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BYPASS FLOW AND SODIUM TRANSPORT IN RICE (*ORYZA SATIVA* L.)

BY

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Presented for the degree of Doctor of Philosophy in the School of Life Sciences at the University of Sussex

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DECLARATION

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Signature:……

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UNIVERSITY OF SUSSEX

BUALUANG FAIYUE **DPHIL**

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ABSTRACT

An apoplastic pathway, the so-called bypass flow, is important for Na^+ uptake in rice under saline conditions. The primary aim of this thesis was to identify the point of entry for bypass flow into rice roots subjected to salinity. Investigations using lateral rootless mutants (*lrt1*, *lrt2*), a crown rootless mutant (*crl1*), their wild types (Oochikara, Nipponbare and Taichung 65, respectively) and seedlings of rice cv. IR36 showed that the entry point, quantified using trisodium-8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS), was not at the sites of lateral root emergence. However, PTS was identified in the vascular tissue of lateral roots using both epifluorescence microscopy and confocal laser scanning microscopy. Cryo-scanning electron microscopy and epifluorescence microscopy of sections stained with berberine-aniline blue and Fluorol Yellow 088 revealed that an exodermis was absent in the lateral roots, suggesting that the lack of the exodermis allowed PTS to pass through the cortical layers, enter the stele and be transported to the shoot via the transpiration stream. These findings suggest a role for the lateral roots of rice in bypass flow. The addition of polyethylene glycol (PEG) and silicon (Si) to the culture solution significantly reduced Na^+ uptake to the shoot by reducing bypass flow through the lateral roots. PEG was found to be more effective than Si. It was also shown that changing the relative humidity in the air around the shoots had a significant effect on the magnitude of bypass flow and the flux of water across the roots: the greater the flux of water through the roots, the greater the Na^+ uptake and bypass flow. Furthermore, results showed that recombinant inbred lines of rice with low Na^+ transport possessed low magnitudes of bypass flow, whereas lines with high Na^+ transport had a high degree of bypass flow, indicating that bypass flow could be used as a criterion for screening salt resistance in rice varieties.

LIST OF PUBLICATIONS

1. Faiyue B., Vijayalakshmi C., Nawaz S., Nagato Y., Taketa S., Ichii M., Al-Azzawi M.J. & Flowers T.J. (2010) Studies on sodium bypass flow in lateral rootless mutants *lrt1* and *lrt2*, and crown rootless mutant *crl1* of rice (*Oryza sativa* L.). *Plant, Cell and Environment* **33**, 687-701.

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ABBREVIATIONS

2,4-D: 2,4-Dichlorophenoxyacetic acid

ABA: Abscisic acid

AFLP: Amplified fragment length polymorphism

AKT: *Arabidopsis* K^+ transporter

CLSM: Confocal laser scanning microscopy

crl1: Crown rootless1

Cryo-SEM: Cryo-scanning electron microscopy

DIDS: 4,4´-diisothiocyano-2,2´-disulfonic acid

EC: Electrical conductivity

EDTA: Ethylenediaminetetraacetic acid

gDW: Gram dry weight

g RDW: Gram root dry weight

H^+-ATPase: H^+-translocating adenosine triphosphatase

H^+-PPiase or H^+-PPase: H^+-translocating inorganic pyrophosphatase

HKTs: High-affinity K^+ transporters

IBA: Indole-3-butyric acid

KIRCs: K^+ inward rectifying channels

KUP/HAK/KT: K^+ uptake permease/high-affinity K^+/K^+ transporters

LAR: Leaf area ratio

LEV: Leaf elongation velocity

lrt1: Lateral rootless1

lrt2: Lateral rootless2

NHX: Na^+/H^+ exchanger or Na^+/H^+ antiporter

NSCCs : Non-selective cation channels

P: Probability level

PEG: Polyethylene glycol

PTS: Trisodium-8-hydroxy-1,3,6-pyrenetrisulphonic acid

RH: Relative humidity

TEA^+: Tetraethylammonium

WUE: Water-use efficiency

CHAPTER 1

GENERAL INTRODUCTION

1.1 Soil salinity and its impact on food supply

Soil salinity is one of the major agricultural problems affecting crop productivity worldwide (Shannon & Grieve 1999; Ashraf *et al*. 2008; Qadir *et al*. 2008; Rozema & Flowers 2008). It has been estimated that more than 800 million hectares (Mha) of land throughout the world are salt affected either by sodicity or salinity (Munns 2005; Rengasamy 2006). The majority of salt-affected soils are dominated by sodium (Na^+), but sodic soils are characterised by an excess of carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-), whereas saline soils are dominated by a high concentration of chloride (Cl^-) and sulphate (SO_4^{2-}) (Ghassemi, Jakeman & Nix 1995). Soils are classified as saline when the electrical conductivity (EC) is 4 dS/m or greater; 4 dS/m is equivalent to approximately 40 mM NaCl.

Most naturally salt-affected soils (primary salinisation) are caused by the weathering of parental rocks (Flowers & Flowers 2005; Munns & Tester 2008). However, these areas are not particularly important to agriculture as they are mostly coastal salt marshes and inland deserts; the major problem for agriculture is secondary salinisation caused by irrigation and forest clearance (Flowers & Flowers 2005). Excessive irrigation, beyond that required for plant growth, raises the water table and carries salts that have accumulated in the soil to the surface. When the water evaporates, the salts dissolved in the water are left behind in the soil, thus increasing salinity. Approximately 1500 Mha of land are cultivated without irrigation and of this only about 2% is salt affected, whereas of the 230 Mha of irrigated land about 20% is thought to be affected by salinisation (Munns & Tester 2008). Although irrigated land covers only 15% of total cultivated land of the world, it is the irrigated land that is the most productive in world agriculture by producing one third of the world's food (Munns & Tester 2008). Approximately 10 Mha of irrigated land worldwide are abandoned every year due to secondary salinisation (Flowers & Yeo 1995). Moreover, it has been anticipated that the sea level will rise by 1.0 m in the next 500 years because of the melting of the Antarctic

ice sheet as a result of global climate change (O'Farrell *et al.* 1997). This rising sea level will adversely affect land used in coastal areas, increase saline soils and consequently affect food production. Thus, salinity could seriously threaten to food supply for the world population.

Rice (*Oryza sativa* L.) is one of the most important food crops in the world in terms of both dietary and monetary value, with about one third of world population consuming rice as a staple food (Sengupta & Majumder 2010). About 70% of the world's rice production is exported from Asia, especially from countries like Thailand, India and Pakistan (IRRI 2009). Currently, cultivated areas in those countries are affected by salinity. Recent studies have estimated that 2.8 Mha in Thailand (Yuvaniyama *et al.* 2008), 6.7 Mha in India (Singh 2009) and 6.8 Mha in Pakistan (Awan *et al.* 2007) are salt affected. Unfortunately, rice is a salt-sensitive crop, which is ineffective in controlling the influx of Na^+ across the roots, leading to the rapid accumulation of toxic Na^+ concentrations in the shoots (Flowers & Yeo 1981; Yeo & Flowers 1982, 1983; Yeo, Yeo & Flowers 1987; Flowers, Salama & Yeo 1988; Yeo *et al*. 1999; Roshandel & Flowers 2009). Both seedlings and grain yields of rice are extremely susceptible to salinity (Flowers & Yeo 1981; Khatun & Flowers 1995a, b; Khatun, Rizzo & Flowers 1995; Lutts, Kinet & Bouharmont 1995). Although salt tolerance in rice has been investigated for many years, the physiological response of rice to this stress is not fully understood, especially the uptake and transport of Na^+ (Singh & Flowers in press). Therefore, it is necessary to continue studying the pathway of Na^+ transport in rice, as an approach to increasing its salt tolerance, together with methods to identify salt tolerance in rice varieties in order to combat the adverse effects of salinity on food production and guarantee food supply for future generations.

1.2 The effects of salinity on plants with an emphasis on rice

Generally, the effects of salinity on plants are associated with osmotic effects, ionic effects and nutritional imbalance (Shannon & Grieve 1999; Tester & Davenport 2003; Ashraf & Harris 2004; Fricke *et al.* 2004, 2006; Flowers & Flowers 2005; Rengasamy 2006; Qadir *et al*. 2008; Shabala & Cuin 2008; Singh & Flowers in press).

The osmotic effect is induced by the effect of NaCl outside the plant. Raising the external concentration of NaCl reduces the ability of plants to take up water by decreasing the external water potential; this can lead to the reduction of plant growth (Munns 1993, 2002; Fricke 2004a; Fricke *et al.* 2004; Munns & Tester 2008). Yeo *et al.* (1991) reported that an addition of 50 mM NaCl to the culture solution stopped leaf elongation of rice genotypes IR2153 and Pokkali for 20 min, after which the elongation rate resumed to the non-salinised control rate by 24 h. It was hypothesised that stopping leaf elongation was due to a limitation of water supply to the growing zone caused by salinisation. A similar result was observed in barley (*Hordeum vulgare* L.) leaves under salt stress. Fricke (2004a) and Fricke *et al.* (2004, 2006) reported that leaf elongation velocity (LEV) of barley leaf 3[1] was zero or close to zero within 10 min of the addition of 100 mM NaCl to the culture solution. This rapid cessation in growth of the leaf under saline stress was due to the reduction of xylem water potential in the elongation zone, generated by NaCl in the culture solution. Then the LEV recovered to about 40-50% of the original value between 20 and 30 min as a result of a decrease in the transpiration and stomatal conductivity caused by an increase (by 3-6 times compared with non-salinised plants) in leaf abscisic acid (ABA), leading to an increase in the xylem water potential and growth resumption (Fricke 2004a; Fricke *et al.* 2004, 2006). A study in barley, bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L.) by Munns, Schachtman & Condon (1995) indicated that genotypes differing in salt tolerance did not differ in the osmotic effect of salinity.

Excessive Na^+ could accumulate in the cell walls and dehydrate the cells. For example, Yeo, Caporn & Flowers (1985) reported that the concentration of Na^+ in a leaf causing a 50% reduction in photosynthesis was approximately 120 mM on a tissue water basis (or 0.5 mmol/gDW on a tissue dry weight basis) in the rice genotype IR2153 after exposure to 25 mM NaCl for 7 d. However, such a concentration did not affect chloroplast structure (Flowers *et al.* 1985) and inhibited only 25% of the activities of nitrate reductase and malate dehydrogenase from leaves of IR2153 (Yeo & Flowers 1983). Therefore, Yeo *et al.* (1985a) suggested that the reduction of photosynthesis in rice under salinity was possibly caused by water deficit in the leaf cells due to an accumulation of Na^+ in the apoplast. Subsequent study by Flowers, Hajibagheri & Yeo

[1] Leaf number was counted from the oldest leaf (1) and this counting system has been applied throughout the thesis.

(1991) found a negative correlation between water and Na^+ concentrations in the leaves of rice seedlings of genotypes Amber and IR2153 hydroponically grown in 50 mM NaCl for 9 d. The authors hypothesised that there would be substantial ion accumulation in the leaf apoplast (e.g. cell wall) which would remove water from protoplasts that would then be lost to the atmosphere by evaporation. Their subsequent measurement of tissue ion contents by X-ray microanalysis showed that the Na^+ + potassium (K^+) concentration was 691 and 488 mM in the cell wall of Amber and IR2153, respectively, whereas it was 355 and 291 mM in the cytoplasm when Na^+ concentration in the leaf was 1 mmol/gDW. These findings led to the conclusions that an extracellular accumulation of salt in the apoplast dehydrated the cell (Flowers *et al.* 1991).

The ionic effect is induced by the effect of NaCl within the plant. When NaCl taken up via the transpiration stream exceeds the ability of cells to compartmentalise it in the vacuole, excessive Na^+ and Cl^- rapidly builds up in the cytoplasm and inhibits enzyme activity (Munns 2002; Tester & Davenport 2003; Munns & Tester 2008). Although the concentration at which Na^+ in cytoplasm becomes toxic is not well defined, *in vitro* studies showed that most enzymes started to be inhibited at a concentration above 100 mM NaCl or about 0.5 mmol/gDW (Flowers 1972; Greenway & Osmond 1972; Flowers, Troke & Yeo 1977; Munns, James & Läuchli 2006; Munns & Tester 2008). For example, Yeo & Flowers (1983) found that the activities of nitrate reductase and malate dehydrogenase extracted from leaves of rice IR2153 were inhibited by 50% when Na^+ concentration was 150 and 265 mM, respectively. They have also reported that there was no evidence of enzymatic adaptation to function specifically under saline conditions in rice as the activities of those enzymes from both control and salt-stressed plants (50 mM NaCl for 8 d) were inhibited more than 50% at Na^+ concentrations in excess of 200 mM. Also, enzymes extracted from salt-tolerant halophyte, *Suaeda maritima* L. were not more tolerant to Na^+ than those extracted from salt-sensitive glycophyte, *Pisum sativum* L. (Flowers 1972; Flowers *et al.* 1977).

Nutritional imbalance is another effect of salinity. For example, Bernstein, Silk & Läuchli (1995) reported that K^+ and calcium (Ca^{2+}) concentrations were dramatically decreased in the elongation zone of sorghum (*Sorghum bicolor* L.) leaf 6 salinised with 100 mM NaCl 8 d after germination. Hu & Schmidhalter (1998) found that the treatment of 120 mM NaCl for 3 d significantly reduced nitrate (NO_3^-) concentration by

2-3 times in the elongation zone of leaf 4 of bread wheat as compared to untreated control plants. Similarly, Neves-Piestun & Bernstein (2005) reported that K^+ and Ca^{2+} concentrations were reduced throughout the growing zone of leaf 4 of maize (*Zea mays* L.) subjected to 80 mM NaCl for 7 d. The nutritional imbalance occurred because Na^+ inhibited the uptake of other nutrients by interfering ion transporters (e.g. K^+ channels) (Tester & Davenport 2003; Hu & Schmidhalter 2005; Shabala & Cuin 2008), inhibiting root growth (Tester & Davenport 2003) or limiting the translocation of water and solutes to growing cells by reducing the numbers of veins (Hu, Fricke & Schmidhalter 2005). More than 50 enzymes require K^+ as a cofactor for cellular functions and those enzymes are susceptible to Na^+ (Munns *et al.* 2006). A high Na^+ level in cytoplasm competes with K^+ for the binding sites of many enzymes, thus inhibiting several metabolic processes (Maathuis & Amtmann 1999; Tester & Davenport 2003; Sengupta & Majumder 2010).

In rice, there is much evidence to support the view that salinity damage is mainly caused by the ionic effect of NaCl rather than osmotic effects or nutritional imbalance (Flowers & Yeo 1981; Yeo & Flowers 1982, 1983; Yeo *et al.* 1985a). For example, the survival of rice genotype IR2153 was significantly higher in seedlings treated with 50 mM NaCl plus 70 g/l polyethylene glycol (PEG) 1540 for 22 d than the ones treated only with 50 mM NaCl (Yeo & Flowers 1984a, b). Although the combined treatment of NaCl and PEG (- 460 kPa) had a lower water potential than the treatment of NaCl alone (- 230 kPa), the combined treatment demonstrated lower leaf Na^+ concentrations and better survival. As a result, the authors pointed out that the injurious effect of salinity on rice was primarily due to excessive ion entry. Similarly, Matoh, Kairusmee & Takahashi (1986) reported that the growth of rice seedlings cv. Kinmaze hydroponically grown in 100 mM NaCl (– 460 kPa) for 30 d was more severe than those grown in iso-osmotic PEG 6000 and concluded that the growth reduction in rice under salinity was mainly due to ionic toxicity. In an investigation of the effect of salinity (25 mM NaCl for 7 d) on photosynthesis in leaves 3-5 of rice IR2153, Yeo *et al.* (1985a) reported that photosynthesis was not reduced in the plant as a whole, but was reduced only when Na^+ accumulated in the leaves; photosynthesis was decreased by 50% in the oldest leaf 3 with highest leaf Na^+ concentration, whereas it was not affected in the youngest leaf 5 with lowest leaf Na^+ concentration. Consequently, the authors indicated that the reduction in photosynthesis and salt damage in rice under salinity were attributed to the

accumulation of Na^+ within the tissue and not to external water deficit. Recently, it was found that three genes from amplified fragment length polymorphism (AFLP) bands, encoding for a proline-rich protein, senescence-associated protein and heat-shock protein, were up-regulated in the shoots of salt-tolerant[2] rice IR4630, but not in salt-sensitive rice IR15324 and only in seedlings treated with 50 mM NaCl, but not in those treated with iso-osmotic mannitol (85 mM). The results highlighted the fact that differential expression of genes in response to salinity between salt-tolerant and salt-sensitive rice genotypes was induced by the ionic rather than the osmotic effect of NaCl (Roshandel & Flowers 2009).

According to the ionic effect of salinity, both Na^+ and Cl^- are toxic to plant tissues, if they are accumulated at high concentrations in the cytoplasm (Teakle & Tyerman 2010). Although it is difficult to determine whether the effects are due to Na^+ or Cl^-, studies on rice have reported that Na^+ is likely more toxic than Cl^-. For example, Lin & Kao (2001) demonstrated that the inhibitory effect of 150 mM NaCl for 5 d on root growth of rice seedlings Taichung Native 1 was similar to the effect of 150 mM NaCl plus 0.05 mM 4,4′-diisothiocyano-2,2′-disulfonic acid (DIDS, a Cl^- uptake inhibitor), and the reduction of root growth was not different between treatments of 100 mM NaCl and 50 mM Na^+-gluconate ($NaC_6H_{11}O_7$) in which both treatments had similar Na^+ concentrations in the roots, but the treatment of Na^+-gluconate had much less Cl^- concentration, implying that only Na^+ was involved in NaCl-induced growth reduction in roots of rice seedlings. Hong *et al.* (2009) reported that the activity of an antioxidant enzyme glutathione reductase (GR) and the expression of glutathione reductase genes (*OsGR2, OsGR3*) were increased in roots of rice Taichung Native 1 either subjected to 150 mM NaCl or 150 mM $NaNO_3$ for 8 h but not in iso-osmotic stress of 276 mM mannitol; the expression of *OsGR2, OsGR3* genes and activity of GR were similar between NaCl and $NaNO_3$ treatments. As a result, the authors concluded that Na^+ but not Cl^- or osmotic stress induced the expression of *OsGR2, OsGR3* and GR activity in response to salt stress. Consequently, it was only Na^+ that was investigated in this thesis.

[2] Rice is a sensitive species and the term ‘tolerant’ is used in a relative sense to describe differences between genotypes.

1.3 The pathways of Na^+ uptake in rice

The pathways of Na^+ uptake by roots of higher plants have been frequently reviewed (e.g. Amtmann & Sanders 1999; Maathuis & Amtmann 1999; Blumwald, Aharon & Apse 2000; Tester & Davenport 2003; Apse & Blumwald 2007; Flowers & Colmer 2008; Plett & Moller 2010; Zhang, Flowers & Wang 2010). For rice, the pathways for Na^+ uptake can be divided into the symplastic and apoplastic.

1.3.1 Symplastic pathway

The symplastic pathway is a pathway in which Na^+ moves through plasma membranes facilitated by membrane transporter proteins, namely channels and carriers (Maathuis & Amtmann 1999; Taiz & Zeiger 2006; Maathuis 2007).

1.3.1.1 Channels

Channels are transporter proteins that function as membrane pores and their specificity is determined by the pore size (Taiz & Zeiger 2006). The transport of ions through channels is always passive in the direction of electrochemical gradient at rates of about 10^6-10^8 ions per second (Maathuis & Amtmann 1999; Taiz & Zeiger 2006; Maathuis 2007). Golldack *et al.* (2003) reported that K^+ inward rectifying channels (KIRCs) such as OsAKT1 (homologous to *Arabidopsis* K^+ transporter) could mediate Na^+ uptake in rice under saline conditions. They comparatively analysed the expression of *OsAKT1* in salt-sensitive rice cv. IR29, and salt-tolerant rice Pokkali and BK after a 48 h exposure to 150 mM NaCl. Their results showed that *OsAKT1* transcripts disappeared from roots of Pokkali and BK, whereas they were present in IR29. As a result, the authors concluded that differential expression of *OsAKT1* was involved in the regulation of Na^+ uptake in rice genotypes differing in salinity resistance.

Non-selective cation channels (NSCCs) have been reported to mediate the uptake of Na^+ in rice. For example, in a study of Na^+ uptake into leaf protoplasts of salt-sensitive rice cv. BRRI Dhan29 and salt-tolerant Pokkali under 5, 50 and 100 mM NaCl for 10 min, Kader & Lindberg (2005) found that pre-treatment of protoplasts with K^+ channel blockers (1 mM Tetraethylammonium: TEA^+, 10 mM Cs^+ and 1 mM Ba^{2+}) and NSCC inhibitors (1 mM Ca^{2+}, 1 mM Zn^{2+} and 1 mM La^{3+}) for 10 min significantly reduced

Na^+ concentrations in the cytoplasm of BRRI Dhan29, whereas the cytoplasmic Na^+ concentration in Pokkali was reduced only by NSCC inhibitors. As a result, they concluded that both K^+ channels and NSCCs contributed to the Na^+ uptake in BRRI Dhan29, whereas NSCCs were the main pathways for Na^+ uptake in Pokkali. However, according to recently published articles, *NSCC* genes remain unknown and no *NSCC* defective mutants have been isolated in any plant species to show a reduction in Na^+ influx into the roots (Horie *et al.* 2007; Yao *et al.* 2010).

1.3.1.2 Carriers

Unlike channels, carriers transport substrates by binding the transported molecules on one side of the membrane and releasing them on another side (Taiz & Zeiger 2006; Maathuis 2007). Carriers can mediate either passive or active transport with the rate of about 10^2-10^3 per second (Maathuis & Amtmann 1999; Taiz & Zeiger 2006; Maathuis 2007). Carriers involved in Na^+ transport in rice have been reported such as high-affinity K^+ transporters (HKTs) and K^+ uptake permease/high-affinity K^+/K^+ transporters (KUP/HAK/KT).

OsHKT2;1 was first identified in roots of salt-tolerant Pokkali and an intermediate tolerant Nipponbare by Horie *et al.* (2001). By characterising transport properties using an HKT deficient mutant (CY162) and a Na^+ hypersensitive mutant (G19) of yeast (*Saccharomyces cerevisiae*) as well as electrophysiological techniques in *Xenopus laevis* oocytes, OsHKT2;1 was found to function as a Na^+ uniporter without mediating K^+ flux (Horie *et al.* 2001). A similar function of OsHKT2;1 was also confirmed by others in tobacco (*Nicotiana tabacum* L. cv. Bright Yellow 2) cells (Yao *et al.* 2010). An analysis of Na^+ and K^+ concentrations in a suspension culture of the HKT deficient mutant yeast (W303) demonstrated that Na^+ uptake mediated by OsHKT2;1 was inhibited by K^+ and this was consistent with the inhibitory effect seen in roots of Nipponbare (Garciadeblas *et al.* 2003). Recently, Horie *et al.* (2007) successfully isolated, from a population of Nipponbare, an *oshkt2;1* mutant which is defective in the *OsHKT2;1* gene. The *oshkt2;1* mutant plants showed normal growth and were indistinguishable from the wild type under the control conditions, but grown hydroponically in 0.5 mM NaCl for 2 or 9 d, the *oshkt2;1* mutant plants accumulated significantly less Na^+ concentration in roots, shoots and xylem than the wild type. An analysis of influx of $^{22}Na^+$ in the *oshkt2;1* mutant and wild-type plants at 0.1 and 1 mM

external NaCl indicated that OsHKT2;1 mediated Na^+ influx into the roots of wild-type plants grown in low-K^+ conditions and the influx was dramatically inhibited when the growth medium was supplemented with either 5 mM KCl or 30 mM NaCl (Horie *et al.* 2007). Consequently, it has been concluded that OsHKT2;1 is responsible for mediating Na^+ uptake into rice roots under K^+-starvation and this transport was inhibited under high Na^+ concentrations (Horie *et al.* 2001, 2007; Rodriguez-Navarro & Rubio 2006).

OsHKT2;2 has been identified in salt-tolerant Pokkali (Horie *et al.* 2001) and in salt-sensitive BRRI Dhan29 (Kader *et al.* 2006). By studying transport properties in mutant yeasts (CY162 and G19), *Xenopus* oocytes and tobacco cells, OsHKT2;2 was characterised as a Na^+/K^+ symporter (Horie *et al.* 2001; Yao *et al.* 2010). Growth inhibition of the Na^+ hypersensitive mutant yeast G19 expressing *OsHKT2;2* was completed at 150 mM NaCl, suggesting that OsHKT2;2 mediated Na^+ influx (Horie *et al.* 2001). However, Kader *et al.* (2006) reported that *OsHKT2;2* was up-regulated in root tissue of salt-tolerant Pokkali in response to 150 mM NaCl for 72 h with an approximately 177% higher level of expression than the control, whereas it was only 50% higher than the control in salt-sensitive BRRI Dhan29. Although Kader *et al.* (2006) suggested that OsHKT2;2 might be involved in K^+ uptake into the cells to maintain a high level of cytoplasmic K^+/Na^+ ratio, the function of OsHKT2;2 in relation to salt tolerance in rice is still unclear (Horie *et al.* 2008).

OsHAK1-17 genes have been identified encoding KUP/HAK/KT type transporters in the rice cv. Nipponbare by Banuelos *et al.* (2002). An investigation of each gene in rice roots under salinity stress of 50 mM NaCl for 24 h showed that the expression of *OsHAK1*, *OsHAK7* and *OsHAK16* was higher than the other genes, implying that those three genes might be involved in response to salinity stress (Okada *et al.* 2008). Similarly, Wu, Hu & Xu (2009) reported that *OsHAK1* transcription in roots of Nipponbare was up-regulated when exposed to 25 mM NaCl for 9 d, which was associated with an increase in Na^+ concentration in roots and shoots; however, at high levels of salt stress (100 mM NaCl), *OsHAK1* expression was down-regulated. To date, *OsHAK18-27* genes have also been identified in the rice genome, but their functions in response to salinity are unknown (Gupta *et al.* 2008).

1.3.2 Apoplastic pathway (Bypass flow)

The apoplastic pathway, the so-called bypass flow, is a pathway in which Na^+ from outside moves through cell walls and intercellular spaces to the xylem without crossing plasma membranes (Yeo *et al.* 1987; Yeo 1992; Singh & Flowers in press). Tracing the pathway by which water and dissolved ions pass through roots has challenged physiologists for many years (Yeo *et al.* 1987; Skinner & Radin 1994; Hose *et al*. 2001) because of the unique properties of water and the difficulties of using tritiated water as a tracer (Garland 1980). Consequently, a variety of other tracers, very dissimilar in physical properties to water, have been tested. One of these, trisodium-8-hydroxy-1,3,6-pyrenetrisulphonic acid (PTS), is a water soluble fluorescent, non-toxic substance that does not cross cell membranes or adhere to cell walls (Strugger 1939; Peterson, Emanuel & Humphreys 1981; Yeo *et al.* 1987; Skinner & Radin 1994; Vesk *et al*. 2000). Hence, PTS has been used as a tracer in the studies of bypass flow in plants such as maize and broad bean (*Vicia faba* L.) (Peterson *et al.* 1981), red pine (*Pinus resinosa* Ait.) (Hanson, Sucoff & Markhart 1985), grey mangrove (*Avicennia marina*) (Moon *et al*. 1986), cotton (*Gossypium hirsutum* L.) (Skinner & Radin 1994), *Opuntia ficus-indica* (North & Nobel 1996), onion (*Allium cepa* L.) (Cholewa & Peterson 2001) and rice (Yeo *et al.* 1987, 1999; Yadav, Flowers & Yeo 1996; Garcia *et al.* 1997; Ochiai & Matoh 2002; Anil *et al*. 2005; Bridges 2005; Roshandel 2005; Gong, Randall & Flowers 2006; Sobahan *et al.* 2009). PTS can be detected and identified by its fluorescence using spectrofluorometry (Yeo *et al*. 1987), epifluorescence microscopy or confocal laser scanning microscopy (CLSM) (Cholewa & Peterson 2001).

Yeo *et al*. (1987) investigated bypass flow and Na^+ uptake in rice subjected to salinity stress of 25 mM NaCl for 24 h and reported a strong positive correlation between the transport of Na^+ and a bypass flow tracer, PTS, in rice genotypes IR2153 and IR26. Similarly, Yadav *et al*. (1996) found a significant relationship between Na^+ and PTS transport in the shoots of rice within the cv. IR36 subjected to 25 mM NaCl for 24 h. The authors indicated that the high-Na^+-transporting line (IR36-43-13-2-3-3-1) took up more PTS than the low-Na^+-transporting line (IR36-59-7-5-1-1-1) and suggested using bypass flow as a selection criterion for salinity resistance in rice. Garcia *et al*. (1997) showed that the magnitude of bypass flow in rice cv. IR36 after exposure to 50 mM NaCl for 48 h was 5.5%, which was about 10 times greater than that of bread wheat

(cvs. Kharchia-65 and Punjab-85) subjected to the same salt stress conditions and confirmed a strong relationship between Na^+ and PTS uptake in rice, but not in wheat. A significant correlation between Na^+ and PTS concentrations in the shoots of IR36 has also been seen in rice seedlings grown in 50 mM NaCl for 24 h (Bridges 2005). Although bypass flow represents a small percentage of the transpiration stream, usually about 1-6%, in 25-50 mM NaCl stress, it is enough to account for toxic concentrations of Na^+ in the shoots and could be an important pathway in Na^+ transport at high transpiration rates and at high external concentrations (Yeo *et al.* 1987, 1999; Yeo 1992; Garcia *et al.* 1997). Anil *et al.* (2005) reported that the percentage of bypass flow in salt-tolerant Pokkali and salt-sensitive rice Jaya was approximately 54 and 68%, respectively, when subjected to 200 mM NaCl for 48 h. A comparative study amongst rice, barley and maize showed that rice had much higher accumulation of PTS in the shoots than barley (by approximately 2000 times) and maize (by 27 times) when exposed to 50 mM NaCl for 24 h (Bridges 2005).

The presence of Casparian bands and suberin lamellae on the walls of exodermal and endodermal cells has been reported to be a barrier to bypass flow of water, solutes and fluorescent dyes (Slatyer 1967; Peterson *et al*. 1981; Peterson & Enstone 1996; Steudle & Frensch 1996; Steudle & Peterson 1998; Steudle 2000a, b, 2001; Hose *et al*. 2001; Miyamoto *et al*. 2001; Enstone, Peterson & Ma 2003; Ma & Peterson 2003; Taiz & Zeiger 2006). For example, Enstone & Peterson (1998) showed that the inward movement of a bypass flow tracer, berberine-thiocyanate, was blocked by the exodermal Casparian bands in roots of maize. Cholewa & Peterson (2001) found that PTS fluorescence was observed in the cortex of onion roots where the exodermal Casparian bands were not developed, whereas the fluorescence had disappeared in the root zone that had Casparian bands. The existence of the exodermal and endodermal layers was also shown to reduce the ability to take up water (hydraulic conductivity) of rice roots cvs. Azucena and IR64 (Miyamoto *et al*. 2001). Similarly, Armstrong & Armstrong (2005) reported that the suberisation of exodermal and endodermal layers, induced by sodium sulphide (Na_2S), reduced water uptake and bypass flow of ferrous ion (Fe^{2+}) in roots of rice cv. Norin36. Soukup *et al*. (2007) found that the penetration of periodic acid (HIO_4), a bypass flow tracer, to the root cortex of common reed (*Phragmites australis*) was prevented by suberin lamellae of the exodermis. In rice, Krishnamurthy *et al.* (2009) demonstrated that salt-tolerant Pokkali developed more

extensive Casparian bands and suberin lamellae on the walls of exodermal and endodermal cells than salt-sensitive rice cv. IR20 in response to 100 mM NaCl for 7 d, and Pokkali accumulated a lower shoot Na^+ concentration than IR20. As a result, it was concluded that the development of Casparian bands and deposition of suberin lamellae on exodermal and endodermal walls of rice roots were correlated with reduced Na^+ uptake to the shoots and increased survival of seedlings in saline conditions. Apart from the exodermis and endodermis, the thickening of walls of the sclerenchymatous layer with lignin, suberin and/or other phenolic compounds (Kondo *et al*. 2000; Insalud *et al*. 2006) is also believed to be a barrier to bypass flow of water and ions (Clark & Harris 1981; Galamay *et al.* 1991; Kondo *et al*. 2000; Miyamoto *et al.* 2001; Ranathunge, Steudle & Lafitte 2003).

