Physiological evidence for a sodium-dependent high-affinity phosphate and nitrate transport at the plasma membrane of leaf and root cells of *Zostera marina* L.

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Abstract

*Zostera marina* L. is an angiosperm that grows in a medium in which inorganic phosphate (Pi) and nitrate (NO$_3^-$) are present in micromolar concentrations and must be absorbed against a steep electrochemical potential gradient. The operation of a Na$^+$-dependent NO$_3^-$ transport was previously demonstrated in leaf cells of this plant, suggesting that other Na$^+$-coupled systems could mediate the uptake of anions. To address this question, Pi transport was studied in leaves and roots of *Z. marina*, as well as NO$_3^-$ uptake in roots. Electrophysiological studies demonstrated that micromolar concentrations of Pi induced depolarizations of the plasma membrane of root cells. However, this effect was not observed in leaf cells. Pi-induced depolarizations showed Michaelis–Menten kinetics ($K_m$=1.5$\pm$0.6 mM Pi; $D_{max}$=7.8$\pm$0.8 mV), and were not observed in the absence of Na$^+$. However, depolarizations were restored when Na$^+$ was resupplied. NO$_3^-$ additions also evoked depolarizations of the plasma membrane of root cells only in the presence of Na$^+$. Both NO$_3^-$ and Pi-induced depolarizations were accompanied by an increase in cytoplasmic Na$^+$ activity, detected by Na$^+$-sensitive microelectrodes. Pi net uptake (measured in depletion experiments) was stimulated by Na$^+$. These results strongly suggest that Pi uptake in roots of *Z. marina* is mediated by a high-affinity Na$^+$-dependent transport system. Both NO$_3^-$ and Pi transport systems exploit the steep inwardly directed electrochemical potential gradient for Na$^+$, considering the low cytoplasmic Na$^+$ activity (10.7$\pm$3.3 mM Na$^+$) and the high external Na$^+$ concentration (500 mM Na$^+$).

Key words: Nitrate uptake, phosphate uptake, sodium-dependent transport, sodium-selective microelectrodes, *Zostera marina*.

Introduction

Seagrasses are unique angiosperms that grow in the sea, *Zostera marina* L. being one of the most common seagrasses along coasts worldwide (Larkum and den Hartog, 1989). *Zostera marina* lives in a medium with a high Na$^+$ concentration (around 0.5 M). Leaf cells of this species exhibit a plasma membrane potential ($E_m$) of $\approx$255 mV (Fernández et al., 1999), maintained by the activity of a H$^+$-ATPase (Fukuhara et al., 1996; Fernández et al., 1999; Muramatsu et al., 2002). With this highly negative $E_m$, the uptake of essential anions such as inorganic phosphate (Pi), which usually occurs in seawater at concentrations below 10$^{-7}$M (Riley and Chester, 1971), and whose concentration in the cytoplasm of aquatic plant cells is considered to be between 1 and 10 mM, must be energized (Raven, 1984).

Most studies on vascular plants have reported that Pi uptake is powered by the electrochemical potential for H$^+$ present across the plasma membrane of plant cells (Schachtman et al., 1998; Rausch and Bucher, 2002). Several lines of evidence support the presence of an H$^+$/Pi co-transport in the plasma membrane of plant cells: Pi transport transiently depolarizes the plasma membrane, which indicates the inward movement of positive charges (Ullrich-Eberius et al., 1984); Pi uptake is accompanied by an increase in extracellular pH while the cytoplasm acidifies (Sakano, 1990; Ullrich and Novacky, 1990; Mimura et al., 1992; Sakano et al., 1992) and inhibitors that dissipate the...
electrochemical gradient for H⁺, also inhibit Pᵢ uptake (Lin, 1979). More recent reports using heterologous expression have shown that protonophores inhibit Pᵢ uptake in cells expressing a distinct Pᵢ transporter gene (Mitsukawa et al., 1997; Liu et al., 1998).

Both high- and low-affinity Pᵢ uptake systems have been detected in plants by different methods. The progress on molecular techniques has allowed the identification of H⁺/Pᵢ symporters included in the Ph1 family, using models such as Arabidopsis, tomato, barley, Catharanthus, Medicago truncatula, and Lupinus albus (Grossman and Takahashi, 2001; Rausch and Bucher, 2002; Smith et al., 2003). The Ph1 family comprises both high- and low-affinity Pᵢ transport systems (Kᵢₚᵢ≈10 μM Pᵢ and Kᵢᵢ≈400 μM Pᵢ, respectively; Rae et al., 2003). A second family, Pht2, includes H⁺-coupled Pᵢ transporters with low affinity (Kᵢₚᵢ≈400 μM) that show similarities with Na⁺-coupled Pᵢ transporters found in fungal species and animals (Daram et al., 1999).

