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(54) Title: SALT TOLERANT PLANTS

(57) Abstract: The present invention relates to polypeptides, and polynucleotides encoding therefor, with cation transporter activity. In particular, the present invention relates to methods for producing, identifying, and/or breeding transgenic or non-transgenic plants, especially wheat or barley plants, with enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant. Also provided are plants produced using these methods.

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SALT TOLERANT PLANTS

FIELD OF THE INVENTION

The present invention relates to polypeptides, and polynucleotides encoding therefor, with cation transporter activity. In particular, the present invention relates to methods for producing, identifying, and/or breeding transgenic or non-transgenic plants, especially wheat or barley plants, with enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant. Also provided are plants produced using these methods.

BACKGROUND OF THE INVENTION

Soil salinity causes significant reductions in plant productivity, and consequent economic losses associated with reduced grain quality and yield of agricultural crops (Pitman and Läuchli, 2002). Over 6% of the world's land is affected by either salinity or sodicity. A large proportion of the Australian wheat belt is at risk of salinisation due to rising water tables, and a further and larger part has soils that are sodic, and underlain with subsoil salinity (Rengasamy, 2002). This subsoil salinity is formed in semi-arid zones (with annual rainfall less than 450 mm), and is transient in nature as it moves in and out of the root zone according to soil wetting and drying cycles (Rengasamy, 2002).

Cultivars of durum wheat are more salt sensitive than bread wheat (Gorham et al., 1990; Rawson et al., 1988), and may yield less when grown on saline soils (Francois et al., 1986; Maas and Grieve, 1990). The usual high price of durum wheat on the international market can bring a better return to farmers than bread wheat and other crops, so, breeding new cultivars of durum wheat with improved salt tolerance can allow growers more options in dealing with subsoil salinity. Marker assisted selection is potentially the most efficient approach to developing cultivars that can tolerate saline soils.

There are three avenues by which to introduce salt-tolerance into durum wheat: traditional breeding techniques using physiologically-based phenotyping, marker-assisted selection, and through transformation of genes known to improve Na^+ exclusion or tissue tolerance. To increase salt tolerance of crops in terms of yield increases and associated economic gains, there is great potential for the introduction of salt tolerance traits into durum wheat using marker-assisted selection (Munns et al., 2002). This approach has successfully been used to introduce various agronomic traits into cereals, and overcomes the problems associated with wheat transformation and market acceptance (Koorneef and Stam, 2001). Plant breeding using marker-assisted selection has a proven track-record of successfully incorporating a stable trait into the

genome of the target species. However, marker development is dependent on accurate phenotyping, requiring a quantitative measure of a specific trait. An understanding of physiological mechanisms is needed to identify such a trait.

Salt tolerance in the Tritiaceae is associated with sodium exclusion, which limits the entry of sodium into the plant and its transport to leaves. Sodium exclusion from the transpiration stream reaching the leaves is controlled at three stages: (1) selectivity of the root cells taking up cations from the soil solution, (2) selectivity in the loading of cations into the xylem vessels in the roots, and (3) removal of sodium from the xylem in the upper part of the roots and the lower part of the shoot (Munns et al., 2002; Tester and Davenport, 2003).

Bread wheat (hexaploid) cultivars are able to exclude Na^+ from the leaves, however, durum wheat (tetraploid) cultivars lack this trait (Dubcovsky et al., 1996). The *Kna1* locus on chromosome 4DL of hexaploid wheat is linked to Na^+ exclusion and K^+/Na^+ discrimination, and subsequently, improved salt tolerance (Dvořák et al., 1994; Shah et al., 1987). Hexaploid wheat has three genomes, A, B and D, but tetraploid wheat has only the A and B genomes. A homoeologue of the *Kna1* locus has not yet been found on the A or B genomes.

Recently, a novel source of Na^+ exclusion was identified in a durum landrace (Munns et al., 2000). The landrace had very low rates of Na^+ accumulation in the leaf blade, as low as bread wheat cultivars, and maintained a high rate of K^+ accumulation, with consequent high K^+/Na^+ discrimination. The low-Na^+ durum landrace had a K^+/Na^+ ratio of 17 whereas the durum cultivars Wollaroi, Tamaroi and Langdon had K^+/Na^+ ratios of 1.5, 0.7 and 0.4 respectively (Munns et al., 2000). The bread wheat cultivars Janz and Machete had K^+/Na^+ ratios of 10 and 8 respectively. The low Na^+ trait was shown to confer a significant yield advantage at moderate soil salinity (Husain et al., 2003), indicating that this novel germplasm provides the opportunity to improve the salt tolerance of cultivated durum wheat. Markers for identifying the *Nax1* locus from durum landrace wheat which is partially responsible for the sodium exclusion phenotype have recently been described (WO 2005/120214).

Methods for selection of Na^+ excluding individuals in wheat breeding populations are time-consuming and expensive. In one case, the method involves growing plants in pots using a sub-irrigation system to provide a gradual and uniform exposure to NaCl to the plant, and the harvesting of a given leaf for Na^+ accumulation. Although this screening method is very reproducible, it is labour intensive and requires a controlled environment. It is not possible to screen plants in the field or with large numbers of individual lines using this method. QTL mapping and marker-assisted selection is a technique that has many advantages over phenotypic screening as a selection tool. Marker-assisted selection is non-destructive

and can provide information on the genotype of a single plant without exposing the plant to the stress. The technology is capable of handling large numbers of samples. Although developing a QTL map is laborious, the markers identified may prove to be sufficiently robust to use as the sole selection tool for a specific trait in a breeding program. PCR-based molecular markers have the potential to reduce the time, effort and expense often associated with physiological screening. In order to use marker-assisted selection in breeding programs, the markers must be closely linked to the trait, and work across different genetic backgrounds.

There is a need for the identification of further genes and/or markers thereof which can be used to produce plants, particularly wheat or barely plants, with enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant.

SUMMARY OF THE INVENTION

The present inventors have identified a family of wheat genes which encode cation transporters. At least some alleles of these genes have been shown to confer upon a wheat plant enhanced tolerance to saline and/or sodic soils.

In a first aspect, the present invention provides a tetraploid or hexaploid wheat plant comprising a gene on chromosome 2A which hybridises under stringent conditions to a nucleic acid molecule having nucleotides in a sequence as provided in one or both of SEQ ID Nos: 3 or 4, wherein said chromosome 2A comprises a recombination event between said gene and one or more genetic markers present on chromosome 2A of wheat Line 149.

Preferably, the gene encodes a cation transporter comprising amino acids having a sequence as provided in SEQ ID NO:1 or SEQ ID NO:2, or an amino acid sequence which is at least 73% identical to, more preferably at least 90% identical, SEQ ID NO:1 and/or SEQ ID NO:2. More preferably, the gene encoding a cation transporter comprising amino acids having a sequence as provided in SEQ ID NO:1 or SEQ ID NO:2.

Preferably, chromosome 2A comprises less than 75%, more preferably less than 50%, and even more preferably less than 25%, of chromosome 2A of wheat Line 149.

Preferably, the recombination event is proximal to the gene.

Preferably, the chromosome does not comprise a yield penalty locus present on chromosome 2A of wheat Line 149.

In one embodiment, the wheat plant comprises a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first

nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 319 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 29-348 of SEQ ID NO: 6 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149.

In another embodiment, the wheat plant comprises a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 415 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 41-455 of SEQ ID NO: 7 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149.

In a further embodiment, the wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 387 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 19-405 of SEQ ID NO: 8 or its complement, wherein said second nucleotide sequence is comprised in a *Hin*dIII fragment that is different in size to the corresponding *Hin*dIII fragment present on chromosome 2A of wheat Line 149.

In a further embodiment, the wheat plant comprises a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement

thereof, and a second nucleotide sequence of at least 301 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 11-311 of SEQ ID NO: 9 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149.

In a further embodiment, the wheat plant comprises a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 336 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 13-348 of SEQ ID NO: 10 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149.

In a further embodiment, the wheat plant comprises a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 427 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 30-456 of SEQ ID NO: 11 or its complement, wherein said second nucleotide sequence is comprised in a *Eco*RV fragment that is different in size to the corresponding *Eco*RV fragment present on chromosome 2A of wheat Line 149.

With regard to the above embodiments, the wheat plant may have any combination of said second sequences. Furthermore, preferably the first and one or more of said second nucleotide sequences are on the same chromosome. Preferably, the wheat plant is of the species *Triticum aestivum ssp aestivum* or *Triticum durum*.

The (approximate) sizes of the *Nco*I, *Hin*dIII, or *Eco*RV fragments in wheat Line 149 comprising the second nucleotide sequences of the above aspects are as described in Table 8. In an embodiment, the sizes of the restriction enzyme fragments

are determined or compared by Southern blot hybridisation or RFLP analysis such as, for example, as described herein. In an embodiment, the difference in size of the restriction enzyme fragment comprising the second nucleotide sequence of the above aspects relative to the corresponding restriction enzyme fragment derived from chromosome 2A of wheat Line 149 is at least 10 basepairs, preferably at least 100 basepairs, more preferably at least 200, at least 250 or at least 500 basepairs.

The first nucleic acid probe of the above aspects can be replaced with any nucleic acid probe comprising a wheat HKT7 gene, or portion thereof which is at least 25 nucleotides in length. Examples include the wheat HKT7 cDNAs provided as SEQ ID NO's 3 and 4, as well as the corresponding genes provided as SEQ ID NO's 18 and 19.

Preferably, the first nucleotide sequence is comprised in the A genome of the wheat plant. More preferably, the first nucleotide sequence is comprised in chromosome 2A of the wheat plant.

Preferably, the first nucleotide sequence comprises a *Nax1* gene which confers enhanced tolerance to saline and/or sodic soils to the plant, and/or reduced sodium accumulation in an aerial part of the plant. Preferably, the *Nax1* gene encodes a polypeptide comprising amino acids having a sequence as provided in SEQ ID NO:1 or SEQ ID NO:2, or an amino acid sequence which is at least 73% identical to SEQ ID NO:1 or SEQ ID NO:2, wherein the polypeptide has cation transporter activity when produced in a cell. More preferably, the first nucleotide sequence comprises a nucleotide sequence as provided in SEQ ID NO: 3 or SEQ ID NO: 4 or a nucleotide sequence which is at least 90% identical to SEQ ID NO: 3 or SEQ ID NO: 4, or even more preferably, a nucleotide sequence that is at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, or at least 97% identical to a nucleotide sequence as provided in SEQ ID NO: 3 or SEQ ID NO: 4.

In a preferred embodiment, said first nucleotide sequence is derived from durum wheat Line 149, *T. monococcum* C68-01, or a progenitor or progeny plant thereof comprising said first nucleotide sequence.

Preferably, the wheat plant is homozygous for said first nucleotide sequence. Furthermore, it is preferred that the wheat plant is homozygous for one or more of said second nucleotide sequences.

In an embodiment, the wheat plant is growing in a field.

In yet another embodiment, the grain yield of said plant is at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, and more preferably at least 100%, of the grain yield of an isogenic plant lacking said first nucleotide sequence when said plants are grown under equivalent conditions.

In a further embodiment, the number of heads of said plant is at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, and more preferably at least 100%, of the number of heads compared to an isogenic plant lacking said first nucleotide sequence.

In a further aspect, the present invention provides a wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 319 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 29-348 of SEQ ID NO: 6 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

In a further aspect, the present invention provides a wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 415 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 41-455 of SEQ ID NO: 7 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

In a further aspect, the present invention provides a wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 387 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe

consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 19-405 of SEQ ID NO: 8 or its complement, wherein said second nucleotide sequence is comprised in a *Hin*dIII fragment that is different in size to the corresponding *Hin*dIII fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

In a further aspect, the present invention provides a wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 301 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 11-311 of SEQ ID NO: 9 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

In a further aspect, the present invention provides a wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 336 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 13-348 of SEQ ID NO: 10 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

In a further aspect, the present invention provides a wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 427 nucleotides on chromosome 2A, said second nucleotide sequence being

capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 30-456 of SEQ ID NO: 11 or its complement, wherein said second nucleotide sequence is comprised in a *Eco*RV fragment that is different in size to the corresponding *Eco*RV fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

With regard to the above aspects relating to a tetraploid or hexaploid wheat plant, the wheat plant may have any combination of said second sequences. Furthermore, preferably the first and one or more of said second nucleotide sequences are on the same chromosome. Preferably, the wheat plant is of the species *Triticum aestivum ssp aestivum* or *Triticum durum*.

In one embodiment, the wheat plant is non-transgenic. In another embodiment, the wheat plant is transgenic for said first nucleotide sequence.

In another aspect the present invention provides a substantially purified and/or recombinant polypeptide comprising amino acids having a sequence as provided in SEQ ID NO:1 or SEQ ID NO:2, a biologically active fragment thereof, or an amino acid sequence which is at least 73% identical to SEQ ID NO:1 or SEQ ID NO:2, wherein the polypeptide has cation transporter activity when produced in a cell.

In an embodiment, the polypeptide comprises amino acids having a sequence which is at least 90% identical to SEQ ID NO:1 or SEQ ID NO:2.

In another embodiment, the polypeptide is from or in wheat or barley.

In a further embodiment, the cation is sodium and/or potassium.

In an embodiment, the polypeptide comprises at least one membrane spanning domain. In another embodiment, the polypeptide comprises at least four membrane spanning domains.

In a further embodiment, the polypeptide is a fusion protein further comprising at least one other polypeptide sequence.

The at least one other polypeptide may be, for example, a polypeptide that enhances the stability of a polypeptide of the present invention, or a polypeptide that assists in the purification of the fusion protein.

In another aspect, the present invention provides an isolated and/or exogenous polynucleotide comprising nucleotides having a sequence as provided in, or complementary to, SEQ ID NO:3 or SEQ ID NO:4, a sequence which is at least 73% identical to SEQ ID NO:3 or SEQ ID NO:4, a sequence which hybridizes to one or more of SEQ ID NO:3 or SEQ ID NO:4, or a sequence which encodes a polypeptide of the invention, wherein the polynucleotide is not SEQ ID NO:5.

In an embodiment, the polynucleotide comprises nucleotides having a sequence which are at least 90% identical to one or more of SEQ ID NO:3 or SEQ ID NO:4.

In a further embodiment, the polynucleotide comprises nucleotides having a sequence which hybridizes to one or more of SEQ ID NO:3 or SEQ ID NO:4 under stringent conditions.

Preferably, the polynucleotide is operably linked to a promoter capable of directing expression of the polynucleotide in a cell, preferably a plant cell and more preferably in a cereal plant. In one embodiment, the cell is a root cell. In a further embodiment, the cell is a leaf sheath cell. In yet another embodiment, the cell is a xylem parenchyma cell.

Preferably, the polynucleotide encodes a polypeptide having cation transporter activity when expressed in a cell.

In a further aspect, the present invention provides a method of producing the polypeptide of the invention, comprising expressing in a cell the polynucleotide of the invention.

In one embodiment, the cell is a recombinant cell. In another embodiment, the cell is non-recombinant.

Preferably, the cell is a plant cell.

Preferably, the cell is comprised in a plant which may be growing in the field under saline and/or sodic conditions.

In a further aspect, the present invention provides an isolated and/or exogenous polynucleotide which, when present in a cell of a cereal plant, decreases the expression of at least one gene that hybridises to a nucleic acid molecule encoding a wheat HKT7 polypeptide under stringent conditions, said decreased expression being relative to an otherwise isogenic cell of a cereal plant that lacks said polynucleotide.

This aspect of the invention is particularly useful when it is desirable to preferentially express a *Nax1* gene that confers enhanced tolerance to saline and/or sodic soils to a cereal plant, and/or reduced sodium accumulation in an aerial part of a cereal plant, and at the same time down-regulate mRNA levels of a *Nax1* gene family member that does not confer one or both of these traits. Thus, in an embodiment, the polynucleotide does not confer enhanced tolerance to saline and/or sodic soils to a cereal plant, and/or reduced sodium accumulation in an aerial part of a cereal plant.

Preferably, the HKT7 gene is on the B or D genome of tetraploid or hexaploid wheat.

Preferably, the polynucleotide of this aspect is operably linked to a promoter capable of directing expression of the polynucleotide in a cell of a cereal plant.

Preferably, the polynucleotide of this aspect is an antisense polynucleotide, a sense polynucleotide, a catalytic polynucleotide, an artificial microRNA or a duplex RNA molecule.

In a further aspect, provided is a vector comprising a polynucleotide of the invention.

Preferably, the polynucleotide is operably linked to a promoter. In one embodiment, the promoter confers expression of the polynucleotide preferentially in the root and/ leaf sheath of a cereal plant relative to at least one other tissue or organ of said cereal plant. In another embodiment, the promoter confers expression of the polynucleotide preferentially in xylem parenchyma cells of a cereal plant.

Also provided is a cell comprising a polypeptide of the invention, a polynucleotide of the invention, or a vector of the invention.

In an embodiment, the polypeptide, polynucleotide or vector was introduced into the cell or a progenitor of the cell. Examples of such cells include, but are not limited to, a bacterial cell, plant cell or animal cell.

In an embodiment, the cell is an *E. coli* cell, an *Agrobacterium* cell or a cereal plant cell.

In an embodiment, the polynucleotide is integrated into the genome of the cell.

In a further embodiment, the cell comprises a polynucleotide of the invention encoding at least one *Nax1* gene that confers enhanced tolerance to saline and/or sodic soils, to a cereal plant and a polynucleotide which, when present in a cell of a cereal plant, decreases the expression of at least one *Nax1* gene family member which does not confer one of these phenotypes relative to a cell of a cereal plant that lacks said polynucleotide. As noted above, this embodiment of the invention is particularly useful when it is desirable to preferentially express a *Nax1* gene that confers enhanced tolerance to saline and/or sodic soils to a cereal plant, and/or reduced sodium accumulation in an aerial part of a cereal plant, and at the same time down-regulate mRNA levels of a *Nax1* gene family member that does not confer one or both of these traits.

In another embodiment, the *Nax1* gene encodes a polypeptide comprising an amino acid sequence which is at least 80% identical to SEQ ID NO:1 and/or SEQ ID NO:2.

In a further aspect, the present invention provides a plant comprising the cell according to the invention. Preferably, all of the cells of the plant comprise the polypeptide, polynucleotide or vector of the invention.

Preferably, the plant is a cereal plant. More preferably, the plant is a wheat plant.

In one embodiment, the wheat plant is of the species *Triticum aestivum* ssp *aestivum*.

In another embodiment, the wheat plant is of the species *Triticum durum*.

In a further embodiment, the plant has a genetic background comprising less than 50% of the genetic complement of durum Line 149, 5049 or of the cultivar Tamaroi.

In yet another embodiment, the plant further comprises an allele of the *Nax2* gene which confers enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant.

Preferably, the *Nax2* gene is non-transgenic.

In a further embodiment, the gene is on chromosome 5A.

In yet another embodiment, the plant further comprises an allele of the *Kna1* gene which confers enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant.

Preferably, the plant has enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant, relative to a corresponding plant lacking said cell.

In yet another aspect, the present invention provides a genetically modified plant having increased expression and/or activity of a polypeptide of the invention relative to a corresponding non-modified plant, wherein the polypeptide of the invention is expressed from a polynucleotide of the invention encoding said polypeptide.

In yet a further aspect, the present invention provides a genetically modified hexaploid wheat plant comprising a transgenic *Nax1* gene that confers enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant, relative to a corresponding plant not having the gene.

Preferably, the *Nax1* gene is obtained from durum wheat. Preferably, the *Nax1* gene is expressed in xylem parenchyma cells.

In yet a further aspect, the present invention provides a method of producing the cell of the invention, the method comprising the step of introducing the polynucleotide of the invention, or a vector of the invention, into a cell.

Preferably, the method further comprises the step of regenerating a transgenic plant from the cell.

In a further aspect, the present invention provides for the use of a polynucleotide of the invention or vector of the invention to produce a recombinant cell.

Also provided is a method of obtaining a wheat plant, the method comprising;

i) crossing two parental wheat plants of which at least one plant comprises a *Nax1* locus comprising a first nucleotide sequence as defined herein,

ii) screening progeny plants from the cross for the presence or absence of said *Nax1* locus, and

iii) screening progeny plants from the cross for the presence or absence of a second nucleotide sequence as defined herein,

wherein at least one of the parental wheat plants is a tetraploid or hexaploid wheat plant.

Preferably, the method further comprising a step of selecting a progeny wheat plant which has enhanced tolerance to saline and/or sodic soils, reduced sodium accumulation in an aerial part of the plant, and/or a grain yield of at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, and more preferably at least 100%, of the grain yield of an isogenic plant lacking said first nucleotide sequence when said plants are grown under equivalent conditions.

In an embodiment, the method further comprises the step of selecting a plant with the desired genotype or of analysing the plant for at least one other genetic marker.

In a further embodiment, at least one of the parental wheat plants is a hexaploid wheat plant.

In yet another embodiment, the cross is between a durum wheat plant comprising said *Nax1* locus and a hexaploid wheat plant lacking said *Nax1* locus.

Preferably, one of the wheat plants is durum wheat Line 149, *T. monococcum* C68-01, or a progenitor or progeny plant thereof comprising said first nucleotide sequence.

In yet a further aspect, the present invention provides a method of introducing a *Nax1* locus into the genome of a wheat plant lacking said locus, the method comprising;

i) crossing a first parent wheat plant with a second parent wheat plant, wherein the second plant comprises a first nucleotide sequence as defined herein, and a second nucleotide sequence as defined herein,

ii) backcrossing the progeny of the cross of step i) with plants of the same genotype as the first parent plant for a sufficient number of times to produce a plant with a majority of the genotype of the first parent but comprising said first nucleotide sequence and said second nucleotide sequence, and

iii) selecting a progeny wheat plant which has enhanced tolerance to saline and/or sodic soils, reduced sodium accumulation in an aerial part of the plant, and/or a grain yield of at least 95%, more preferably at least 96%, more preferably at least

97%, more preferably at least 98%, more preferably at least 99%, and more preferably at least 100%, of the grain yield of an isogenic plant lacking said first nucleotide sequence when said plants are grown under equivalent conditions.

In yet a further aspect, the present invention provides a method of identifying a wheat plant with enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant, the method comprising detecting a first nucleic acid molecule of the plant as defined herein or a second nucleotide sequence as defined herein.

In one embodiment, the method comprises:

i) hybridising a third nucleic acid molecule to a nucleic acid which is obtained from said plant,

ii) optionally hybridising at least one other nucleic acid molecule to said nucleic acid molecule which is obtained from said plant; and

iii) detecting a product of said hybridising step(s) or the absence of a product from said hybridising step(s).

Preferably, the third nucleic acid molecule is used as a primer to reverse transcribe or replicate at least a portion of the nucleic acid molecule.

The nucleic acid can be detected using a technique known in the art. Examples include, but are not limited to, restriction fragment length polymorphism analysis, amplification fragment length polymorphism analysis, microsatellite amplification and/or nucleic acid sequencing.

In one embodiment, the method comprises nucleic acid amplification.

In yet a further aspect, the present invention provides a method of enhancing tolerance to saline and/or sodic soils in a cereal plant, the method comprising genetically manipulating said plant such that the production of a polypeptide is increased when compared to a wild-type plant, wherein the polypeptide has cation transporter activity, wherein the polypeptide is a polypeptide of the invention.

In another aspect, the present invention provides a method of reducing sodium accumulation in an aerial part of a cereal plant, the method comprising genetically manipulating said plant such that the production of a polypeptide is increased when compared to a wild-type plant, wherein the polypeptide has cation transporter activity, wherein the polypeptide is a polypeptide of the invention.

In a further aspect, the present invention provides a method for identifying a plant comprising:

(i) obtaining a nucleic acid sample from each plant in a population of plants,

(ii) screening each nucleic acid sample for the presence or absence of a gene which hybridises under stringent conditions to a nucleic acid molecule having nucleotides in a sequence as provided in SEQ ID Nos: 3 or 4,

(iii) screening each nucleic acid sample for the presence or absence of a genetic marker which genetic marker is different to the gene of part (ii),

(iv) identifying a plant from the population of plants which comprises the gene and which lacks the genetic marker.

Preferably, the plant is a cereal plant other than rice such as wheat or barely.

Preferably, the plant is a wheat plant, and step (iii) comprises screening each nucleic acid sample for the presence or absence of a genetic marker present on chromosome 2A of wheat Line 149, which genetic marker is different to the gene of part (ii).

Preferably, step (ii) comprises screening the nucleic acid samples for the presence or absence of a genetic marker which is genetically linked to the gene on chromosome 2A and different to the gene.

Preferably, the method further comprises

(v) selecting a plant comprising a recombination event between the gene and said genetic marker.

Preferably, the plant has enhanced tolerance to saline and/or sodic soils.

In another aspect, the present invention provides a method for identifying a nucleic acid molecule which confers to a plant enhanced tolerance to saline and/or sodic soils comprising:

(i) obtaining a nucleic acid molecule operably linked to a promoter, the nucleic acid molecule encoding a polypeptide comprising amino acids having a sequence that is at least 50% identical to SEQ ID NO: 1 and/or SEQ ID NO:2,

(ii) introducing the nucleic acid molecule into a cell in which the promoter is active,

(iii) determining whether the sodium or potassium concentration in the cell is modified when compared to an isogenic cell lacking the nucleic acid molecule,

(iv) optionally, selecting a nucleic acid molecule which confers modified sodium or potassium concentration in the cell, and

(v) optionally, obtaining a plant comprising the nucleic acid molecule selected in step (iv), wherein the plant has enhanced tolerance to saline and/or sodic soils.

Preferably, the cell is a cell of a wheat plant.

In a further aspect, the present invention provides a method for identifying a nucleic acid molecule which confers to a plant enhanced tolerance to saline and/or sodic soils comprising:

(i) obtaining a nucleic acid molecule operably linked to a promoter, the nucleic acid molecule encoding a polypeptide comprising amino acids having a sequence that is at least 50% identical to SEQ ID NO: 1 and/or SEQ ID NO:2,

(ii) introducing the nucleic acid molecule into at least one cell of a plant in which the promoter is active,

(iii) cultivating a plant comprising the nucleic acid molecule in a saline and/or sodic soil and determining whether the plant has enhanced tolerance to the saline and/or sodic soil compared to an isogenic plant lacking the nucleic acid molecule, and

(iv) optionally, selecting a nucleic acid molecule which confers to a plant enhanced tolerance to saline and/or sodic soils.

Preferably, the cell is a xylem parenchymal cell.

Preferably, the plant is a wheat plant.

Also provided is a plant, or progeny thereof, produced using a method of the invention.

In yet another aspect, the present invention provides a wheat plant, or progeny thereof, identified or obtained using a method of the invention.

In a further aspect the present invention provides a method of producing seed, the method comprising;

a) growing a plant of the invention, and

b) harvesting the seed.

Also provided is a seed or grain of a plant of the invention.

In yet a further aspect, the present invention provides a method of producing flour, wholemeal, starch or other product obtained from seed, the method comprising;

a) obtaining seed of the invention, and

b) extracting the flour, wholemeal, starch or other product.

Also provided is a product produced from a plant of the invention.

In a further aspect, the present invention provides a product produced from a seed of the invention. The product may be a food or non-food product.

Examples of food products include, but are not limited to, flour, starch, leavened or unleavened breads, pasta, noodles, animal fodder, beer, malt, breakfast cereals, snack foods, cakes, malt, beer, pastries and foods containing flour-based sauces.

Examples of non-food products include, but are not limited to, films, coatings, adhesives, building materials and packaging materials.

In a further aspect, the present invention provides a method of preparing a food product of the invention, the method comprising mixing seed, or flour, wholemeal or starch from said seed, with another ingredient.

Also provided is a method of preparing malt, comprising the step of germinating seed of the invention.

In another aspect, the present invention provides a substantially purified antibody, or fragment thereof, that specifically binds a polypeptide of the invention.

As will be apparent, preferred features and characteristics of one aspect of the invention are applicable to many other aspects of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1. Frequency distribution for leaf Na^+ concentrations of BC_5F_2 wheat family, after plants were grown at 150 mM NaCl for 10 d, showing the numbers of plants in the family having leaf 3 Na+ concentrations in each 25 μmol g^{-1}DW class. The black bars represented homozygotes for Nax1, the grey bars heterozygous lines, the open bars homozygous nulls. Arrows indicate parental leaf Na^+ concentration means (μmol g^{-1}DW, n=6). Line 149: 141 ± 14, P_1 (BC_4F_3): 233 ± 39, Tamaroi: 811 ± 31.

Figure 2. Schematic genetic maps of wheat chromosome 2AL and rice chromosome 4 using the low resolution mapping family. *Left*: Physical/genetic map of rice chromosome 4 constructed from the sequence annotations of rice genes as shown on Gramene (http://www.gramene.org). The solid line connects non-colinear markers. *Middle*: Genetic map of wheat chromosome 2AL in low resolution mapping family showing relative positions of wEST markers. The top region (grey highlight) represented Tamaroi chromatin in the BC_4F_2 parent. *Right*: Physical mapping of markers into deletion bins on wheat chromosome 2AL.

Figure 3. Schematic genetic map of wheat chromosome 2AL compared to rice chromosome 4 using the high resolution mapping family. *Left*: Physical/genetic map of rice chromosome 4 constructed from the sequence annotations on Gramene (http://www.gramene.org). The solid lines highlight the re-arrangement in wheat relative to rice. *Middle*: Genetic map of *Nax1* region using high resolution mapping family, indicating the relative positions of the wEST markers used. *Right*: Physical mapping of markers into deletion bins of wheat chromosome 2AL. The broad grey arrow in the left side indicates the interstitial inversion event.

Figure 4. DNA gel blot hybridised with wEST BE604162 corresponding to *OsHKT7*. The genomic DNA was digested by *EcoR*V. The arrows on right side indicates polymorphic allelic bands between Line 149 and Tamaroi which co-segregated with

Nax1 in high resolution mapping family. The polymorphic and monomorphic alleles in A genome between Line 149 and Tamaroi were named as *TaHKT7*-A1 and *TaHKT7*-A2, respectively.

Figure 5. Full length sequence of *TmHKT7*-A1 gene (intron highlighted with grey colour) (SEQ ID NO: 18).

Figure 6. Full length sequence of *TmHKT7*-A2 gene (intron highlighted with grey colour) (SEQ ID NO: 19).

