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A major QTL controlling the tolerance to manganese toxicity in barley (*Hordeum vulgare* L.)

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Abstract Waterlogging stress disturbs plant metabolism through increased ion (manganese and iron) toxicity resulting from the changes in the soil redox potential under hypoxic conditions. Our previous study found a significant correlation between the tolerance to Mn^{2+} toxicity and waterlogging stress tolerance in barley, suggesting that waterlogging tolerance could be increased by improving the tolerance to Mn^{2+} toxicity. In this study, a doubled-haploid (DH) population from the cross between barley varieties Yerong and Franklin (waterlogging-tolerant and -sensitive, respectively) was used to identify QTL controlling tolerance to Mn^{2+} toxicity based on chlorophyll content and plant survival as selection criteria. Four significant QTL for plant survival under Mn^{2+} stress (*QSur.yf.1H*, *QSur.yf.3H*, *QSur.yf.4H*, and *QSur.yf.6H*) were identified in this population at the seedling stage. Two significant QTL (*QLC.yf.3H* and *QLC.yf.6H*) controlling leaf chlorosis under Mn^{2+} stress were identified on chromosomes 3H and 6H close to *QSur.yf.3H* and *QSur.yf.6H*. The major QTL *QSur.yf.3H*, located near the marker Bmag0013, explained 21% of the phenotypic variation. The major QTL for plant survival on 3H was validated in a different DH population (TX9425/Naso Nijo). This major QTL could potentially be used in breeding programmes to enhance tolerance to both manganese toxicity and waterlogging.

Keywords Barley (*Hordeum vulgare* L.) · Mn^{2+} toxicity · Waterlogging tolerance · QTL mapping

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Introduction

Waterlogging is one of the most hazardous natural constraints affecting agricultural crop production. Based on soil moisture or water levels, waterlogging may refer to flood, submergence, soil saturation, anoxia, or hypoxia (Ahmed et al. 2012). Soil waterlogging is generally caused by the prolonged rain or flooding in combination with poor soil drainage. The yield loss can vary with duration of the stress, soil types, and the tolerance of different species and genotypes (Bailey-Serres and Voesenek 2008; Shabala 2011).

Waterlogging stress affects plant metabolism in multiple ways, with plants showing a broad range of morphological and physiological responses to waterlogging. The phenotypic features associated directly with waterlogging tolerance remain unclear and controversial (Pezeshki et al. 1999; Smethurst et al. 2005; Teakle et al.

2010), even though the severity of leaf chlorosis after waterlogging has been proven to be one of the reliable indicators of waterlogging tolerance (Li et al. 2008; Zhou 2011). Other symptoms caused by ion toxicity or accumulation of toxic organic metabolites in roots under waterlogging conditions (Pang et al. 2007) may also occur (Khabaz-Saberi et al. 2006; Bailey-Serres and Voesenek, 2008; Khabaz-Saberi and Rengel 2010; Shabala 2011). The availability of chemical elements in soils is affected by multiple interacting factors, including soil pH, redox potential, cation exchange capacity, and microbial activity. With the redox potential declining after waterlogging onset, metals such as Mn^{4+} and Fe^{3+} are utilised as alternative electron acceptors when oxygen is depleted, resulting in increased concentrations of soluble Mn^{2+} and Fe^{2+} (Kirk et al. 2003) that may exceed plant requirements and cause toxicity. Genotypes with tolerance to Mn^{2+} or Fe^{2+} toxicity generally perform better than the sensitive ones in waterlogged soils (Pang et al. 2007; Khabaz-Saberi and Rengel 2010; Khabaz-Saberi et al. 2012). Enhanced tolerance to multiple ion toxicities was reported to improve wheat performance in waterlogging-affected acid soils (Khabaz-Saberi and Rengel 2010). The significant correlation between tolerance to Mn^{2+} toxicity and tolerance to waterlogging in barley (Huang et al. 2015) suggests that plant breeding for tolerance to waterlogging traits may be advanced by targeting tolerance to Mn^{2+} toxicity.

