Physiological Characterization of Two Genes for Na^+ Exclusion in Durum Wheat, *Nax1* and *Nax2*[1]

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Durum wheat (*Triticum turgidum* L. subsp. *durum* Desf.) Line 149 contains two novel major genes for excluding Na^+ from leaf blades, named *Nax1* and *Nax2*. The genes were separated into families containing a single gene and near-isogenic homozygous lines were selected. Lines containing either *Nax1* or *Nax2* had lower rates of Na^+ transport from roots to shoots than their near-isogenic pairs due to lower rates of net loading of the xylem, not to lower rates of net uptake from the soil or higher rates of retranslocation in the phloem. *Nax1* and *Nax2* lines also had higher rates of K^+ transport from root to shoot, resulting in an enhanced discrimination of K^+ over Na^+. Lines containing *Nax1* differed from those containing *Nax2* by unloading Na^+ from the xylem as it entered the shoot so that Na^+ was retained in the base of the leaf, leading to a high sheath to blade ratio of Na^+ concentration. Gradients in tissue concentrations of Na^+ along the leaf suggested that Na^+ was continually removed from the xylem. The *Nax2* line did not retain Na^+ in the base of the leaf, suggesting that it functioned only in the root. The *Nax2* gene therefore has a similar function to *Kna1* in bread wheat (*Triticum aestivum*).

Salt tolerance in wheat (*Triticum aestivum*) and many other species is associated with the ability to exclude Na^+ so that high Na^+ concentrations do not occur in leaves, particularly in the leaf blade (Läuchli, 1984; Munns, 2005). High leaf Na^+ concentrations can cause premature leaf senescence and loss of photosynthetic activity (James et al., 2002), which reduces the rate of carbon assimilation and ultimately grain yield (Husain et al., 2003).

Durum (pasta) wheat (*Triticum turgidum* L. subsp. *durum* Desf.) is more salt sensitive than bread wheat, probably because of its poorer ability to exclude Na^+ from the leaf blade (Gorham et al., 1990). In monocotyledonous species, the leaf is composed of the blade (top part) and the sheath (bottom part). The bulk of the leaf's photosynthesis and transpiration occurs in the blade. An unusual source of Na^+ exclusion was found in durum wheat Line 149 (Munns et al., 2000). A screen of a large number of durum-related genotypes for leaf blade Na^+ accumulation showed that Line 149 had an unusually low concentration, as low as bread wheat (Munns et al., 2000). However, the concentration in shoots as a whole was not as low as bread wheat (Husain et al., 2004), suggesting that Na^+ was retained in the leaf sheath. The Na^+ transport characteristics of Line 149 were compared with the durum cv Tamaroi, which has the high Na^+ concentrations typical of durum wheat, by measurement of $^{22}Na^+$ transport and net Na^+ accumulation (Davenport et al., 2005). The genotypes did not differ in unidirectional root uptake of Na^+. The major differences in Na^+ transport between the genotypes were in the rate of transfer to the shoot (net root xylem loading) and the preferential accumulation of Na^+ in the leaf sheath versus the leaf blade (Davenport et al., 2005).

Genetic analysis of a cross between Line 149 and cv Tamaroi indicated two genes of major effect for Na^+ exclusion (Munns et al., 2003). A quantitative trait locus for low Na^+ concentration in leaf blades was mapped to the distal region on the long arm of chromosome 2A and named *Nax1*. This quantitative trait locus accounted for 38% of the phenotypic variation in the F_2 generation, suggesting that it was associated with one of the two major genes. A microsatellite marker, *gwm312*, was closely linked to the trait and has been used to accelerate the transfer of this trait into commercial varieties of durum wheat (Lindsay et al., 2004). The presence of a second gene for Na^+ exclusion was confirmed by the observation that some plants without the Line 149 allele of *gwm312* still had moderately low Na^+ concentrations in the leaf blade. A second gene, independent of *Nax1*, was suggested to contribute to the full expression of the Na^+ exclusion trait (Lindsay et al., 2004). It was named *Nax2*.

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To distinguish the physiological mechanisms of *Nax1* and *Nax2*, families were developed containing only one of these genes. This article describes the separation of the two genes, their different functions, and their possible origin. We show that both *Nax* genes restrict the transport of Na^+ from roots to shoots and result in enhanced K^+-Na^+ discrimination in the leaf blade. *Nax2* has a similar mechanism to that described for *Kna1* in bread wheat. *Nax1* differs from *Nax2* in removing Na^+ from the xylem in the lower part of the leaf, as well as in the root, and represents a function not present in bread wheat. A wild wheat ancestor, *Triticum monococcum*, is the original source of both *Nax* genes.

RESULTS

Separation of *Nax1* and *Nax2*

Near-isogenic lines with and without the *Nax1* or *Nax2* gene were developed by backcrossing Line 149 with cv Tamaroi to obtain single-gene BC_5F_2 families. These were selected using the phenotype of Na^+ concentration in the leaf blade and a codominant microsatellite marker *gwm312* that is closely linked to *Nax1* (Lindsay et al., 2004). Putative single-gene *Nax1* families showed a 1:2:1 distribution for leaf blade Na^+ concentration (Fig. 1A), indicating the segregation of a single codominant gene. Progeny testing of the BC_5F_3 lines validated the single plant F_2 phenotype (data not