However, such barriers may be interrupted during lateral root formation and create the area for leakage of solutes into the main root (Peterson *et al*. 1981; Enstone & Peterson 1992, 1998; Ranathunge, Steudle & Lafitte 2005a, b). For example, Peterson *et al.* (1981) reported that the dyes Tinopal CBS and PTS, tracers for bypass flow, moved along the margins of lateral roots into the stele of the primary roots of maize and broad bean. Enstone & Peterson (1992) found that the emergence of lateral roots provided the area for the inward movement of bypass flow tracer berberine-thiocyanate into the cortex of sunflower (*Helianthus annuus* L.). Similarly, Enstone & Peterson (1998) noticed that the berberine-thiocyanate tracer entered the primary root cortex of maize along the side of emerged lateral roots. Ranathunge *et al*. (2005a, b) reported that the emergence of lateral roots in rice cvs. IR64 and Azucena created leakage in the exodermal and endodermal Casparian bands and allowed copper (Cu^{2+}) and ferrocyanide ($[Fe(CN)_6]^{4-}$) ions as well as water to move apoplastically from the cortex into the stele. There is also evidence to suggest that bypass flow is not only located where lateral roots emerge, but also in the lateral roots themselves. For example, North & Nobel (1996) reported that PTS was observed in the xylem of lateral roots as well as in the main roots of *Opuntia ficus-indica* L. grown in drying soil for 30 d before rewetting for 3 d. Enstone & Peterson (1998) reported that, in maize, the bypass flow tracer berberine moved into the xylem of the main root through the xylem of the lateral roots. Similarly, others have reported that bypass flow tracers such as HIO_4 and Fe^{2+} reached the stele of nodal roots growing from rhizomes of common reed via the vascular tissues of the lateral roots (Soukup, Votrubova & Cizkova 2002).

Although it has been shown that bypass flow is a significant pathway for Na^+ uptake in rice under saline conditions (Yeo *et al.* 1987, 1999; Yeo 1992; Yadav *et al.* 1996; Garcia *et al.* 1997; Tester & Davenport 2003; Anil *et al*. 2005; Bridges 2005; Gong *et al*. 2006; Krishnamurthy *et al*. 2009; Sobahan *et al.* 2009; Plett & Moller 2010; Singh & Flowers in press), the physical location of this pathway has not been established.

1.4 Ontogenetic changes in effects of NaCl on rice

The sensitivity of rice to salinity is dependent on the developmental stage under investigation (Yoshida 1981; Lutts *et al.* 1995; Singh & Flowers in press). Germination is relatively tolerant to salinity. A study on the effect of NaCl on germination of nine genotypes of rice (Kalijira, Shakkorkhora, Khaskani, Pokkali, Kalobail, Ashami, IPK37008, IPK37011 and BR11) showed that more than 80% of germination was observed in 150 mM NaCl (Khan, Hamid & Karim 1997). Even at a concentration as high as 200 mM NaCl, the landrace Saunji showed 92% germination (Agnihotri, Palni & Pandey 2006).

The early seedling stage (1-3 weeks) is considered to be salt-sensitive. Pearson, Ayers & Eberhard (1966) reported that the growth of six genotypes of rice (Bluebonnet, Caloro, Zenith, Agami Montakhab I, Asahi No. 1 and Kala-Rata) was reduced by 75% when 3-day-old seedlings were treated with 45 mM NaCl for 28 d in sand. Flowers & Yeo (1981) found that 50% of rice IR2153 plants were dead after 11 d when 7-day-old seedlings were salinised with 50 mM NaCl, but half the seedlings survived for 25 and more than 45 d when salinised at the age of 14 or 35 d, respectively.

The tillering stage is relatively tolerant to salinity. For instance, a study reported by Lutts *et al.* (1995) indicated that the number of leaves of the main culm was not different amongst varieties and survival percentage was about 90-100% when 10 genotypes of rice (I Kong Pao, Aiwu, Tainung 67, IR4630, IR2153, IR31785, Pokkali, Nona Bokra, Buhra Rata and Panwell) were treated with 20, 30, 40 and 50 mM NaCl for 20 d at the age of 55 d. Their results also showed that the salinity treatment did not significantly affect the number of tillers in the majority of varieties examined as compared to non-salinised control plants.

The reproductive (panicle initiation, anthesis and fertilisation) stage has been shown to be salt-sensitive. Khatun & Flowers (1995a) reported that seed setting was markedly reduced when IR36 was salinised with 10, 25 or 50 mM NaCl between 1-month-old and when the main tiller flowered. Although pollen viability, determined by staining with tetrazolium salt 3-(4,5-dimethylithyazolyl)-2,5-diphenyl monotetrazolium bromide, was significantly decreased by approximately 20 and 30% in IR36 subjected to 25 or 50 mM NaCl at panicle initiation (Khatun & Flowers 1995b), the authors (Khatun & Flowers 1995a) found that the adverse effect of salinity was more pronounced on stigmatic receptivity than pollen viability. For example, the percentage of seed set was dramatically reduced by 38, 72 and 100% when the female plants were grown in 10, 25 and 50 mM NaCl, respectively, whereas it was reduced by only 10 and 20% when the male plants were subjected to 10 and 25 mM NaCl, respectively (Khatun & Flowers 1995a). In the same way, in a study of five rice genotypes (IR15324, IR2153, IR26, IR54 and BR6) subjected to 50 mM NaCl at panicle initiation (and remaining at this salinity until maturity), Khatun *et al.* (1995) observed that salinity significantly delayed (by 5 d) or inhibited flowering, reduced the number of productive tillers (by 50%), decreased seed set per panicle (by 80%), reduced grain yield per plant (by 90%) and decreased grain weight (by 40%). A significant reduction in flowering percentage, the number of fertile tillers, pollen viability, seed setting and grain weight was also reported by Lutts *et al.* (1995) when seven rice genotypes (IR2153, IR31785, IR4630, Aiwu, I Kong Pao, Tainung 67 and Panwell) were treated with 50 mM NaCl at the age of 75 d and remained under saline condition up to harvest. A study by Abdullah, Khan & Flowers (2001) of 1-month-old rice cv. IR28 subjected to 50 mM NaCl until maturity revealed that the sterility in seed set under NaCl stress was correlated with a reduction in soluble carbohydrate concentrations and an inhibition of starch synthetase, an important enzyme in developing rice grain, as a result of a high accumulation of Na^+ in rice spikelets.

1.5 Factors affecting Na^+ tolerance in rice

The mechanisms employed by plants in general to tolerate salinity have been frequently reviewed (e.g. Flowers *et al*. 1977; Greenway & Munns 1980; Amtmann & Sanders 1999; Munns 2002; Munns *et al.* 2002; Tester & Davenport 2003; Flowers & Colmer

2008; Munns & Tester 2008; Plett & Moller 2010; Zhang *et al.* 2010). For rice, salinity tolerance is a complex issue, which includes restriction of entry of Na^+ into the root xylem, reducing Na^+ uptake to the shoots, leaf-to-leaf distribution, compartmentation of Na^+ within and between cells and plant vigour (Yeo & Flowers 1984a, 1986; Yeo *et al.* 1990; Yeo 1992; Flowers *et al.* 2000; Singh & Flowers in press).

1.5.1 Restriction of entry of Na^+ into the root xylem

Restriction of entry of Na^+ into the root xylem (also known as Na^+ exclusion; Munns 2005) has been suggested to be the most efficient mechanism to control Na^+ accumulation in plants, since if the Na^+ uptake is reduced, other mechanisms do not need to be involved (Zhang *et al.* 2010). It was reported that when grown in 50 mM NaCl, bread wheat (cvs. Janz, Chinese Spring, Kharchia-65 and Punjab-85) and durum wheat (cvs. Wollaroi, Langdon and Line 149) excluded approximately 97% of the Na^+ in culture solution, whereas rice cv. IR36 excluded 91% (Garcia *et al.* 1997; Munns 2005; Gong *et al.* 2006); the most salt-tolerant variety of rice (Pokkali) was found to exclude only 94% (Flowers *et al.* 1988). Furthermore, when Pokkali was grown in higher salinity stress such as 200 mM NaCl, its ability to exclude Na^+ from the transpiration stream was only 59% of the Na^+ in the culture solution (Anil *et al.* 2005). Consequently, rice is considered as a poor Na^+ excluder, leading to the rapid accumulation of a lethal Na^+ concentration in the shoots (Flowers & Yeo 1981; Yeo & Flowers 1982, 1983; Flowers *et al.* 1988; Yeo *et al*. 1999; Roshandel & Flowers 2009). Interestingly, barley, the most salt-tolerant cereals, was found to exclude a similar proportion of the Na^+ in the culture solution (~ 94 to 95%) to rice (Munns 2005; Shabala *et al.* 2010), but it shows much higher tolerance to Na^+ than rice, indicating the complexity of salt tolerance and that a single mechanism does not determine the tolerance (Singh & Flowers in press).

1.5.2 Reducing Na^+ uptake to the shoots

Na^+ and other solutes reach the shoots of rice, as in other plants, by way of the transpiration stream (Yeo & Flowers 1984b; Yeo *et al.* 1987, 1999; Flowers *et al.* 1988; Leigh & Tomos 1993; Fricke *et al*. 1995; Fricke, Leigh & Tomos 1996; Robinson *et al.* 1997; Fricke 2004b; Backhausen *et al*. 2005; Gong *et al*. 2006; Plett & Moller 2010). Therefore, reducing the transpiration rate should lower the net transport of Na^+ to the

shoot. An ability to control transpiration has been proposed as an important mechanism for plants growing in saline conditions (Yeo 1992; Tester & Davenport 2003) and the ability to control transpiration can be expressed as either an absolute transpiration volume or the ratio between growth and transpiration, namely water-use efficiency, WUE (Flowers *et al.* 1988; Yeo 1992). In a study on the effect of salinity (50 mM NaCl for 7 d) on WUE of seven rice genotypes (Pokkali, Bhura Rata, Amber, IR2153, IR4630, IR36 and IR28), a positive correlation between WUE and salinity resistance was observed: the greater the WUE, the greater the resistance to salinity (Flowers *et al.* 1988). Consistently, Yadav *et al.* (1996) reported that a low-Na^+-transporting line of rice cv. IR36 had a higher WUE than a high-Na^+-transporting line.

Generally, transpiration rates in all plants tend to decrease with increasing salinity levels in the culture solution (Robinson *et al.* 1997; Tester & Davenport 2003). It has been hypothesised that low root and shoot water potential triggers ABA synthesis in the roots which is then released to the xylem before being transferred to leaves to induce stomatal closure, and thus decrease transpiration (Asch, Dorffling & Dingkuhn 1995). Moradi & Ismail (2007) also showed that salt-tolerant rice lines IR632 and IR651 expressed lower transpiration than salt-sensitive rice cv. IR29 in response to a salinity of 100 mM NaCl for 14 d and that reduction of the transpiration volume was correlated with less Na^+ accumulation in the shoots of IR632 and IR651 than IR29.

1.5.3 Leaf-to-leaf distribution

When Na^+ was transported to the shoot of rice, Na^+ was not accumulated uniformly between the leaves, but higher concentrations occurred in the older than the younger leaves (Yeo & Flowers 1982, 1984a, 1986; Yeo *et al.* 1985a, b). An ability to keep Na^+ in the older leaves and maintain the younger leaves at low Na^+ concentrations is crucial for plant survival under saline conditions (Yeo & Flowers 1982). It was found that when exposed to 50 mM NaCl for 12 d, the concentration of Na^+ in leaves of salt-sensitive rice cv. IR28 increased rapidly in both the older and younger leaves, whereas it was filled up only in the older leaves of salt-tolerant Pokkali and IR4630; both Pokkali and IR4630 were able to maintain Na^+ concentrations in the younger leaves below 1 mmol/gDW for 12 d, whereas IR28 was able to achieve this only for 3-4 d (Yeo & Flowers 1982). As a consequence, it was claimed that salt-tolerant varieties were better

at maintaining lower Na^+ concentrations in their younger leaves than salt-sensitive ones (Yeo & Flowers 1986; Singh & Flowers in press). The loss of a few old leaves does not affect the growth of a large plant, but if old leaves die more quickly than new ones develop, then the growth rate would be dramatically reduced and eventually the plant will die (Yeo & Flowers 1982; Yeo *et al.* 1991; Munns 2005; Munns & Tester 2008). Thus, maintaining the leaf-to-leaf distribution is beneficial for rice in order to retain photosynthesis for growth and maintain rates of leaf initiation at least equal to the rate of leaf death (Yeo & Flowers 1982, 1984a, 1986; Yeo *et al.* 1985a, b).

The discontinuous distribution of Na^+ from leaf to leaf in rice could not be explained by a dilution by growth in the younger leaves or by integrating time of exposure to salt, leading to more Na^+ in older leaves (Yeo & Flowers 1984a, 1986; Singh & Flowers in press). The evidence came from a tracer study using radio-labelled $^{22}Na^+$ by Yeo & Flowers (1982). Their results showed that after short-term exposure of 14-day-old rice seedlings IR2153 to 50 mM NaCl (labelled with $^{22}Na^+$) for 48 h, $^{22}Na^+$ increased rapidly in the older leaves 2 and 3, while the increase in the younger leaves 4 and 5 was slow. The youngest leaf 5 accumulated less Na^+ than the oldest leaf 2 in both concentration and content of $^{22}Na^+$, indicating that the low $^{22}Na^+$ concentration in leaf 5 was due to low $^{22}Na^+$ input and not a consequence of dilution by growth (Yeo & Flowers 1982, 1984a).

The distribution pattern of Na^+ from leaf to leaf in rice is not positively correlated with their transpiration rates (Yeo *et al.* 1985b; Yeo & Flowers 1986; Singh & Flowers in press). Yeo *et al.* (1985b) simultaneously compared transpiration rates and Na^+ accumulation in leaves 1 to 4 of rice IR2153 subjected to 50 mM NaCl for 72 h using ^{14}C-ethane diol as a tracer for transpiration volume flow and $^{22}Na^+$ for Na^+. The results showed that transpiration rates of the young leaves 3 and 4 were higher than the older leaves 1 and 2, whereas the young leaves accumulated lower concentrations of Na^+ than the older ones (Yeo *et al.* 1985b).

It has been suggested that the distribution of Na^+ between leaves of rice is related to the properties of the roots supplying different leaves (Yeo *et al.* 1985b; Yeo & Flowers 1986; see Chapter 2 for root morphology and anatomy of rice). A recent study by Bridges (2005) supported that suggestion. It was shown that when the seminal root of

rice IR36 was treated with 50 mM NaCl for 16, 23 or 42 h, the oldest leaf 1 accumulated a higher Na^+ concentration than the youngest leaf 3; however, when adventitious roots were treated, no significant difference in Na^+ concentration between leaves 1 and 3 was observed. Consistently, when the seminal root was removed, a dramatic decrease in the concentration of Na^+ in leaf 1 was apparent, whereas Na^+ concentrations in leaf 3 slightly increased. As a result, the author concluded that Na^+ concentrations in old leaves were supplied dominantly by the seminal root, whereas younger leaves were dominantly fed by adventitious roots (Bridges 2005). Although the results implied that the seminal root is responsible for the discontinuous distribution of Na^+ from leaf to leaf in rice under salinity, it is still unclear how such a phenomenon is achieved (Singh & Flowers in press).

Apart from the discontinuous distribution between leaves, a gradient of Na^+ concentration along the leaf sheath and leaf blade has also been observed in rice. It was found that Na^+ concentration in leaf sheaths was higher than that of leaf blades in rice IR2153 when exposed to 50 mM NaCl for 40 d (Yeo & Flowers 1982). Similarly, Matsushita & Matoh (1991) reported that the shoot base region, including the mesocotyl and leaf sheaths, of rice cv. Kinmaze growing hydroponically in 10, 25 or 50 mM NaCl for 7 d accumulated more Na^+ than leaf blades. It was also seen in rice cv. Nipponbare when subjected to 257 mM NaCl for 2 weeks in soil (Mitsuya *et al.* 2002). Gong *et al.* (2006) also reported similar results for discontinuous distribution between leaf sheath and leaf blade in rice IR36 grown hydroponically in 50 mM NaCl for 2 d. Taken together, an ability to retain Na^+ in leaf sheaths rather than leaf blades could be beneficial for rice subjected to salinity as this could reduce the quantity of Na^+ reaching the photosynthetic area and subsequently extend seedling survival.

1.5.4 Compartmentation of Na^+ within and between cells

Once Na^+ has entered the cells, it has to be compartmentalised in the vacuoles in order to maintain low cytoplasmic Na^+ concentrations and a high K^+/Na^+ ratio (Flowers *et al.* 1977; Fricke *et al.* 1996; Munns & Tester 2008; Plett & Moller 2010). Na^+ in the cytoplasm is toxic to the cells because it will inhibit enzyme activities and interrupt metabolic processes by competing with K^+ for the binding sites (see Section 1.2).

The compartmentation of Na^+ from the cytoplasm into the vacuole is mediated by the vacuolar Na^+/H^+ antiporter (NHX) that is driven by the proton gradient generated by the vacuolar H^+-translocating enzymes, H^+-ATPase and H^+-PPiase (Blumwald *et al.* 2000; Apse & Blumwald 2007). The compartmentation of Na^+ into the vacuole reduces the deleterious effects of Na^+ in the cytoplasm, creates an osmotic potential for driving water into the cells and thus extends survival (Blumwald *et al.* 2000). A gene *OsNHX1* encoding the vacuolar Na^+/H^+ antiporter in rice has been identified from Nipponbare (Fukuda, Nakamura & Tanaka 1999). Transgenic rice lines 95-3-2 and 95-4-12 of cv. Nipponbare overexpressing *OsNHX1* could maintain growth at 100 mM NaCl for 7 weeks, while the wild-type line 97-1-29 eventually died (Fukuda *et al.* 2004). Similarly, over-expression of *OsNHX1* increased salt tolerance in transgenic rice plants (IRAT109) growing in 200 mM NaCl for 18 d, whereas wild-type plants did not survive (Chen *et al.* 2007). Also, Verma *et al.* (2007) found that over-expression of *PgNHX1* from a halophytic plant, *Pennisetum glaucum*, increased salt tolerance in transgenic rice plants (PB1) which were able to complete their life cycle and produce seeds at 150 mM NaCl, a salt concentration that the wild-type plants could not endure.

In addition to being compartmentalised in the vacuoles, Na^+ from the cytoplasm may be extruded to the apoplast by the plasma membrane Na^+/H^+ antiporter (also known as salt overly sensitive 1, SOS1; Singh & Flowers in press) that is energised by the proton gradient generated by the plasma membrane H^+-ATPase (Blumwald *et al.* 2000; Apse & Blumwald 2007). Although OsSOS1 has been identified in rice (Martinez-Atienza *et al.* 2007), its role in salt tolerance is still unclear (Singh & Flowers in press) as extruding Na^+ back to the cell walls would cause cell dehydration and reduce plant survival (see Section 1.2).

Once Na^+ is sequestered in the vacuoles, compatible solutes (also known as osmoprotectants) that are compatible with metabolic activity and do not inhibit biochemical reactions even at high concentrations should be accumulated in the cytoplasm to balance the osmotic potential of Na^+ in the vacuoles (Flowers *et al.* 1977; Tester & Davenport 2003; Ashraf *et al.* 2008; Munns & Tester 2008; Singh & Flowers in press). The most common of these compatible solutes are glycinebetaine, proline, mannitol and sucrose (Flowers *et al.* 1977; Greenway & Munns 1980; Ashraf & Harris 2004; Ashraf *et al.* 2008; Singh & Flowers in press).

Rice does not synthesise glycinebetaine due to lacking of a functional gene for choline monooxygenase (CMO), which catalyses choline to betaine aldehyde before conversion to glycinebetaine by betaine aldehyde dehydrogenase (BADH) (Rathinasabapathi *et al.* 1993; Luo *et al.* 2007; Singh & Flowers in press). Although rice does not accumulate glycinebetaine, Shirasawa *et al.* (2006) reported that transgenic rice lines of cv. Sasanishiki transformed with a *CMO* gene from spinach (*Spinacia oleracea* L.) accumulated significant amount of glycinebetaine and grew better than the wild type when exposed to 150 mM NaCl for 5 d in soil. Others have also shown that exogenous application of glycinebetaine increased the concentrations of glycinebetaine in the shoots and enhanced salt tolerance in rice by preventing the loss of relative water content (Cha-um, Supaibulwatana & Kirdmanee 2006), alleviating membrane damage (Demiral & Turkan 2004) and reducing bypass flow (Sobahan *et al.* 2009).

Rice accumulates proline under saline conditions (Singh & Flowers in press). Kumar *et al.* (2008) reported that proline content increased in both shoots and roots of rice cvs. Karjat-3 and Panvel-3 soil grown in 50-300 mM NaCl for 7 d and the magnitude of proline accumulation was greater in salt-tolerant Panvel-3 than salt-sensitive Karjat-3. Similarly, Chutipaijit, Cha-um & Sompornpailin (2009) found that proline content in the shoots of salt-tolerant rice cv. Sangyod was higher than that of salt-sensitive Pathumthani 1 after exposure to 100 mM NaCl for 4 d under phototrophic conditions and the proline level was positively correlated with relative water content, but was negatively correlated with malondialdehyde content, a product of lipid peroxidation of cell membrane. Over-accumulation of proline increased biomass production in transgenic rice seedlings of cv. Kenfong transformed with an important enzyme in proline synthesis pathway, Δ^1-pyrroline-5-carboxylate synthetase (*p5cs*) cDNA (from *Vigna aconitifolia* L.) when exposed to 300 mM NaCl for 30 d in soil (Su & Wu 2004). Exogenous application of proline has also been shown to reduce Na^+ concentration in the shoot of rice subjected to NaCl stress (Sobahan *et al.* 2009).

Mannitol has been claimed to enhance salt tolerance in rice. For example, Pujni, Chaudhary & Rajam (2007) reported that transgenic lines of rice Pusa Basmati 1 expressing an *E. coli* mannitol-1-phospho dehydrogenase gene accumulated higher mannitol concentration and exhibited greater tolerance to salinity than the wild type when salinised with 150 mM NaCl for 20 d. In contrast, the role of sucrose in rice under

saline conditions is unclear, although it was found to increase in some genotypes under salinity. For example, Pattanagul & Thitisaksakul (2008) reported that increased accumulation of sucrose was seen in leaves of salt-sensitive rice Khao Dawk Mali 105 subjected to 50-150 mM NaCl in soil for 9 d, whereas it was unchanged in salt-tolerant Pokkali. The authors speculated that an increase in sucrose concentration in salt-sensitive variety was due to the limitation of growth caused by salinity.

Apart from the compartmentation of Na^+ in the vacuoles and the synthesis of compatible solutes in the cytoplasm, it has been reported that different cell types of rice leaves accumulated different concentrations of Na^+ when subjected to salt stress. For example, X-ray microanalysis on leaf 3 of rice cv. Cigaron after 24 h exposure to 25 mM NaCl and 48 h with 50 mM NaCl showed that the highest Na^+/K^+ ratio was found in the bundle sheath cells, whereas this ratio was lowest in the mesophyll, indicating that the bundle sheath cells were the first tissue to accumulate Na^+ and probably less sensitive to Na^+ than other cells (Yeo & Flowers 1984a).

1.5.5 Plant vigour

Plant vigour contributes to salinity resistance in rice by providing dilution of Na^+ by growth (Yeo & Flowers 1984a; Yeo *et al.* 1990; Yeo 1992). This characteristic has been seen in the tall landraces of rice, Pokkali and Bhura Rata (Yeo & Flowers 1984a; Flowers *et al.* 1988). Yeo & Flowers (1984a) studied the time course of net Na^+ transport to the shoot (Na^+ content in the shoot per root dry weight per unit of time), shoot Na^+ concentration and shoot dry weight between salt-tolerant Pokkali and salt-sensitive rice cv. IR22 exposed to 50 mM NaCl for 8 d. Their results clearly showed that Pokkali and IR22 had the same initial net Na^+ transport to the shoot and Pokkali had a grater rate than IR22 in later state, but shoot Na^+ concentration in Pokkali was significantly lower than IR22. As a result, the authors explained that the low shoot Na^+ concentration in Pokkali was attributed to the dilution by growth as it was shown that shoot dry weight of Pokkali was greater than IR22. Yeo *et al.* (1990) also reported that vigour was more strongly correlated with survival of seedlings under saline conditions than shoot Na^+ concentration.

1.6 Approaches to enhancing salt tolerance in rice

1.6.1 Screening

As pointed out earlier, many physiological characteristics contribute to salt tolerance in rice, including restriction of entry of Na^+ into the root xylem, reducing Na^+ uptake to the shoots, maintaining a leaf-to-leaf distribution, compartmentation of Na^+ within and between cells and plant vigour (see Section 1.5). Yeo & Flowers (1986) suggested that salt tolerance of rice could be enhanced by selecting for each physiological characteristic and then combining them. Although the procedure is conceptually simple, it requires suitable screening criteria (Flowers & Yeo 1995). An exposure of 14-day-old seedlings of rice genotypes IR2153 and IR28 to different NaCl concentrations (50, 100 and 150 mM) for 10 d revealed that the concentrations of 100 and 150 mM NaCl markedly reduced the growth, indicated by shoot dry weight, of both salt-tolerant IR2153 and salt-sensitive IR28 by 70-80%, whereas a concentration of 50 mM NaCl showed a significant difference between salt-tolerant and salt-sensitive genotypes, in which IR2153 grew better than IR28 by approximately 60% (Flowers & Yeo 1981). Therefore, it was established that 50 mM NaCl, equivalent to an EC of 6 dS/m or an osmotic potential of about – 230 kPa, was a useful concentration, producing a wide range of varietal responses in rice (Flowers & Yeo 1981; Yeo & Flowers 1984a, 1986) and a concentration of 50 mM NaCl has been reported to reduce the grain yield of many varieties of rice by 50% (Yeo & Flowers 1986; Khatun *et al.* 1995).

A number of traits have been used for screening salt tolerance in rice, including leaf Na^+ concentration (Yeo & Flowers 1983; Yeo *et al.* 1988, 1990), leaf area per total dry weight (Akita & Cabuslay 1990), leaf-to-leaf distribution, tissue tolerance and plant vigour (Yeo *et al.* 1990), shoot fresh weight (Aslam, Qureshi & Ahmed 1993), K^+/Na^+ ratio (Asch *et al.* 2000), relative water content (Suriya-arunroj *et al.* 2004) and floret fertility (Khatun *et al.* 1995; Rao *et al.* 2008), but very few salt-tolerant varieties have been released (Flowers & Yeo 1995; Rozema & Flowers 2008) and no genotype of rice possesses a favourable combination of all those characteristics (Garcia *et al.* 1995). One of the main reasons for this is the lack of reliable and reproducible techniques for identifying salt tolerance (Yeo *et al.* 1990; Gregorio & Senadhira 1993; Garcia *et al*. 1995; Xie *et al.* 2000; Koyama *et al*. 2001; Yadav *et al.* 2008).

1.6.2 Wide crossing

One possible approach to increase salt tolerance in rice is the introduction of resistance genes from an already tolerant species via wild crossing (Flowers *et al.* 1990; Yeo *et al.* 1990; Latha *et al.* 2004; Sengupta & Majumder 2010). *Porteresia coarctata* Tateoka is highly salt-tolerant wild rice and is a relative of *Oryza sativa* L. (Flowers *et al.* 1990; Yeo *et al.* 1990). This species is native to coastal salt-marshes of India, Pakistan and Bangladesh and can withstand total submergence with seawater for 10 h /d (Flowers *et al.* 1990; Latha *et al.* 2004). Studies undertaken in the greenhouse showed that *P. coarctata* was able to survive in Na^+ concentration as high as 20% artificial seawater (approximately 200 mM NaCl) for 28 d without affecting growth in terms of shoot fresh and dry weight (Flowers *et al.* 1990). Anatomical study using scanning electron microscopy revealed that *P. coarctata* developed epidermal unicellular hairs on the adaxial surface of the leaf and salinisation could increase the number of these hairs (Flowers *et al.* 1990). X-ray microanalysis also showed that the vacuolar concentrations of Na^+ and Cl^- in these hairs were higher than those of the mesophyll and neighbouring epidermal cells and the concentrations of Na^+ and Cl^- in the hair vacuoles increased with increasing external NaCl concentration (Flowers *et al.* 1990). It would appear that these hairs act as salt glands to secrete excess salt from the leaves of rice to maintain a low leaf Na^+/K^+ ratio and to survive under extremely saline conditions (Flowers *et al.* 1990; Yeo *et al.* 1990).

Jena (1994) reported the successful production of a hybrid between female parents of *O. sativa* L. (cvs. IR28, IR36 and Tellahamsa) and a male parent of *P. coarctata*. However, it was found that the crossability of these rice varieties with *P. coarctata* was very low with only 0.46, 0.30 and 0.19% seed set for IR28, IR36 and Tellahamsa, respectively. Only 2 and 8 hybrid plants were generated from IR28 and IR36, respectively, and all of the hybrids were male sterile; crossing between Tellahamsa and *P. coarctata* did not produce any viable hybrid plants (Jena 1994).

Protoplast fusion of somatic cells between *O. sativa* L. (Taipei 309) and *P. coarctata* has been successfully claimed with 11% of plant generation from calli (Jelodar *et al.* 1999). The somatic hybrid lines were reported as being assessed in breeding programmes to determine their potential for the introduction of salt tolerance genes into

rice (Jelodar *et al.* 1999). The absence of recent publications related to these hybrid plants suggests the process is slow (Bridges 2005).

1.6.3 Genetic engineering

This is another approach to increase salt tolerance in rice. As pointed out above (see Section 1.5.4), transgenic rice lines have been reported to be more resistant to salinity than the wild-type control plants. Those transgenic characteristics include over-expression of the vacuolar Na^+/H^+ antiporter (*NHX1*) genes (Fukuda *et al.* 2004; Chen *et al.* 2007; Verma *et al.* 2007), over-accumulation of proline (Su & Wu 2004), glycinebetaine (Shirasawa *et al.* 2006) and mannitol (Pujni *et al.* 2007). The progress in the understanding of the mechanism of salt tolerance in the halophytic wild rice, *P. coarctata*, will also provide sources of salt tolerance genes to be transferred to salt-sensitive cultivated rice (Section 1.6.2). However, any transgenic plants need to be assessed for their tolerance in the field since the conditions in the field are very different from those in the laboratory where there is often little or no transpiration (Flowers 2004).

1.6.4 Reducing Na^+ transport to the shoot

As pointed out above (Section 1.3.2), it is clear that bypass flow is an important pathway for Na^+ uptake in rice under saline conditions. Therefore, if bypass flow were restricted, Na^+ transport to the xylem would be decreased. It has been reported that an addition of PEG and silicon (Si) to the culture solution in which rice plants were growing significantly reduced shoot Na^+ concentrations in seedlings growing under salinity (Yeo & Flowers 1984b; Matoh *et al*. 1986; Yeo *et al.* 1999; Ochiai & Matoh 2004; Gong *et al.* 2006). Relative humidity (RH) has also been shown to reduce Na^+ uptake in rice subjected to salinity (Yeo & Flowers 1984b; Asch *et al.* 1999, 2000). However, it is unknown how RH interacts with the bypass flow.

1.7 Research objectives

The aim of this thesis was (1) to investigate whether the possible entry sites for Na^+ during bypass flow in rice under saline conditions are where lateral roots emerge or in the lateral roots themselves, (2) to study the effects of PEG, Si and RH on Na^+ uptake and bypass flow in rice and (3) to evaluate the possibility of using the bypass flow as a new screening technique for salt resistance in rice varieties.

It is, therefore, hypothesised in this thesis that under saline conditions, bypass flow in rice possibly occurs in regions of the roots where the lateral roots emerge from the main root or in lateral roots themselves. Chapters 2 and 3 thoroughly investigate these two hypotheses. Chapter 4 reports the relative effectiveness of PEG and Si in the reduction of bypass flow of Na^+ in rice varieties with different salt tolerance, and reports the results of an investigation of the effects of different RH levels on Na^+ uptake and bypass flow in rice subjected to salinity stress. Chapter 5 reports the results of an evaluating the possibility of using the bypass flow as a new screening technique for salt resistance in rice varieties.

CHAPTER 2

STUDIES ON SODIUM BYPASS FLOW IN LATERAL ROOTLESS MUTANTS *lrt1*, *lrt2* AND CROWN ROOTLESS MUTANT *crl1* OF RICE (*ORYZA SATIVA* L.)

2.1 INTRODUCTION

Rice is a salt-sensitive crop, especially during the seedling stage; under saline conditions, it is ineffective in controlling the influx of Na^+ to its shoots, leading to the accumulation of toxic concentrations in the leaves (Chapter 1, sections 1.1 and 1.4). Na^+ reaches the leaves via the transpiration stream, which it enters either symplastically or apoplastically (Chapter 1, section 1.3). Neither the mechanism by which Na^+ enters the symplast (Chapter 1, section 1.3.1), nor the precise pathway of bypass flow is yet clear in rice, although the latter has been shown to be significant for Na^+ uptake under saline conditions (Chapter 1, section 1.3.2). It has been postulated that the magnitude of bypass flow in rice depends on the anatomical and morphological developments of the roots (Yeo *et al.* 1987; Yeo 1992; Yadav *et al.* 1996; Garcia *et al.* 1997; Gong *et al.* 2006; Krishnamurthy *et al.* 2009). However, our understanding of the specific roles of particular roots in bypass flow is still poor.

