Toward Map-based Cloning of a Na⁺ Exclusion Gene From Barley 
*(Hordeum vulgare L.)*

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ABSTRACT

Salinity is a major abiotic stress reducing crop productivity around the world. Mechanisms of salt tolerance are still largely unknown, and more work is needed to unravel the systems plants use to tolerate toxic levels of salt in their growing environment. The diploid species barley is relatively salt tolerant and therefore represents a useful model for studying salinity tolerance in the cereals. The ability to exclude Na\(^+\) from the shoot is one component of salinity tolerance, and can be studied by measuring Na\(^+\) accumulation in the shoot using ICPOES (inductively coupled plasma optical emission spectrometer) or flame photometry.

A population of 150 barley doubled-haploid (DH) lines generated from a cross between Australian cultivar Clipper and the Algerian landrace Sahara 3771 was developed at the Waite Campus of the University of Adelaide. Four separate soil-based experiments by three researchers found that this population segregated for Na\(^+\) accumulation in the shoot, and that this trait was controlled by a major QTL (quantitative trait locus) on the long arm of chromosome 1H, which was named Hv\textit{Nax3} (Chapter 3). The locus accounted for 80, 84, 77 and 40% of the total variation in shoot Na\(^+\) concentration in these experiments. Interestingly, another experiment revealed no Na\(^+\) accumulation effect of this QTL at all (Quinn, 2003), indicating that expression of the QTL was strongly dependent on the environmental conditions. Considering the phenotype distribution of non-recombinants for the Hv\textit{Nax3} interval and the phenotype distribution of recombinants, it was possible to map Hv\textit{Nax3} as a Mendelian locus.

Sequences of markers in the Hv\textit{Nax3} region were used to identify a 6.6 Mb corresponding interval on rice chromosome 5 (Chapter 4). Co-linearity between the rice and barely genomes was used to identify barley ESTs and rice genes to generate new CAPS markers in the Hv\textit{Nax3} region. Mapping and polymorphisms surveys with the marker fragments suggested that the Hv\textit{Nax3} region is a region of high recombination frequency but low polymorphism. Mapping newly generated markers reduced the interval on rice chromosome 5 to about 2 Mb. Comparative mapping revealed that co-linearity was interrupted by duplication, inversions and transpositions. The existence of these
inversions and the low polymorphism frequency in this region hampered the generation of markers.

Experiments in hydroponics, sand and soils were carried out to characterize the effect of the HvNax3 locus on Na⁺, K⁺ accumulation in organs or whole shoot (Chapter 5). The experiments were conducted in growth chambers, glasshouse and in the field. Individual organs or the whole shoot were analysed using flame photometry or ICPOES. Conditions under which the HvNax3 was expressed were identified, and one such setup was used in a subsequent exercise to fine-map the locus.

HvNax3 was fine-mapped using an F₂ population of 125 plants developed by crossing two Clipper × Sahara (CS) doubled haploid lines (Chapter 6). The HvNax3 genotypes of the eleven F₂ recombinants were determined by scoring F₃ progeny families for shoot Na⁺ accumulation and markers. The combined information was used to further delimit HvNax3 to a 2.8 cM marker interval in barley and a corresponding interval in rice of 222 kb on rice chromosome 5. Marker HvCLP, a close homologue of AtSOS3 which contributes to salinity tolerance in Arabidopsis, co-segregated perfectly with the HvNax3 locus, and was therefore considered as a plausible candidate for the HvNax3 gene.

HvCLP RNA expression was studied using Clipper (Na⁺ excluder) and Sahara (Na⁺ non-excluder) parents; CS DH line 134, Golden promise and BC₁F₂ derived sib lines carrying contrasting HvNax3 alleles (Chapter 7). These studies revealed that HvCLP expression was higher in Clipper than Sahara and higher in root than shoot. However, further experiments on BC₁F₂-derived lines suggested that the HvCLP alleles do not differ inherently in their expression levels.

BAC (bacterial artificial chromosomes) clones containing candidate genes were identified and full length cDNA and genomic sequences of the HvSOS3 homologue (HvCLP) from Clipper and Sahara were obtained using 5’ RACE PCR and polymorphisms were identified (Chapter 7). One amino acid difference (Alanine to Threonine) was identified between the Clipper and Sahara sequences, which could
potentially account for phenotypic differences (Na⁺ exclusion) between the Sahara (non-excluding) and Clipper (excluding) HvNax3 alleles, consistent with HvCLP being the HvNax3 gene. 3D modelling of the Clipper and Sahara HvSOS3 proteins using the known Arabidopsis SOS3 structure as a template indicated that the overall shape and the distribution of the secondary structure elements were highly conserved in these proteins (Appendix C).

Twenty-four BC₁F₂ -derived lines containing mostly a Clipper genetic background, but homozygous for either the Clipper or Sahara allele of HvNax3 (12 of each type), were generated. In two field trial sites in South Australia, the lines carrying the HvNax3 Clipper allele averaged 48.5 and 38% less leaf Na⁺ accumulation and 30 and 18.5% more grain yield, than the lines carrying the Sahara allele, respectively. Therefore, the Na⁺ exclusion HvNax3 allele appears to have considerable value for improving the salinity tolerance of barley in commercial South Austrian field conditions. This gene could be better utilized in breeding programs by using the linked PCR markers to select for the gene, or could be used to engineer tolerant varieties by transformation.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Alireza Rivandi and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Alireza Rivandi: ..............................