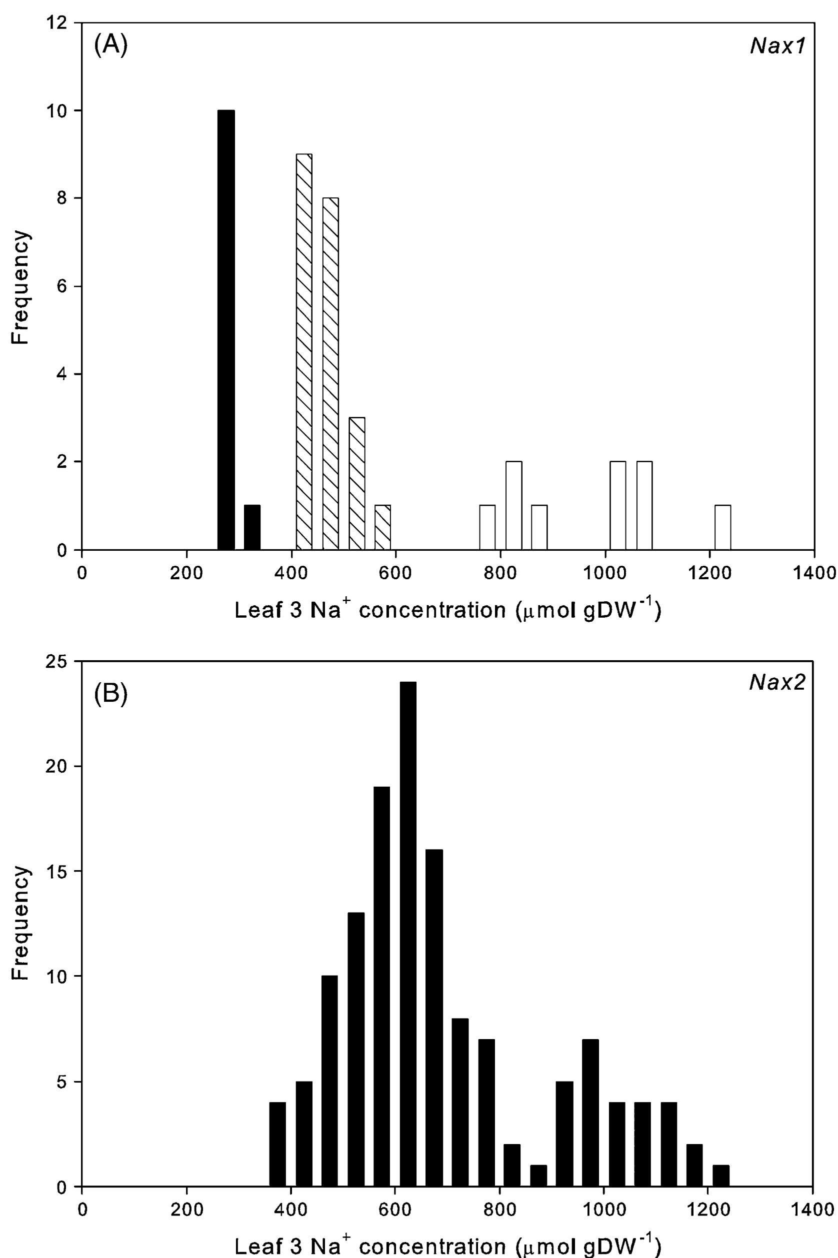

Figure 1. Frequency distributions for Na^+ concentrations in the leaf blade of single-gene BC_5F_2 families grown at 150 mM NaCl for 10 d. A, *Nax1* family (n = 41). Individuals in the low Na^+ class (black bars) were homozygous for the Line 149 allele of *gwm312*; individuals in the high Na^+ class (white bars) were homozygous for the cv Tamaroi allele of *gwm312*; and individuals in the intermediate class (hatched bars) were heterozygous. Parental means were 141 ± 14 μmol g^{-1} dry weight for Line 149 and 811 ± 31 for cv Tamaroi (n = 6). B, *Nax2* family (n = 140). Parental means were 278 ± 37 μmol g^{-1} dry weight for Line 149 and 1,193 ± 48 for cv Tamaroi.

shown). Homozygous BC_5F_3 lines with or without *Nax1* are designated [+]*Nax1* and [−]*Nax1*, respectively.

Putative single-gene *Nax2* families showed a 3:1 distribution for leaf blade Na^+ concentration (Fig. 1B), indicating the segregation of a single dominant gene. This was confirmed by progeny testing (C. Byrt, unpublished data). Homozygous *Nax2* BC_5F_3 lines with or without *Nax2* are called [+]*Nax2* and [−]*Nax2*, respectively.

Effects of *Nax1* and *Nax2* on Na^+ and K^+ Concentrations in Leaf Blade and Sheath

The presence of either *Nax1* or *Nax2* reduced the Na^+ concentration in the leaf blade and the leaf sheath (Table I). The leaf sheath in a cereal is the lower part of the leaf and is delineated from the blade by the ligule. Neither gene on its own reduced the Na^+ concentration as much as when present together in parent Line 149 (Table I). The physiological mechanism by which the two genes achieved low Na^+ concentrations in the blade differed. *Nax1* was distinguished by a higher Na^+ concentration in the leaf sheath than the leaf blade so that the sheath to blade ratio was similar to that of parent Line 149 (Table I). All lines lacking *Nax1* had the same Na^+ concentration in sheath and blade. *Nax1* was also associated with a higher root Na^+ concentration, as was Line 149 (Table I). The sheath is only a small proportion of the total leaf, making up only 23% of the total dry weight of the leaves. However, the amount of Na^+ sequestered in this tissue was considerable and, in lines containing the *Nax1* gene, 50% of the Na^+ in the leaf as a whole was retained in this tissue (Table I).

Nax2 greatly reduced Na^+ concentrations in both leaf blade and sheath (Table I). There was no preferential retention of Na^+ in the leaf sheath, so the reduced Na^+ uptake into the leaf blade was determined predominantly by the roots.

K^+ concentration in both leaf blade and sheath was enhanced by the presence of either *Nax1* or *Nax2* (Table II). In the blade, the presence of both genes in Line 149 had a greater effect on K^+ concentration than either gene alone, but in the sheath *Nax2* alone produced as high a K^+ concentration as Line 149. Thus, the *Nax2* gene resulted in a higher concentration of K^+ in the total shoot than did *Nax1* (data not shown). The K^+ to Na^+ discrimination ratio was enhanced by both genes in the leaf blade, but, in the leaf sheath, the presence of *Nax2* resulted in a much higher K^+ to Na^+ ratio than did *Nax1* (Table II).

In the absence of NaCl, there was no difference in either Na^+ or K^+ concentration in the leaf blade between Line 149 and cv Tamaroi (Rivelli et al., 2002). Cl^- concentrations were not measured because previous experiments had shown there was little difference between Line 149 and cv Tamaroi (Rivelli et al., 2002).

Nax1 and *Nax2* Control of Na^+ Transport from Root to Shoot

Time-course experiments using $^{22}Na^+$ showed that the *Nax1* and *Nax2* genes reduced the rate of unidirectional transport from root to shoot and thereby accounted for the difference in total accumulation of Na^+ over time in the shoot as a whole in the experiments described above. Reduced transport in the xylem, rather than higher retranslocation in the phloem, was therefore the likely function of the *Nax* genes.

In the *Nax1* lines, net $^{22}Na^+$ uptake by roots was higher in [+]*Nax1* than [−]*Nax1* lines after 15 min (Fig. 2A), and this difference was maintained after 48 h of $^{22}Na^+$ feeding (data not shown). This difference was consistent with the root total Na^+ concentrations shown in Table I, with roots of the [+]*Nax1* line containing more Na^+ than those of the [−]*Nax1* line. $^{22}Na^+$ appeared in the shoots after 15 min and, after 30 min, the uptake by the [+]*Nax1* and [−]*Nax1* lines differed significantly (Fig. 2B). At 4 h, the calculated Na^+ uptake rate of [+]*Nax1* was two-thirds that of [−]*Nax1* (Fig. 2B). The rapid appearance of $^{22}Na^+$ in the shoot and the time course of root uptake established previously (Davenport et al., 2005) indicated rapid labeling of the root cytoplasmic pool with the external solution, leading to a steady rate of transport to the shoot by 30 min. If maintained over 10 d, this would completely account for the differences in total

Table I. *Na^+ concentration in leaf blades, leaf sheaths, and total shoot and roots in Line 149, cv Tamaroi, and Nax1 and Nax2 near-isogenic lines grown in 50 mM NaCl for 10 d*

Values are means ± SE (n = 6). Fresh weight:dry weight ratios are 7.2 for leaf blades and 8.8 for leaf sheaths.

Plant Material	Category	Na^+ Concentration					Sheath Na^+ % of Total Shoot
		Leaf Blades	Leaf Sheaths	Sheath to Blade Ratio	Total Shoot	Roots	
		μmol g⁻¹ dry weight			*μmol g⁻¹ dry weight*		*μmol*
Parents	Line 149	104 ± 7	271 ± 12	2.6	160 ± 9	983 ± 23	50
	cv Tamaroi	579 ± 17	559 ± 15	1.0	572 ± 16	811 ± 26	28
Nax1 lines	[+]*Nax1*	183 ± 6	427 ± 8	2.3	260 ± 6	886 ± 17	51
	[−]*Nax1*	599 ± 15	581 ± 21	1.0	593 ± 16	836 ± 17	26
Nax2 lines	[+]*Nax2*	210 ± 8	209 ± 20	1.0	209 ± 11	819 ± 20	28
	[−]*Nax2*	583 ± 18	529 ± 12	0.9	565 ± 16	805 ± 13	29
	$LSD_{(0.05)}$	34	43	0.1	35	58	3

Table II. *K^+ concentration in leaf blades, leaf sheaths, and roots in Line 149, cv Tamaroi, and Nax1 and Nax2 near-isogenic lines grown in 50 mM NaCl for 10 d*

Values are means ± SE (n = 6).