Morphologically, the rice root system is composed of a seminal root, crown roots and lateral roots (Galamay *et al*. 1991; Yao, Taketa & Ichii 2002; Inukai *et al*. 2005; Suralta, Inukai & Yamauchi 2008). The seminal root of a rice plant emerges directly from the seed, becomes visible 2 or 3 d after germination and functions until the seventh leaf stage with a maximum length of about 150 mm (Yoshida 1981; Hochholdinger *et al*. 2004). Subsequently, the crown roots (also known as adventitious roots or nodal roots) that emerge from nodes of the culm are dominant in the root system (Yoshida 1981; Hochholdinger *et al.* 2004; Inukai *et al.* 2005). In rice, the seminal and crown roots normally consist of an outermost rhizodermis, an exodermis, a sclerenchymatous layer, cortical cell layers, the endodermis and stele (Clark & Harris 1981; Yoshida 1981; Ranathunge *et al*. 2003, 2004, 2005a; Schreiber *et al*. 2005; Gong *et al.* 2006;

Krishnamurthy *et al.* 2009). The exodermis and endodermis in the primary state are characterised by Casparian bands on the radial and transverse walls of the cells, with the deposition of suberin lamellae in tangential walls occurring during secondary development (Clark & Harris 1981; Ranathunge *et al.* 2003, 2004; Schreiber *et al.* 2005; Krishnamurthy *et al.* 2009). The endodermal Casparian bands have been observed as close as 5 and 20 mm to the root tip in seminal and crown roots, respectively, while exodermal Casparian bands were detectable at a distance of 30 mm from the tip (Clark & Harris 1981; Ranathunge *et al.* 2003; Krishnamurthy *et al.* 2009). The deposition of suberin lamellae started at distances of about 20 and 30 mm from the tip in endodermis and exodermis, respectively, and such depositions were fully matured at around 50-70 mm (Ranathunge *et al.* 2003, 2004; Schreiber *et al.* 2005). However, under salt stress (100 mM NaCl) it has been reported that suberin lamellae in rice (Pokkali and IR20) developed closer to the root tip and more extensively than in control plants, and the amount of suberin was significantly higher in soil-grown than in hydroponically grown plants (Krishnamurthy *et al.* 2009).

The presence of Casparian bands and suberin lamellae on the walls of exodermal and endodermal cells has been reported to be a barrier to bypass flow of water, solutes and fluorescent dyes (Chapter 1, section 1.3.2). Furthermore, the thickened walls of the sclerenchymatous layer with lignin, suberin and/or other phenolic compounds are believed to be a barrier to any apoplastic continuum that allows transport of water and ions (Chapter 1, section 1.3.2) or diffusion of oxygen (Colmer *et al.* 1998; Insalud *et al.* 2006); the sclerenchymatous layer functions as a protective coat when rhizodermis and exodermis are lost in mature roots (Clark & Harris 1981; Galamay *et al.* 1991).

Rice roots are unlike the roots of the majority of plants because of the presence of gas-filled spaces (aerenchyma) in the cortical layer (Armstrong 1971; Clark & Harris 1981; Yoshida 1981; McDonald, Galwey & Colmer 2002; Colmer, Cox & Voesenek 2006). This modification of the root cortex facilitates gas diffusion inside the plants between shoots and roots in response to hypoxic conditions caused by flooding (Armstrong 1971; Colmer *et al*. 1998; Jackson & Armstrong 1999; Colmer 2003; Voesenek *et al*. 2006; Colmer & Flowers 2008). The mechanism of aerenchyma formation in rice is known to be lysigenous (Clark & Harris 1981; Justin & Armstrong 1991; Jackson & Armstrong 1999; Voesenek *et al.* 2006) and enhanced by low oxygen or high ethylene

levels (Justin & Armstrong 1991; Colmer *et al.* 2006). As a consequence of the presence of aerenchyma, the area of the apoplast available for bypass flow is much reduced.

Barriers to bypass flow may be interrupted during lateral root emergence and create an opportunity for leakage of solutes into the main root (Chapter 1, section 1.3.2). Lateral roots originate from the pericycle of seminal and crown roots and play important roles in providing the absorptive area for water and other nutrients as well as to anchor the plant in the soil (Banoc *et al*. 2000; Ma *et al*. 2001; Bailey, Currey & Fitter 2002; Kamoshita *et al*. 2004).

The structure of the root system is controlled by endogenous factors such as genetic programmes and hormones and exogenous environmental factors such as nutrients and moisture (Reed, Brady & Muday 1998; Malamy & Ryan 2001; Al-Ghazi *et al*. 2003; Hochholdinger *et al.* 2004; Chhun *et al*. 2007; Suralta *et al.* 2008). Auxins have been reported to play a crucial role in regulating root architecture in rice (Hao & Ichii 1999; Inukai *et al*. 2001, 2005; Chhun *et al*. 2003a, b, 2004; Wang *et al*. 2006). Hao & Ichii (1999) isolated a 2,4-dichlorophenoxyacetic acid (2,4-D) resistant mutant from a population of the rice cv. Oochikara and named it lateral rootless1 (*lrt1*). The mutant *lrt1* produces no lateral roots, but shows no significant difference in seminal root length compared with the wild-type Oochikara (Hao & Ichii 1999).

Inukai *et al*. (2001) successfully isolated a rice mutant defective in crown root formation using *N*-methyl-*N*-nitrosourea in populations of cv. Taichung 65, and designated the mutant as crown rootless1 (*crl1*). The number of crown roots of *crl1* was significantly lower than that of the wild type, but no significant difference was observed for other morphological characteristics (Inukai *et al.* 2001). The defect in crown root formation of *crl1* is likely caused by the lack of ability to accumulate or respond to auxin in nodal regions (Inukai *et al.* 2001). Subsequent investigation by Inukai *et al*. (2005) concluded that the mutant *crl1* also had a decreased lateral root number on its seminal root, and reported that the *Crl1* gene was expressed in cells of the pericycle, endodermis of seminal and crown roots and in parenchyma cells adjacent to the peripheral vascular cylinder of the culm.

More recently, Wang *et al.* (2006) isolated a new lateral rootless mutant in rice cv. Nipponbare, namely lateral rootless2 (*lrt2*). The mutant *lrt2* was isolated by screening for resistance to 0.5 μM 2,4-D from M_2 lines of Nipponbare. *lrt2* exhibits several altered root phenotypes such as lack of lateral roots, reduced root gravity response and a longer seminal root as compared to the wild-type Nipponbare (Wang *et al.* 2006). However, no significant differences were found in plant height, number of crown roots and crown root length between the mutant and the wild type. By genetic analysis it was indicated that the recessive *lrt2* gene was localised on the short arm of chromosome 2 (Wang *et al.* 2006).

In this Chapter, I hypothesised that if bypass flow of Na^+ in rice seedlings originates at the sites where lateral roots emerge from the main root, then bypass flow and Na^+ concentration will be higher in plants with numerous lateral roots than ones with fewer laterals. I investigated bypass flow and Na^+ concentration in lateral rootless mutants (*lrt1*, *lrt2*), a crown rootless mutant (*crl1*), their wild types (Oochikara, Nipponbare and Taichung 65, respectively) and in seedlings of the rice cv. IR36 in which the number of lateral and crown roots had been reduced through physical and hormonal manipulations. The study sought to correlate the numbers of lateral roots with Na^+ accumulation in an attempt to shed more light on the possible sites of entry for Na^+ during bypass flow in rice.

2.2 MATERIALS AND METHODS

2.2.1 Plant material and growth conditions

Caryopses of rice (*Oryza sativa* L.) cvs. Oochikara, *lrt1*, Nipponbare, *lrt2*, Taichung 65, *crl1* and IR36 were imbibed in aerated water for 24 h before being sown on nylon mesh floated on modified Yoshida culture solution (Appendix 2.1). Uniform 7-day-old seedlings were transplanted into individual black-painted tubes (50 ml) filled with culture solution without aeration (Figure 2.1). Plants were held in place with non-absorbent cotton wool and grown in a plant growth cabinet (a modified Conenviron Model E15; Winnipeg, MB, Canada), in which the photoperiod was 12 h with photosynthetically active radiation of 375-400 μmol m^{-2} s^{-1} (Osram Powerstar HQ1-BT

lamps, Berlin, Germany) at 29±1 °C and the RH was 40-50%. During the dark period (12 h), the temperature was 19.5±0.5 °C and the RH was 50-60%.

Figure 2.1 Seedlings of rice (*Oryza sativa* L.) growing in individual 50ml-black-painted tubes filled with modified Yoshida culture solution. Seedlings were held in place with non-absorbent cotton wool.

2.2.2 NaCl and PTS treatment in wild-type and mutant rice

Twenty-day-old rice seedlings of Oochikara, *lrt1*, Nipponbare, *lrt2*, Taichung 65 and *crl1* (with four fully developed leaves) were treated with 50 mM NaCl and 100 mg/l PTS (0.2 mM). Plants were allowed to take up Na^+ and PTS for 72 h, after which the treated solution was replaced by culture solution for a chase period of 48 h to allow transpiration to carry any Na^+ and PTS in the root xylem to the shoot. Shoots were harvested and dried in an oven at 80 °C for 72 h.

2.2.3 NaCl and PTS treatment in reduced lateral root and crown root seedlings using mechanical manipulation

Seminal roots and those crown roots with laterals were cut (above the solution) from 19-day-old seedlings of IR36 to eliminate all laterals, leaving only crown roots lacking laterals. In another set of plants of IR36, all the crown roots were cut, leaving only seminal roots. Seedlings were left in culture solution for 24 h to recover from the damage due to experimental handling by sealing the cut ends of xylem vessels (Yeo *et al.* 1987). Then, seedlings of lateral-root-cut, crown-root-cut and the controls (uncut) were treated with 50 mM NaCl and 100 mg/l PTS for 72 h. Shoots were harvested after a chase period of 48 h in culture solution before being dried in an oven at 80 °C for 72 h.

2.2.4 NaCl and PTS treatment in 2,4-D treated seedlings

Caryopses of rice cv. IR36 were imbibed in aerated water for 24 h before being sown on nylon mesh floated on culture solution for 4 d. Uniform seedlings were transferred to black-painted boxes (2 l) in culture solution with or without 0.05 µM 2,4-D. After 8 d in solutions, seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 72 h. Then, shoots were harvested after a chase period as above and dried in an oven at 80 °C for 72 h.

2.2.5 Morphological characteristics of seedlings

The lengths of seminal and crown roots were measured with a ruler. Root surface area per plant was analysed using Delta-T SCAN software (version 2.04nc, Delta-T Devices Ltd., UK). The numbers of lateral roots and crown roots per plant were counted either with Delta-T SCAN software or by eye, while the root system was laid out on a tray and submerged in distilled water.

2.2.6 Root anatomy

Seminal roots at a distance of 45 – 55 mm from the root tip were cross-sectioned (freehand) with a sharp razor blade. Root sections were stained for 1 h with 0.01% (w/v) Fluorol Yellow 088 (Brundrett, Kendrick & Peterson 1991) to check the presence of suberin lamellae. The sections were observed with an epifluorescence microscope using an ultraviolet filter set (excitation filter G 365, chromatic beam splitter FT 395, barrier filter LP 420; Zeiss Axiophot, Oberkochen, Germany). The number of central conducting vessels (late metaxylem) was counted, and diameters of roots and vessels were measured with software available on the microscope (AxioVision Rel. 4.6, Carl Zeiss, Göttingen, Germany).

2.2.7 Determination of ions and PTS fluorescence in the shoots

Shoots were immersed in 5-10 ml distilled water and extracted for 2 h at 90 °C. Na^+ and K^+ in the extracts were measured by atomic absorption spectroscopy (Unicam 919, Cambridge, UK). PTS florescence was analysed at $\lambda_{excitation}$ = 403 nm and $\lambda_{emission}$ = 510 nm with a fluorescence spectrometer (Perkin-Elmer LS-3B; Beaconsfield, Buckinghamshire, UK).

2.2.8 Transpiration, WUE and bypass flow

Whole-plant transpiration was measured gravimetrically and corrected for water loss by evaporation (Murray & Ayres 1986; Yeo *et al.* 1999; Gong *et al.* 2006) during treatment with NaCl and PTS and subsequent chase periods. Briefly, the individual tubes containing a rice seedling and culture solution were weighed at the beginning of NaCl treatment and at the end of the chase periods on an electronic balance (HF-300G, A&D Instruments, Oxfordshire, UK) with an accuracy of 0.001 g. Control tubes filled with culture solution without plants were also weighed to check evaporation from the tubes. Transpiration was calculated as the difference in weight loss between tubes with and without plants. WUE was determined as shoot dry weight after 25 d per unit of water transpired for 25 d (Flowers *et al.* 1988; Yadav *et al.* 1996).

Bypass flow (J_{VB}) was calculated as a percentage of the total volume flow during the experimental period following methods described by Flowers and co-workers (Yeo *et al.* 1987; Garcia *et al.* 1997; Gong *et al.* 2006) as shown in equation 1. PTS content in the shoot ($PTS_{[cont]}$) was divided by water transpired (Jv) to give the apparent PTS concentration in the transpiration stream ($PTS_{[xyl]}$). Dividing this by PTS concentration in the external medium ($PTS_{[ext]}$) gave the leakage of PTS ($PTS_{[xyl]}/PTS_{[ext]}$). An empirical correction factor (7.57, Yeo *et al.* 1987) for the relative mobilities of PTS and water was applied to estimate the water bypass flow.

$$J_{VB}(\%) = \frac{(PTS_{[cont]}/Jv)}{PTS_{[ext]}} \times 7.57 \times 100 \qquad \text{Equation 1}$$

The Na^+ content in the shoot was divided by water transpired to give the apparent Na^+ concentration in xylem ($Na_{[xyl]}$). This was then divided by the concentration of Na^+ in the external medium to give the leakage of Na^+ ($Na_{[xyl]}/Na_{[ext]}$).

2.2.9 Phloem sap collection and analysis

Phloem sap was obtained using the phloem exudation technique described by King & Zeevaart (1974) and Berthomieu *et al*. (2003). Leaves 2, 3 and 4 were cut at their leaf sheath-blade junctions. The leaf blades were then re-cut under a solution of 20 mM K_2EDTA (pH 7.5) at approximately 5 mm from the original cut and the cut ends were kept in the same solution for 1 min. Then, the leaves were transferred to 1 ml of a

solution of 15 mM K_2EDTA (pH 7.5) and left for 4 h in an illuminated plant growth cabinet (SANYO MLR-350HT, Osaka, Japan) with photosynthetically active radiation of 250-300 μmol m^{-2} s^{-1} supplied by fluorescent lamps (40W x 15 lamps), at 30±1 °C and 90% RH to reduce transpiration. The concentration of Na^+ in the EDTA solution was determined by atomic absorption spectroscopy (Unicam 919, Cambridge, UK). Then, the original concentration of Na^+ in the EDTA solution was subtracted from this concentration of Na^+ in the EDTA solution to get the quantity of Na^+ in phloem sap.

2.2.10 Analysis of data

The experiments were repeated at least twice. The results presented in this Chapter are averages between the repeat experiments by pooling individual data and subjected to statistical analysis by the Mann-Whitney test as the data were not distributed normally about the mean.

2.3 RESULTS

2.3.1 Morphological and anatomical characteristics of seedlings

As shown in Table 2.1 and Appendix 2.2, the mutant *lrt1* produced no lateral roots (100% reduction as compared to the respective wild-type Oochikara), whereas no significant differences were observed in other morphological characteristics (such as seminal and crown root length, number of crown roots). The mutant *lrt2* exhibited only a few lateral roots per plant (99% reduction) and had a longer seminal root length as compared to the wild-type Nipponbare (Table 2.1); no significant difference in length and number of crown roots was detected. For the mutant *crl1*, the number and length of crown roots were reduced and there were significantly fewer lateral roots than in the wild-type Taichung 65, whereas the length of the seminal root was not significantly different between mutant and wild type (Table 2.1). For IR36, the number of lateral roots of 2,4-D-treated seedlings was significantly reduced, by 63%, compared with control plants (Table 2.1), whereas the reduction was 57% by mechanical cutting (the number of lateral roots was 446±28.0 and 192±9.86 for control and mechanical cutting, respectively, means ± standard errors of 20 plant analyses). Moreover, 2,4-D treatment significantly decreased the number of crown roots and length of crown and seminal roots (Table 2.1).

Table 2.1 Morphological and anatomical characteristics of lateral rootless mutants (*lrt1*, *lrt2*), a crown rootless mutant (*crl1*) and their respective wild types (Oochikara, Nipponbare and Taichung 65, respectively), and 2,4-D treated seedlings and control plants of rice cv. IR36.

	Oochikara	*lrt1*	Nipponbare	*lrt2*	Taichung65	*crl1*	IR36	
							control	2,4-D treatment
Number of lateral roots	1003±35.9a	0.0±0.0b	585±21.7a	6.90±1.68b	932±14.9a	212±14.3b	100±6.14a	32.4±1.92b
Seminal root length (cm)	10.0±0.62a	9.50±0.21a	11.5±0.35a	13.4±0.78b	13.4±0.55a	12.9±0.24a	7.55±0.33a	3.45±0.09b
Crown root length (cm)	12.0±0.35a	11.2±0.35a	9.91±0.31a	10.8±0.33a	9.46±0.32a	0.0±0.0b	5.81±0.24a	0.61±0.17b
Number of crown roots	12.3±0.72a	13.2±0.57a	11.7±0.50a	10.3±0.45a	13.1±0.30a	0.0±0.0b	8.50±0.37a	2.80±0.94b
Seminal root diameter (μm)	370±23.0a	284±12.3b	373±11.7a	249±8.81b	332±6.12a	381±11.4b	269±20.6a	507±4.61b
Number of central conducting vessel	1.00±0.0a	9.60±1.36b	1.00±0.0a	5.80±0.99b	1.00±0.0a	1.00±0.0a	1.00±0.0a	1.30±0.15a
Largest vessel diameter(μm)	28.6±1.12a	22.7±1.37b	27.8±0.57a	20.0±0.75b	26.7±0.71a	28.6±0.59b	22.2±1.07a	39.7±3.12b
Shoot dry mass (mg)	183±3.61a	74.8±1.38b	127±3.26a	87.2±2.16b	153±3.92a	146±5.15a	29.4±1.00a	17.4±0.64b
Root dry mass (mg)	43.9±1.05a	17.9±0.39b	32.5±0.99a	16.6±0.53b	66.4±2.44a	9.32±0.73b	6.63±0.26a	4.99±0.21b
Root surface area (mm^2)	1773±78.0a	382±21.7b	953±69.9a	392±23.7b	659±24.4a	88.5±7.30b	339±13.3a	98.8±4.19b

The age of plants sampled was 25 d for mutants and their wild types, and 17 d for IR36. Values of each characteristic followed by the same letters (either between mutant and its wild type or between 2,4-D treatment and control) are not significantly different at $P<0.05$ according to the Mann-Whitney test. The data are means and standard errors (n = 8-105).

The seminal root diameters of *lrt1* and *lrt2* were smaller than those of Oochikara and Nipponbare, whereas this diameter in *crl1* was larger than Taichung 65 (Table 2.1). The most striking anatomical difference between lateral rootless mutants and the wild types was the difference in the number of central conducting vessels. Oochikara and Nipponbare had only one central conducting vessel, whereas this number was about 10 in *lrt1* and six in *lrt2* (Table 2.1 & Figure 2.2). The number of central conducting vessels did not differ between *crl1* and Taichung 65, but the *crl1* mutant had significantly larger vessel diameters than Taichung 65 (Table 2.1). The 2,4-D treatment of IR36 seedlings significantly increased seminal root diameter and vessel diameter (by 89 and 79%, respectively) compared with untreated control plants (Table 2.1).

2.3.2 Development of suberin lamellae

At a distance of 45 – 55 mm from the seminal root tip, an intense yellow fluorescence of Fluorol Yellow 088 was clearly observed in the endodermis of Oochikara, whereas the fluorescence was very faint in the endodermis of *lrt1* (Figure 2.2a, b), indicating a fully developed endodermis with suberin lamellae in Oochikara. Oochikara had more suberin lamellae in its exodermis and produced more layers of sclerenchyma than *lrt1* (Figure 2.2c, d). The endodermis of Nipponbare was also more fully developed with suberin lamellae than in *lrt2* (Figure 2.2e, f): suberin was deposited on both inner and outer tangential walls of Nipponbare, whereas it was mainly visible on the outer tangential walls of *lrt2* (Figure 2.2e, f). Suberin lamellae in the exodermis and the thickening of the walls of the sclerenchymatous layer were also more developed in Nipponbare compared with *lrt2* (Figure 2.2g, h). No significant difference in suberin lamellae development was noticed in the exodermis or endodermis of Taichung 65 and *crl1* (Appendix 2.3).

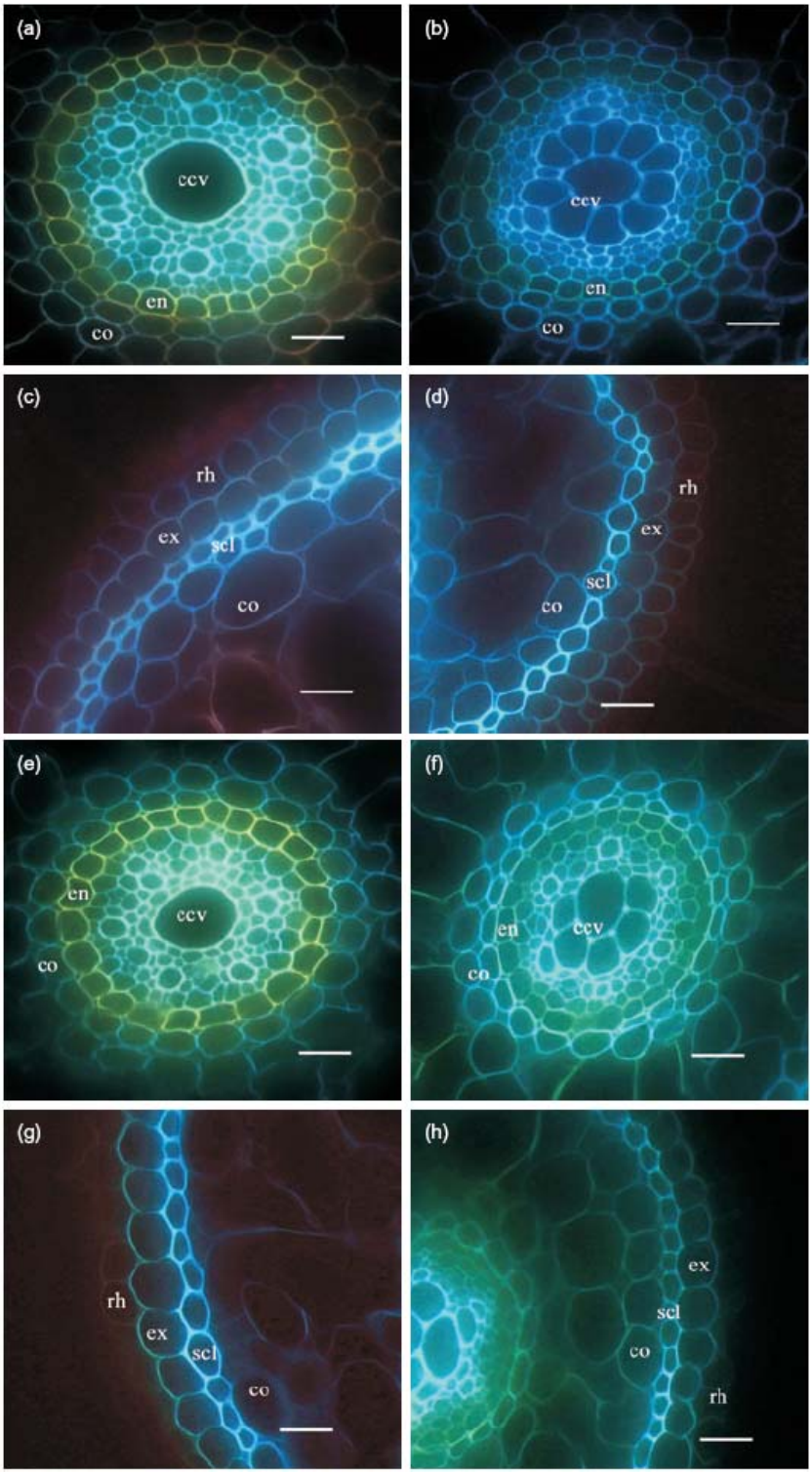

Figure 2.2 Freehand cross-sections of seminal roots of rice cvs. Oochikara (a, c), *lrt1* (b, d), Nipponbare (e, g) and *lrt2* (f, h). Sections taken at a distance of 45 – 55 mm from the root tip of 20-day-old seedlings were stained for 1 h with 0.01% (w/v) Fluorol Yellow 088 to detect suberin lamellae and observed with an epifluorescence microscope. Scale bars = 20 µm. ccv, central conducting vessel; co, cortex; en, endodermis; ex, exodermis; rh, rhizodermis; scl, sclerenchyma.

2.3.3 Ion concentrations in shoots

Shoot Na^+ concentrations in mutants, *lrt1* and *lrt2*, were significantly lower (by 23 and 22%, respectively) than those of their respective wild types, Oochikara and Nipponbare (Figure 2.3a & Appendix 2.4). Although the concentration of Na^+ in *crl1* was 80% of that in wild-type Taichung 65, the difference was not statistically different (Figure 2.3a). The K^+ concentration was significantly higher, by 14%, in *lrt2* as compared to Nipponbare (Figure 2.3b) and while the concentration of K^+ in shoots of *lrt1* was higher by 9%, this was not statistically different from that of Oochikara (Figure 2.3b). The concentration of K^+ in *crl1* was lower by 15% compared with Taichung 65 (Figure 2.3b). K^+/Na^+ ratios in *lrt1* and *lrt2* were about 1.6 and 1.5 times greater than those of Oochikara and Nipponbare (Figure 2.3c), respectively, whereas this ratio was not significantly changed in *crl1* as compared to Taichung 65 (Figure 2.3c).

For cv. IR36, the Na^+ concentration in shoots of lateral-root-cut and 2,4-D treated seedlings was significantly higher (by 58 and 74%, respectively) than that of the controls (Figure 2.4a), whereas no significant difference was observed between crown-root-cut and the uncut control plants (Figure 2.4a). Cutting lateral roots and 2,4-D treatment did not significantly affect K^+ concentration in shoots, but cutting crown roots slightly decreased this concentration (by 5%) as compared to the control (Figure 2.4b). The K^+/Na^+ ratios were significantly lower by 1.4-1.6 times in lateral-root-cut and 2,4-D treated seedlings than the controls (Figure 2.4c), but this ratio was not significantly different between crown-root-cut and control plants (Figure 2.4c).

2.3.4 Transpiration and WUE

Transpiration expressed per plant of all mutants was significantly lower than that of the wild types, but when expressed per unit root surface area this rate was highly significantly higher in *lrt1* and *crl1* compared with that of their wild types (Table 2.2). WUE in *lrt2* and *crl1* was significantly (2-2.3 times) higher than that of Nipponbare and Taichung 65 (Table 2.2). In contrast, there was no significant difference between *lrt1* and Oochikara in WUE, although the WUE of *lrt1* was 1.3 times greater than that of Oochikara (Table 2.2).

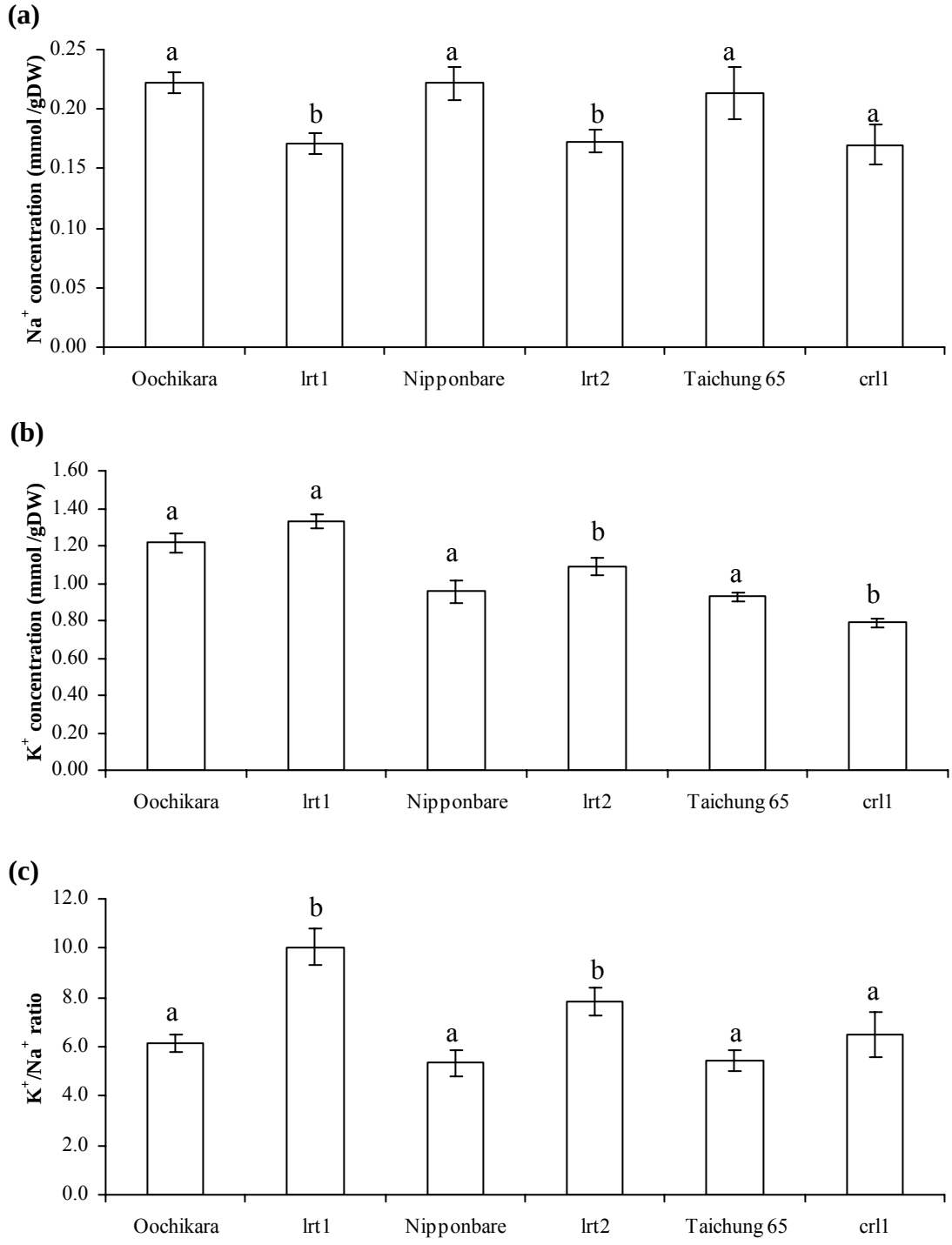

Figure 2.3 Concentrations of Na^+ (a), K^+ (b), and K^+/Na^+ ratio on a molar basis (c) in shoots of lateral rootless mutants (*lrt1*, *lrt2*), their respective wild types (Oochikara, Nipponbare), a crown rootless mutant (*crl1*) and its wild type (Taichung 65). Twenty-day-old rice seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 72 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters (between each mutant and its wild type) are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 30-105).

