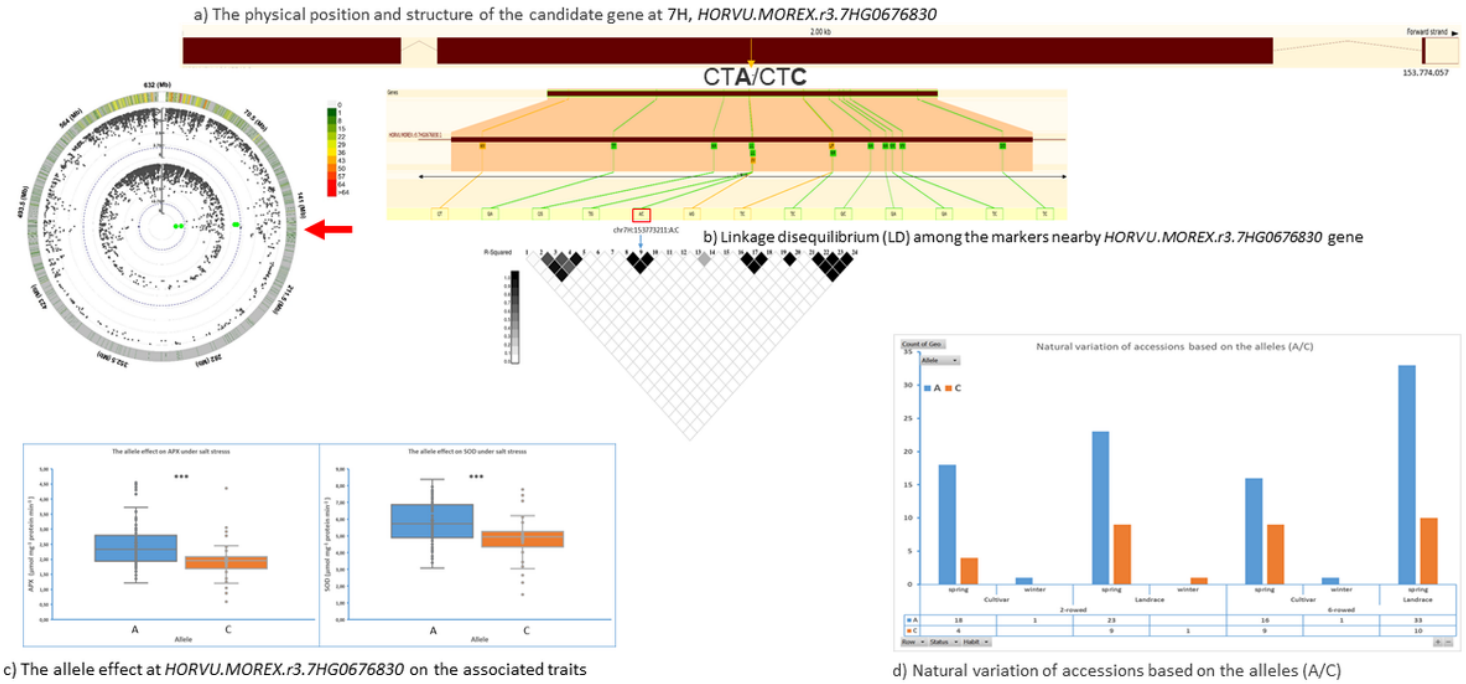

Figure 6

a) The physical position and structure of the candidate gene at 7H, *HORVU.MOREX.r3.7HG0676830* with allelic variation in the exon, b) linkage disequilibrium (LD) among the markers close to *HORVU.MOREX.r3.7HG0676830* gene presents as R-square (R^2). Arrow indicated the SNP located physically inside the *HORVU.MOREX.r3.7HG0676830.* c) allele effect on the accessions at the natural genetic variant (A/C), (d) natural variation of accessions based on the alleles (A/C).

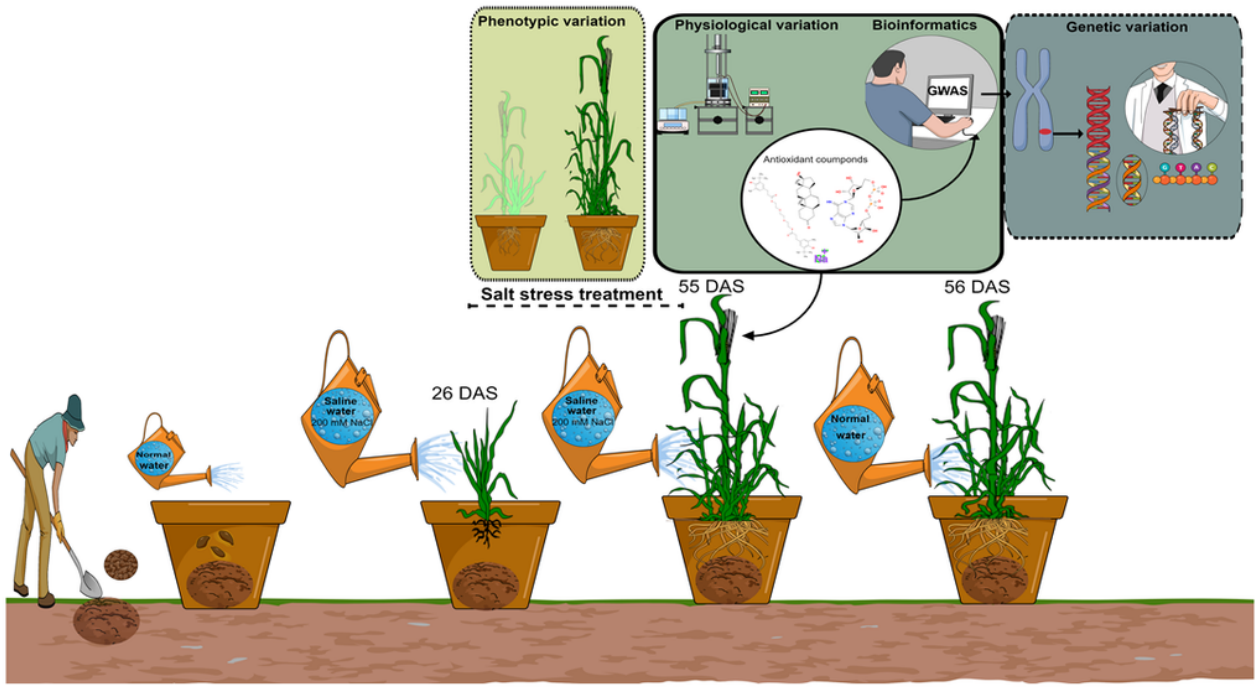

Figure 7

General description of experiment.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.pdf
- Supplementarytables.xlsx