Plant Material	Category	K^+ Concentration			K to Na Ratio	
		Leaf Blades	Leaf Sheaths	Roots	Leaf Blades	Leaf Sheaths
			μmol g^{-1} dry weight			
Parents	Line 149	1,390 ± 14	1,386 ± 16	730 ± 23	13.8 ± 1.0	5.2 ± 0.3
	cv Tamaroi	941 ± 15	1,099 ± 20	949 ± 48	1.6 ± 0.0	2.0 ± 0.1
Nax1 lines	[+]*Nax1*	1,180 ± 18	1,217 ± 17	842 ± 21	6.5 ± 0.2	2.9 ± 0.1
	[−]*Nax1*	901 ± 11	1,072 ± 12	828 ± 35	1.5 ± 0.0	1.9 ± 0.1
Nax2 lines	[+]*Nax2*	1,110 ± 19	1,324 ± 20	844 ± 36	5.4 ± 0.2	6.5 ± 0.6
	[−]*Nax2*	874 ± 19	1,075 ± 12	918 ± 33	1.5 ± 0.1	2.0 ± 0.1
	$LSD_{(0.05)}$	40	49	75	1.4	0.7

shoot Na^+ concentration between lines with and without *Nax1* (Table I).

In the *Nax2* lines, root $^{22}Na^+$ uptake rates were the same for the [+]*Nax2* and [−]*Nax2* lines over the whole period studied (Fig. 3A). This finding was consistent with their having the same total Na^+ concentrations in roots after 10 d in 50 mM NaCl (Table I). Shoot uptake of $^{22}Na^+$ was apparent at 15 min, by which time [+]*Nax2* plants had a lower rate of uptake than [−]*Nax2* plants (Fig. 3B). The difference was statistically significant at 30 min. At 4 h, the shoot Na^+ uptake rate for the [+]*Nax2* line was one-half that for the [−]*Nax2* line (Fig. 3B) and was sufficient to account for differences in shoot Na^+ concentrations (Table I). Because the rates of root Na^+ uptake were identical in the lines with and without *Nax2*, differences in shoot uptake are due to the net rate of xylem loading in the root.

Nax1 and *Nax2* Increase Withdrawal of Na^+ from the Root Xylem

Control of shoot Na^+ uptake could be due to either tight control of xylem loading or high rate of withdrawal of Na^+ from the transpiration stream into the upper part of the roots. Evidence for xylem withdrawal of Na^+ in the roots of both [+]*Nax1* and [+]*Nax2* lines was obtained in a separate compartmental loading experiment. When the lower part of the root was exposed to $^{22}Na^+$, the [+]*Nax1* line withdrew more of the total transported $^{22}Na^+$ into the upper roots (88%) than the [−]*Nax1* line (51%; Fig. 4). Similarly, the [+]*Nax2* line withdrew more $^{22}Na^+$ into the upper roots (91%) than the [−]*Nax2* line (44%). These differences were associated with a 4-fold higher shoot $^{22}Na^+$ content in both the [−]*Nax1* and [−]*Nax2* lines than their respective isogenic pairs (data not shown).

Net Na^+ and K^+ Transport Rates from Root to Shoot and Xylem Concentrations

The net Na^+ and K^+ transport rates from root to shoot were quantified from the increase in Na^+ and K^+ in roots and shoots between 6 and 10 d after exposure to 50 mM NaCl. Very low net Na^+ transport rates were found in Line 149 and the [+]*Nax1* line, with slightly higher rates in the [+]*Nax2* line. Lines lacking *Nax1* and *Nax2* had high net Na^+ transport rates similar to the recurrent parent cv Tamaroi (Table III). There was less variation in K^+ transport rates between genotypes;

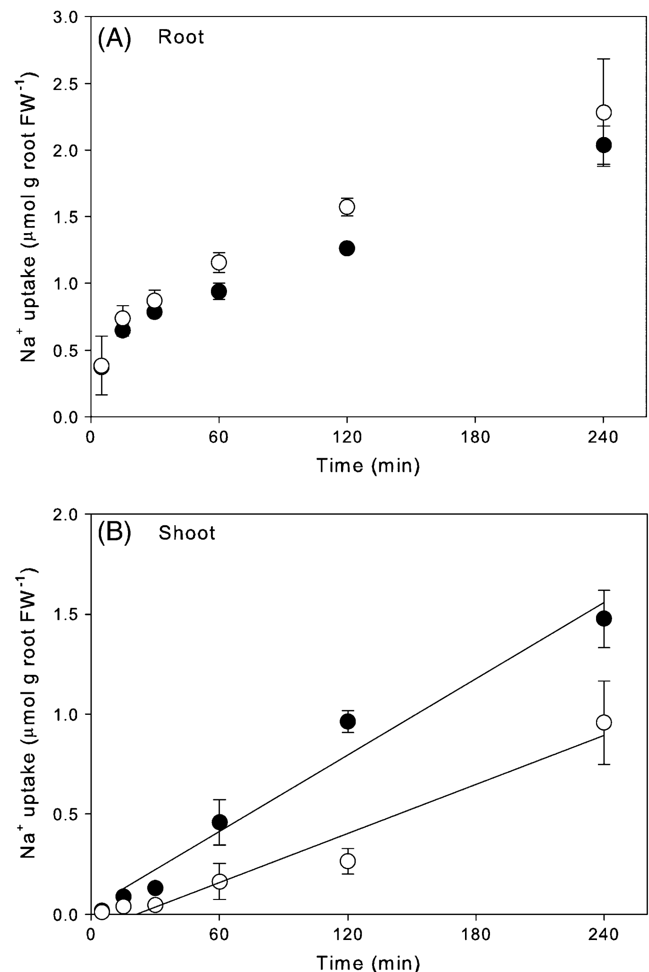

Figure 2. Rate of $^{22}Na^+$ accumulation in *Nax1* near-isogenic lines grown in 25 mM NaCl in root (A) and shoot (B); [+]*Nax1* (○), [−]*Nax1* (●). Values are means ± SE (n = 4). Fitted linear regressions for $^{22}Na^+$ uptake by the shoot are [+]*Nax1*: $y = 4.08 \times 10^{-3}x - 0.09$ (r^2 = 0.96); [−]*Nax1*: $y = 6.36 \times 10^{-3}x + 0.03$ (r^2 = 0.96).