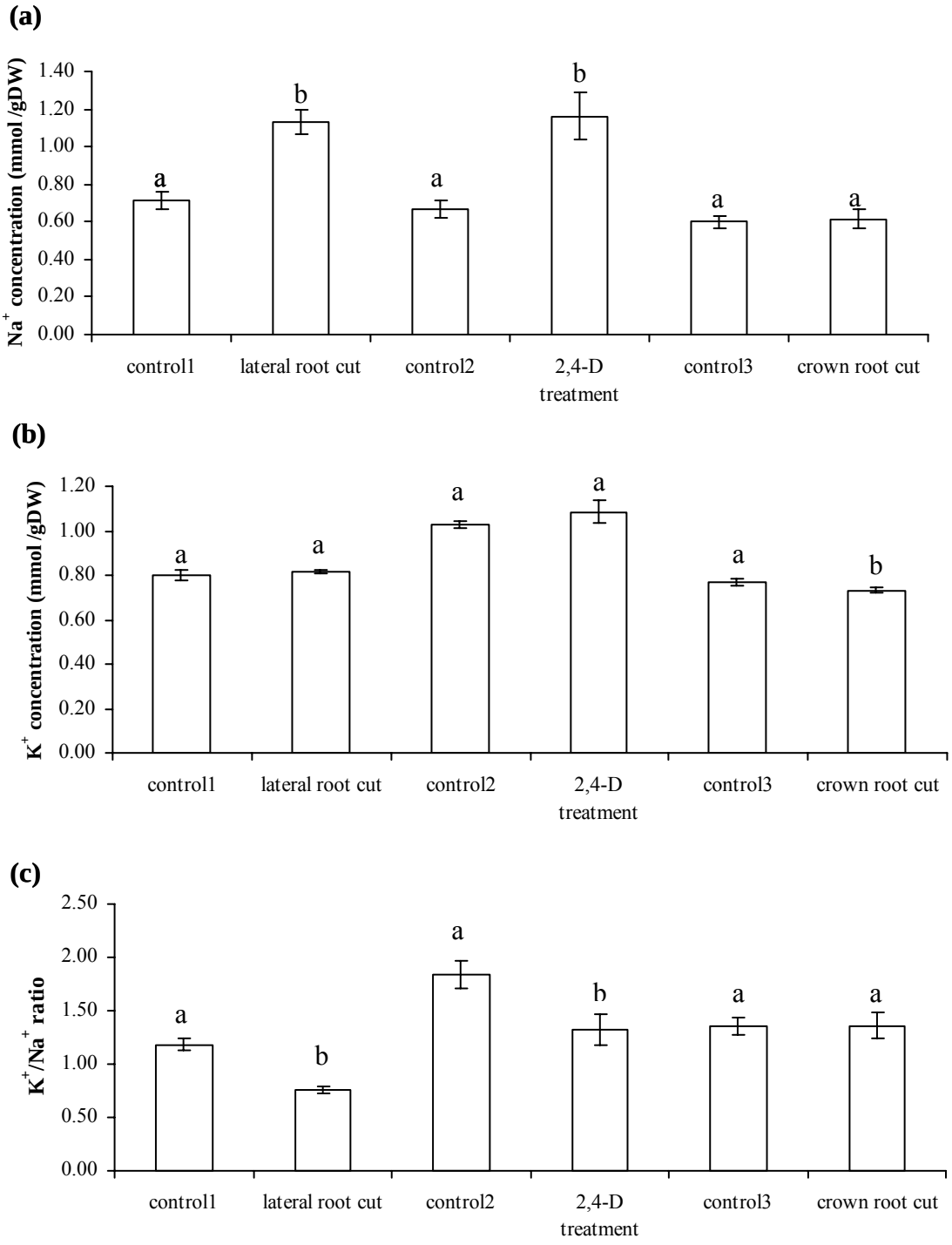

Figure 2.4 Concentration of Na^+ (a), K^+ (b) and K^+/Na^+ ratio on a molar basis (c) in shoots of lateral-root-cut, 2,4-D treated, crown-root-cut seedlings of rice cv. IR36 and their control plants. Seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 72 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters (between each treatment and its control) are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 20-36).

Table 2.2 Transpiration rate, transpiration per unit root surface area and WUE of lateral rootless (*lrt1*, *lrt2*), crown rootless (*crl1*) mutants and their respective wild types.

Parameter(units)	Oochikara	*lrt1*	Nipponbare	*lrt2*	Taichung65	*crl1*
Transpiration rate (g water /d)	7.48±0.15a	2.93±0.09b	4.72±0.13a	1.84±0.11b	4.73±0.18a	2.36±0.14b
Transpiration per unit root surface (g water /mm^2)	0.022±0.001a	0.031±0.004b	0.028±0.002a	0.018±0.002b	0.037±0.001a	0.155±0.014b
Shoot dry weight at 25 d (mg)	183±3.61a	74.8±1.38b	127±3.26a	87.2±2.16b	153±3.92a	146±5.15a
WUE (g DW /kg water)	0.99±0.02a	1.25±0.09a	1.09±0.03a	2.51±0.28b	1.33±0.05a	2.68±0.15b

Twenty-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 72 h and harvested after a chase period of 48 h in culture solution. Whole-plant transpiration was measured gravimetrically and corrected for water loss by evaporation during treated and chase periods. WUE was determined as shoot dry weight after 25 d per unit of water transpired for 25 d. Values of a parameter followed by the same letters (between each mutant and its wild type) are not significantly different at $P<0.05$ according to the Mann-Whitney test. The data are means and standard errors (n = 30-105).

2.3.5 Bypass flow and Na^+ uptake

Analysis of data on the uptake of water, PTS and Na^+ by whole shoots of rice seedlings showed that bypass flow was 0.9, 1.6 and 0.7% in wild types of Oochikara, Nipponbare and Taichung 65, respectively (Table 2.3). Bypass flow was increased to 2.0, 5.9 and 1.8% in *lrt1*, *lrt2* and *crl1*, respectively (Table 2.3). Similarly, bypass flow was significantly increased in lateral-root-cut, 2,4-D treated and crown-root-cut seedlings of IR36 (by 75, 265 and 97%, respectively) as compared to the controls (Table 2.3). The apparent Na^+ concentration in the transpiration stream (xylem) of *lrt2* and *crl1* was significantly higher than that of their wild types, whereas this concentration was not significantly changed in *lrt1* compared with Oochikara (Table 2.3).

There was a highly significant correlation between bypass flow and the apparent Na^+ concentration in the xylem of *lrt1*, *lrt2* and *crl1* (Figure 2.5). Interestingly, when no bypass flow (x = 0), the apparent Na^+ concentration in the xylem of Oochikara and Nipponbare (y-intercept) was slightly higher than that of *lrt1* and *lrt2* (Figure 2.5a, b), suggesting that Na^+ taken up to the shoots through a symplastic pathway of wild types was greater than that of the lateral rootless mutants. In contrast to *lrt1* and *lrt2*, the crown rootless mutant *crl1* had a higher symplastic transport of Na^+ than the wild-type Taichung 65 (Figure 2.5c).

Using the data in Table 2.3, it was possible to calculate the Na^+ delivered to the shoots via bypass flow in each variety (T.J. Flowers personal communication) - the water bypass flow (J_{VB}) multiplied by the concentration of Na^+ in the external medium ($Na_{[ext]}$) and water transpired (Jv) – and compare this with the total Na^+ accumulated in the same time: bypass flow delivered 36% of the shoot Na^+ in the wild-type Oochikara, 49% in Nipponbare and 27% in Taichung 65, figures that rose to 58% in the mutant *lrt1*, 89% in *lrt2* and 44% in *crl1*.

2.3.6 Na^+ in phloem sap

The quantity of Na^+ in phloem sap of rice seedlings ranged from 30 to 340 μmol in leaf 2 (Figure 2.6a), from 22 to 144 μmol in leaf 3 (Figure 2.6b) and from 7 to 36 μmol in leaf 4 (Figure 2.6c) of the seven genotypes examined. The mutant *lrt1* showed the highest amount of Na^+ in phloem sap of leaves 2, 3 and 4 (Figure 2.6).

Table 2.3 Na^+, PTS uptake and bypass flow (J_{VB}) by lateral rootless mutants (*lrt1*, *lrt2*), a crown rootless mutant (*crl1*), their respective wild types (Oochikara, Nipponbare, Taichung 65), and lateral-root-cut, crown-root-cut, 2,4-D treated seedlings and their control plants of cv. IR36.

Cultivar or treatment	Jv (ml)	PTS (μg)	$PTS_{[xyl]}$ (μM)	$PTS_{[xyl]}/PTS_{[ext]}$ (%)	J_{VB} (%)	Na (μmol)	$Na_{[xyl]}$ (mM)	$Na_{[xyl]}/Na_{[ext]}$ (%)
Oochikara	37.4±0.77a	4.03±0.45a	0.22±0.03a	0.11±0.01a	0.86±0.10a	40.4±1.74a	1.09±0.05a	2.17±0.09a
lrt1	14.6±0.47b	2.07±0.28b	0.51±0.12a	0.27±0.07a	2.03±0.49a	12.7±0.61b	1.11±0.11a	2.22±0.22a
Nipponbare	23.6±0.67a	4.60±0.43a	0.40±0.04a	0.21±0.02a	1.61±0.17a	39.8±2.14a	1.74±0.10a	3.48±0.20a
lrt2	9.21±0.56b	4.25±0.45a	1.48±0.23b	0.77±0.14b	5.86±1.09b	20.7±1.04b	2.88±0.32b	5.76±0.63b
Taichung 65	23.7±0.89a	2.07±0.17a	0.18±0.02a	0.09±0.01a	0.70±0.07a	30.8±2.37a	1.33±0.11a	2.66±0.22a
crl1	11.8±0.71b	2.51±0.28a	0.46±0.07b	0.24±0.03b	1.84±0.26b	24.2±2.19a	2.24±0.24b	4.48±0.48b
Control 1	15.5±1.07a	15.0±1.32a	2.11±0.27a	1.11±0.14a	8.38±1.07a	69.8±3.44a	4.99±0.47a	9.98±0.94a
Lateral root cut	9.56±0.64b	18.2±2.38a	3.70±0.37b	1.94±0.19b	14.7±1.45b	89.3±5.13b	9.97±0.69b	19.9±1.38b
Control 2	6.22±0.46a	2.04±0.26a	0.63±0.08a	0.33±0.04a	2.48±0.31a	18.8±1.09a	3.03±0.18a	6.06±0.35a
2,4-D treatment	1.78±0.29b	2.13±0.31a	2.28±0.37b	1.20±0.18b	9.06±1.34b	18.5±1.51a	10.4±0.85b	20.8±1.70b
Control 3	9.73±0.53a	9.40±1.00a	2.08±0.30a	1.09±0.16a	8.26±1.18a	37.9±1.65a	4.23±0.37a	8.45±0.74a
Crown root cut	7.21±0.71b	10.9±1.21a	4.11±0.92b	2.15±0.48b	16.3±3.65b	33.0±2.84a	5.65±0.87a	11.3±1.74a

Values of a parameter followed by the same letters (either between each mutant and its wild type or between each manipulation and its control) are not significantly different at $P<0.05$ according to the Mann-Whitney test. The data are means and standard errors (n = 20-105). See section 2.2.8 for the details of calculation.

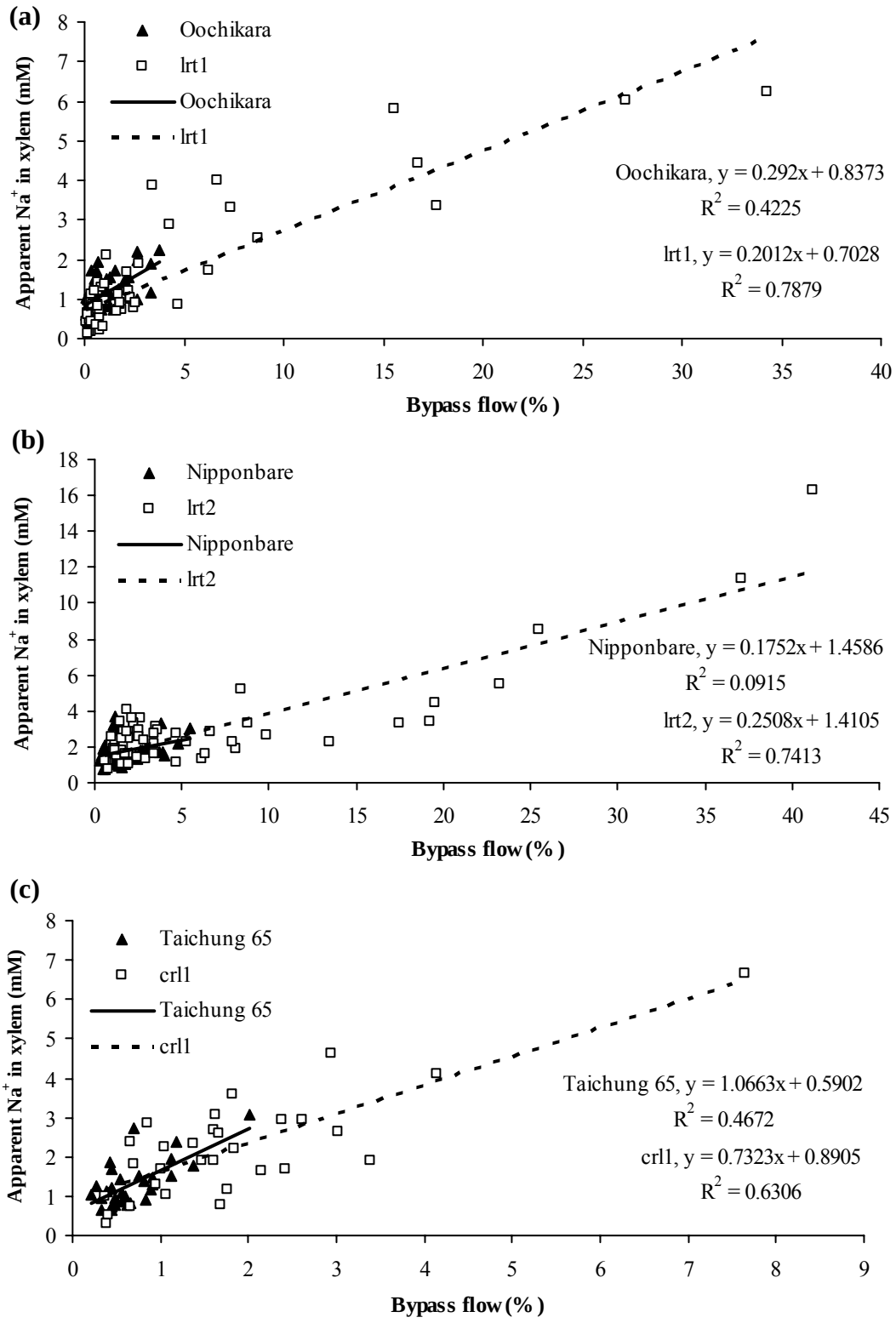

Figure 2.5 Relationships between bypass flow and the apparent Na^+ concentration in the xylem in shoots of Oochikara, *lrt1* (a), Nipponbare, *lrt2* (b) and Taichung 65, *crl1* (c). Twenty-day-old rice seedlings were exposed to 50 mM NaCl and 100 mg/l PTS for 72 h followed by a chase period of 48 h in culture solution. (n = 30-105).

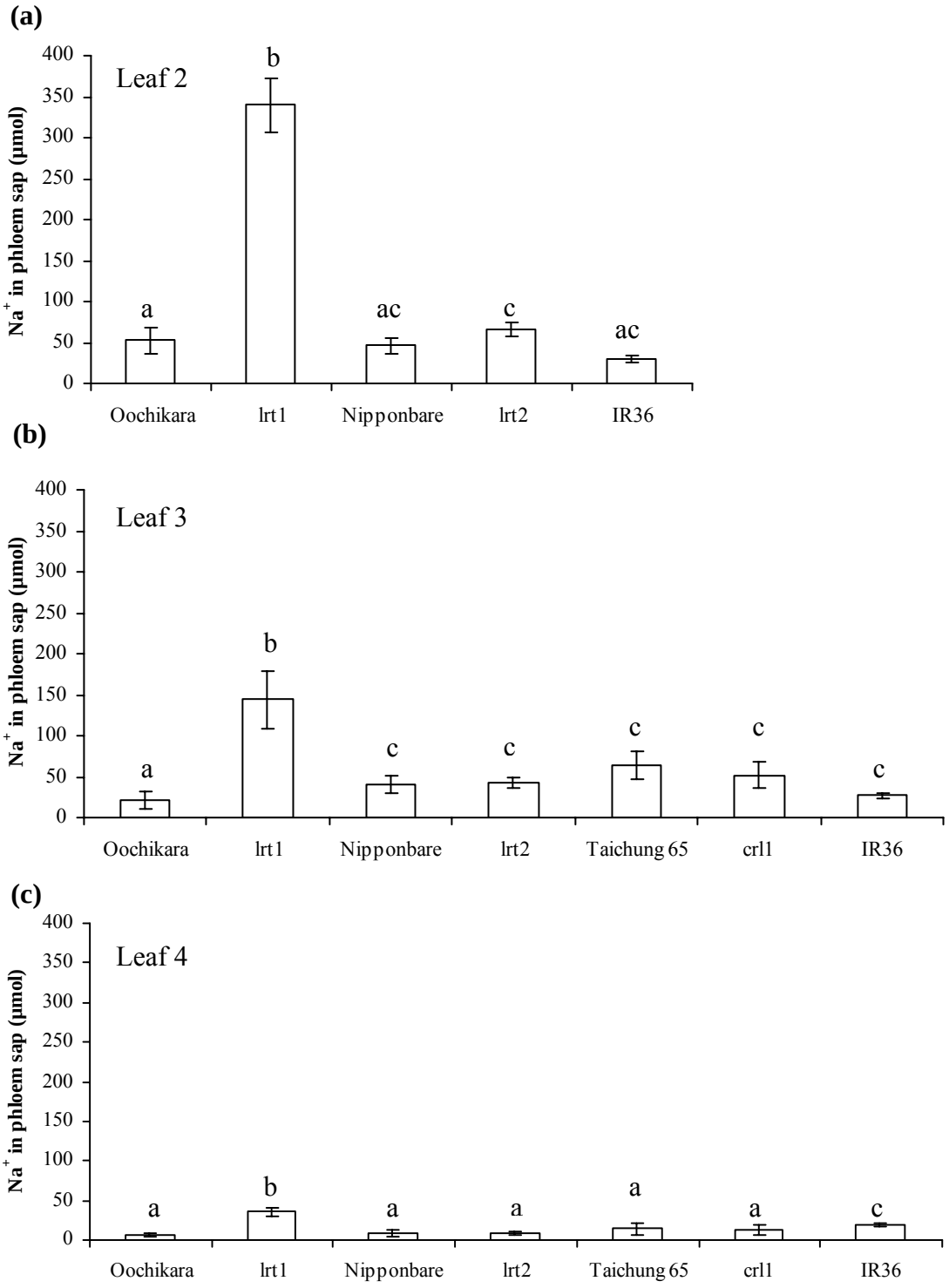

Figure 2.6 The quantity of Na^+ in phloem sap of leaves 2 (a), 3 (b) and 4 (c) of rice cvs. Oochikara, *lrt1*, Nipponbare, *lrt2*, Taichung 65, *crl1* and IR36. Twenty-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 72 h and harvested after a chase period of 48 h in culture solution. Na^+ in phloem sap was collected in EDTA solutions. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 10-30).

2.4 DISCUSSION

The defective characteristics in lateral root formation of *lrt1*, *lrt2* and *crl1*, and in crown root formation of *crl1* (Table 2.1) are consistent with other reports (Hao & Ichii 1999; Inukai *et al.* 2001, 2005; Wang *et al.* 2006). Auxins such as indole-3-butyric acid (IBA) and 2,4-D regulate root architecture in rice by stimulating or inhibiting the lateral and crown root formations (Chhun *et al.* 2003a, b, 2004; Inukai *et al.* 2005; Wang *et al.* 2006). Exogenous application of IBA (0.01-10 μM) increased the number of lateral roots in Oochikara, *lrt1* and IR8 (Chhun *et al*. 2003a, 2004, 2005). In contrast to IBA, application of 2,4-D (0.01-1.0 μM) decreased lateral root number of Nipponbare, Taichung 65 and *crl1* (Inukai *et al.* 2005; Wang *et al.* 2006). In the present study, the inhibitory effect of 2,4-D (0.05 μM) on lateral and crown root production, and on seminal and crown root lengths was seen in rice cv. IR36 (Table 2.1). These results are consistent with previous observations (Inukai *et al.* 2005; Wang *et al.* 2006). Although the mechanism by which 2,4-D inhibits lateral and crown root formation in rice is rarely investigated, it has been shown that the uptake of IBA (carrier-mediated uptake) in rice root of an auxin-resistant mutant (*arm2*) is blocked (~ 48%) by application of 1 mM 2,4-D (Chhun *et al.* 2005).

The present results showed that bypass flow was increased by 136% in the mutant *lrt1* compared with its wild-type Oochikara (Table 2.3), although the mutant did not produce any lateral roots (Table 2.1). Similarly, the bypass flow in *lrt2* was dramatically increased by 264% compared with its wild-type Nipponbare (Table 2.3), while the mutant produced few lateral roots - about 1% as compared to the wild type (Table 2.1). Also, in the mutant *crl1*, which produced fewer lateral roots (~ 23%) than wild-type Taichung 65 (Table 2.1), the bypass flow was increased by 163% (Table 2.3). Using mechanical cutting and 2,4-D treatment to reduce the number of lateral roots (by 57-63%) in IR36 also increased the bypass flow by 75-265% (Table 2.3) and this result adds weight to the view that the bypass flow did not originate at the bases of lateral roots as I hypothesised; if this were the case, I would expect to see higher bypass flow in wild types than in the lateral rootless mutant *lrt1* and *lrt2*, and in the control plants than in lateral-root-cut (where the roots were cut – see methods) and 2,4-D treated seedlings of IR36.

My results are not consistent with those of Peterson and co-workers (Peterson *et al.* 1981; Enstone & Peterson 1992, 1998) and Ranathunge *et al*. (2005a) who found that bypass flow occurred in the region of lateral root emergence. Moon *et al*. (1986) reported that PTS concentration in xylem sap of undisturbed grey mangrove root systems was less than 0.17% of the concentration in the external medium, but the concentration increased to 13% (by at least 75 times) in disturbed root systems during removal from sand. Disturbance is a possible explanation for the results observed by Peterson and co-workers (Peterson *et al.* 1981; Enstone & Peterson 1992, 1998) and Ranathunge *et al*. (2005a), allowing the bypass flow tracer dyes and ions to move into the primary root. That this was a possible explanation of their data was implied by Enstone & Peterson (1992). Furthermore, Enstone & Peterson (1998) considered that their results in leakage of berberine-thiocyanate tracer into the parent root at the sites of emerged lateral roots of maize might be an artefact of handling.

The increase in bypass flow of mutants led to an increase in Na^+ delivered to the shoots via bypass flow by 59, 83 and 62% in *lrt1*, *lrt2* and *crl1*, respectively, as compared to the wild types. A highly significant correlation between bypass flow and the apparent Na^+ concentration in xylem of *lrt1*, *lrt2* and *crl1* (Figure 2.5) indicated that a significant proportion of Na^+ in shoots was taken up via this pathway and is compatible with earlier finding (Yeo *et al.* 1987, 1999; Yeo 1992; Yadav *et al.* 1996; Garcia *et al.* 1997; Ochiai & Matoh 2002; Anil *et al.* 2005; Bridges 2005; Gong *et al.* 2006; Krishnamurthy *et al.* 2009). Phloem exudation estimated using the EDTA technique (King & Zeevaart 1974; Berthomieu *et al.* 2003) revealed that there was a substantial amount of Na^+ in phloem sap of *lrt1* (Figure 2.6). This finding implies that Na^+ taken up to the shoot of the mutant via bypass flow leaked into the phloem. The result is in line with the view that salt-sensitive species simply cannot keep Na^+ out of the phloem (Flowers, Hajibagheri & Clipson 1986; Munns, Fisher & Tonnet 1986; Munns *et al*. 1988, 2002; Munns & Tester 2008).

The importance of lateral roots in providing the area for rice to absorb water and nutrients from the soil has been previously discussed (Banoc *et al*. 2000; Ma *et al.* 2001; Kamoshita *et al.* 2004). As a result, any reduction in the number of lateral roots in *lrt1*, *lrt2* and *crl1* may severely affect growth of seedlings. The mutants, however, displayed several associated morphological and anatomical characteristics that could affect their

ability to take up and transport nutrients and water (Fukai & Cooper 1995; Maggio *et al*. 2001; Manschadi *et al*. 2006; Watt, Magee & McCully 2008). For example, *lrt1* had more central conducting vessels compared with Oochikara (Table 2.1 & Figure 2.2a, b). Similarly, *lrt2* had a longer seminal root and more central conducting vessels than Nipponbare (Table 2.1 & Figure 2.2e, f). Likewise, *crl1* had larger diameter of xylem vessel than Taichung 65 (Table 2.1). By using the Hagen-Poiseuille's law to estimate the axial conductance of water flow in roots (Varney *et al*. 1991; Watt *et al.* 2008), I calculated that the axial flow of water in mutant *lrt1* was not statistically different from that of Oochikara, whereas the axial flow was considerably increased (by 1.3 times) in mutant *crl1* compared with Taichung 65 (Figure 2.7). The mutant *lrt1*, *lrt2* and *crl1* had 1.3-2.3 times greater WUE than that of the wild types (Table 2.2).

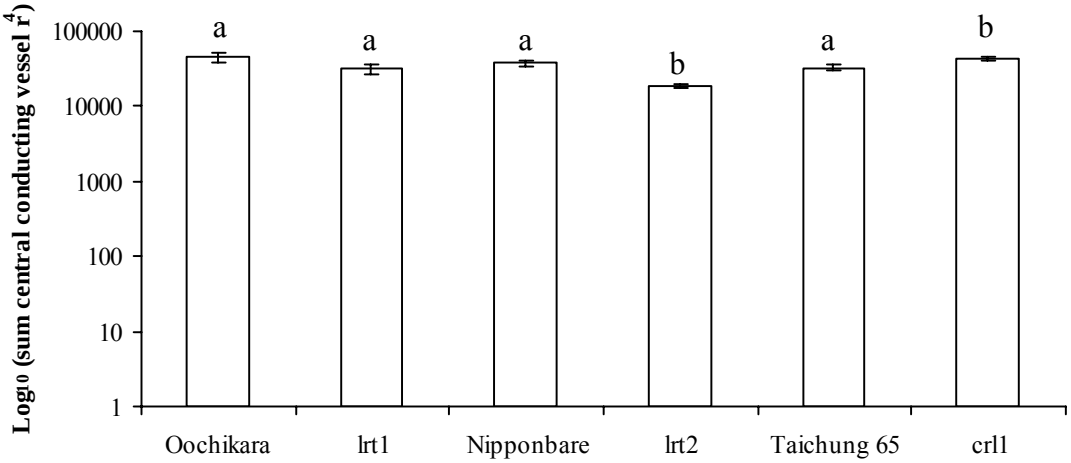

Figure 2.7 Estimation of axial water flow of lateral rootless (*lrt1*, *lrt2*), crown rootless (*crl1*) mutants and their respective wild types calculated using the Hagen-Poiseuille's law and presented as $\log_{10}$ of $\Sigma(r^4)$, where r is the vessel radius and the variable determining flux (Watt *et al*. 2008). Bars with the same letters (between each mutant and its wild type) are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 8-10).

The presence of suberin lamellae in exodermal and endodermal cells, and the thickened walls of the outer sclerenchymatous cells have been reported to be barriers to bypass flow of water, solutes and fluorescent dyes (Clark & Harris 1981; Feldman 1984; Galamay *et al.* 1991; Enstone & Peterson 1998; Kondo *et al*. 2000; Cholewa & Peterson 2001; Miyamoto *et al.* 2001; Enstone *et al.* 2003; Ma & Peterson 2003; Armstrong &

Armstrong 2005; Soukup *et al.* 2007; Krishnamurthy *et al.* 2009). In rice under saline conditions, suberisation of the exodermis and endodermis in roots has been demonstrated to reduce Na^+ uptake to the shoots and enhance survival; the more suberin deposition in the roots, the less Na^+ accumulation in the shoots (Krishnamurthy *et al.* 2009). In the present study, suberin lamellae were less developed in the exodermis and endodermis of seminal roots of *lrt1* and *lrt2* compared with Oochikara and Nipponbare (Figure 2.2). *lrt1* had fewer sclerenchymatous layers than Oochikara (Figure 2.2c, d), and the sub-exodermal sclerenchyma in *lrt2* had thinner cell walls than that in Nipponbare (Figure 2.2g, h). These anatomical differences could facilitate bypass flow in *lrt1* and *lrt2*. It is interesting to note that although suberin lamellae in both exodermis and endodermis did not appear (by microscopy) different between Taichung 65 and *crl1* (Appendix 2.3), the mutant *crl1* exhibited greater bypass flow than the wild type (Table 2.3). Apart from the larger vessel diameter in *crl1*, another possible explanation would be the difference in structure of suberin lamellae between these two varieties. Aliphatic suberin rather than aromatic suberin has been proposed to be barriers to water and solutes (Schreiber *et al.* 1999, 2005; Hose *et al*. 2001; Franke & Schreiber 2007). A recent study by Krishnamurthy *et al*. (2009) showed that the amount of aliphatic suberin was higher in salt-tolerant Pokkali than in salt-sensitive IR20 in both control and saline conditions.

Although the mutants had much higher bypass flow than the wild types (Table 2.3), the lower Na^+ concentration in shoots of mutant *lrt1*, *lrt2* and *crl1* than that of their respective wild types (Figure 2.3a) suggests a reduction in Na^+ uptake via the symplastic pathway. Many transporters such as OsAKT1, NSCCs, OsHKT2;1, OsHKT2;2 and OsHAK1 have been reported to mediate Na^+ influx into rice roots (Chapter 1, section 1.3.1). These transporters are present in plasma membranes of roots such as in epidermis, exodermis, endodermis and the vascular cylinder (Golldack *et al.* 2002, 2003; Kader *et al.* 2006). Consequently, any reduction in number of lateral roots of the mutants would inevitably reduce Na^+ uptake via such transporters. The OsHKT2;1 transporter has been reported to be inhibited by K^+ (Garciadeblas *et al.* 2003; Rodriguez-Navarro & Rubio 2006; Wu *et al.* 2009).

In conclusion, the results showed that bypass flow of Na^+ was increased in lateral rootless mutants (*lrt1*, *lrt2*), a crown rootless mutant (*crl1*), and in rice seedlings cv.

IR36 where the number of lateral and crown roots was reduced by physical and hormonal manipulations. These findings present direct evidence that: (1) the path of bypass flow is not at the sites of lateral root emergence; (2) hormonal treatment such as 2,4-D increases bypass flow; and (3) the magnitude of bypass flow in rice depends on the morphology and anatomy of roots. The results in this study also encourage the use of mutants as tools in the genetic investigation of ion transport in rice under saline environment. It is likely that lateral roots themselves are the sites of bypass flow in rice as I have evidence that PTS can be identified in the vascular tissue of the lateral roots of rice seedlings cv. IR36 using epifluorescence microscopy and CLSM (see Chapter 3).

CHAPTER 3

THE ROLE OF LATERAL ROOTS IN BYPASS FLOW IN RICE (*ORYZA SATIVA* L.)

3.1 INTRODUCTION

There is substantial evidence that bypass flow is a significant pathway for Na^+ movement from roots to shoots in rice growing under saline conditions (Chapter 1, section 1.3.2). Since the sensitivity of rice to salinity is associated with the accumulation of toxic concentrations of Na^+ in the leaves, any reduction in bypass flow could increase the resistance of rice to NaCl. For example, adding Si to the culture solution reduced bypass flow, reduced Na^+ uptake and extended the survival of seedlings growing in salt (50 mM NaCl) (Chapter 1, section 1.6.4). Bypass flow is a small percentage of the transpirational volume flow, usually 1-6%, but it becomes important in ion transport at high transpiration rates and at high external concentrations (Chapter 1, section 1.3.2). Although bypass flow is a significant pathway for Na^+ uptake in rice, the physical location of the pathway has yet to be established.

PTS has been widely used as a tracer in studies of bypass flow in plants (Chapter 1, section 1.3.2). Its fluorescence intensity can be detected and identified by using spectrofluorometry, epifluorescence microscopy or CLSM (Chapter 1, section 1.3.2). However, the fluorescence intensity of PTS is dependent on the degree of ionisation of the 8-hydroxyl group (Kano & Fendler 1978; Clement & Gould 1981; Kondo, Miwa & Sunamoto 1982) and so its fluorescence excitation maximum is affected by pH: it is at about 400 nm in acidic solutions and 460 nm at alkaline pH values; the emission maximum at 510 nm is unchanged with change in pH (Kano & Fendler 1978; Kondo *et al.* 1982; Nunogaki & Kasai 1986; Pino, Campos & Lissi 2003). In this Chapter, I have also checked the fluorescence excitation and emission spectra of PTS using spectrofluorimetry and CLSM in order to be confident that any changes in fluorescence that I detected were a result of changes in PTS.

The apoplast is a continuum in plants, whose transport capacity is limited by pore size (Carpita *et al*. 1979) and the deposition of water-impermeable materials such as Casparian bands and suberin lamellae on the walls of the exodermal and endodermal cell layers - and which act as barriers to bypass flow (Chapter 1, section 1.3.2). However, barriers to bypass flow may be interrupted during lateral root formation and create the area for leakage of solutes into the main root (Chapter 1, section 1.3.2). There is also evidence to suggest that the pathway of bypass flow is located in the lateral roots themselves (Chapter 1, section 1.3.2).

In Chapter 2, I investigated Na^+ and bypass flow in lateral rootless mutants (*lrt1*, *lrt2*) and a crown rootless mutant (*crl1*) of rice, and presented evidence that the start of the path of bypass flow is not at the sites of lateral root emergence. In this Chapter, I report the results of my investigation into the possible role of lateral roots in bypass flow in rice. I hypothesised that bypass flow occurs through the lateral roots and used epifluorescence microscopy, CLSM and fluorescence spectrometry to follow the uptake of the bypass flow tracer PTS. The results show that a lack of an exodermis in the lateral roots of rice was correlated with the entry of PTS into the stele and its transport to the shoot via the transpiration stream. Pre-treating seedlings with PEG and Si reduced PTS in the lateral roots and uptake into rice shoots. These findings indicate a possible role for the lateral roots of rice in bypass flow.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

Caryopses of rice (*Oryza sativa* L.) cv. IR36 were imbibed in aerated water for 24 h before being sown on nylon mesh floated on modified Yoshida culture solution (see Chapter 2). Uniform 7-day-old seedlings were divided into three groups. Each group was transplanted into individual black-painted tubes (50 ml) filled with either culture solution, culture solution with 10 g/l PEG 1500 (-33 kPa; Sigma-Aldrich Company Ltd., Gillingham, UK), or culture solution with 0.67 ml/l (~3 mM) Si from Na_2SiO_3 (BDH, 25.5-28.5% SiO_2), respectively. Plants were held in place with non-absorbent cotton wool and grown for another 7 d in a glasshouse at temperatures of 28±2 °C during the day and 18±2 °C at night with the RH range of 30-70%; the day length was 12 h with a

minimum photosynthetically active radiation of 250-300 μmol m^{-2} s^{-1} supplied by high-pressure sodium lamps (Osram 400W VIALOX NAV-T SON-T, Foshan, China).

