

Identification of salt tolerance QTL in a wheat RIL mapping population using destructive and non-destructive phenotyping

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Abstract. Bread wheat (*Triticum aestivum* L.) is one of the most important food crops, however it is only moderately tolerant to salinity stress. To improve wheat yield under saline conditions, breeding for improved salinity tolerance of wheat is needed. We have identified nine quantitative trait loci (QTL) for different salt tolerance sub-traits in a recombinant inbred line (RIL) population, derived from the bi-parental cross of Excalibur × Kukri. This population was screened for salinity tolerance subtraits using a combination of both destructive and non-destructive phenotyping. Genotyping by sequencing (GBS) was used to construct a high-density genetic linkage map, consisting of 3236 markers, and utilised for mapping QTL. Of the nine mapped QTL, six were detected under salt stress, including QTL for maintenance of shoot growth under salinity ($QG_{(1-5).asl-5A}$, $QG_{(1-5).asl-7B}$) sodium accumulation ($QNa.asl-2A$), chloride accumulation ($QCl.asl-2A$, $QCl.asl-3A$) and potassium : sodium ratio ($QK:Na.asl-2DS2$). Potential candidate genes within these QTL intervals were shortlisted using bioinformatics tools. These findings are expected to facilitate the breeding of new salt tolerant wheat cultivars.

Additional keywords: chloride, non-destructive phenotyping, potassium, salinity, shoot ion-independent tolerance, sodium, quantitative trait locus, wheat.

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Introduction

Wheat plays a major role in food security across the globe and the demand of wheat is expected to increase by 60% by 2050 (Nelson *et al.* 2010; Shiferaw *et al.* 2013). However, one of the major constraints for the productivity of wheat is soil salinity, which greatly reduces yield (Rengasamy 2002, 2006; Roy *et al.* 2014; Munns and Gilliam 2015). In Australia, it is estimated that 69% of the Australian wheatbelt is affected by salinity to some degree (Rengasamy 2002). Hence, to meet the growing demands of wheat consumption, there is an urgent

need to identify novel salt tolerance subtraits and develop salinity tolerant wheat cultivars.

To survive saline conditions, plants rely on a range of different tolerance mechanisms, including, but not limited to: excluding Na^+ and Cl^- from the shoot (Rashid *et al.* 1999; Poustini and Siosemardeh 2004; Roy *et al.* 2014; Ismail and Horie 2017); maintaining a high $K^+ : Na^+$ ratio in the leaves (Shabala and Cuin 2008; Shabala and Pottosin 2014; Hanin *et al.* 2016; Ali and Yun 2017); vacuolar sequestration of toxic ions (Hasegawa *et al.* 2000; Flowers and Colmer 2008; Munns *et al.* 2016; Ismail and

Horie 2017); compatible solutes synthesis (Munns *et al.* 2016, 2020; van Zelm *et al.* 2020); and maintenance of plant growth (Roy *et al.* 2014; Al-Tamimi *et al.* 2016; Tilbrook *et al.* 2017; Asif *et al.* 2018). Reductions in plant growth have been shown to occur during the first few minutes of salt stress, before ions can accumulate to high levels in the shoot tissue, and the ability of plants to maintain shoot growth is called shoot ion-independent tolerance or osmotic tolerance (Roy *et al.* 2014; Asif *et al.* 2018). Of the described tolerance mechanisms, little is known about the genes that control the mechanisms of shoot ion-independent tolerance and/or Cl⁻ accumulation in bread wheat, although several candidate genes have been proposed in recent studies (Genc *et al.* 2014; Asif *et al.* 2018). Shoot ion-independent tolerance is considered a complex mechanism and long-distance signalling (reactive oxygen species and Ca²⁺ signalling or long distance electrical signalling) is believed to be involved in this tolerance mechanism (Kudla *et al.* 2010; Roy *et al.* 2014; Al-Tamimi *et al.* 2016; van Zelm *et al.* 2020). Chloride, in contrast, is an essential micronutrient with metabolic functions in enzyme activation, photosynthesis, osmoregulation and movement of stomata, but it is toxic at high concentrations and can result in leaf chlorosis, growth reduction and yield loss (White and Broadley 2001; Tavakkoli *et al.* 2010; Li *et al.* 2017; Wege *et al.* 2017; Geilfus 2018). Identification and exploitation of genetic variation for these tolerance mechanisms have great potential to breed new salt tolerant wheat cultivars. Therefore, a forward genetics approach to identify quantitative trait loci (QTL) followed by fine mapping could lead to the detection of novel genes for these tolerance mechanisms.

QTL mapping is a valuable tool for studying complex polygenic traits like salinity tolerance in various crops (Ortiz 1998; Ruttan 1999; Roy *et al.* 2011). QTL for salinity tolerance have been identified in a large number of plant species including bread wheat (Asif *et al.* 2019); however, limited success has been made so far regarding the identification of candidate genes and development of new salt tolerant wheat cultivars (Gillilham *et al.* 2017; Asif *et al.* 2019). Hence, more studies are needed to identify and understand salt tolerance mechanisms and the underlying genes responsible for such traits. Here, we describe the genetic characterisation of a new recombinant inbred line (RIL) population and QTL associated with different salt tolerance subtraits. We speculate on the potential candidate genes within the QTL intervals which could be tested to develop new salt tolerant cultivars.