The existence of Na⁺-dependent transport systems has been demonstrated in several fungi, cyanobacteria, and algae. Na⁺-dependent uptake of glucose, amino acids, and nitrate (NO₃⁻) has been shown in marine diatoms (Hellebust, 1978; Rees et al., 1980), and the existence of a Na⁺-dependent NO₃⁻ transport has also been described in cyanobacteria (Lara et al., 1993). Na⁺-coupled Pᵢ uptake has been reported in fungal species (Versaw and Metzenberg, 1995; Martínez and Persson, 1998; Zvyagilskaya et al., 2000). Pᵢ uptake in the green alga Ankistrodesmus found to be stimulated by Na⁺ (Ulrich and Glaser, 1982), as well as in some other green algae (Raven, 1984). More recently, Na⁺ and Pᵢ uptake measurements and voltage-clamp experiments carried out in another green alga, Chara corallina, have reported the activity of a high-affinity Na⁺/Pᵢ transporter in this plant (Kᵢₚᵢ≈4 μM; Reid et al., 2000).

However, there are few reports about Na⁺-coupled transport systems in vascular plants. A Na⁺-dependent K⁺ uptake has been described in some freshwater angiosperms such as Egeria, Elodea, and Vallisneria (Walker, 1994; Maathuis et al., 1996). The HKT1 transporter from wheat was initially described as a K⁺/Na⁺ symporter using heterologous expression (Rubio et al., 1995), although this activity has not been detected in intact plants (Maathuis et al., 1996; Rubio et al., 1996).

As suggested by Rausch and Bucher (2002), although the dependence on Na⁺ of a Pᵢ uptake system has so far not been demonstrated in vascular plants, the existence of such a transport system cannot be excluded, for example, in halophytes or in plants living in alkaline media, in which the presence of an inwardly directed electrochemical potential difference for Na⁺ can potentially be exploited for energization of solute transport. Interestingly, the first physiological evidence of a Na⁺-coupled NO₃⁻ transport in an angiosperm has been reported in Z. marina, where a high-affinity NO₃⁻ transport operates in mesophyll leaf cells (García-Sánchez et al., 2000). This result points to the potential relevance of Na⁺-coupled transport in this halophytic species and raises the question whether other anions, such as Pᵢ, could be transported in the same way.

Zostera marina, as other submerged aquatic angiosperms, is able to take up Pᵢ from the surrounding water by the leaves, as well as from the interstitial water in the sediment through the roots (Pérez-Lloréns and Niell, 1995). Nevertheless, since nutrient concentrations can vary between the seawater surrounding the leaves and the substrate where the roots are anchored (Touchette and Burkholder, 2000), several differences could be found between the Pᵢ uptake characteristics of leaf and root cells.

The aim of this work was to test the existence of a Na⁺-dependent Pᵢ transport in both leaves and roots of *Z. marina*. Pᵢ-transport has been investigated at the plasma membrane of leaf and root cells to determine the possible interactions between Pᵢ and Na⁺. The results obtained are compared with previous and new contributions on Na⁺-dependent NO₃⁻ transport in *Z. marina*.

### Materials and methods

#### Plant material

*Zostera marina* L. plants were collected off the coast of Málaga (Spain) at a depth of 5 m. Plants were maintained in the laboratory in natural seawater (NSW) at 15 °C and at a light intensity of 150 μmol m⁻² s⁻¹, with a photoperiod of 16/8 h light/dark. The NSW was renewed every 3 d. Under these conditions, leaves were suitable for electrophysiological experiments for at least 2 months, while roots from the collected plants necrotized in 2 weeks and even more quickly if the plants were submitted to nutrient starvation. However, some plants developed new secondary roots in the laboratory, which were used in electrophysiological experiments.

In addition, secondary roots from seedlings were also used in electrophysiological experiments. Reproductive shoots containing seeds of *Z. marina* were collected at low tide from the Eems estuary (The Netherlands) in August 2002, and also from the *Z. marina* population located on the coast of Málaga in June 2003. The shoots collected were maintained in NSW until mature seeds were released. The seeds were stored before germination in NSW at 4 °C in darkness. Seeds were germinated at 20 °C in distilled water and, after 1 or 2 d, the seed coats opened and cotyledons appeared. Immediately, germinated seeds were sequentially transferred to NSW adjusted to increasing salinities, i.e. 0.1, 1, and 10%o, and finally to NSW at 35%o. After 1 month of culture in NSW, the first pair of secondary roots was suitable for electrophysiological experiments.

The criteria for experimental viability of the cells from both mature plants and seedlings were the membrane potential (*Eₘ*), and the response to the addition of 1 mM sodium cyanide (NaCN) and 1 mM salicylhydroxamic acid (SHAM), two inhibitors of respiration, which consequently depolarized the membrane to the diffusion potential (*Eₐ*), with a photoperiod of 16/8 h light/dark. The NSW was renewed every 3 d. Under these conditions, leaves were suitable for electrophysiological experiments for at least 2 months, while roots from the collected plants necrotized in 2 weeks and even more quickly if the plants were submitted to nutrient starvation. However, some plants developed new secondary roots in the laboratory, which were used in electrophysiological experiments.
Membrane potential ($E_{m}$) was measured using the standard glass microelectrode technique as described by Felle (1981). Leaf pieces ($\approx 2$ cm length), in which the epidermis had been partially removed, or excised roots ($\approx 2$ cm length) were mounted in plexiglass chambers (volume $\approx 1.1$ ml). Continuous perfusion of the assay medium was maintained at a constant flux rate of $\approx 10$ ml min$^{-1}$. Mesophyll leaf cells or epidermal root cells (located at $\approx 0.5$ cm from the root tip) were impaled with single-barrelled microelectrodes. Microelectrodes were backfilled with 500 mM KCl and fixed to electrode holders, containing an Ag/AgCl pellet, that were connected to a high-impedance differential amplifier (FD-223, World Precision Instruments, Sarasota, FL, USA).