Date: ..............................
This thesis is dedicated to my family and all my teachers.
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B.2 Analysis of Variance table – First Glenthorne soil experiment

B.3 Analysis of Variance table – Second Glenthorne soil experiment

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B.5 Analysis of Variance table – Minnipa soil experiment

Appendix: C

Phylogenetic analysis and construction of SOS3 models from barley and rice proteins.

Figure 1: An un-rooted radial phylogenetic tree of selected SOS3 proteins. Amino acid sequences were aligned with ClustalX (Thompson et al., 1997) and branch lengths are drawn to scale. Circle colors indicate selected branches of cereal (yellow and pink) and plant dicotyledonous SOS3 proteins (green). Two letter prefixes for sequence identifiers indicate species of origin and a full list of all SOS3 proteins is specified in Materials and Methods. The tree was bootstrapped using N-J algorithm (Thompson et al., 1997). The barley Clipper and Sahara, rice and Arabidopsis sequences (in bold) were used for construction of molecular models and are highlighted in dark pink (Fig. 1).
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(B), Stereoview of ribbon representations of the Clipper (steel blue) and Sahara (pink) SOS3 models shows the disposition of secondary structure elements. The two models are superposed with rmsd value of 0.29 Å in Cα positions over 185 residues.

(C), Stereoview of ribbon representations of the Clipper (steel blue) and Sahara (pink) SOS3 models shows the position of a central helix that contains Ala111 (Clipper, in cpk sticks), while in the Sahara model this residue is substituted (green arrow) by Thr111 (green sticks). For clarity the last 12 residues in both models are not shown.

(D), Stereoview of ribbon representations of the Sahara (pink) and rice (cyan) SOS3 models shows the amino acid residues forming the central helix that are critical to SOS3 protein fold. Note that both models contain Thr one one of the central helices (Thr111 in Sahara and Thr109 in rice, in green and cyan sticks, respectively), while the rice model also contains Lys instead of Glu; the latter forms a typical signature of the barley sequences. The residues are shown in sticks and colored in cpk (Sahara) and cyan (rice). The two models are superposed with rmsd value of 0.63 Å in Cα positions over 185 residues.

Appendix: D

ANOVA was conducted using GenStat program (edition 6.1).

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**D.2**: Analysis of variance table - Bird proof cage experiment

**D.3**: Analysis of variance table – experiments in the field

Georgetown field trial
Whitwarta field trial
<table>
<thead>
<tr>
<th>Acronyms/symbols</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABI:</td>
<td>Applied Biosystems Incorporated</td>
</tr>
<tr>
<td>ACPFG:</td>
<td>Australian Centre for Plant Functional Genomics</td>
</tr>
<tr>
<td>AFLP:</td>
<td>Amplified Fragment Length Polymorphism</td>
</tr>
<tr>
<td>AGRF:</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>ANOVA:</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP:</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAC:</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>BC₁F₂:</td>
<td>Backcross one F₂ Derived Lines</td>
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<td>bp:</td>
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<td>BSA:</td>
<td>Bovine Serum Albumin</td>
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<td>HC:</td>
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<td>HS:</td>
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</tr>
<tr>
<td>ICPOES:</td>
<td>Inductively Coupled Plasma Optical Emission Spectrophotometry</td>
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<tr>
<td>PCR:</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pH:</td>
<td>Negative Logarithm of Hydrogen Ion Concentration</td>
</tr>
<tr>
<td>PVPP:</td>
<td>Polyvilylpolypyrroldone</td>
</tr>
<tr>
<td>QTL:</td>
<td>Quantitative Trait Loci</td>
</tr>
<tr>
<td>R²:</td>
<td>Measure of Association</td>
</tr>
<tr>
<td>RACE:</td>
<td>Rapid Amplification of cDNA Ends</td>
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</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>RAPD</td>
<td>Randomly Amplified Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse Osmosis</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>SARDI</td>
<td>South Australian Research and Development Institute</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
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<td>s</td>
<td>Second</td>
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<td>SOS</td>
<td>Salt Overly Sensitive</td>
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<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<td>SSC</td>
<td>Sodium Chloride Citrate</td>
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<td>SSR</td>
<td>Simple Sequence Repeats</td>
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<tr>
<td>SMART (5')</td>
<td>Switching Mechanism at 5' End of RNA Transcript</td>
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<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
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<td>TAIR</td>
<td>The <em>Arabidopsis</em> Information Resources</td>
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<td>TE</td>
<td>Tris-EDTA</td>
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<td>TILLING</td>
<td>Targeting Induced Local Lesions In Genomes</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
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<td>YAC</td>
<td>Yeast Artificial Chromosome</td>
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<tr>
<td>µL</td>
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<td>µM</td>
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<td>µg</td>
<td>Microgram</td>
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<td>Millilitre</td>
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<td>mM</td>
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