Figure 7. Gene structures of *TaHKT7*-A1, A2 and *OsHKT7*. The grey triangles represent introns in the genes. The small arrows indicate the relative positions of primers designed for gene expression analysis.

Figure 8. The alignment of amino acid sequences of proteins encoded by *TaHKT7*-A1, -A2 and *OsHKT7* (SEQ ID NO's 1, 2 and 64 respectively). The highlighted black boxes indicate the identical amino acids and the highlighted grey box indicate similar but not identical amino acids.

Figure 9. Topological structures of TaHKT7-A1, A2 and OsHKT7 predicted by TopPred software (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) using the predicted amino acid sequences. The membrane-spanning regions were predicted by a hydrophobicity value in the range 0.6 to 1.0 as indicated by arrows.

Figure 10. Expression analysis by RT-PCR of *TaHKT7*-A1 and -A2 in roots, sheaths and blades of T. *monococcum*, Line 149 and Tamaroi using gene specific primers (A1F/A1R and A2F/A2R; Table 5). The expected sizes of genomic DNA (gDNA) and cDNA of *TaHKT7*-A1 were 292 and 138 bp respectively. The expected sizes of gDNA and cDNA of *TaHKT7*-A2 were 2361 and 451 bp respectively. There was no amplification of gDNA of *TaHKT7*-A2 due to the presence of a large intron.

Figure 11. Schematic map of selected recombinants close to *Nax1*. Filled horizontal bars represent homozygous chromosomal regions derived from Line 149, open bars represent homozygous chromosomal regions derived from Tamaroi, grey bars represent heterozygous regions.

Figure 12. Production of double recombinants with a minimal introgressed region including *Nax1*.

Figure 13. Diagram of matches between sequences of specific probes for *HKT1/2*-like and *HKT3/9*-like genes and sequences of *OsHKT1/2* and *OsHKT3/9*. *OsHKT1/2* and *OsHKT3/9* have some similarity (73-76% identity) in three regions of *OsHKT1* at the positions of 669-855, 1016-1170 and 1366-1502. The sequence of specific probe for *HKT1/2*-like genes matched *OsHKT1/2* (Table 9) and was 100% identical to *TaHKT1* but had no similarity to *OsHKT3/9*. The sequence of specific probe for *HKT3/9*-like genes matched *OsHKT3/9* (Table 9) but had no similarity to *OsHKT1/2* and *TaHKT1*.

Figure 14. Diagram showing detected chromosome arm locations of *HKT* genes using Southern blot analyses in hexaploid bread wheat Chinese Spring (AABBDD) and barley cultivar Betzes. A, B and D represent the three different genomes of bread wheat, and numbers 1-7 correspond to chromosomes 1-7 of each genome. The black circles represent centromeres.

KEY TO THE SEQUENCE LISTING

SEQ ID NO: 1 - *T. monococcum* TaHKT7-A1 polypeptide.
SEQ ID NO: 2 - *T. monococcum* TaHKT7-A2 polypeptide.
SEQ ID NO: 3 - Polynucleotide encoding *T. monococcum* TaHKT7-A1 polypeptide.
SEQ ID NO: 4 - Polynucleotide encoding *T. monococcum* TaHKT7-A2 polypeptide.
SEQ ID NO: 5 - Wheat EST (Genbank Accession No. BE604162).
SEQ ID NO: 6 - Wheat EST (Genbank Accession No. BF474590).
SEQ ID NO: 7 - Wheat EST (Genbank Accession No. BE403863).
SEQ ID NO: 8 - Wheat EST (Genbank Accession No. BE423738).
SEQ ID NO: 9 - Wheat EST (Genbank Accession No. AL817940).
SEQ ID NO: 10 - Wheat EST (Genbank Accession No. BE403217).
SEQ ID NO: 11 - Wheat EST (Genbank Accession No. BG262791).
SEQ ID NO: 12 - Wheat EST (Genbank Accession No. BE498441).
SEQ ID NO: 13 - Wheat EST (Genbank Accession No. BM137419).
SEQ ID NO: 14 - Wheat EST (Genbank Accession No. CK205077).
SEQ ID NO: 15 - Wheat EST (Genbank Accession No. CA498418).
SEQ ID NO: 16 - Barley EST (Genbank Accession No. BJ472463).
SEQ ID NO: 17 - Wheat EST (Genbank Accession No. BE423738).
SEQ ID NO: 18 - Gene sequence encoding *T. monococcum* TaHKT7-A1 polypeptide.
SEQ ID NO: 19 - Gene sequence encoding *T. monococcum* TaHKT7-A2 polypeptide.
SEQ ID NO's 20 to 63 – Oligonucleotide primers.
SEQ ID NO:64 – Rice HKT7.

DETAILED DESCRIPTION OF THE INVENTION

General Techniques

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, immunology, immunohistochemistry, protein chemistry, and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al., (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan et al., (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

Selected Definitions

As used herein, the term "*Nax1* gene" refers to a gene of a wheat plant which confers enhanced tolerance to saline and/or sodic soils and/or reduced sodium accumulation in an aerial part of the plant comprising the gene. This gene is naturally located on chromosome 2AL of certain diploid wheat genotypes but does not naturally occur on chromosome 2AL of tetraploid and hexaploid wheat genotypes. However, the *Nax1* gene has been introgressed into certain tetraploid and hexaploid wheat genotypes as taught in WO2005/120214, herein incorporated by reference. As taught herein, *Nax1* is a HKT7 gene family member. It will readily be understood by those skilled in the art that a *Nax1* gene can be introduced into cells other than wheat cells, preferably other plant cells and more preferably cereal plant cells, and such cells are said to comprise a *Nax1* gene. Homoeologues of the *Nax1* gene are found in the B and/or D genomes of hexaploid wheat, on chromosomes 2B and 2D. The "*Nax1* gene family" or "wheat HKT7 gene family" as used herein therefore refers to members of the gene family including such homoeologues encoding polypeptides (referred to herein as Nax1 polypeptides) comprising amino acids having a sequence as provided

in SEQ ID NO:1 or SEQ ID NO:2, or an amino acid sequence which is at least 73% identical to SEQ ID NO:1 or SEQ ID NO:2, wherein the polypeptide has cation transporter activity when produced in a cell. As exemplified herein, members of the *Nax1* gene family contribute to enhanced salt tolerance and/or reduced sodium accumulation, although some members do so to a greater extent than others. However, it is to be understood that all of the *Nax1* gene family members in a plant may contribute together to the observed salt tolerance phenotype of the plant.

As used herein, a "*Nax1* locus" refers to a region (locus) of the genome of a plant encompassing a *Nax1* gene. Typically, this includes a region of the genome extending up to about 2cM on either side of the *Nax1* gene. An allelic variant (allele) of a *Nax1* locus present on the A genome has been shown herein to be linked to enhanced tolerance to saline and/or sodic soils as well as reduced sodium accumulation in an aerial part of a plant. Examples of markers of alleles of the *Nax1* locus which confer enhanced tolerance to saline and/or sodic soils as well as reduced sodium accumulation, or which are genetically linked thereto, include RFLP markers based on wEST sequences BF474590, BE403863, CK205077, BE423738, AL817940, BE604162, BG262162, BE403217 as taught herein, as well as the microsatellite marker Xgwm312 (also referred to herein as gwm312) (see the Examples section for further details). Particularly preferred markers of alleles of the *Nax1* locus linked to enhanced tolerance to saline and/or sodic soils, as well as reduced sodium accumulation, are based on the SEQ ID Nos: 3 or 4.

As used herein, the term "cation transporter activity" refers to the ability of a polypeptide to form part of the membrane of a plant cell (especially a wheat cell) and play a role in the active transport of a cation(s), particularly sodium and/or potassium, across the cell membrane.

As used herein, the phrase "enhanced tolerance to saline and/or sodic soils" is considered as a relative term. A saline soil is defined as having a high concentration of soluble salts, high enough to affect plant growth. Salt concentration in a soil is measured in terms of its electrical conductivity. As used herein a "saline soil" has an EC_e of at least 1 dS/m, more preferably at least 2 dS/m, more preferably at least 3 dS/m, and even more preferably at least 4 dS/m. EC_e is the electrical conductivity of the 'saturated paste extract', that is, of the solution extracted from a soil sample after being mixed with sufficient water to produce a saturated paste. Sodic soils have a low concentration of soluble salts, but a high percent of exchangeable Na^+; that is, Na^+ forms a high percent of all cations bound to the negative charges on the clay particles that make up the soil complex. Sodicity is defined in terms of the threshold ESP (exchangable sodium percentage) that causes degradation of soil structure. As used herein a "sodic soil" has an ESP greater than 5, more preferably an ESP greater than

7, more preferably an ESP greater than 9, more preferably an ESP greater than 11, more preferably an ESP greater than 13, and even more preferably an ESP greater than 15. A wheat plant with enhanced tolerance to saline and/or sodic soils is defined as a wheat plant which is more capable of growing, and/or reproducing, in saline and/or sodic conditions when compared to a plant with the same, or similar, genotype but lacking the salt tolerance allele. Indicators of enhanced tolerance to saline and/or sodic soils linked to loci of the invention include, but are not limited to, reduced sodium uptake and/or lower levels of sodium in seeds (whether grown in saline and/or sodic soils or not).

As used herein, the term "a field under saline and/or sodic conditions" refers to an area of land where the soil is a "saline soil" and/or "sodic soil" as defined above.

As used herein, the term "reduced sodium accumulation in an aerial part of the plant" is considered a relative term. More specifically, the present inventors have identified genes and markers of wheat plants linked to a low rate of Na^+ accumulation in, for example, the leaf blade. A wheat plant with "reduced sodium accumulation" is defined as a wheat plant which accumulates less sodium in an aerial part of the plant when compared to a plant with the same, or similar, genotype but lacking the salt tolerance allele. Preferably, the aerial part of the plant is selected from the leaf sheaths, leaf blades, inflorescence, developing seeds and/or mature seed. "Reduced sodium accumulation" can be determined using any method known in the art.

An aspect of the invention relates to a method of introducing a *Nax1* allele which confers enhanced tolerance of saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of a plant, into the genome of a wheat species lacking said allele, without introducing a second nucleotide sequence (as defined herein) which is naturally linked to the *Nax1* allele in certain diploid wheat genotypes. The aim of this aspect is to produce a plant with a majority of the genotype of a first parent plant but comprising said *Nax1* allele introduced from a second parent plant, without the second nucleotide sequence. As used in this context, the term "majority" means that the product of the breeding comprises greater than 50% of the genome of the first parent. However, the product preferably comprises at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, and even more preferably at least 99% of the genome of the first parent. In an embodiment, the product comprising the *Nax1* gene does not comprise one or more of chromosomes 1A, 3A, 4A, 5A 6A and 7A from the second parent plant, preferably most or all of these. In a particular embodiment, the product does not contain chromosomes 1A, 3A, 4A, 5A, 6A and 7A from durum lines Line 149, Line 5049 or variety Tamaroi.

As used herein, the term "wheat" refers to any species of the Genus *Triticum,* including progenitors thereof, as well as progeny thereof produced by crosses with other species. Wheat includes "hexaploid wheat" which has genome organization of AABBDD, comprised of 42 chromosomes, and "tetraploid wheat" which has genome organization of AABB, comprised of 28 chromosomes. Hexaploid wheat includes *T. aestivum*, *T. spelta*, *T. macha*, *T. compactum*, *T. sphaerococcum*, *T. vavilovii*, and interspecies cross thereof. A preferred species of hexaploid wheat is *T. aestivum* ssp *aestivum* (also termed "breadwheat"). Tetraploid wheat includes *T. durum* (also referred to herein as durum wheat or *Triticum turgidum* ssp. *durum)*, *T. dicoccoides*, *T. dicoccum*, *T. polonicum*, and interspecies cross thereof. In addition, the term "wheat" includes potential progenitors of hexaploid or tetraploid *Triticum sp.* such as *T. uartu*, *T. monococcum* or *T. boeoticum* for the A genome, *Aegilops speltoides* for the B genome, and *T. tauschii* (also known as *Aegilops squarrosa* or *Aegilops tauschii*) for the D genome. Particularly preferred progenitors are those of the A genome, even more preferably the A genome progenitor is *T. monococcum*. A wheat cultivar for use in the present invention may belong to, but is not limited to, any of the above-listed species. Also encompassed are plants that are produced by conventional techniques using *Triticum sp.* as a parent in a sexual cross with a non-*Triticum* species (such as rye [*Secale cereale*]), including but not limited to *Triticale*.

As used herein, the term "barley" refers to any species of the Genus *Hordeum,* including progenitors thereof, as well as progeny thereof produced by crosses with other species. It is preferred that the plant is of a *Hordeum* species which is commercially cultivated such as, for example, a strain or cultivar or variety of *Hordeum vulgare* or suitable for commercial production of grain.

The term "plant" as used herein as a noun refers to a whole plants such as, for example, a plant growing in a field for commercial wheat production. A "plant part" refers to vegetative structures (for example, leaves, stems), roots, floral organs/structures, seed (including embryo, endosperm, and seed coat), plant tissue (for example, vascular tissue, ground tissue, and the like), cells and progeny of the same.

A "transgenic plant", "genetically modified plant" or variations thereof refers to a plant that contains a gene construct ("transgene") not found in a wild-type plant of the same species, variety or cultivar. A "transgene" as referred to herein has the normal meaning in the art of biotechnology and includes a genetic sequence which has been produced or altered by recombinant DNA or RNA technology and which has been introduced into the plant cell. The transgene may include genetic sequences derived from a plant cell. Typically, the transgene has been introduced into the plant

by human manipulation such as, for example, by transformation but any method can be used as one of skill in the art recognizes.

As used herein, the term "corresponding non-modified plant" refers to a wild-type plant. "Wild type", as used herein, refers to a cell, tissue or plant that has not been modified according to the invention. Wild-type cells, tissue or plants may be used as controls to compare levels of expression of an exogenous nucleic acid or the extent and nature of trait modification with cells, tissue or plants modified as described herein. Wild-type varieties that are suitable as a reference standard include durum cv. Tamaroi and breadwheat cv. Westonia and Chinese Spring.

The terms "seed" and "grain" are used interchangeably herein. "Grain" generally refers to mature, harvested grain but can also refer to grain after imbibition or germination, according to the context. Mature grain commonly has a moisture content of less than about 18-20%.

"Nucleic acid molecule" refers to a polynucleotide such as, for example, DNA, RNA or oligonucleotides. It may be DNA or RNA of genomic or synthetic origin, double-stranded or single-stranded, and combined with carbohydrate, lipids, protein, or other materials to perform a particular activity defined herein.

As used herein, the term "nucleic acid amplification" refers to any *in vitro* method for increasing the number of copies of a nucleic acid molecule with the use of a DNA polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA molecule or primer thereby forming a new DNA molecule complementary to a DNA template. The newly formed DNA molecule can be used a template to synthesize additional DNA molecules.

"Operably linked" as used herein refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. Typically, it refers to the functional relationship of transcriptional regulatory element (promoter) to a transcribed sequence. For example, a promoter is operably linked to a coding sequence, such as a polynucleotide defined herein, if it stimulates or modulates the transcription of the coding sequence in an appropriate cell. Generally, promoter transcriptional regulatory elements that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are *cis*-acting. However, some transcriptional regulatory elements, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

As used herein, the term "gene" is to be taken in its broadest context and includes the deoxyribonucleotide sequences comprising the protein coding region of a structural gene and including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of at least about 2 kb on either end and which are involved in expression of the gene. The sequences which are located 5' of the coding

region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region which may be interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide. The term "gene" includes a synthetic or fusion molecule encoding all or part of the proteins of the invention described herein and a complementary nucleotide sequence to any one of the above. The HKT7 genes disclosed herein typically contain one or two introns.

As used herein, "haplotype" means the genotype for multiple loci or genetic markers in a haploid gamete. Generally, these loci or markers reside within a relatively small and defined region of a chromosome. A preferred haplotype comprises a region which is at most a 10 cM region or a 5 cM region or a 2 cM region surrounding a *Nax1* gene which confers enhanced tolerance to saline and/or sodic soils and/or reduced sodium accumulation in an aerial part of the plant comprising the gene. More preferred haplotypes comprise the region of at most 1cM or 0.5cM surrounding *Nax1*.

As used herein, the term "genetically linked" or similar refers to a marker locus and a second locus being sufficiently close on a chromosome that they will be inherited together in more than 50% of meioses, e.g., not randomly. This definition includes the situation where the marker locus and second locus form part of the same gene. Furthermore, this definition includes the embodiment where the marker locus comprises a polymorphism that is responsible for the trait of interest (in other words the marker locus is directly "linked" or "perfectly linked" to the phenotype). In another embodiment, the marker locus and a second locus are different, yet sufficiently close on a chromosome that they will be inherited together in more than 50% of meioses. The percent of recombination observed between genetically linked loci per generation (centimorgans (cM)), will be less than 50. In particular embodiments of the invention, genetically linked loci may be 45, 35, 25, 15, 10, 5, 4, 3, 2, or 1 or less cM apart on a chromosome. Preferably, the markers are less than 5 cM apart and most preferably about 0 cM apart.

An "allele" refers to one specific form of a genetic sequence (such as a gene) within a cell, an individual plant or within a population, the specific form differing from other forms of the same gene in the sequence of at least one, and frequently more than one, variant sites within the sequence of the gene. The sequences at these variant sites that differ between different alleles are termed "variances", "polymorphisms", or "mutations".

A "polymorphism" as used herein denotes a variation in the nucleotide sequence between alleles of the loci of the invention, of different species, cultivars, strains or individuals of a plant. A "polymorphic position" is a preselected nucleotide position within the sequence of the gene. In some cases, genetic polymorphisms are reflected by an amino acid sequence variation, and thus a polymorphic position can result in location of a polymorphism in the amino acid sequence at a predetermined position in the sequence of a polypeptide. In other instances, the polymorphic region may be in a non-polypeptide encoding region of the gene, for example in the promoter region such may influence expression levels of the gene. Typical polymorphisms are deletions, insertions or substitutions. These can involve a single nucleotide (single nucleotide polymorphism or SNP) or two or more nucleotides.

As used herein, the term "restriction enzyme" has its usual meaning in the field of biotechnology, as is well known in the art. Examples of restriction enzymes are *Hin*dIII, *Nco*I and *Eco*RV. Each restriction enzyme cleaves double-stranded DNA molecules at sequence-specific sites, for example *Hin*dIII cleaves at the sequence 5'AACGTT3', *Nco*I cleaves at the sequence 5'CCATGG3" and *Eco*RV cleaves at the sequence 5'GATATC3'. Reference to a restriction enzyme fragment herein is taken to mean an essentially double-stranded DNA molecule which is the product of cleavage by the particular restriction enzyme, the cleavage having continued essentially to completion in the absence of methylation of the sequence-specific sites. Restriction enzyme fragments have a defined length (in nucleotide basepairs) that can be measured by methods well known in the art such as, for example, gel electrophoresis with molecular size markers. As used herein, the term "fragment that is different in size" refers to the comparison of the size of particular cleavage products obtained by treatment of two different nucleic acid samples with a specific restriction enyme (endonuclease), where the corresponding fragments consist of a different number of nucleotide basepairs. Thus, the two fragments differ in length by at least one nucleotide. Preferably, the two fragments differ in length by at least about 10 basepairs, at least 100 basepairs, at least 250 basepairs, more preferably at least about 500 bases, such that differences in fragment length can be detected by, for example, Southern blot hybridisations, well known in the art. Whilst restriction enzyme digestion of each nucleic acid sample will result in multiple bands, the fragments

which are to be analysed for their length hybridize at least one nucleic probe as defined herein.

The term "isogenic" refers to a cell, tissue or plant that has the same genotype as a cell, tissue or plant of the invention but without a gene as defined herein. Typically, the plant will be a non-transgenic wheat plant of the same variety or cultivar as the plant into which an exogenous nucleic acid was introduced. Plants isogenic to those of the invention can be used as controls to compare levels of exogenous nucleic acid expression, or the extent and nature of trait modification with cells, tissue or plants modified as described herein.

As used herein, the "other genetic markers" may be any molecules which are linked to a desired trait of a cereal plant such as wheat. Such markers are well known to those skilled in the art and include molecular markers linked to genes determining traits such disease resistance, yield, plant morphology, grain quality, dormancy traits such as grain colour, gibberellic acid content in the seed, plant height, flour colour and the like. Examples of such genes in wheat are stem-rust resistance genes *Sr2, Sr21* or *Sr38*, the stripe rust resistance genes *Yr10* or *Yr17*, the nematode resistance genes such as *Cre1* and *Cre3*, alleles at glutenin loci that determine dough strength such as *Ax*, *Bx*, *Dx*, *Ay*, *By* and *Dy* alleles, the *Rht* genes that determine a semi-dwarf growth habit and therefore lodging resistance (Eagles et al., 2001; Langridge et al., 2001; Sharp et al., 2001). In a preferred embodiment, the other genetic marker is a marker other than *Sr21*. The other gene may also confer enhanced tolerance to saline and/or sodic soils to the plant, and/or reduced sodium accumulation in an aerial part of the plant, examples of such genes include *Nax2* and *KnaI*. As shown herein, a combination of alleles of genes which enhanced tolerance to saline and/or sodic soils to the plant, and/or reduced sodium accumulation in an aerial part of the plant, can have an additive effect on these traits.

As used herein, the term "*KnaI*" refers to a region (locus) on the long arm of chromosome 4 of the genome of a wheat plant. An allelic variant (allele) of the *Kna1* locus has been shown to be linked to enhanced tolerance to saline and/or sodic soils as well as reduced sodium accumulation (Dvořák et al., 1994).

As used herein, the term "*Nax2*" refers to a gene on the A genome of a wheat plant which confers enhanced tolerance to saline and/or sodic soils and/or reduced sodium accumulation in an aerial part of the plant comprising the gene. This gene is located on chromosome 5AL of certain diploid, tetraploid and hexaploid wheat genotypes. It is ancestrally located on chromosome 4AL. *Nax2* is a HKT8 gene family member. An example of an allele of HKT8 which confers enhanced tolerance to saline and/or sodic soils and/or reduced sodium accumulation in an aerial part of the plant comprising the gene (namely, a *Nax2* gene) is *TmHKT8*. Platten et al., (in

preparation) propose to call this new gene family HKT1;5. It will readily be understood by those skilled in the art that a *Nax2* gene can be introduced into cells other than wheat cells, preferably other plant cells and more preferably cereal plant cells, and such cells are said to comprise a *Nax2* gene. Examples of markers of alleles of the *Nax2* locus which confer enhanced tolerance to saline and/or sodic soils as well as reduced sodium accumulation, or which are genetically linked thereto, include genomic regions amplified using the primer pair CATCACCGTCGAGGTTATCAG (SEQ ID NO: 20) and TTGAGGTACTCGGCATA (SEQ ID NO: 21), as well as microsatellite markers Xgwm291, Xgwm410 and gpw2181.

As used herein, the phrase "yield penalty locus" refers to a region, typically but not necessarily encoding a polypeptide, that when present in a cell of a plant has a negative affect on the production of grain compared to plants lacking said locus. In an embodiment, the yield penalty is a reduction, for example by at least 5%, in average yield (t/ha). In another embodiment, the yield penalty is a reduction, for example by at least 5%, in the number of heads per square metre.

As used herein, the phrase "proximal to the gene" refers to the recombination event being closer to the centromere than the gene.

Polypeptides

By "substantially purified polypeptide" or "purified" we mean a polypeptide that has been separated from one or more lipids, nucleic acids, other polypeptides, or other contaminating molecules with which it is associated in its native state. It is preferred that the substantially purified polypeptide is at least 60% free, more preferably at least 75% free, and more preferably at least 90% free from other components with which it is naturally associated.

The term "recombinant" in the context of a polypeptide refers to the polypeptide when produced by a cell, or in a cell-free expression system, in an altered amount or at an altered rate compared to its native state. In one embodiment the cell is a cell that does not naturally produce the polypeptide. However, the cell may be a cell which comprises a non-endogenous gene that causes an altered, preferably increased, amount of the polypeptide to be produced. A recombinant polypeptide of the invention includes polypeptides which have not been separated from other components of the transgenic (recombinant) cell, or cell-free expression system, in which it is produced, and polypeptides produced in such cells or cell-free systems which are subsequently purified away from at least some other components.

The terms "polypeptide" and "protein" are generally used interchangeably and refer to a single polypeptide chain which may or may not be modified by addition of non-amino acid groups. It would be understood that such polypeptide chains may

associate with other polypeptides or proteins or other molecules such as co-factors. The terms "proteins" and "polypeptides" as used herein also include variants, mutants, modifications, analogous and/or derivatives of the polypeptides of the invention as described herein.

The % identity of a polypeptide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. The query sequence is at least 25 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 25 amino acids. More preferably, the query sequence is at least 50 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 50 amino acids. More preferably, the query sequence is at least 100 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 100 amino acids. Even more preferably, the query sequence is at least 250 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 250 amino acids. Even more preferably, the GAP analysis aligns the two sequences over their entire length.

As used herein a "biologically active" fragment is a portion of a polypeptide of the invention which maintains a defined activity of the full-length polypeptide, namely be able to transport ions across a cell membrane of a plant cell, preferably Na^+ and/or K^+ ions. Biologically active fragments can be any size as long as they maintain the defined activity. Preferably, biologically active fragments are at least 100, more preferably at least 200, and even more preferably at least 350 amino acids in length.

With regard to a defined polypeptide, it will be appreciated that % identity figures higher than those provided above will encompass preferred embodiments. Thus, where applicable, in light of the minimum % identity figures, it is preferred that the polypeptide comprises an amino acid sequence which is at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

In an embodiment, a polypeptide of the invention is not a polypeptide encoded by a polynucleotide provided as Accession No. BE604162 (SEQ ID NO:5).

In a further preferred embodiment, the polypeptide is SEQ ID NO:2 or a polypeptide at least 90% identical thereto.

Amino acid sequence mutants of the polypeptides of the present invention can be prepared by introducing appropriate nucleotide changes into a nucleic acid of the present invention, or by *in vitro* synthesis of the desired polypeptide. Such mutants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final polypeptide product possesses the desired characteristics.

Mutant (altered) polypeptides can be prepared using any technique known in the art. For example, a polynucleotide of the invention can be subjected to *in vitro* mutagenesis. Such *in vitro* mutagenesis techniques may include sub-cloning the polynucleotide into a suitable vector, transforming the vector into a "mutator" strain such as the *E. coli* XL-1 red (Stratagene) and propagating the transformed bacteria for a suitable number of generations. In another example, the polynucleotides of the invention are subjected to DNA shuffling techniques as broadly described by Harayama (1998). These DNA shuffling techniques may include genes related to those of the present invention, such as other HKT family members. Products derived from mutated/altered DNA can readily be screened using techniques described herein to determine if they are able to confer enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part, to a plant expressing said mutated/altered gene.

In designing amino acid sequence mutants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting other residues adjacent to the located site.

Amino acid sequence deletions generally range from about 1 to 15 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Substitution mutants have at least one amino acid residue in the polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as important for function. Other sites of interest are those in which particular residues obtained from various strains or species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are preferably substituted in a relatively

conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "exemplary substitutions".

Table 1. Exemplary substitutions

Original Residue	**Exemplary Substitutions**
Ala (A)	val; leu; ile; gly
Arg (R)	lys
Asn (N)	gln; his
Asp (D)	glu
Cys (C)	ser
Gln (Q)	asn; his
Glu (E)	asp
Gly (G)	pro, ala
His (H)	asn; gln
Ile (I)	leu; val; ala
Leu (L)	ile; val; met; ala; phe
Lys (K)	arg
Met (M)	leu; phe
Phe (F)	leu; val; ala
Pro (P)	gly
Ser (S)	thr
Thr (T)	ser
Trp (W)	tyr
Tyr (Y)	trp; phe
Val (V)	ile; leu; met; phe; ala

In a preferred embodiment a mutant/variant polypeptide has one or two or three or four or five conservative amino acid changes when compared to a naturally occurring polypeptide. Details of conservative amino acid changes are provided in Table 1. Sites of particular interest to alter are those which are not conserved between two or all three of the polypeptides provided in Figure 8. As the skilled person would be aware, such minor changes can reasonably be predicted not to alter the activity of the polypeptide when expressed in a recombinant cell.

Furthermore, if desired, unnatural amino acids or chemical amino acid analogues can be introduced as a substitution or addition into the polypeptides of the present invention. Such amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogues in general.

Also included within the scope of the invention are polypeptides of the present invention which are differentially modified during or after synthesis, e.g., by biotinylation, benzylation, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. These modifications may serve to increase the stability and/or bioactivity of the polypeptide of the invention.

Polypeptides of the present invention can be produced in a variety of ways, including production and recovery of natural polypeptides, production and recovery of recombinant polypeptides, and chemical synthesis of the polypeptides. In one embodiment, an isolated polypeptide of the present invention is produced by culturing a cell capable of expressing the polypeptide under conditions effective to produce the polypeptide, and recovering the polypeptide. A preferred cell to culture is a recombinant cell of the present invention. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit polypeptide production. An effective medium refers to any medium in which a cell is cultured to produce a polypeptide of the present invention. Such medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Polynucleotides and Oligonucleotides

By an "isolated polynucleotide", including DNA, RNA, or a combination of these, single or double stranded, in the sense or antisense orientation or a combination of both, dsRNA or otherwise, we mean a polynucleotide which is at least partially

separated from the polynucleotide sequences with which it is associated or linked in its native state. Preferably, the isolated polynucleotide is at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other components with which they are naturally associated. Furthermore, the term "polynucleotide" is used interchangeably herein with the term "nucleic acid".

The term "exogenous" in the context of a polynucleotide refers to the polynucleotide when present in a cell, or in a cell-free expression system, in an altered amount compared to its native state. In one embodiment, the cell is a cell that does not naturally comprise the polynucleotide. However, the cell may be a cell which comprises a non-endogenous polynucleotide resulting in an altered, preferably increased, amount of production of the encoded polypeptide. An exogenous polynucleotide of the invention includes polynucleotides which have not been separated from other components of the transgenic (recombinant) cell, or cell-free expression system, in which it is present, and polynucleotides produced in such cells or cell-free systems which are subsequently purified away from at least some other components. The exogenous polynucleotide (nucleic acid) can be a contiguous stretch of nucleotides existing in nature, or comprise two or more contiguous stretches of nucleotides from different sources (naturally occurring and/or synthetic) joined to form a single polynucleotide. Typically such chimeric polynucleotides comprise at least an open reading frame encoding a polypeptide of the invention operably linked to a promoter suitable of driving transcription of the open reading frame in a cell of interest.