Materials and methods

Plant materials

An F₂:F₆ population of RILs, derived from a cross between single plants of two Australian wheat cultivars Excalibur-198 (RAC177/Unicula492/RAC311S) and Kukri-199 (76ECN44/76ECN36/RAC549/Madden/6*RAC177) were utilised for this study. Excalibur is a salt- and drought-tolerant cultivar with a high yield potential under South Australian conditions, but it has low grain quality and is susceptible to rust. Kukri produces excellent quality grain and is resistant to rust (Izanloo *et al.* 2008; Asif *et al.* 2018). Seeds of RILs and parents

(Excalibur and Kukri) were provided by Australian Centre for Plant Functional Genomics (ACPFPG), Australia.

Non-destructive glasshouse-based phenotyping

A phenotyping experiment was conducted on 128 RILs using the automated high-throughput imaging facility at The Plant Accelerator, Adelaide, South Australia (longitude 138.64, latitude -34.97). The 128 RILs and parents (Excalibur and Kukri) were phenotyped during late winter to early spring, 14 August to 16 September 2013 using a partially replicated (20%) split-plot design, using the same method described by Asif *et al.* (2018).

Plants were germinated and planted as described by Asif *et al.* (2018) with some minor changes. Salt treatment was applied by adding 212 mL of 170 mM NaCl at 27% (w/w) soil water content (573 ml) to the saucer of each salt stress pot to a final concentration of 100 mM NaCl in the soil after drying down to 17% (w/w) soil water (361 mL). Control pots received 212 mL of water on the same day to reach a soil water content of 27% (w/w) and allow drying down to 17% (w/w), the same as salt treated pots. After salt application, each pot was weighed and watered automatically, on a daily basis on the electronic conveyor system to maintain the soil water content at 17% (w/w) and 100 mM NaCl concentration in soil. Plant imaging, shoot ion-independent tolerance calculations and statistical analysis were performed as described previously by Asif *et al.* (2018).

Measurement of leaf Na⁺, K⁺ and Cl⁻ concentration

The fourth leaf, which was the fully developed leaf blade under salt stress treatment, was harvested 13 days after salt treatment and used to measure Na⁺, K⁺ and Cl⁻ contents. Fresh weight of leaf samples was recorded before samples were dried in an oven for two days at 65°C and the dry weight recorded. Samples were digested in 10 mL of 1% (v/v) HNO₃ at 85°C for 4 h in a 54-well Hotblock (Environmental Express). The concentration of Na⁺ and K⁺ were measured using a flame photometer (Model 420 Sherwood), while Cl⁻ was measured using a chloride analyser (Model 926 Sherwood).

Genotyping of the RIL population

Genomic DNA was extracted from leaf material using a phenol/chloroform method described previously by Rogowsky *et al.* (1991) and Pallotta *et al.* (2000). The DNA concentration was quantified by PicoGreen (Ahn *et al.* 1996). 272 RILs were genotyped using genotyping by sequencing (GBS) to identify single nucleotide polymorphism (SNP) markers for high density genetic map construction. GBS libraries were prepared using protocols described in Elshire *et al.* (2011) and Poland *et al.* (2012). DNA samples of all the RILs and parents were digested with two restriction enzymes (*Pst*I – CTGCAG and *Msp*I – CCGG) for complexity reduction and barcoded with DNA adapters, designed following the criteria described by Poland *et al.* (2012) (Table S1, available as Supplementary Material to this paper). Three multiplex GBS libraries, each having 96 samples (93 RILs, two parents and one negative control), were sequenced using the Illumina NextSeq500 platform at the Australian Genome

Research Facility (AGRF, Adelaide, South Australia). Sequencing data was processed using the Universal Network Enabled Analysis Kit (UNEAK), which is the non-reference GBS SNP calling pipeline and an extension of the Java program of TASSEL (Lu *et al.* 2012). The heterozygous SNP calls were assigned as missing data and only the SNP markers which contained less than 20% missing data were used for map construction.

RILs were further genotyped for the phenology genes *Ppd-2A*, *Ppd-2B* and *Vrn-A1* and 16 Kompetitive Allele Specific PCR (KASP) polymorphic markers by a KASP assay (<https://www.biosearchtech.com/products/pcr-kits-and-reagents/genotyping-assays/kasp-genotyping-chemistry>, accessed 4 August 2020) (Table S2).

Genetic map construction

Map construction was performed using R/ASMap following the instructions outlined by Taylor (2015). Genotypic data was checked for lines with missing data (>25%), segregation distortion (an allele frequency of either parent at <0.4 and >0.6, at $P = 0.05$) and clonal individuals (similarity of >90%) using the appropriate functions in R/ASMap (Taylor 2015). The data outside the threshold ranges were removed and remaining markers and lines were used for map construction. Recombination fractions were converted to cM distances using the Kosambi mapping function and the final map was constructed using 3236 markers for the 128 RILs that were phenotyped. The total length of the genetic map was 3084 cM, with a marker density of 0.95 cM per marker (Table 1). Chromosome numbers were assigned to each linkage group (LG) using the sequence reads that were outputted from the UNEAK pipeline for each of the SNPs that were identified. These sequences were assigned to each of the chromosomes using an in-house BLAST portal with a BLASTn performed against the IWGSC RefSeq v1.0 (IWGSC, <https://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies>, accessed 2 August 2020). The limit of acceptance of assignment was based on the percentage of similarity (>96%) and the final percentage of matches (80–100%) between the query (the SNP markers) and the hit from the sequence database.