3.2.2 Bypass flow tracer uptake

PTS was purchased from Acros Organics (Geel, Belgium) and used as a tracer for bypass flow as previously described (Chapter 2). For long-term experiments (24 h), 100-200 mg/l PTS was used (Peterson *et al*. 1981; Yeo *et al*. 1987; Yadav *et al*. 1996), whereas for a short-term experiment (2 h), 1 g/l PTS was used to localise the development of exodermal Casparian bands (Cholewa & Peterson 2001). In very short-term (0.5 h) experiments reported in this Chapter, the concentration of PTS was increased to 50 g/l (95.3 mM) to allow detection of the tracer within tissues. Roots of 14-day-old rice seedlings were submerged in PTS (a 50 g/l aqueous solution, pH 5.6) for 0.5 h and then this solution was replaced by distilled water for a chase period of 0 (no chase), 1 or 3 h. The roots were then quickly washed with distilled water to remove excess dye from the outer surfaces, gently blotted dry with paper towel and separated from the shoots. The seminal root region, 45-55 mm from the root apex with 8-10 lateral roots (with an average length of 6.4 ± 0.4 mm, means ± standard errors of 18 plant analyses), of both treated and untreated plants was cut with a shape razor blade, placed on a 1-mm-thick glass microscope slide in non-fluorescent mounting medium (IMMU-MOUNT, Thermo Shandon, USA), covered with a 22x22 mm coverslip for observations under CLSM (see Section 3.2.3) and an epifluorescence microscope (see Section 3.2.4). Fresh weights of shoots were recorded before they were dried in an oven at 80 °C for 72 h.

3.2.3 Fluorescence spectra of PTS as detected with spectrofluorometry and CLSM

The pH of a solution of 20 mg/l PTS (0.04 mM in distilled water) was adjusted to between 5.2 and 12.5 with 1 N HNO_3 or 1 N KOH. Then, the fluorescence intensity of PTS solutions at different pH values was measured using a fluorescence spectrometer (Perkin-Elmer LS-3B; Beaconsfield, Buckinghamshire, UK) at different emission wavelengths between 450 and 600 nm when the excitation wavelength was fixed at 403 nm. After that, the fluorescence intensity of the same solutions of PTS was measured with a fixed emission at 510 nm, but varied excitation wavelengths between 350 and

500 nm. A standard PTS solution at a concentration of 50 g/l in distilled water (pH 5.6) was used to investigate its emission spectrum using a Zeiss LSM510 META Axiovert 200M microscope supported by LSM software. For this CLSM work, fluorescence was excited using light with a wavelength of 458 or 488 nm produced by an argon ion laser with a 380-700 nm emission filter.

3.2.4 Epifluorescence microscopy

PTS in the root segments was observed using an epifluorescence microscope (Zeiss Axiophot, Germany) with a blue-violet filter set (excitation filter BP 395-440, chromatic beam splitter FT 460, barrier filter LP 470).

3.2.5 Cryo-scanning electron microscopy (Cryo-SEM)

The seminal root region of 14-day-old rice seedlings, 45 to 55 mm from the root apex with several lateral roots, was cut with a sharp razor blade, loaded into two cryo-rivets (hollow rivets with external and internal diameters of 2 mm and 1.3 mm made of aluminium) stuck together with superglue and placed in the cryostage of a scanning electron microscope (LEO 420 Stereoscan; Carl Zeiss SMT Ltd., Cambridge, UK). The whole assembly was plunged into nitrogen slush (-210 °C), then quickly transferred to the cryo-unit (EMITECH K1150, Emitech Ltd., Ashford, UK) in the frozen state. Once in the SEM, the uppermost rivet was knocked off causing the roots inside to fracture. The sample was etched by subliming ice at -80 °C for 20-30 min to expose the fractured surface of the roots. The sample was then re-cooled to -140 °C and viewed at an accelerating voltage of 10 kV and a probe current of 15 pA.

3.2.6 Detection of Casparian bands and suberin lamellae

Lateral and seminal roots in the region 45-55 mm from the root tip of 14-day-old rice seedlings were cross-sectioned (freehand) with a sharp razor blade. The root sections were stained for 1 h with 0.01% (w/v) Fluorol Yellow 088 (Brundrett *et al.* 1991) to check the presence of suberin lamellae and passage cells. For a comparative study to ensure the adequacy of the staining procedures, aerial roots of an orchid (*Phalaenopsis* sp. provided by Dr. P. Scott of the University of Sussex) were also cross-sectioned and stained with Fluorol Yellow 088. To view the Casparian bands, sections were stained for 1 h with 0.1% (w/v) berberine hemisulfate in distilled water and counterstained for

another hour with 0.5% (w/v) aniline blue in distilled water (Brundrett, Enstone & Peterson 1988). The sections were observed with the epifluorescence microscope using an ultraviolet filter set (excitation filter G 365, chromatic beam splitter FT 395, barrier filter LP 420; Zeiss Axiophot, Germany).

3.2.7 Determination of ions and PTS fluorescence in the shoots

After recording the dry weight, shoots were extracted in 5-10 ml distilled water for 2 h at 90 °C. Na^+ and K^+ in the extracts were measured by atomic absorption spectroscopy (Unicam 919, Cambridge, UK). PTS florescence was analysed at $\lambda_{excitation}$ = 403 nm and $\lambda_{emission}$ = 510 nm with a fluorescence spectrometer (Perkin-Elmer LS-3B; Beaconsfield, Buckinghamshire, UK).

3.2.8 Analysis of data

The experiments were repeated at least twice. The results presented in this Chapter are averages between the repeat experiments and subjected to statistical analysis by the Mann-Whitney test as the data were not distributed normally about the mean.

3.3 RESULTS

3.3.1 PTS fluorescence spectra

Figure 3.1a shows that when fluorescence of a solution of PTS was excited at 403 nm, the wavelength at which emission was maximal remained at 510 nm over the pH range 5.2 to 12.5. In contrast, when the fluorescence was measured at 510 nm, the wavelength of light exciting maximal fluorescence varied with the pH of the solution. There were two significant excitation maxima (Figure 3.1b); the first at 400 nm when the PTS solution was acidic and the second at 460 nm when the solution was alkaline.

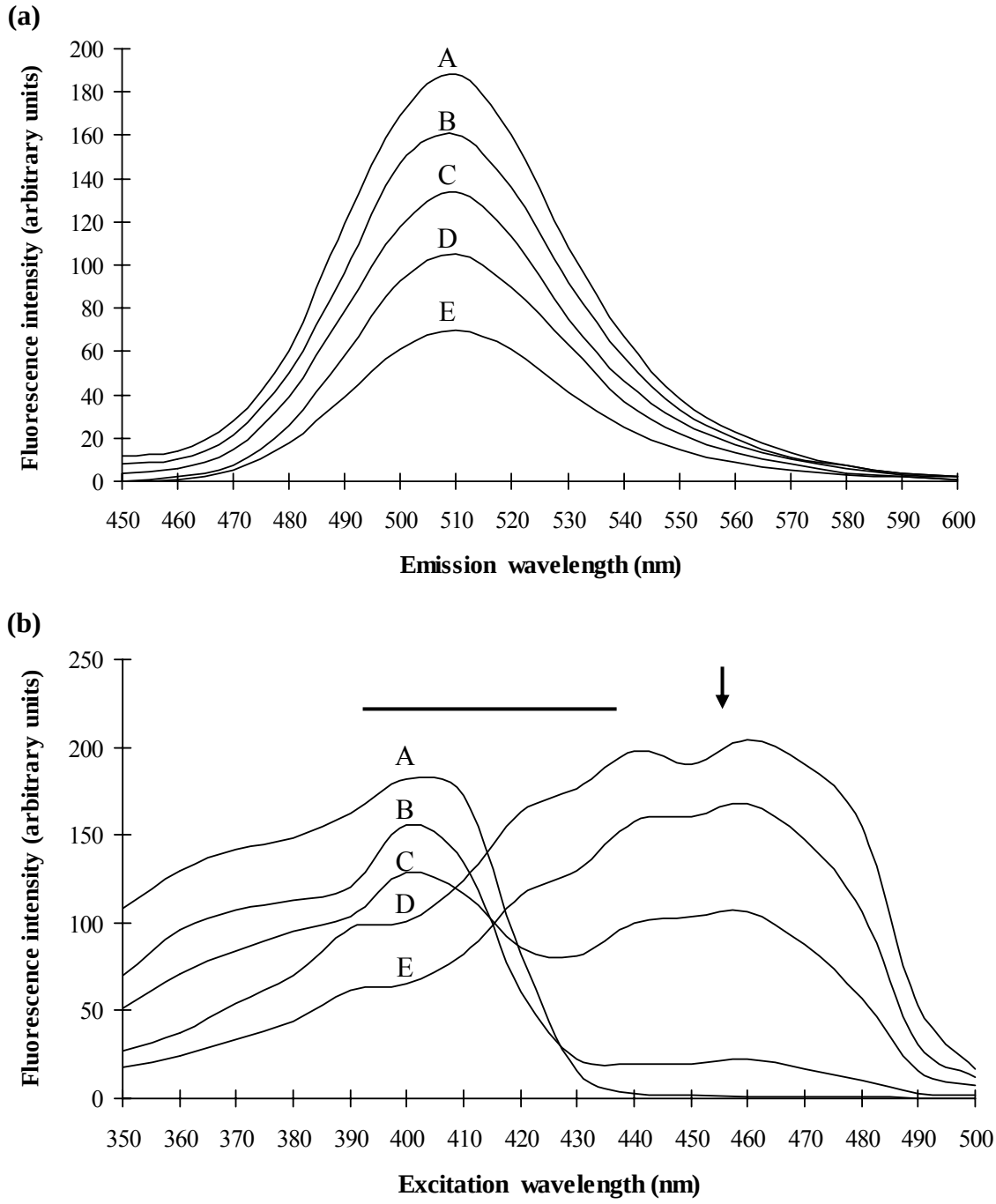

Figure 3.1 (a) Fluorescence spectra of PTS (20 mg/l) in distilled water at various pH values. The spectra were obtained with $\lambda_{excitation}$ = 403 nm. (b) Excitation spectra measured with $\lambda_{emission}$ = 510 nm. The pH of the medium was 5.2 (A), 6.0 (B), 7.0 (C), 10.5 (D) and 12.5 (E). The bar and the arrow indicate the excitation wavelengths of the epifluorescence microscope (395-440 nm) and the CLSM (458 nm), respectively.

Although the spectral results in Figure 3.1 showed the emission and excitation maxima of PTS were 510 and 400 or 460 nm (depending on the pH), respectively, the CLSM had a limited range of lasers available to excite fluorescence. So, a PTS solution (at a pH of 5.6) was excited at 458 and 488 nm and emission data collected for comparison using the CLSM: the emission spectrum of PTS excited at 458 nm (Figure 3.2a) showed a much larger emission peak at 510 nm than when excited at 488 nm (Figure 3.2b), when a second peak at 560 nm was also apparent. As a result, for CLSM, I detected PTS fluorescence in root tissue using fluorescence excited at 458 nm with emission at 485-560 nm. For epifluorescence microscopy, PTS in the root segments was detected using an excitation filter BP 395-440 and barrier filter LP 470.

3.3.2 PTS in rice root

After 0.5 h exposure to PTS (50 g/l), fluorescence was observed using the epifluorescence microscope in the cortex and the stele of the lateral roots (Figure 3.3a, b). A high intensity of fluorescence was evident in the vascular tissue of the lateral root (Figure 3.3b) as compared to PTS-untreated root (Figure 3.3c). CLSM images revealed PTS in the stele of the laterals up to the parental roots (Figure 3.3d, e). PTS was not observed between the root tip and 30 μm from it (Figure 3.3a) nor in the main root around the lateral root and parent root junction (Figure 3.3e).

3.3.3 PTS in lateral roots and shoots during time-chase periods

The lateral roots of plants treated with PTS emitted bright fluorescence (Figure 3.4a). The fluorescence was fainter after a 1h-chase period, although in the stele, the fluorescence intensity was still bright (Figure 3.4b). After a 3h-chase period (Figure 3.4c) it was hardly possible to differentiate between PTS fluorescence and autofluorescence (in roots that had not been treated with PTS, Figure 3.4d), indicating that the dye was washed out from the lateral roots. Without a chase, the PTS concentration in shoot extracts was about 85.9 μg/gDW and the concentration rose as the length of the chase period increased from 1 to 3 h (Figure 3.5) as the PTS in the lateral roots declined (Figure 3.4).

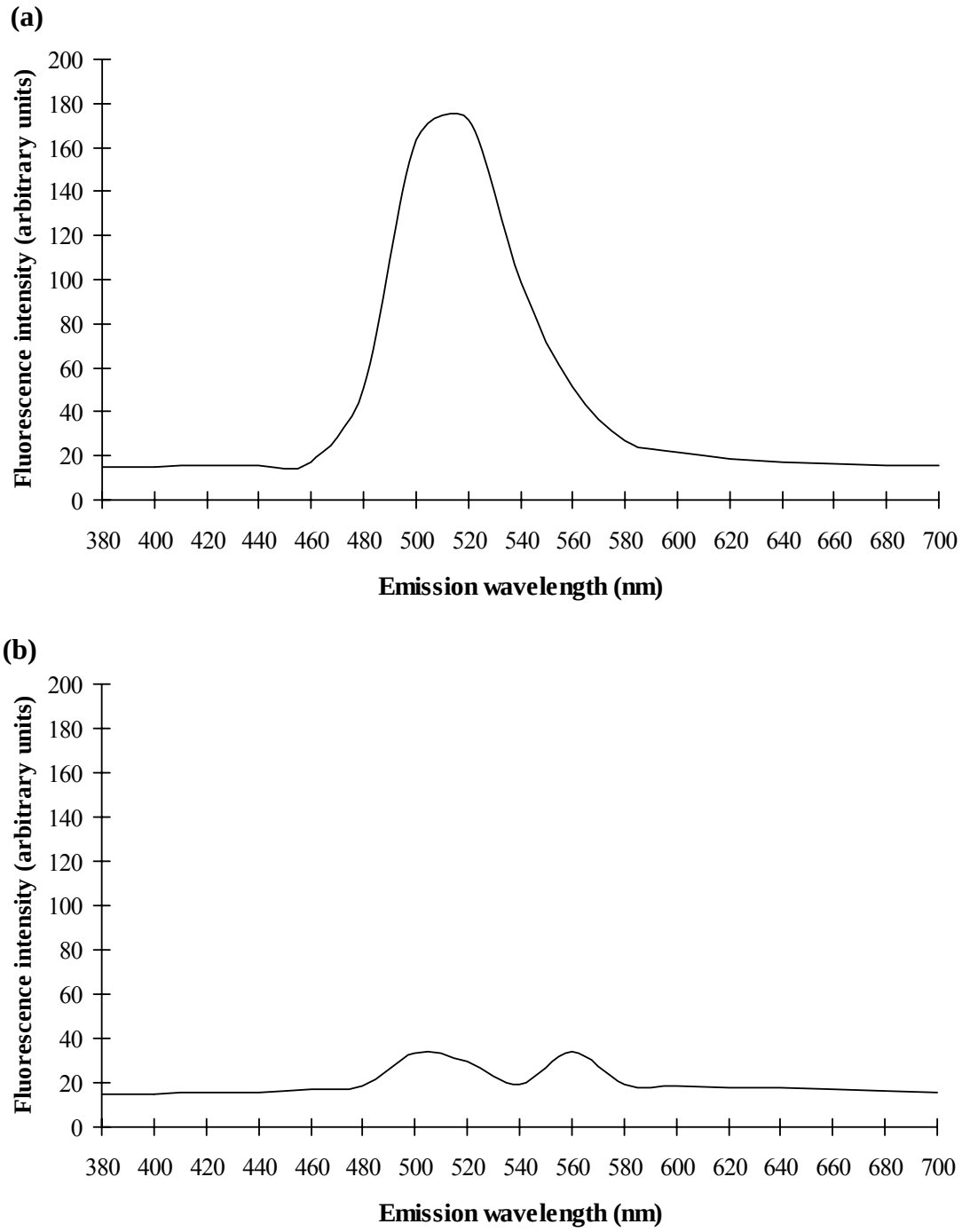

Figure 3.2 Emission spectra of PTS (50 g/l, pH 5.6) obtained with the CLSM excited at either 458 nm (a) or 488 nm (b).

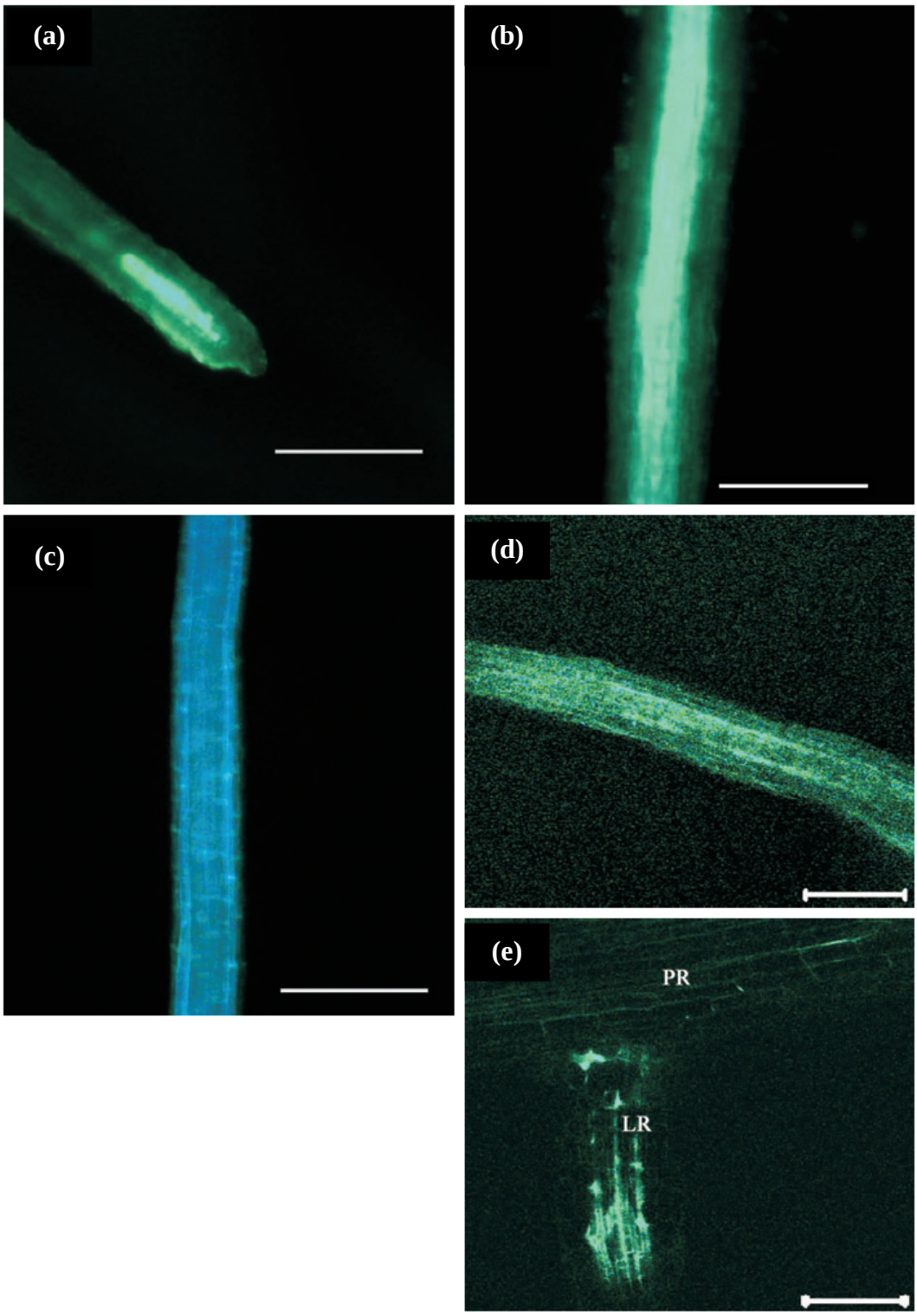

Figure 3.3 Localisation of PTS in lateral roots of rice (IR36) viewed with an epifluorescence microscope under blue-violet light using an excitation filter BP 395-440, chromatic beam splitter FT 460 and barrier filter LP 470 (a-c), or CLSM with 458 nm for excitation and emission at 485-560 nm (d-e). Samples of lateral root treated with PTS (50 g/l, pH 5.6) for 0.5 h (a-b, d-e) compared with PTS-untreated root (c). Scale bars = 100 µm. LR, lateral root; PR, parental root.

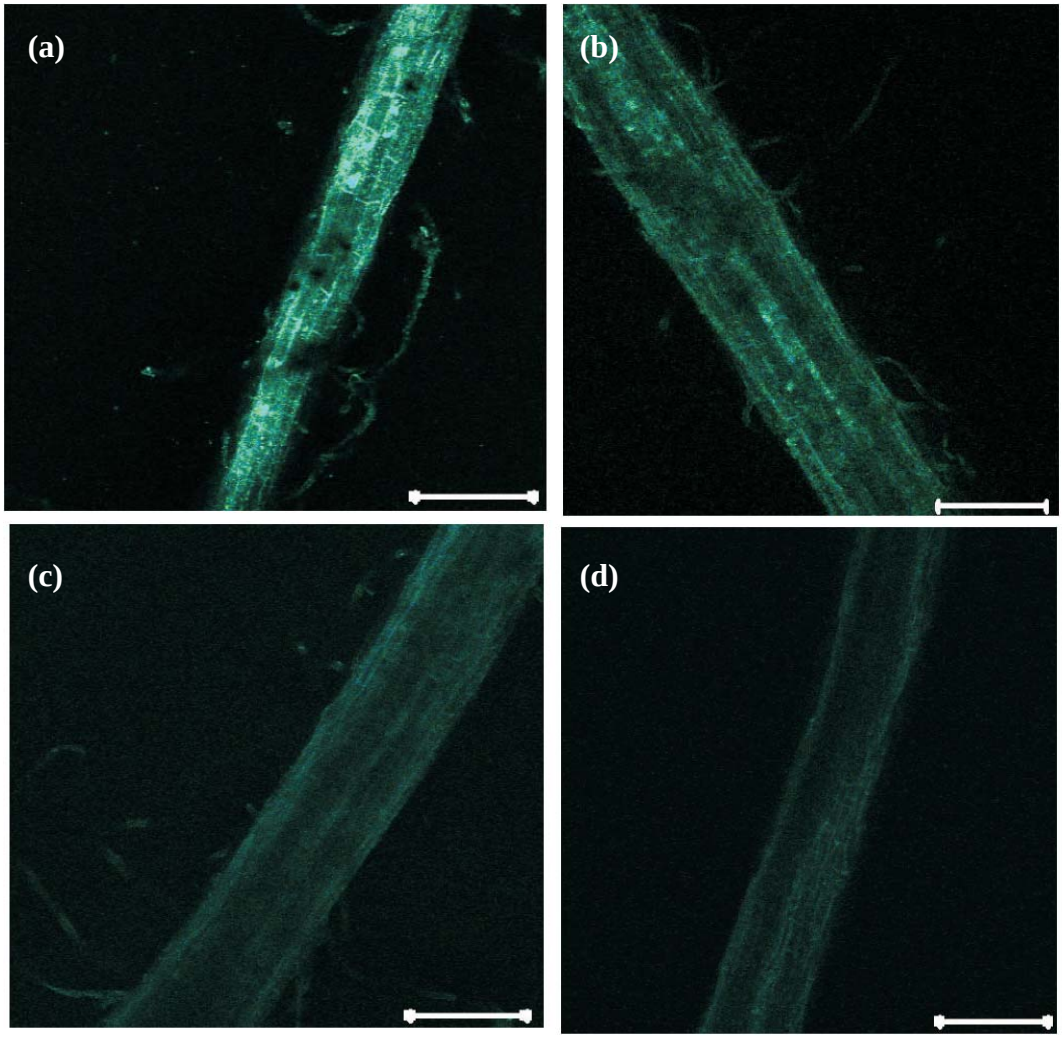

Figure 3.4 PTS intensity in lateral roots of rice (IR36) treated with PTS (50 g/l, pH 5.6) for 0.5 h viewed with CLSM with no-chase period (a), 1h- chase period (b) or 3h-chase period (c) in distilled water compared with autofluorescence in PTS-untreated roots (d). Scale bars = 100 µm.

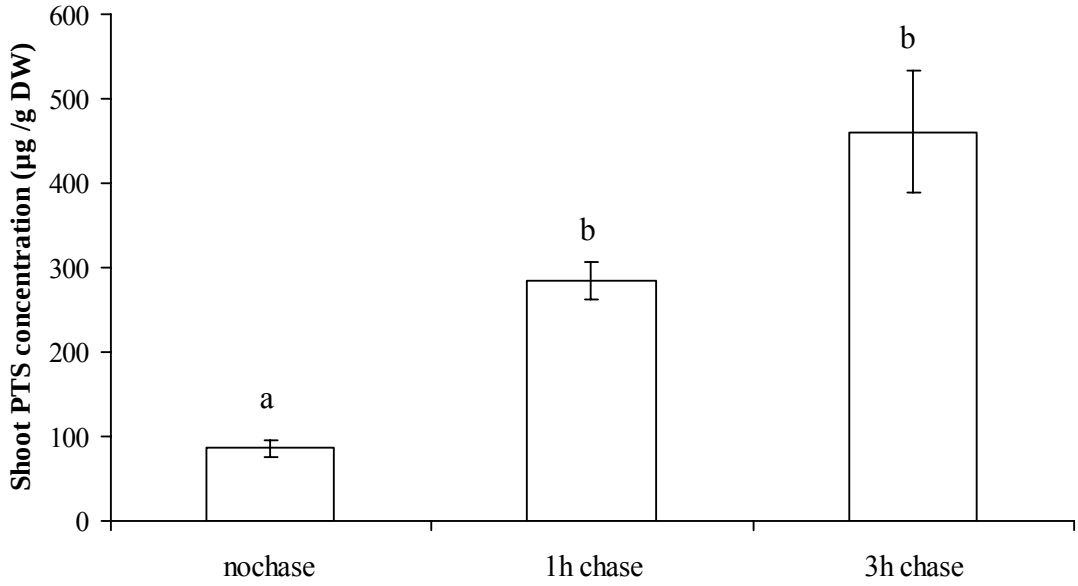

Figure 3.5 Effect of chase periods on PTS concentration in shoots. Seedlings of rice (IR36) were treated with PTS (50 g/l, pH 5.6) for 0.5 h and then transferred to distilled water for 0, 1 or 3 h. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 9) are presented.

3.3.4 Root anatomy

At a distance of 45 – 55 mm from the root tip, rhizodermis, exodermis, sclerenchyma, cortical cells and endodermis were clearly observed in seminal roots using Cryo-SEM (Figure 3.6a). In these seminal roots, suberin lamellae were fully developed in the endodermis and exodermis (Figure 3.6b, c): there were no obvious passage cells as seen in the aerial root of an orchid (*Phalaenopsis* sp.) in which 10-15 passage cells were seen in the endodermis (Figure 3.6d). However, in the lateral roots of rice the exodermal and sclerenchymatous layers were absent as compared to those of the seminal root (Figure 3.6e). This is particularly clear with roots stained with berberine-aniline blue (Figure 3.6f) or with Fluorol Yellow 088 (Appendix 3.1). Endodermal Casparian bands in lateral root were not evident (Figure 3.6f).

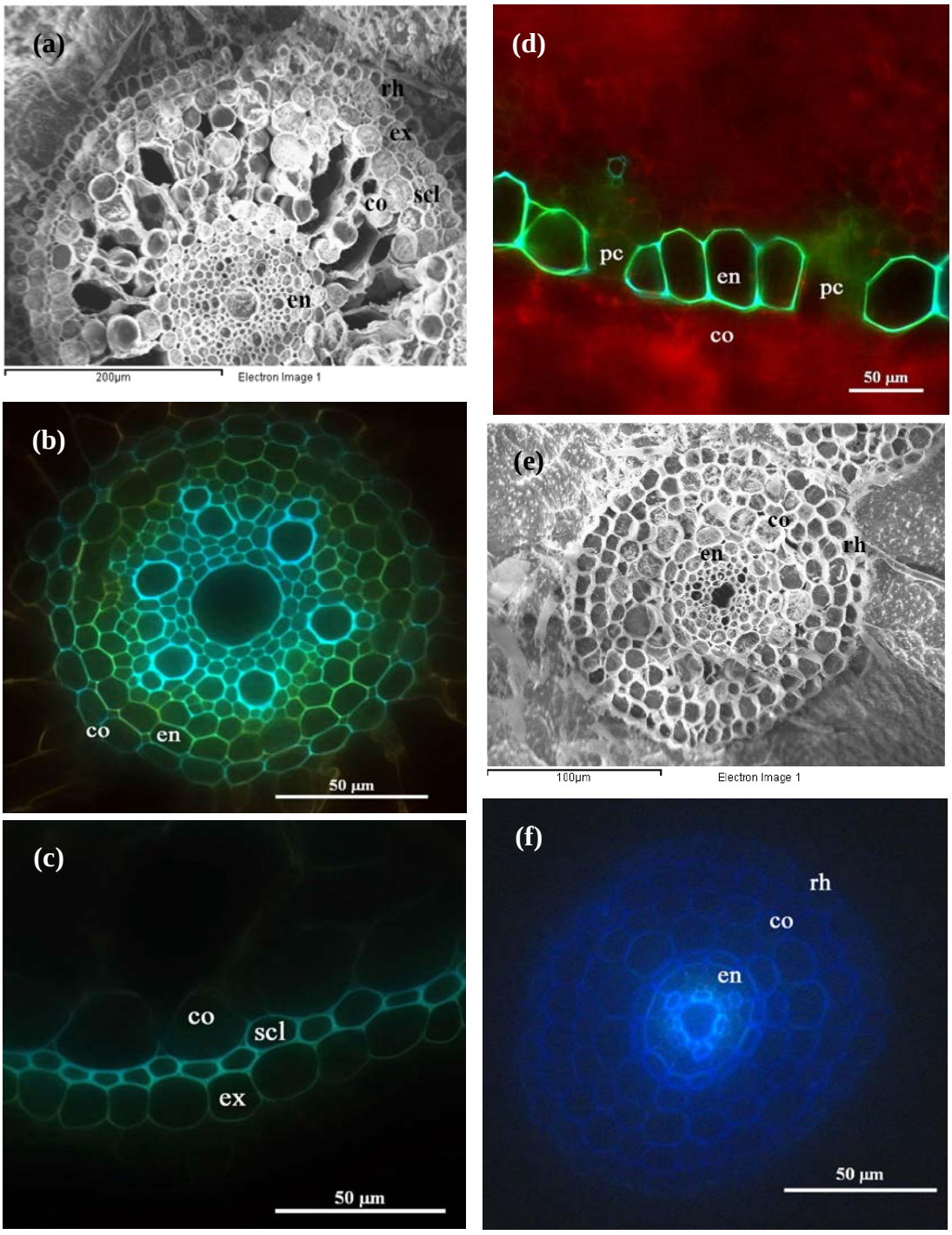

Figure 3.6 Cryo-SEM micrographs showing seminal root (a) and lateral root (e) anatomy of the rice variety IR36. Freehand cross-sections of seminal root of rice (b, c) and aerial root of an orchid (*Phalaenopsis* sp.) (d) stained with Fluorol Yellow 088 are also shown together with a freehand cross-section of a lateral root of rice stained with berberine-aniline blue (f). co, cortex; en, endodermis; ex, exodermis; rh, rhizodermis; pc, passage cell; scl, sclerenchyma. The orchid was used as a control to demonstrate the adequacy of the staining procedure.

3.3.5 Pre-treated PEG and Si on PTS in lateral roots and shoots

PEG and Si have been reported to reduce bypass flow of Na^+ uptake in the seminal root of rice, but the effect has not been tested specifically in the lateral roots (Chapter 1, section 1.6.4). In this experiment, seedlings pre-treated with PEG or Si for 7 d prior to the treatment with PTS showed reduced PTS intensity in their lateral roots (Figure 3.7a, b) as compared to untreated seedlings (Figure 3.7c). PTS, where present, was located in the surface layers of the root in both the PEG- and Si-treated roots (Figure 3.7a, b): the intensity of PTS in PEG-treated lateral roots was virtually indistinguishable from the autofluorescence of control (no PTS-treatment) roots (Figure 3.7a, d), suggesting that PEG might reduce bypass flow more effectively than Si.