The % identity of a polynucleotide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. Unless stated otherwise, the query sequence is at least 45 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 45 nucleotides. Preferably, the query sequence is at least 150 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 150 nucleotides. More preferably, the query sequence is at least 300 nucleotides in length and the GAP analysis aligns the two sequences over a region of at least 300 nucleotides. Even more preferably, the GAP analysis aligns the two sequences over their entire length.

With regard to the defined polynucleotides, it will be appreciated that % identity figures higher than those provided above will encompass preferred embodiments. Thus, where applicable, in light of the minimum % identity figures, it is preferred that a polynucleotide of the invention comprises a sequence which is at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%,

more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

As used herein, the term "hybridizes" refers to the ability of two single stranded nucleic acid molecules being able to form at least a partially double stranded nucleic acid through hydrogen bonding.

As used herein, the phrase "stringent conditions" refers to conditions under which a polynucleotide, probe, primer and/or oligonucleotide will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures than shorter sequences. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. Since the target sequences are generally present at excess, at Tm, 50% of the probes are occupied at equilibrium. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes, primers or oligonucleotides (e.g., 10 nt to 50 nt) and at least about 60°C for longer probes, primers and oligonucleotides. Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

Stringent conditions are known to those skilled in the art and can be found in Ausubel et al., (*supra*), Current Protocols In Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6, as well as the Examples described herein. Preferably, the conditions are such that sequences at least about 65%, 70%, 75%, 85%, 90%, 95%, 98%, or 99% homologous to each other typically remain hybridized to each other. A non-limiting example of stringent hybridization conditions are hybridization in a high salt buffer comprising 6xSSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 mg/ml denatured salmon sperm DNA at 65°C, followed by one or more washes in 0.2.xSSC, 0.01% BSA at 50°C. In another embodiment, a nucleic acid sequence that is hybridizable to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO's 10 to 18, under conditions of

moderate stringency is provided. A non-limiting example of moderate stringency hybridization conditions are hybridization in 6xSSC, 5xDenhardt's solution, 0.5% SDS and 100 mg/ml denatured salmon sperm DNA at 55°C, followed by one or more washes in 1xSSC, 0.1% SDS at 37°C. Other conditions of moderate stringency that may be used are well-known within the art, see, e.g., Ausubel et al., (*supra*), and Kriegler, Gene Transfer And Expression, A Laboratory Manual, Stockton Press, 1990. In yet another embodiment, a nucleic acid that is hybridizable to the nucleic acid molecule comprising any one of the nucleotide sequences SEQ ID NO's 10 to 18, under conditions of low stringency, is provided. A non-limiting example of low stringency hybridization conditions are hybridization in 35% formamide, 5xSSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 mg/ml denatured salmon sperm DNA, 10% (wt/vol) dextran sulfate at 40°C., followed by one or more washes in 2xSSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS at 50°C. Other conditions of low stringency that may be used are well known in the art, see, e.g., Ausubel et al., (*supra*) and Kriegler, Gene Transfer And Expression, A Laboratory Manual, Stockton Press, 1990, as well as the Examples provided herein.

In an embodiment, a polynucleotide of the invention is not a polynucleotide provided as Accession No. BE604162 (SEQ ID NO:5).

Polynucleotides of the present invention may possess, when compared to naturally occurring molecules, one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the nucleic acid).

Oligonucleotides of the present invention can be RNA, DNA, or derivatives of either. Although the terms polynucleotide and oligonucleotide have overlapping meaning, oligonucleotide are typically relatively short single stranded molecules. The minimum size of such oligonucleotides is the size required for the formation of a stable hybrid between an oligonucleotide and a complementary sequence on a target nucleic acid molecule. Preferably, the oligonucleotides are at least 15 nucleotides, more preferably at least 18 nucleotides, more preferably at least 19 nucleotides, more preferably at least 20 nucleotides, even more preferably at least 25 nucleotides in length.

Usually, monomers of a polynucleotide or oligonucleotide are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a relatively short monomeric units, e.g., 12-18, to several hundreds of monomeric units. Analogs of phosphodiester linkages include: phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate.

The present invention includes oligonucleotides that can be used as, for example, probes to identify nucleic acid molecules, or primers to produce nucleic acid molecules. Oligonucleotide of the present invention used as a probe are typically conjugated with a detectable label such as a radioisotope, an enzyme, biotin, a fluorescent molecule or a chemiluminescent molecule.

Probes and/or primers can be used to clone homologues of the polynucleotides of the invention from other species. Furthermore, hybridization techniques known in the art can also be used to screen genomic or cDNA libraries for such homologues.

A variant of an oligonucleotide described herein includes molecules of varying sizes of, and/or are capable of hybridising to the genome close to that of, the specific oligonucleotide molecules defined herein. For example, variants may comprise additional nucleotides (such as 1, 2, 3, 4, or more), or less nucleotides as long as they still hybridise to the target region. Furthermore, a few nucleotides may be substituted without influencing the ability of the oligonucleotide to hybridise the target region. In addition, variants may readily be designed which hybridise close (for example, but not limited to, within 50 nucleotides) to the region of the genome where the specific oligonucleotides defined herein hybridise.

Antisense Polynucleotides

The term "antisense polynucletoide" shall be taken to mean a DNA or RNA, or combination thereof, molecule that is complementary to at least a portion of a specific mRNA molecule encoding a polypeptide of the invention and capable of interfering with a post-transcriptional event such as mRNA translation. The use of antisense methods is well known in the art (see for example, G. Hartmann and S. Endres, Manual of Antisense Methodology, Kluwer (1999)). The use of antisense techniques in plants has been reviewed by Bourque, 1995 and Senior, 1998. Bourque, 1995 lists a large number of examples of how antisense sequences have been utilized in plant systems as a method of gene inactivation. She also states that attaining 100% inhibition of any enzyme activity may not be necessary as partial inhibition will more than likely result in measurable change in the system. Senior, 1998 states that antisense methods are now a very well established technique for manipulating gene expression.

An antisense polynucleotide of the invention will hybridize to a target polynucleotide under physiological conditions. As used herein, the term "an antisense polynucleotide which hybridises under physiological conditions" means that the polynucleotide (which is fully or partially single stranded) is at least capable of forming a double stranded polynucleotide with mRNA encoding a protein, such as

those provided in SEQ ID NO: 3 or SEQ ID NO:4 under normal conditions in a cell, preferably a wheat cell.

Antisense molecules may include sequences that correspond to the structural genes or for sequences that effect control over the gene expression or splicing event. For example, the antisense sequence may correspond to the targeted coding region of the genes of the invention, or the 5'-untranslated region (UTR) or the 3'-UTR or combination of these. It may be complementary in part to intron sequences, which may be spliced out during or after transcription, preferably only to exon sequences of the target gene. In view of the generally greater divergence of the UTRs, targeting these regions provides greater specificity of gene inhibition.

The length of the antisense sequence should be at least 19 contiguous nucleotides, preferably at least 50 nucleotides, and more preferably at least 100, 200, 500 or 1000 nucleotides. The full-length sequence complementary to the entire gene transcript may be used. The length is most preferably 100-2000 nucleotides. The degree of identity of the antisense sequence to the targeted transcript should be at least 90% and more preferably 95-100%. The antisense RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule.

Catalytic Polynucleotides

The term catalytic polynucleotide/nucleic acid refers to a DNA molecule or DNA-containing molecule (also known in the art as a "deoxyribozyme") or an RNA or RNA-containing molecule (also known as a "ribozyme") which specifically recognizes a distinct substrate and catalyzes the chemical modification of this substrate. The nucleic acid bases in the catalytic nucleic acid can be bases A, C, G, T (and U for RNA).

Typically, the catalytic nucleic acid contains an antisense sequence for specific recognition of a target nucleic acid, and a nucleic acid cleaving enzymatic activity (also referred to herein as the "catalytic domain"). The types of ribozymes that are particularly useful in this invention are the hammerhead ribozyme (Haseloff and Gerlach, 1988, Perriman et al., 1992) and the hairpin ribozyme (Shippy et al., 1999).

The ribozymes of this invention and DNA encoding the ribozymes can be chemically synthesized using methods well known in the art. The ribozymes can also be prepared from a DNA molecule (that upon transcription, yields an RNA molecule) operably linked to an RNA polymerase promoter, e.g., the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention is a nucleic acid molecule, i.e., DNA or cDNA, coding for a catalytic polynucleotide of the invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced *in vitro* upon incubation

with RNA polymerase and nucleotides. In a separate embodiment, the DNA can be inserted into an expression cassette or transcription cassette. After synthesis, the RNA molecule can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase.

As with antisense polynucleotides described herein, catalytic polynucleotides of the invention should also be capable of hybridizing a target nucleic acid molecule (for example an mRNA encoding a polypeptide provided in SEQ ID NO:1 or SEQ ID NO:2 under "physiological conditions", namely those conditions within a cell (especially conditions in a plant cell such as a wheat or barley cell).

RNA interference

RNA interference (RNAi) is particularly useful for specifically inhibiting the production of a particular protein. Although not wishing to be limited by theory, Waterhouse et al., (1998) have provided a model for the mechanism by which dsRNA (duplex RNA) can be used to reduce protein production. This technology relies on the presence of dsRNA molecules that contain a sequence that is essentially identical to the mRNA of the gene of interest or part thereof, in this case an mRNA encoding a polypeptide according to the invention. Conveniently, the dsRNA can be produced from a single promoter in a recombinant vector or host cell, where the sense and anti-sense sequences are flanked by an unrelated sequence which enables the sense and anti-sense sequences to hybridize to form the dsRNA molecule with the unrelated sequence forming a loop structure. The design and production of suitable dsRNA molecules for the present invention is well within the capacity of a person skilled in the art, particularly considering Waterhouse et al., (1998), Smith et al., (2000), WO 99/32619, WO 99/53050, WO 99/49029, and WO 01/34815.

In one example, a DNA is introduced that directs the synthesis of an at least partly double stranded RNA product(s) with homology to the target gene to be inactivated. The DNA therefore comprises both sense and antisense sequences that, when transcribed into RNA, can hybridize to form the double-stranded RNA region. In a preferred embodiment, the sense and antisense sequences are separated by a spacer region that comprises an intron which, when transcribed into RNA, is spliced out. This arrangement has been shown to result in a higher efficiency of gene silencing. The double-stranded region may comprise one or two RNA molecules, transcribed from either one DNA region or two. The presence of the double stranded molecule is thought to trigger a response from an endogenous plant system that destroys both the double stranded RNA and also the homologous RNA transcript from the target plant gene, efficiently reducing or eliminating the activity of the target gene.

The length of the sense and antisense sequences that hybridise should each be at least 19 contiguous nucleotides, preferably at least 30 or 50 nucleotides, and more preferably at least 100, 200, 500 or 1000 nucleotides. The full-length sequence corresponding to the entire gene transcript may be used. The lengths are most preferably 100-2000 nucleotides. The degree of identity of the sense and antisense sequences to the targeted transcript should be at least 85%, preferably at least 90% and more preferably 95-100%. The RNA molecule may of course comprise unrelated sequences which may function to stabilize the molecule. The RNA molecule may be expressed under the control of a RNA polymerase II or RNA polymerase III promoter. Examples of the latter include tRNA or snRNA promoters.

Preferred small interfering RNA ('siRNA") molecules comprise a nucleotide sequence that is identical to about 19-21 contiguous nucleotides of the target mRNA. Preferably, the target mRNA sequence commences with the dinucleotide AA, comprises a GC-content of about 30-70% (preferably, 30-60%, more preferably 40-60% and more preferably about 45%-55%), and does not have a high percentage identity to any nucleotide sequence other than the target in the genome of the plant (preferably wheat or barley) in which it is to be introduced, e.g., as determined by standard BLAST search.

microRNA

MicroRNA regulation is a clearly specialized branch of the RNA silencing pathway that evolved towards gene regulation, diverging from conventional RNAi/PTGS. MicroRNAs are a specific class of small RNAs that are encoded in gene-like elements organized in a characteristic inverted repeat. When transcribed, microRNA genes give rise to stem–looped precursor RNAs from which the microRNAs are subsequently processed. MicroRNAs are typically about 21 nucleotides in length. The released miRNAs are incorporated into RISC-like complexes containing a particular subset of Argonaute proteins that exert sequence-specific gene repression (see, for example, Millar and Waterhouse, 2005; Pasquinelli et al., 2005; Almeida and Allshire, 2005).

Cosuppression

Another molecular biological approach that may be used is co-suppression. The mechanism of co-suppression is not well understood but is thought to involve post-transcriptional gene silencing (PTGS) and in that regard may be very similar to many examples of antisense suppression. It involves introducing an extra copy of a gene or a fragment thereof into a plant in the sense orientation with respect to a promoter for its expression. The size of the sense fragment, its correspondence to

target gene regions, and its degree of sequence identity to the target gene are as for the antisense sequences described above. In some instances the additional copy of the gene sequence interferes with the expression of the target plant gene. Reference is made to WO 97/20936 and EP 0465572 for methods of implementing co-suppression approaches.

Nucleic Acid Constructs, Vectors and Host Cells

The present invention includes the production of various transgenic plants. These include, but are not limited to, i) plants that express a polynucleotide of the invention which encodes a polypeptide having cation transporter activity, ii) plants where the expression level of at least one endogenous *Nax1* gene has been increased relative to a corresponding non-transgenic plant, and iii) plants that express a polynucleotide which, when present in a cell of a cereal plant, down-regulates the level of *Nax1* activity in the cell when compared to a cell that lacks said polynucleotide.

Nucleic acid constructs useful for producing the above-mentioned transgenic plants can readily be produced using standard techniques.

When inserting a region encoding an mRNA the construct may comprise intron sequences. These intron sequences may aid expression of the transgene in the plant. The term "intron" is used in its normal sense as meaning a genetic segment that is transcribed but does not encode protein and which is spliced out of an RNA before translation. Introns may be incorporated in a 5'-UTR or a coding region if the transgene encodes a translated product, or anywhere in the transcribed region if it does not. However, in a preferred embodiment, any polypeptide encoding region is provided as a single open reading frame. As the skilled addressee would be aware, such open reading frames can be obtained by reverse transcribing mRNA encoding the polypeptide.

To ensure appropriate expression of the gene encoding an mRNA of interest, the nucleic acid construct typically comprises one or more regulatory elements such as promoters, enhancers, as well as transcription termination or polyadenylation sequences. Such elements are well known in the art.

The transcriptional initiation region comprising the regulatory element(s) may provide for regulated or constitutive expression in the plant. Preferably, expression at least occurs in cells of the root and/or leaf sheath. More preferably, expression at least occurs in xylem parenchyma cells. The regulatory elements may be selected be from, for example, root-specific promoters, leaf sheath-specific promoters or promoters not specific for root or leaf sheath cells.

A number of constitutive promoters that are active in plant cells have been described. Suitable promoters for constitutive expression in plants include, but are not limited to, the cauliflower mosaic virus (CaMV) 35S promoter, the Figwort mosaic virus (FMV) 35S, the sugarcane bacilliform virus promoter, the commelina yellow mottle virus promoter, the light-inducible promoter from the small subunit of the ribulose-1,5-bis-phosphate carboxylase, the rice cytosolic triosephosphate isomerase promoter, the adenine phosphoribosyltransferase promoter of *Arabidopsis*, the rice actin 1 gene promoter, the mannopine synthase and octopine synthase promoters, the Adh promoter, the sucrose synthase promoter, the R gene complex promoter, and the chlorophyll α/β binding protein gene promoter. These promoters have been used to create DNA vectors that have been expressed in plants; see, e.g., PCT publication WO 8402913. All of these promoters have been used to create various types of plant-expressible recombinant DNA vectors.

An example of a root specific promoter is the promoter for the acid chitinase gene. Expression in root tissue could also be accomplished by utilizing the root specific subdomains of the CaMV 35S promoter that have been identified.

The promoter may be modulated by factors such as temperature, light or stress. Ordinarily, the regulatory elements will be provided 5' of the genetic sequence to be expressed. The construct may also contain other elements that enhance transcription such as the nos 3' or the ocs 3' polyadenylation regions or transcription terminators.

The 5' non-translated leader sequence can be derived from the promoter selected to express the heterologous gene sequence of the polynucleotide of the present invention, and can be specifically modified if desired so as to increase translation of mRNA. For a review of optimizing expression of transgenes, see Koziel et al., (1996). The 5' non-translated regions can also be obtained from plant viral RNAs (Tobacco mosaic virus, Tobacco etch virus, Maize dwarf mosaic virus, Alfalfa mosaic virus, among others) from suitable eukaryotic genes, plant genes (wheat and maize chlorophyll a/b binding protein gene leader), or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. The leader sequence could also be derived from an unrelated promoter or coding sequence. Leader sequences useful in context of the present invention comprise the maize Hsp70 leader (U.S. 5,362,865 and U.S. 5,859,347), and the TMV omega element.

The termination of transcription is accomplished by a 3' non-translated DNA sequence operably linked in the chimeric vector to the polynucleotide of interest. The 3' non-translated region of a recombinant DNA molecule contains a polyadenylation signal that functions in plants to cause the addition of adenylate nucleotides to the 3'

end of the RNA. The 3' non-translated region can be obtained from various genes that are expressed in plant cells. The nopaline synthase 3' untranslated region, the 3' untranslated region from pea small subunit Rubisco gene, the 3' untranslated region from soybean 7S seed storage protein gene are commonly used in this capacity. The 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes are also suitable.

Typically, the nucleic acid construct comprises a selectable marker. Selectable markers aid in the identification and screening of plants or cells that have been transformed with the exogenous nucleic acid molecule. The selectable marker gene may provide antibiotic or herbicide resistance to the wheat cells, or allow the utilization of substrates such as mannose. The selectable marker preferably confers hygromycin resistance to the wheat cells.

Preferably, the nucleic acid construct is stably incorporated into the genome of the plant. Accordingly, the nucleic acid comprises appropriate elements which allow the molecule to be incorporated into the genome, or the construct is placed in an appropriate vector which can be incorporated into a chromosome of a plant cell.

One embodiment of the present invention includes a recombinant vector, which includes at least one polynucleotide molecule of the present invention, inserted into any vector capable of delivering the nucleic acid molecule into a host cell. Such a vector contains heterologous nucleic acid sequences, that is nucleic acid sequences that are not naturally found adjacent to nucleic acid molecules of the present invention and that preferably are derived from a species other than the species from which the nucleic acid molecule(s) are derived. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a virus or a plasmid.

A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, supp. 1987; Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; and Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990. Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Another embodiment of the present invention includes a recombinant cell comprising a host cell transformed with one or more recombinant molecules of the

present invention. Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed nucleic acid molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained. Preferred host cells are plant cells, more preferably cells of a cereal plant, more preferably barley or wheat cells, and even more preferably a wheat cell.

Transgenic Plants

Plants contemplated for use in the practice of the present invention include both monocotyledons and dicotyledons. Target plants include, but are not limited to, the following: cereals (for example, wheat, barley, rye, oats, rice, sorghum and related crops); beet (sugar beet and fodder beet); pomes, stone fruit and soft fruit (apples, pears, plums, peaches, almonds, cherries, strawberries, raspberries and black-berries); leguminous plants (beans, lentils, peas, soybeans); oil plants (rape, mustard, poppy, olives, sunflowers, coconut, castor oil plants, cocoa beans, groundnuts); cucumber plants (marrows, cucumbers, melons); fibre plants (cotton, flax, hemp, jute); citrus fruit (oranges, lemons, grapefruit, mandarins); vegetables (spinach, lettuce, asparagus, cabbages, carrots, onions, tomatoes, potatoes, paprika); lauraceae (avocados, cinnamon, camphor); or plants such as maize, tobacco, nuts, coffee, sugar cane, tea, vines, hops, turf, bananas and natural rubber plants, as well as ornamentals (flowers, shrubs, broad-leaved trees and evergreens, such as conifers). Preferably, the plant is a cereal plant, more preferably wheat or barley, even more preferably wheat.

Transgenic plants, as defined in the context of the present invention include plants (as well as parts and cells of said plants) and their progeny which have been genetically modified using recombinant techniques to cause production of at least one polypeptide of the present invention in the desired plant or plant organ. Transgenic plants can be produced using techniques known in the art, such as those generally described in A. Slater et al., Plant Biotechnology - The Genetic Manipulation of Plants, Oxford University Press (2003), and P. Christou and H. Klee, Handbook of Plant Biotechnology, John Wiley and Sons (2004).

In a preferred embodiment, the transgenic plants are homozygous for each and every gene that has been introduced (transgene) so that their progeny do not segregate for the desired phenotype. The transgenic plants may also be heterozygous for the

introduced transgene(s), such as, for example, in F1 progeny which have been grown from hybrid seed. Such plants may provide advantages such as hybrid vigour, well known in the art.

Four general methods for direct delivery of a gene into cells have been described: (1) chemical methods (Graham et al., 1973); (2) physical methods such as microinjection (Capecchi, 1980); electroporation (see, for example, WO 87/06614, US 5,472,869, 5,384,253, WO 92/09696 and WO 93/21335); and the gene gun (see, for example, US 4,945,050 and US 5,141,131); (3) viral vectors (Clapp, 1993; Lu et al., 1993; Eglitis et al., 1988); and (4) receptor-mediated mechanisms (Curiel et al., 1992; Wagner et al., 1992).

Acceleration methods that may be used include, for example, microprojectile bombardment and the like. One example of a method for delivering transforming nucleic acid molecules to plant cells is microprojectile bombardment. This method has been reviewed by Yang et al., Particle Bombardment Technology for Gene Transfer, Oxford Press, Oxford, England (1994). Non-biological particles (microprojectiles) that may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like. A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly transforming monocots, is that neither the isolation of protoplasts, nor the susceptibility of *Agrobacterium* infection are required. An illustrative embodiment of a method for delivering DNA into *Zea mays* cells by acceleration is a biolistics α-particle delivery system, that can be used to propel particles coated with DNA through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with corn cells cultured in suspension. A particle delivery system suitable for use with the present invention is the helium acceleration PDS-1000/He gun is available from Bio-Rad Laboratories.

For the bombardment, cells in suspension may be concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the microprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded.

Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the microprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more foci of cells transiently expressing a marker gene. The number of cells in a focus that express the exogenous gene product 48 hours post-bombardment often range from one to ten and average one to three.

In bombardment transformation, one may optimize the pre-bombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

In another alternative embodiment, plastids can be stably transformed. Method disclosed for plastid transformation in higher plants include particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination (U.S. 5, 451,513, U.S. 5,545,818, U.S. 5,877,402, U.S. 5,932479, and WO 99/05265.

Accordingly, it is contemplated that one may wish to adjust various aspects of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One may also minimize the trauma reduction factors by modifying conditions that influence the physiological state of the recipient cells and that may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art in light of the present disclosure.

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art (see, for example, US 5,177,010, US 5,104,310, US 5,004,863, US 5,159,135). Further, the integration of the T-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border sequences, and intervening DNA is usually inserted into the plant genome.

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee et al., Plant DNA Infectious Agents, Hohn and Schell, (editors), Springer-

Verlag, New York, (1985): 179-203). Moreover, technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant varieties where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single genetic locus on one chromosome. Such transgenic plants can be referred to as being hemizygous for the added gene. More preferred is a transgenic plant that is homozygous for the added structural gene; i.e., a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants for the gene of interest.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in Fehr, Breeding Methods for Cultivar Development, J. Wilcox (editor) American Society of Agronomy, Madison Wis. (1987).

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments. Application of these systems to different plant varieties depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (Fujimura et al., 1985; Toriyama et al., 1986; Abdullah et al., 1986).

Other methods of cell transformation can also be used and include but are not limited to introduction of DNA into plants by direct DNA transfer into pollen, by direct injection of DNA into reproductive organs of a plant, or by direct injection of DNA into the cells of immature embryos followed by the rehydration of desiccated embryos.

The regeneration, development, and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach et al., Methods for Plant Molecular Biology, Academic Press, San Diego, (1988)). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene is well known in the art. Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired exogenous nucleic acid is cultivated using methods well known to one skilled in the art.

Methods for transforming dicots, primarily by use of *Agrobacterium* tumefaciens, and obtaining transgenic plants have been published for cotton (U.S. 5,004,863, U.S. 5,159,135, U.S. 5,518,908); soybean (U.S. 5,569,834, U.S. 5,416,011); Brassica (U.S. 5,463,174); peanut (Cheng et al., 1996); and pea (Grant et al., 1995).

Methods for transformation of cereal plants such as wheat and barley for introducing genetic variation into the plant by introduction of an exogenous nucleic acid and for regeneration of plants from protoplasts or immature plant embryos are well known in the art, see for example, Canadian Patent Application No. 2,092,588, Australian Patent Application No 61781/94, Australian Patent No 667939, US Patent No. 6,100,447, International Patent Application PCT/US97/10621, U.S. Patent No. 5,589,617, U.S. Patent No. 6,541,257, and other methods are set out in Patent specification WO99/14314. Preferably, transgenic wheat or barley plants are produced by *Agrobacterium tumefaciens* mediated transformation procedures. Vectors carrying the desired nucleic acid construct may be introduced into regenerable wheat cells of tissue cultured plants or explants, or suitable plant systems such as protoplasts.

The regenerable wheat cells are preferably from the scutellum of immature embryos, mature embryos, callus derived from these, or the meristematic tissue.

To confirm the presence of the transgenes in transgenic cells and plants, a polymerase chain reaction (PCR) amplification or Southern blot analysis can be performed using methods known to those skilled in the art. Expression products of

the transgenes can be detected in any of a variety of ways, depending upon the nature of the product, and include Western blot and enzyme assay. One particularly useful way to quantitate protein expression and to detect replication in different plant tissues is to use a reporter gene, such as GUS. Once transgenic plants have been obtained, they may be grown to produce plant tissues or parts having the desired phenotype. The plant tissue or plant parts, may be harvested, and/or the seed collected. The seed may serve as a source for growing additional plants with tissues or parts having the desired characteristics.

Marker Assisted Selection

Marker assisted selection is a well recognised method of selecting for heterozygous plants required when backcrossing with a recurrent parent in a classical breeding program. The population of plants in each backcross generation will be heterozygous for the gene of interest normally present in a 1:1 ratio in a backcross population, and the molecular marker can be used to distinguish the two alleles of the gene. By extracting DNA from, for example, young shoots and testing with a specific marker for the introgressed desirable trait, early selection of plants for further backcrossing is made whilst energy and resources are concentrated on fewer plants. To further speed up the backcrossing program, the embryo from immature seeds (25 days post anthesis) may be excised and grown up on nutrient media under sterile conditions, rather than allowing full seed maturity. This process, termed "embryo rescue", used in combination with DNA extraction at the three leaf stage and analysis of at least one *Nax1* gene or allele that confers enhanced tolerance to saline and/or sodic soils to the plant, and/or reduced sodium accumulation in an aerial part of the plant, allows rapid selection of plants carrying the desired trait, which may be nurtured to maturity in the greenhouse or field for subsequent further backcrossing to the recurrent parent.

Any molecular biological technique known in the art which is capable of detecting alleles of a *Nax1* gene can be used in the methods of the present invention. Such methods include, but are not limited to, the use of nucleic acid amplification, nucleic acid sequencing, nucleic acid hybridization with suitably labeled probes, single-strand conformational analysis (SSCA), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis (HET), chemical cleavage analysis (CCM), catalytic nucleic acid cleavage or a combination thereof (see, for example, Lemieux, 2000; Langridge et al., 2001). The invention also includes the use of molecular marker techniques to detect polymorphisms linked to alleles of (for example) a *Nax1* gene which confers enhanced tolerance to saline and/or sodic soils to the plant, and/or reduced sodium accumulation in an aerial part of a plant. Such methods include the

detection or analysis of restriction fragment length polymorphisms (RFLP), RAPD, amplified fragment length polymorphisms (AFLP) and microsatellite (simple sequence repeat, SSR) polymorphisms. The closely linked markers can be obtained readily by methods well known in the art, such as Bulked Segregant Analysis, as reviewed by Langridge et al., (2001).

The "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using a "pair of primers" or "set of primers" consisting of "upstream" and a "downstream" primer, and a catalyst of polymerization, such as a DNA polymerase, and typically a thermally-stable polymerase enzyme. Methods for PCR are known in the art, and are taught, for example, in "PCR" (M.J. McPherson and S.G Moller (editors), BIOS Scientific Publishers Ltd, Oxford, (2000)). PCR can be performed on cDNA obtained from reverse transcribing mRNA isolated from plant cells expressing a *Nax1* gene or allele which confers enhanced tolerance to saline and/or sodic soils to the plant, and/or reduced sodium accumulation in an aerial part of the plant. However, it will generally be easier if PCR is performed on genomic DNA isolated from a plant.

A primer is an oligonucleotide sequence that is capable of hybridising in a sequence specific fashion to the target sequence and being extended during the PCR. Amplicons or PCR products or PCR fragments or amplification products are extension products that comprise the primer and the newly synthesized copies of the target sequences. Multiplex PCR systems contain multiple sets of primers that result in simultaneous production of more than one amplicon. Primers may be perfectly matched to the target sequence or they may contain internal mismatched bases that can result in the introduction of restriction enzyme or catalytic nucleic acid recognition/cleavage sites in specific target sequences. Primers may also contain additional sequences and/or contain modified or labelled nucleotides to facilitate capture or detection of amplicons. Repeated cycles of heat denaturation of the DNA, annealing of primers to their complementary sequences and extension of the annealed primers with polymerase result in exponential amplification of the target sequence. The terms target or target sequence or template refer to nucleic acid sequences which are amplified.

Methods for direct sequencing of nucleotide sequences are well known to those skilled in the art and can be found for example in Ausubel et al., (*supra*) and Sambrook et al., (*supra*). Sequencing can be carried out by any suitable method, for example, dideoxy sequencing, chemical sequencing or variations thereof. Direct sequencing has the advantage of determining variation in any base pair of a particular sequence.