To analyse the effect of $P_i$ additions on $E_{m}$ of both leaf and root cells, experiments were carried out in P-free ASW buffered to pH 8 with 10 mM MOPS/tris tripropane and containing 0.01 mM NaNO$_3$. Increasing concentrations of NaH$_2$PO$_4$ from 0.01 to 25 $\mu$M were sequentially added to the assay medium. To study the effect of Na$^+$, experiments were performed with Na$^+$-free ASW (sorbitol-ASW) containing 800 mM sorbitol, 55 mM MgCl$_2$, 12 mM CaCl$_2$, 10 mM KCl, 2 mM KHCO$_3$, and adjusted to pH 8 with 10 mM MOPS/tris tripropane. $P_i$ was added as KH$_2$PO$_4$ and Na$^+$ was supplemented as NaCl. Both ASW and sorbitol-ASW showed similar osmolality (1.09 osmol kg$^{-1}$) measured with a cryoscopic osmometer (Osmomat, model 030, Gonotec GmbH, Germany).

To analyse the effect of $P_i$ additions on $E_{m}$ of epidermal root cells, experiments were carried out in N-free ASW buffered to pH 8 with 10 mM MOPS/tris tripropane, but containing 0.01 mM NaH$_2$PO$_4$. Increasing concentrations of NaNO$_3$ from 0.01 to 100 $\mu$M were sequentially added to the assay medium. To study the effect of Na$^+$, experiments were carried out in sorbitol-ASW, NO$_3^-$ was added as KNO$_3$, and Na$^+$ was supplemented as NaCl.

In order to measure cytosolic Na$^+$ activity ($a_{Na^+}$) single- and double-barrelled microelectrodes containing Na$^+$-selective ionophore ETH227 were used. Preparation of Na$^+$-selective microelectrodes was similar to the protocol described for pH microelectrodes by Fernández et al. (1999), with slight modifications (Carden et al., 2001). After 30 min heating at 180 °C, the microelectrodes were slialized by the addition of one or two drops of dimethyldichlorosilane/benzene solution (0.1% v/v) to the blunt end of the microelectrode. The microelectrodes were then heated again for 60 min at 180 °C. Once cold, the microelectrodes were backfilled with the Na$^+$-sensor up to 4–5 mm from the tip. The Na$^+$ sensor was prepared by mixing 1 vol. of Sodium Ionophore I-cocktail A (Fluka no. 71176) in ~6 vols of tetrahydrofuran (THF) containing solid polivinylchloride (2% w/v). All chemicals were from Fluka, Sigma-Aldrich (St Louis, MO, USA).

Once filled, the microelectrodes were stored vertically in a desicator at room temperature to allow the loss of air bubbles and THF to evaporate. The microelectrode barrel containing the Na$^+$ sensor was backfilled with a 500 mM NaCl solution using a 70-mm-long Microtite needle. The tip was also dipped into 500 mM NaCl for ~30 min in order to condition the microelectrode prior to the experiments. The voltage-barrel was filled with 500 mM KCl. The signals from the Na$^+$-selective and voltage barrels were measured and simultaneously subtracted by the high impedance differential amplifier. The difference was calibrated before and after the experiments with different NaCl solutions (from 1 to 500 mM NaCl) containing a fixed background KCl concentration (96 mM KCl), as described by Carden et al. (2001). Calibration slopes were 52 mV/pNa, similar to that reported for the Na$^+$-selective ionophore ETH227 (Carden et al., 2001). Moreover, no interference effects were observed on Na$^+$ measurements when Na$^+$-selective microelectrodes were tested for pH and K$^+$ using a NaCl solution (100 mM NaCl) adjusted to different pH values with 10 mM MOPS/tris tripropane (pH 5, 6, 7, 8, 9), or using a 100 mM NaCl solution containing different KCl concentrations (1, 10, 50, and 100 mM KCl).

To determine $P_i$ net uptake rates, whole plants were submitted for 8 d to P-free ASW. Experiments were carried out after 1 h of preincubation in the assay media. The composition of the assay media was the same as for impalements, except for the osmoticum used in Na$^+$-free ASW. Since the presence of sorbitol interfered with $P_i$ analytical determination, Na$^+$-free ASW was made with 0.5 M Cl-choline (choline-ASW). Excised leaf and roots (0.3–0.6 g fresh weight) were placed separately in 250 ml flasks. The assay was carried out with gentle and constant agitation at 25 °C. Three replicates were assayed for each treatment (ASW, choline-ASW, and choline-ASW supplemented with 20 mM NaCl). At the beginning of the experiment, 10 $\mu$M KH$_2$PO$_4$ was added to the assay medium, and samples were taken at 0, 0.5, 1, 2, 4, 8, 12, and 24 h. $P_i$ was analysed colorimetrically (Fernández et al., 1985) and P$_n$ uptake rates were estimated as the slope of the $P_i$ depletion curves.