The A and B genome were well represented among all of the LGs. A total of 1478 markers were assigned to the A genome, which consisted of 1415 cM of the genetic map (0.96 cM per marker) (Table 1). The B genome accounted for 1007 cM with 1459 markers (0.69 cM per marker) (Table 1). The D genome was the least represented group with 299 markers spanning 662 cM (2.22 cM per marker) (Table 1).

QTL analysis

Composite interval mapping (CIM) was performed on 128 RILs using WinQTLCart-vers. 2.5 (Model 6 standard analysis with five control markers and a window size of 10 cM) (Wang *et al.* 2012) (Table S3). Log of odds (LOD) value thresholds were determined with 1000 times permutations (Churchill and Doerge 1994) at a 1 cM walk speed ($P = 0.05$). Significant QTL were summarised with their position on a linkage group,

Table 1. Marker distribution, density and length of the Excalibur × Kukri RIL genetic linkage map

Linkage groups	Number of markers	Length (cM)	Marker density (cM/markers)
1A	251	196.14	0.78
1B	130	84.25	0.65
1BL	33	47.54	1.44
1D	66	148.53	2.25
2A	183	191.45	1.05
2B	384	196.77	0.51
2DS1	17	65.9	3.88
2DS2	33	35.66	1.08
3A	193	197.08	1.02
3B	234	187.2	0.80
3D	5	66.64	13.33
3DL	25	3.09	0.12
4A	227	191.34	0.84
4B	0	0	0.00
4D	6	17.03	2.84
4DL	3	13.88	4.63
5A	182	234.08	1.29
5B	199	112.61	0.57
5BL	59	73.72	1.25
5D	10	46.28	4.63
5DL	25	26.06	1.04
6A	201	155.96	0.78
6B	225	151.27	0.67
6D	10	22.6	2.26
6DS	10	7.03	0.70
6DL	32	18.73	0.59
7A	241	248.9	1.03
7B	195	153.51	0.79
7D	41	187.6	4.58
7DS	16	3.29	0.21
A genome	1478	1414.95	0.96
B genome	1459	1006.87	0.69
D genome	299	662.32	2.22
Total	3236	3084.14	0.95

LOD score, magnitude and directions of their estimated additive effects and their contribution to the genetic variance. Map graphics and QTL positions were drawn using MapChart 2.1 (Voorrips 2002). The notation for individual QTL followed the format previously described by Asif *et al.* (2018). A QTL region was defined as unique if it was further than 15 cM from a neighbouring QTL (Sewell *et al.* 2000; Sewell *et al.* 2002).

Physical mapping of the QTL

To determine the potential candidate genes within the QTL intervals, all the markers (Tables S4, S5) that were up to two LOD drops from the maximum likelihood value of selected QTL were used for BLASTn against the IWGSC RefSeq v1.0 (IWGSC, as above) using an in-house BLAST portal as described previously by Asif *et al.* (2018). Only the query sequence having a cumulative identity percentage of similarity (>96%) and a cumulative alignment length

percentage of matches (90–100%) to the hit from the sequence database were shortlisted. All the scaffolds that were within the QTL intervals were retrieved from the BLAST results and used to find expressed genes on the scaffolds, using DAWN (diversity among wheat genomes) (Watson-Haigh et al. 2018) and POTAGE (PopSeq Ordered *Triticum aestivum* Gene Expression) (Suchecki et al. 2017). DAWN integrates data from the *Triticum aestivum* Chinese Spring IWGSC RefSeq v1.0 genome with public whole genome sequencing and exome data from 17 and 62 bread wheat accessions, respectively (Watson-Haigh et al. 2018) and RNA-Seq expression data from five wheat tissues (root, leaf, stem, spike and grain), taken at three developmental stages (seedling, vegetative and flowering). POTAGE integrates map location with gene expression and inferred functional annotation and visualises these data through a web browser interface (Suchecki et al. 2017).

Results

Glasshouse phenotyping of RILs

To assess the responses of plants to salinity, we exposed a total of 128 RILs and parents (Excalibur and Kukri) to 100 mM NaCl salt stress for 13 days; their growth during this period was monitored using high-throughput, non-destructive imaging. Images were used to extract the projected shoot area (PSA), which in turn was used to estimate the relative growth rate (RGR) of plants in the interval of 1–5 days after salt treatment. The ratio of RGR (RGR salt/RGR control) during this period was used to calculate the shoot ion-independent tolerance. The range of shoot ion-independent tolerance within the RIL population varied from 0.51 to 1.16 (Table 2).