Similar to other waterlogging-related traits, various Mn^{2+} tolerance mechanisms (e.g. avoidance versus tissue tolerance) operate in different tolerant genotypes (Huang et al. 2015). Direct selection for tolerance to Mn^{2+} toxicity is affected by environmental factors and is thus largely ineffective. Marker-assisted selection (MAS) is based on distinct genetic differences that can easily be scored and mapped in most segregating populations (Kearsey and Farguhar 1998). During past decades, many QTL have been reported for tolerance to waterlogging or submergence based on various agronomic and morphological characteristics in different doubled-haploid (DH) populations (Qiu et al. 2007; Li et al. 2008; Zhou 2011; Zhang et al. 2013). However, to the best of our knowledge, no QTL for tolerance to Mn^{2+} toxicity has been reported so far.

In the present study, one DH population was used to identify QTL controlling tolerance to Mn^{2+} toxicity, and the QTL were validated in another DH population. Chlorophyll content and plant survival under Mn^{2+} toxicity were used as the indicators of Mn^{2+} tolerance.

Materials and methods

Plant materials and growth condition

A total of 177 lines from a DH population originated from the cross between Yerong (waterlogging tolerant) and Franklin (waterlogging sensitive) were used to identify QTL conferring Mn^{2+} tolerance. Another 188 DH lines from the cross between TX9425 (waterlogging tolerant) and Naso Nijo (waterlogging sensitive) were used to validate the QTL identified in the Yerong/ Franklin population.

The DH lines and parents were grown as reported previously (Huang et al. 2015). Seeds were surface-sterilised using 0.5% v/v sodium hypochlorite for 15 min and then thoroughly washed in running water for 30 min. After 1-day germination in a Petri dish, seeds were transferred to a floating mesh in 60-L ($60 \times 40 \times 25$) plastic containers with nutrient solution. The solution was bubbled with air for the duration of the experiment. The nutrient solution contained 1.5-mM KNO_3, 1.0-mM $Ca(NO_3)_2$, 0.25-mM $MgSO_4$, 0.25-mM $(NH_4)_2HPO_4$, 0.25-mM $NH_4H_2PO_4$, and 4-mM MES hydrate buffer (modified Rygol-Johnson solution) (Shabala et al. 2010). Aliquots of freshly prepared 1-M manganese ($MnSO_4$) stock solution were added to the nutrient solution to attain 1-mM final concentration. Nutrient solutions were replaced every 2 days. The pH of nutrient solutions was adjusted to 4.8 daily using 1-M HCl or 1-M NaOH. Distilled water was added to maintain the volume of the solution to compensate for the loss by transpiration.

Evaluating severity of Mn^{2+} toxicity by SPAD chlorophyll measurements

A combined visual scoring system including plant survival (dead leaf percentage) and leaf chlorosis was used in the trial. The scoring (0 = not affected and 10 = all dead) was conducted after 10 days of Mn^{2+} treatment. Chlorophyll content of the oldest fully expanded leaves was determined using a SPAD-520 chlorophyll meter (Konica Minolta Sensing Inc., Sakai, Japan) as a measure of Mn^{2+} tolerance.

Two independent trials were conducted for each of the two DH populations. Both trials had three replications, with each replicate having three plants. Each replication was placed in single plastic containers, and the genotypes were randomly arranged.

Map construction

The genetic linkage map of the Yerong/Franklin population has been published earlier by Li et al. (2008), comprising 496 DArT markers and 28 microsatellite (SSR) markers. For the TX9425/Naso Nijo population, the DH lines and the two parental varieties were genotyped with DArTSeq (http://www.diversityarrays.com/dart-application-dartseq). Due to the large number of DNA markers (~ 30,000 SNP and DArTSeq markers), markers with the same positions or with greater distortion and missing data were removed from the map construction. These markers were combined with previous genotypic data (DArT and SSR markers) (Xu et al. 2012; Wang et al. 2014). A total of around 2500 markers were selected to construct the genetic map.