Hybridization based detection systems include, but are not limited to, the TaqMan assay and molecular beacons. The TaqMan assay (US 5,962,233) uses allele specific (ASO) probes with a donor dye on one end and an acceptor dye on the other end such that the dye pair interact via fluorescence resonance energy transfer (FRET). A target sequence is amplified by PCR modified to include the addition of the labeled ASO probe. The PCR conditions are adjusted so that a single nucleotide difference will effect binding of the probe. Due to the 5' nuclease activity of the Taq polymerase enzyme, a perfectly complementary probe is cleaved during PCR while a probe with a single mismatched base is not cleaved. Cleavage of the probe dissociates the donor dye from the quenching acceptor dye, greatly increasing the donor fluorescence.

An alternative to the TaqMan assay is the molecular beacon assay (US 5,925,517). In the molecular beacon assay, the ASO probes contain complementary sequences flanking the target specific species so that a hairpin structure is formed. The loop of the hairpin is complimentary to the target sequence while each arm of the hairpin contains either donor or acceptor dyes. When not hybridized to a donor sequence, the hairpin structure brings the donor and acceptor dye close together thereby extinguishing the donor fluorescence. When hybridized to the specific target sequence, however, the donor and acceptor dyes are separated with an increase in fluorescence of up to 900 fold. Molecular beacons can be used in conjunction with amplification of the target sequence by PCR and provide a method for real time detection of the presence of target sequences or can be used after amplification.

TILLING

Plants of the invention can be produced using the process known as TILLING (Targeting Induced Local Lesions IN Genomes). In a first step, introduced mutations such as novel single base pair changes are induced in a population of plants by treating seeds (or pollen) with a chemical mutagen, and then advancing plants to a generation where mutations will be stably inherited. DNA is extracted, and seeds are stored from all members of the population to create a resource that can be accessed repeatedly over time.

For a TILLING assay, PCR primers are designed to specifically amplify a single gene target of interest. Specificity is especially important if a target is a member of a gene family or part of a polyploid genome. Next, dye-labeled primers can be used to amplify PCR products from pooled DNA of multiple individuals. These PCR products are denatured and reannealed to allow the formation of mismatched base pairs. Mismatches, or heteroduplexes, represent both naturally occurring single nucleotide polymorphisms (SNPs) (i.e., several plants from the population are likely to carry the same polymorphism) and induced SNPs (i.e., only

rare individual plants are likely to display the mutation). After heteroduplex formation, the use of an endonuclease, such as Cel I, that recognizes and cleaves mismatched DNA is the key to discovering novel SNPs within a TILLING population.

Using this approach, many thousands of plants can be screened to identify any individual with a single base change as well as small insertions or deletions (1-30 bp) in any gene or specific region of the genome. Genomic fragments being assayed can range in size anywhere from 0.3 to 1.6 kb. At 8-fold pooling, 1.4 kb fragments (discounting the ends of fragments where SNP detection is problematic due to noise) and 96 lanes per assay, this combination allows up to a million base pairs of genomic DNA to be screened per single assay, making TILLING a high-throughput technique.

TILLING is further described in Slade and Knauf, 2005, and Henikoff et al., (2004).

In addition to allowing efficient detection of mutations, high-throughput TILLING technology is ideal for the detection of natural polymorphisms. Therefore, interrogating an unknown homologous DNA by heteroduplexing to a known sequence reveals the number and position of polymorphic sites. Both nucleotide changes and small insertions and deletions are identified, including at least some repeat number polymorphisms. This has been called Ecotilling (Comai et al., 2004).

Each SNP is recorded by its approximate position within a few nucleotides. Thus, each haplotype can be archived based on its mobility. Sequence data can be obtained with a relatively small incremental effort using aliquots of the same amplified DNA that is used for the mismatch-cleavage assay. The left or right sequencing primer for a single reaction is chosen by its proximity to the polymorphism. Sequencher software performs a multiple alignment and discovers the base change, which in each case confirmed the gel band.

Ecotilling can be performed more cheaply than full sequencing, the method currently used for most SNP discovery. Plates containing arrayed ecotypic DNA can be screened rather than pools of DNA from mutagenized plants. Because detection is on gels with nearly base pair resolution and background patterns are uniform across lanes, bands that are of identical size can be matched, thus discovering and genotyping SNPs in a single step. In this way, ultimate sequencing of the SNP is simple and efficient, made more so by the fact that the aliquots of the same PCR products used for screening can be subjected to DNA sequencing.

Antibodies

The invention also provides monoclonal or polyclonal antibodies to polypeptides of the invention or fragments thereof. Thus, the present invention

further provides a process for the production of monoclonal or polyclonal antibodies to polypeptides of the invention.

The term "binds specifically" refers to the ability of the antibody to bind to a polypeptide of the present invention but not other known proteins, for example, cation transporters such as those from rice. It is preferred that an antibody of the invention does not bind other polypeptides found in a wheat cell producing the polypeptide (with the exception of related polypeptides that have cation transporter activity).

As used herein, the term "epitope" refers to a region of a polypeptide of the invention which is bound by the antibody. An epitope can be administered to an animal to generate antibodies against the epitope, however, antibodies of the present invention preferably specifically bind the epitope region in the context of the entire polypeptide.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide such as that provided as SEQ ID NO:1 or SEQ ID NO:2. Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides peptides of the invention or fragments thereof haptenised to another peptide for use as immunogens in animals.

Monoclonal antibodies directed against polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced can be screened for various properties; i.e., for isotype and epitope affinity.

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv). Furthermore, the antibodies and fragments thereof may be humanised antibodies, for example as described in EP-A-239400.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

Preferably, antibodies of the present invention are detectably labeled. Exemplary detectable labels that allow for direct measurement of antibody binding include radiolabels, fluorophores, dyes, magnetic beads, chemiluminescers, colloidal particles, and the like. Examples of labels which permit indirect measurement of binding include enzymes where the substrate may provide for a coloured or fluorescent product. Additional exemplary detectable labels include covalently bound enzymes capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art. Further exemplary detectable labels include biotin, which binds with high affinity to avidin or streptavidin; fluorochromes (e.g., phycobiliproteins, phycoerythrin and allophycocyanins; fluorescein and Texas red), which can be used with a fluorescence activated cell sorter; haptens; and the like. Preferably, the detectable label allows for direct measurement in a plate luminometer, e.g., biotin. Such labeled antibodies can be used in techniques known in the art to detect polypeptides of the invention.

EXAMPLES

Example 1. Identification of candidate genes for *Nax1*

Previously, *Nax1*, a major gene for low Na^+ concentration in leaf blades of durum wheat was identified in a durum wheat line designated Line 149 (Munns et al., 2000). Line 149 (Australian Winter Cereals Collection, Tamworth, NSW, Australia - Accession No. AUS17045) was derived from a cross between *Triticum monococcum* L. (AA) accession C68-101 and the durum cultivar Marrocos (The, 1973). The locus containing the *Nax1* gene was mapped as a QTL and shown to be genetically linked to the microsatellite marker *gwm312* on chromosome 2AL (Lindsay et al., 2004). Durum and hexaploid wheats do not naturally contain the *Nax1* gene on chromosome 2A.

To develop a more detailed genetic linkage map, a backcross-derived mapping family of 41 BC_5F_2 lines was generated and used. Line 149 was crossed with the Australian durum cultivar Tamaroi (salt-sensitive, high Na^+ accumulation) and backcrossed four generations with Tamaroi followed by selfing to produce a homozygous low Na^+ BC_4 line that was used as the parent in an additional backcross to generate a low resolution mapping family (James et al., 2006). Plants were

phenotyped for Na^+ accumulation as follows. Plants were grown according to the method of Munns and James (2003). At 8 d after seedling emergence, 25 mM NaCl salt solution was added to the irrigation solution twice daily until a final NaCl concentration was reached of 150 mM. Na^+ concentration in the blade of the third leaf, 10 d after emergence, was measured in all plants according to Munns et al., (2000). Leaves were dried at 70°C for 3 d, extracted in 500 mM HNO_3 at 80°C for 1.5 h, and Na^+ concentration was measured by an Inductively Coupled Plasma-Atomic Emission Spectrometer (Varian Vista Pro, Melbourne, Australia).

The data for Na^+ accumulation in leaf 3 for the 41 BC_5F_2 lines is shown in Figure 1. The difference in leaf 3 Na^+ concentration between the low Na^+ BC_4F_3 parent line and Tamaroi was nearly four-fold. In the family of 41 BC_5F_2 lines, the segregation of the sodium exclusion trait fitted the expected ratio for a single major gene (designated *Nax1*) (Expected 10:21:10; Observed: 11:21:9; χ^2 =0.22, $P_{0.05}$=6.00).

To extract DNA for genotyping, plants were transplanted from the salt tanks into soil and allowed to grow for approximately 4 weeks before DNA was extracted as described by Lagudah et al., (1991). Four microliters of extracted DNA was used to perform PCR. Primer sequences of flanking microsatellite marker *gwm*312 were as described in Röder et al., (1998). Amplifications were performed in 20 µL aliquots containing 1.5 mM $MgCl_2$, 2 µM of each primer, 200 µM dNTPs, 200 µM 1× PCR buffer, 2 units Taq DNA polymerase and 100 ng genomic DNA. The PCR program and gel running conditions were as described by Lindsay et al., (2004).

The mapping family was used to position *Nax1* to a single genetic locus (Figure 2) as follows. The *gwm312* marker, known to be linked to *Nax1*, was mapped to the deletion bin "Flow Length (FL) 0.77-0.85" in a genetic background cultivar Chinese Spring indicating that *Nax1* was located proximal (towards the centromere) to the deletion breakpoint FL 0.85 on chromosome 2AL (Figure 2).

Wheat ESTs (wESTs) previously mapped to chromosome 2AL were therefore examined as a source of potential markers. On the basis that the region containing the *Nax1* locus on wheat chromosome 2AL was syntenic with chromosome 4 of rice, wheat ESTs (wESTs) previously mapped into the deletion bin FL 0.00-0.85 with respect to the cultivar 'Chinese Spring' wheat were examined for homology to rice genes in the syntenic region of 17.8 to 34.4 Mb on rice chromosome 4L. A total of 347 sequences for wESTs previously mapped to the deletion bin 2AL 0.0-0.85 were downloaded from the Graingenes web site (http://wheat.pwusda.gov/NSF/progress_mapping.html). BlastN analysis, using as selection parameters an E value of $\leq 10^{-15}$ and a length of identity greater than 100 bp, was used to search the NCBI and Gramene databases (http://www.ncbi.nlm.nih.gov/; http://www.gramene.org). A subset of 74 of the wEST sequences were identified as

having closely related genes on rice chromosome 4L between the positions 17.8 Mb and 34.4 Mb. Table 2 lists wESTs with close matches to rice genes, which were used to develop RFLP or PCR-based markers as described below.

Initially, two wESTs, BE498441 and BM137419, were selected for mapping. These were closely related to rice genes located at 24.1 and 24.2 Mb on rice chromosome 4L, respectively (Table 2). Primers (Table 3) were designed on the basis of published wheat ESTs sequences, and PCR reactions carried out to amplify the gene fragments for use as probes. The amplified products were cloned using the pGEM T-Easy Vector system (Promega, USA) and the nucleotide sequences of the gene fragments confirmed by sequencing. DNA probes were amplified by PCR and labelled with ^{32}P using the Megaprime DNA Labelling System (Amersham Biosciences, UK).

For RFLP analysis, DNA samples prepared from plants of wheat cultivar Chinese Spring, a series of 2AL deletion lines (Endo and Gill 1996), *T. monococcum* accession C68-101, parental lines and F_2 lines of the mapping family as described above were digested with six restriction enzymes (*Dra*I, *EcoR*I, *EcoR*V, *Hind*III, *Nco*I and *Xba*I) and electrophoresed on agarose gels before Southern blotting. DNA hybridisation analysis on the blots was conducted according to Seah et al., (1998).

Both wEST BE498441 and BM137419 probes detected polymorphic markers between the donor Line 149 and the recurrent parent Tamaroi. However, only the Tamaroi allele was present for both markers in the BC_4 parental line and in the BC_5F_2 family, indicating that the chromosomal region containing BE498441 and BM137419 was replaced by the Tamaroi alleles during the process of backcrossing. Another wEST, BF474590, with sequence relatedness to a rice gene located at 26.9 Mb was also polymorphic between the parents. In contrast to BE498441 and BM137419, BF474590 segregated in the BC_5F_2 family and mapped 7.3 cM from *Nax1* (Figure 2). To identify additional markers, we therefore focused on wESTs that were closely related to rice genes located distal to 26.9 Mb on chromosome 4L.

Table 2. BlastN search results of selected wESTs located in wheat deletion bin 2AL (0.0-0.85) with rice chromosome 4.

EST	BlastN	Locus	Distance (Mb)	BlastX
BE498441	5.00E-15	LOC_Os04g41040	24.14	oj991113_30.8 [*Oryza sativa* (japonica cultivar-group)]
BM137419	5.00E-27	LOC_Os04g41200	24.21	oj991113_30.22 [*Oryza sativa* (japonica cultivar-group)]
BF474590	7.00E-16	LOC_Os04g45800	26.89	putative sphingosine kinase [*Oryza sativa*]
BE403863	9.00E-44	LOC_Os04g48130	28.42	membrane protein; protein id: At5g07250.1, supported by cDNA: gi_16648761, [*Arabidopsis thaliana*]
BG262791	2.00E-57	LOC_Os04g49570	29.35	ligand-gated ion channel, putative; protein id: At1g42540.1 [*Arabidopsis thaliana*]
BE423738* (CK205077)	5.00E-58	LOC_Os04g52390	30.91	putative potasium transporter [*Oryza sativa* (japonica cultivar-group)]
BE403217	3.00E-61	LOC_Os04g52900	31.29	MRP-like ABC transporter [*Oryza sativa* (japonica cultivar-group)]

* BE423738 and CK205077 matched the same rice gene of a putative potassium transporter.

Table 3. Primer pairs of markers selected for mapping *Nax1* gene in chromosome 2AL of durum wheat.

Markers	Forward primer	Reverse primer
Wheat ESTs		
BE498441	AGGAGAACGTCAACAATGGCATA (SEQ ID NO: 22)	AGCGTCTTCTTCTGCTCCTTTGT (SEQ ID NO: 23)
BM137419	GGCTCAGACATCTTCTGGGAAAT (SEQ ID NO: 24)	TCAGCAAAATGTTAGAGCGGAAC (SEQ ID NO: 25)
BF474590	GGTTAAACCTGGTGCAAATACCC (SEQ ID NO: 26)	TGGAGGCATCCCTCTATAATCAC (SEQ ID NO: 27)
BE403863	ACATGATCAGCCTCATCTTCGTC (SEQ ID NO: 28)	CAAGCCGTGTACTTGGACTTTGT (SEQ ID NO: 29)
BG262791	TGTGGTGCATCACAGGGCTGTTC (SEQ ID NO: 30)	AGCGCTTGCATACTCGTCCGG (SEQ ID NO: 31)
BE423738	GGTAGCAGATATCGTGGCTTGAC (SEQ ID NO: 32)	GTCGGTGCACTGAACATACATACA (SEQ ID NO: 33)
CK205077	ACGTTTCCAGGAACCTGATTTGT (SEQ ID NO: 34)	GTTAGAAGAATTTCCCCGCCTTC (SEQ ID NO: 35)

BE403217	AGCAATGAGGATGGTGCTTTCTC (SEQ ID NO: 36)	TGTGAGCGACTCCTCGATTTCAG (SEQ ID NO: 37)
CA498418	GATTTTGAGGGAAGGTGATCCAG (SEQ ID NO: 38)	AGTAAAACCGGAATGTGTGCTGA (SEQ ID NO: 39)
BE604162	ATTCAGGCAACACCTAATCATGC (SEQ ID NO: 40)	GCATCACAAGAATGAGGATGAGC (SEQ ID NO: 41)
AL817940	CTGGCTTCTTGTTTGGGCTTTAT (SEQ ID NO: 42)	TTCAGTTTGGTTCCGTAGTTCCA (SEQ ID NO: 43)
Barley ESTs		
BJ472463	TTAAAAATATTCGGGCCAACACC (SEQ ID NO: 44)	TGGGGTAAGCAGAAGAAGGAAAG (SEQ ID NO: 45)
Microsatellites		
gwm312	ATCGCATGATGCACGTAGAG (SEQ ID NO: 46)	ACATGCATGCCTACCTAATGG (SEQ ID NO: 47)

Five additional wESTs (BE403863, BG262791, CK205077, BE403217, CA498418) were identified which corresponded to rice genes located in the distal region of chromosome 4 (28.4 Mb to 32.5 Mb) (Figure 2). Portions of these genes were amplified using the primer pairs listed in Table 3 and labelled to provide probes for RFLP analysis. Primer sequences for CK205077 were as follows: Forward primer, 5'-ACGTTTCCAGGAACCTGATTTGT-3' (SEQ ID NO: 34); Reverse primer, 5'-GTTAGAAGAATTTCCCCGCCTTC-3' (SEQ ID NO: 35). PCR products amplified from both parents contained different *Rsa*I restriction sites. This polymorphism was used to develop a CAPS marker using *Rsa*I to digest the amplification products.

Consistent with the physical location of the corresponding rice genes, BE403863 was mapped as an RFLP proximal to *Nax1* (6.1 cM from *Nax1*), while CK205077 co-segregated with *Nax1* in this mapping family (Figure 2); no recombinants were identified between CK205077 and *Nax1* in the mapping population. The wEST CK205077 had sequence homology to a putative potassium transporter in rice (OsHAK11) (Table 2). Another wEST BE403217 corresponding to a rice gene near the distal end of chromosome 4L was also located on the distal side of *Nax1* and co-segregated with *gwm312* (Figure 2). A break in co-linearity was observed with wEST BG262791; this gene co-segregated with *gwm312*, although its predicted map location was on the proximal side of *Nax1*.

The genetic order of the wESTs was confirmed by their physical location within one of three deletion bins on chromosome 2AL, including wEST CA498418 which was placed into the distal deletion bin (FL 0.85-1.00) consistent with the location of the corresponding rice gene (Figure 2). These results suggest that an interstitial region was re-arranged on wheat chromosome 2AL relative to rice chromosome 4L. Based on these results, *Nax1* was located within a 7 cM genetic interval flanked by wEST markers BE403863 and BE403217. This genetic interval corresponded to a physical interval between 28.4 and 31.3 Mb on rice chromosome 4L.

To identify rice genes which might have been related to candidate genes for *Nax1*, the 3 Mb interval (28.4-31.3 Mb) was searched for genes encoding putative potassium or sodium transporters. Beside *OsHAK11*, three additional rice genes were identified, one with homology to a putative potassium transporter (AL817940, homologous to *OsHAK15*) and two with homology to sodium transporters in wheat and barley (BE604162, homologous to *OsHKT7*; BJ472463, homologous to *OsHKT4*) (Table 4).

Table 4. List of candidate genes for *Nax1* on rice chromosome 4L*.

Locus	Distance (Mb)	Wheat or Barley EST	E-value	BlastX
OsHKT4 (LOC_Os04g51820)	30.51**	BJ472463	2E-43	Putative Na^+ transporter
OsHKT7 (LOC_Os04g51830)	30.52	BE604162	3E-67	Putative Na^+ transporter
OsHAK15 (LOC_Os04g52120)	30.73	AL817940	3E-97	Putative K^+ transporter
OsHAK11 (LOC_Os04g52390)	30.91	CK205077	1E-79	Putative K^+ transporter
SKOR (LOC_Os04g55080)	32.53	CA498418	2E-99	Cyclic nucleotide and calmodulin-regulated K^+ channel

* The *HKT* and *HAK* gene families in rice were as named by Garciadeblás et al., (2003) and Bañuelos et al., (2002), respectively.

** Based on search results from Gramene database (http://www.gramene.org), *OsHKT4* and *OsHKT7* were located side by side separated by ~3Kb on chromosome 4.

Example 2. Map position of candidate genes relative to *Nax1*

Based on the initial genetic mapping, a high resolution genetic map was generated by screening a mapping population of 864 BC_5F_2 seeds (equivalent to 1728 gametes) to identify recombinants between *Nax1* and markers corresponding to the rice genes identified in Example 1, as follows. DNA was extracted from half of each seed, with each second half seed (containing the embryo) being retained for regeneration of plants so that the *Nax1* phenotype could be assayed and the plant line retained. For the DNA extraction, the method according to Mago et al., (2005) was used. For the phenotyping, a concentration of 50mM NaCl was used rather than 150mM in order to reduce the stress on the seedlings derived from half-seeds which were less vigorous. There was little difference in levels of shoot Na^+ accumulation between the 150 and 50 mM NaCl treatments (Husain et al., 2004). Additional $CaCl_2$ was added to give a final Na^+:Ca^{2+} ratio of 15:1.

DNA samples isolated from the 864 individual F2 half seeds were screened with the flanking *gwm312* marker and a cleavage amplification polymorphism

sequence (CAPS) marker derived from wEST CK205077 as described above, as it was thought these markers might flank *Nax1*.

The great majority of the 864 individuals were parental for both markers, having either both markers from Tamaroi or both markers from Line 149. Twenty two F_2 individuals, however, were recombinants, showing a non-parental combination of the two markers and therefore these individuals contained recombination events in the marker interval *gwm312 - caCK205077*. These plants constituted a high resolution mapping family.

To establish the *Nax1* phenotype for each of these plants, they were selfed and at least 8 F_3 progeny from each F_2 plant were phenotyped for the Na^+ accumulation trait to confirm the phenotypic scores obtained at the F_2 generation.

The high resolution family of 22 F_2 lines was genotyped for markers to the putative potassium transporter genes CK205077 (homologous to *OsHAK11*) and AL817940 (*OsHAK15*). The primers used to prepare probes for the RFLP analysis are listed in Table 3. Recombinants between *Nax1* and each of these markers were identified, ruling them out as candidate genes (Figure 3). Because there were no matching wheat EST sequences available in the database corresponding to the putative sodium transporter from rice, *OsHKT4*, a closely related barley EST (BJ472462) was isolated and used as DNA probe. When this probe was used, it failed to hybridise to genomic DNA of *T. monococcum*, the donor of *Nax1* in Line 149, using a range of 5 restriction enzymes (*EcoR*I, *EcoR*V, *Hind*III, *Nco*I and *Xba*I). However, the same barley gene probe hybridised to at least one or two gene members in tetraploid and hexaploid wheats, respectively, indicating that the B and D genomes contained *HKT4*-like members. This also showed that the probe was functional under the hybridisation conditions used. The lack of a closely related BJ472463 member on the A genome of *T. monococcum* eliminated the *OsHKT4*-like gene from further investigation as a possible candidate for *Nax1*.

The partial wEST BE604162, which was closely related to a putative sodium transporter in rice (OsHKT7), co-segregated with *Nax1* in the high resolution mapping family (Figure 3) suggesting that a *HKT7*-like gene was a strong candidate for *Nax1*. When used as a probe on genomic DNA of *T. monococcum*, an amplified region of BE604162 hybridised to at least two gene members (Figure 4). Line 149 contained both gene members, but only one member (designated *TaHKT7*-A1) was polymorphic between Line 149 and Tamaroi when examined with the five restriction enzymes *EcoR*I, *EcoR*V, *Hind*III, *Nco*I and *Xba*I. This marker co-segregated perfectly with *Nax1* in the family of 22 recombinants. The second gene member (designated *TaHKT7*-A2) was monomorphic between parents with a range of restriction enzymes and was present in the same deletion bin (FL 0.27-0.77) as *TaHKT7*-A1 (Figure 4). It

was concluded that both *TaHKT7*-A1 and *TaHKT-A2* were strong candidate genes for *Nax1,* in contrast to the other cation transporters examined. The same probe hybridised to at least 4 bands in tetraploid and six bands in hexaploid wheat, suggesting that the B and D genomes also each carry 2 copies of *OsHKT7*-like genes, respectively (Figure 4).

Structure of the interstitial region on wheat chromosome 2AL

The genetic order of wESTs CK205077, AL817940, BE604162, BG262791, BE403217 was supported by their physical positions in deletion bins on chromosome 2AL (Figure 3) when the Chinese Spring 2AL deletion lines were probed in Southern blot hybridisations. The three proximal wESTs (CK205077, AL817940, BE604162) on the genetic map were also located in the proximal deletion bin FL 0.27-0.77, while wESTs BG262791 and BE403217 from the distal part of the map were present in the distal deletion bin FL 0.77-0.85 (Figure 3). The physical order of rice genes corresponding to wESTs CK205077, AL817940, BE604162 and BG262791 was inverted in wheat relative to rice. It was concluded that the chromosomal segment between approximately 29.4 and 30.9 Mb was reversed in orientation in wheat relative to rice. It was concluded that the wESTs BE403867 and BE403217 corresponding to rice genes at 28.4 and 31.3 Mb, respectively, flanked this interstitial inversion event (Figure 3).

Example 3. Isolation of full length *HKT7* genes from wheat

A *T. monococcum* BAC library (Lijavetzky et al., 1999) was screened with a probe from wEST BE604162 to isolate full length sequences corresponding to both of the *TaHKT7-A1* and *-A2* gene members. The *T. monococcum* accession DV92, which was the source for the BAC library, produced the same DNA hybridisation pattern with a BE604162 probe in Southern blots as the salt tolerant *T. monococcum* donor line C68-101, indicating that DV92 also contained *TaHKT7-A1* and *-A2*. High-density filters for the BAC library were screened with the probe matching wEST BE604162.

Nine positive BAC clones were isolated. These could be separated into two groups using a gene specific probe based on the 3' intron present in *TaHKT7-A1* but not *TaHKT7-A2*, to distinguish clones carrying *TaHKT7-A1* from those carrying *TaHKT7-A2*. However, the presence of some fragments in common following digestion with *Hind*III and gel electrophoresis suggested that some of the BAC clones containing *TaHKT7*-A1 and *TaHKT7*-A2 were overlapping. It was concluded that the physical distance between *TaHKT7*-A1 and *TaHKT7*-A2 was less than approximately

145 kb based on the estimation of the sizes of BAC clone inserts and the sum of the sizes of the overlapping fragments.

Protein coding regions (open reading frames, ORFs) in each of *TaHKT7*-A1 and *TaHKT7*-A2 were identified by direct BAC clone sequencing and examination of the nucleotide sequences. The predicted ORF of *TaHKT7*-A1 was 1692 bp long and contained two introns (Figures 5 and 7), while the ORF for *TaHKT7*-A2 was 1665 bp long and contained only one intron (Figures 6 and 7). The coding regions were 88% identical in nucleotide sequence. The amino acid sequences of the predicted proteins (TaHKT7-A1 and TaHKT7-A2) were 70% and 72% identical to OsHKT7, respectively (Figure 8). Further amino acid sequence comparisons revealed that TaHKT7-A2 had nine fewer amino acids than TaHKT7-A1 while the OsHKT7 sequence was shorter by 46 amino acids at the N terminus (Figure 8). When the predicted protein structures were examined, TaHKT7-A1 and A2 shared very similar topological structures except in the N-terminal hydrophilic region (Figure 9). OsHKT7 had a similar topological structure to TaHKT7-A1 and A2 but lacked the hydrophilic N-terminal tail (Figure 9). All of the proteins had at least eight, possibly ten, hydrophobic domains that were likely to correspond to trans-membrane spanning domains. Such structures are often seen in cation transporters.

Example 4. Expression of the *TaHKT7*-A1 and *TaHKT7*-A2 genes in wheat

To determine where the TaHKT7 genes might be expressed in wheat plants, RT-PCR experiments were carried out on RNA samples prepared from different tissues. RNA was extracted using the Trizol method (Invitrogen, Australia) from roots, leaf sheaths and leaf blades of 8-day-old plants treated with 50 mM NaCl for 48 h. RT-PCR procedures were performed using a OneStep RT-PCR Kit (Qiagen, Australia) with the following cycling conditions: 50°C for 30 min; 95°C for 15min; 35 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 50 sec, and then 72°C for 5 min, 25°C for 1 min. The specific primers for *TaHKT7*-A1 and A2 for RT-PCR analysis are listed in Table 5.

Table 5. Primer pairs spaning intron region for expression analysis of *TmHKT7*-A1 and A2 using RT-PCR.

Member	Forward primer	Reverse primer
TmHKT7-A1	A1F GAGAGGGAAAAGCTCAAGGAG (SEQ ID NO: 48)	A1R GTCCACCGGTCAGTGCACATCCC (SEQ ID NO: 49)
TmHKT7-A2	A2F GAGCTCAAGGAGACTTTGAAGCA (SEQ ID NO: 50)	A2R TGACGAAGATGGTGAGGTAGGAG (SEQ ID NO: 51)

Using these gene specific primers, no cDNA product was detected in an initial experiment corresponding to *TaHKT7*-A1 in root, leaf sheaths or blades of T. *monococcum*, Line 149 and Tamaroi (Figure 10). However, the expected cDNA product for *TaHKT7*-A2 was detected in root and leaf sheaths of *T. monococcum* and Line 149 but not in Tamaroi (Figure 10). *TaHKT7*-A2 was not expressed in leaf blades of *T. monococcum* or Line 149, consistent with the physiological role of *Nax1* in reducing the Na^+ concentration in blades by retaining Na^+ in the sheaths (James et al., 2006). Therefore, *TaHKT7*-A2 was considered most likely to correspond to *Nax1*. However, it is possible that low level expression of *TaHKT7*-A1 might also contribute to the total salt tolerant phenotype of the *Nax1* locus in *T. monococcum* and Line 149 and therefore in wheat plants that are bred to contain this introgressed locus.

Example 5. Field trials on wheat containing *Nax1*

To determine the field performance of wheat lines containing *Nax1*, BC_4F_4 seeds that were homozygous for *Nax1* or lacking *Nax1*, obtained from the cross between Line 149 and Tamaroi as described above with subsequent bulking up, were sown at Ginninderra Experimental Station (GES) in June 2004. In order to compare the performance to that of a near isogenic line lacking *Nax1*, the parental variety Tamaroi lacking *Nax1* was also sown. Soils at GES were of low salinity and therefore no advantage was expected for the presence of *Nax1*.

The field plots were harvested in December 2004 and various parameters relating to yield were measured. Surprisingly and unexpectedly, it was observed that there was a substantial yield penalty associated with the presence of *Nax1*. Table 6 shows a compilation of the data. Average yield (t/ha) for the *Nax1* containing lines was 5.56 compared to 6.07 for Tamaroi. Yield component analysis showed that the yield reduction was associated primarily with a reduced number of heads per square metre (Table 6). Harvest index (HI) and the number of grains per head were not affected. The average seed weight (100 seed wt) was higher in the *Nax1* lines but did not compensate for the reduced heads per square metre, with the result that the yield was reduced by about 9% (Table 6). The yield of the BC_4F_4 lines which lacked *Nax1* was on average not different to Tamaroi.