The ionic contents (Na^+ , K^+ , and Cl^-) in the fourth leaf of the RILs growing in 100 mM saline soil for 13 days revealed considerable variation between the lines (Table 2). The mean and standard error of shoot Na^+ accumulation for this population was $215 \pm 21 \mu\text{mol g}^{-1} \text{DW}$. The majority of lines had a concentration between 50 and $600 \mu\text{mol g}^{-1} \text{DW}$, with seven lines having a leaf Na^+ concentration greater than $600 \mu\text{mol g}^{-1} \text{DW}$ and the highest concentration observed at $1877 \mu\text{mol g}^{-1} \text{DW}$ (Table 2). By contrast, K^+ accumulation in

the leaf followed a closer to normal distribution and had a population mean of $1013 \pm 14 \mu\text{mol g}^{-1} \text{DW}$ and ranged from 364 to $1377 \mu\text{mol g}^{-1} \text{DW}$ (Table 2). The fourth leaf Cl^- accumulation exhibited a distribution similar to that of Na^+ with a population mean of $458 \pm 21 \mu\text{mol g}^{-1} \text{DW}$ (Table 2).

QTL mapping

A total of nine QTL at eight unique locations on seven linkage groups were detected under salt stress (100 mM NaCl; six QTL) and control (0 mM NaCl; three QTL) treatments (Table 3; Fig. 1). The phenotypic variation explained by a single QTL ranged between 8.7 and 14.6% (Table 3).

The ability to maintain growth under salinity (shoot ion-independent tolerance) was mapped to two QTL on chromosomes 5A (*QG₍₁₋₅₎.asl-5A*) and 7B (*QG₍₁₋₅₎.asl-7B*) (Table 3; Fig. 1). The LOD score of the 5A QTL was 3.8 and explained 10.9% of the phenotypic variation. The second QTL on chromosomes 7B had a LOD score of 3.4 and accounted for 8.7% of the phenotypic variation. The 5A QTL had a positive effect from the Excalibur parent, while the favourable allele of the 7B QTL was from Kukri (Table 3). Under control treatment a significant QTL for relative growth rate (RGR) was also identified in another region on chromosome 5A (*QCRGR.asl-5A*) with a LOD score of 4.1 and explaining 11.2% of the phenotypic variation. The additive effect of this QTL was very small (0.003) with the positive allele inherited from the Excalibur parent (Table 3).

Table 2. Frequency distribution of shoot ion-independent tolerance (RGR salt/RGR control, Days 1–5, fourth leaf Na^+ ($\mu\text{mol g}^{-1} \text{DW}$), K^+ ($\mu\text{mol/g DW}$) and Cl^- ($\mu\text{mol g}^{-1} \text{DW}$) accumulation in the Excalibur \times Kukri RIL population. RGR, relative growth rate

Trait	Excalibur	Kukri	RIL population	
			Mean \pm s.e.	Range
Growth ₍₁₋₅₎	0.91	0.93	0.90 ± 0.007	0.51–1.16
Na^+ ($\mu\text{mol g}^{-1} \text{DW}$)	208	178	215 ± 21	47–1877
K^+ ($\mu\text{mol g}^{-1} \text{DW}$)	899	1007	1013 ± 14	364–1377
Cl^- ($\mu\text{mol g}^{-1} \text{DW}$)	513	412	458 ± 21	187–2010

Table 3. Quantitative trait loci (QTL) for salt tolerance traits determined in the Excalibur \times Kukri RIL mapping population under control and salt stress (100 mM NaCl for 13 days) conditions

Trait name, QTL name, treatment, chromosome number (Chr), position (cM), physical position (bp) based on IWGSC RefSeq v1.0, marker (most significant marker), log of odds (LOD), additive effect and phenotypic variation (R^2) explained by the QTL (% variation) are shown. CRGR, control relative growth rate (Days 1 to 5), Growth₍₁₋₅₎, RGR of plants between 1–5 days after salt stress (RGR salt/RGR control)

Trait	QTL	Treatment	Chr	Position	Physical position	Marker	LOD	Additive effect	R^2
Growth ₍₁₋₅₎	<i>QG₍₁₋₅₎.asl-5A</i>	Salt	5A	229.6	696479487	TP14539	3.8	0.03	10.9
Growth ₍₁₋₅₎	<i>QG₍₁₋₅₎.asl-7B</i>	Salt	7B	119.9	689968548	TP211556	3.4	-0.03	8.7
RGR	<i>QCRGR.asl-5A</i>	Control	5A	143.1	584618687	TP141568	4.1	0.003	11.2
Na^+ ($\mu\text{mol g}^{-1} \text{DW}$)	<i>QNa.asl-2A</i>	Salt	2A	141.6	716221845	TP81191	3.5	-128.2	10.3
Cl^- ($\mu\text{mol g}^{-1} \text{DW}$)	<i>QCl.asl-2A</i>	Salt	2A	141.6	716221845	TP81191	3.4	-80.8	9.7
Cl^- ($\mu\text{mol g}^{-1} \text{DW}$)	<i>QCl.asl-3A</i>	Salt	3A	159.7	718544292	TP87398	3.6	-83.7	10.6
Cl^- ($\mu\text{mol g}^{-1} \text{DW}$)	<i>QCl.asl-1A</i>	Control	1A	108.3	520571878	TP112034	3.2	15.7	9.2
$\text{Na}^+ : \text{K}^+ \text{ DW}$	<i>QNa:K.asl-2B</i>	Control	2B	131.7	696278400	TP9739	4.9	0.02	14.6
$\text{K}^+ : \text{Na}^+ \text{ DW}$	<i>QK:Na.asl-2DS2</i>	Salt	2DS2	1.97	14379318	TP37342	3.6	1.7	10.2