QTL mapping and statistical analysis

The average values from each experiment were used for the identification of QTL associated with Mn^{2+} tolerance. The software package MapQTL6.0 (Van Ooijen 2006, 2009) was used to detect QTL that were first analysed by interval mapping (IM). The closest marker at each putative QTL identified using IM was selected as a cofactor and the selected markers were used as genetic background controls in the approximate multiple QTL model (MQM). The logarithm of the odds (LOD) value thresholds, used to declare the presence of a QTL, were estimated by performing the genome-wide permutation tests using at least 1000 permutations of the original data set for each trait, resulting in a 95% significance at LOD threshold 3.0. Two LOD support intervals around each QTL were established by taking the two positions around the peak that had the LOD values two less than the maximum (Van Ooijen 2009) after performing restricted MQM mapping. The percentage of variance explained by each QTL (R^2) was obtained using restricted MQM mapping implemented with MapQTL 6.0. Graphical representation of linkage groups and QTL was generated by using MapChart 2.2 (Voorrips 2002). All other statistical analyses, including calculation of mean values, standard errors, and analysis of variance (ANOVA), were performed using IBM SPSS Statistics 20 (Chicago, IL, USA).

Results

### Response of parents and DH lines to Mn^{2+} toxicity

The presence of high concentration (1 mM) of Mn^{2+} in the growth solution had a significant effect on plant growth. Occurrence of tiny brown spots was the common symptom on older leaf tips at the seedling stage (Zadoks score of 11). The symptoms were later spread over the whole leaf and resulted in pronounced leaf chlorosis. The symptom development was faster on susceptible than tolerant genotypes (data not shown). One day after applying excess Mn^{2+}, the susceptible parents Franklin and Naso Nijo already showed numerous tiny brown spots, and leaf chlorosis occurred 2 days later. At the end of the 1-mM Mn^{2+} treatment, both sensitive parents showed severe leaf chlorosis (score of 7). In contrast, both Yerong and TX9425 showed much better tolerance than Franklin and Naso Nijo. At the end of the Mn^{2+} treatment, only brown spots appeared on the leaves of tolerant parents Yerong and TX9425 with no obvious chlorosis. TX9425 was less tolerant than Yerong (Fig. 1).

A wide variation (2.0–7.5) in plant survival index (mainly leaf chlorosis) after the Mn^{2+} treatment was found in the DH lines from both populations (Fig. 2A, C). The Yerong × Franklin population also showed a wide variation in chlorophyll content, with the SPAD reading ranging from 14 to 31 (Fig. 2B). The plant survival scores in both populations (Fig. 2A, C) and the chlorophyll content of the Yerong × Franklin population (Fig. 2B) showed normal distribution. Transgressive segregation in the plant survival scores was found in both populations, with some lines surpassing their parents. A significant regression ($R^2 = 0.62$) between leaf chlorophyll content and plant survival index was found in the Yerong × Franklin population (Fig. 2D).

QTL for chlorophyll content and plant survival under Mn^{2+} stress

Four significant QTL for plant survival under Mn^{2+} stress (*QSur.yf.1H*, *QSur.yf.3H*, *QSur.yf.4H*, and *QSur.yf.6H*) were identified in the Yerong/Franklin DH population at the seedling stage (Table 1, Fig. 3). The Yerong alleles contributed to the tolerance at all the loci, except for the *QSur.yf.1H* locus where the Franklin allele improved the plant survival. These four QTL explained up to 40% of the phenotypic variation, with