To determine NO$_3^-$ net uptake rates in Z. marina roots, whole plants were incubated for 3 d in N-free ASW. As above, experiments were carried out after 1 h of preincubation in the assay media, whose compositions were the same as used for impalements (ASW, sorbitol-ASW, and sorbitol-ASW supplemented with 20 mM NaCl). Excised roots (0.3–0.6 g fresh weight) were incubated in 250 ml flasks and three replicates were assayed for each treatment. The assay was carried out with gentle constant agitation at 25 °C. At the onset of the experiment 100 $\mu$M KNO$_3$ was added to the assay media and samples were taken at 0, 0.5, 1, 2, 4, 8, 12, and 24 h. NO$_3^-$ was analysed colorimetrically using the Shinn method (Strickland and Parsons, 1972) and NO$_3^-$ uptake rates were estimated as the slope of the NO$_3^-$ depletion curves.

Epidermal root cells of both plants and seedlings showed basic electrical membrane characteristics similar to those found in mesophyll leaf cells (Fernández et al., 1999). The resting plasma membrane potential ($E_{m}$), in both NSW and ASW was $-150 \pm 11$ mV ($n = 17$), and the membrane rapidly depolarized after the addition of 1 mM CN$^-$ and 1 mM SHAM, reaching the diffusion potential, $E_{D} = -72 \pm 8$ mV ($n = 15$).

The addition of micromolar $P_i$ concentrations (0.1–25 $\mu$M NaH$_2$PO$_4$) evoked rapid depolarizations in the plasma membrane of epidermal root cells from P-starved seedlings (Fig. 1). This effect was also observed in epidermal root cells from P-starved mature plants, on the few occasions in which healthy secondary roots were available (data not shown). However, the $E_{m}$ of mesophyll leaf cells from both seedlings and mature plants did not show any shift following $P_i$ additions (data not shown). Membrane depolarizations, which are an estimate of the transport activity, showed saturation kinetics and were fitted to the Michaelis–Menten
model (Fig. 1). Curve fitting of the seedling data rendered a $K_m$ value of $1.5 \pm 0.6 \mu M$ and a maximum depolarization ($D_{max}$) of $7.8 \pm 0.8$ mV.

The effect of $P_i$ additions on the $E_m$ of epidermal root cells was also analysed in Na+-free ASW, in which NaCl was substituted by sorbitol (sorbitol-ASW). Replacement of NaCl for isosmotic sorbitol produced a slight depolarization of the membrane (data not shown). In sorbitol-ASW, $P_i$-induced depolarizations were not observed. However, once the medium was supplemented with 20 mM NaCl, $P_i$-induced depolarizations were restored (Fig. 2).

**Effect of NO$_3^-$ additions on the membrane potential of epidermal root cells**

Since the maximum depolarizations induced by saturating $P_i$ concentrations in epidermal root cells were not higher than 10 mV, and no response could be detected in leaf cells, the effect of NO$_3^-$ additions on the $E_m$ of epidermal root cells from mature plants was analysed to check if this nutrient also showed an electrophysiological response in these cells different from that observed in mesophyll cells from these plants (García-Sánchez et al., 2000). The addition of micromolar concentrations of NO$_3^-$ from 0.1 to 100 $\mu M$ NaNO$_3$ induced plasma membrane depolarizations in root cells of N-starved plants. These membrane depolarizations showed saturation kinetics (Fig. 3) as detected previously in leaf cells (García-Sánchez et al., 2000). Curve fitting of the data to the Michaelis–Menten model rendered a $K_m$ value of $8.9 \pm 3.9 \mu M$ NO$_3^-$, higher than that reported for leaf cells ($K_m=2.3 \pm 0.78 \mu M$ NO$_3^-$; García-Sánchez et al., 2000). Furthermore, NO$_3^-$-induced $D_{max}$ in epidermal root cells was $7.0 \pm 0.8$ mV, only 45% of the $D_{max}$ value observed in leaf cells ($15.6 \pm 0.9$ mV; García-Sánchez et al., 2000). This response was also detected in epidermal root cells from seedlings which showed similar $K_m$ and $D_{max}$ values (data not shown).

As in the case of leaf cells, NO$_3^-$-induced depolarizations were abolished in Na+-free medium (sorbitol-ASW). Nevertheless, if the Na+-free medium was supplemented with

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**Fig. 1.** Membrane potential depolarizations ($\Delta E_m$) induced by increasing $P_i$ concentrations in epidermal root cells from P-starved plants incubated in ASW. Values were fitted to the Michaelis–Menten equation, as shown by the curve. Data are means ± standard deviation ($n=5$).