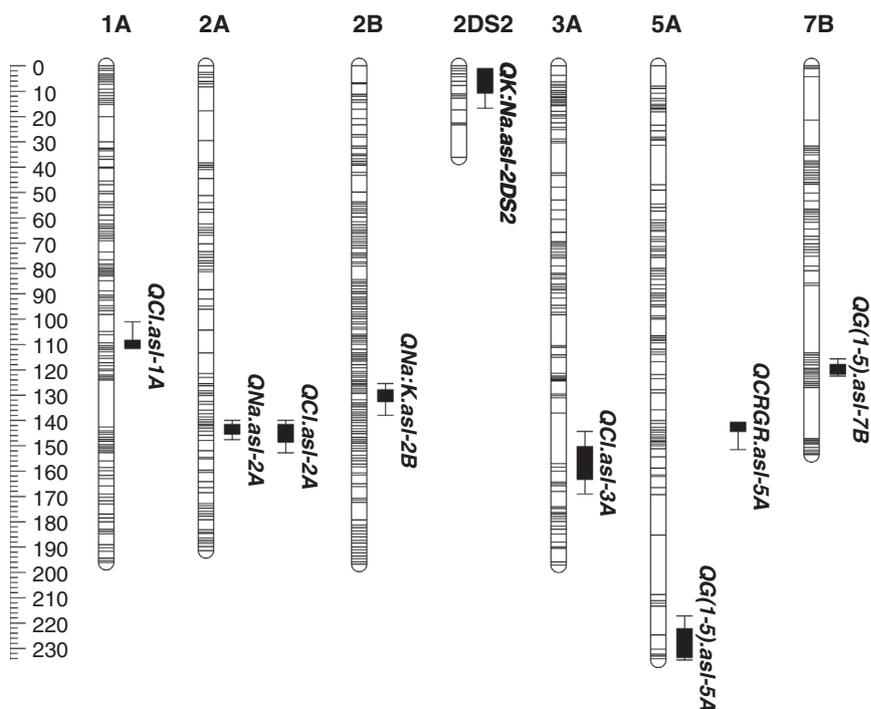


Fig. 1. Position of quantitative trait loci (QTL) detected in Excalibur × Kukri recombinant inbred lines (RILs) population under control and salt stress (100 mM NaCl for 13 days) conditions. The vertical QTL bars represent the one and two log of odds (LOD) drops from the QTL maximum likelihood value. QTL and their position are indicated: for Growth₍₁₋₅₎ under salinity (*QG*₍₁₋₅₎.*asl-5A*, *QG*₍₁₋₅₎.*asl-7B*), relative growth rate in control conditions (*QCRGR.asl-5A*), fourth leaf Na⁺ (μmol g⁻¹ DW) accumulation in 100 mM NaCl (*QNa.asl-2A*), Cl⁻ (μmol g⁻¹ DW) accumulation in 100 mM NaCl (*QCl.asl-2A*, *QCl.asl-3A*) and control conditions (*QCl.asl-1A*), Na⁺:K⁺ (DW) in control conditions (*QNa:K.asl-2B*) K⁺:Na⁺ (DW) in 100 mM NaCl conditions (*QK:Na.asl-2DS2*).

A single QTL for fourth leaf Na⁺ accumulation under salt treatment was mapped on chromosome 2A (*QNa.asl-2A*) with a LOD score of 3.5. It accounted for 10.3% of the phenotypic variation with the allele for Na⁺ exclusion linked to Excalibur (Table 3).

A total of three QTL were associated with fourth leaf Cl⁻ exclusion including two QTL in salt treated plants and one under control (Table 3; Fig. 1). The QTL detected under the salt treatment were mapped on chromosome 2A (*QCl.asl-2A*) and 3A (*QCl.asl-3A*) with LOD scores of 3.4 and 3.6, respectively. These two QTL accounted for 9.7 and 10.6% of the phenotypic variation and the Cl⁻ exclusion allele for both was inherited from the Excalibur parent (Table 3). A single QTL detected on chromosome 1A under control treatment had a LOD score of 3.2 which accounted for 9.2% of the phenotypic variation with the favourable allele for Cl⁻ exclusion derived from Kukri (Table 3).

One QTL was identified for Na⁺:K⁺ DW under control treatment on chromosome 2B (*QNa:K.asl-2B*). This QTL had a LOD score of 4.9 and explained 14.6% of the phenotypic variation with the allele for Na⁺ exclusion derived from the Kukri parent (Table 3). One QTL for K⁺:Na⁺ DW was detected under salt stress on chromosome 2DS2 (*QK:Na.asl-2DS2*) with a LOD score of 3.6 and phenotypic variance

of 10.2%. The beneficial allele for this QTL was inherited from the Excalibur parent (Table 3).