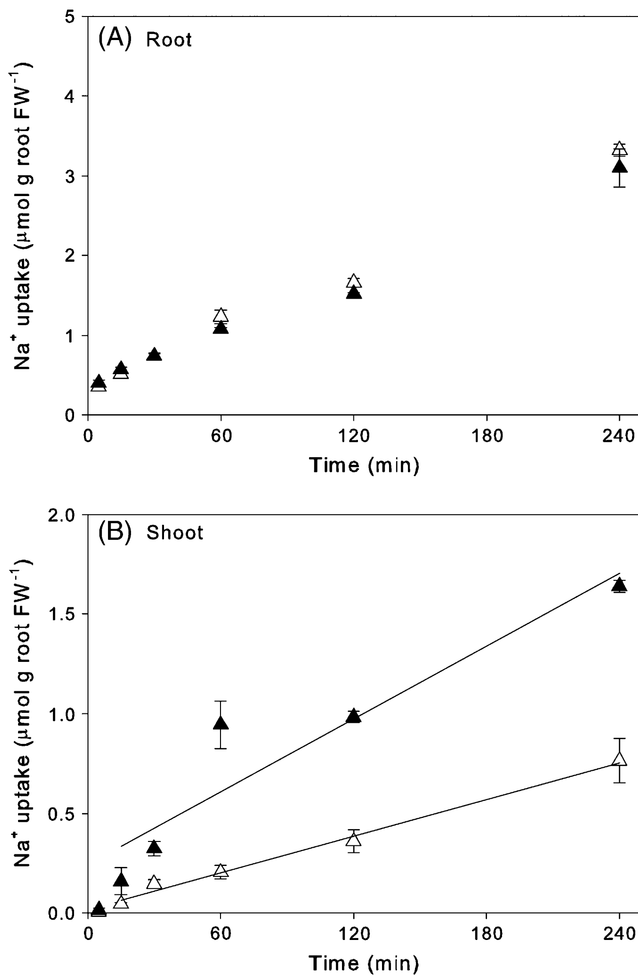

Figure 3. Rate of $^{22}Na^+$ accumulation in *Nax2* near-isogenic lines grown in 25 mM NaCl in root (A) and shoot (B); [+]*Nax2* (△); [−]*Nax2* (▲). Values are means ± SE ($n = 4$). Linear regressions for $^{22}Na^+$ uptake by the shoot are [+]*Nax2*: $y = 3.07 \times 10^{-3}x + 0.02$ ($r^2 = 0.99$); [−]*Nax2*: $y = 6.07 \times 10^{-3}x + 0.25$ ($r^2 = 0.88$).

however, the highest K^+ transport rates were found in Line 149 and the [+]*Nax2* line, suggesting that the *Nax2* mechanism may involve the exchange of K^+ and Na^+ in net xylem loading in the root, possibly a replacement of K^+ for Na^+ in removal of Na^+ from the xylem.

Na^+ concentrations in the xylem were calculated from the net Na^+ transport rate and the transpiration rate, assuming that there was little retranslocation of Na^+ in the phloem. Transpiration measured over 24 h varied from 0.55 to 0.60 mL h^{-1} $plant^{-1}$ with no significant difference between lines. Na^+ concentrations in the xylem were calculated to be about 1 mM for lines containing either *Nax1* and *Nax2*, and 2.5 mM or more if both genes were lacking (Table III). The extent of exclusion of Na^+ from the soil solution (50 mM) in lines containing either *Nax1* or *Nax2* was therefore 98%. However, lines without these genes still excluded 94% of the NaCl in the solution (Table III). Thus, relatively small percentage differences in Na^+ exclusion capability led to profound differences in the accumulation of Na^+ into the shoot.

Ignoring the extent of retranslocation of K^+ in the phloem, K^+ concentrations in the xylem were calculated to be 6.5 mM if *Nax2* was present, but 5 mM or less if it was absent (Table III). The real concentrations are probably twice this because the phloem can retranslocate 50% of the K^+ carried in the xylem to the shoots (Wolf et al., 1990). Presuming that the recirculation of K^+ is similar for all lines, the data indicate that the *Nax2* gene is associated with enhanced K^+ uptake.

Gradients in Na^+ and K^+ along the Leaf in the [+]*Nax1* Line

To examine in more detail the unusual phenotype of *Nax1*, namely, the high Na^+ sheath to blade ratio, the distribution of Na^+ along the entire length of a given leaf was measured. This would show whether the ability of cells lining the xylem to withdraw Na^+ and sequester it was confined to the sheath (i.e. whether the property of xylem parenchyma cells was different in sheath and blade) or whether the ligule could act as a barrier to Na^+ movement in the xylem from sheath to blade.

Leaves of the [+]*Nax1* line, as well as parent Line 149, showed a gradient of Na^+ concentrations, highest in the leaf base and lowest in the leaf tip, from the time the salt was added. There was no discontinuity at the junction between the sheath and the blade, indicating that there was no barrier to Na^+ movement in the xylem at the ligule. Na^+ concentrations in sheath and leaf blade segments of leaf 2 in the [+]*Nax1* line after 2 and 5 d in 50 mM NaCl are shown in Figure 5. The increase in Na^+ concentration with time in both sheath and blade indicated that the xylem parenchyma cells in the leaf sheath did not differ from those in the blade in the ability to withdraw Na^+ from the xylem. After 5 d

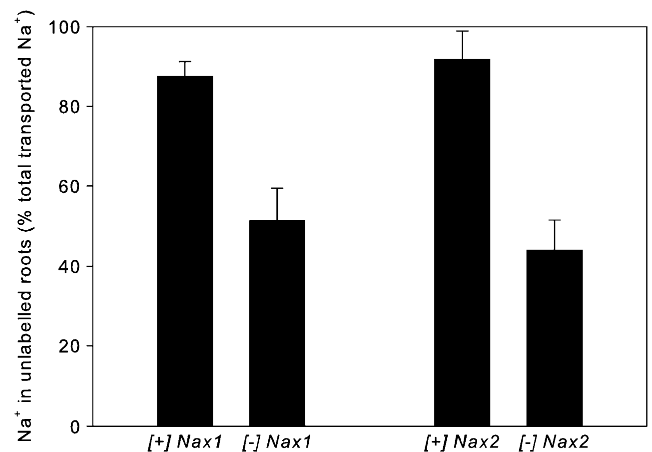

Figure 4. Withdrawal of $^{22}Na^+$ from the xylem by roots of *Nax1* and *Nax2* near-isogenic lines grown in 25 mM NaCl. Withdrawal was calculated as the amount of $^{22}Na^+$ in the unlabeled upper roots as a percentage of total $^{22}Na^+$ transported from the labeled lower roots after 2 h. Bars are means ± SE ($n = 5$).

Table III. *Na^+ and K^+ net transport rates to the shoot, Na^+ and K^+ concentration in the xylem stream, and percentage exclusion of Na^+ by the roots in Line 149, cv Tamaroi, and Nax1 and Nax2 near-isogenic lines grown in 50 mM NaCl*

Values are calculated over a 6- to 10-d period from two experiments and are means ± SE (n = 12).