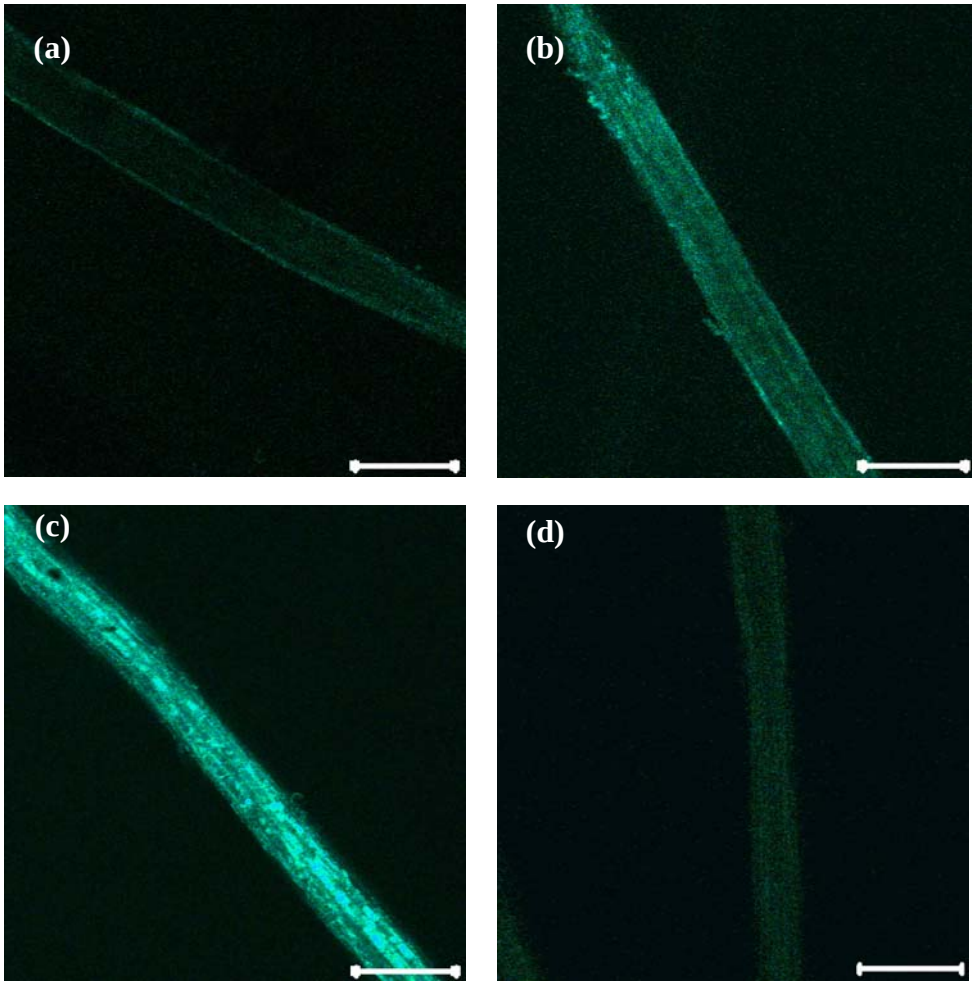

Figure 3.7 PTS intensity in lateral roots viewed with CLSM. Seven-day-old seedlings of rice (IR36) were pre-treated with 10 g/l PEG 1500 (a) or 3 mM Si (b) for 7 d before being treated with PTS (50 g/l, pH 5.6) for 0.5 h compared with PEG and Si-untreated root (c) and autofluorescence in PTS-untreated root (d). Scale bars = 100 µm.

Pre-treatment with PEG or Si dramatically reduced bypass flow as indicated by the shoot concentrations of PTS. As shown in Figure 3.8, seedlings pre-treated with PEG and Si had dramatically reduced PTS concentrations in their shoots as compared to the control - reductions of 92% and 88% in PEG and Si-treated seedlings, respectively. It is interesting to note that PEG reduced the PTS in shoots to a greater extent than Si, although the differences were not statistically significant (at P=0.05; Figure 3.8).

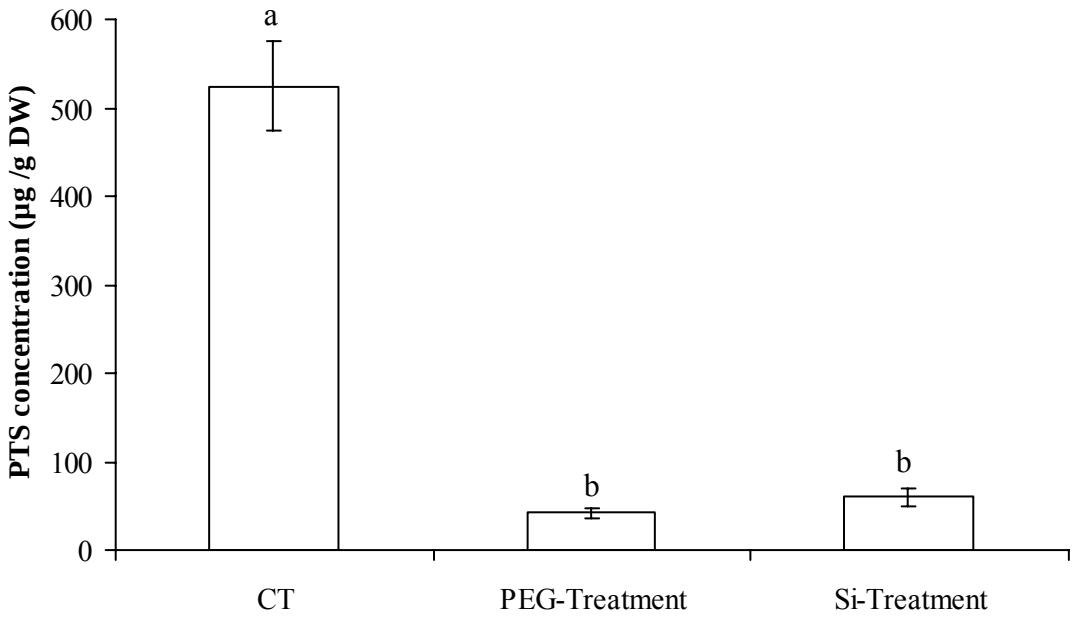

Figure 3.8 Effect of pre-treatment with PEG or Si on PTS concentration in shoots of rice (IR36). Seven-day-old seedlings were pre-treated for 7 d with 10 g/l PEG 1500 (PEG-Treatment) or 3 mM Si (Si-Treatment) before being treated with PTS (50 g/l, pH 5.6) for 0.5 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 9) are presented. CT, control (without added PEG or Si).

3.4 DISCUSSION

PTS was identified in the stele of lateral roots both with the epifluorescence microscope (Figure 3.3a, b) and CLSM (Figure 3.3d, e). The emission spectrum of PTS with its peak at 510 nm was independent of the pH value, whereas the excitation spectra were highly pH-dependent (Figure 3.1). These results are consistent with other reports (Kano & Fendler 1978; Kondo *et al.* 1982; Nunogaki & Kasai 1986; Pino *et al.* 2003). The excitation spectra at 400 nm are due to the protonated form of PTS, while the spectra at 460 nm are mainly because of the deprotonated form (Kondo *et al.* 1982; Nunogaki & Kasai 1986; Pino *et al.* 2003). The emission stability of PTS at 510 nm over a wide pH range indicates a fast equilibration between protonated and deprotonated forms (Pino *et al.* 2003). When PTS was excited at 458 nm, the emission intensity between pH 5.2 and pH 6.0 did not change significantly (Figure 3.1b). Since the pH of xylem sap in rice ranged between 5.6 – 6.0 (Brugidou *et al*. 2002; Mitani, Ma & Iwashita 2005) and is similar to the remainder of the apoplast and the culture solution, the PTS intensity observed in roots in the present experiments is likely to be a true refection of its localisation rather than of any differences in pH in different compartments. North & Nobel (1996) also reported that PTS was observed in the lateral root xylem of *Opuntia ficus-indica*. Other bypass flow tracers such as berberine, HIO_4 and Fe^{2+} have also been observed in the xylem of lateral roots of maize and common reed (Enstone & Peterson 1998; Soukup *et al.* 2002). Given the taxonomic range of these species, these data suggest that lateral roots are, in general, permeable to bypass flow tracers and a possible pathway of bypass flow through roots.

When exposure to PTS was followed by its removal from the solution, PTS fluorescence in the stele of the lateral roots was weaker when the chase period was longer (Figure 3.4), while PTS concentration in shoots increased the longer the chase period (Figure 3.5). PTS was washed out of the lateral roots and transported to the shoots through the transpiration stream, as the time course required mass flow rather than diffusion. The results obtained here confirm the existence of bypass flow from the root to the shoot in rice seedlings and are in line with earlier finding by Flowers and co-workers (Yeo *et al.* 1987, 1999; Yadav *et al.* 1996; Garcia *et al.* 1997; Gong *et al.*

2006), who reported that bypass flow is a significant component of Na^+ uptake in rice under saline conditions.

No PTS was observed in the lateral roots prior to the development of the xylem (approximately 0-30 μm from the tip; Figure 3.3a). Such a finding is consistent with previous studies by Peterson and co-workers (Peterson *et al.* 1981; Enstone & Peterson 1992; Cholewa & Peterson 2001) who reported that PTS and berberine did not penetrate into the vascular tissues of maize, broad bean, pea, onion and sunflower at the root tip. The authors speculated that the restriction of bypass flow in the apical regions was due to the small intermicrofibrillar pore spaces in young cell walls. The absence of PTS in the root apex in rice seedlings in this study indicated that PTS did not enter the vascular tissue of the laterals at the apical region of the root.

Although PTS can be seen at the junction of a lateral root and the main root axis (Figure 3.3e), the dye is within the stele of the lateral root, not the cortex of the main root. This finding agrees with that of Peterson and co-workers (Moon *et al.* 1986; Peterson & Moon 1993) who found that fluorescent dye Cellufluor, used to trace bypass flow, did not enter the cortex via the lateral/parent root junctions in mature grey mangrove and onion. The same authors explained that the cortical parenchyma cells surrounding the lateral root formed a collar-like ring impregnated with suberin and lignin to seal any discontinuity between exodermis and epidermis at the lateral root emergence. The thickening of walls with suberin lamellae in the exodermis of seminal roots shown in Figure 3.6c could support this explanation. In a previous study (Chapter 2), I found that bypass flow in mutants *lrt1*, *lrt2* and *crl1* was higher than that of their wild-types Oochikara, Nipponbare and Taichung 65, respectively, although the mutant *lrt1* did not produce any lateral roots and the other mutants (*lrt2*, *crl1*) produced few lateral roots compared with their wild types, indicating that bypass flow in rice does not originate at the sites where lateral roots emerge from the main roots. In contrast to my results, Ranathunge *et al.* (2005a, b) found that Cu^{2+} and $[Fe(CN)_6]^{4-}$ moved apoplastically from the cortex into the stele of rice cvs. IR64 and Azucena where the lateral roots emerged from the parent root. The leakage observed by Ranathunge *et al*. (2005a) could have been transient because the authors noted that no brown crystals of $Cu_2[Fe(CN)_6]$ were observed in basal parts of mature lateral roots. Peterson & Lefcourt (1990) pointed out that in broad bean, where the Casparian band is continuous from the main root to the

lateral root, the presence or absence of bypass flow at the bases of lateral roots depended on the growth rates of the laterals.

It is generally accepted that the exodermis is a barrier to bypass flow of ions and fluorescent dyes. For example, Peterson, Emanuel & Wilson (1982) and Peterson & Perumalla (1984) reported that the fluorescent dyes Tinopal CBS and Calcofluor white M2R were prevented from entering the cortex of onion and maize roots by exodermal Casparian bands. Similarly, the inward movement of berberine-thiocyanate into the cortex of onion, maize, sunflower and pickerel weed (*Pontederia cordata* L.) roots was stopped by the Casparian bands of the exodermis (Enstone & Peterson 1992, 1998; Seago, Peterson & Enstone 2000). Krishnamurthy *et al*. (2009) demonstrated that the development of Casparian bands and deposition of suberin lamellae on exodermal and endodermal walls of rice roots were correlated with reduced Na^+ uptake to the shoots and increased survival of seedlings in saline conditions. In the study of the mutants *lrt1*, *lrt2* and *crl1*, a higher percentage of bypass flow in the mutants than that of their wild types was correlated with a reduction in the deposition of suberin lamellae on exodermal and endodermal walls of the seminal root of the mutants (Chapter 2, sections 2.3.2, 2.3.5). Since there was no preventive exodermal layer in the lateral roots of rice, PTS could move freely through the cortical layers, enter the stele and be transported to the shoot via the transpiration stream. In the present study, I could see PTS in the stele of the lateral roots (Figure 3.3), although an endodermis was present (Figure 3.6e, f & Appendix 3.1), indicating that the endodermis is not a complete barrier to bypass flow: either the Casparian bands in the laterals are permeable to PTS or there are areas where they do not, or have not yet, formed barriers. Such a finding is in line with the result obtained by Ranathunge *et al*. (2005b) who reported that Cu^{2+} could cross the endodermis apoplastically either in immature or in mature parts of the roots.

Pre-treatment with either PEG or Si reduced PTS uptake either detected by the fluorescence intensity in lateral roots with CLSM (Figure 3.7) or measured fluorometrically in shoot extracts (Figure 3.8). The results are consistent with previous reports that PEG and Si reduced bypass flow in rice seedlings (Yeo & Flowers 1984b; Yeo *et al.* 1999; Gong *et al.* 2006). Since the transport of solutes from the root to the shoot is related to the transpirational volume flow (Chapter 1, section 1.5.2), any reduction in transpiration could also reduce the uptake of solutes. In the present

experiment, seedlings were treated in PTS solution for only 0.5 h, so it was impossible to measure transpiration volume. However, PEG and Si, used at similar concentrations, did not affect transpiration in rice seedlings (Yeo *et al.* 1999; Gong *et al.* 2006), indicating that PEG and Si did not reduce PTS transport as a consequence of reduced transpiration; any effect of PEG and Si on water potential was very small because of the low concentrations used. As far as the effect of Si is concerned, Yeo *et al.* (1999) inferred that adding Si to culture solution minimised Na^+ uptake in rice by reducing bypass flow. A subsequent study by Gong *et al.* (2006) showed that Si deposition in the endodermis and exodermis of seminal roots decreased Na^+ uptake by diminishing bypass flow. My results reported here (Figures 3.7, 3.8) strongly confirm that Si reduced bypass flow in rice and the mechanisms by which PEG and Si reduce the bypass flow were evident in the lateral roots.

Yeo & Flowers (1984b) demonstrated that PEG binds to root plasma membranes and suggested that the binding may reduce the leakage of Na^+ through membranes, arguing "An explanation based solely on the bypass flow requires that this contributes at least 70% of the shoot Na^+ at 10 mM NaCl and that -33 kPa of PEG blocks it specifically and effectively". However, PEG did reduce not only the uptake of Na^+ but also of K^+ from relatively high (10 mM) but not from low (0.5 or 2.0 mM) concentrations (Yeo & Flowers 1984b), a phenomenon consistent with an effect on bypass flow. The results of Yeo & Flowers (1984b) taken together with my evidence of reduced bypass flow suggest that PEG might affect both bypass flow and symplastic transport of Na^+ (T.J. Flowers personal communication).

In conclusion, the results show that PTS entered the vascular tissue of the lateral roots in rice and that it was transported to the shoots. These findings indicate a possible role for the lateral roots of rice in bypass flow and salt tolerance. PEG and Si reduced PTS uptake to the shoot by reducing the bypass flow through the lateral roots. These results together with the previous findings (Chapter 2) suggest that salt tolerance in rice would be increased, if breeding efforts reduced the bypass flow by reducing the proportion of lateral roots and/or increasing the deposition of suberin lamellae in the root system. The following Chapter reports the results of investigations into the effect of PEG, Si and RH on Na^+ uptake and bypass flow in rice seedlings under saline conditions.

CHAPTER 4

THE EFFECT OF POLYETHYLENE GLYCOL, SILICON AND RELATIVE HUMIDITY ON ION UPTAKE AND BYPASS FLOW IN RICE (*ORYZA SATIVA* L.)

4.1 INTRODUCTION

PEG is a water-soluble, non-toxic, non-ionic and non-penetrating chemical made from polymerised ethylene oxide and available in a wide range of molecular weights (Lawlor 1970; Meurk, Yanez & Bergstrom 2001; Ahmad, Javed & Ashraf 2007; Radhouane 2007; Munir & Aftab 2009). Because of these properties, PEG has been widely used as an osmotic agent to create water stress by decreasing the water potential of the culture solution in studies of plant response to drought (Radhouane 2007; Basu *et al.* 2010; Zhao *et al.* 2010) and salinity stress (Yamane *et al.* 2003; Ahmad *et al.* 2007; Veselov *et al.* 2009).

PEG has also been reported to ameliorate the adverse effects of plants under saline conditions (Chapter 1, section 1.6.4). For example, Yeo & Flowers (1984b) demonstrated that addition of PEG 1540 (at –33 kPa to -230 kPa or 10-70 g/l) to the culture solution reduced Na^+ concentration in shoots of rice genotypes Bhura Rata, IR4630, IR2153, IR28, IR20 and MI 48 by 30-80%. Their results showed that the effect of PEG on reduction of Na^+ uptake was independent of the transpiration rate. Using [^{14}C]PEG 4000, they found that PEG bound to root plasma membranes and suggested that the binding might block the leakage of Na^+ through plasma membranes (Yeo & Flowers 1984b). Subsequent explanation by Yeo *et al.* (1999) suggested that PEG blocked bypass flow at the cell walls, thus reducing Na^+ transport from roots to shoots of rice seedlings under salinity. Similarly, Ochiai & Matoh (2004) reported that addition of 0.5% PEG 500000 (w/v) to the culture solution significantly reduced Na^+ concentration in the shoots of rice cv. IR36 subjected to 100 mM NaCl for 9 h, but did not affect the transpiration rate. The authors speculated that PEG interacted with the cell

wall components through hydrogen bonding, modified the pore size and reduced the quantity of Na^+ uptake. They also found that an exogenous supply of 0.5% PEG 500000 (w/w) reduced shoot Na^+ concentration in rice seedlings by 86% after growing in saline soil contained 40 mmol NaCl for 2 months.

In tomato (*Lycopersicon esculentum* L.), Balibrea *et al.* (1999) reported that a complete immersion of 5-day-old seedlings in PEG 6000 solutions (at -500 and -750 kPa) for 12 h increased salt tolerance in adult plants treated with 100 mM NaCl (at 30 d) for 22 or 43 d. Their results showed that shoot and root fresh weights of PEG-treated plants were higher by 28-59% than those of PEG-untreated control plants. Moreover, PEG-treated plants had increased leaf K^+ concentrations and K^+/Na^+ ratios when compared with the controls. Likewise, Munir & Aftab (2009) reported that 60-day-old calli of sugarcane (*Saccharum officinarum* L. cvs. SPF 234 and HSF 240) pre-incubated with 1% PEG 4000 (w/v) for 5 d before culture in media containing 80, 100, 120 or 140 mM NaCl for 60 d reduced callus necrosis, increased the activity of antioxidant enzymes such as peroxidase (POD) and superoxide dismutase (SOD) and increased the regeneration potential as compared to non-incubated control plants. Other functions of PEG have also been reported. For instance, PEG has been used to promote growth of pollen tubes in a culture medium (Read, Clarke & Bacic 1993) and stimulate protoplast fusion (Lentz 2007).

In addition to PEG, a number of studies have shown that Si alleviates salinity stress of plants (Epstein 1994; Ma 2004; Liang *et al.* 2007; Ma & Yamaji 2008). For example, addition of 1.0 mM Si from K_2SiO_3 into a nutrient solution was found to decrease Na^+, but increase K^+ concentrations in shoots and roots of barley cvs. Jian 4 (salt tolerant) and Kepin No.7 (salt sensitive) exposed to 120 mM NaCl for 30 d (Liang 1999). Also, adding Si was found to increase plasma membrane H^+-ATPase activity in roots of salt-stressed barley (Liang 1999). In addition, inclusion of 1.0 mM Si to a culture solution increased the activity of antioxidant enzymes such as POD, SOD, GR and catalase (CAT) and the activity of vacuolar H^+-ATPase and H^+-PPase in roots of barley subjected to 120 mM NaCl for 2, 4 and 6 d (Liang *et al.* 2003; Liang *et al.* 2005).

Saqib, Zorb & Schubert (2008) reported that adding 1.0 mM Si from Na_2SiO_3 to the culture solution significantly improved the shoot growth of bread wheat cvs. SARC-1

(salt tolerant) and 7-Cerros (salt sensitive) subjected to 125 mM NaCl for 21 d by reducing shoot Na^+ concentration, but increasing shoot K^+/ Na^+ ratio. Tuna *et al.* (2008) also reported that supplementary Si from Na_2SiO_3 at 0.25 and 0.5 mM increased salinity tolerance in both bread wheat cv. Izmir-85 and durum wheat cv. Gediz-75, hydroponically grown in 100 mM NaCl for 7 weeks. They found that Si treatments increased the concentrations of K^+ in both shoots and roots of wheat, whereas Na^+ concentrations were significantly decreased. Ashraf *et al.* (2010) also showed that addition of different levels of Si as $CaSiO_3$ at 1.4, 2.1 and 2.8 mM to the growth medium significantly improved shoot growth and juice quality (% sucrose in juice) in sugarcane cvs. SPF 213 (salt-sensitive) and HSF 240 (salt-tolerant) grown in gravel at 100 mM NaCl for 60 or 330 d, by reducing Na^+ concentration, but increasing K^+ concentration and K^+/Na^+ ratio in shoots. Those alleviative effects were more pronounced in salt-sensitive than salt-tolerant genotypes (Ashraf *et al.* 2010).

The effect of Si on the growth of rice growing under saline conditions has also been investigated (Chapter 1, section 1.6.4). Matoh *et al.* (1986) found that salinity severely reduced shoot dry weight by 57% in cv. Kinmaze hydroponically grown in 100 mM NaCl for 30 d, but the reduction of shoot dry weight was only 35% when Si was added at 0.89 mM (from Na_2SiO_3) to the culture solution. Na^+ concentration in shoots of Si-treated rice was only 54% of that in rice grown without adding Si. The authors suggested that Si deposition in cell walls of leaves decreased the transpiration, decreased Na^+ transport to the shoot and reduced salinity damage. Later, Yeo *et al.* (1999) showed that Si did not reduce salinity damage in rice by a reduction in the transpiration volume flow. Their results revealed that adding Si at 3 mM (from Na_2SiO_3) to the culture solution either significantly increased whole-plant transpiration volume in rice cvs. IR36 and GR4, or did not significantly affect this parameter in cv. CSR10 subjected to 50 mM NaCl for 48 h, but the addition of Si significantly reduced Na^+ concentration in the shoots by 29, 47 and 54% for CSR10, IR36 and GR4, respectively, as compared to Si-untreated seedlings. By using a bypass flow tracer, PTS, Yeo *et al.* (1999) found that Si reduced bypass flow from roots to the shoots by 39, 58 and 70% for CSR10, IR36 and GR4, respectively. As a result, the authors proposed that the deposition of Si in the roots could be the mechanism by which Si reduced the bypass flow of Na^+ in rice seedlings under saline conditions. A subsequent study by Gong *et al.* (2006) using scanning electron microscopy and X-ray microanalysis demonstrated that

Si was deposited in the exodermal and endodermal layers of the seminal roots and these depositions were correlated with a reduction of shoot Na^+ and bypass flow in Si-treated rice. Gong *et al.* (2006) also confirmed that application of Si to the culture solution did not affect the transpiration rate, but it directly inhibited the bypass flow at the roots of seedlings, decreased the transport of Na^+ to the shoot and reduced the transport of Na^+ in the xylem.

In Chapter 3, it was shown that the mechanisms by which PEG and Si reduced bypass flow were evident in the lateral roots and the results obtained from CLSM of the lateral roots and fluorescence spectrometry of shoot extracts of PTS also suggested that PEG might reduce bypass flow more effectively than Si (Chapter 3, section 3.3.5). However, shoot Na^+ concentration and the magnitude of bypass flow were not reported in Chapter 3 since the main objective was to present evidence of the site of bypass flow. Consequently, in the first part of this Chapter, Na^+ concentration in the shoots and the percentage of bypass flow have been quantified in order to compare the effectiveness of PEG and Si in reducing bypass flow in rice seedlings growing under salt stress (50 mM NaCl for 96 h). Na^+ concentrations in the xylem were determined using the xylem-feeding insect *Philaenus spumarius* L. (Malone, Watson & Pritchard 1999; Watson, Pritchard & Malone 2001; Malone, Herron & Morales 2002; Ponder *et al.* 2002; Bridges 2005; Roshandel 2005; Gong *et al.* 2006) and the quantity of Na^+ in the phloem sap estimated using the EDTA technique (King & Zeevaart 1974; Berthomieu *et al.* 2003).

Another parameter that could reduce Na^+ transport to the shoot, hence reducing a plant's susceptibility to salinity, is humidity (Yeo & Flowers 1984b; Lauter & Munns 1987; Salim 1989; Yeo *et al.* 1990; Asch *et al.* 1999, 2000; An *et al.* 2001, 2002; Flowers *et al.* 2010). Yeo & Flowers (1984b) reported that an increase in RH from 70 to 90% reduced the concentration of Na^+ in the shoot of rice genotype IR2153 by 64% when treated with 50 mM NaCl for 10 d. Asch *et al.* (1999) also showed that Na^+ uptake to the panicle of rice growing in 30 mM NaCl until maturity (~ 5 months) was lower in the wet season with 60-100% RH than in the hot dry season with 10-65% RH.

Increasing RH has also been reported to mitigate the adverse effects of salinity in other plant species. For example, Lauter & Munns (1987) studied the effect of different RH

(55, 75, 88 and 95%) and NaCl levels (0, 12, 24 and 36 mM) on the accumulation of Na^+ in leaves of chickpea (*Cicer arietinum* L.) cvs. L-550 (salt resistant) and E-100 (salt sensitive) hydroponically grown for 35 d. The authors reported that increasing RH levels decreased the leaf Na^+ concentration in both genotypes examined. An *et al.* (2001) reported that Na^+ concentration in leaves of soybean (*Glycine max* L. cv. Tachiyutaka) subjected to 40 and 80 mM NaCl for 3 weeks was lower in 70% RH than in 30% RH. Similarly, an increase in RH from 30 to 70% significantly decreased the concentration of Na^+ in leaves of muskmelon (*Cucumis melo* L. cv. Revigal C-8) exposed to 80 mM NaCl for 15 d (An *et al*. 2002). Backhausen *et al*. (2005) also found that potato (*Solanum tuberosum* L. cv. Desiree) plants subjected to 300 mM NaCl under 60% RH were damaged within 5 d, while the plants grown in 85% RH could survive at least for 3 weeks under the same salt-stressed condition. The authors reported that the rate of Na^+ accumulation in leaves of potato was very low at high RH as compared to the low RH levels.

Since the transport of solutes to the shoot in general is related to the transpiration stream (Chapter 1, section 1.5.2), it has been suggested that increasing RH levels would decrease salt accumulation in the plants by a reduction of the transpirational volume flow (Lauter & Munns 1987; Asch *et al*. 1999; An *et al*. 2001; Backhausen *et al*. 2005). However, Yeo & Flowers (1984b) reported that although the transpiration volume was reduced by 65% in 90% RH as compared to 70% RH, the Na^+ concentration in the xylem sap of rice was reduced by only 32%. Also, Salim (1989) found that an increase in RH from 30 to 90% decreased the transpiration rate and shoot Na^+ concentration in salt-sensitive mung bean (*Vigna raiata* L.) subjected to salt stress (100 mM NaCl for 7 d), whereas increased RH reduced shoot Na^+ concentration in salt-tolerant *Atriplex spongiosa* L. without affecting the transpiration rate under the same salt conditions. Taken together, it is still unclear how RH interacts with salt stress.

A model of water and solute transport in plants, involving cell-to-cell and apoplastic pathways (bypass flow) has been discussed over many years (see, for example, Weatherley 1965; Slatyer 1967; Steudle & Frensch 1996; Steudle & Peterson 1998; Fricke 2000, 2002; Steudle 2000a, b, 2001). Water and solutes can move from cell-to-cell via a pathway that has a symplastic and a transcellular component: water and solutes move symplastically from one cell to the next via the plasmodesmata, whereas

in transcellular movement, water and solutes have to cross cell walls and then cell membranes. In contrast to the cell-to-cell pathway, water and solutes can move apoplastically over both short and long distances through intercellular spaces and cell walls (Sanderson 1983; Hanson *et al.* 1985; Yeo *et al.* 1987; Steudle & Frensch 1996; Steudle & Peterson 1998; Fricke 2000, 2002; Steudle 2000a, b, 2001). However, it is generally accepted that the apoplastic transport of water and solutes across roots is stopped or reduced by the deposition of hydrophobic material in radial walls of the exodermal and endodermal layers of the root (Chapter 1, section 1.3.2) as well as in the mestome sheath of the leaves (e.g. barley, Fricke 2002). The volume flow through the cell-to-cell pathway and the bypass flow can be flexibly switched from one to another depending on the hydraulic and osmotic driving forces on water (Steudle & Frensch 1996; Steudle & Peterson 1998; Steudle 2000a, b, 2001). The driving force for water movement is affected by a number of environmental stresses such as drought, salinity, temperature, low oxygen or heavy metals (Steudle & Frensch 1996; Steudle & Peterson 1998; Steudle 2000a, b, 2001; Fricke 2002).

In the first part of this Chapter, I compare the effectiveness of PEG and Si in reducing bypass flow in rice growing in saline conditions: the results showed that PEG was the more effective. In the second part, I report the results of tests of my hypothesis that changing the RH levels would influence the uptake of Na^+ and the bypass flow in rice by changing the proportion of water flowing through the cell-to-cell pathway and the bypass flow of the root. The results obtained revealed that a change in RH levels had a significant effect on the flux of solution across the root and this was correlated with a change in the magnitude of the bypass flow.

4.2 MATERIALS AND METHODS

4.2.1 Plant materials

Caryopses of rice (*Oryza sativa* L.) cvs. CSR10 (salt tolerance), IR36 (intermediate tolerance) and GR4 (salt sensitive) were soaked in aerated water for 24 h and germinated on nylon mesh supported on Perspex grids floating on modified Yoshida culture solution (Chapter 2). At day 7 after sowing, single seedlings were transplanted into 50 ml black-painted glass tubes and into 3l-black-painted boxes filled with culture

solution. The plants were held in place with non-absorbent cotton wool or with sponge and grown in the glasshouse under the same conditions as described in Chapter 3.

4.2.2 NaCl, PEG and Si treatments

Fourteen-day-old seedlings of CSR10, IR36 and GR4 were treated with either 50 mM NaCl and 100 mg/l PTS (NaCl), 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500 (NaCl+PEG), or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si (NaCl+Si) in the form of Na_2SiO_3 (BDH, 25.5-28.5% SiO_2). Plants were allowed to take up Na^+ and PTS for 96 h, after which the treatment solution was replaced by culture solution for a chase period of 48 h to carry any remaining Na^+ and PTS in the root xylem to the shoot. After the chase period, shoots were harvested and were then dried at 80 °C for 72 h. Whole-plant transpiration was measured gravimetrically and corrected for water loss by evaporation during treatment with NaCl and PTS and a subsequent chase period (see Chapter 2, section 2.2.8 for more details). Bypass flow and Na^+ delivered to the shoots via bypass flow was calculated as described previously in Chapter 2, sections 2.2.8 and 2.3.5.

4.2.3 Xylem sap collection and analysis

Xylem sap was collected from leaves 2 and 3 of CSR10, IR36 and GR4 subjected to salt treatments with PEG and Si as stated above (Section 4.2.2) using adults of the xylem-feeding insect *P. spumarius* (Malone *et al.* 1999, 2002; Watson *et al.* 2001; Ponder *et al.* 2002; Bridges 2005; Gong *et al.* 2006). Adults of *P. spumarius* were collected (July-September) in a meadow near Devil's Dyke in West Sussex, UK, using a sweep net and an aspirator. An Eppendorf tube (1.5 ml) was modified to form a cage by making small holes on the lid and a slit through the side. The insects were placed in the cages (two insects per cage) and the cages fitted midway along leaves 2 and 3 of rice seedlings by sliding the leaf through the slit in the side of the tube (Appendix 4.1). Excreta were collected from the bottom of the cage with a Hamilton syringe (Hamilton Bonaduz, Schweiz, Switzerland) through holes on the lid at 24, 48, 72 and 96 h after initiation of salt stress (50 mM NaCl and 100 mg/l PTS). Only cages that had at least 40 µl of excreta were used for collection to avoid an error due to evaporation as indicated by Malone *et al*. (2002). Na^+ and K^+ concentrations in the excreta were measured by atomic absorption spectroscopy (Unicam 919, Cambridge, UK). PTS fluorescence was

analysed with a fluorescence spectrometer (Perkin-Elmer LS-3B; Beaconsfield, Buckinghamshire, UK) at $\lambda_{excitation}$ = 403 nm and $\lambda_{emission}$ = 510 nm.

4.2.4 Phloem sap collection and analysis

Phloem sap was obtained using the EDTA technique (see Chapter 2, section 2.2.9 for more details). After the end of treatment at 96 h, leaves 2 and 3 were cut at their leaf sheath-blade junctions and leaf blades were transferred into EDTA solutions to allow phloem exudation. The quantity of Na^+ in the EDTA solution was determined by atomic absorption spectroscopy (Unicam 919, Cambridge, UK).

4.2.5 The effect of RH on ion uptake and bypass flow

Fourteen-day-old seedlings of cv. IR36 were subjected to 50 mM NaCl and 100 mg/l PTS for 96 h at different RH levels: 30, 50, 70 or 90% in a plant growth cabinet (SANYO MLR-350HT, Osaka, Japan) with 12 h photoperiod of photosynthetically active radiation (250-300 μmol m^{-2} s^{-1}) supplied with fluorescent lamps (40W x 15 lamps), at 30±1 °C; the average temperature during dark period was 25±1 °C. Shoots were harvested and dried in an oven at 80 °C for 72 h. Whole plant transpiration was measured gravimetrically and corrected for water loss by evaporation (Chapter 2, section 2.2.8). Bypass flow and Na^+ delivered to the shoots via bypass flow was calculated as described previously in Chapter 2, sections 2.2.8 and 2.3.5.

4.2.6 Determination of ions and PTS fluorescence in the shoots

After recording the dry weight, shoots were extracted in 10 ml distilled water for 2 h at 90 °C. Na^+ and K^+ in the extracts were measured by atomic absorption spectroscopy (Unicam 919, Cambridge, UK). PTS florescence was analysed at $\lambda_{excitation}$ = 403 nm and $\lambda_{emission}$ = 510 nm with a fluorescence spectrometer (Perkin-Elmer LS-3B; Beaconsfield, Buckinghamshire, UK).

4.2.7 Analysis of data

The experiments were repeated at least twice. The results presented in this Chapter are averages between the repeat experiments by pooling individual data. Statistical analysis was carried out using the Mann-Whitney test as the data were not distributed normally about the mean.

4.3 RESULTS

4.3.1 Ion and PTS concentrations in seedlings harvested at the beginning of experiments (T-0)

The average (across varieties) concentration of ions in shoots of rice seedlings grown in culture solution without added NaCl, PEG or Si was 0.07 mmol/gDW for Na^+ and 1.2 mmol/gDW for K^+ (Figure 4.1a, b). Statistical analysis showed that salt-tolerant CSR10, intermediate-tolerant IR36 and salt-sensitive GR4 were similar in Na^+ and K^+ concentrations at the beginning of the experiments (Figure 4.1a, b). Autofluorescence of shoot extracts at excitation/emission wavelength used to estimate PTS was equivalent to a concentration of 0.0026 mg/gDW (Figure 4.1c). This concentration was negligible compared with PTS fluorescence of PTS-treated seedlings (Appendix 4.2).

4.3.2 The effect of added PEG and Si to culture solution during NaCl stress of rice seedlings

4.3.2.1 Shoot and root dry weight measurements

Addition of PEG to the culture solution did not affect shoot dry weight of rice seedlings in any of the genotypes growing in 50 mM NaCl for 96 h (Figure 4.2a & Appendix 4.3). Although added Si slightly increased shoot dry weight, the effect was not statistically significant (Figure 4.2a). Adding PEG and Si did not change root dry weight in CSR10 and GR4, but in IR36, adding Si produced a significant reduction in root dry weight (Figure 4.2b).

4.3.2.2 Na^+ and K^+ concentrations in the shoots

After exposure to 50 mM NaCl for 96 h, shoot Na^+ concentrations of seedlings without added PEG or Si to culture solution increased to 0.11, 0.35 and 0.35 mmol/gDW for CSR10, IR36 and GR4, respectively (Figure 4.3a & Appendix 4.3). Addition of PEG and Si significantly decreased shoot Na^+ concentrations in all genotypes (Figure 4.3a). PEG reduced shoot Na^+ concentration by 33, 20 and 36%, whereas Si reduced this concentration by 28, 29 and 39% for CSR10, IR36 and GR4, respectively, as compared to NaCl-stressed plants without added PEG or Si. No significant difference was

observed between PEG and Si treatments in the reduction of shoot Na^+ concentration in the three genotypes examined (Figure 4.3a).

Shoot K^+ concentrations in PEG-treated seedlings were significantly higher than those for Si-treated and the control seedlings (Figure 4.3b & Appendix 4.3). In contrast, Si did not change the concentration of K^+ in IR36 and GR4, but slightly reduced the concentration in CSR10 (Figure 4.3b & Appendix 4.3). The addition of PEG and Si significantly increased shoot K^+/Na^+ ratio in all genotypes compared with the controls, but the effect was not different between PEG and Si treatments (Figure 4.3c).

4.3.2.3 Na^+ and K^+ contents and transport from the root to the shoot

Na^+ content in the shoot of seedlings grown in 50 mM NaCl for 96 h (without added PEG and Si) was 7.0, 22.4 and 22.8 µmol for CSR10, IR36 and GR4, respectively (Figure 4.4a & Appendix 4.3). This content significantly decreased when seedlings were subjected to salt stress together with either PEG or Si (Figure 4.4a). The addition of PEG and Si increased K^+ content in all rice genotypes, although it was statistically significant only in IR36 with the addition of PEG (Figure 4.4b).

In the absence of additional PEG and Si, the net Na^+ transport from the root to the shoot was 2.9, 6.9 and 10.2 µmol/g RDW/h for CSR10, IR36 and GR4, respectively (Figure 4.5a). The addition of PEG to the culture solution decreased the net transport to 1.8, 5.6 and 6.3 µmol/g RDW/h, while adding Si reduced it to 2.3, 6.0 and 6.8 µmol/g RDW/h for CSR10, IR36 and GR4, respectively (Figure 4.5a & Appendix 4.3). Both PEG and Si significantly reduced the net transport of Na^+ to the shoot, although their effect was not statistically different (Figure 4.5a). In contrast to Na^+ transport, the addition of PEG increased the net K^+ transport to the shoot in all genotypes, although it was statistically significant only in IR36 (Figure 4.5b). Similarly, the addition of Si to the culture solution increased the net K^+ transport in rice genotypes and the results were significant in IR36 and GR4 (Figure 4.5b).

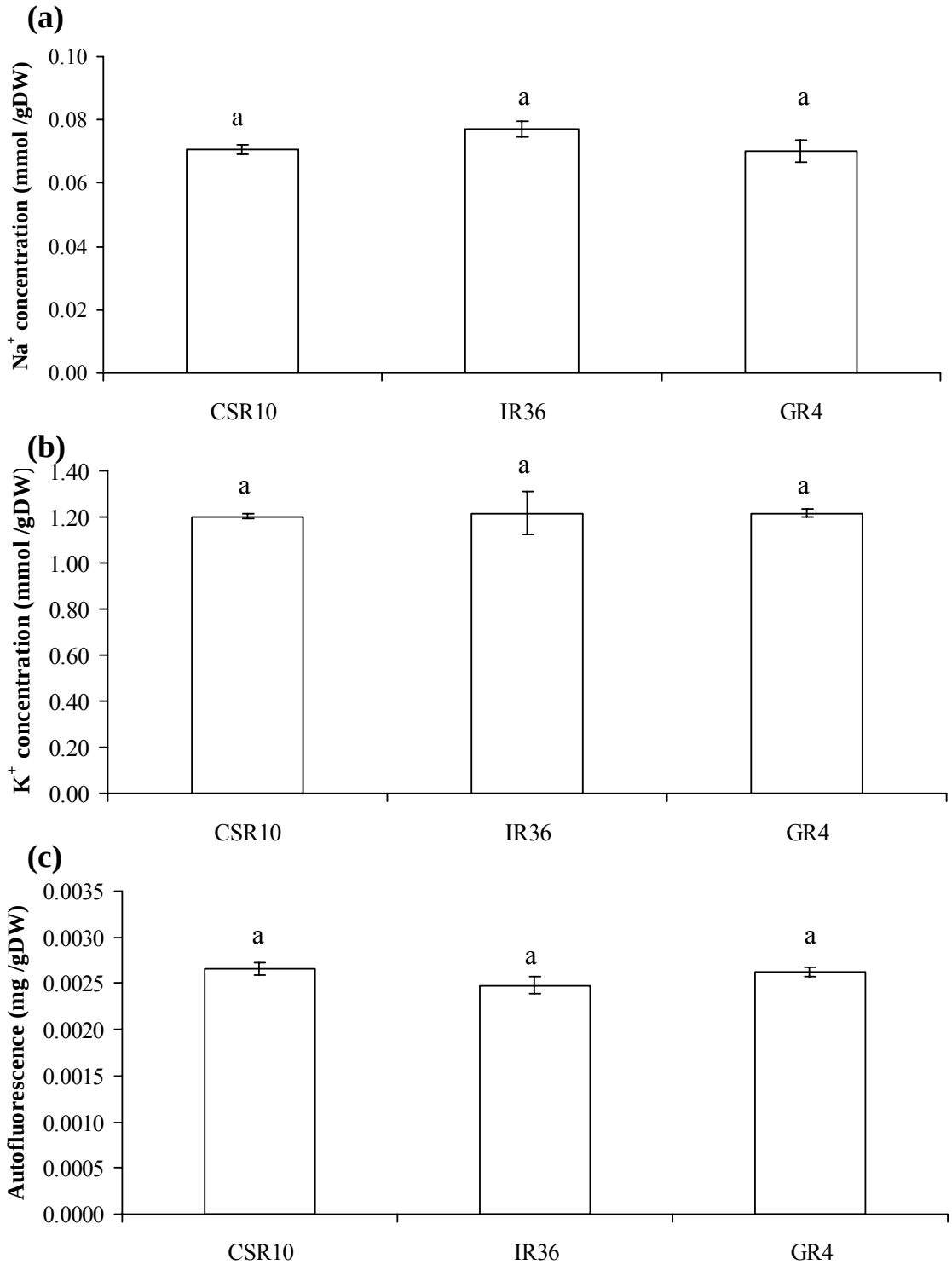

Figure 4.1 Concentrations of Na^+ (a), K^+ (b), and autofluorescence (c) in shoots of rice seedlings cvs. CSR10, IR36 and GR4 harvested at the beginning of experiments (T-0). Seedlings were grown in modified Yoshida culture solution without added NaCl, PEG or Si for 14 d. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 20).

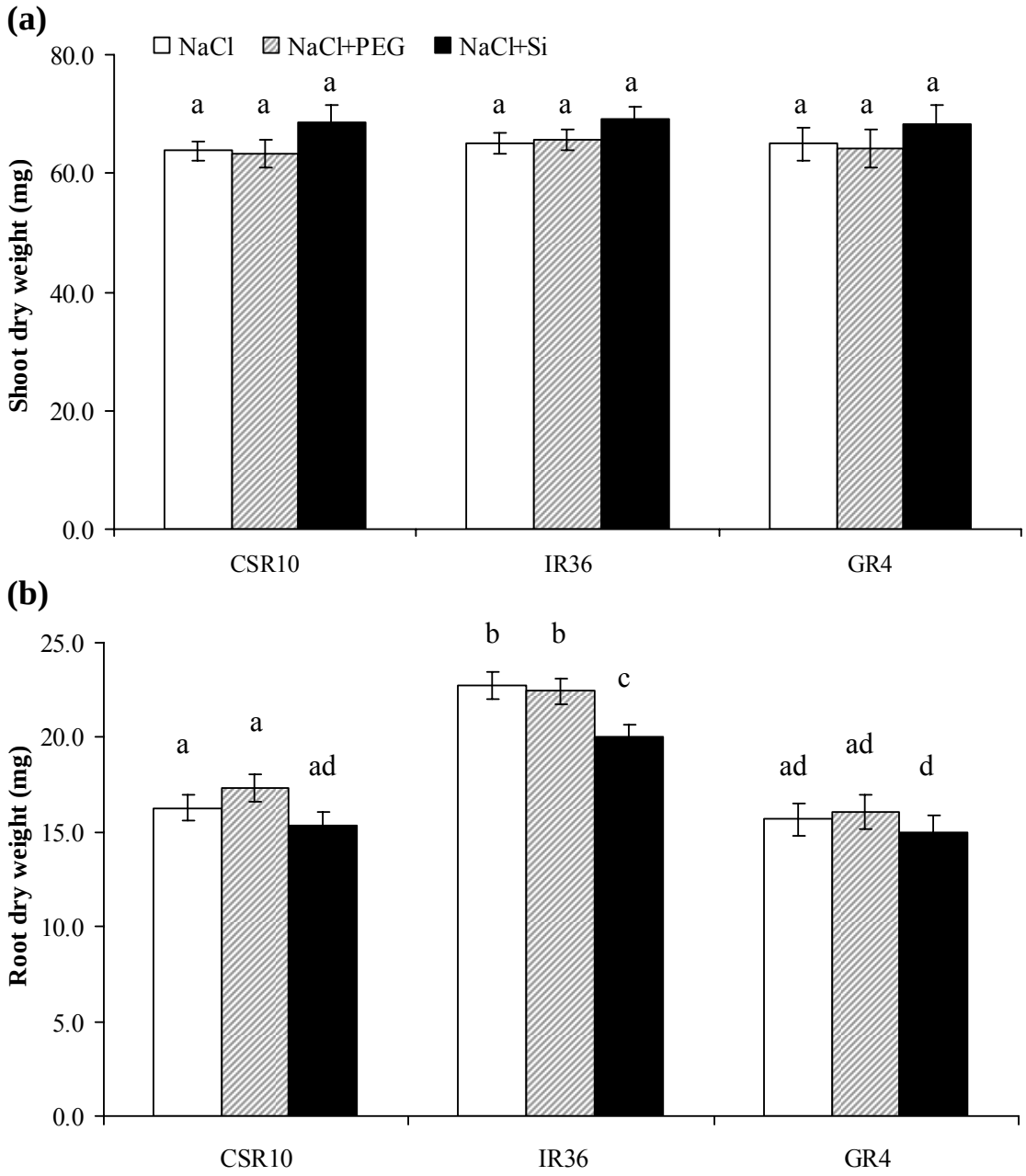

Figure 4.2 Effect of PEG and Si on shoot dry weight (a) and root dry weight (b) of rice cvs. CSR10, IR36 and GR4 under NaCl stress. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS (NaCl), 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500 (NaCl+PEG), or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si (NaCl+Si) for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 20).

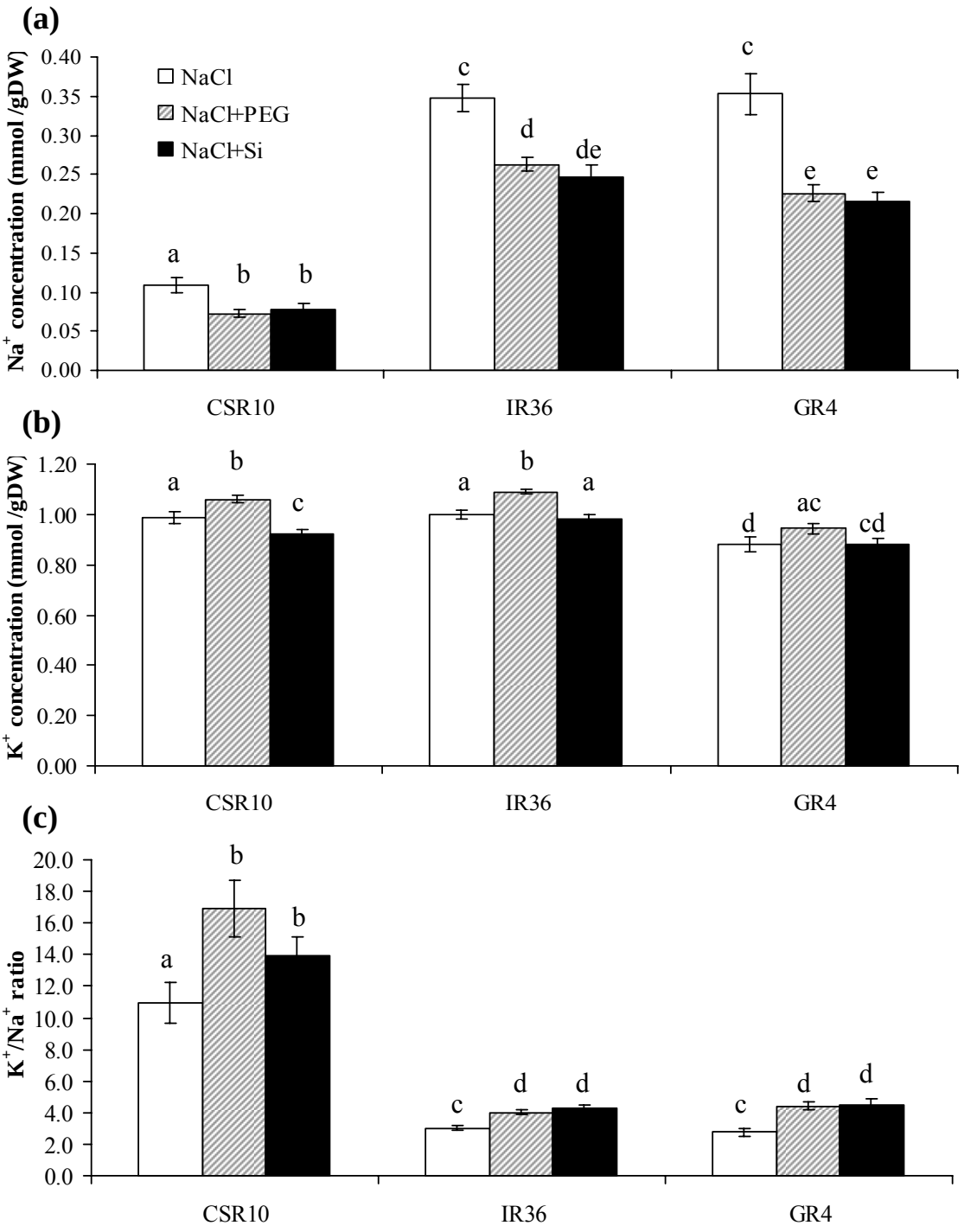

Figure 4.3 Effect of added PEG and Si on concentrations of Na^+ (a), K^+ (b), and K^+/Na^+ ratio on a molar basis (c) in shoots of rice cvs. CSR10, IR36 and GR4. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS (NaCl), 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500 (NaCl+PEG), or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si (NaCl+Si) for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 20).

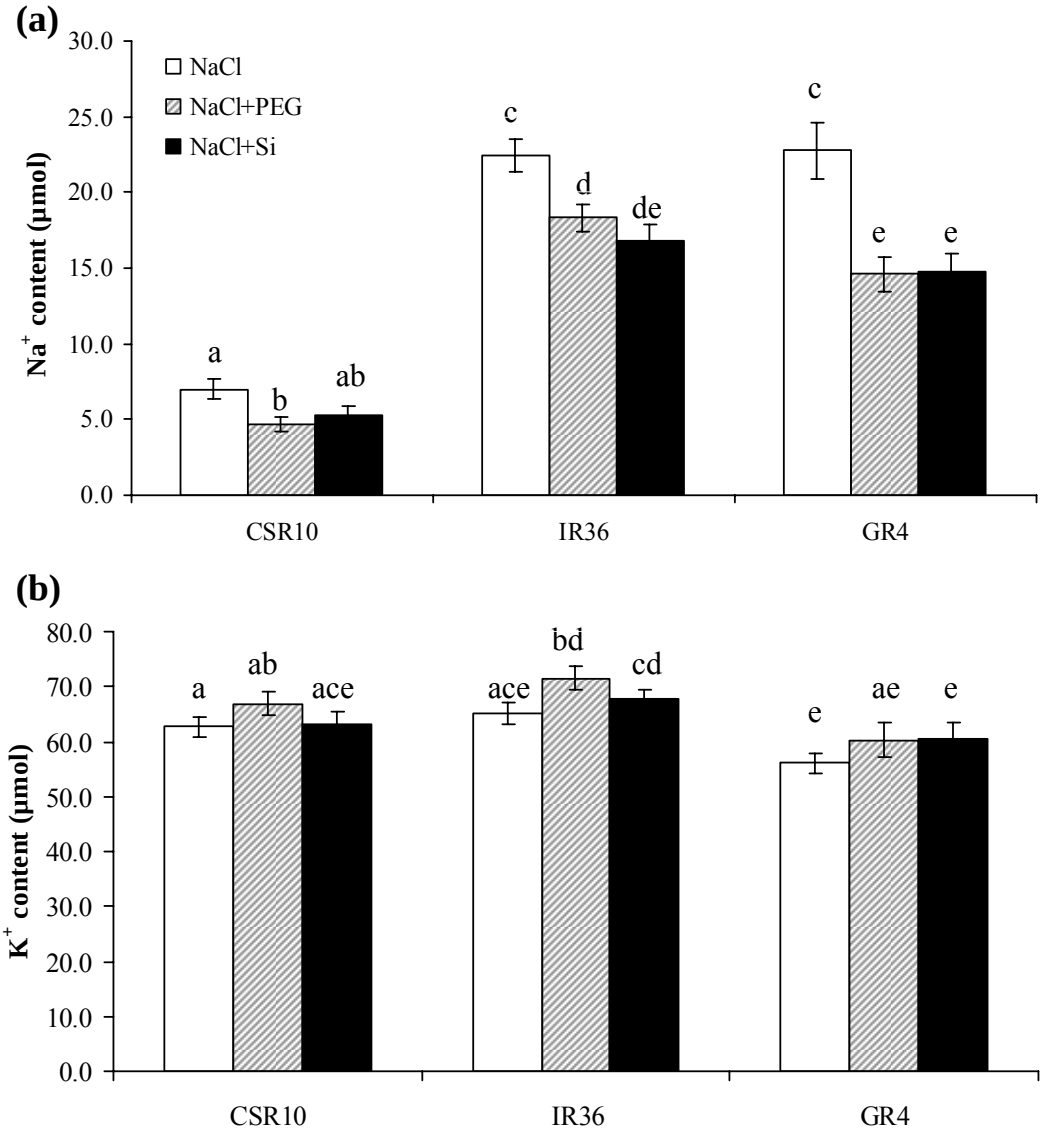

Figure 4.4 Effect of added PEG and Si on contents of Na^+ (a) and K^+ (b) in shoots of rice seedlings cvs. CSR10, IR36 and GR4. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS (NaCl), 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500 (NaCl+PEG), or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si (NaCl+Si) for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 20).

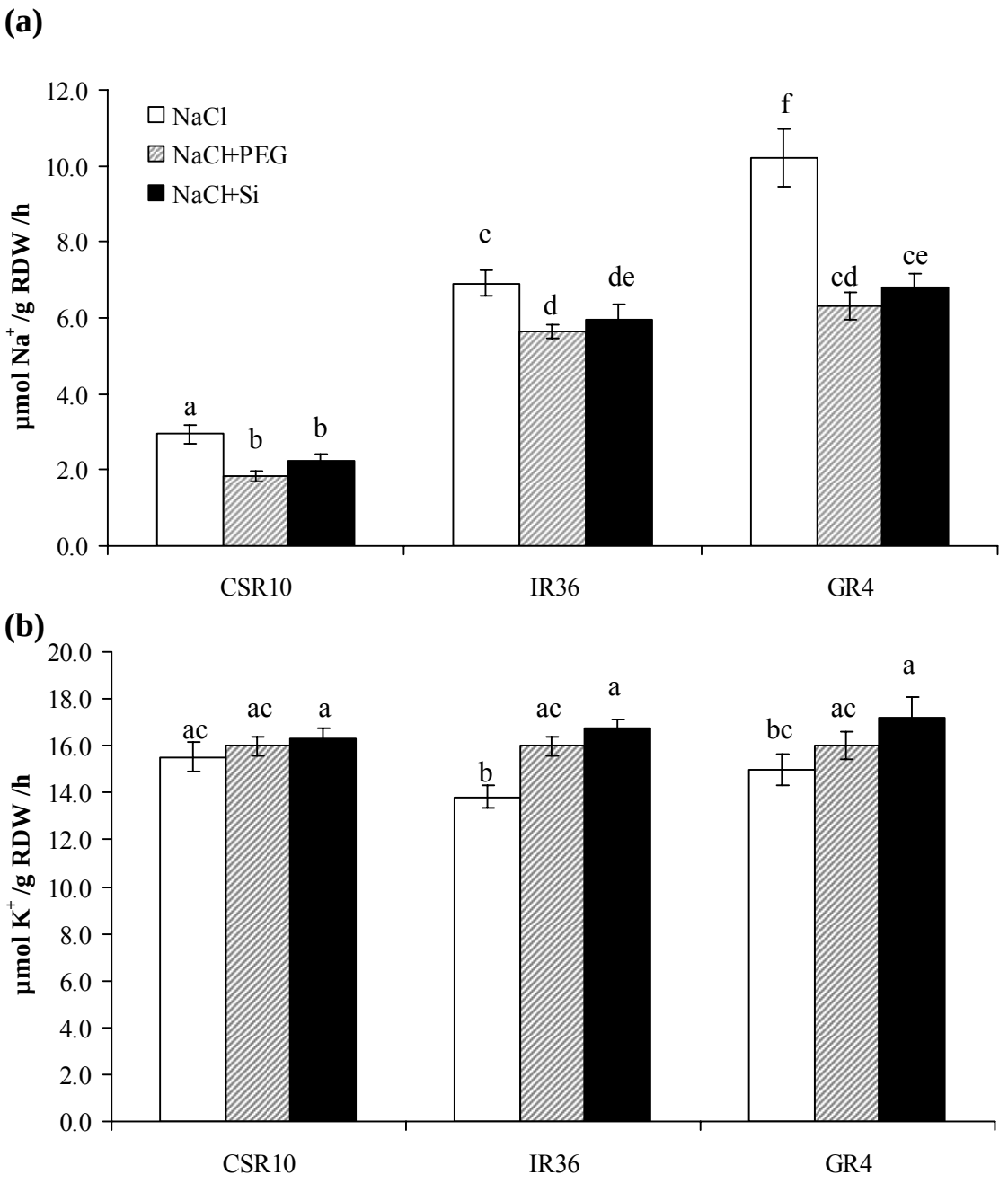

Figure 4.5 Effect of added PEG and Si on net Na^+ (a) and K^+ (b) transport from root to shoot in rice seedlings cvs. CSR10, IR36 and GR4 growing in the presence of NaCl. Fourteen-day-old rice seedlings were treated with 50 mM NaCl and 100 mg/l PTS (NaCl), 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500 (NaCl+PEG), or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si (NaCl+Si) for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 20).

4.3.2.4 Transpiration and bypass flow

Data analysis of the uptake of water, PTS and Na^+ by whole shoots of rice seedlings revealed that PEG and Si did not affect transpiration volume in any of the genotypes tested (Table 4.1). Without added PEG and Si, bypass flow was 1.2, 2.7 and 4.1% in salt-stressed seedlings of CSR10, IR36 and GR4, respectively. Adding PEG to the culture solution reduced the percentage of bypass flow to 0.6, 0.8 and 1.4% for CSR10, IR36 and GR4, respectively, whereas addition of Si reduced the bypass flow to 0.9, 1.5 and 2.3% for CSR10, IR36 and GR4, respectively (Table 4.1). Interestingly, bypass flow was significantly lower in PEG-treated than in Si-treated seedlings in all rice genotypes (Table 4.1). The same result was also seen in seedlings pre-incubated with PEG or Si for 7 d before exposure to salt stress (Appendix 4.4). The apparent PTS and Na^+ concentrations in the transpiration stream of PEG- and Si-treated seedlings were significantly lower than those of the controls (Table 4.1), confirming that PEG and Si reduced the bypass flow from root to the shoot in the xylem sap.

Using the data of the water bypass flow (J_{VB}), Na^+ concentration in the external medium ($Na_{[ext]}$) and transpiration volume (Jv), it was possible to calculate the Na^+ delivered to the shoots via bypass flow in each variety (see Chapter 2, section 2.3.5 for more details). The results revealed that in seedlings growing without addition of PEG and Si to the culture solution, the bypass flow delivered 5.9, 17.5 and 21.4 μmol of Na^+ to the shoots of CSR10, IR36 and GR4, respectively (Figure 4.6 & Appendix 4.3). The addition of PEG significantly reduced Na^+ delivered by bypass flow to 8.3 μmol in IR36 (by 52.6%) and 6.2 μmol in GR4 (by 71.2%). Similarly, adding Si significantly decreased Na^+ bypass flow to 12.1 μmol (by 30.7%) in IR36 and 7.8 μmol (by 63.4%) in GR4 (Figure 4.6). Although PEG and Si reduced Na^+ bypass flow in shoots of CSR10 to 3.5 and 4.7 μmol (by 41.1 and 19.8%), respectively, the results were not statistically different from PEG and Si-untreated plants (Figure 4.6).

Comparison between added PEG and Si showed that the addition of PEG had a more pronounced effect than added Si in the reduction of the Na^+ delivered to the shoots via bypass flow in all rice genotypes, although the effect was statistically different only in the intermediate-tolerant IR36 (Figure 4.6).

Table 4.1 Na^+, PTS uptake and bypass flow (J_{VB}) by rice seedlings cvs. CSR10, IR36 and GR4.

	CSR10			IR36			GR4		
	NaCl	NaCl+PEG	NaCl+Si	NaCl	NaCl+PEG	NaCl+Si	NaCl	NaCl+PEG	NaCl+Si
Jv (ml)	13.9±0.67a	12.3±0.68a	13.2±0.58a	13.5±0.60a	13.3±0.63a	12.9±0.42a	14.1±1.05a	13.0±1.07a	13.1±0.76a
PTS (µg)	2.29±0.22a	0.98±0.08b	1.73±0.24cf	4.79±0.68d	1.51±0.18c	2.96±0.19d	6.70±0.64e	2.10±0.23f	4.62±0.69d
$PTS_{[xyl]}$(µM)	0.32±0.03a	0.16±0.01b	0.23±0.02c	0.71±0.10d	0.22±0±03bc	0.39±0.02ae	1.09±0.19d	0.36±0.05ac	0.61±0.07de
$PTS_{[xyl]}/PTS_{[ext]}$ (%)	0.16±0.02a	0.08±0.01b	0.12±0.01c	0.35±0.05de	0.11±0.01bc	0.20±0.01ae	0.55±0.09d	0.18±0.03ac	0.31±0.04e
Jvb (%)	1.22±0.11a	0.60±0.05b	0.89±0.09c	2.69±0.38de	0.85±0.10bc	1.48±0.07af	4.14±0.71e	1.37±0.20ac	2.32±0.29f
Na (µmol)	6.99±0.69a	4.73±0.48b	5.33±0.49ab	22.5±1.12c	18.3±0.91d	16.9±0.98de	22.8±1.87c	14.6±1.14e	14.8±1.14e
$Na_{[xyl]}$ (mM)	0.50±0.04a	0.37±0.03b	0.38±0.03b	1.74±0.13c	1.42±0.08d	1.33±0.08de	1.79±0.19cd	1.19±0.09e	1.20±0.13e
$Na_{[xyl]}/Na_{[ext]}$ (%)	1.00±0.09a	0.75±0.05b	0.74±0.05b	3.48±0.26c	2.83±0.16d	2.58±0.16de	3.57±0.38cd	2.38±0.18e	2.33±0.26e

PTS fluorescence in the shoot was divided by water transpired (Jv) to give the apparent PTS concentration in the transpiration stream ($PTS_{[xyl]}$). Dividing this by PTS concentration in the external medium gave the leakage of PTS ($PTS_{[xyl]}/PTS_{[ext]}$). An empirical correction factor (7.57, Yeo *et al.* 1987) for the relative mobilities of PTS and water was applied to estimate the water bypass flow (J_{VB}). Na^+ content in the shoot was divided by water transpired to give the apparent Na^+ concentration in xylem ($Na_{[xyl]}$). This was then divided by the concentration of Na^+ in the external medium to give the leakage of Na^+ ($Na_{[xyl]}/Na_{[ext]}$). PTS concentration in the external medium was 100 mg/l (0.2 mM). Na^+ concentration in the external medium was 50 mM for control (NaCl) and PEG treatment (NaCl+PEG), and 51.5 mM for Si treatment (NaCl+Si). Values of a parameter within a row followed by the same letters are not significantly different at $P<0.05$ according to the Mann-Whitney test. The data are means and standard errors (n = 20).