It was noteworthy that two *Nax1* lines, 5038 and 5020, had yields higher than Tamaroi. It was also noteworthy that two lines which lacked *Nax1*, 5004 and 5042, had yields equal to or higher than Tamaroi. These were subsequently shown to be homozygous for *Nax2*.

A second field trial was carried out at Two Wells, near Adelaide, South Australia, at what was considered to be a moderately saline site, and a third trial at Roseworthy, South Australia. The Two Wells trial showed an average yield penalty

associated with *Nax1* of 24% (Table 7). Notably one *Nax1* containing line (5040) yielded significantly higher than Tamaroi. Also, lines 5004 and 5042 were ranked 1st and 2nd in yield of the lines lacking *Nax1*, yielding much higher than Tamaroi. At Roseworthy, the yield penalty associated with *Nax1* was about 10%.

Further field trials were conducted in 2005. By this time, molecular markers linked to the *Nax2* gene had been obtained and therefore accurate *Nax2* genotyping of the "high Na+" BC_4F_4 lines was possible. This analysis showed that some of the lines tested in 2004 that were classified as lacking *Nax1* had contained *Nax2*, including some that were homozygous for *Nax2* and some that were heterozygous. Field trials in 2005 at two sites showed that almost every line carrying *Nax1* had a yield penalty, whether or not it carried *Nax2*. From this it was concluded that it was *Nax1*, not *Nax2*, that was associated with the observed yield penalty.

Table 6. Field data for Nax1 BC4F4 lines grown at Ginninderra Experimental Station.

Na+ status	Family	Height (cm)	100 seed wt (g)	HI	Grain (per head)	Heads (per m^2)	Plot Yield (g)	Yield (t/ha)
Low	5038	110	5.62	0.43	40.8	346	4876	7.36
	5020	100	4.16	0.44	50.4	366	4975	7.20
	5057	120	5.09	0.42	35.0	403	4569	6.61
	5044	125	5.06	0.40	40.4	355	4374	6.60
	5053	130	5.19	0.44	43.4	313	4522	6.54
	5013	105	5.17	0.41	41.3	332	4493	6.50
	5058	110	5.17	0.44	45.1	288	4392	6.22
	5065	105	3.32	0.41	59.6	343	4594	6.14
	5021	110	5.66	0.48	40.9	290	4026	6.08
	5033	105	5.05	0.43	46.3	274	4434	5.92
	5008	105	5.35	0.45	36.1	338	4163	5.90
	5051	110	3.74	0.40	52.7	321	3694	5.83
	5012	105	4.77	0.49	40.8	315	4030	5.71
	5049	100	4.79	0.45	36.6	357	4259	5.69
	5052	105	4.70	0.39	37.4	337	3778	5.35
	5034	115	4.75	0.44	41.7	289	3688	5.34
	5066	105	4.82	0.40	34.9	327	3635	5.26
	5005	110	4.23	0.45	36.7	361	3568	5.16
	5048	105	5.07	0.43	40.9	268	3564	5.16

	5039	105	4.11	0.37	38.6	352	3880	5.08
	5043	110	6.20	0.41	43.6	205	3568	4.96
	5040	105	4.67	0.41	43.2	262	3287	4.86
	5018	105	4.03	0.38	43.4	303	3408	4.83
	5017	120	4.80	0.47	44.3	245	3203	4.73
	5009	100	5.20	0.42	39.6	241	3250	4.51
	5011	100	5.09	0.42	42.3	221	3101	4.31
	5050	110	3.89	0.39	47.0	243	2670	3.95
	5035	95	4.78	0.41	46.1	186	2566	3.87
	average:	**108**	**4.80**	**0.42**	**42.5**	**303**		**5.56**
High	5029	95	3.92	0.43	54.5	401	5418	7.84
	5001	100	4.64	0.44	42.8	424	5134	7.75
	5025	95	3.75	0.38	40.4	519	4886	7.22
	5004	100	5.36	0.48	42.3	342	4900	7.09
	5047	100	4.57	0.42	44.7	370	4966	7.04
	5006	100	5.16	0.47	51.4	284	4389	6.93
	5019	105	4.04	0.39	44.3	400	4289	6.47
	5056	105	3.64	0.38	47.5	408	4400	6.37
	5042	105	4.99	0.38	33.3	405	4689	6.26
	5026	95	3.76	0.42	49.6	360	4173	6.17
	5036	100	5.45	0.45	40.2	296	4166	6.03
	5027	95	4.88	0.35	28.0	477	4142	5.99
	5028	100	4.77	0.41	43.5	310	3778	5.96
	5041	100	3.59	0.34	39.3	456	4437	5.93
	5007	100	3.99	0.46	37.1	427	3778	5.70
	5059	95	3.94	0.41	39.2	396	4194	5.60
	5054	95	3.94	0.36	39.9	389	4029	5.60
	5031	100	4.86	0.44	36.5	335	3849	5.57
	5023	105	3.60	0.38	43.7	380	3842	5.56
	5032	95	3.55	0.39	50.2	313	3442	5.09
	5030	100	4.19	0.35	33.7	368	3428	4.76
	5002	95	4.04	0.33	34.7	370	3348	4.65
	5003	100	4.00	0.53	53.9	230	3468	4.54
	average:	**99**	**4.29**	**0.41**	**42.2**	**376**		**6.09**

(High)	Tamaroi	95	4.72	0.42	46.4	337	4734	6.85
		92	4.06	0.42	40.4	414	4828	6.45
		95	4.88	0.46	47.0	293	4715	6.18
		95	3.92	0.36	41.9	323	3392	4.81
	average:	**94**	**4.40**	**0.42**	**43.9**	**342**		**6.07**

Table 7. Yield data for Nax1 BC4F4 lines grown at Two Wells.

Na+ status	**Line no.**	**Raw Yield averages**	**(% Tamaroi)**	**Line no.**	**Adj. yield (RxC) averages**	**(% Tamaroi)**
High Na+ families	5004	2.97	165	5004	2.85	157
	5042	2.69	150	5019	2.48	137
	5019	2.60	145	5054	2.41	133
	5054	2.52	140	5042	2.40	132
	5030	2.49	138	5031	2.39	132
	5036	2.41	134	5046	2.36	130
	5059	2.40	134	5030	2.33	129
	5027	2.39	133	5059	2.33	128
	5031	2.31	129	5036	2.32	128
	5026	2.28	127	5003	2.25	124
	5001	2.27	127	5027	2.24	123
	5028	2.27	126	5056	2.23	123
	5056	2.27	126	5032	2.20	121
	5003	2.26	126	5028	2.16	119
	5025	2.24	125	5006	2.16	119
	5041	2.20	122	5026	2.13	118
	5047	2.20	122	5007	2.13	117
	5029	2.18	121	5029	2.12	117
	5046	2.15	120	5001	2.11	116
	5002	2.06	115	5047	2.08	115
	5006	2.04	113	5025	2.07	114

	5032	1.99	111	5002	1.96	108
	5023	1.96	109	5041	1.85	102
	5007	1.94	108	5023	1.82	100
	average:	**2.29**	**128**		**2.22**	**123**
Low Na+ families	5040	2.08	116	5020	2.08	115
	5066	2.08	116	5058	1.94	107
	5038	2.00	111	5012	1.94	107
	5020	1.90	106	5066	1.93	107
	5048	1.88	105	5048	1.92	106
	5012	1.84	103	5038	1.91	105
	5013	1.83	102	5040	1.90	105
	5011	1.82	101	5013	1.82	100
	5005	1.81	100	5051	1.76	97
	5049	1.80	100	5065	1.76	97
	5051	1.79	100	5005	1.69	93
	5033	1.75	98	5057	1.58	87
	5058	1.74	97	5034	1.58	87
	5017	1.71	95	5017	1.57	87
	5008	1.70	95	5050	1.57	86
	5052	1.69	94	5043	1.56	86
	5043	1.69	94	5011	1.56	86
	5034	1.67	93	5052	1.55	85
	5057	1.65	92	5033	1.54	85
	5065	1.58	88	5008	1.54	85
	5009	1.54	86	5049	1.53	84
	5053	1.52	85	5035	1.52	84
	5018	1.50	84	5053	1.45	80
	5050	1.48	83	5021	1.43	79
	5021	1.37	76	5018	1.41	78
	5035	1.30	72	5009	1.35	75
	5044	1.24	69	5044	1.33	73
	5039	1.17	65	5039	1.32	73
	average:	**1.68**	**94**		**1.64**	**91**

Field trials were also carried out at sites having saline soils. Comparisons for yield at 8 saline sites ranging from low salinity (Winulta) to high salinity (Buckleboo, Port Pirie) showed that the presence of either *Nax1* or *Nax2* provided a yield benefit. The yield penalty of the lines containing *Nax1* was as great as 20% on the less saline sites, and became less in relation to Tamaroi in the more saline soils. In the lowest yielding sites (most saline), the yield penalty due to *Nax1* was only 5-10% compared to Tamaroi.

In order to measure the levels of Na^+ uptake and accumulation in field grown plants, leaf samples were collected from plants growing at Garah, in northern NSW, Australia in 2005. This field exhibited subsoil salinity during the early grain filling stage of development. It was of interest to measure Na^+ accumulation since glasshouse studies indicated that the effect of the *Nax1* and *Nax2* genes on excluding Na^+ from leaves became greater as the plants grew older, and that the flag leaf of plants of Line 149 had almost no Na^+ even in the highest salinity treatment. Flag leaf samples from the field were analysed for Na^+ levels. Lines having *Nax1* had only 2-8 μmol Na^+ per gram leaf tissue compared to Tamaroi which had 150 μmol per gram, a 30-fold difference. The levels were similarly low when both *Nax1* and *Nax2* were present. Lines having only *Nax2* had 10-30 μmol Na^+ per gram, corresponding to about 2-6mM NaCl on a fresh weight basis. Uptake of Na^+ into grain of these plants was also decreased in the presence of *Nax1* or *Nax2*.

Example 6. Removal of the yield penalty associated with *Nax1*

It was considered that the yield penalty associated with *Nax1* as described in Example 5 was not due to the *Nax1* gene itself but to a linked gene (or genes) present on the introgressed region from Line 149. This type of phenomenon is often termed linkage drag. Line 149 was developed from *T. monococcum* C68-101 and the durum line Marrocos as a possible tetraploid bridge to transfer the gene *Sr21,* which confers stem rust resistance, from *T. monococcum* into hexaploid wheat (The, 1973). *Sr21* is located on chromosome 2AL, the same chromosome as *Nax1*. *Sr21* is also associated with a yield penalty similar in degree to that of the *Nax1* gene as described above and was therefore considered to be a good candidate for the gene associated with *Nax1* causing the observed yield penalty.

Testing of some of the BC4 lines described above showed they contained *Sr21*. An extensive survey of all of the lines is being carried out. *Sr21* has not been mapped precisely, but one report placed it on the proximal region of 2AL toward the centromere. Fortuitously, it was noticed that a rice gene having a genome position at 27.2Mb on 4L (LOC_Os04g46300, Accession No. AL731613) was a good candidate

for a homolog of a wheat resistance gene such as *Sr21*. The protein encoded by the rice gene shows high similarity (809/998, 81% identity) in amino acid sequence to a barley resistance gene analog (RGA S-120, Accession No. CAD45036) which has been mapped to barley chromosome 2H, close to the centromere. 2H is syntenic with wheat chromosome 2AL. It was therefore considered that a good candidate for *Sr21* homologous to the rice and barley genes would lie between the wheat markers corresponding to BF474590 and BE403863.

The recombinant wheat lines developed for the genetic mapping of *Nax1* as described in Example 2 will be tested for yield parameters in field trials. It is predicted that at least some recombinants will have lost the alleles from Line 149 that cause the yield penalty, particularly the recombinants that have breakpoints closer to *Nax1* and therefore having a smaller introgressed region from Line 149. Figure 11 shows a schematic diagram of the recombinants close to *Nax1*. Furthermore, recombinants on the proximal and distal sides of *Nax1* can be combined by crossing to provide a double recombinant with a minimal introgressed region (Figure 12). Markers such as those provided in Table 8 can be used to select lines which provide the advantages of the salinity tolerance conferred by *Nax1* without the yield penalty, and so will be suitable for use in either saline or non-saline conditions.

Table 8. Enzymes for RFLP marker development.

Markers	**Restriction Enzymes**	**Marker type**	**Approximate fragment size**	
			Line 149	**Tamaroi**
Wheat ESTs				
BF474590	NcoI	RFLP marker	~10kb	~8kb
BE403863	NcoI	RFLP marker	~7kb	~10kb
BG262791	EcoRV	RFLP marker	~14kb	~9kb
BE423738 (**HAK11**)	HindIII	RFLP marker	~10kb	~12kb
CK205077 (**HAK11**)	(CAPS marker, digested with RsaI)	PCR marker		
BE403217	NcoI	RFLP marker	~10kb	~7kb
CA498418	NcoI	RFLP marker	~3.5kb	~4kb
BE604162 (**HKT7**)	EcorV	RFLP marker	~3kb	~6kb
AL817940 (**HAK15**)	NcoI	RFLP marker	~10kb	~9kb

Example 7. Crossing of sodium-exclusion allele of *Nax1* lacking yield penalty locus into hexaploid wheat

The sodium exclusion alleles of *Nax1* from recombinant lines lacking the yield penalty locus will be introduced into representative hexaploid wheat varieties by backcrossing. The hexaploid varieties will be chosen as representative of the genetic backgrounds of bread wheats currently grown across the Australian wheat belt. Bread wheats generally have lower Na^+ uptake than durum wheats and therefore have superior salt tolerance. However, there is about a 2-fold variation in Na^+ uptake in varieties of bread wheat, which will also be represented in the varieties to be used. *Nax1* (on chromosome 2A) conferring salt tolerance will be introduced into bread wheat because the genes controlling the retention of Na^+ in the leaf sheath are lacking in bread wheat. The presence of this gene may be particularly important where salinity is associated with waterlogging or any soil abiotic/biotic stress that impairs root function. *Nax2* (on chromosome 5A) will also be introduced, even though a salt tolerance gene with similar mechanism is already known to exist in many varieties of bread wheat, *Kna1* (on chromosome 4D - Dvořák et al., 1994).

Initial crosses will be made between the hexaploid cultivars Chara, Camm, Carnamah and Westonia and the recombinant lines in the durum Tamaroi background as described herein. F_1 pentaploids from these direct crosses will be backcrossed with the hexaploid (male) to produce BC_1F_1 plants, and the progeny then selfed.

The BC_1F_2 seedlings will be screened for the presence of the Na^+ exclusion allele from the tetraploid parent using any one of the markers as described herein such as, for example, the *Xgwm312* marker or markers based on SEQ ID NO: 3 or SEQ ID NO: 4. Robust analyses may be performed using the *Xgwm31 mod* primers as described in WO2005/120214.

The hexaploid BC_1F_3 selections containing the tetraploid Na^+ exclusion allele will be backcrossed again into Westonia and also top-crossed with the hexaploid cultivars Sunstate, Carnamah and Chara. Further backcross/top-crosses will be completed without selection using BC_2F_1 plants, and additional top crosses performed into the hexaploid cultivars Janz and Yitpi. BC_3F_2 populations of these crosses will be screened using one of the molecular markers and selections made, thus generating BC_3F_3 homozygous families containing the tetraploid Na^+ exclusion allele in 6 different hexaploid backgrounds.

The homozygous lines will be tested in both greenhouse and field trials under saline and non-saline conditions for Na^+ accumulation in leaf and grain, growth rate, biomass accumulation and grain yield parameters including the number of heads per square meter. For example, Na^+ uptake in cultivars will be substantially decreased in

the presence of the Na^+ exclusion alleles from the tetraploid parent, and associated with improved salt tolerance and yield.

Example 8. Constructs for transgenic expression of wheat HKT7 genes in heterologous species

For heterologous expression in yeast cells, the coding sequence of the HKT7-A2 gene including native translation start and termination codon was amplified from a cDNA clone by PCR using primers in the 5'- and 3'-UTRs. The PCR products were cloned into pGEMT-easy and sequenced to identify clones free from PCR-induced errors. A gene insert having error-free coding sequence was then introduced into the yeast vector pYES2 and used to transform yeast strains lacking a high affinity cation transporter (*trk1/trk2* knockout mutant). Control yeast strains contained pYES2 without the HKT7 gene insert. The yeast transformants were plated on a minimal medium lacking uracil, to maintain selection for the presence of the pYES2 derived vectors, and containing a low concentration of Na^+ (2 mM) and either 8 mM K^+ or 60 mM K^+. In the presence of the lower level of potassium ion, the *trk1/trk2* cells containing the HKT7-A2 insert grew very slowly and were inhibited in growth compared to the *trk1/trk2* cells containing the control vector pYES2, indicating a sensitivity to Na^+ conferred by HKT-A2 to the cells under these conditions. This growth inhibition was reduced by the addition of the higher level of potassium ions (60 mM). From these data, it was concluded that the HKT7-A2 clone encoded a functional cation transporter.

To further examine the function of the *HKT7* genes described above, experiments will be performed to express the coding region in heterologous species. Two model plant species will be chosen for this purpose: the dicotyledonous plant *Arabidopsis thaliana* due to its ease of transformation and the monocotyledonous plant barley (*Hordeum vulgare*) which is more closely related to wheat and therefore is expected to respond similarly to wheat. Transformation systems for barley are routine, for example using *Agrobacterium*, and while barley is considered relatively salt-tolerant compared to wheat, it may lack the Na^+-exclusion mechanism mediated by the *Nax1* locus. These model plants can therefore be used to characterise the identified *HKT7* genes from wheat and compare their function.

Arabidopsis expression constructs will be produced based on the pART7 and pART27 vector systems (Gleave, 1992). The coding sequence of each gene including native translation start and termination codons will be amplified from cDNA clones by PCR using primers in the 5'- and 3'-UTRs. PCR products will be cloned into standard cloning vectors such as pGEMT-easy and sequenced to identify clones free from PCR-induced errors. Gene inserts having error-free coding sequences will be excised from the cloning vectors using flanking *Eco*RI restriction sites, and

introduced into the *Eco*RI site within the multiple cloning region of the pART7 vector. Once the coding region is subcloned into pART7, the orientation of the insert relative to the promoter will be determined via restriction digests and clones with the coding region in the forward (sense) orientation will be identified. The 35S promoter-coding region-OCS terminator cassette will then be excised with *Not*I and ligated into the *Not*I site in the multiple cloning region of the pART27 binary vector. The orientation of the insert will again be determined with restriction digests, and clones with the cassette in the forward orientation will be purified. These will be used to transform *Agrobacterium* cells of the GV3101 strain via an electroporation method. Transformed cells will be selected on LB-rifampicin-spectinomycin media and grown at 28°C. Several colonies will be picked and cultured in liquid medium. To confirm that the correct plasmid had been introduced and that no rearrangements or deletions had occurred, plasmid DNA will be extracted using standard protocols, transformed into *E. coli* and tested with restriction digests and sequencing to confirm the correct structures within the vector. *Agrobacterium* colonies thus identified will be grown up and used for transformation.

Arabidopsis plants of the ecotype Columbia will be used for transformation. Plants will be grown under standard conditions until approximately 1 week after the first flower buds begin to open. Transformation will be carried out by a floral dip method, well known in the art. T_1 seed thus produced will be plated on MS-kanamycin medium to select transformants. PCR will be performed on DNA extracted from tissue samples of these plants to confirm the presence of the *HKT7* transgene and/or the Kan^R selectable marker. T_2 seed will collected and sown to produce transgenic progeny which will be used to assay Na^+ and K^+ uptake.

Barley transformation will be carried out using the pWUbi – pVec8 vector system (Murray et al., 2004). Coding regions of the *HKT7* genes will be excised with *Eco*RI and ligated into the *Eco*RI site of pWUbi as described above for pART7. The expression cassette will then be excised with *Not*I and ligated into the appropriate site of pVec8. Correctly oriented (sense) clones will be electrotransformed into *Agrobacterium* strain AGL0, and colonies with plasmids of the correct structure will be identified. Tissue culture and transformation will be carried out using tissue from immature embryos of barley as described (Murray et al., 2004) using hygromycin as the selection agent, based on the presence of a hygromycin resistance selectable marker gene on pVec8.

Arabidopsis and barley transformants will be obtained. Na^+ and K^+ uptake will be measured in T_2 segregating families overexpressing the *HKT7* genes under the control of the 35S promoter. Under saline or non-saline conditions, soil-grown plants

overexpressing the genes are expected to show an increased Na^+ content in leaves compared to wild-type plants of cv. Columbia.

Example 9. Genetic mapping of HKT genes in wheat, barley and rice

Materials and Methods

Genetic materials used for *HKT* gene mapping in wheat, barley and rice included the hexaploid bread wheat cv. Chinese Spring, the tetraploid durum wheat cv. Langdon and Tamaroi, the diploid wheats *Triticum urartu* AUS1789 and AUS1790, *Triticum monococcum* C68-101 and DV92 and *Aegilops tauschii* AUS18913, and the barley cv. Betzes. For wheat chromosome mapping, nullitetrasomic and ditelosomic aneuploid stocks developed in Chinese Spring were used (Sears et al., 1954). In each nullitetrasomic line, a deleted pair of chromosomes was compensated for by two copies of a pair of homoeologous chromosomes. Ditelosomic lines carried a centromeric deletion of one chromosome arm (Sears et al., 1954). For barley chromosome mapping, wheat–barley addition lines were used. These were developed by adding one barley chromosome from Betzes barley (chromosome addition line) or chromosome arm (ditelosomic addition line) into Chinese Spring (Islam et al., 1981).

DNA Extraction and Southern Hybridisation

Plants were grown in soil for four weeks. The leaves were harvested for DNA extraction as described by Lagudah et al., (1991). DNA was digested with different restriction enzymes (*EcoR*I, *EcoR*V, *Hind*III, *Nco*I) and electrophoretically fractionated in 1% agarose gel and transferred to Hybond N^+ nylon membranes (Amersham) by capillary transfer. Prehybridization and hybridization were performed in a rotary hybridization chamber at 65°C in a solution containing 1% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl (pH8.0), 10 mM EDTA, 3.3xSSC buffer, 10% dextran sulphate, 0.1% BSA, 0.1% PVP, 0.1% Ficoll-400 and 0.03% salmon DNA. The immobilized DNAs were hybridized overnight in buffer at 65°C with probes [^{32}P]-labeled by the random primer method using Megaprime DNA Labelling Kit (Amersham). The membranes were washed at 65°C twice, 20 min each time, in 2x SSC/0.1%SDS, once for 20 min in 1x SSC/0.1% SDS and once for 15 min in 0.5xSSC/0.1%SDS. These hybridisation and washing conditions correspond to high stringency hybridisation conditions. Autoradiograms were exposed for 1-3 days at -80°C with intensifying screens.

Results

Database searching of the rice genome confirmed that rice had 8 *HKT* like genes, extending previous reports. These have been designated genes *OsHKT1-4; 6-9*; (Garciadeblás et al., 2003; Horie et al., 2001). *OsHKT1* was annotated in the Nipponbare genome sequence as Os06g48810 on chromosome 6 and shared 93% identity at the nucleotide level with *OsHKT2* (AB061313), a gene isolated from salt tolerant cultivar Pokkali but which could not be identified in the *japonica* or *indica* rice genome sequences. *OsHKT3* (AJ491820; Os01g34850) was positioned on chromosome 1 and was 95% identical to *OsHKT9* (AJ491855; Os06g48800) on chromosome 6. *OsHKT9* was therefore tightly linked to *OsHKT1* but had only 73-76% sequence identity in three regions of *OsHKT1* at the position of 669-855, 1016-1170 and 1366-1502 (Figure 13). *OsHKT4* on chromosome 4 (Os4g51820) was separated by approx 3 kb from a pseudo gene *OsHKT5*. *OsHKT4* and *OsHKT5* gene sequences were approximately 80% identical at the nucleotide level. *OsHKT6* was located on chromosome 2 (Os02g07830), *OsHKT7* on chromosome 4 (Os4g51830) and *OsHKT8* on chromosome 1 (Os01g20160). Garciadeblás et al., (2003) described the sequence similarity between *OsHKT* genes using phylogenetic trees. NCBI (www.ncbi.nlm.nih.gov) and the Gramene (www.gramene.org.) database were used to search closely related wheat or barley sequences for probe design (Table 9). The sequences of the rice genes as referred to above are herein incorporated by reference.

Cloning and sequencing of wheat or barley ESTs related to HKT genes in rice

Primers were designed on the basis of wheat or barley EST sequences that were closely related to rice *HKT* genes (Table 9). The amplified products from wheat or barley were cloned using pGEM-T Easy vector system (Promega) and confirmedby sequencing. These cloned fragments were then radioactively labeled by standard procedures to be used as probes for Southern blot hybridisation analysis of wheat, barley and rice DNA. The probe developed from the wheat ortholog *TaHKT1* was not expected to hybridise to *HKT*3 or *HKT*9 like genes in wheat, although *OsHKT1* had some similarity (73-76% identity) at the positions 669-855, 1016-1170 and 1366-1502 with *OsHKT3/9* in rice.

Chromosome mapping

DNA probes were used to localise each *HKT*-like gene to a specific chromosome or chromosome arm in wheat and barley, utilising the nullitetra-disomic and ditelosomic aneuploid stocks developed in Chinese Spring hexaploid wheat. The mapping results for each *HKT*-like gene are given in detail below and are summarised in Table 10 and shown schematically in Figure 14.

Table 9. Probes developed for detection of *HKT* genes in wheat and barley genomes.

***HKT* genes**	**New name**	**Matched wheat/barley sequence**	**Probe size (bp)**	**E-value**	**Identity**	**Primers**
HKT1/2		U16709	318	7E-34	78%	TATGTGATGAGTCGCAGCTTGAA (SEQ ID NO: 52)
	HKT2;1			1E-35	78%	
	HKT2;2					GCAACAAGAGGCCTGAATTCTTT (SEQ ID NO: 53)
HKT3/9	*HKT2;3*	DR73356	387	2E-89	82%	TCTTAGTTCGGCAAGGCATATCA (SEQ ID NO: 54)
	HKT2;4	2		6E-101	83%	
						TGCACGGTAACCGATGTAACTCT (SEQ ID NO: 55)
HKT4/5	*HKT1;1*	BJ472463	431	4E-11	76%	TTAAAAATATTCGGGCCAACACC (SEQ ID NO: 56)
	HKT1;2			1E-54	79%	
						TGGGGTAAGCAGAAGAAGGAAAG (SEQ ID NO: 57)
HKT6	*HKT1;3*	BJ473256	368	1E-91	85%	CTATTTTGCCAAATCTGCACAGC (SEQ ID NO: 58)

						TCTGGTCCCTTCTGTTGAATGAA (SEQ ID NO: 59)
HKT7	*HKT1;4*	BE604162	453	3E-30	83%	ATTCAGGCAACACCTAATCATGC (SEQ ID NO: 60)
						GCATCACAAGAATGAGGATGAGC (SEQ ID NO: 61)
HKT8	*HKT1;5*	DQ64634 2	315	3E-55	87%	CGTGCTAGCGCAGCTGTCGCTCT (SEQ ID NO: 62)
						ATCATACCATTAGATGCGTCATG (SEQ ID NO: 63)

Table 10. Summary of *HKT*-like genes detected in barley and wheat genomes by probes using genomic DNA Southern hybridization.

***HKT* genes**	**New name***	**Rice genome**	**Barley genome**	**Wheat genome***					
				A^mA^m	A^uA^u	AA	BB	DD	D^tD^t
HKT1/2	*HKT2;1* *HKT2;2*	**1-2**	**1**	**1**	**2**	**2**	**2** ***TaHKT1***	**1**	**2**
HKT3/9	*HKT2;3* *HKT2;4*	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**
HKT4/5	*HKT1;1* *HKT1;2*	**1**	**1**	**0**	**0**	**0**	**1**	**1**	**1**
HKT6	*HKT1;3*	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**
HKT7	*HKT1;4*	**1**	**2**	**2** ***Nax1***	**2**	**2**	**3**	**3**	**3**
HKT8	*HKT1;5*	**1** ***SKC1***	**1**	**1** ***Nax2***	**0**	**0**	**3**	**1** ***Kna1***	**1**

*: A^mA^m represent A genome from *Triticum monococcum*. A^uA^u represent A genome from *Triticum urartu*. D^tD^t represent D genome of *Ae. tauschii*.

HKT1/2-like genes in wheat and barley

The *HKT1/2* probe which was developed from *TaHKT1* (Genbank accession: U16709) hybridised to 5 bands in genomic DNA of hexaploid wheat suggesting that up to 5 members of the *HKT1/2*-like family could be present in the bread wheat genome, which was consistent with results of Laurie et al., (2002). Up to two bands were mapped on the long arm of chromosome 7A and 7B, while only one band mapped to chromosome 7D (Figure 14). In barley, only one band was detected (Table 10), which was mapped on chromosome 7H (Figure 14). Two bands were detected in the A genome of the diploid *T. urartu*, but only one band was present in the A genome of the diploid *T. monococcum* (Table 10). In the D genome of the diploid *Ae. tauschii*, two bands were detected. The location of *HKT1/2*-like genes within the syntenic wheat region to rice chromosome 6 suggested that these genes were orthologous to *OsHKT1* (Sorrells et al., 2003).

HKT3/9-like genes in wheat and barley

In rice, *OsHKT3* (AJ491820) and *OsHKT9* (AJ491855) were 95% identical at the nucleotide sequence level and were located on chromosomes 1 and 6, respectively. Using a probe developed from a closely related barley EST (DR733562), 3 bands were detected in hexaploid wheat with one band mapping to each of chromosomes 7A, 7B and 7D. In barley, two bands were detected and mapped to chromosome 7H (Table 10). There was one band present in DNA from *T. monococcum, T. urartu* and *Ae. tauschii*, indicating that only one member of *HKT3/9*-like genes were present in those diploid wheats (Table 10). The location of *HKT3/9*-like genes on the long arm of wheat chromosome 7 suggested that these genes were orthologs of *OsHKT9* (Os06g48800) located within the syntenic region on rice chromosome 6 (Sorrells et al., 2003) but not of *OsHKT3* (Os01g34850) on chromosome 1. An *HKT3*-like ortholog could be absent from the wheat genome.