**Fig. 2.** Membrane potential depolarizations induced by addition of $P_i$ in ASW containing different Na$^+$ concentrations. Traces show the response of $E_m$ of a single epidermal root cell to the addition of 10 $\mu M$ KH$_2$PO$_4$ (downward arrow). The cells were incubated sequentially in different media: ASW containing 0.5 M NaCl (ASW), Na$^+$-free ASW (sorbitol-ASW), and sorbitol-ASW supplemented with 20 mM NaCl. Upward arrows show onset of $P_i$ wash.

**Fig. 3.** Membrane potential depolarizations induced by increasing NO$_3^-$ concentrations in epidermal root cells from N-starved plants incubated in ASW. Values were fitted to the Michaelis–Menten equation, as shown by the curve. Data are means ± standard deviation ($n=5$).
20 mM NaCl, the NO₃-induced depolarizations were restored (Fig. 4).

**Effect of Pi or NO₃ additions on the cytoplasmic Na⁺ activity of epidermal root cells**

Figure 5A and B illustrates two examples of simultaneous measurements of $E_m$ and cytoplasmic Na⁺ activity ($aNa_+^c$) in epidermal root cells of *Z. marina* using double-barrelled microelectrodes. The impalements were stable for at least 30 min, the measured membrane potentials being similar to those recorded with single microelectrodes ($-153 \pm 14$ mV, $n=5$). The mean value of $aNa_+^c$, calculated from the calibration curves (Fig. 5C), was $10.7 \pm 3.3$ mM ($n=5$).

It should be noted that the addition of saturating concentrations of Pi (Fig. 5A) or NO₃ (Fig. 5B) produced the depolarization of the plasma membrane (around 8 mV in both cases) and simultaneously an increase of the $aNa_+^c$. Calibrations curves showed that $aNa_+^c$ increased a maximum of 0.6 mM after the addition of 10 $\mu$M KH₂PO₄, and the initial $aNa_+^c$ value was restored after Pi was washed from the medium (Fig. 5A). On the other hand, the addition of 50 $\mu$M KNO₃ evoked an increase of 0.4 mM in the $aNa_+^c$, followed by the restoration of the initial $aNa_+^c$ value, once NO₃ was washed from the medium (Fig. 5B).

**Pi and NO₃ net uptake rates in the presence and absence of Na⁺**

Pi depletion experiments revealed that both leaves and roots from mature plants take up Pi from the medium (Table 1), although Pi net uptake rates were 3-fold higher in roots than in leaves in ASW containing 0.5 M NaCl (ANOVA, $\alpha=0.05$). In both cases, Pi net uptake rates were higher in the presence than in the absence of Na⁺. In Na⁺-free ASW (choline-ASW), Pi net uptake rates decreased both in leaves and roots by at least 90% compared with the rates obtained in ASW. When choline-ASW was supplemented with 20 mM

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**Fig. 4.** Membrane potential depolarizations induced by the addition of NO₃ in ASW containing different Na⁺ concentrations. Traces show the response of $E_m$ of a single epidermal root cell to the addition of 100 $\mu$M KNO₃ (downward arrow). The cells were incubated sequentially in different media: ASW containing 0.5 M NaCl (ASW), Na⁺-free ASW (sorbitol-ASW), and sorbitol-ASW supplemented with 20 mM NaCl. Upward arrows show onset of NO₃ wash.

**Fig. 5.** Effect of the addition of saturating concentrations of Pi (A) and NO₃ (B) on the membrane potential ($E_m$) and cytoplasmic Na⁺ activity ($aNa_+^c$) of epidermal root cells. Epidermal root cells were impaled in ASW with double-barrelled microelectrodes. Downward arrows indicate the addition of 10 $\mu$M KH₂PO₄ or 50 $\mu$M KNO₃. Upward arrows show onset of NO₃ or Pi wash. Traces are representative examples of three equivalent experiments. A calibration curve of the Na⁺-microelectrode is also included (C).
NaCl, P₃ was depleted from the medium at higher rates, almost to 50% of the rate observed in ASW (ANOVA, α=0.05).

NO₃⁻ depletion experiments were also carried out in Z. marina roots and NO₃⁻ net uptake rates were estimated in media with different Na⁺ concentrations (Table 1). NO₃⁻ net uptake rates were always higher in the presence than in the absence of Na⁺ (ANOVA, α=0.05). The highest net uptake rate was measured in ASW containing 0.5 M NaCl. In Na⁺-free ASW (sorbitol-ASW), NO₃⁻ uptake rates were always higher in the presence than in the absence of Na⁺ (ANOVA, α=0.05). The highest net uptake rate was measured in ASW containing 0.5 M NaCl. In Na⁺-free ASW (sorbitol-ASW), NO₃⁻ uptake rates were always higher in the presence than in the absence of Na⁺ (ANOVA, α=0.05). The highest net uptake rate was measured in ASW containing 0.5 M NaCl. In Na⁺-free ASW (sorbitol-ASW), NO₃⁻ uptake rates were always higher in the presence than in the absence of Na⁺ (ANOVA, α=0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pᵢ net uptake rates (µmol Pᵢ fresh wt g⁻¹ h⁻¹)</th>
<th>NO₃⁻ net uptake rates (µmol NO₃⁻ fresh wt g⁻¹ h⁻¹)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Roots</td>
</tr>
<tr>
<td>ASW (Na⁺=500 mM)</td>
<td>0.54±0.09</td>
<td>1.42±0.44</td>
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<tr>
<td>Na⁺-free ASW</td>
<td>0.07±0.02</td>
<td>0.06±0.01</td>
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<tr>
<td>Na⁺-free ASW</td>
<td>0.23±0.07</td>
<td>0.67±0.13</td>
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<td>(Na⁺=20 mM)</td>
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**Discussion**

Electrophysiological experiments performed in Z. marina have shown that the addition of Pᵢ micromolar concentrations induces rapid membrane depolarizations in epidermal root cells from both P-starved seedlings and mature plants (data not shown). It seems then that there is no developmental difference in the response of the cells to Pᵢ. The induced depolarizations indicate that Pᵢ uptake is coupled with the inward movement of positive charge and several lines of evidence suggest that Pᵢ transport is coupled with the entrance of Na⁺. Thus, Pᵢ-induced depolarizations were only recorded in media containing Na⁺ and there was an increase of aNa⁺ concurrent to the Pᵢ-induced depolarizations. In addition, depletion experiments have shown that, in Na⁺-free media, Pᵢ net uptake rates were only 4% of the rates measured in ASW containing normal seawater Na⁺ concentrations (0.5 M NaCl).

A Na⁺-coupled transport system has been described previously in this plant, where a Na⁺-dependent high-affinity NO₃⁻ transport operates in mesophyll leaf cells (García-Sánchez et al., 2000). This high-affinity system also seems to be present in epidermal root cells of Z. marina. In fact, NO₃⁻ micromolar concentrations induced membrane depolarizations in epidermal root cells of N-starved plants and these depolarizations disappeared in the absence of Na⁺ in the medium. In addition, there was an increase of aNa⁺ accompanying the NO₃⁻-induced depolarizations. On the other hand, NO₃⁻ net uptake rates were higher in the presence than in the absence of Na⁺.

Maximum Pᵢ-induced depolarizations were not higher than 10 mV and the calculated Dₘₐₓ value was 7.8±0.8 mV. These Pᵢ-induced depolarizations are low compared with the values obtained in frond cells of the aquatic higher plant Lemna gibba, where the depolarizations were in the range 18–66 mV depending on the Eₘ value (Ullrich-Eberius et al., 1981), or in root hairs of Limnobiurn stoloniferum, where depolarizations of about 60 mV were recorded (Ullrich and Novacky, 1990). These values were obtained with 1–1.8 mM Pᵢ concentrations; however, Pᵢ-depolarizations were saturated at 50–100 µM Pᵢ in Lemna gibba (Ullrich-Eberius et al., 1981). These saturating values are higher than those found in epidermal root cells of Z. marina, which are around 10 µM Pᵢ. In this species, the addition of 1 mM Pᵢ did not produce higher membrane depolarizations (data not shown) and the low Pᵢ saturation value could be considered an adaptation to the diluted Pᵢ concentrations in seawater.

Pᵢ-induced depolarizations in Z. marina could only be observed after 8 d of P starvation. This result agrees with the observation of maximum P influx in Chara corallina after 10 d of P starvation (Mimura et al., 1998) and also with the P-induced depolarizations observed in Lemna gibba submitted to P starvation for 10 d (Ullrich-Eberius et al., 1981). In the same way, N-starvation was necessary for NO₃⁻ to induce membrane depolarizations, but only for 3 d. This contrasts with the results observed in other plants where some NO₃⁻ transporters increased their activity after exposure to NO₃⁻ (Kronzucker et al., 1995; Glass et al., 2002).

Although maximum Pᵢ-induced depolarizations values are low, they are quite similar to those produced by NO₃⁻ in epidermal root cells (Dₘₐₓ=7.01±0.8 mV). However, this Dₘₐₓ value was half of the value of the Dₘₐₓ induced by NO₃⁻ in leaf cells (Dₘₐₓ=15.6±0.9 mV, García-Sánchez et al., 2000), although the range of assayed concentrations and the Eₘ of the cells were similar in both tissues. On the contrary, no Pᵢ induced depolarizations were detected in mesophyll leaf cells following Pᵢ additions. In this way, the difference in Dₘₐₓ values induced by NO₃⁻ suggests that the number of active transporters could be lower in root than in leaf cells. On the other hand, there are also differences in the affinity of the transporter for NO₃⁻ that is lower in root (Kₘₐₓ=8.9±3.9 µM NO₃⁻) than in leaf cells (Kₘₐₓ=2.3±0.78 µM NO₃⁻; García-Sánchez et al., 2000). These results indicate that leaves and roots could have a different role in Pᵢ and NO₃⁻ transport.
Despite the fact that sediment pore water is generally considered the primary nutrient source for seagrasses (McRoy and McMillan, 1977; Marschner, 1995; Pérez-Lloréns and Niell, 1995), recent evidence suggests that uptake of both NO$_3^-$ and P$_i$ by below-ground tissues can be limited by diffusion, and that roots may lack the capacity to support the total nutrient requirement (Touchette and Burckholder, 2000). Thus, in *Z. marina*, as in most seagrasses, NO$_3^-$ supplies would be mainly provided by leaf absorption from the water column (Terrados and Williams, 1997; Lee and Dunton, 1999) and P$_i$ supplies would rely upon P$_i$ uptake from the sediment only when P$_i$ is negligible in the water column (Touchette and Burckholder, 2000). Depletion experiments in *Z. marina* have shown that roots have higher P$_i$ and NO$_3^-$ net uptake rates than leaves; however, as the depletion experiments were carried out in artificial seawater and not in the real substrate where the roots are anchored, the uptake capacity of the roots could have been overestimated. In fact, the very low $K_m$ for NO$_3^-$ in leaves points to the importance of NO$_3^-$ uptake from the surrounding seawater. On the contrary, the small $K_m$ value for P$_i$ observed in epidermal root cells (1.5 ± 0.6 μM P$_i$), and the lack of any P$_i$-induced depolarization in leaf cells, suggests the relevance of P$_i$ uptake through the roots.

While the $K_m$ value calculated for P$_i$ transport in epidermal root cells is in the range of high-affinity values described for P$_i$ transporters in higher plants ($K_m$ = 10 μM P$_i$; Mirnima, 2001; Rae et al., 2003), it is lower than the reported value for system I of *Lemna gibba* ($K_m$ = 7.3 μM P$_i$; Ullrich-Eberius et al., 1989) and even lower than the $K_m$ (3.1 μM P$_i$) measured in cultured tobacco cells expressing the gene encoding for the Pth1 transporter of *Arabidopsis* (Mitsukawa et al., 1997). The $K_m$ for P$_i$ in *Z. marina* is closer to the values observed in some fungal species found in mycorrhizal associations such as *Gigaspora margarita* ($K_m$ = 1.8–3.1 μM P$_i$ for the high-affinity transporter; Thomson et al., 1990) or in the unicellular alga *Chlamydomonas reinhardtii* (0.1–0.5 μM P$_i$; Shimogawara et al., 1999). *Zostera marina* is an angiosperm that evolved from a terrestrial to a marine habitat. High-affinity NO$_3^-$ transport systems of terrestrial plants, such as barley or maize, exhibit $K_m$ values ranging from 10 to 100 μM (Guo et al., 2002), which are slightly higher than the $K_m$ showed by epidermal root cells of *Z. marina* and much higher than the value observed in leaf cells (García-Sánchez et al., 2000). A compilation of data on the nutrient environment of seagrass meadows worldwide (Hemminga, 1998) shows that the average P$_i$ concentration is only 1 μM in the water column and 12 μM in pore water, while in the case of NO$_3^-$ average concentrations are not much different between water column and pore water (2.7 μM and 3.4 μM NO$_3^-$, respectively). Thus, the high affinity of both P$_i$ and NO$_3^-$ transporters in *Z. marina* could be related to the development of mechanisms to improve survival in a P- and N-diluted medium like seawater.

On the other hand, the Na$^+$-dependence of both P$_i$ and NO$_3^-$ transporters could have been evolved as an adaptation to a medium with a high salinity and alkaline pH, considering the high electrochemical potential for Na$^+$ that can be developed in cells of *Z. marina*. The free energy relationship ($\Delta G'/F$) for a plasma membrane cation-coupled transport system operating with a stoichiometry of $n$ cations per transported anion is given (in millivolts) as:

$$ \Delta G'/F = (n + z_a)E_m + 59 \cdot \log\left(\left[\frac{[C^+]_o}{[C^+]_c}\right] \cdot \left[A^-_c\right] / \left[A^-_o\right]\right) $$

where $z_a$ defines the electrical charge of the anion, C$^+$ is the coupling cation (either Na$^+$ or H$^+$), A is the transported anion, $E_m$ is the membrane potential, and subscripts ‘o’ and ‘c’ refer to external medium and cytoplasm, respectively.

P$_i$ concentration in the cytoplasm of aquatic plants is in the range of 1–10 mM (Raven, 1984), similar to the values reported for terrestrial plants (5–10 mM P$_i$; Mimura, 2001). Nevertheless, P$_i$ exists in different forms (H$_2$PO$_4^-$, H$_3$PO$_4^-$, HPO$_2^{4-}$, and PO$_3^{3-}$) and the concentration of each species varies as a function of pH. The cytosolic pH value measured both in leaf (Fernández et al., 1999) and in root cells (data not shown) of *Z. marina* is around 7.3. Since the pH value for the dissociation of H$_2$PO$_4^-$ into HPO$_2^{4-}$ is 7.2, the most abundant species of P$_i$ in the cytoplasm are H$_2$PO$_4^-$ (44% P$_i$) and HPO$_2^{4-}$ (56% P$_i$). On the other hand, under the alkaline conditions of seawater (pH 8) the most abundant form is HPO$_2^{4-}$ (86% P$_i$).

As discussed in García-Sánchez et al. (2000), cytosolic concentrations of NO$_3^-$ in *Z. marina* could be considered to be around 3 mM NO$_3^-$. The mean value of aNa$^+$ reported in this work is 10.7 mM, which is in agreement with the reported range for some marine plants (1–50 mM Na$^+$; Raven, 1984) and is close to the values measured in a salt-tolerant variety of barley (2–28 mM Na$^+$) using Na$^+$-selective microelectrodes (Carden et al., 2003).

The Na$^+$ concentration of natural seawater and seawater in the sediments can be taken as 500 mM Na$^+$ (Siever et al., 1965; Riley and Chester, 1971) and the pH is typically 8. However, it should be considered that the external Na$^+$ concentration and pH could be different surrounding the cells. On the other hand, external P$_i$ and NO$_3^-$ concentrations could be considered to be around 10 μM (Riley and Chester, 1971). Figure 6 shows the free energy relationship ($\Delta G'$) for P$_i$ and NO$_3^-$ transport across the plasma membrane of epidermal root cells calculated from equation 1. The cation:P$_i$ or cation:NO$_3^-$ stoichiometry values ($n$) which render a negative free energy ($\Delta G'<0$) are the only stoichiometries that make the transport thermodynamically feasible. In the case of P$_i$ the free energy relationship was calculated for both species, H$_2$PO$_4^-$ and HPO$_2^{4-}$.

It should be noted that Na$^+$-coupled transport is strongly favoured, in both H$_2$PO$_4^-$ or HPO$_2^{4-}$ and NO$_3^-$ transport.
systems. A stoichiometry of $2\text{Na}^+ : \text{H}_2\text{PO}_4^-$ renders sufficient free energy for transport ($\Delta G = -13 \text{ kJ mol}^{-1}$) in Z. marina, while the same stoichiometry for $\text{H}^+ (2\text{H}^+ : \text{H}_2\text{PO}_4^-)$ is not sufficient to drive net uptake of $\text{P}_i (\Delta G > 0$, Fig. 6A). Furthermore, if $\text{P}_i$ is transported as $\text{HPO}_2^-$, a stoichiometry of $3\text{Na}^+ : \text{HPO}_2^-$ is sufficient to allow the transport, while a stoichiometry of $5\text{H}^+ : \text{HPO}_2^-$ yields only a modest inward driving force of $-8 \text{ kJ mol}^{-1}$ (Fig. 6B). This stoichiometry could be different in leaf cells, where $\text{P}_i$ net uptake has been detected by depletion experiments, as $\text{P}_i$ transport seems to be electrically silent in mesophyll cells. On the other hand, as estimated for the $\text{Na}^+$-coupled $\text{NO}_3^-$ transport system in leaf cells (García-Sánchez et al., 2000), a stoichiometry of $2\text{Na}^+ : \text{NO}_3^-$ renders sufficient energy for transport ($\Delta G = -19 \text{ kJ mol}^{-1}$), while a stoichiometry higher than $4\text{H}^+ : \text{NO}_3^-$ is required to generate a similar inward driving force (Fig. 6C).

In vascular plants there have not been reports of functional analysis of $\text{Na}^+$-coupled $\text{P}_i$ transporters, although several genes of putative $\text{Na}^+$-coupled $\text{P}_i$ transporters have been identified (Smith et al., 2003). The first quantitative physiological demonstration of such a transport system in plant cells is that described in Chara corallina. This freshwater charophyte plant showed a $K_m$ value for $\text{P}_i$ around $10 \mu \text{M}$ and a stoichiometry as high as $6\text{Na}^+$ for each $\text{P}_i$, estimated by the influx of positive charge in voltage-clamp experiments (Reid et al., 2000). The estimated stoichiometry for the $\text{Na}^+$-coupled $\text{P}_i$ transport in Z marina is much lower, $2\text{Na}^+$ or $3\text{Na}^+$. $\text{Na}^+ / \text{P}_i$ transport in C. corallina seems to be induced by low $\text{P}_i$ concentrations (0.5–1 $\mu \text{M}$ $\text{P}_i$) and is inactivated by a 6 d treatment with 1 $\mu \text{M}$ $\text{P}_i$ (Mimura et al., 2002). However, in Z. marina, $\text{P}_i$-induced depolarizations could be observed only in plants submitted to $\text{P}$ starvation for at least 8 d and no depolarizations were ever detected in plants maintained in natural seawater containing 0.5–1 $\mu \text{M}$ $\text{P}_i$.

In conclusion, several lines of evidence indicate that a $\text{Na}^+$-coupled high-affinity $\text{P}_i$ transport system operates in epidermal root cells of Z. marina. This is the first report of a $\text{Na}^+$-dependent $\text{P}_i$ transport in an angiosperm and, together with the presence a $\text{Na}^+$-coupled $\text{NO}_3^-$ transport in this plant, it highlights the importance of $\text{Na}^+$-dependent transport in halophytic species. The increment in the $\text{aNa}^+$ accompanying both $\text{NO}_3^-$ and $\text{P}_i$ transport, as well as the low $\text{aNa}^+$ measured in Z. marina epidermal root cells, suggest that very efficient $\text{Na}^+$ homeostatic mechanisms would have been developed in this plant, and studies on this topic are now in progress.

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References