Fig. 1 Toxicity symptoms on the oldest fully expanded leaf of the parent genotypes at the end of the 1-mM Mn^{2+} treatment (11-day-old plants). Genotypes tolerant to Mn^{2+} toxicity: Yerong (**a**, chlorosis score 2.9), TX9425 (**b**, chlorosis score 3.2). Genotypes sensitive to Mn^{2+} toxicity: Franklin (**c**, chlorosis score 6.6), Naso Nijo (**d**, chlorosis score 6.7)
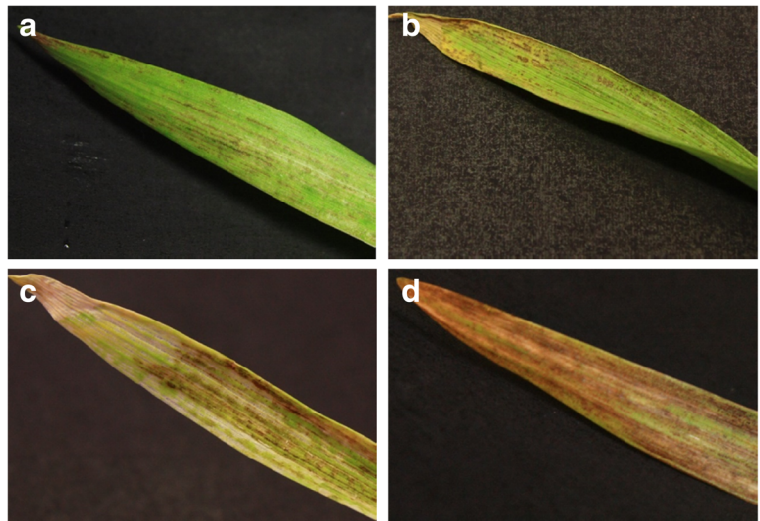
individual QTL explaining between 6.1 and 21% of the phenotypic variation. The major QTL for plant survival (*QSur.yf.3H*) was located on chromosome 3H, with the closest marker being Bmag0013 (Table 1).

Two significant QTL (*QLC.yf.3H* and *QLC.yf.6H*) controlling the severity of leaf chlorosis under Mn^{2+} stress were identified on chromosomes 3H and 6H, respectively (Table 1, Fig. 3). The Yerong alleles were
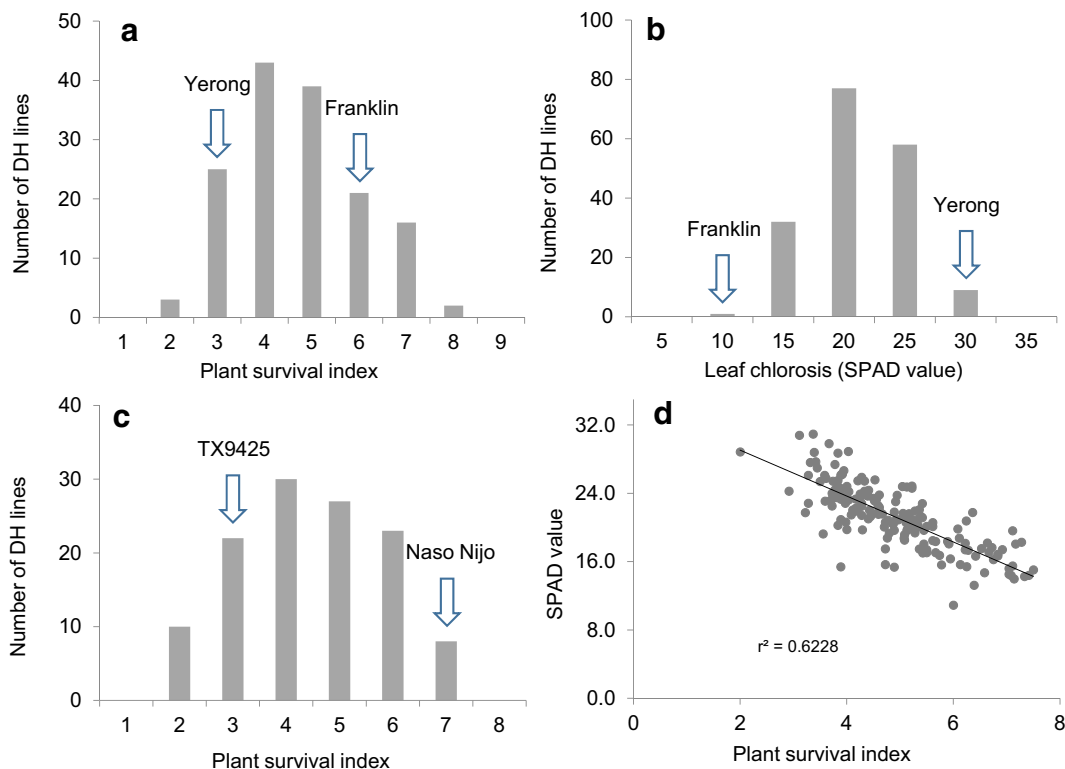

Fig. 2 Frequency distribution of Mn^{2+} toxicity tolerance of the Yerong/Franklin DH lines (**a**, **b**) and the TX9425/Naso Nijo DH lines (**c**). **d** Correlation between SPAD values and the plant survival index of both populations. Values are the means of two independent experiments

Table 1 QTL associated with tolerance to Mn^{2+} identified in the DH populations Yerong × Franklin and TX9425 × Naso Nijo (*yf* and *tn*, respectively, in the QTL name)

Trait	QTL	Chr.	Nearest marker	One LOD support interval (cM)	LOD	R^2 (%)
Leaf chlorosis (chlorophyll content)	QLC.yf.3H	3H	Bmag0013	118–122	10.5	22.1
	QLC.yf.6H	6H	bPb-6607	69–80	3.97	7.6
Plant survival	Qsur.yf.1H	1H	HvHVA1	101–102	3.73	6.1
	QSur.yf.3H	3H	Bmag0013	118–122	9.81	20.9
	QSur.yf.4H	4H	bPb-5265	136–139	3.9	7.7
	QSur.yf.6H	6H	bPb-3230	77–79	3.44	6.8
Plant survival	Qsur.tn.3H	3H	3276026	114–133	3.86	14.6

responsible for mild leaf chlorosis (higher chlorophyll content) at both QTL. The major QTL (*QLC.yf.3H*) explained 22% of the phenotypic variation (Table 1). This QTL was co-located with *QSur.yf.3H* for plant survival under Mn^{2+} stress (Fig. 3). The minor QTL (*QLC.yf.6H*) explained 7.5% of the phenotypic variation. This QTL was

located at a similar position to that for plant survival QTL (*QSur.yf.6H*). To test the relationship between chlorophyll content and plant survival scores, QTL analysis was conducted for the chlorophyll content using the plant survival scores as covariate, resulting in no significant QTL for the chlorophyll content.
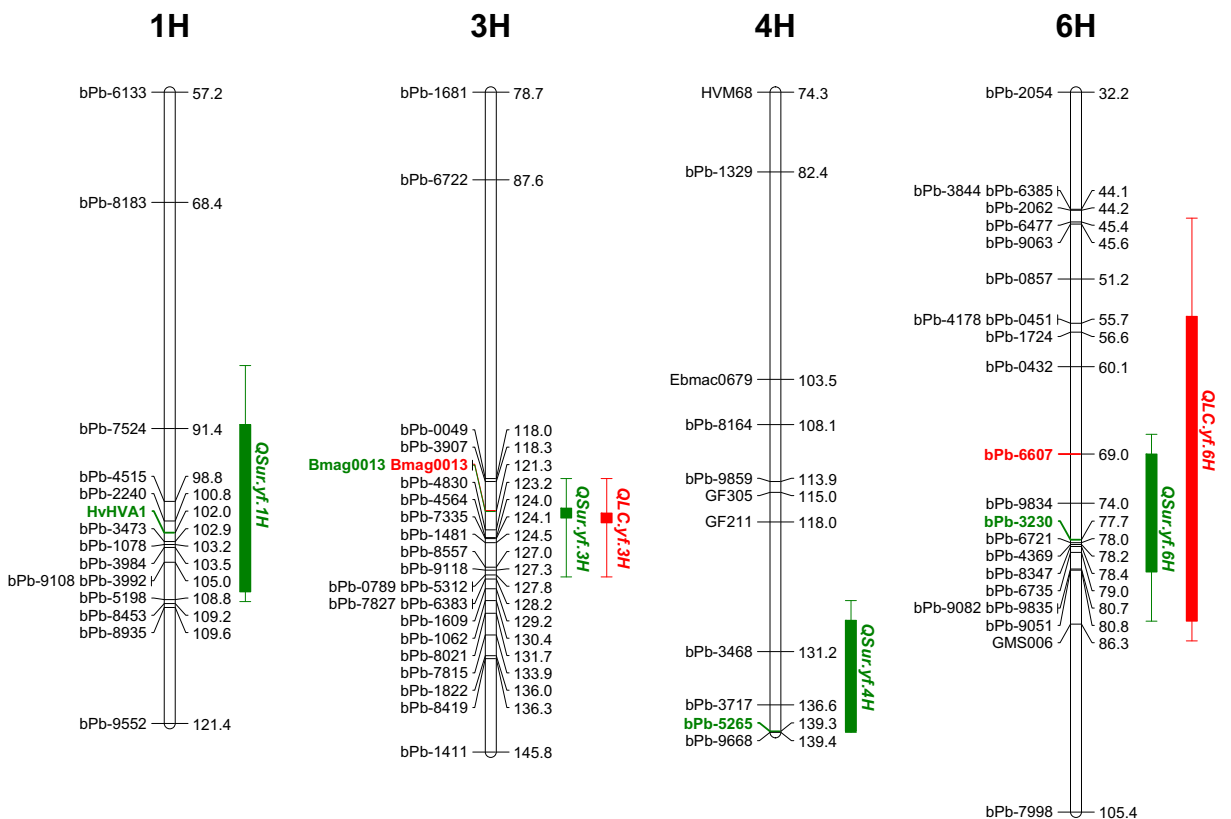