HKT4/5 like genes in wheat and barley

The barley probe which was derived from EST BJ472463 detected two members of the *HKT4/5*-like gene family present in hexaploid wheat. Those two members were mapped to the long arm of chromosome 2B and 2D, respectively, but no *HKT4/5*-like gene was detected in the A genome of hexaploid wheat. Likewise, no *HKT4/5*-like gene was found in the A genome of *T. monococcum* and *T. urartu*. In the D genome of Ae. *tauschii*, one copy of an *HKT4/5*-like gene was detected. In barley, one band was present and mapped to the long arm of chromosome 2H. The map location of *HKT4/5*-like genes in wheat was syntenic to rice chromosome 4 (Sorrells et al., 2003) containing *OsHKT4* (Os04g51820) and *OsHKT5*.

HKT6-like genes in wheat and barley

Two members of the *HKT6*-like genes were detected in hexaploid wheat and mapped to the short arm of chromosomes 6B and 6D, respectively. No *HKT6*-like gene was detected in the A genome of bread wheat, however in the A genomes of *T. monococcum* and *T. urartu*, up to one copy of the *HKT6*-like gene was present in each genome. This result differed from that of the *HKT4/5*-like genes, which were absent from all homoeologous A genomes of bread wheat, *T. monococcum* and *T. urartu*. In *Ae. tauschii,* one *HKT6*-like gene was found. In barley, one copy of an *HKT6*-like gene was present and mapped to the short arm of chromosome 6H. The location of *HKT6*-like genes on wheat chromosome 6 was syntenic to rice chromosome 2 (Sorrells et al., 2003) that contained *OsHKT6* (Os02g07830).

HKT7-like genes in wheat and barley

The *HKT7* probe showed that up to 8 bands were present in hexaploid wheat. Three hybridisation bands were mapped to each of the long arms of chromosomes 2B and 2D and two bands were mapped to the long arm of chromosome 2A. Two bands were present in the A genomes of *T. monococcum* and *T. urartu*, the same number as in the A genome of hexaploid wheat. One *HKT7*-like gene in *T. monococcum* (EF062819) was a strong candidate for *Nax1*, see Examples above. In *Ae. tauschii*, three bands were detected, the same number as for the D genome of hexaploid wheat. In barley, two bands were detected and mapped to the long arm of chromosome 2H. The location of *HKT7*-like genes on the long arm of chromosome 2 was syntenic to rice chromosome 4 (Sorrells et al., 2003) containing *OsHKT7* (Os04g51830).

HKT8-like genes in wheat and barley

Up to four bands of *HKT8*-like genes were detected in hexaploid wheat using an *HKT8* probe (Table 10). Three hybridisation bands were mapped to the long arm of chromosome 4B and one band was mapped on the long arm of chromosome 4D (Figure 14). Notably, no *HKT8*-like gene was detected in the A genome of hexaploid wheat. A single copy *HKT8*-like gene was present in *T. monococcum* while no member was found in the accession of *T. urartu* used in the study. The single copy *HKT8*-like gene found in *T. monococcum* was located in the distal region of long arm of chromosome 5A reflecting the ancient reciprocal translocation which occurred between the distal segment of the long arm of chromosome 4A and 5A in an ancestral wheat genome. The *HKT8*-like gene in *T. monococcum* (DQ646339) was considered a strong candidate for *Nax2*, a gene conferring sodium exclusion in durum wheat (Byrt et al., 2007). The *HKT8*-like gene in the D genome of hexaploid wheat was a

candidate for *Kna1* (DQ646342), a gene conferring sodium exclusion in hexaploid wheat (Byrt et al., 2007; Dubcovsky et al., 1996; Gorham et al., 1990b). One band was also detected in *Ae. tauschii*. In barley, one band was detected and mapped to the long arm of chromosome 4H (Table 10 and Figure 14). No syntenic relationship between *HKT8*-like genes in wheat and rice was found since *OsHKT8* (Os01g20160) is located on rice chromosome 1, which was syntenic to wheat chromosome 3 (Sorrells et al., 2003).

Discussion

In *Arabidopsis thaliana*, there is only one *HKT* gene (Uozumi et al., 2000). In rice (*Oryza sativa*), there are eight *HKT* genes (Garciadeblás et al., 2003; Horie et al., 2001). Based on amino acid sequence similarity, *HKT* genes have been grouped into two main subfamilies (Platten et al., 2006). The division into the two subfamilies is associated with differences in a key amino acid in the first pore loop of the protein (Garciadeblás et al., 2003; Mäser et al., 2002); all gene members of subfamily 1 have a serine residue which is replaced by glycine in most members of subfamily 2. The division is also associated with differences in Na^+ and K^+ selectivity (Horie et al., 2001; Garciadeblás et al., 2003; Mäser et al., 2002).

The Na^+-specific transporters – Subfamily 1

Gene members of subfamily 1 are all low-affinity Na^+ specific transporters. Some of them are specifically expressed in the plasma membrane of cells in the stele of roots, particularly the xylem parenchyma cells, rather than the cortex, where they retrieve Na^+ from the xylem sap and so prevent it reaching the shoots. The rice gene *OsHKT8* (renamed *OsHKT1;5*), first identified as the quantitative trait locus *SKC1* (Lin et al., 2004), controls unloading of Na^+ from the root xylem (Ren et al., 2005). In wheat, two *HKT8*-like (*HKT1:5*-like) genes are likely candidates for *Nax2*, a major gene controlling Na^+ exclusion in durum wheat Line 149, as well as for *Kna1* in bread wheat (Byrt et al., 2007). *Nax2* controls Na^+ exclusion from leaves via xylem unloading in roots (James et al., 2006). *Kna1* also controls Na^+ exclusion from leaves at the point of xylem loading in roots (Gorham et al., 1990). An *HKT7*-like (*HKT1;4*-like) gene was shown in the Examples above to be a candidate for the quantitative trait locus *Nax1* identified in durum wheat Line 149 by Lindsay et al., (2004). *Nax1* controls unloading of Na^+ from the xylem in roots and leaf bases (James et al. 2006).

OsHKT4 can be expressed in rice shoots and roots (Garciadeblás et al., 2003). In a transformed yeast system, *OsHKT4* mediated low-affinity Na^+ uptake (Garciadeblás et al., 2003). In the barley genome and wheat B and D genomes, there is only one copy of the *HKT4/5*-like gene (Table 10). It is not clear which gene

member is absent from the wheat and barley genome. In wheat, *HKT4/5*-like ESTs (CJ594572, CJ700470, CJ594562 and CJ700475) were isolated from the shoots, showing that the *HKT4/5*-like gene can be expressed in wheat shoots. In barley, *HKT4/5*-like ESTs (BJ472463, BM816866, CD058368, BF262602 and DN17794) were isolated from leaves or leaf epidermis. Those barley ESTs could come from different regions of the same gene because they have 100% or 99% (sequence variation) identity in the overlapped region. This may provide additional information that there is only one *HKT4/5*-like gene in barley. Future research is required to test any tissue specific expression of *HKT4/5*-like gene in barley.

OsHKT6 in rice was found to be mainly expressed in shoots, with little expression in roots (Garciadeblás et al., 2003). The present study found no wheat EST matching *OsHKT6*, although a single copy of an *HKT6*-like gene was present in the B and D genomes of bread wheat (Table 10). In barley, 18 *HKT6*-like ESTs (e.g. BJ476674) were isolated from one cDNA library from the leaves of adult plants, indicating that an *HKT6*-like gene may highly expressed in barley leaves. Garciadeblás et al., (2003) working with transformed yeast observed that the gene product of *OsHKT6* might not target the plasma membrane but an internal membrane. In barley, any *HKT6*-like gene products targeted to tonoplast deserve further investigation, which may be related to tissue tolerance.

OsHKT7 in rice was mainly expressed in shoots (Garciadeblás et al., 2003). A barley *HKT7*-like gene (BQ739876) was also expressed in the leaves of drought-stressed plants (Ozturk et al., 2002). The matched wheat EST BE604162 was isolated from a drought-stressed wheat leaf cDNA library, indicating it was expressed in leaf tissues. As described herein, an *HKT7*-like gene, *TmHKT7*-A2, was cloned from *Triticum monococcum* as the candidate for *Nax1* conferring sodium exclusion and salt tolerance to durum wheat. *TmHKT7*-A2 co-segregated with *Nax1* and its expression pattern in roots and leaf sheath was consistent with its proposed physiological role in removing Na^+ from the xylem of the roots and leaf sheaths.

OsHKT8 (*SKC1*) has been shown to maintain high shoot K^+ and low Na^+ accumulation under salt stress in a salt-tolerant rice cultivar by controlling the unloading of Na^+ from the root xylem (Ren et al., 2005). *OsHKT8* (*SKC1*) was preferentially expressed in the parenchyma cells surrounding the xylem vessels. Voltage-clamp analysis showed that OsHKT8 functions as a Na^+-selective transporter. In wheat, two *HKT8*-like genes were considered as strong candidates for *Nax2* in durum wheat and *Kna1* in bread wheat (Byrt et al., 2007), which confer salt tolerance via Na^+ exclusion (James., 2006; Gorham et al., 1990). There are five phenotypic characteristics in common conferred by *SKC1*, *Nax2* and *Kna1*: (1) low Na^+ concentration in the leaves; (2) enhanced discrimination of K^+ over Na^+ in transport

from the roots to the shoots; (3) regulation of K^+/Na^+ ratio the leaves; (4) no effect on root Na^+ concentration; and (5) no effect on the sheath-to-blade Na^+ ratio (Gorham et al., 1990; Davenport et al., 2005; James et al., 2006). Two *HKT8*-like genes, candidates for *Nax2* and *Kna1*, could control the unloading of Na^+ from the root xylem contributing to Na^+ exclusion (Gorham et al., 1990; Byrt et al., 2007; James et al., 2006). Of all the chromosome substitution lines in the durum wheat Langdon that were analysed for leaf Na^+ and K^+, the lines with chromosome 4A or 4 B substituted by 4D, that is, the lines containing *Kna1,* were the only lines to show substantial reduction of Na^+ in the shoots, with associated high K^+/Na^+ selectivity (Gorham et al., 1990). This indicates that the transporter on 4D was more effective at reducing Na^+ transport to the shoot than one on any other chromosome. This transporter is likely to be HKT8.

The Na^+-K^+ co-transporters – Subfamily 2

Gene members of subfamily 2 are Na^+-K^+ co-transporters except *OsHKT2* (renamed *OsHKT2;2*). These are high-affinity transporters of K^+ and/or Na^+, and are important in K^+-deficient conditions where they may take up Na^+ and thereby promote growth. Some of them are specifically expressed in plasma membrane of cells in the epidermis and cortex of roots and their expression could be down-regulated in conditions of salinity. This was recently shown to be the case for *OsHKT1* (renamed *OsHKT2;1*) (Horie et al., 2007). *OsHKT1* regulated the transport of Na^+ into roots of K^+-starved plants and enhanced their growth, but was downregulated when plants were exposed to 30 mM NaCl (Horie et al., 2007). In wheat and barley roots, *TaHKT1* (renamed *TaHKT2;1*) and *HvHKT1* (renamed *HvHKT2;1*) also mediated Na^+ uptake particularly under conditions of K^+ deficiency (Haro et al., 2005; Laurie et al., 2003).

TaHKT1 was the first *HKT* gene isolated from the higher plants (Schachtman and Schroeder 1994). Bread wheat has 5 copies of *HKT1*-like genes on the basis of DNA hybridisation described above and the previous report (Laurie et al., 2002). *TaHKT1* is probably one of the two copies located on the B genome as (1) the wheat EST (BE428877) isolated from roots of tetraploid durum wheat was 100% identical to *TaHKT1* (U16709), and (2) primers designed on the basis of *TaHKT1* amplified a product only from the long arm of chromosome 7B (Mullan et al., 2007). *TaHKT1* was found to be expressed in cortical cells of bread wheat roots by *in situ* hybridisation (Schachtman and Schroeder 1994), but this finding may be confounded by potential cross hybridisation with other *HKT1*-like members in bread wheat.

In barley, there was only a single copy of the *HKT1*-like gene (*HvHKT1*, AM000056) (Haro et al., 2005), which was consistent with data described above (Table 10). *HvHKT1* and *TaHKT1* had 92% identity at nucleotide sequence level and

both functioned as a Na^+-K^+ co-transporters in a yeast transformation system (Haro et al., 2005; Rubio et al., 1995). In a root uptake system, *HvHKT1* and *TaHKT1* functioned as a putative Na^+ uniport (Haro et al., 2005). This hypothesis was supported by another study using a *TaHKT1* anti-sense transgenic line (Laurie et al., 2002). The down-regulation of *TaHKT1* in wheat increased shoot fresh weight by 50% to 100% in 200 mM NaCl under conditions of K^+ deficiency (Laurie et al., 2002). Following the down-regulation of *TaHKT1*, the transgenic wheat had smaller Na^+-induced depolarization in root cortical cells than the control, and lower $^{22}Na^+$ influx, indicating that *TaHKT1* mediated Na^+ influx.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

This application claims priority from AU 2006904749, the entire contents of which are incorporated herein by reference.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS

1. A tetraploid or hexaploid wheat plant comprising a gene on chromosome 2A which hybridises under stringent conditions to a nucleic acid molecule having nucleotides in a sequence as provided in one or both of SEQ ID Nos: 3 or 4, wherein said chromosome 2A comprises a recombination event between said gene and one or more genetic markers present on chromosome 2A of wheat Line 149.

2. The wheat plant of claim 1, wherein the gene encodes a cation transporter comprising amino acids having a sequence as provided in SEQ ID NO:1 or SEQ ID NO:2, or an amino acid sequence which is at least 73% identical to SEQ ID NO:1 and/or SEQ ID NO:2.

3. The wheat plant of claim 1 or claim 2, wherein the chromosome 2A comprises less than 75% of chromosome 2A of wheat Line 149.

4. The wheat plant of claim 3, wherein the chromosome comprises less than 25% of chromosome 2A of wheat Line 149.

5. The wheat plant of any one of claims 1 to 4 wherein the recombination event is proximal to the gene.

6. The wheat plant according to any one of claims 1 to 5 which comprises a gene encoding a cation transporter comprising amino acids having a sequence as provided in SEQ ID NO:1 or SEQ ID NO:2.

7. The wheat plant according to any one of claims 1 to 6, wherein the chromosome does not comprise a yield penalty locus present on chromosome 2A of wheat Line 149.

8. The wheat plant according to any one of claims 1 to 7 comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 319 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe

consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 29-348 of SEQ ID NO: 6 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149.

9. The wheat plant according to any one of claims 1 to 8 comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 415 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 41-455 of SEQ ID NO: 7 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149.

10. The wheat plant according to any one of claims 1 to 9 comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 387 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 19-405 of SEQ ID NO: 8 or its complement, wherein said second nucleotide sequence is comprised in a *Hin*dIII fragment that is different in size to the corresponding *Hin*dIII fragment present on chromosome 2A of wheat Line 149.

11. The wheat plant according to any one of claims 1 to 10 comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 301 nucleotides on chromosome 2A, said second nucleotide sequence being

capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 11-311 of SEQ ID NO: 9 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149.

12. The wheat plant according to any one of claims 1 to 11 comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 336 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 13-348 of SEQ ID NO: 10 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149.

13. The wheat plant according to any one of claims 1 to 12 comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 427 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 30-456 of SEQ ID NO: 11 or its complement, wherein said second nucleotide sequence is comprised in a *Eco*RV fragment that is different in size to the corresponding *Eco*RV fragment present on chromosome 2A of wheat Line 149.

14. The wheat plant according to any one of claims 1 to 13 which is of the species *Triticum aestivum ssp aestivum* or *Triticum durum*.

15. The wheat plant according to any one of claims 8 to 14, wherein the first nucleotide sequence is comprised in the A genome of the wheat plant.

16. The wheat plant according to any one of claims 8 to 15, wherein the first nucleotide sequence is comprised in chromosome 2A of the wheat plant.

17. The wheat plant according to any one of claims 8 to 16, wherein the first and one or more of said second nucleotide sequences are on the same chromosome.

18. The wheat plant according to any one of claims 8 to 17, wherein the first nucleotide sequence comprises a *Nax1* gene which confers enhanced tolerance to saline and/or sodic soils to the plant, and/or reduced sodium accumulation in an aerial part of the plant.

19. The wheat plant according to any one of claims 8 to 18, wherein said first nucleotide sequence is derived from durum wheat Line 149, *T. monococcum* C68-01, or a progenitor or progeny plant thereof comprising said first nucleotide sequence.

20. The wheat plant according to any one of claims 8 to 19 which is homozygous for said first nucleotide sequence.

21. The wheat plant according to any one of claims 8 to 20 which is homozygous for one or more of said second nucleotide sequences.

22. The wheat plant according to any one of claims 1 to 23 which is growing in a field.

23. The wheat plant according to any one of claims 8 to 24, wherein the grain yield of said plant is at least 95% of the grain yield of an isogenic plant lacking said first nucleotide sequence when said plants are grown under equivalent conditions.

24. The wheat plant according to any one of claims 8 to 25, wherein the number of heads of said plant is at least 95% of the number of heads compared to an isogenic plant lacking said first nucleotide sequence.

25. A wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 319 nucleotides on chromosome 2A, said

second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 29-348 of SEQ ID NO: 6 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

26. A wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 415 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 41-455 of SEQ ID NO: 7 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

27. A wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 387 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 19-405 of SEQ ID NO: 8 or its complement, wherein said second nucleotide sequence is comprised in a *Hin*dIII fragment that is different in size to the corresponding *Hin*dIII fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

28. A wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide

sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 301 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 11-311 of SEQ ID NO: 9 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

29. A wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 336 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 13-348 of SEQ ID NO: 10 or its complement, wherein said second nucleotide sequence is comprised in a *Nco*I fragment that is different in size to the corresponding *Nco*I fragment present on chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

30. A wheat plant comprising a first nucleotide sequence of at least 453 nucleotides on a chromosome other than chromosome 2B or 2D, said first nucleotide sequence being capable of hybridising under stringent conditions to a first nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 2 to 455 of SEQ ID NO: 5 or the complement thereof, and a second nucleotide sequence of at least 427 nucleotides on chromosome 2A, said second nucleotide sequence being capable of hybridising under stringent conditions to a second nucleic acid probe consisting essentially of a nucleic acid molecule having the sequence corresponding to nucleotides 30-456 of SEQ ID NO: 11 or its complement, wherein said second nucleotide sequence is comprised in a *Eco*RV fragment that is different in size to the corresponding *Eco*RV fragment present on

chromosome 2A of wheat Line 149, and wherein the wheat plant is a tetraploid or hexaploid wheat plant.

31. The wheat plant according to any one of claims 25 to 30 which is non-transgenic.

32. The wheat plant according to any one of claims 25 to 30 which is transgenic for said first nucleotide sequence.

33. A substantially purified and/or recombinant polypeptide comprising amino acids having a sequence as provided in SEQ ID NO:1 or SEQ ID NO:2, a biologically active fragment thereof, or an amino acid sequence which is at least 73% identical to SEQ ID NO:1 or SEQ ID NO:2, wherein the polypeptide has cation transporter activity when produced in a cell.

34. The polypeptide of claim 33, wherein the polypeptide comprises amino acids having a sequence which is at least 90% identical to SEQ ID NO:1 or SEQ ID NO:2.

35. The polypeptide of claim 33 or claim 34, wherein the polypeptide is from or in wheat or barley.

36. The polypeptide according to any one of claims 33 to 35, wherein the cation is sodium and/or potassium.

37. The polypeptide according to any one of claims 33 to 36 which comprises at least eight membrane spanning domains.

38. The polypeptide according to any one of claims 33 to 37, which is a fusion protein further comprising at least one other polypeptide sequence.

39. An isolated and/or exogenous polynucleotide comprising nucleotides having a sequence as provided in, or complementary to, SEQ ID NO:3 or SEQ ID NO:4, a sequence which is at least 73% identical to SEQ ID NO:3 or SEQ ID NO:4, a sequence which hybridizes to one or more of SEQ ID NO:3 or SEQ ID NO:4, or a sequence which encodes a polypeptide according to any one of claims 1 to 6, wherein the polynucleotide is not SEQ ID NO:5.

40. The polynucleotide of claim 39, wherein the polynucleotide comprises nucleotides having a sequence which are at least 90% identical to one or more of SEQ ID NO:3 or SEQ ID NO:4.

41. The polynucleotide of claim 39 which comprises nucleotides having a sequence which hybridizes to one or more of SEQ ID NO:3 or SEQ ID NO:4 under stringent conditions.

42. The polynucleotide according to any one of claims 39 to 41 which is operably linked to a promoter capable of directing expression of the polynucleotide in a cell of a cereal plant.

43. The polynucleotide of claim 42, wherein the cell is a root or leaf sheath cell.

44. The polynucleotide of claim 42 or claim 43, wherein the cell is a xylem parenchyma cell.

45. The polynucleotide according to any one of claims 39 to 44 which encodes a polypeptide having cation transporter activity when expressed in a cell.

46. A method of producing the polypeptide according to any one of claims 33 to 38, comprising expressing in a cell the polynucleotide according to any one of claims 39 to 44.

47. The method of claim 46, wherein the cell is a recombinant cell.

48. The method of claim 46, wherein the cell is non-recombinant.

49. The method according to any one of claims 46 to 48, wherein the cell is a plant cell.

50. The method according to any one of claims 46 to 49, wherein the cell is comprised in a plant growing in the field under saline and/or sodic conditions.

51. An isolated and/or exogenous polynucleotide which, when present in a cell of a cereal plant, decreases the expression of at least one gene that hybridises to a nucleic acid molecule having the sequence of SEQ ID NO: 3 or SEQ ID NO: 4 under

stringent conditions, said decreased expression being relative to an otherwise isogenic cell of a cereal plant that lacks said polynucleotide.

52. The polynucleotide of claim 51, wherein the gene does not confer enhanced tolerance to saline and/or sodic soils to a cereal plant, and/or reduced sodium accumulation in an aerial part of a cereal plant.

53. The polynucleotide of claim 51 or claim 52 which is operably linked to a promoter capable of directing expression of the polynucleotide in a cell of a cereal plant.

54. The polynucleotide according to any one of claims 51 to 53, wherein the polynucleotide is an antisense polynucleotide, a sense polynucleotide, a catalytic polynucleotide, an artificial microRNA or a duplex RNA molecule.

55. A vector comprising the polynucleotide according to any one of claims 39 to 46 or claims 51 to 54.

56. The vector of claim 55, wherein the polynucleotide is operably linked to a promoter.

57. The vector of claim 56, wherein the promoter confers expression of the polynucleotide preferentially in the root and/or leaf sheath of a cereal plant relative to at least one other tissue or organ of said cereal plant.

58. The vector according to any one of claims 55 to 57, wherein the promoter confers expression of the polynucleotide preferentially in xylem parenchyma cells of a cereal plant.

59. A cell comprising the polypeptide according to any one of claims 1 to 6, the polynucleotide according to any one of claims 39 to 46 or claims 51 to 54, or the vector according to any one of claims 55 to 58.

60. The cell of claim 59, wherein the polypeptide, polynucleotide or vector was introduced into the cell or a progenitor of the cell.

61. The cell of claim 59 or claim 60 which is a bacterial cell, plant cell or animal cell.

62. The cell of claim 61 which is an *E. coli* cell, an *Agrobacterium* cell or a cereal plant cell.

63. The cell according to any one of claims 59 to 62, wherein the polynucleotide is integrated into the genome of the cell.

64. The cell according to any one of claims 59 to 63, wherein the cell comprises a polynucleotide according to any one of claims 39 to 46 encoding at least one *Nax1* gene that confers enhanced tolerance to saline and/or sodic soils, to a cereal plant and a polynucleotide according to any one of claims 51 to 54.

65. The cell of claim 64, wherein the *Nax1* gene encodes a polypeptide comprising an amino acid sequence which is at least 80% identical to SEQ ID NO:1 and/or SEQ ID NO:2.

66. A plant comprising the cell according to any one of claims 59 to 65.

67. The plant of claim 66, wherein the plant is a cereal plant.

68. The plant of claim 67 which is a wheat plant.

69. The plant of claim 68 which is of the species *Triticum aestivum* ssp *aestivum*.

70. The plant of claim 68 which is of the species *Triticum durum*.

71. The plant of claim 70 which has a genetic background comprising less than 50% of the genetic complement of durum Line 149, 5049 or of the cultivar Tamaroi.

72. The plant according to any one of claims 68 to 71 which further comprises an allele of the *Nax2* gene which confers enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant.

73. The plant of claim 72, wherein the *Nax2* gene is non-transgenic.

74. The plant of claim 73, wherein the gene is on chromosome 5A.

75. The plant according to any one of claims 68 to 74 which further comprises an allele of the *Kna1* gene which confers enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant.

76. The plant according to any one of claims 66 to 75, which has enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant, relative to a corresponding plant lacking said cell.

77. A genetically modified plant having increased expression and/or activity of a polypeptide relative to a corresponding non-modified plant, wherein the polypeptide is as defined in any one of claims 33 to 38 and is expressed from a polynucleotide as defined in any one of claims 39 to 46.

78. A genetically modified hexaploid wheat plant comprising a transgenic *Nax1* gene that confers enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant, relative to a corresponding plant not having the gene.

79. The plant of claim 78, wherein the *Nax1* gene is obtained from durum wheat.

80. The plant of claim 78 or claim 79, wherein the *Nax1* gene is expressed in xylem parenchyma cells.

81. A method of producing the cell according to any one of claims 27 to 33, the method comprising the step of introducing the polynucleotide according to any one of claims 39 to 46 or claims 51 to 54, or a vector according to any one of claims 55 to 58, into a cell.

82. The method of claim 81 further comprising the step of regenerating a transgenic plant from the cell.

83. Use of the polynucleotide according to any one of claims 39 to 46 or claims 51 to 54 or vector according to any one of claims 55 to 58 to produce a recombinant cell.

84. A method of obtaining a wheat plant, the method comprising;

i) crossing two parental wheat plants of which at least one plant comprises a *Nax1* locus comprising a first nucleotide sequence as defined in any one of claims 25 to 32,

ii) screening progeny plants from the cross for the presence or absence of said *Nax1* locus, and

iii) screening progeny plants from the cross for the presence or absence of a second nucleotide sequence as defined in any one of claims 25 to 32,

wherein at least one of the parental wheat plants is a tetraploid or hexaploid wheat plant.

85. The method of claim 84, wherein the method further comprising a step of selecting a progeny wheat plant which has enhanced tolerance to saline and/or sodic soils, reduced sodium accumulation in an aerial part of the plant, and/or a grain yield of at least 95% of the grain yield of an isogenic plant lacking said first nucleotide sequence when said plants are grown under equivalent conditions.

86. The method of claim 84 or claim 85, wherein the method further comprises the step of selecting a plant with the desired genotype or of analysing the plant for at least one other genetic marker.

87. The method according to any one of claims 84 to 86, wherein at least one of the parental wheat plants is a hexaploid wheat plant.

88. The method of claim 87, wherein the cross is between a durum wheat plant comprising said *Nax1* locus and a hexaploid wheat plant lacking said *Nax1* locus.

89. The method according to any one of claims 84 to 88, wherein one of the wheat plants is durum wheat Line 149, *T. monococcum* C68-01, or a progenitor or progeny plant thereof comprising said first nucleotide sequence.

90. A method of introducing a *Nax1* locus into the genome of a wheat plant lacking said locus, the method comprising;

i) crossing a first parent wheat plant with a second parent wheat plant, wherein the second plant comprises a first nucleotide sequence as defined in any one of claims 25 to 32, and a second nucleotide sequence as defined in any one of claims 25 to 32,

ii) backcrossing the progeny of the cross of step i) with plants of the same genotype as the first parent plant for a sufficient number of times to produce a plant with a majority of the genotype of the first parent but comprising said first nucleotide sequence and said second nucleotide sequence, and

iii) selecting a progeny wheat plant which has enhanced tolerance to saline and/or sodic soils, reduced sodium accumulation in an aerial part of the plant, and/or a

grain yield of at least 95% of the grain yield of an isogenic plant lacking said first nucleotide sequence when said plants are grown under equivalent conditions.

91. A method of identifying a wheat plant with enhanced tolerance to saline and/or sodic soils, and/or reduced sodium accumulation in an aerial part of the plant, the method comprising detecting a first nucleic acid molecule of the plant as defined in any one of claims 25 to 32 or a second nucleotide sequence as defined in any one of claims 25 to 32.

92. The method of claim 91 which comprises:

i) hybridising a third nucleic acid molecule to a nucleic acid which is obtained from said plant,

ii) optionally hybridising at least one other nucleic acid molecule to said nucleic acid molecule which is obtained from said plant; and

iii) detecting a product of said hybridising step(s) or the absence of a product from said hybridising step(s).

93. The method of claim 92, wherein the third nucleic acid molecule is used as a primer to reverse transcribe or replicate at least a portion of the nucleic acid molecule.

94. The method according to any one of claims 91 to 93, wherein the nucleic acid is detected using a technique selected from the group consisting of: restriction fragment length polymorphism analysis, amplification fragment length polymorphism analysis, microsatellite amplification and/or nucleic acid sequencing.

95. The method according to any one of claims 91 to 94 which comprises nucleic acid amplification.

96. A method of enhancing tolerance to saline and/or sodic soils in a cereal plant, the method comprising genetically manipulating said plant such that the production of a polypeptide is increased when compared to a wild-type plant, wherein the polypeptide has cation transporter activity, wherein the polypeptide is a polypeptide according to any one of claims 33 to 38.

99. A method of reducing sodium accumulation in an aerial part of a cereal plant, the method comprising genetically manipulating said plant such that the production of a polypeptide is increased when compared to a wild-type plant, wherein the

polypeptide has cation transporter activity, wherein the polypeptide is a polypeptide according to any one of claims 33 to 38.