Predicted genes within the QTL intervals

All the scaffolds from the bread wheat IWGSC RefSeq v1.0 (IWGSC, as above) that were within two LOD drops from the maximum likelihood value of the QTL observed under salt treatment (*QG*₍₁₋₅₎.*asl-5A*, *QG*₍₁₋₅₎.*asl-7B*, *QNa.asl-2A*, *QCl.asl-2A*, *QCl.asl-3A*, *QK:Na.asl-2DS2*) were retrieved from BLAST results and used to investigate the presence of potential candidate genes using DAWN (Watson-Haigh *et al.* 2018) and POTAGE (Sucheck *et al.* 2017). Potential candidate genes were selected based on their role in salinity tolerance as published in literature.

The first QTL for shoot ion-independent tolerance or maintenance of growth under salinity on chromosome 5A (*QG*₍₁₋₅₎.*asl-5A*) spanned six scaffolds (scaffold45192, scaffold15805, scaffold4453, scaffold43014, scaffold21530, scaffold34444–3) and contained 184 expressed genes (Table S6) which contained several potential candidates for the phenotype including the purple acid phosphatases (*PAPs*) and a nitrate transporter (*NRT1.1*) (Table 4). The second QTL for this tolerance sub-trait identified on chromosome 7B (*QG*₍₁₋₅₎.

Table 4. List of potential candidate genes within the interval of each quantitative trait locus

For each gene, Gene ID, Gene name, Munich Information Centre for Protein Sequences (MIPS) annotation hit ID and rice annotation hit ID is given

QTL	Gene ID	Gene name	MIPS annotation Hit ID	Rice annotation Hit ID
<i>QG₍₁₋₅₎,asl-5A</i>	Traes_5AL_F7270AC94	Purple acid phosphatase	AT5G50400.1	LOC_Os08 g41880.1
	Traes_5AL_394CDB9BC	Purple acid phosphatase	AT5G50400.1	LOC_Os08 g41880.1
	Traes_5AL_F792D0298	Purple acid phosphatase	AT1G52940.1	LOC_Os12 g44010.1
	Traes_5AL_0AB5FEA83	Nitrate transporter 1.1	AT1G12110.1	LOC_Os03 g01290.1
<i>QG₍₁₋₅₎,asl-7B</i>	Traes_7BL_AABF91B01	Calmodulin like protein	AT3G01830.1	LOC_Os01 g72550.1
	Traes_2AL_B854D3399	V-type proton ATPase	sp Q8AVM5 VPP1_XENLA	LOC_Os10 g10500.1
<i>QNa.asl-2A and QCl.asl-2A</i>	Traes_2AL_3E61D4DE2	V-type proton ATPase	sp O13742 VPH1_SCHPO	LOC_Os10 g10500.1
	Traes_2AL_30BE9A333	Calcium/calmodulin-dependent protein kinases	AT2G17290.1	LOC_Os04 g49510.3
<i>QCl.asl-3A</i>	Traes_3AL_509408B05	MATE efflux family protein	AT3G21690.1	LOC_Os03 g37640.1
	Traes_3AL_9FC13B618	MATE efflux family protein	AT3G21690.1	LOC_Os03 g37640.1
	Traes_3AL_61D8E92DB	MATE efflux family protein	AT3G21690.1	LOC_Os03 g37640.1
	Traes_3AL_39236690F	MATE efflux family protein	AT1G61890.1	LOC_Os03 g37640.1
	Traes_3AL_3AA7AE317	MATE efflux family protein	AT3G21690.1	LOC_Os0 g37640.1
<i>QK:Na.asl-2DS2</i>	Traes_2DS_981F332F2	Sodium/hydrogen exchanger 7	sp Q9 LKW9 NHX7_ARATH	LOC_Os12 g44360.4

asl-7B) was located on four scaffolds (scaffold101790, scaffold101708, scaffold138378, scaffold85256) containing a total of 88 expressed genes with a calmodulin like (CML) protein shortlisted as a potential candidate (Table S6).

Two scaffolds (scaffold52744, scaffold78889) were found within the QTL region for *QNa.asl-2A*, which contained 280 expressed genes (Table S6). Candidate genes selected within this interval include those coding for a vacuolar type H⁺-ATPase (V-ATPase) and calcium/calmodulin-dependent protein kinases (CaMKs) based on their role in salt tolerance (Kirsch *et al.* 1996; Dietz *et al.* 2001; Pandey *et al.* 2002; Beyenbach and Wiczorek 2006; Yang *et al.* 2011; Lv *et al.* 2017) (Table 4).

The Cl⁻ accumulation QTL (*QCl.asl-2A*) identified under salinity stress was located across four scaffolds (scaffold52744, scaffold78889, scaffold13951, scaffold21915) containing 317 expressed genes (Table S6). There are an additional 37 genes located within this interval compared with *QNa.asl-2A*, however none of these genes appear as additional candidates to those listed above. For *QCl.asl-3A*, a total of four scaffolds (scaffold87465, scaffold60640, scaffold10162, scaffold29358) were identified having 108 expressed genes (Table S6). A Multidrug and Toxic Compound Extrusion (MATE) protein was shortlisted as a candidate gene based on its role in Cl⁻ transport (Zhang *et al.* 2017) (Table 4).

For the K⁺:Na⁺ DW QTL (*QK:Na.asl-2DS2*), a total of five scaffolds (scaffold65451, scaffold 42730, scaffold87109, scaffold32556, scaffold38944) with 170 expressed genes were retrieved (Table S6). Within this QTL region a sodium/hydrogen exchanger 7 (*NHX7*) or a salt overly sensitive 1 (*SOS1*) was selected as a potential candidate due to its role in Na⁺ homeostasis (Zhu 2003; Olías *et al.* 2009; Ullah *et al.* 2016) (Table 4).