Plant Material	Category	Transport Rate from Root to Shoot		Ion Concentration in Xylem		Exclusion by Roots
		Na^+	K^+	Na^+	K^+	Na^+
		μmol g_{root} fresh weight^{-1} d^{-1}		mM		%
Parents	Line 149	6.0 ± 0.6	45 ± 3	0.9 ± 0.1	6.7 ± 0.3	98.3 ± 0.2
	cv Tamaroi	22.7 ± 0.8	41 ± 3	2.8 ± 0.1	5.0 ± 0.4	94.4 ± 0.3
Nax1 lines	[+]*Nax1*	6.5 ± 0.3	35 ± 3	1.0 ± 0.1	5.2 ± 0.3	98.0 ± 0.1
	[−]*Nax1*	22.5 ± 1.5	37 ± 3	2.8 ± 0.3	4.7 ± 0.5	94.4 ± 0.6
Nax2 lines	[+]*Nax2*	8.7 ± 0.9	42 ± 2	1.3 ± 0.1	6.3 ± 0.4	97.4 ± 0.2
	[−]*Nax2*	17.9 ± 1.3	33 ± 3	2.5 ± 0.1	4.5 ± 0.3	95.0 ± 0.2
	$LSD_{(0.05)}$	2.9	7	0.5	1.1	0.9

in 50 mM NaCl, Na^+ concentrations in lower sheath segments reached a maximum of about 250 mM on a tissue-water content basis (Fig. 5) or 1,400 μmol g^{-1} dry weight, possibly indicating a threshold in the storage capacity of the cells. No gradient was found along the leaf in [−]*Nax1* lines, which displayed an even profile of Na^+ concentration across both the sheath and blade, with slightly higher concentrations in the upper (older) portions of both sheath and blade, probably reflecting the age of the tissue and a slightly longer exposure to salinity with the resultant increased deposition of Na^+ (data not shown).

Measurements of K^+ in leaves at 2 and 5 d indicated that K^+ was displaced by Na^+ over time. K^+ was initially at high concentrations (400 mM) in all parts of the sheath and blade, but decreased over time as Na^+ increased (Fig. 5). The decrease of K^+ in the leaf sheath over time, while Na^+ increased, indicated that K^+ was either entering the xylem in exchange for Na^+ and moving toward the leaf tip or entering the phloem and moving out of the leaf.

Retranslocation of Na^+ from Shoots to Roots

The rate of retranslocation of Na^+ from the shoot to the root was estimated using a split-root system, where one-half of the roots were fed $^{22}Na^+$ for 48 h and the appearance of radioactivity in the unlabeled roots and solution was used to estimate retranslocation. Retranslocation of Na^+ was a relatively small component of shoot Na^+ uptake (2%–6%; Fig. 6). These values are likely to be an underestimate of the total retranslocation because shoot $^{22}Na^+$ had not yet come to an equilibrium with the feeding solution. Comparisons between lines showed that the [+]*Nax1* line had twice the rate of retranslocation than [−]*Nax1* and both [+]*Nax2* and [−]*Nax2* lines (Fig. 6). This difference may not be an intrinsic property of the *Nax1* gene but a result of the higher Na^+ in the sheath tissue of these lines (Table I; Fig. 5).

Origin of *Nax1* and *Nax2*

Durum Line 149 was derived from a cross between durum cv Marrocos and an accession of the wheat progenitor *T. monococcum*, C68-101 (The, 1973). C68-101 also has the Line 149 allele of *gwm312*, indicating that it was the source of the *Nax1* gene.

Na^+ transport rates from root to shoot in C68-101 were similar to Line 149, but cv Marrocos had a much higher transport rate (Table IV). C68-101 had the same low Na^+ concentrations in leaves as Line 149, the same high K^+-Na^+ discrimination, as well as the characteristic of *Nax1*, which is the high sheath to blade ratio (Table IV). Cultivar Marrocos had a sheath to blade ratio of 1:1. These data indicate that C68-101 is the source of both *Nax1* and *Nax2* genes.

DISCUSSION

The two genes for Na^+ exclusion, *Nax1* and *Nax2*, reduce Na^+ transport from root to shoot, as evidenced by the time course of $^{22}Na^+$ transport. Net Na^+ transport in the xylem accounted for the differences in shoot concentrations, not retranslocation in the phloem. However, there were differences in the mechanisms of action of the two genes. First, *Nax1* had a higher rate of deposition of Na^+ in the leaf sheath than *Nax2* and, consequently, a higher ratio of Na^+ concentration in the sheath to blade ratio. Second, *Nax1* had a lower rate of K^+ transport from root to shoot than *Nax2*, but the displacement of K^+ in the sheath led to an equal deposition in the leaf blade. Thus, both *Nax1* and *Nax2* lead to Na^+ exclusion from the leaf blade and a high K to Na^+ ratio, but by different mechanisms.

Nax1 and *Nax2* Withdraw Na^+ from the Xylem in the Roots

That Na^+ can be withdrawn from the xylem in the roots was shown by feeding the lower parts of roots with $^{22}Na^+$ and the appearance of a large proportion of the labeled Na^+ in the upper roots. The proportion was 2-fold greater in lines containing *Nax1* or *Nax2* than their respective isogenic pairs. The *Nax2* mechanism was confined to the roots and had the effect of reducing the transport of Na^+ from root to shoot while increasing the transport of K^+, and so resulted in a net exchange of Na^+ for K^+. Removal of Na^+ from the

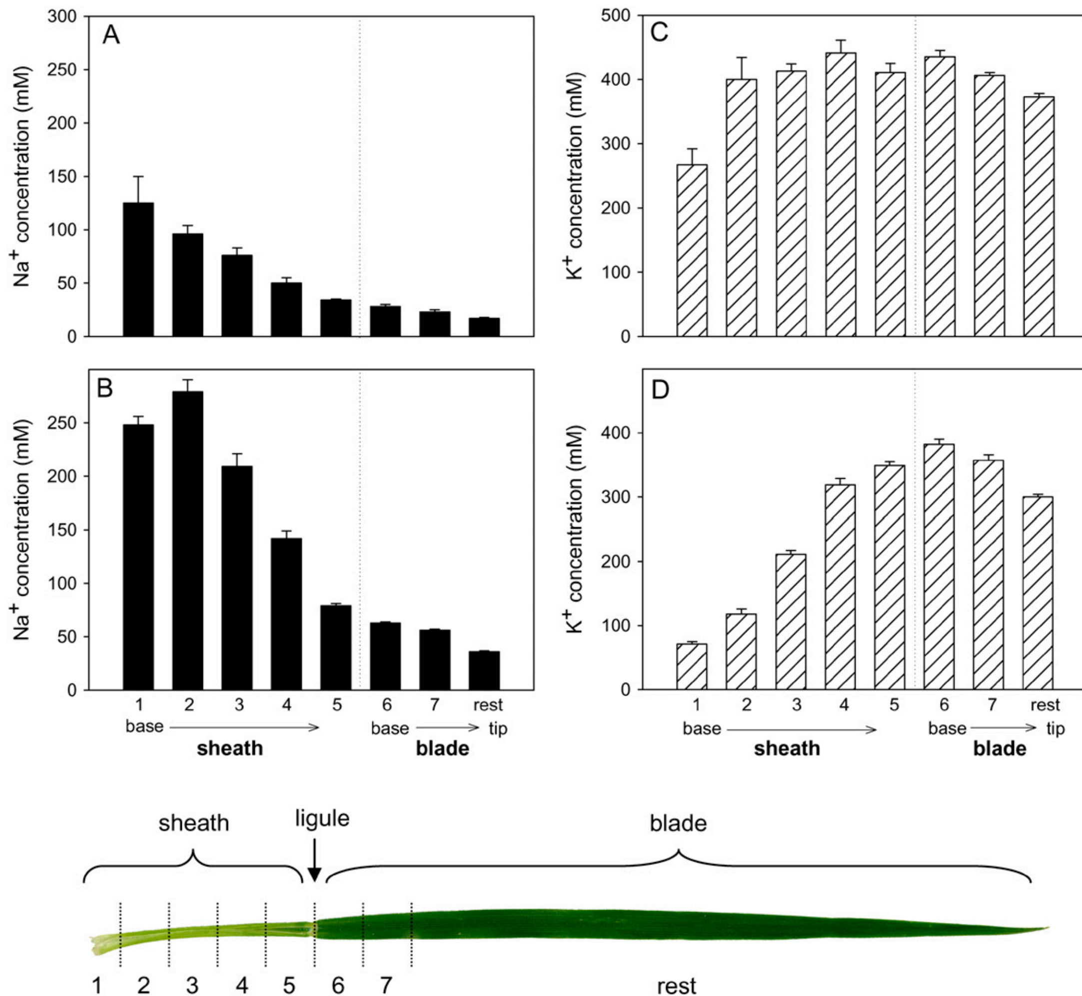

Figure 5. Na^+ (A and B) and K^+ (C and D) concentrations in sheath and leaf segments of leaf 2 from [+]*Nax1* seedlings grown in 50 mM NaCl for 2 d (A and C) and 5 d (B and D). Na^+ and K^+ concentrations were calculated on a tissue-water basis. The dotted line indicates the ligule. Bars are bulked (× 3) means ± SE ($n = 4$). The bottom diagram is an image of leaf 2 (total length 180 mm) with lines indicating where the sheath and blade were sectioned for the ion analysis shown above.

xylem by the upper part of the root could induce a consequent influx of K^+ into the xylem to restore the electrical potential. Alternatively, or additionally, higher K^+ translocation in [+]*Nax2* plants could indicate greater K^+-Na^+ selectivity in loading of xylem in the roots. Our data do not allow us to discriminate between these possibilities. The phenotype of *Nax2* is very similar to that of *Kna1* in bread wheat, which is considered to discriminate between Na^+ and K^+ at the point of xylem loading (Gorham et al., 1990).

Reabsorption of Na^+ from the xylem in the upper part of the root system has been described for maize (*Zea mays*; Shone et al., 1969; Johanson and Cheeseman, 1983), soybean (*Glycine max*; Lacan and Durand, 1996), common bean (*Phaseolus vulgaris*; Jacoby, 1964), and scarlet runner bean (*Phaseolus coccineus*; Kramer et al., 1977). The studies with soybean indicated an exchange of K^+ for Na^+, energized by H^+-ATPases, and the authors suggested that Na^+-H^+ and K^+-H^+ antiporters at the plasma membrane of the xylem parenchyma might be involved (Lacan and Durand, 1996).

Na^+ reabsorption from the xylem in the upper part of scarlet runner bean roots was associated with cells having the appearance of transfer cells. These are xylem parenchyma cells with a wall labyrinth that increases the surface area of the plasma membrane, suggesting a function in transport processes (Kramer et al., 1977). Transfer cells have been described in the roots of other species (Kramer, 1983) but have not been found in wheat (A. Läuchli, personal communication).

Nax1 Also Withdraws Na^+ from the Xylem in the Leaf

The function of *Nax1* in removing Na^+ was not restricted to the root because the transport of Na^+ to the shoot as a whole was lower in the [+]*Nax1* than the [−]*Nax1* family.

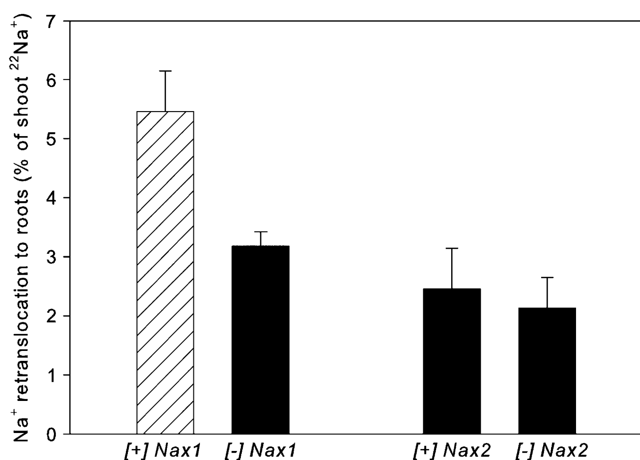

Figure 6. Estimate of retranslocation of $^{22}Na^+$ from shoot to roots in *Nax1* and *Nax2* near-isogenic lines after 48 h of labeling with $^{22}Na^+$ in seedlings grown in a split-root system in 25 mM NaCl. Bars are means ± SE ($n = 4$).

The mechanism conferred by *Nax1*, which was characterized by the deposition of Na^+ in the leaf sheath, is not confined to wheat germplasm containing the *Nax1* gene. Preferential deposition of Na^+ in the leaf base has been described for rice (*Oryza sativa*), common reed (*Phragmites communis*; Matsushita and Matoh, 1991), and sorghum (Lacerda et al., 2003). An equivalent of the *Nax1* gene may be present in these other species. However, it may not be widespread. For instance, barley (*Hordeum vulgare*) does not have preferential retention of Na^+ in the leaf sheath (Munns et al., 1988).

The gradients in Na^+ and K^+ concentration along the leaf and their change over time (Fig. 5) indicate that the cells lining the xylem were removing Na^+ from the xylem stream, storing it in parenchyma cells in the sheath, and causing a displacement of the K^+ there. It is possible to explain the gradient of Na^+ along the leaf with a model incorporating the passive movement of Na^+ from the xylem and possibly a subsequent active scavenging of Na^+ as the concentration falls. Na^+ can move passively from the xylem into the xylem parenchyma cells, against a concentration gradient, due to the negative electrical potential of the cells, which might be −100 to −200 mV. Na^+ in the xylem could initially move passively via a Na^+-permeable channel or a Na^+ uniporter into the xylem parenchyma in the basal sheath tissue, leading to high rates of retrieval in these cells compared to the cells in the upper sheath and leaf blade, which would experience progressively lower Na^+ concentrations in the xylem stream and subsequently lower rates of Na^+ retrieval. Active uptake might be necessary to scavenge Na^+ at very low concentrations, depending on the cytosolic Na^+ concentration of cells near the leaf tip. Alternatively, passive uptake could act to maintain apoplastic leaf Na^+ at low levels.

Candidate Genes

The major candidates for transporters that could withdraw Na^+ from the xylem are nonselective cation channels and high-affinity K^+ transporters (HKTs) that function as Na^+ uniporters (Tester and Davenport, 2003). A nonselective cation channel can be ruled out for *Nax1* because it appears to be Na^+-selective in withdrawing cations from the xylem in the sheath. The concentration of Na^+ in the sheath increased over time, whereas that of K^+ diminished (Fig. 5). This selectivity for Na^+ over K^+ was also shown by Davenport et al. (2005), where unidirectional uptake of $^{22}Na^+$, but not $^{86}Rb^+$, into leaf sheaths was elevated in Line 149 compared to cv Tamaroi. A nonselective cation channel cannot be ruled out for *Nax2*.

HKT transporters characterized so far are Na^+-selective or function in Na^+-coupled K^+ symport (although the latter may be an artifact of heterologous expression in at least some cases; Haro et al., 2005). The rice OsHKT8 transporter is Na^+-selective and is proposed to withdraw Na^+ from the xylem (Ren et al., 2005). The Arabidopsis (*Arabidopsis thaliana*) Na^+-selective ortholog AtHKT1 appears to withdraw Na^+ from the xylem along the length of the transpiration stream (Sunarpi et al., 2005). It is possible that *Nax1* is a HKT transporter involved in Na^+ withdrawal from the xylem and expressed in root and leaf vasculature. Na^+-selective HKTs have been implicated in Na^+ withdrawal from the xylem with concomitant enhancement of K^+ uptake to the shoot, but it is not clear whether the HKTs affect K^+ transport directly, or indirectly, via

Table IV. *Na^+ and K^+ concentrations and transport rates in Line 149 and its parents, the wheat progenitor T. monococcum C68-101, and durum cv Marrocos grown in 50 mM NaCl*

Transport rates are calculated over 6 to 10 d and are means ± SE ($n = 6$).

Genotype	Leaf Blade Na^+	Leaf Blade K^+	K^+ to Na^+ Ratio in Blades	Leaf Sheath Na^+	Leaf Sheath K^+	K^+ to Na^+ Ratio in Sheaths	Na^+ Ratio Sheath:Blade	Na^+ Shoot Transport Rate
	μmol g⁻¹ dry weight			*μmol g⁻¹ dry weight*				*μmol g_{root} fresh weight^{-1} d^{-1}*
C68-101	116 ± 37	1,126 ± 29	9.7	342 ± 63	1,132 ± 38	3.3	2.9	8.6 ± 2.3
cv Marrocos	678 ± 34	1,158 ± 20	1.7	619 ± 27	1,235 ± 15	2.0	0.9	21.9 ± 0.5
Line 149	104 ± 7	1,426 ± 9	13.7	404 ± 18	1,294 ± 8	3.2	3.9	6.2 ± 0.4
$LSD_{(0.05)}$	75	62	6.2	101	61	1.2	0.6	4.4

an influence on cation homeostasis (Rus et al., 2004; Ren et al., 2005).

Other Mechanisms Control Root Na^+ Concentrations

Although lines containing either *Nax1* or *Nax2* excluded 98% of the Na^+ from entering the shoot, in the absence of both genes, 94% was excluded. This means that other genes control the net uptake of Na^+ from the soil solution and possibly the net loading of the xylem. Control of Na^+ concentrations in wheat roots is quite remarkable. In experiments when the external NaCl concentration ranged up to 150 mM, the maximal Na^+ concentration in roots was no more than 50 mM, even in durum wheat lines lacking *Nax1* and *Nax2* (Husain et al., 2004). There was little genotypic difference in root concentration but a large difference in shoot concentration. This was also observed by Gorham et al. (1990) for a wider range of wheat species.

The physiological mechanism for this control of root Na^+ concentrations is not just restriction of unidirectional uptake, which is quite high in relation to the net rates of Na^+ uptake (Davenport et al., 1997), but to Na^+ efflux, as shown by a significant amount of $^{22}Na^+$ efflux found in roots of both Line 149 and cv Tamaroi (Davenport et al., 2005). Lines without *Nax1* and *Nax2* also withdrew one-half of Na^+ from xylem (Fig. 4), which presumably was then effluxed.

Retranslocation of Na^+ from Shoot to Root Is Small

The experiment involving labeling of a split-root system with $^{22}Na^+$ indicates that shoot export of Na^+ was only a small proportion of the import, likely to be no more than 10%. This is similar to the value of 10% for barley grown in 100 mM NaCl, obtained from direct measurements of phloem sap collected by aphid stylets (Wolf et al., 1990).

Nax2 clearly does not control loading of Na^+ into the phloem because the rate of retranslocation was the same for [+]*Nax1* and [−]*Nax2* lines. However, the [+]*Nax1* line had twice the rate of retranslocation as the [−]*Nax1* line (Fig. 6). This result was surprising because we expected higher rates of retranslocation in the phloem to be associated with higher shoot Na^+ concentrations and, consequently, to be greater in the [−]*Nax1* and [−]*Nax2* lines, which had higher Na^+ concentrations in both blade and sheath than their isogenic pairs (Table I). It is possible that higher rates of retranslocation of Na^+ to the roots in the [+]*Nax1* line are a function of high tissue concentrations at the base of the leaf sheath (Fig. 5) and possibly specific localization of Na^+ in the cells in the vascular bundles that might be involved in loading of the phloem. Recirculation of $^{22}Na^+$ that was deposited in the shoot base was shown in common bean (Jacoby, 1979) and common reed (Matsushita and Matoh, 1991). We conclude that enhanced recirculation in the [+]*Nax1* family is an indirect effect of the *Nax1* gene, not the primary effect.

Relationship of *Nax* Genes to Other Na^+-Excluding Genes in Wheat

It is apparent that both *Nax1* and *Nax2* genes come from *T. monococcum* C68-101, a diploid A genome species, and not from the durum parent of Line 149, cv Marrocos.

The function of the *Nax* genes is generally similar to the *Kna1* gene in bread wheat, which is on chromosome 4D (Dubcovsky et al., 1996). However, *Nax1* varies from *Kna1* in phenotype as well as in homoeologous chromosomal location. *Nax1* is located on chromosome 2A (Lindsay et al., 2004) and carries the phenotype of retention of Na^+ in the leaf sheath and a high sheath to blade Na^+ concentration ratio. In contrast, the phenotype of *Nax2* is the same as *Kna1*, as described by Gorham et al. (1990). Like *Kna1*, *Nax2* results in low Na^+ and high K^+ concentrations in the leaf blades of plants growing in 50 mM NaCl and does not cause preferential deposition of Na^+ in the leaf sheath. We have examined several bread wheat cultivars and found the same concentration in sheath and blade (R. Munns and R. James, unpublished data). It is possible that *Nax2* and *Kna1* may be homoeologous genes.

In summary, both *Nax* genes restrict the transport of Na^+ from roots to shoots with a high selectivity for K^+ over Na^+. Both result in enhanced K^+-Na^+ discrimination in the leaf blade, although by different mechanisms. The *Nax1* gene promotes withdrawal of Na^+ from the xylem in the base of the leaf as well as the root. This gene could serve a unique function in reducing the movement of Na^+ into the leaf blade at high salinity or in conditions when root function is impaired, such as in waterlogged soil. The *Nax2* gene is likely to perform a similar function to *Kna1*.

MATERIALS AND METHODS

Plant Materials

Parental material used in crossing and in Na^+ uptake and flux experiments were durum wheat (*Triticum turgidum*) Line 149 and cv Tamaroi, and the parents of Line 149, *Triticum monococcum* C68-101 and durum cv Marrocos. Seeds were provided by Dr. Ray Hare of the Tamworth Agricultural Institute, New South Wales Department of Primary Industries.

Development of Near-Isogenic *Nax1* and *Nax2* Lines

An F_2 family derived from a cross between Line 149 and cv Tamaroi previously identified a microsatellite marker (*gwm312*) closely linked to the *Nax1* gene (Lindsay et al., 2004). From this F_2 family, individuals with leaf Na^+ concentrations as low as parent Line 149 were selected and backcrossed to cv Tamaroi four times. Each backcross was selfed, and individual F_2 plants with the lowest leaf Na^+ concentrations were used for the next backcross. The BC_4F_2 family of 100 individuals was used to isolate the *Nax1* and *Nax2* genes into separate BC_5F_2 single-gene families. Selections were based on allelic variation of the *gwm312* marker in combination with a Na^+ phenotype screen. The presence of *Nax2* was evident in lines that carried the cv Tamaroi allele for *gwm312* but were intermediate for Na^+. Whereas plants that were homozygous for the Line 149 *gwm312* allele usually had a low Na^+ phenotype, some plants were intermediate for Na^+, indicating the possible absence of *Nax2*.