100. A method for identifying a plant comprising:

(i) obtaining a nucleic acid sample from each plant in a population of plants,

(ii) screening each nucleic acid sample for the presence or absence of a gene which hybridises under stringent conditions to a nucleic acid molecule having nucleotides in a sequence as provided in SEQ ID Nos: 3 or 4,

(iii) screening each nucleic acid sample for the presence or absence of a genetic marker which genetic marker is different to the gene of part (ii),

(iv) identifying a plant from the population of plants which comprises the gene and which lacks the genetic marker.

101 The method of claim 100, wherein the plant is a wheat plant, step (iii) comprises screening each nucleic acid sample for the presence or absence of a genetic marker present on chromosome 2A of wheat Line 149, which genetic marker is different to the gene of part (ii).

102. The method of claim 101, wherein the step (ii) comprises screening the nucleic acid samples for the presence or absence of a genetic marker which is genetically linked to the gene on chromosome 2A and different to the gene.

103. The method of claim 102 which further comprises

(v) selecting a plant comprising a recombination event between the gene and said genetic marker.

104. The method according to any one of claims 100 to 103, wherein the plant has enhanced tolerance to saline and/or sodic soils.

105. A method for identifying a nucleic acid molecule which confers to a plant enhanced tolerance to saline and/or sodic soils comprising:

(i) obtaining a nucleic acid molecule operably linked to a promoter, the nucleic acid molecule encoding a polypeptide comprising amino acids having a sequence that is at least 50% identical to SEQ ID NO: 1 and/or SEQ ID NO:2,

(ii) introducing the nucleic acid molecule into a cell in which the promoter is active,

(iii) determining whether the sodium or potassium concentration in the cell is modified when compared to an isogenic cell lacking the nucleic acid molecule,

(iv) optionally, selecting a nucleic acid molecule which confers modified sodium or potassium concentration in the cell, and

(v) optionally, obtaining a plant comprising the nucleic acid molecule selected in step (iv), wherein the plant has enhanced tolerance to saline and/or sodic soils.

106. The method of claim 105, wherein the cell is a cell of a wheat plant.

107. A method for identifying a nucleic acid molecule which confers to a plant enhanced tolerance to saline and/or sodic soils comprising:

(i) obtaining a nucleic acid molecule operably linked to a promoter, the nucleic acid molecule encoding a polypeptide comprising amino acids having a sequence that is at least 50% identical to SEQ ID NO: 1 and/or SEQ ID NO:2,

(ii) introducing the nucleic acid molecule into at least one cell of a plant in which the promoter is active,

(iii) cultivating a plant comprising the nucleic acid molecule in a saline and/or sodic soil and determining whether the plant has enhanced tolerance to the saline and/or sodic soil compared to an isogenic plant lacking the nucleic acid molecule, and

(iv) optionally, selecting a nucleic acid molecule which confers to a plant enhanced tolerance to saline and/or sodic soils.

108. The method of claim 107, wherein the cell is a xylem parenchymal cell.

109. The method of claim 107 or claim 108, wherein the plant is a wheat plant.

110. A plant, or progeny thereof, produced using a method according to any one of claims 84 to 90.

111. A wheat plant, or progeny thereof, identified or obtained using a method according to any one of claims 91 to 104.

112. A method of producing seed, the method comprising;

a) growing a plant according to any one of claims 1 to 32, 66 to 80, 110 or 111, and

b) harvesting the seed.

113. A seed or grain of a plant according to any one of claims 1 to 32, 66 to 80, 110 or 111.

114. A method of producing flour, wholemeal, starch or other product obtained from seed, the method comprising;

a) obtaining seed according to claim 113, and

b) extracting the flour, wholemeal, starch or other product.

115. A product produced from a plant according to any one of claims 1 to 32, 66 to 80, 110 or 111.

116. A product produced from a seed according to claim 113.

117. The product of claim 115 or claim 116, wherein the product is a food product.

118. The product of claim 117, wherein the food product is selected from the group consisting of: flour, starch, leavened or unleavened breads, pasta, noodles, animal fodder, breakfast cereals, snack foods, cakes, malt, beer, pastries and foods containing flour-based sauces.

119. The product of claim 117, wherein the product is beer or malt.

120. The product of claim 115 or claim 116, wherein the product is a non-food product.

121. The product of claim 120, wherein the non-food product is selected from the group consisting of: films, coatings, adhesives, building materials and packaging materials.

122. A method of preparing a food product of claim 117, the method comprising mixing seed, or flour, wholemeal or starch from said seed, with another ingredient.

123. A method of preparing malt, comprising the step of germinating seed of claim 113.

124. A substantially purified antibody, or fragment thereof, that specifically binds a polypeptide according to any one of claims 33 to 38.

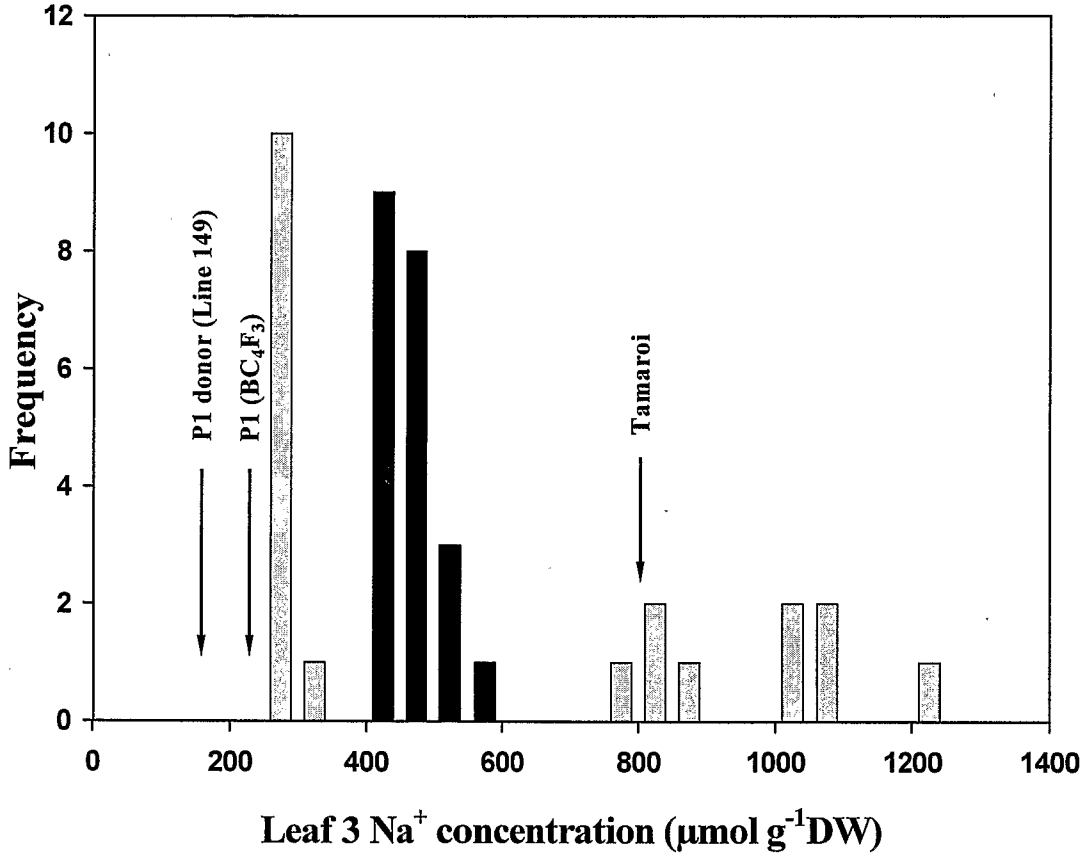

Frequency
Leaf 3 Na+ concentration (µmol g-1DW)
P1 donor (Line 149)
P1 (BC4F3)
Tamaroi
0
2
4
6
8
10
12
0
200
400
600
800
1000
1200
1400

Figure 1

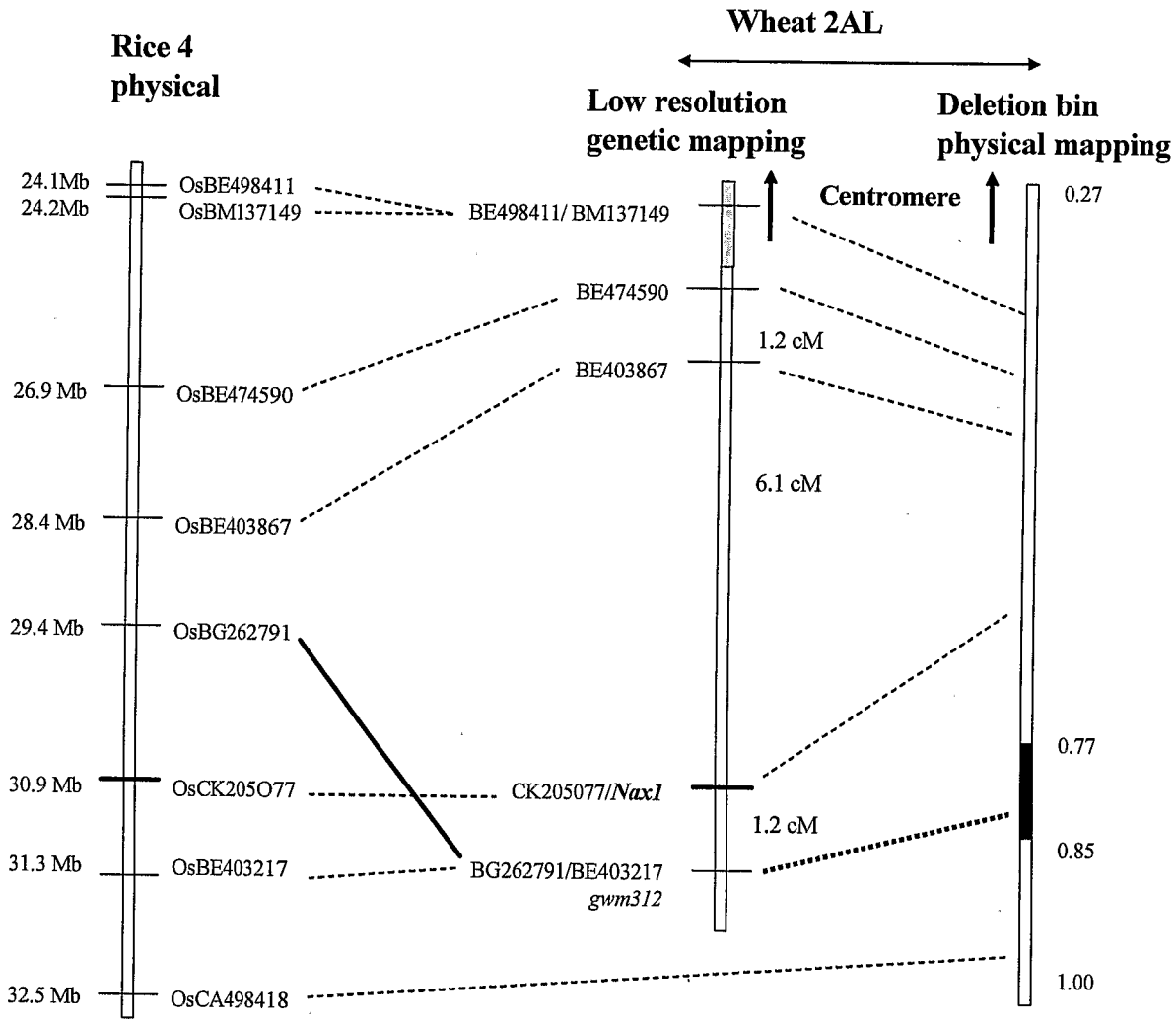

Wheat 2AL
Rice 4 physical
Low resolution genetic mapping
Deletion bin physical mapping
Centromere
24.1Mb
24.2Mb
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OsBM137149
BE498411/ BM137149
0.27
BE474590
1.2 cM
26.9 Mb
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BE403867
6.1 cM
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Figure 2

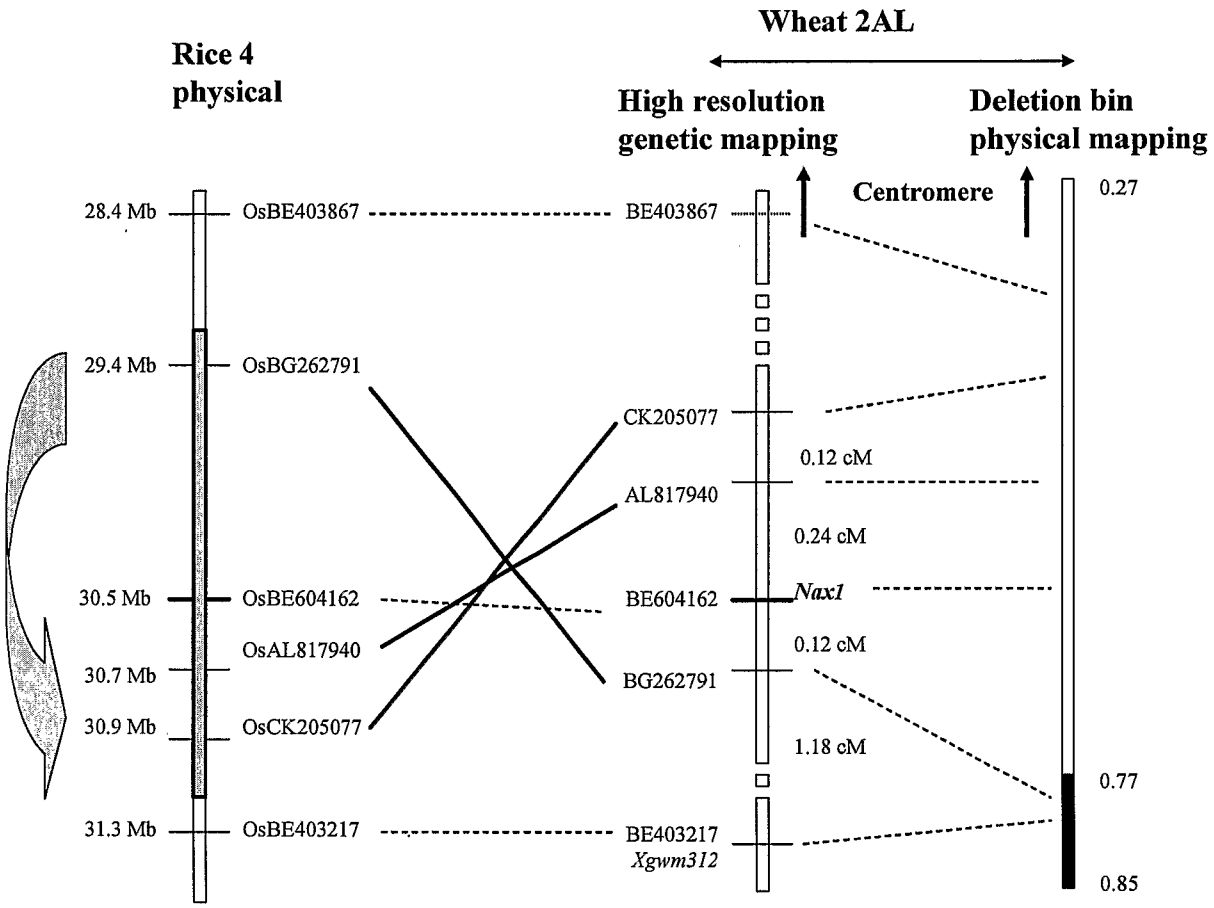

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Rice 4
physical
High resolution
genetic mapping
Deletion bin
physical mapping
Centromere
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Figure 3

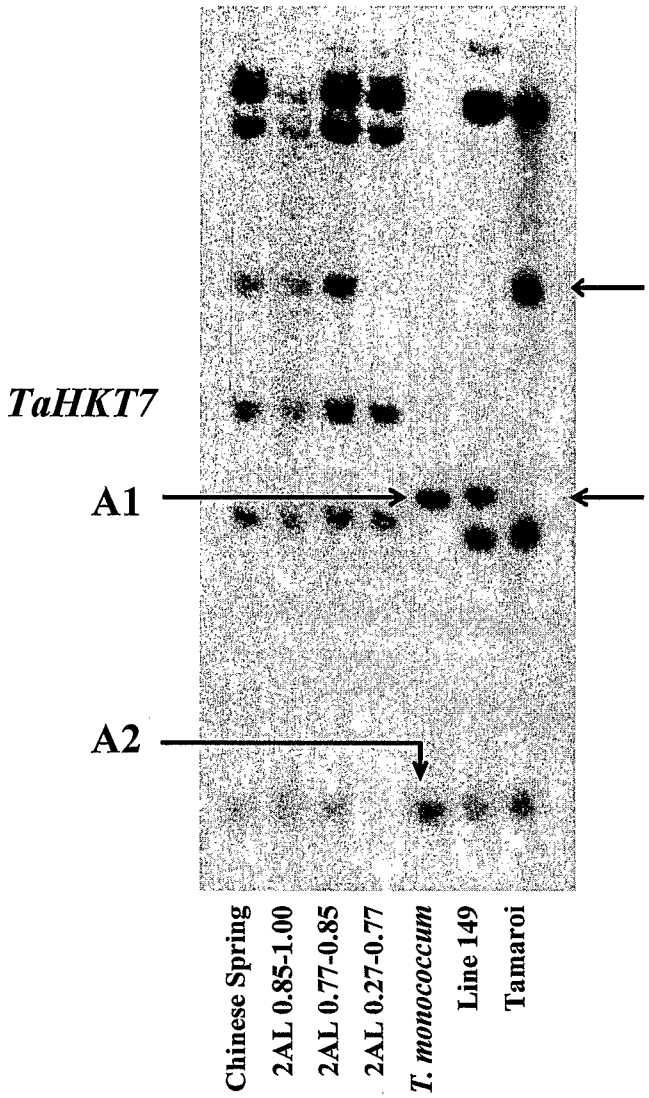

TaHKT7
A1
A2
Chinese Spring
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2AL 0.77-0.85
2AL 0.27-0.77
T. monococcum
Line 149
Tamaroi

Figure 4

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CGTGGTCTATGAGAAATCGTGACCGTATGTATATAGGCAGCGAATCCGGACCAGTATCTGTG
CCATTCCATGAGAATGTTTCCGCTCGCCGGAATGTCCTTGCACGTCGACGTCTGGATACTGA
AAGTCTGGTAGTAGGTGCCATTTTTGGGATCCAGAAGAGCCTTGGTAACTTGTGTCCAAGTT
TGATCATAGATTTAACTAACAAAATGTTAATGCATATCATAAAAAATTATACCATTGCATTC

Figure 5a

GTATTTGAACATAGTTTTCAATGATATAATTTCTGCTTACATGCATTGATATTTTGCTAGTT
AAATCTACGATTAAAATTTGACACTAAATATAAAGGGGACCAATAAACTAGGACGCGGGTAG
TAGAACTGAAGCTCCAGCACGCAAGTGCTTTCATACTGCATATCTACAATTGGACGCTCTTC
TCAATGTTCTCATTATCCTCTCGACACAATCCCAACGCGTTTCTCAACGAGTTTCGCGTACC
TCATCAAAGTAAAGTAGATAAAGCGTGTTAGTGCACTCTTTGCAGCTACACATTATCACCAC
TCACGTTCTGCTTCTTCTTGCTTGCATGCAGGTACCTGCCTCCATACACGACGTGGTTTCCA
TTTGAAGAGAGCTCCGGCGTCAAGGACCAACCCAGGGAGGAGACCCAGGGGGTCAGGCTGCT
CAAGAGCACGCTTCTGTCACAACTCTCCTACCTTGCCATCTTTGTCATCGCCATCTGCATCA
CCGAGAGGGAAAAGCTCAAGGAGGACCCTCTCAACTTCAGCTTGCTCAGCATCCTCGTCGAA
GTCGTCAGGCAAGTCCCCTCTAACTCTAAGCAATCCAAAGCTTGAAAAAGAAAATAACAATA
TACTCCTTTTTCCTTCTTTTTTCAAGTATATACTACACGATAAGAATTGCAAGTCGCGTCCT
TGAGGGAAGCTGACATTTTTCAGTGTAACCATCTGCAGCGCTTATGGGAATGTGGGCTTCTC
CATGGGCTACAGCTGCAGTAGGCAGATCGGCCCGGACGGGATGTGCACTGACCGGTGGACTG
GCTTTGCTGGGAGGTGGAGCGATTCCGGCAAGCTCATCCTCATTCTTGTCATGCTCTTCGGG
AGGCTGAAAAAGTTCAGCATGAAAGCGGGCAAAGCCTGGAAGCTTAGTTAG

Figure 5b

ATGGCCGGAGCTCATCATAAGGTCGGCGAGCTGCTGCACCACACGCCGCGCAGCTCGACGGC
CGCGGTCGACAAGGCAATGCCCTCGCCCTCCAGTTCATACGCGCGGCACCACGCCGTCAAGG
AGCGCGTGGCGCGGTGGCGGCGCGCGCTCGCCGGCCGGTTCTGGCCGCGCGTCGGCTCGCTG
CTCGTCCACGTCGCCTACTTCCTCACCGTCTCCTGGCTCGGGTACCTCCTACTCGCGCAGCT
CAGGTTCCGCGCCGGCGGCGACGGGACGCGGCGGCCCCGCGGCATCGACCTGTTCTTCACCG
CCGTCTCGGCCGCGACGGTGTCCAGCATGTCCACCATCGAGATGGAGGTGTTCTCCAACGGG
CAGCTCCTCGTCCTGACCGTCCTCATGCTCGTCGGCGGCGAGGTGTTTTTATCCCTCGTAGG
CCTCGCGTCCAAGTGGTCCAAGCTGAGGAAGCAGGCTGCACACAAATCCCGGCGCGTGGAGA
TCCACCACGTCGCCGAGCTTGAGATGCTGCCGGCCGCCGCCGCCGACATCGACAACCCAACG
TCTACCGCGGATGATGAGACGAGCAAGTCGTTGGAGCACGTACCTGACACGAGGCTGCGGCG
TGACGCGGTGCGGTCGCTGTTCTTCGTCGTCCTCGCCATCTTCCTGGTGGTGCACGTCCTCG
GCGCCGGGGCCATCGCGGCGTACATCCTGCACGCGTCTCCGGCGGCGAGGCGGACGCTGCGG
GACAAGGCCCTGAACGTGTGGACCTTCGCCGTGTTCACGACGGTGTCCACGTTCTCGAACTG
CGGGTTCATGCCGACGAACGAGAACATGGCAGTGTTCCAGCGGGACACCGGGCTGCAGCTGC
TGCTCGTGCCGCAGGTGCTGGTCGGGAACACGCTGTTCGCGCCGCTGCTGGCCGCGTGCGTG
CGCGCCGCCGCCGCGGCGACCAGGCGCGTGGAGCTCAAGGAGACTTTGAAGCAGGGCAGGGA
GCTGACGGGGTACTCCCACCTGCTCCCGGCGCGGCGGTGCGGGATGCTGGCGGCCACGGTGG
CCGGTTTCCTCGCCGTGCAGGTGGCGATGCTGTGCGGCATGGAGTGGAGCGGGGCGCTGCGG
GGGATGAGCGCGTGGGAGAAGGTGTCGAACGCGGTGTTCCTGGCGGTGAACTCCCGCCACGC
CGGCGAGTCGACCCTCGACATCTCCACCCTCGCGCCGGCCATCCTCGTGCTCTTCGTGCTCA
TGATGAGTCCTGCTCGATCTCTCTCTCTCTTTCTCCAGCTGCGGGTCGATGCTTACGAGAAT
GCTGTTAAAACTAACCTGGGAAATGGCATACGTAGTTTGTTTTCTTCCCATAACACTGAGTT
CCCTACGTTGAACGCCAACAAGAATCTAGAACGTAGCACTAAGATTCTTTTTTCCCGTGCTT
CCCAACTAAATGTCATCTCAGATTCCCTAAAATTTCATGTCACGTTCCGAAGCTTTTGATCA
ACACCTAATCATGCTCTTTCGCAGAAAAAAAAACACACACACCTATCATGTTCATCTCAT
AGTCGACCTACTTGACCATCTTTGTCATGCGATCTGTGCTCTTTTTTTTTTACGAATACTTG
CTATCGGTGCTCAACTTTAAGCCTAGCAGAGATTTTTAACAATTTCATGCCAAAGGTAAATG
TAATTATAGGCTAAGCTTACACTGCCCCCCATTTAATAAACAAGTGGCATTTTGAATATATA
CATAGTAAAACTTTGAACATTTTGACTTATGGTACCATTTTATTAAAAACTTGGAGATCATA
TATACATTTTTCCCTGGAAATATTCGAGATTTTGGTTTTTGGAATGGAGAATATTGTTATTT
GGTTGGGGTAATATTATAGATATCTACCCTAAGTCAAATATCTTAAGTTTGACCAAGTCTAT
AGAAAAATATACTAAGATGTGCCACGCCAAATTAATTTCATTTGATTTACCACAGTATATAT
TTTCCCGTAGTATTTTTTCATACTAGAATAAACTAATTGAAGGAAGTGCTGGTATCCAAAGC
CATGGGTATTGCTGCAAGACTTGCAATCATTCTTCAAGAAACTCAAATTGTATGTTAATTTT
ATTTTATTTTTTGCGAATAAAAATGTATGTTAATTAACTATGGGGAGAGTATAAATGTATAA
TAACCGATGAGACTAGTAAATCTGCATAATTTGTCATGCATGTTGCTCATCAAGTACCATAA
CCTGATCATCACTGAATACTCCTTACCAAGTACTGCATGGGGAAAAAGAATTCACGTTAACT
ATGGATTAGTACTACCTCCTTTCCCTTCGTATTTTAGGCCAGACTTGACCATGTATAGACAA
TTAAAATATAAAGTATATGCTATAAAAAATTATACTGTTGGATTTGTATTTGAAAGAGCTTT
CCAATAATATAATTTTTATAACATATGATTTACATTTTGTTAGTCAAATTTTTAGGTCAAAT
TTTAGCCCAAAATAAGAGGGGGCCTACCGCGTAATAAACCGGGATGGAGGGAGTATTTATTA
ATAAGTGCTCCAGGTGCCCTTTTGTCCATAATGCTAGCCCTTAACTAATGAAACTAGGAAAT
GGCGTTCAATAATAATTGAACAACGAACCGGCTGAGGAATCTTCCCTCCCCGCGGAACGGAC
AGGCCGAAAGTATTCAAGCTCACGCGCAGCTGTGACCGCACCTTGGCCAGGAAGAAGAGGCA
AGAAAAACACGAGGAAATTACCGCCAAATTAAGCACCCAAAACCAAAATCCTGGACCAGCTG
CCCATCGGTTCATGCGGAGCCACGAAATTGCGTGTTCCATAGTCCATAGTACGACATTGCCA
ACCACGATCGAGCATCGATCGCCACAGGTTCATGATCGGAATGCCGATCGATGGATTGATCG

Figure 6a

GTTCGGAACTATTCTCTAGATTCGGATTCCATGCATATATGAAGATACGGTAGTGCCAGTGC
AGTGGTCCATGAGAAACTGAGACCATATGTAGGCCGCGAGAGAATCAGAACCTGCATATGTG
CCGCGCCGTTGATTTCCTGAGAATATTGCCGGTCACCAGAATGTCCTTGCAGCACGTCATCC
TGAGAATCGATACTATCCGTCTCAGTTTTCTCACGTTCTGCTTGCTTCCTCTTGCTTCAGGT
ATCTGCCTTCATACACCACGTGGTTTCCATTTGCGGGGAGCTCCGGCACAACGGATCACGCC
GAGGAGACACAGGGTATCAGGCTGATCAAGAGCACGGCCTTGTCGCAGCTCTCCTACCTCAC
CATCTTCGTCATCGCCATCTGCGTCACCGAGAGGGAGAAGCTCACGGAAGACCCCCTCAACT
TCAACCTGCTCAGCATCGTCGTCGAAGTTGTCAGCGCTTATGGAAATGTGGGATTCTCCATG
GGCTACAGTTGCAACAGGCAGATCAGCCCGGACCAGCTGTGCACCGACAGGTGGACCGGCTT
CGTCGGGAGGTGGAGCGATTCCGGCAAGCTCGTCCTCATTCTCGTGATGCTCTTCGGGAGGC
TCAAGAAGTTCAGCATGAAAGGAGGCAAAGCCTGGAAGCTTAGTTAG

Figure 6b

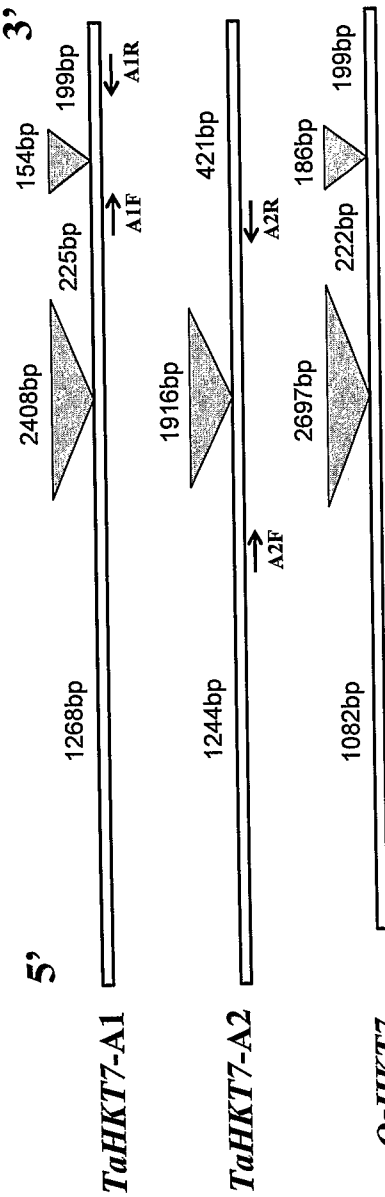

5'
3'
TaHKT7-A1
TaHKT7-A2
OsHKT7
1268bp
2408bp
225bp
154bp
199bp
A1F
A1R
1244bp
1916bp
421bp
A2F
A2R
1082bp
2697bp
222bp
186bp
199bp