Fig. 3 The Yerong/Franklin chromosomes showing the locations of QTL for the traits analysed. Green: QTL for plant survival; red: QTL for leaf chlorophyll content

Validation of the major QTL for plant survival in the TX9425/Naso Nijo population

Given that chlorophyll content is closely correlated with plant survival score (Fig. 2D), we used plant survival as a suitable proxy to validate the QTL in a different population. In the TX9425/Naso Nijo DH population, only one significant QTL for plant survival under Mn^{2+} stress was identified. The TX9425 alleles increased tolerance to manganese toxicity at *QSur.tx.3H* locus, explaining 15% of the phenotypic variation (Table 1). This QTL was co-located with *QSur.yf.3H* (cf. Fig. 3).

Discussion

Waterlogging stress is one of the major abiotic yield-limiting factors in crops, and tolerance to this stress is likely to be a complex trait controlled by several mechanisms and complicated by various confounding factors. Because of the low efficiency of direct selection for waterlogging-tolerant plants in the field (Zhou 2010), various indirect criteria have been investigated. Among them, the severity of leaf chlorosis has been used to estimate the capacity for plant survival under waterlogging stress (Li et al. 2008; Zhou 2011). In our previous report, the severity of leaf chlorosis showed a significant inverse correlation with Mn^{2+} tolerance, and Mn^{2+} tolerance was significantly correlated with waterlogging tolerance in field conditions (Huang et al. 2015). In this experiment, a significant regression ($R^2 = 0.62$) between the severity of leaf chlorosis and plant survival under Mn^{2+} stress was also found in the Yerong × Franklin DH population (Fig. 2).

An understanding of the genetic background and mechanisms enhancing Mn^{2+} tolerance is crucial in the research and breeding programmes. Several transporters have been reported to be responsible for Mn accumulation and sequestration (Pittman 2005; Pedas et al. 2008; Cailliatte et al. 2010; Sasaki et al. 2012; Chen et al. 2013). However, no QTL has been reported for the tolerance to Mn^{2+} toxicity in barley. Most of the previous studies have targeted Mn^{2+}-use efficiency (Pallotta et al. 2000; McDonald et al. 2001; Hebbern et al. 2005), with a QTL identified on the short arm of chromosome 4H (Pallotta et al. 2000). In the study reported here,

barley tolerance to Mn^{2+} toxicity was investigated. Four significant QTL for plant survival (*QSur.yf.1H*, *QSur.yf.3H*, *QSur.yf.4H*, and *QSur.yf.6H*) and two for the severity of leaf chlorosis (*QLC.yf.3H* and *QLC.yf.6H*) under Mn^{2+} stress in the seedling stage were identified in the Yerong/Franklin population. Among them, *QSur.yf.3H* contributed more than 20% of the phenotypic variation for tolerance to Mn toxicity in the Yerong/Franklin population, whereas other QTL each explained less than 10%. To our surprise, only one minor QTL controlling plant survival under Mn^{2+} stress (*QSur.yf.4H*) was located at a similar position as the major QTL for waterlogging tolerance in the Yerong/Franklin population based on the plant survival scores after 9 weeks of waterlogging (Zhou 2011). The major QTL on chromosome 3H (*QSur.yf.3H*) and the minor QTL on 6H (*QSur.yf.6H*) are likely to be responsible for waterlogging tolerance at the early treatment stages because QTL for waterlogging tolerance on 3H and 6H were identified after 3 weeks and 2–5 weeks of waterlogging treatment, respectively (Zhou 2011).