To develop Lines containing only *Nax1*, BC_4F_2 individuals were selected that were homozygous for the Line 149 allele of *gwm312* but had an intermediate Na^+ concentration. To develop lines containing only *Nax2*, BC_4F_2

individuals were selected that were homozygous for the cv Tamaroi allele of *gwm312* but had an intermediate Na^+ concentration. These selections were backcrossed to the recurrent parent cv Tamaroi and selfed in the BC_5F_1. The resulting BC_5F_2 families were scored for leaf Na^+ concentration. Plants were grown in supported hydroponics as described previously (Munns and James, 2003). At approximately 6 d after emergence, 25 mM NaCl was added twice a day to a final concentration of 150 mM, and $CaCl_2$ was added to give a final concentration of 10 mM. Plants were grown in a controlled environment chamber with a 10-h photoperiod and photosynthetic photon flux density of 800 μmol m^{-2} s^{-1} at 25°C during the day and 18°C during the night. After 10 d, the blade of leaf 3 was harvested and Na^+ concentration was measured (Lindsay et al., 2004). Progeny testing of the resulting BC_5F_3 lines using leaf Na^+ concentration score confirmed zygosity.

Homozygous BC_5F_3 low and high Na^+ near-isogenic *Nax1* and *Nax2* lines used in Na^+ uptake studies were given the annotations [+]*Nax1*, [−]*Nax1*, [+]*Nax2*, and [−]*Nax2*, respectively.

Na^+ and K^+ Transport Rates and Gradients in Na^+ Concentrations along the Leaf

Plants were grown as described above, except that the final NaCl concentration was 50 mM NaCl, and $CaCl_2$ was added to give a final concentration of 4 mM. To measure transport rates, plants were harvested after 6 and 10 d in 50 mM NaCl, six replicates per harvest. Previous studies had shown that Na^+ net uptake rates reached steady state in parental lines cv Tamaroi and Line 149 at 5 d in 50 mM NaCl (Davenport et al., 2005). Shoots were separated into leaf blades and leaf sheaths. Roots were washed in a cold solution of 10 mM $Ca(NO_3)_2$ for 10 to 15 s, blotted, and weighed. All plant material was then dried at 70°C for 3 d, weighed, and extracted in 500 mM HNO_3 at 80°C for 1.5 h and analyzed for Na^+ and K^+ by an inductively coupled plasma-atomic emission spectrometer (Varian Vista Pro). Net Na^+ and K^+ transport rates (roots to shoots) were calculated on a root fresh-weight basis according to Pitman (1988) and Storey (1995). The rate of net ion uptake, J (mol g_{root} fresh weight^{-1} d^{-1}) was calculated as:

$$J = \frac{M_2 - M_1}{t_2 - t_1} \times \frac{\ln WR_2 - \ln WR_1}{WR_2 - WR_1} \quad (1)$$

and ion transport rates from roots to shoots, Js (mol g_{root} fresh weight^{-1} d^{-1}), as:

$$J_S = \frac{M_{S2} - M_{S1}}{t_2 - t_1} \times \frac{\ln WR_2 - \ln WR_1}{WR_2 - WR_1} \quad (2)$$

where M_2 and M_1 are the ion contents of the whole plant (mol), M_{s2} and M_{s1} are the shoot ion contents (mol) at times t_1 and t_2 (d), and WR_1 and WR_2 are the corresponding root fresh weights (g). Rates and standard errors were calculated on paired plants after ranking the six plants at each harvest in order of increasing root dry weight.

Transpiration rates, E (g_{water} g_{root} fresh weight^{-1} d^{-1}), were estimated from the measured leaf area and whole-plant water loss of a corresponding set of seedlings grown in pots containing coarse sand, which were watered and flushed daily with 50 mM NaCl in one-half-strength modified Hoagland solution. The xylem concentration (mol L^{-1}) was estimated from the uptake rate and the transpiration rate as:

$$\text{Ion concentration in xylem} = J_s/E \quad (3)$$

This estimation presumes there is no retranslocation from shoots to roots.

For the analysis of ion gradients along leaves, plants were harvested at 2 and 5 d in 50 mM NaCl. Leaf sheaths were divided into five equal segments (between 1–3 mg dry weight) from the basal tissue (connecting to the root-shoot junction) to the upper tissue (connecting to the ligule). Leaf blades were divided into three segments: two basal segments, similar in size to sheath segments, and the remainder of the leaf blade.

$^{22}Na^+$ Uptake

Na^+ uptake to the root and shoot was measured using $^{22}Na^+$ as described previously (Davenport et al., 2005). Seeds were germinated and then transferred to Eppendorf tubes with the base removed and suspended over hydroponic solution. Seedlings were exposed to one-half-strength modified Hoagland solution (P concentration reduced from 1 mM to 100 μM) for 5 d and then transferred to one-half-strength modified Hoagland plus 25 mM NaCl and 2 mM $CaCl_2$ for 5 d before experiments.

$^{22}Na^+$ Retranslocation

$^{22}Na^+$ retranslocation into roots was measured with a split-root system. Three-day-old seedlings were transferred to a pretreatment solution of 25 mM NaCl and 2 mM $CaCl_2$ in one-half-strength modified Hoagland solution. After 7 d, the roots were divided evenly and placed in two beakers that were covered with foil and connected by tape, each containing 120 mL of pretreatment solution. The shoot was supported between the two beakers in an upright position and placed on a rotating shaker under a light bank with a photosynthetic photon flux density of 150 μmol m^{-2} s^{-1} and a 16-h photoperiod for 20 h before solutions were refreshed and $^{22}Na^+$ added to one beaker. Labeled and unlabeled roots and shoots were harvested after 48 h and $^{22}Na^+$ was measured in the unlabeled root and surrounding solution. Na^+ retranslocation was calculated as a percentage of total shoot $^{22}Na^+$, taking into account the size of the labeled and unlabeled roots.

Withdrawal of $^{22}Na^+$ from Xylem by Upper Parts of Roots

Seedlings were grown as described for $^{22}Na^+$ uptake and transferred to a flat Perspex chamber (15 × 2.5 × 2.5 cm) with two unequal-sized compartments separated by a movable Perspex barrier pierced with a hole for the root. The seedling was secured in the larger compartment so that the shoot was upright and the lower portion (4–5 cm) of a single root was sealed into the smaller compartment with silicon grease. The small compartment was filled with 5 mL of 25 mM NaCl in one-half-strength Hoagland solution and the rest of the root system was covered in filter paper wetted with the same solution (identical to the saline growth solution). Both compartments were sealed to maintain high humidity. Plants were placed under a light bank on a slowly rotating shaker for 1 h before the solution was replaced with $^{22}Na^+$-labeled solution of the same composition. After 2 h, the plant was harvested and divided into three parts: the labeled root, the upper part of the root and contacting paper, and the shoot. Withdrawal of Na^+ from xylem in the upper part of the root was calculated as the amount of $^{22}Na^+$ there as a percentage of the total amount of $^{22}Na^+$ transported from the labeled root (i.e. $^{22}Na^+$ in the upper root and shoot).

Statistical Analysis

Data summarized in Tables I to IV were analyzed using ANOVA and LSDS ($P = 0.05$) were used to compare genotype means.

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