Figure 7

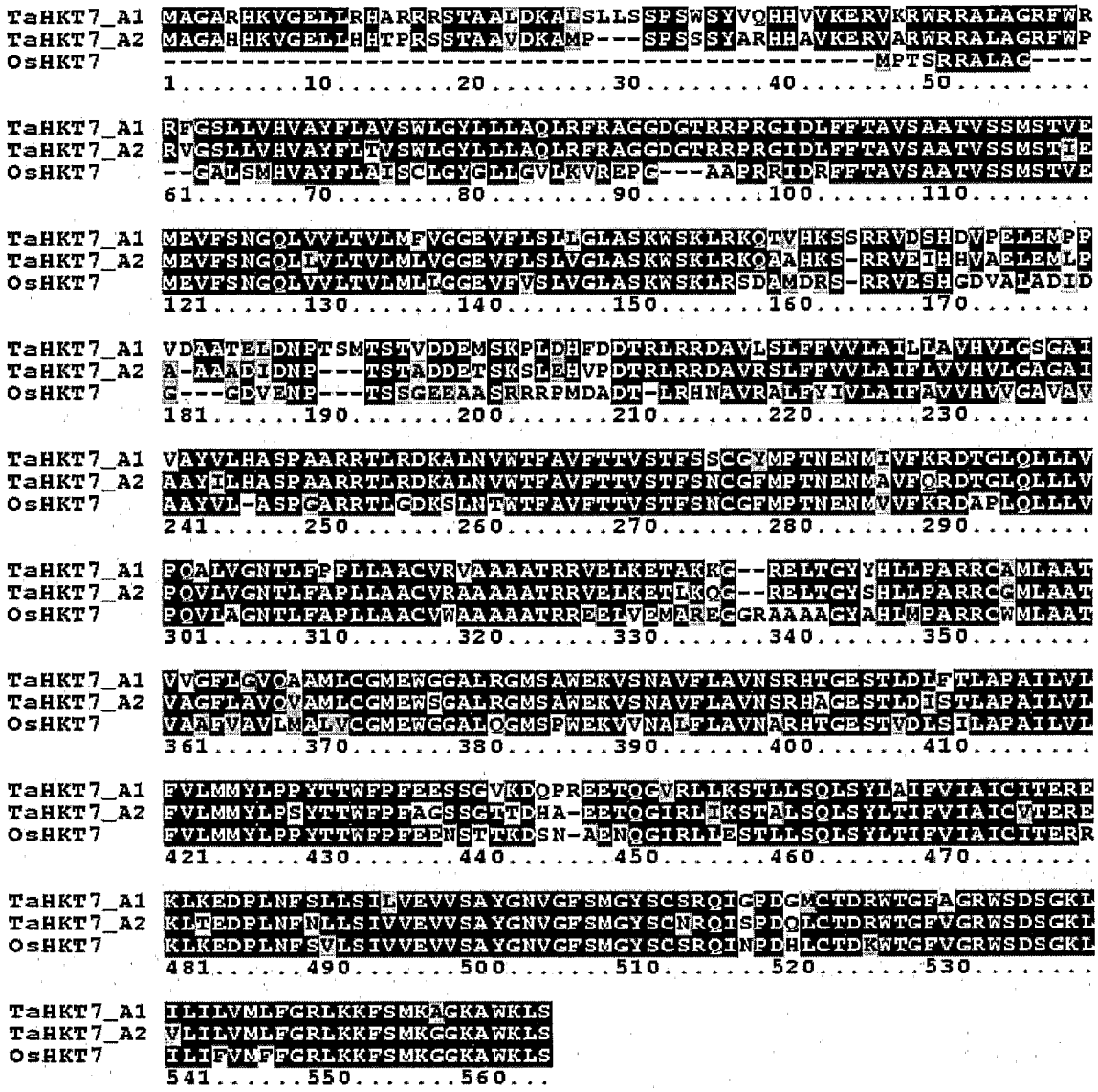
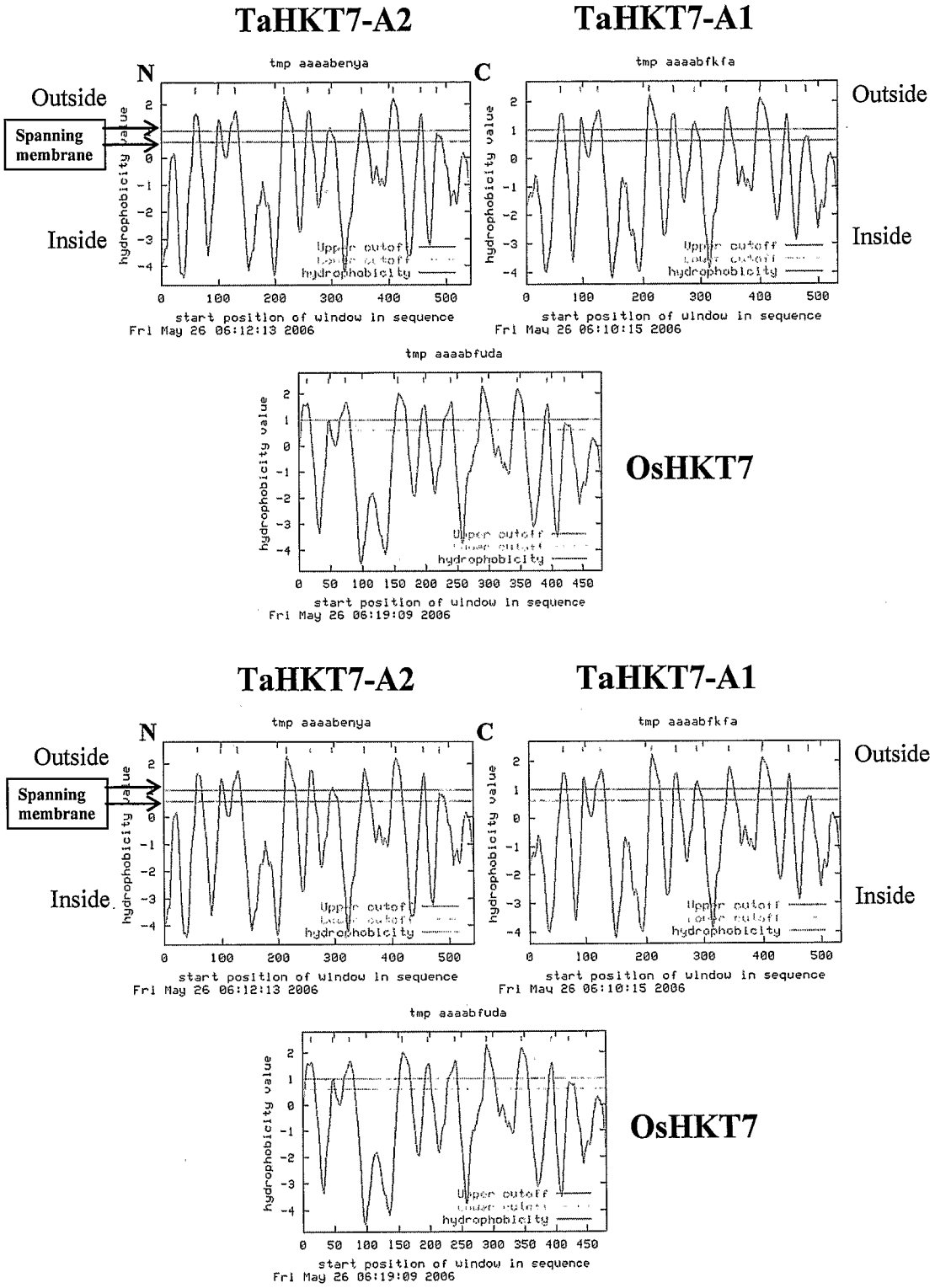


Figure 8



TaHKT7-A2
TaHKT7-A1
Outside
Inside
OsHKT7

Figure 9

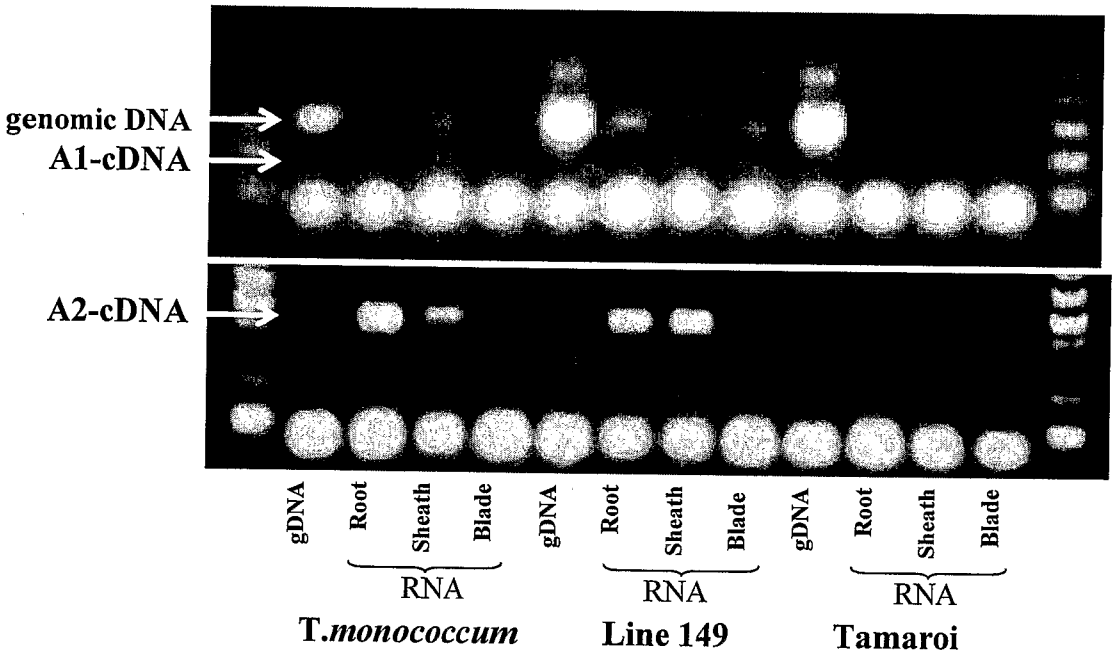

genomic DNA
A1-cDNA
A2-cDNA
gDNA
Root
Sheath
Blade
gDNA
Root
Sheath
Blade
gDNA
Root
Sheath
Blade
RNA
RNA
RNA
T.monococcum
Line 149
Tamaroi

Figure 10

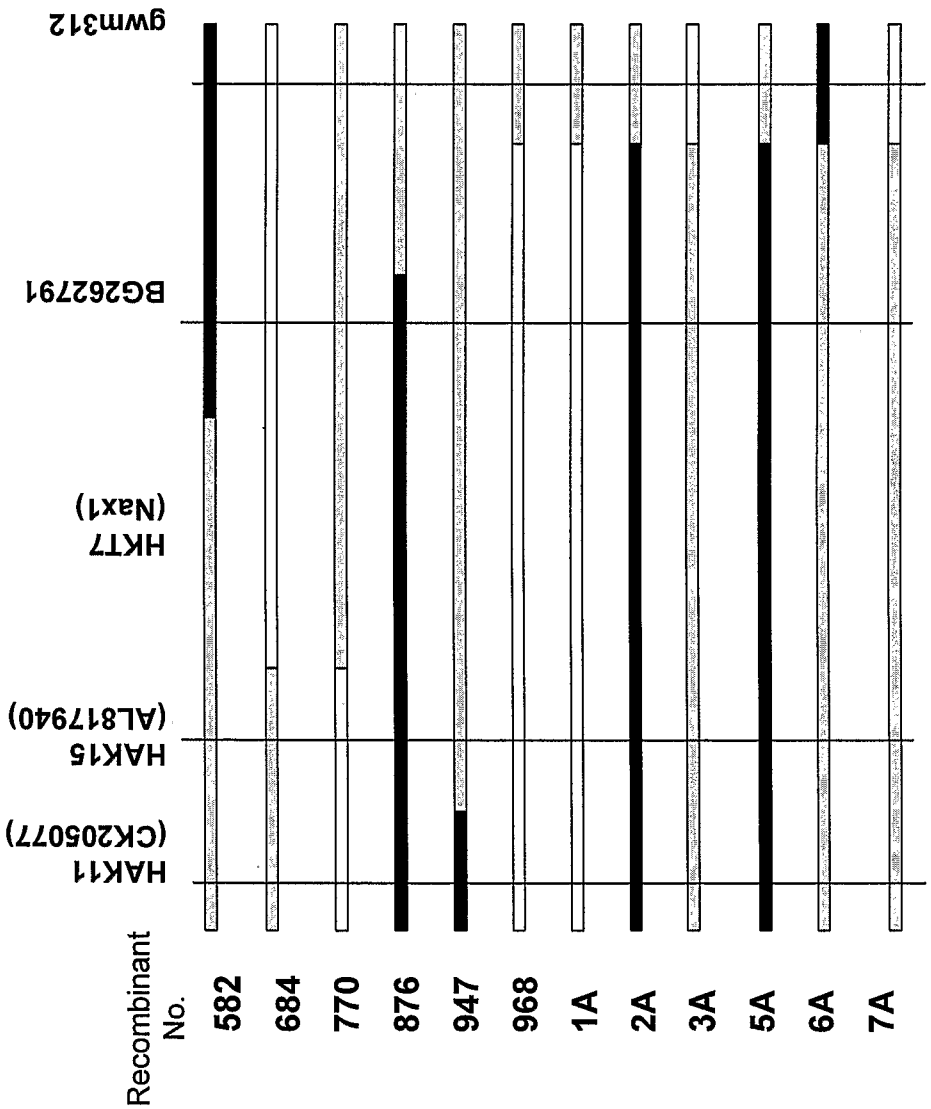

Recombinant No.
582
684
770
876
947
968
1A
2A
3A
5A
6A
7A
HAK11 (CK205077)
HAK15 (AL817940)
HKT7 (Nax1)
BG626791
gwm312

Figure 11

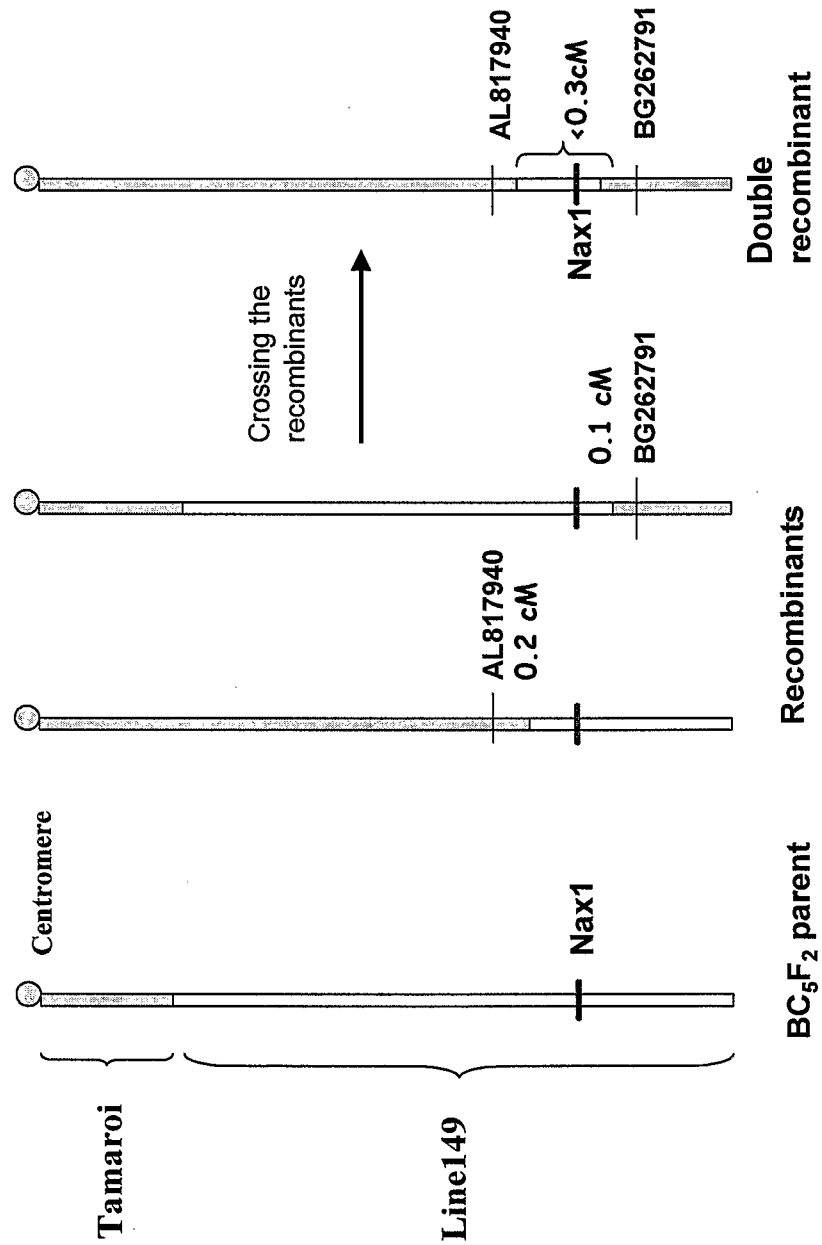

Tamaroi
Line149
Centromere
Nax1
AL817940
0.2 cM
0.1 cM
BG262791
AL817940
<0.3cM
Nax1
BG262791
Crossing the recombinants
BC5F2 parent
Recombinants
Double recombinant

Figure 12

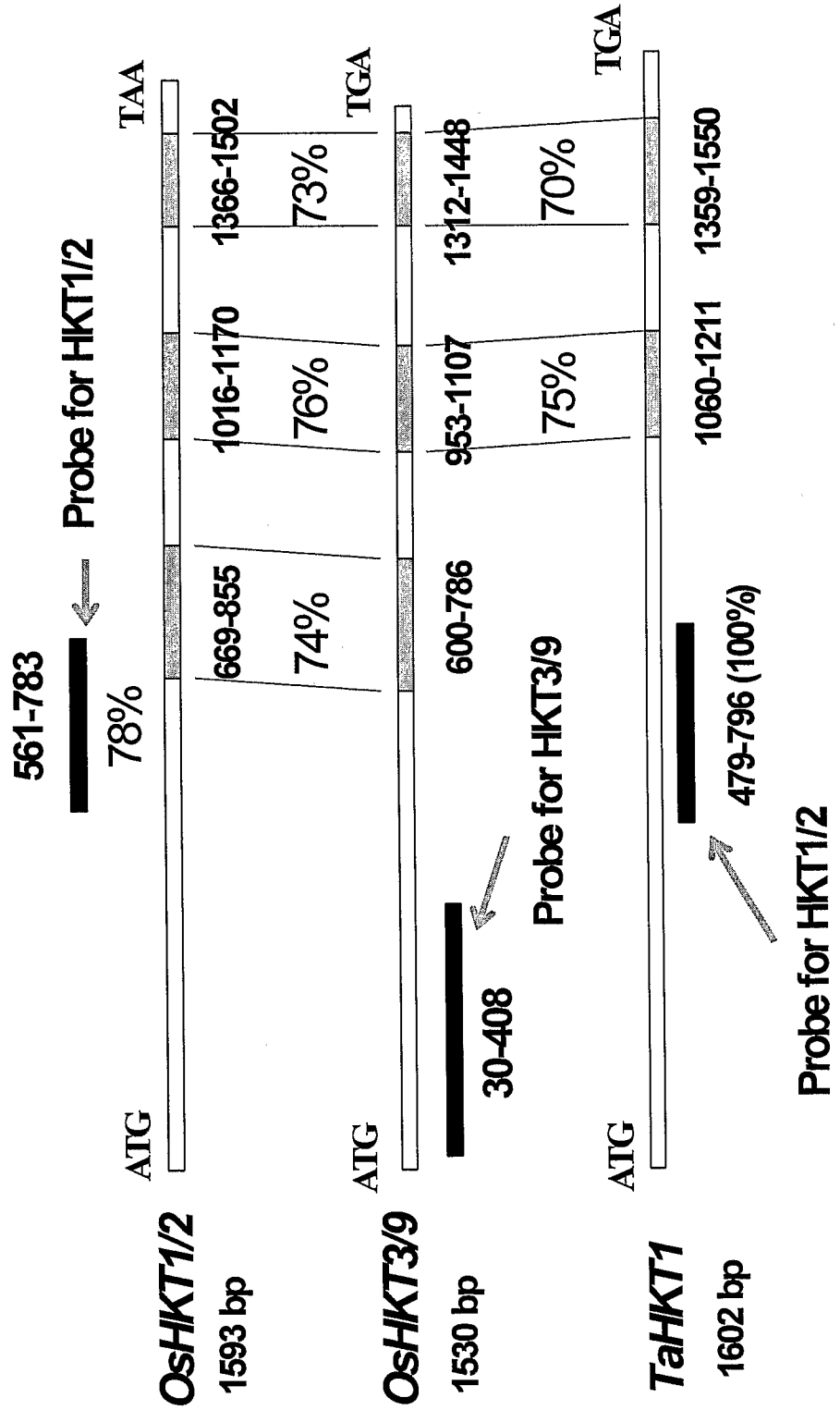

OsHKT1/2
1593 bp
ATG
TAA
561-783
Probe for HKT1/2
78%
669-855
74%
1016-1170
76%
1366-1502
73%
OsHKT3/9
1530 bp
ATG
TGA
30-408
600-786
Probe for HKT3/9
953-1107
75%
1312-1448
70%
TaHKT1
1602 bp
ATG
TGA
479-796 (100%)
Probe for HKT1/2
1060-1211
1359-1550

Figure 13

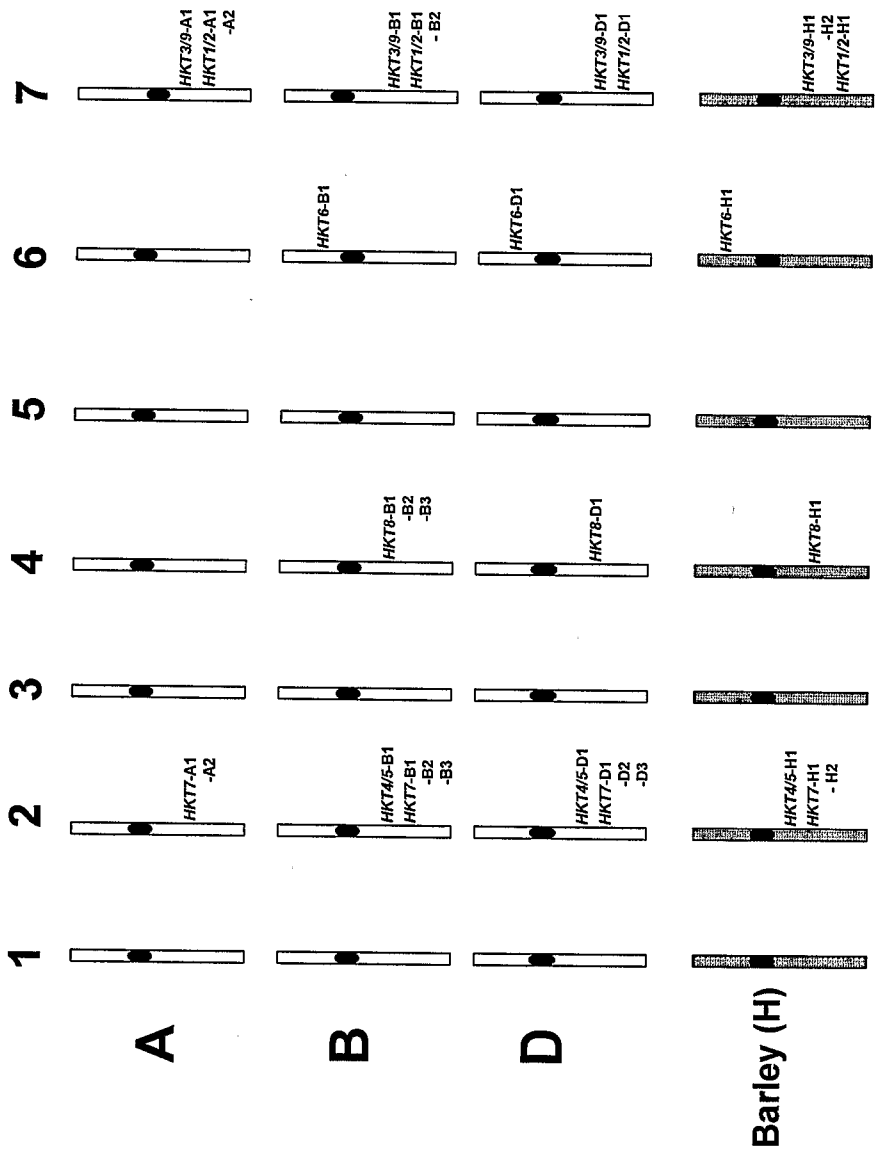

1
2
3
4
5
6
7
HKT7-A1
-A2
HKT1/2-A1
HKT3/9-A1
HKT4/5-B1
HKT7-B1
-B2
-B3
HKT8-B1
HKT6-B1
HKT1/2-B1
HKT3/9-B1
HKT4/5-D1
HKT7-D1
-D2
-D3
HKT8-D1
HKT6-D1
HKT1/2-D1
HKT3/9-D1
HKT4/5-H1
HKT7-H1
- H2
HKT8-H1
HKT6-H1
HKT1/2-H1
HKT3/9-H1
-H2
A
B
D
Barley (H)

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001280

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A01H 1/00 (2006.01) ***A01H 1/02*** (2006.01) ***A01H 1/04*** (2006.01) ***A01H 5/00*** (2006.01)

C12N 15/29 (2006.01) ***C12Q 1/68*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, WPIDS, CAPLUS, AGRICOLA, GENOMEQUEST: nax?, hkt?, athkt?, oshkt?, salt toleran?, SEQ ID NO: 1, SEQ ID NO 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MUNNS, R et al, "Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits", Plant and Soil, 2002, Vol. 247(1), p 93-105 Whole document	1-32, 84-95
X	LINDSAY M P et al, "A locus for sodium exclusion (*Nax1*), a trait for salt tolerance, mapped in durum wheat", Functional Plant Biology, 2004, Vol. 31, p 1105-1114 Whole document	84-95
X	WO 2005/120214 A1, (CSIRO et al), 22 December 2005 Whole document	84-95

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 October 2007	- 7 NOV 2007
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **SUSAN MACKENZIE** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : (02) 6283 2690

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001280

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/045829 A1, (CROPDESIGN N.V.), 4 May 2006 SEQ ID 30 is shares 64.9% and 66.2% identity with SEQ ID 1 and 2 respectively Pages p2 lines 32-37 - p2 lines 1-10	105-123
Y	US 20040123343, (LA ROSA et al), 24 June 2004 SEQ ID 157020 shares 54.5% and 56% identity with SEQ ID 1 and 2 respectively Claim 3(k)	105-123
Y	Yoshida Kazuya, "Plant biotechnology - genetic engineering to enhance plant salt tolerance", Journal of Bioscience and Bioengineering, (2002) Vol. 94, No. 6, p. 585-590. Whole document	105-123

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001280

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box 1 and 2

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box 1
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: III

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

In assessing whether there is more than one invention claimed, I have given consideration to those features which can be considered to potentially distinguish the claimed combination of features from the prior art. Where different claims have different distinguishing features they define different inventions.

This International Searching Authority has found that there are different inventions as follows:

1. Claims 1-24, 33-77, 124 (completely), 78-123 (partially) are directed to the polypeptides of SEQ ID NOs: 1 and 2, encoding polynucleotides SEQ ID Nos: 3 and 4 respectively, methods of using the polypeptides or polynucleotides and plants comprising the polypeptides or polynucleotides. It is considered that polypeptides of SEQ ID NOs: 1 and 2 comprise a first distinguishing feature.

2. Claims 25-32, 84-95 110-123 (all partially) are directed to the *Nax1* locus marker of SEQ ID NO: 5, methods involving the use of the marker and plants comprising the marker. It is considered that the marker of SEQ ID NO: 5 comprises a second distinguishing feature.

3. Claims 25, 31, 32, 84-90, 110, 112-123 (all partially) are directed to the *Nax1* locus marker of SEQ ID NO: 6, methods involving the use of the marker and plants comprising the marker. It is considered that the marker of SEQ ID NO: 6 comprises a third distinguishing feature.

4. Claims 26, 31, 32, 84-90, 110, 112-123 (all partially) are directed to the *Nax1* locus marker of SEQ ID NO: 7, methods involving the use of the marker and plants comprising the marker. It is considered that the marker of SEQ ID NO: 7 comprises a fourth distinguishing feature.

5. Claims 27, 31, 32, 84-90, 110, 112-123 (all partially) are directed to the *Nax1* locus marker of SEQ ID NO: 8, methods involving the use of the marker and plants comprising the marker. It is considered that the marker of SEQ ID NO: 8 comprises a fifth distinguishing feature.

6. Claims 28, 31, 32, 84-90, 110, 112-123 (all partially) are directed to the *Nax1* locus marker of SEQ ID NO: 9, methods involving the use of the marker and plants comprising the marker. It is considered that the marker of SEQ ID NO: 9 comprises a sixth distinguishing feature.

7. Claims 29, 31, 32, 84-90, 110, 112-123 (all partially) are directed to the *Nax1* locus marker of SEQ ID NO: 10, methods involving the use of the marker and plants comprising the marker. It is considered that the marker of SEQ ID NO: 10 comprises a seventh distinguishing feature.

8. Claims 30, 31, 32, 84-90, 110, 112-123 (all partially) are directed to the *Nax1* locus marker of SEQ ID NO: 11, methods involving the use of the marker and plants comprising the marker. It is considered that the marker of SEQ ID NO: 11 comprises an eighth distinguishing feature.

Continued in Supplemental Box 2

Supplemental Box 2
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: III

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

The only feature common to all of the inventions is that they are all associated with the *Nax1* locus, which is associated with salt tolerance in wheat. However this locus and its involvement in salt tolerance in wheat is not novel in the light of the following documents cited in the International Search Report:

D1 MUNNS, R et al, Plant and Soil, 2002, Vol 247(1), p 93-105

D2 LINDSAY M P et al, Functional Plant Biology, 2004, Vol 31, p 1105-1114

D3 WO 2005/0120214 A1, 22 December 2005

This means that the common feature cannot constitute a special technical feature within the meaning of PCT Rule 13.2, second sentence, since it makes no contribution over the prior art.

Because the common feature does not satisfy the requirement for being a special technical feature it follows that it cannot provide the necessary technical relationship between the identified inventions. Therefore the claims do not satisfy the requirement of unity of invention *a posteriori*.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2007/001280

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2005120214	AU	2005251389	EP	1765056		
WO	2006045829	AU	2005298638	CA	2588281	EP	1807521
US	2004123343	US	2007016974				

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX