



# Cell-Based Phenotyping Reveals QTL for Membrane Potential Maintenance Associated with Hypoxia and Salinity Stress Tolerance in Barley

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Waterlogging and salinity are two major abiotic stresses that hamper crop production world-wide resulting in multibillion losses. Plant abiotic stress tolerance is conferred by many interrelated mechanisms. Amongst these, the cell's ability to maintain membrane potential (MP) is considered to be amongst the most crucial traits, a positive relationship between the ability of plants to maintain highly negative MP and its tolerance to both salinity and waterlogging stress. However, no attempts have been made to identify quantitative trait loci (QTL) conferring this trait. In this study, the microelectrode MIFE technique was used to measure the plasma membrane potential of epidermal root cells of 150 double haploid (DH) lines of barley (*Hordeum vulgare* L.) from a cross between a Chinese landrace TX9425 and Japanese malting cultivar Naso Nijo under hypoxic conditions. A major QTL for the MP in the epidermal root cells in hypoxia-exposed plants was identified. This QTL was located on 2H, at a similar position to the QTL for waterlogging and salinity tolerance reported in previous studies. Further analysis confirmed that MP showed a significant contribution to both waterlogging and salinity tolerance. The fact that the QTL for MP was controlled by a single major QTL illustrates the power of the single-cell phenotyping approach and opens prospects for fine mapping this QTL and thus being more effective in marker assisted selection.

**Keywords:** H<sup>+</sup>-ATPase, *Hordeum vulgare*, hypoxia, membrane potential, salinity tolerance, waterlogging tolerance

## INTRODUCTION

Waterlogging is one of the major abiotic stresses limiting agricultural production around the globe (Setter and Waters, 2003). It imposes several limitations on plants during their life span (Bailey-Serres and Voesenek, 2008; Shabala and Pottosin, 2014). Among them, the major constraint that a plant faces when exposed to waterlogging is either a complete unavailability or an inadequate supply of oxygen to submerged organs of flooding sensitive species (Armstrong and Drew, 2002). As a result, the transport of nutrients from roots to shoots is severely disturbed under waterlogged conditions (Smethurst et al., 2005; Colmer and Voesenek, 2009), which consequently affects plant growth and yield (Malik et al., 2001; Colmer et al., 2011). Salinity is another limiting factor for crop production. According to FAO (2008), almost 800 million hectares of global land area are

affected by salinity which accounts for more than 20% of irrigated land area (Yamaguchi and Blumwald, 2005). Under saline conditions, excessive accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  results in their toxicity. Salinity stress also imposes osmotic and oxidative stress and interferes with the uptake and retention of other mineral elements such as  $\text{K}^+$  (Benito et al., 2014). Taken together, these factors lead to a disturbance of plant metabolism, reduced growth rates and plant yield. To meet the target of more than 70% increase in food production by 2050 (Garnett et al., 2013), it is important to improve the plant's tolerance to cope with different abiotic stresses, including waterlogging and salinity.

Barley is considered to be a waterlogging sensitive (Zhou et al., 2012) and moderately salt tolerant cereal (Ullrich, 2002; Munns et al., 2006), although it shows significant variation over genotypes in waterlogging (Takeda and Fukuyama, 1986; Zhou, 2010) and salinity tolerance (Slavich et al., 1990; Jaradat et al., 2004). Many quantitative trait loci (QTLs) have been reported for waterlogging and salinity tolerance based on different physiological and agronomic traits. For waterlogging tolerance, QTL mapping was done targeting aerenchyma formation (Mano and Omori, 2009; Zhang et al., 2015), root porosity (Broughton et al., 2015), grain yield (Zaidi et al., 2015), leaf chlorosis (Li et al., 2008; Zhou et al., 2012; Ma et al., 2015) and plant biomass (Zhang et al., 2013) as the whole-plant based phenotypic traits. Several QTLs have also been identified for salinity tolerance by using many whole-plant based phenotypic indices such as shoot sodium content (Rivandi et al., 2010; Shavrukov et al., 2010), intercellular  $\text{CO}_2$  concentration (Liu et al., 2017), and germination rate (Mano and Komatsuda, 2002). However, none of these findings led to any major progress in creating stress-tolerant cultivars. Several reasons may explain this (Arzani and Ashraf, 2016). First, the statistical testing of null hypotheses (for example no QTL) is deeply embedded in the probability theory and conditions that create error variance lead to threats to statistical conclusion validity. The LOD threshold value for avoiding a false positive with a given confidence, say 95%, depends on the number of markers and the length of the genome. As a consequence, the literature describing QTL analyses might contain false-positive QTLs at too high a rate. The second major reason is the genotype (QTL) by environment interaction which often confounds with main effects of a QTL. This is specifically true to all field-based studies. Next, the quantitative genetic models are often based on certain (unrealistic) assumptions and also have strong background dependency.

From a physiological point of view, the major shortfall is that in nearly all cases the above phenotyping has been conducted at the whole-plant level, so each of the measured traits was conferred by multiple (and often unrelated) contributing mechanisms. As a result, multiple QTLs have been reported for each of these traits. For example 14 QTLs were associated with leaf chlorosis on chromosomes 1H, 2H, 3H, 4H, 5H, 6H, 7H for waterlogging tolerance (Li et al., 2008; Xu et al., 2012; Zhou et al., 2012) and 10 QTLs associated with plant height on chromosomes 1H, 2H, 4H, 5H, 7H for yield component (Li et al., 2005; Xue et al., 2010; Chutimanitsakun et al., 2011; Wang et al., 2015). The second reason is that very often the phenotypic indices used are not directly related to the mechanisms targeted and are, therefore,

misleading. For example, measuring whole-shoot  $\text{Na}^+$  content (as in all studies; Genc et al., 2007; Haq et al., 2014; Tounsi et al., 2016) fails to account for differential ability of plants to sequester  $\text{Na}^+$  in leaf vacuoles; the trait considered to be the most crucial to confer salinity tissue tolerance. As a result, the amount of  $\text{Na}^+$  measured in the shoot will be the same for highly salt-sensitive species such as pea or rye and highly salt-tolerant halophyte species, but the impact on growth will be drastically different.

Thus, it appears that the real progress in plant breeding can be achieved only when plant phenotyping will directly target a contributing mechanism. This can be achieved only when such phenotyping is conducted at the cellular level.

Waterlogging and salinity tolerances are complex traits that are conferred by numerous physiological mechanisms (Jackson et al., 2009; Qiu et al., 2011; Shabala, 2011; Shabala et al., 2016). Amongst these, the plasma membrane (PM)  $\text{H}^+$ -ATPases play a central role in cell ionic homeostasis and stress signaling and adaptation. Channel-mediated root nutrient acquisition depends on the electric potential difference [membrane potential (MP)] across the PM, which is controlled by the  $\text{H}^+$ -ATPase activity (Palmgren and Nissen, 2011).  $\text{H}^+$  pumps also create a proton motive force for the secondary active ion transport (Shabala et al., 2016). The strong correlation between root PM  $\text{H}^+$ -ATPase activity and an overall salinity stress tolerance was found in many species (Chen et al., 2007; Bose et al., 2014; Lei et al., 2014). The same is true for waterlogging stress. Most of the membrane transporters are voltage gated in nature, and the PM is significantly depolarized (typically by 40 to 70 mV) under oxygen-limited conditions due to insufficient ATP availability (Teakle et al., 2013; Zeng et al., 2014).

$\text{H}^+$ -ATPase-mediated maintenance of a highly negative MP is one of the key elements of maintenance of intracellular  $\text{K}^+$  homeostasis. Potassium ( $\text{K}^+$ ) is an essential and most abundant nutrient which plays significant roles in plant growth.  $\text{K}^+$  is involved in the cell turgor pressure maintenance, cell elongation, stress signaling, and osmoregulation (Dreyer and Uozumi, 2011; Shabala and Pottosin, 2014). Stress-induced membrane depolarization activates outward-rectifying  $\text{K}^+$  efflux channels (GORK in *Arabidopsis*), resulting in a massive  $\text{K}^+$  loss under both hypoxia (Elzenga and van Veen, 2010) and salinity stress conditions (Chen et al., 2005), and leading to a significant reduction in plant  $\text{K}^+$  content (Smethurst et al., 2005; Board, 2008). This decline in  $\text{K}^+$  content results in severe yield penalties (Drew and Sisworo, 1979; Robertson et al., 2009) and, in extreme cases, in the loss of the cell viability (Shabala et al., 2016). The plant's ability to survive under waterlogged and saline conditions could be improved by improving its  $\text{K}^+$  retention capacity (Wang et al., 2013). Interestingly, plants often respond to salinity stress by an increase in the GORK transcript level (Adem et al., 2014; Chakraborty et al., 2016) suggesting that it is a post-translational regulation of GORK channel that is crucial for adaptive responses to stress. As mentioned above, voltage gating is arguably the most essential factor in this regulation. Thus, finding the QTL responsible for such gating may open a novel and previously unexplored avenue for improving abiotic (salinity and waterlogging) stress tolerance via enhanced  $\text{K}^+$  retention.

In this study, we have adopted a new method to phenotype plants at the single-cell level, to account for the tissue-specific expression of transporters, and identify a QTL responsible for the maintenance of negative MP under hypoxic conditions. This method relied on using the microelectrode MIFE technique, and has been applied to screen 150 barley double haploid (DH) lines from a cross between TX9425 and Naso Nijo under hypoxia (waterlogging). Analyses were conducted to identify the linkage between this trait and waterlogging/salinity tolerances. For the first time in the literature, we report a major QTL for the MP. This finding may open new avenues for future breeding programs to develop more tolerant varieties.

## MATERIALS AND METHODS

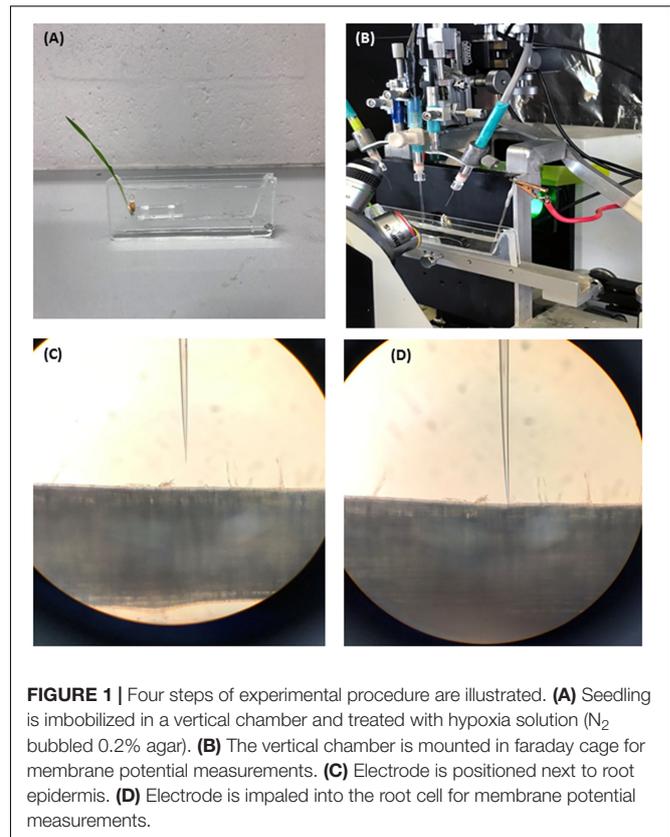
### Plant Material

A total of 150 DH lines from a cross between TX9425 and Naso Nijo (Xu et al., 2012) were used in this study for MP measurements. TX9425 is Chinese, two-rowed barley variety which is tolerant to waterlogging and salinity (Pang et al., 2004; Zhou et al., 2007) and shows a few exceptional agronomic characteristics (Wang et al., 2010) and resistance to some diseases (Li et al., 2009; Li and Zhou, 2011). Naso Nijo is a Japanese malting barley variety with good agronomic characteristics but is sensitive to both waterlogging (Pang et al., 2004) and salinity (Xu et al., 2012).

Seeds were surface sterilized with 10% commercial bleach ( $\text{NaClO}$  42 g  $\text{L}^{-1}$ ; Pental Products, Shepparton, VIC, Australia), thoroughly rinsed with tap water for at least 30 min and then grown in wet paper rolls with basic salt media (BSM) solution (0.5 mM KCl + 0.1 mM  $\text{CaCl}_2$ , pH 5.6) in the dark for 3 days at room temperature ( $25 \pm 1^\circ\text{C}$ ). Two treatments were used in the present experiment: (1) control (BSM, aerated); and (2) hypoxia (BSM solution made with 0.2% agar and bubbled with  $\text{N}_2$  gas). For the treatment with agar, the stagnant solution was prepared by adding agar (Cat. No. LP0011, Oxoid, Hampshire, United Kingdom) to the BSM solution at a ratio of 0.2% (w/v) and boiled, then cooled overnight to room temperature with magnetic stirring to prevent lump formation. The agar solutions were pre-bubbled with high purity  $\text{N}_2$  (Cat. No. 032G, BOC Gases, Hobart, TAS, Australia) for at least 1 h before being used in the experiment.

### Evaluation of the DH Lines for Waterlogging and Salinity Tolerance

All the details related to waterlogging and salt tolerance evaluation experiments are given in our previous publication (Xu et al., 2012). In brief, for waterlogging tolerance evaluation DH lines generated from a cross between TX9425 and Naso Nijo were subjected to waterlogging for 9 weeks until sensitive lines died. A collective scoring system was used, with scoring index 0 indicating no damage and index 10 given to dead plants. Plants with scores 0–5 showed various levels of chlorosis and those with scores 6 or above showing a substantial percentage of necrotic leaves, under waterlogged conditions.



**FIGURE 1** | Four steps of experimental procedure are illustrated. **(A)** Seedling is immobilized in a vertical chamber and treated with hypoxia solution ( $\text{N}_2$  bubbled 0.2% agar). **(B)** The vertical chamber is mounted in faraday cage for membrane potential measurements. **(C)** Electrode is positioned next to root epidermis. **(D)** Electrode is impaled into the root cell for membrane potential measurements.

To evaluate salt tolerance, seeds of the DH lines were sown in 40-L containers filled with a pine bark/loam-based potting mix with premixed slow release fertilizer. After germination (7 days after sowing) 200 mM NaCl treatment was applied and maintained until data collected. Salt tolerance was assessed by combining scores for leaf chlorosis and plant survival after 7 weeks of sowing and conducted on a similar way to the one described above for waterlogging.

### Membrane Potential Measurements

Membrane potential values were measured from root epidermal cells of intact barley seedlings. Conventional microelectrodes (Harvard Apparatus) were filled with 1 M KCl and connected to MIFE electrometer via Ag/AgCl half-cell. During MP measurement, the microelectrode with a tip diameter of  $0.5 \mu\text{m}$  was manually impaled into the epidermal cells of mature root zone (5 mm from shoot base) using a functioned 3D-micromanipulator (MHW-4, Narishige, Tokyo, Japan). MP values were recorded by the MIFE CHART software for at least 2 min after stabilization (Newman, 2001).

Prior to measurement a 3-day old seedling was taken from a paper roll and mounted in a vertical chamber and treated then with hypoxia solution. The measuring chamber was filled with hypoxia solution with the coleoptile being above the surface of the solution. Roots were kept under stagnant conditions for 48 h. The seedlings were then placed into the Faraday cage for MP measurements. For each DH line, MP values were measured

from roots of 5–6 individual seedlings after 48 h of treatment. At least four measurements were taken from each seedling. The overview of the experimental procedure is further illustrated in **Figure 1**.

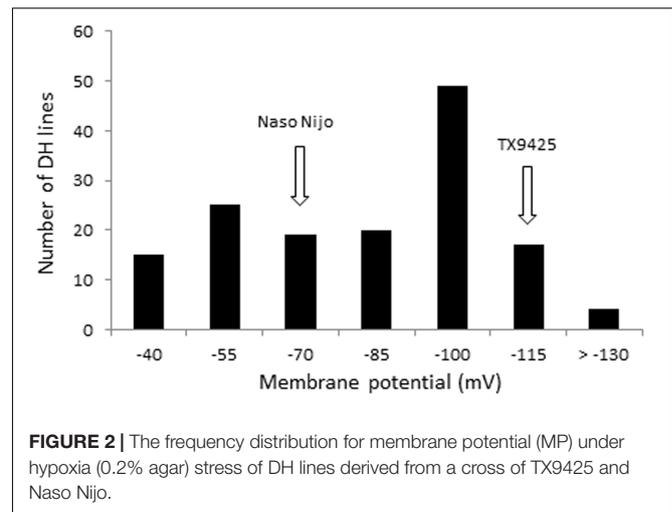
## Map Construction and QTL Analysis

Genomic DNA of the DH population was extracted from the leaf tissue of 4-week old seedlings. A total of 28047 DArT and 8928 SNP markers were used for genotyping. After removing markers with greater distortion and missing data, 4788 markers were chosen for map construction. A new genetic map of the DH population was constructed using the software package JoinMap 4.0 (Van Ooijen, 2006). QTL analysis was conducted using the software package MapQTL 6.0 (Van Ooijen, 2009). Interval mapping (IM) was firstly used to detect the major QTL. The nearest marker at the QTL from IM was chosen as a cofactor in the multiple QTL model (MQM). Logarithm of the odds (LOD) threshold values applied to declare the presence of a QTL were estimated by performing the genome wide permutation tests implemented in MapQTL version 6.0 using at least 1000 permutations of the original data set for each trait, resulting in a 95% LOD threshold around 3.0. To determine the effects of physiological traits on waterlogging and salinity tolerance, QTL for both waterlogging and salinity tolerance were re-analyzed by using various physiological traits as covariates. Maps showing the QTL position and LOD values were generated using MAPCHART (Voorrips, 2002).

## RESULTS

### Membrane Potential Values of Parents and DH Lines under Hypoxia Stress

The protocol for MP measurements from barley roots via microelectrode MIFE technique is shown in **Figure 1**. Both parent cultivars showed a significant difference in MP values when measured from epidermal root cells of barley after 48 h of hypoxia stress. Under hypoxia stress, MP values of the waterlogging tolerant parent TX9425 were significantly more negative ( $-125.3 \pm 3.3$  mV) than of sensitive parent Naso Nijo ( $-83.4 \pm 2.9$  mV) (**Table 1**). The DH lines from the cross between TX9425 and Naso Nijo also showed significant difference in values of MP when exposed to hypoxia for 48 h. **Figure 2** shows the frequency distribution of waterlogging tolerance based on the MP values under hypoxia stress. A continuous distribution



**FIGURE 2** | The frequency distribution for membrane potential (MP) under hypoxia (0.2% agar) stress of DH lines derived from a cross of TX9425 and Naso Nijo.

was found for MP with values ranging from  $-41$  to  $-138$  mV (**Table 1**). Analysis of variance (ANOVA) for MP showed a significant difference ( $P < 0.001$ ) between DH lines under hypoxia stress (**Supplementary Table S1**).

### QTL for Membrane Potential

One major QTL for MP was identified on chromosome 2H which was designated as *QMP.TxNn.2H*. This QTL was detected close to 8613801D2 marker at the position of 8.85 cM and explained 22% of the phenotypic variation (**Table 2**). The position of the QTL identified in this study was the same as that for waterlogging tolerance (Xu et al., 2012) (**Figure 3**).

### Contribution of Membrane Potential to Waterlogging and Salt Tolerance

Membrane potential showed a significant ( $P < 0.05$ ) correlation with waterlogging tolerance (**Figure 4A**). This is further confirmed by QTL analysis for waterlogging tolerance using MP as a covariate. As shown in **Figure 5A**, the LOD value of the QTL on 2H for waterlogging tolerance showed a slight reduction when MP was used as a covariate. The percentage of the phenotypic variation ( $R^2$ ) determined by the QTL also showed a slight reduction, from 21.0 to 18.4% (**Table 2**). MP also showed a close and significant correlation ( $P < 0.001$ ) with salt tolerance (**Figure 4B**). Correlation between MP and salt tolerance is higher than the correlation between MP and waterlogging tolerance. When MP was used as covariate, LOD value and  $R^2$  of the QTL for salt tolerance reduced from 32.8 to 26.3 and 63.7 to 50.8, respectively (**Figure 5B** and **Table 2**).

### QTL for MP When Using Waterlogging and Salt Tolerance As Covariates

The weak correlation with waterlogging tolerance and strong correlation with salt tolerance were further confirmed by reverse QTL analysis, i.e., analysis of QTL for MP using either waterlogging or salt tolerance as a covariate. When such analysis was conducted by using waterlogging damage scores as a covariate, only slight reductions in both LOD and  $R^2$  of the QTL

**TABLE 1** | Effects of hypoxia ( $N_2$  bubbled 0.2% agar) stress on membrane potential values of parents and DH lines.

Cultivars	Membrane potential (mV)
TX9425	$-125.33 \pm 7.34$
Naso Nijo	$-85.42 \pm 6.96$
DH lines average	$-91.17 \pm 14.54$
DH lines range	$-40.97 \pm -137.52$

Data are mean values  $\pm$  SE.

**TABLE 2** | Quantitative trait loci (QTL) on 2HS for membrane potential, salt and waterlogging tolerance detected in the DH population of TX9425 × Naso Nijo.

Traits	Linkage group	Nearest marker	Position (cM)	LOD	R <sup>2</sup> (%)	Co-variate
MP	2H	8613801D2	8.85	6.89	22.0	
	2H	8613801D2	8.85	6.2	19.5	Waterlogging
	2H	8613801D2	8.85	1.99	5.7	Salt
Waterlogging	2H	3258828D2	9.21	7.61	21	
	2H	3258828D2	9.21	5.83	18.4	MP
Salt	2H	3259260S2	7.79	32.79	63.7	
	2H	3259260S2	7.79	26.29	50.8	MP

for MP were found while the QTL for MP became insignificant when salt tolerance scores were used as covariates (**Figure 6**).

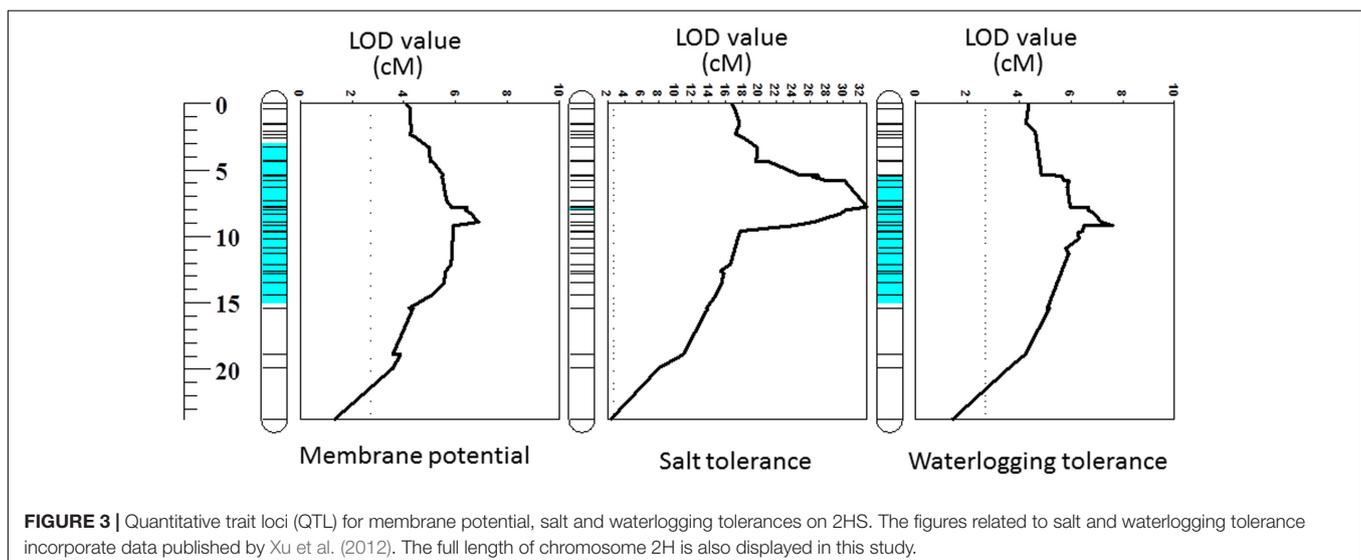
## DISCUSSION

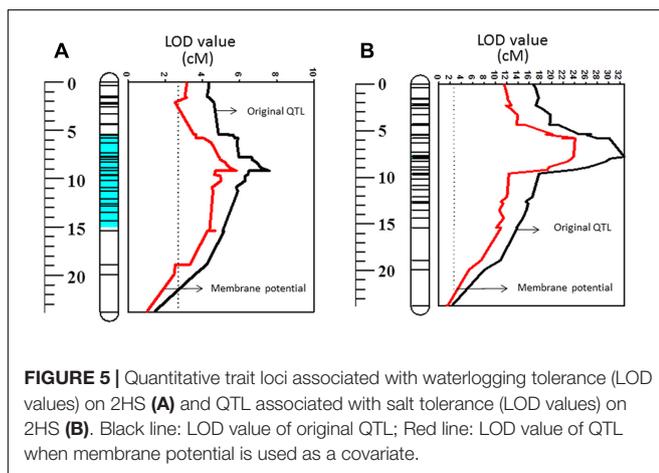
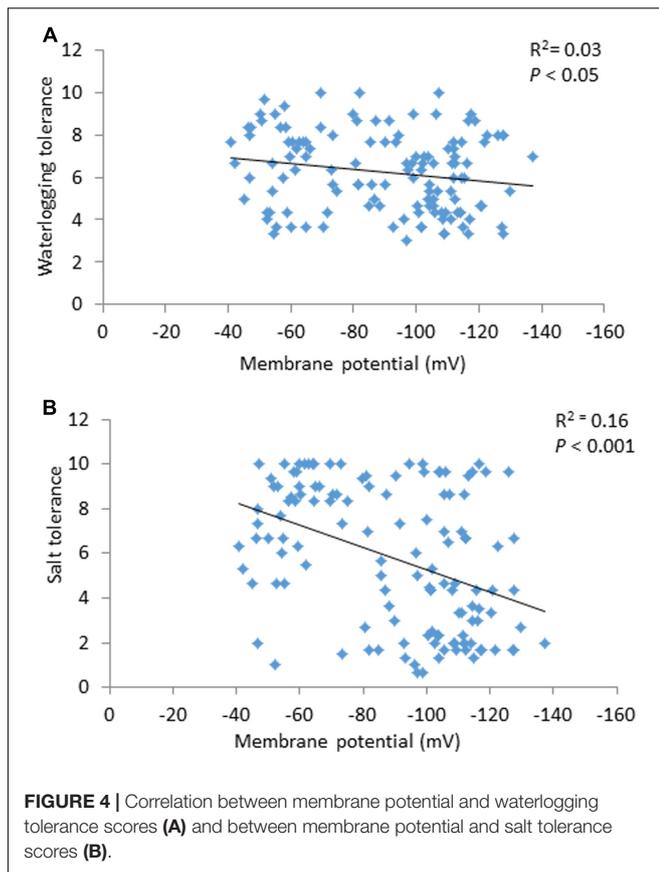
Tolerance to abiotic stresses is an important breeding objective. Great efforts have been made to identify mechanisms conferring waterlogging/salinity tolerance and finding QTL for the tolerance using different screening systems (Aslam et al., 1993; Mano and Takeda, 1997; Foolad et al., 2001; Lee et al., 2006, 2007; Pang et al., 2007; Chen H. et al., 2008; Chen Z. et al., 2008; Farshadfar et al., 2008; Xue et al., 2009; Fan et al., 2015). However, the practical outcomes are still disappointingly small. Both waterlogging and salinity tolerances are highly complicated traits that are controlled by many different mechanisms. Direct selection of the overall tolerance is very hard thus breeders rely on molecular markers linked to the tolerance. Most QTL identified for waterlogging/salinity tolerance are based on plant survival rate, plant healthiness and leaf chlorosis under stress (Li et al., 2008; Xue et al., 2010; Xu et al., 2012; Zhou et al., 2012; Ma et al., 2015; Zhang et al., 2016). While these traits are convenient for high throughput screening, they are not directly related to the mechanisms conferring the tolerance. As a result, fine mapping of these QTLs to provide reliable markers to breeders is very

difficult, even if possible in principle due to the very large number of QTLs involved.

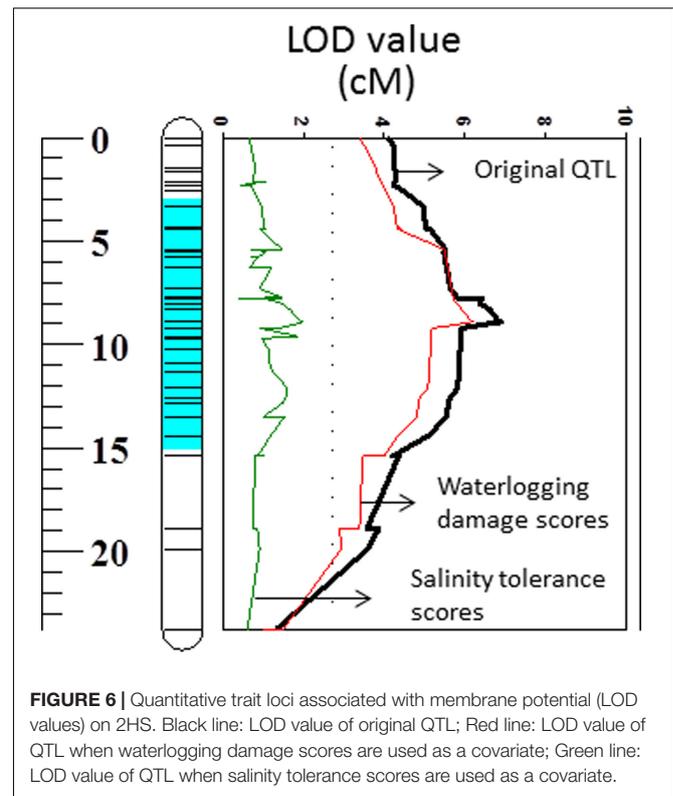
Much more promising is an approach when specific QTLs are linked directly with appropriate mechanisms. Since most of the mechanisms are expected to be controlled by just one or two QTLs, these are much easier to fine map. A good example of this success is for barley waterlogging tolerance, the major QTL for waterlogging tolerance on 4H (Li et al., 2008; Zhou, 2011; Zhou et al., 2012) is due to the formation of aerenchyma under stress which is controlled by a single major QTL (Broughton et al., 2015; Zhang et al., 2016, 2017) and the gene has been fine mapped to a <2 cM region with closely linked markers being available for breeders to use.

The PM is responsible for the maintenance of ionic and electric gradients between the cytosol and external media and thus essential for intracellular ionic homeostasis. It is also an important component of the signal transduction in plants under stress conditions (Kim et al., 2007). PM depolarization is one of the common features between salinity and waterlogging stresses, leading to a substantial disruption in the ionic homeostasis (Palmgren and Nissen, 2011; Shabala et al., 2016) which contributes to metabolic disturbances and ultimately determines the cell's fate. The electrogenic H<sup>+</sup>-ATPase pumps plays a significant role in maintaining negative potential of the PM. Oxygen limited conditions resulted in a significant





depolarization of the PM due to huge decline in ATP availability to fuel  $H^+$ -ATPase. The PM is also depolarized as a result of massive  $Na^+$  uptake under saline conditions. In our experiment, waterlogging/salt tolerant variety, TX9425, showed a much better ability to maintain MP under hypoxia stress than waterlogging/salt sensitive variety, Naso Nijo (Table 1). The DH population from these two varieties showed a wide range of segregation (Figure 2) and a major QTL (*QMP.TxNn.2H*) for MP (Figure 3) was identified. This QTL is located on the short arm of chromosome 2H and explained 22% of phenotypic variation



(Table 2). The fact that only one single major QTL was identified in this population makes it easier to further fine map the gene.

A large number of QTLs for different stress tolerances were reported at this position (Zhang et al., 2017), which include waterlogging (Zhou, 2011; Xu et al., 2012), salinity (Xu et al., 2012), and drought (Fan et al., 2015) with some being identified from the same DH population used in this study. Importantly, all these stresses are known to affect  $H^+$ -ATPase activity and depolarize the PM (Shabala et al., 2016). *Arabidopsis* mutants lacking an  $H^+$ -ATPase isoform showed increased sensitivity to salt and accumulate higher concentrations of  $Na^+$  in leaves compared to wild type plants (Vitart et al., 2001). On the contrary, expressing *Arabidopsis thaliana* V-ATPase subunit C in barley improved plant performance under saline condition by enabling better osmotic adjustment (Adem et al., 2017). When comparing MP with waterlogging/salt tolerance scores from the same population, MP showed significant correlations with both waterlogging and salinity tolerance (Figure 4). Further QTL mapping of different traits was conducted using other related traits as covariates, which has been proved to be effective in confirming the relationship between different traits (Fan et al., 2015). When MP was used as a covariate the LOD value and  $R^2$  of the QTL on 2H for both waterlogging and salt tolerances reduced with less effect on waterlogging tolerance, confirming the weak linkage between MP and waterlogging tolerance and greater contribution of MP to salt tolerance (Figure 5 and Table 2). The reason for the different contribution of MP to the different stresses may be related to the difference in the principal causes for membrane depolarization upon low oxygen and salinity

(e.g., compromised mitochondrial operation for the former, and massive influx of Na<sup>+</sup> for the latter).

It is not clear at this stage what specific gene contributes to the better maintenance of higher MP values in hypoxia-affected barley roots. The nearest marker of the QTL detected in this study was located around 8.28 cM, and the list of all candidate genes within 5 cM distance to 8.28 cM is given in **Supplementary Table S2**. No known subunits of H<sup>+</sup>-ATPase appear to be present in the vicinity of the reported marker suggesting that it was a *regulation* rather than the physical presence of the H<sup>+</sup>-ATPase protein matter for MP maintenance. It was previously shown that the plant's ability to maintain negative MP was not attributed to changes in H<sup>+</sup>-ATPase transcripts or the actual amount of protein (reviewed in Shabala et al., 2016) but rather regulated by the post-translational modifications. This regulation may occur via multiple pathways; one of them involves hormonal signaling. For example, it is known that ABA dephosphorylates the penultimate Thr residue on the H<sup>+</sup>-ATPase, resulting in deactivation of the pump (Hayashi et al., 2014), and the protein kinase PKS5/CIPK11, an important element of ABA signaling cascade (Lumba et al., 2014), reducing the activity of the H<sup>+</sup>-ATPase (Fuglsang et al., 2007). Additionally, an overexpression of a tomato 14-3-3 homolog (GRF9) resulted in an increased H<sup>+</sup>-ATPase activity (Hu et al., 2015). The above annotated contigs for the 3 to 13 cM region on chromosome 2H contains a large number of kinases and kinase-like proteins. Also, two genes related to energy metabolism were detected in the vicinity of the marker. These were ectonucleoside triphosphate diphosphohydrolase 5 (E-NTPDase) and dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex. E-NTPDases break down nucleoside tri- and diphosphates to nucleoside monophosphates and inorganic phosphate (Pi) and perform a wide range of functions. This includes purinergic signaling and control of the ATO concentration in ER and Golgi lumen to regulate ATP-dependent processes (Massalski et al., 2015). It remains therefore a task for a future studies to answer the question which of them is responsible for regulation of H<sup>+</sup>-ATPase activity under stress conditions.

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## CONCLUSION

A major QTL for MP maintenance under hypoxia was identified using cell-based phenotyping involving microelectrode MIFE technique. The QTL is located at a similar position to that for waterlogging and salinity tolerance on chromosome 2H. MP showed a weak but significant linkage with waterlogging tolerance and a strong linkage with salt tolerance. As only one single major QTL was responsible for MP, this makes it easier to fine map this QTL and effectively use this gene in pyramiding different tolerance mechanisms in breeding programs.

## AUTHOR CONTRIBUTIONS

MG conducted phenotyping, data analysis and prepared the draft; YF assisted mapping, QTL analysis and candidate gene search; FZ, LS, and GZ helped in manuscript preparation and revision; SS and MZ conceived and supervised the project, reviewed and revised the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2017.01941/full#supplementary-material>

**TABLE S1** | ANOVA of membrane potential values under waterlogging (hypoxia) stress.

**TABLE S2** | List of annotated proteins search by contig blast from the website (<http://webblast.ipk-gatersleben.de/barley/>).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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