TX9425 is a landrace from China showing better tolerance to waterlogging stress than Naso Nijo as a susceptible genotype (Zhou et al. 2007; Xu et al. 2012). However, the waterlogging tolerance of TX9425 is not as good as that of Yerong (Huang et al. 2015). In addition, TX9425 showed lower tolerance to Mn^{2+} toxicity (Fig. 1) and had higher shoot Mn^{2+} concentration under Mn^{2+} stress than Yerong (Huang et al. 2015). TX9425 appeared to have a high capacity to sequester Mn^{2+}, maintaining higher Mn content in the shoot than susceptible genotype Naso Nijo (Huang et al. 2015). A DH population from the cross of TX9425/ Naso Nijo was tested for Mn^{2+} tolerance in the present study, and only one major QTL for plant survival was identified on chromosome 3H. This QTL was at the similar position to the QTL for plant survival and leaf chlorosis (*QSur.yf.3H* and *QLC.yf.3H*) identified in the Yerong/Franklin population. Therefore, it is likely that the better tolerance of Yerong compared with TX9425 was due to other minor QTL absent in TX9425, i.e. *QSur.yf.1H*, *QSur.yf.4H*, and *QSur.yf.6H*.

Mn^{2+} toxicity can trigger oxidative stress in plant cells (Demirevska-Kepova et al. 2004) because high Mn concentration in the cytosol inhibits the non-cyclic photophosphorylation process (Lidon and Teixeira 2000), thus promoting an increase in the production of

ROS (specifically, hydroxyl radicals as the most aggressive of all ROS species). This Mn^{2+} toxicity-induced ROS accumulation may have consequences not only for structural integrity and organisation of the chloroplast lamellae (and, hence, photosynthetic machinery operation) but also for plant ionic relations. Of specific interest is a potential Mn impact on the cytosolic K^+ homeostasis. ROS (especially hydroxyl radicals) are known to be potent activators of various K^+-permeable channels (e.g. Demidchik et al. 2003, 2007, 2010; Zepeda-Jazo et al. 2011), prompting ROS stress-induced K^+ depletion in the cytosol. Importantly, K^+ is an activator of more than 70 key metabolic enzymes (Dreyer and Uozumi 2011), including those for chlorophyll biosynthesis and photosynthetic CO_2 assimilation. According to the new barley reference genome sequence and annotations (Mascher et al. 2017), a candidate gene (*HORVU3Hr1G068040.1*) that codes for *KAT3* was found in the region of the major QTL identified in the present study on chromosome 3H. *KAT3* is a member of the Shaker family of voltage-gated potassium channels (Sharma et al. 2013) that are expressed in rice guard cells (Hwang et al. 2013).

The other candidate gene *HORVU3Hr1G068200.1* that is also located in this QTL region appears to be more relevant to Mn^{2+} toxicity tolerance. This gene is identified as AK369780 in the GenBank database, a member of heavy metal-associated domain superfamily (HMA). Most of the known HMA transporters were involved in accumulation, transportation, or detoxification of zinc, copper, and cadmium (Takahashi et al. 2012; Deng et al. 2013; Satoh-Nagasawa et al. 2013; Huang et al. 2016). However, *HORVU3Hr1G068200.1* has not been assigned any putative function yet. The gene ontology (GO) data suggest that certain portions of the gene are related to heavy metal ion transport (GO:0030001) and metal ion binding (GO:0046872). This gene remains to be investigated further for its possible involvement in the regulation of Mn accumulation and detoxification.

In conclusion, the major QTL for Mn^{2+} toxicity tolerance was identified on chromosome 3H in the Yerong/Franklin DH population and validated in TX9425/Naso Nijo DH population. Three minor QTL were also found in the Yerong/Franklin population. All the QTL likely contribute to waterlogging tolerance in the early growth stage.

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