Discussion

QTL detection using the newly constructed genetic map revealed a total of nine QTL at eight unique locations on seven different chromosomes for several salt tolerance sub-

traits. These include novel QTL for shoot ion-independent tolerance or maintenance of shoot growth under salinity (*QG₍₁₋₅₎,asl-7B*), Cl⁻ accumulation (*QCl.asl-3A*) and K⁺:Na⁺ DW (*QK:Na.asl-2DS2*) (Table 3; Fig. 1) with mining of the bread wheat reference sequence allowing the identification of candidate genes within these regions.

To date, a limited number of studies have been conducted to identify QTL linked with shoot ion-independent tolerance and/or Cl⁻ accumulation in bread wheat, with the majority of studies focusing on the identification of QTL for shoot ion accumulation (mostly Na⁺ exclusion and K⁺ accumulation) (Dubcovsky *et al.* 1996; Ma *et al.* 2007; Genc *et al.* 2010, 2013; Díaz De León *et al.* 2011; Oyiga *et al.* 2018; Asif *et al.* 2019). Shoot ion-independent tolerance is an important tolerance mechanism and helps plants in maintaining tissue expansion and tillering during the initial phase of salt stress before salt accumulates to toxic levels in the shoot (Roy *et al.* 2014). Recent advancements in non-destructive imaging technology have helped to study this tolerance mechanism in more detail and identifying a QTL in bread wheat on chromosome 7A (Asif *et al.* 2018).

In this study, two QTL were detected for shoot ion-independent tolerance on chromosomes 5A (*QG₍₁₋₅₎,asl-5A*) and 7B (*QG₍₁₋₅₎,asl-7B*) (Table 3; Fig. 1). The physical position of the QTL *QG₍₁₋₅₎,asl-5A* is in the same QTL region identified by Oyiga *et al.* (2018) for shoot DW under salt stress signifying the importance of this locus in maintenance of plant biomass under salinity and is some distance away from the known developmental gene, vernalisation gene *Vrn-A1*. However, another QTL on Chromosome 5A controlling plant biomass (*QCRGR.asl-5A*) was detected in the a region of *Vrn-A1*. This shows that *Vrn-A1* gene could have an effect on plant biomass.

To the best of our knowledge *QG₍₁₋₅₎,asl-7B* is novel, and no other QTL in this region have been reported before under salt treatment. Analysis of shoot ion-independent QTL, *QG₍₁₋₅₎,asl-5A*, in DAWN and POTAGE showed several salt tolerance genes within the region under the QTL, such as, *PAPs*, and *NRT1.1* (Table 4). *PAPs* belong to a diverse group

of acid phosphatases and are found in plants, animals and microorganisms (Schenk *et al.* 2000; Olczak *et al.* 2003). In plants, the majority of PAPs catalyse the hydrolysis of phosphate esters and anhydrides (Zhang *et al.* 2011; Schenk *et al.* 2013); however, recent studies also showed their role in improving plant growth and alleviating oxidative damage during salt stress (Li *et al.* 2008; Deng *et al.* 2014) which makes them attractive candidates from this study. The second candidate, NRT1.1 modulates the nitrate-dependent Na^+ transport in *Arabidopsis* under saline conditions and helps in osmotic adjustment, which prevents water loss and wilting during salt stress (Álvarez-Aragón and Rodríguez-Navarro 2017). Among 88 genes within the interval of $QG_{(1.5),asl-7B}$ a CML protein was shortlisted as a potential candidate (Table 4). CML protein belongs to calcium-binding EF-hand family proteins, which play an important role in cellular calcium signalling cascades through the regulation of numerous target proteins (Ranty *et al.* 2006; Shi and Du 2020). This protein has a role in salinity tolerance and genes related to CML are shown to be upregulated under salt stress (Zeng *et al.* 2015; Dubrovina *et al.* 2019; Shi and Du 2020) and has improved the salt tolerance of *Arabidopsis* by affecting abscisic acid mediated pathways (Magnan *et al.* 2008).

A novel QTL for Cl^- concentration was detected on chromosome 3A $QCl.asl-3A$ under 100 mM salt stress in the glasshouse (Table 3; Fig. 1). Previously a QTL for Cl^- accumulation has been mapped on chromosome 3A under hydroponics and field conditions (Genc *et al.* 2014) but its physical location is different to the $QCl.asl-3A$ reported in this study. For $QCl.asl-3A$, the MATE transporters were identified as a potential candidate (Table 4). MATE are widely accepted as transporters of organic compounds (Li *et al.* 2002; Marinova *et al.* 2007; Dobritzsch *et al.* 2016; Zhang *et al.* 2017); however, a recent study has also shown the role of two tonoplast MATE-type proteins in sequestration of Cl^- into the vacuole which can be helpful in controlling the toxic Cl^- concentration in the cytoplasm under saline conditions (Zhang *et al.* 2017).

Using the new IWGSC reference sequence, (as above), it appears that the QTL for leaf Na^+ accumulation ($QNa.asl-2A$) identified in this study is not in the same position as the *Nax1* locus (35 Mbp away) (Lindsay *et al.* 2004), $Q.Na2A$ locus (88 Mbp away) (Genc *et al.* 2010, 2013) and significant Na^+ concentration SNP locus on chromosome 2A (46 Mbp away) (Genc *et al.* 2019). However, $QNa.asl-2A$ may be the same locus as Na^+ content and $\text{K}^+:\text{Na}^+$ QTL described by (Oyiga *et al.* 2018), as their markers sit on the same IWGSC scaffold (52744). The $QNa.asl-2A$ is also away (74.7 cM) from the photoperiodinsensitive gene *Ppd-A1*, which means this gene has no effect on leaf Na^+ accumulation. The QTL ($QNa.asl-2A$) was associated with fourth leaf Na^+ exclusion under salinity stress in the glasshouse and is co-located with a Cl^- exclusion QTL ($QCl.asl-2A$) (Table 3; Fig. 1). The physical position of both $QNa.asl-2A$ and $QCl.asl-2A$ based on the IWGSC reference sequence 1.0, (as above) indicates that these are on the long arm of chromosome 2A where other QTL have previously been detected for leaf Na^+ accumulation (Genc *et al.* 2010, 2013; Oyiga *et al.* 2018), $\text{K}^+:\text{Na}^+$ (Oyiga *et al.*

2018), Cl^- accumulation (Genc *et al.* 2014), maturity (Díaz De León *et al.* 2011), tiller number (Díaz De León *et al.* 2011) and seedling biomass (Genc *et al.* 2010, 2014) under salt stress in bread wheat. Co-location of ion accumulation (Na^+ , Cl^-) and biomass related QTL indicates that more than one gene for the salt tolerance sub-traits may be present within this region. These may include genes such as *V-ATPase* and *CaMKs* which are shortlisted as a candidate for $QNa.asl-2A$ (Table 4). *V-ATPases* are involved in pumping protons into the vacuole and establishing an electro chemical gradient used by the Na^+/H^+ antiporters to sequester Na^+ into the vacuole which in return improves plant performance under saline conditions (Kirsch *et al.* 1996; Dietz *et al.* 2001; Beyenbach and Wiczorek 2006; Lv *et al.* 2017). *CaMKs* are Ca^{2+} -regulated protein kinases and play a key role in stress signalling (Zhang and Lu 2003; Wang *et al.* 2004). These kinases do not directly bind Ca^{2+} by themselves, but instead interact with a specific Ca^{2+} sensor, such as calmodulin (CaM) or calcineurin B-like protein (CBL) (Zhang and Lu 2003; Wang *et al.* 2004) known for their role in salt tolerance (Pandey *et al.* 2002; Yang *et al.* 2011).

A novel QTL for $\text{K}^+:\text{Na}^+$ DW was detected on the short arm of chromosome 2D ($QK:Na.asl-2DS2$) (Table 3; Fig. 1). Previous studies (Ma *et al.* 2007; Genc *et al.* 2010, 2019; Oyiga *et al.* 2018), found QTL for other salt tolerance subtraits on chromosome 2D including plant biomass, chlorophyll content, leaf chlorosis and leaf Na^+ accumulation, however, at a different region of the chromosome based on the physical position. Of the 170 genes within the region of $QK:Na.asl-2DS2$, a *NHX7* was shortlisted as potential candidate based on its role in salt tolerance by limiting the Na^+ accumulation in plants (Shi *et al.* 2003; Ullah *et al.* 2016) (Table 4).

It should be noted that DAWN (Watson-Haigh *et al.* 2018) and POTAGE (Suchecki *et al.* 2017) bioinformatics tools use databases that have genes expressed only under non-saline conditions, hence, it is possible that there could be other salt responsive genes present in these intervals that have not been detected due to a lack of gene expression data of wheat under salinity stress.

Phenotyping experiments conducted under controlled environments do not always mimic field conditions. Detection of salt stress related traits in the greenhouse under a limited stress period and at a specific growth stage will not necessarily translate to improved yields under field conditions. A single salt tolerance sub-trait cannot always guarantee yield improvement. Traits such as early vigour, at the seedling stage, may be detrimental to final grain yield under field conditions, as the plant may accumulate greater biomass, therefore having reduced water use efficiency. Hence, multiple salinity tolerance subtraits are required over the lifespan of the plant to contribute towards improved yield. Future studies are needed to develop NILs around the key salt tolerance QTL identified in the present study, to evaluate them under a range of salinity levels in the field, and to identify the best alleles for future breeding programs, as was done by Asif *et al.* (2018).

In summary, novel QTL have been identified for shoot ion-independent tolerance ($QG_{(1.5),asl-7B}$), Cl^- accumulation ($QCl.asl-3A$) and $\text{K}^+:\text{Na}^+$ DW ($QK:Na.asl-2DS2$) in bread wheat. The detection of shoot ion-independent tolerance and

Cl⁻ accumulation QTL in this study will help to better understand the genetic control of these mechanisms in bread wheat and has the potential to speed up breeding for these sub-traits. Future work should focus on studying the effect of these loci on yield under saline field conditions followed by fine mapping and differential expression of candidate gene(s) between the two parents.

Conflicts of interest

The authors declare no conflicts of interest.

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