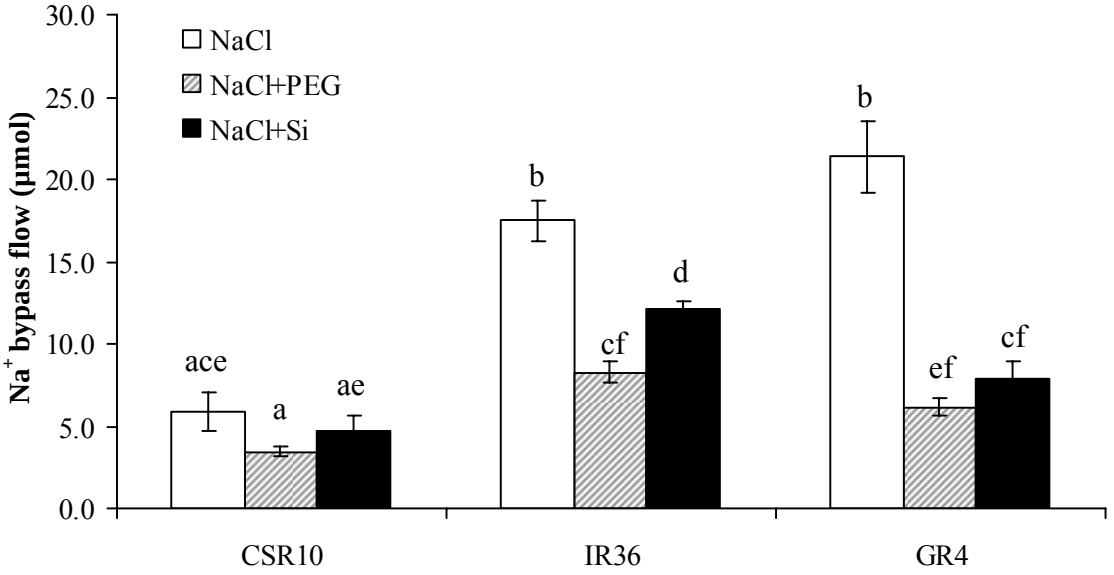

Figure 4.6 Na^+ delivered by bypass flow in shoots of rice seedlings cvs. CSR10, IR36 and GR4 grown in the presence of NaCl. Fourteen-day-old rice seedlings were treated with 50 mM NaCl and 100 mg/l PTS (NaCl), 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500 (NaCl+PEG), or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si (NaCl+Si) for 96 h and harvested after a chase period of 48 h in culture solution. Na^+ bypass flow was calculated as the water bypass flow (J_{VB}) multiplied by $Na_{[ext]}$ and Jv, and compared with total Na^+ content to get percentage of Na^+ bypass flow. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 7-40).

4.3.2.5 Ion and PTS concentrations in the xylem sap

In general, Na^+ and PTS concentrations in the xylem sap of the control seedlings (without added PEG or Si) of all genotypes significantly increased during exposure to 50 mM NaCl for 96 h, whereas K^+ concentrations fluctuated (Figures 4.7 - 4.9 & Appendices 4.4 – 4.6). The addition of PEG and Si to the culture solutions significantly reduced the concentrations of Na^+ and PTS in the xylem sap of both leaves 2 and 3 (Figures 4.7 - 4.9 & Appendices 4.5 – 4.7). Although the xylem concentrations of Na^+ and PTS were lower in PEG than Si treatments, their effects were not statistically different (Appendices 4.5 – 4.7).

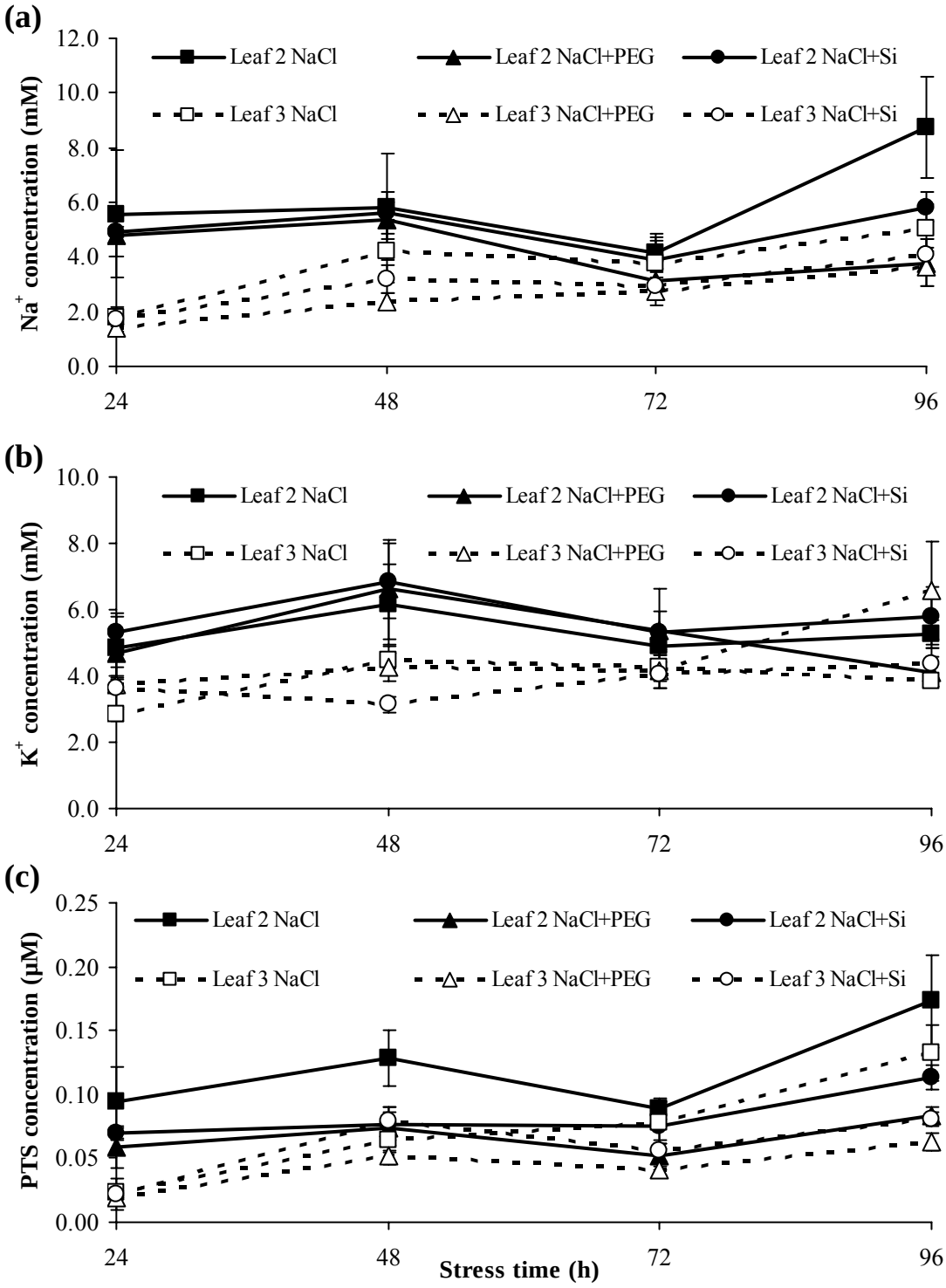

Figure 4.7 Concentrations of Na^+ (a), K^+ (b) and PTS (c) in the xylem sap of leaves 2 and 3 of rice cv. CSR10. Fourteen-day-old rice seedlings were treated with either 50 mM NaCl and 100 mg/l PTS, 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500, or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si for 96 h. The xylem sap was collected using the xylem-feeding insect *P. spumarius*. Means and standard errors (n = 6-14).

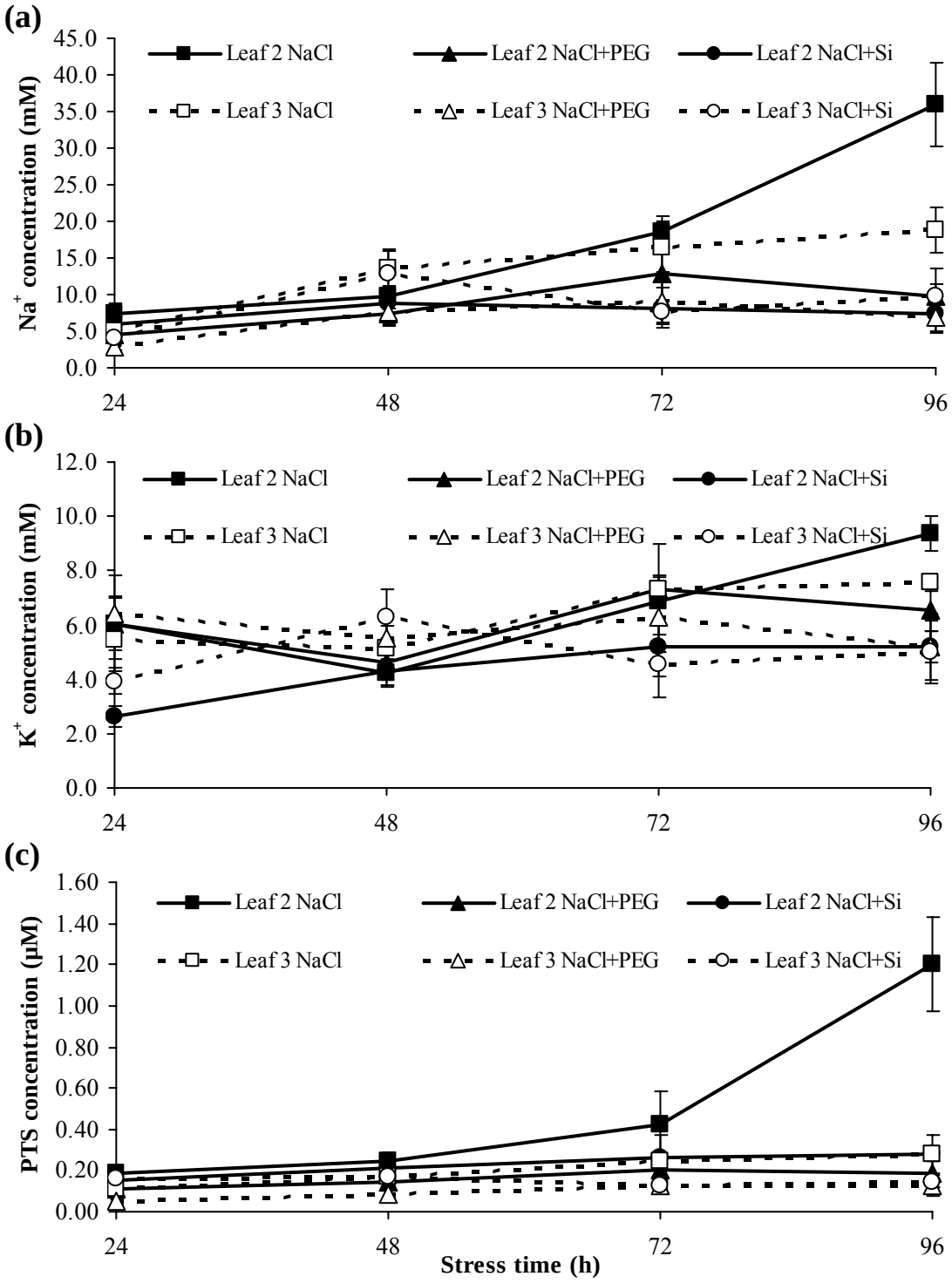

Figure 4.8 Concentrations of Na^+ (a), K^+ (b) and PTS (c) in the xylem sap of leaves 2 and 3 of rice cv. IR36. Fourteen-day-old rice seedlings were treated with either 50 mM NaCl and 100 mg/l PTS, 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500, or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si for 96 h. The xylem sap was collected using the xylem-feeding insect *P. spumarius*. Means and standard errors (n = 5-10).

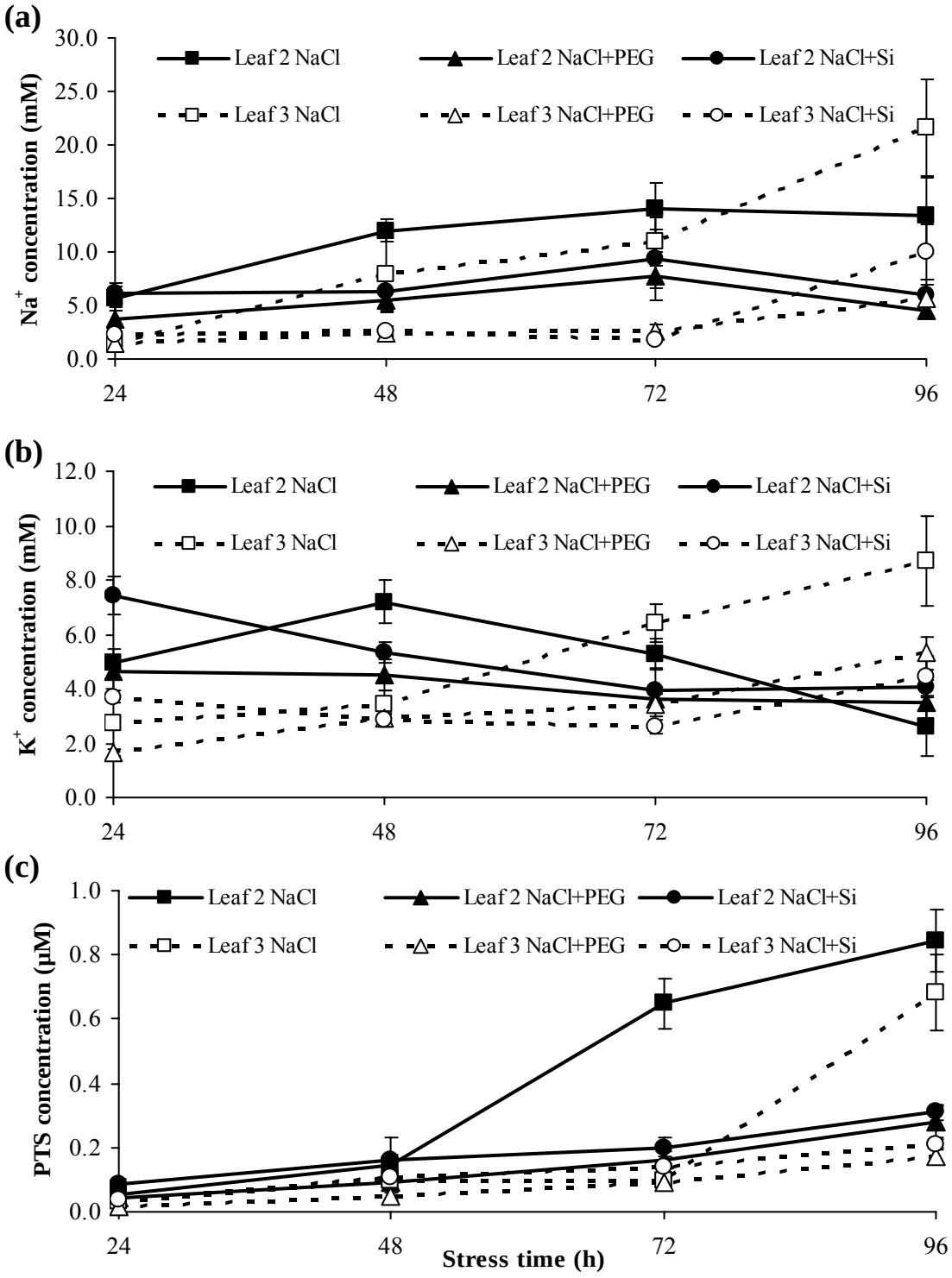

Figure 4.9 Concentrations of Na^+ (a), K^+ (b) and PTS (c) in the xylem sap of leaves 2 and 3 of rice cv. GR4. Fourteen-day-old rice seedlings were treated with either 50 mM NaCl and 100 mg/l PTS, 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500, or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si for 96 h. The xylem sap was collected using the xylem-feeding insect *P. spumarius*. Means and standard errors (n = 5-12).

4.3.2.6 Na^+ in phloem sap

The quantity of Na^+ in phloem sap of CSR10, IR36 and GR4 ranged from 13 to 52 µmol in leaf 2 and 4 to 32 µmol in leaf 3 (Table 4.2). These figures were negligible as compared to those of *lrt1* (340 and 144 µmol for leaves 2 and 3, respectively; see Chapter 2, section 2.3.6), indicating that no significant amount of Na^+ was present in the phloem sap of CSR10, IR36 and GR4.

Table 4.2 The quantity of Na^+ in the phloem sap of leaves 2 and 3 of rice cvs. CSR10, IR36 and GR4

Stress time (h)		Na^+ in phloem sap (µmol)	
		Leaf 2	Leaf 3
CSR10	NaCl	37.8±7.48a	32.3±3.91a
	NaCl+PEG	51.6±7.41b	23.2±1.62a
	NaCl+Si	30.6±4.06a	9.61±2.02c
IR36	NaCl	36.7±12.0ab	19.5±7.90ac
	NaCl+PEG	13.1±4.88a	17.7±14.6a
	NaCl+Si	27.6±5.77b	30.9±7.48bc
GR4	NaCl	39.4±9.72ac	3.65±1.25b
	NaCl+PEG	19.8±5.51ac	4.19±1.03b
	NaCl+Si	20.3±1.47a	12.8±1.77c

Fourteen-day-old rice seedlings were treated with 50 mM NaCl and 100 mg/l PTS, 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500, or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si for 96 h. The phloem sap was collected in EDTA solutions. Statistical comparison was made among leaves and treatments (2x3). Values followed by the same letters are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 20).

4.3.3 The effect of RH on the response of rice to salt

4.3.3.1 Effect of RH on ion uptake

The shoot Na^+ concentration of cv. IR36 dramatically decreased when the RH was 70% or higher, when compared to plants growing in 30% or 50% RH (Table 4.3). The reduction was 22.7 and 42.7% in 70 and 90% RH, respectively. The shoot K^+ concentration (Table 4.3), however, was only reduced at high RH (90%) and then by less than the reduction in Na^+ (11.0% reduction for K^+ and 42.7% for Na^+). The effect on shoot PTS concentration was similar to the effect on Na^+ concentration (Table 4.3).

Table 4.3 The effect of RH on Na^+, K^+ and PTS concentrations in shoots of rice seedlings cv. IR36.

	30%RH	50%RH	70%RH	90%RH
Na^+ concentration (mmol/gDW)	1.03±0.02a	1.05±0.03a	0.80±0.02b	0.60±0.02c
K^+ concentration (mmol/gDW)	1.19±0.02a	1.21±0.02a	1.17±0.02a	1.07±0.03b
PTS concentration (µg/gDW)	85.3±4.76a	82.3±7.48a	55.4±3.22b	28.7±2.76c

Fourteen-day-old seedlings were exposed to NaCl (50 mM) plus PTS (100 mg/l) at 30, 50, 70 or 90% RH and harvested 4 d later. Values within a row followed by the same letters are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 30).

4.3.3.2 Effect of RH on bypass flow

Increasing RH from 30% to 90% significantly decreased the bypass flow of Na^+ to the shoot from 32 to 22% (Table 4.4). Bypass flow of Na^+ in 70% and 90% RH was significantly less statistically than that in 30% RH (Table 4.4). The bypass flow of Na^+ in 90% RH was lower than at 70% RH. The apparent Na^+ and PTS concentrations in the xylem at 90% RH were also lower than those of 70%. There was no difference in the apparent Na^+ and PTS in the xylem between 30% and 50% treatments, although they were statistically higher than those of 70% and 90% RH (Table 4.4). Transpiration volume was not obviously affected by the different RH levels (Table 4.4). However, the flux of water across the roots (expressed as transpiration volume per unit of root dry weight per unit of time) decreased statistically when the RH increased from 30% to 90% (Figure 4.10). The flux of water across the root was highly correlated with shoot Na^+ concentration (Figure 4.11a) and bypass flow (Figure 4.11b).

Table 4.4 The effect of RH on Na^+, PTS uptake and bypass flow (J_{VB}) by rice seedlings cv. IR36.

	30%RH	50%RH	70%RH	90%RH
Jv (ml)	18.8±0.64a	21.2±0.57b	22.3±0.61b	18.6±0.65a
PTS (µg)	7.06±0.40a	7.88±0.74a	6.03±0.42a	3.15±0.30b
$PTS_{[xyl]}$ (µM)	0.73±0.05a	0.70±0.06a	0.51±0.03b	0.34±0.04c
$PTS_{[xyl]}/PTS_{[ext]}$ (%)	0.39±0.02a	0.37±0.03a	0.27±0.02b	0.18±0.02c
J_{VB} (%)	2.91±0.18a	2.80±0.23a	2.04±0.12b	1.33±0.15c
Na (µmol)	85.5±2.55a	100±2.81b	87.2±3.36a	65.6±2.22c
$Na_{[xyl]}$ (mM)	4.68±0.20a	4.79±0.15a	3.93±0.13b	3.60±0.14c
$Na_{[xyl]}/Na_{[ext]}$ (%)	9.36±0.40a	9.59±0.29a	7.86±0.25b	7.20±0.28c
$Na_{[xyl]}/PTS_{[xyl]}$	13.3±0.81a	14.9±0.90ab	15.3±0.57b	23.9±1.37c
Bypass flow of Na (µmol)	26.7±1.51a	29.8±2.79a	22.8±1.60a	14.2±1.38b
Bypass flow of Na (%)	31.8±1.93a	29.4±2.55ab	26.0±1.23b	21.6±1.66c

Values within a row followed by the same letters are not significantly different at $P<0.05$ according to the Mann-Whitney test. The data are means and standard errors (n = 30). See Table 4.1 for the details of calculation.

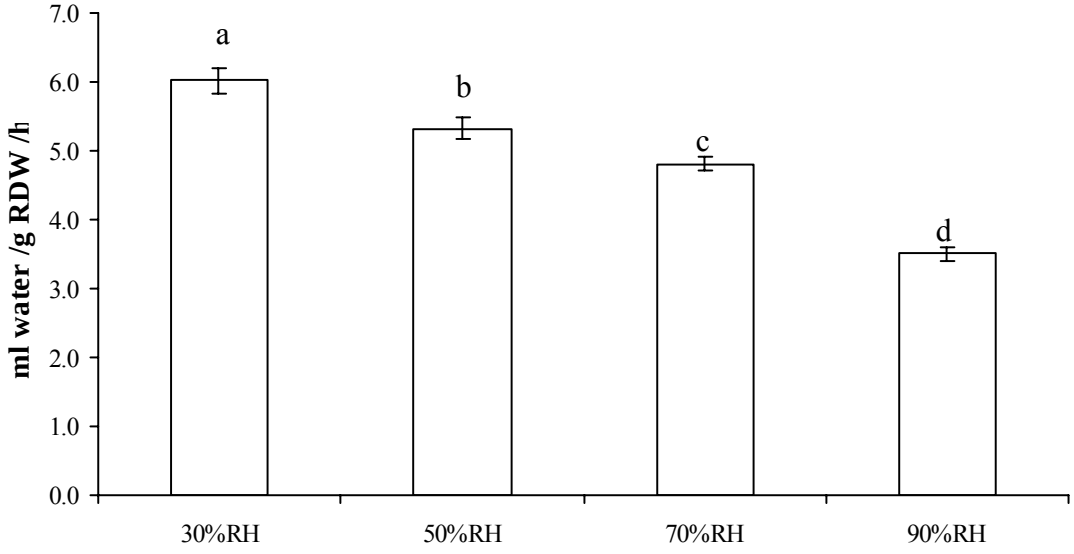

Figure 4.10 The effect of RH on the flux of water across the roots of rice cv. IR36 under a saline condition (50 mM NaCl). Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 30) are presented.

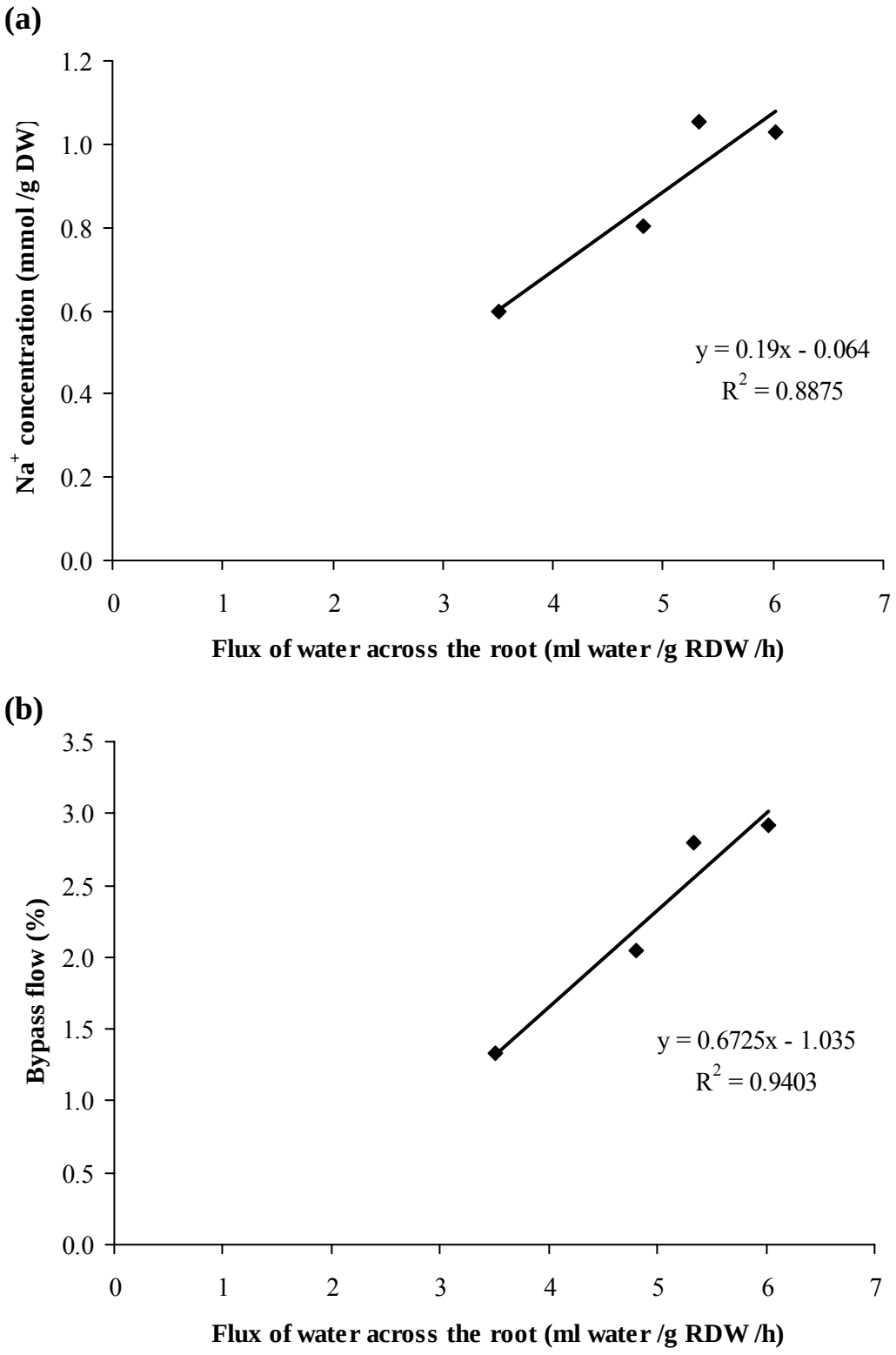

Figure 4.11 The relationships between the flux of water across the root and shoot Na^+ concentration (a) and bypass flow (b) in rice cv. IR36. All values are means (n = 30).

4.4 DISCUSSION

It has been shown that prior to salinisation rice seedlings with some salt tolerance (CSR10), intermediate tolerance (IR36) and showing salt sensitivity (GR4) were similar in their accumulation of Na^+ and K^+ in the shoots; their autofluorescence at wavelengths used to estimate PTS was also similar (Figure 4.1). The addition of PEG or Si to the culture solution did not significantly affect shoot dry weight of any of the three genotypes under salt stress (Figure 4.2a). The results are consistent with previous findings also showing that addition of PEG (Yeo & Flowers 1984b) or Si (Gong *et al.* 2006) did not change shoot dry weight in rice under saline conditions. Although several studies have shown that PEG and Si improved the growth of plants under salinity stress such as in rice (Matoh *et al.* 1986), tomato (Balibrea *et al.* 1999), wheat (Saqib *et al.* 2008) and sugarcane (Ashraf *et al.* 2010), the plants in those studies were treated with higher concentrations of NaCl and for longer times than in my experiments (50 mM NaCl for 4 d); for example, 100 mM NaCl for 30 d in rice (Matoh *et al.* 1986), 100 mM NaCl for 22 d in tomato (Balibrea *et al.* 1999), 125 mM NaCl for 21 d in wheat (Saqib *et al.* 2008) and 100 mM NaCl for 60 d in sugarcane (Ashraf *et al.* 2010). The short-time and low NaCl treatment in the present experiments might explain the different results reported from the other studies.

Shoot Na^+ concentration was significantly increased in the three rice genotypes after exposure to 50 mM NaCl for 4 d, but the increase of Na^+ was greater in GR4 and IR36 compared with CSR10 (Figure 4.3a), suggesting that Na^+ leakage to the shoot of the salt-sensitive variety (GR4) was more than in the salt-tolerant one (CSR10). The addition of 10 g/l PEG to the culture solution showed a significantly decreased shoot Na^+ concentration and Na^+ content in all genotypes (Figures 4.3, 4.4). These results are in agreement with previous studies in rice (Yeo & Flowers 1984b; Ochiai & Matoh 2004), tomato (Balibrea *et al.* 1999) and sugarcane (Munir & Aftab 2009).

The actual mechanism of PEG-enhanced salt tolerance in plants is not well understood (Munir & Aftab 2009). It has been reported that PEG with high molecular weight (more than 1000) did not enter plants (Lawlor 1970; Backhausen *et al.* 2005; Ahmad *et al.* 2007). Therefore, the primary effect of PEG on Na^+ transport should operate outside the

cells. It was speculated that PEG affected bypass flow by blocking the pore size of plant cell walls, and thus reducing Na^+ uptake to the shoots (Yeo & Flowers 1984b; Yeo *et al.* 1999; Ochiai & Matoh 2004). However, the effect of PEG on bypass flow was not previously investigated thoroughly. In the present study, the addition of PEG brought about a significant reduction in bypass flow (Table 4.1) as well as a reduction in the Na^+ delivered to the shoots via bypass flow in the three rice genotypes (Figure 4.6). The reduction of Na^+ bypass flow was more pronounced in the salt sensitive than the salt-tolerant genotype: it was 71.2, 52.6 and 41.1% for GR4, IR36 and CSR10, respectively, compared with PEG-untreated seedlings (Figure 4.6). The reduction of Na^+ concentration and Na^+ bypass flow does not appear to be due to the reduction of the transpiration volume flow because PEG at the concentration used in this study (-33 kPa) did not affect this parameter (Table 4.1). The reduction was a consequence of a decreased Na^+ transport from root to the shoot (Figure 4.5a) and the transport of Na^+ in the xylem (Table 4.1, Figures 4.7 - 4.9 & Appendices 4.5 – 4.7). The results are in agreement with those of Yeo & Flowers (1984b). The apparent Na^+ concentration in the xylem of CSR10, IR36 and GR4 was about 3.5-10 times lower than the concentration of Na^+ obtained from the xylem-feeding insect *P. spumarius* (Table 4.1, Figures 4.7-4.9 & Appendices 4.5-4.7). The result is in agreement with Gong *et al.* (2006) who also found that the apparent Na^+ in the xylem of IR36 was 2.5 times lower than that of *P. spumarius*. The apparent concentration in the xylem represents an average of Na^+ over time and leaf positions, whereas the concentration from *P. spumarius* is localised on a specific xylem vessel. This might explain the differences in concentrations between the two estimates. However, why the apparent PTS concentration in the xylem was similar in IR36, and it was higher in CSR10 and GR4 is unclear (Table 4.1, Figures 4.7-4.9 & Appendices 4.5-4.7), unless this was also due to comparing an average with a specific measurement.

The addition of PEG significantly increased K^+ concentration and K^+/Na^+ ratio (Figures 4.3b, c) as well as increased K^+ content (Figure 4.4b) in the shoots of rice. These findings were also observed in tomato subjected to salt stress (Balibrea *et al.* 1999). However, the mechanism by which PEG increases K^+ transport is rarely discussed. Yeo & Flowers (1984b) suggested that PEG bound to root plasma membranes and modified the selectivity of the membranes by blocking carrier-independent pathways for Na^+ (e.g. membrane leakage, bypass flow), and hence increasing the K^+/ Na^+ ratio. Other effects

of PEG on the plasma membranes have been reported. For example, PEG has been added to the culture medium to stabilise plasma membranes of tobacco pollen tubes by preventing loss of pollen proteins, ions and metabolites to the medium and then promote growth of pollen tubes (Read *et al.* 1993). In protoplast fusion process, PEG was used to bring plasma membranes into contact and trigger their fusion (Lentz 2007).

The addition of 3 mM Si to the culture solution significantly decreased shoot Na^+ concentration (Figure 4.3a). This result is consistent with those of Matoh *et al.* (1986), Yeo *et al.* (1999) and Gong *et al.* (2006) who reported that Si ameliorated salinity effects in rice. Also, the result is compatible with the view that Si alleviates salinity stress of plants in general (Ma 2004; Liang *et al.* 2007; Ma & Yamaji 2008). In the present study, it is clear that the addition of Si significantly decreased the transport of Na^+ to the shoot (Figure 4.5a) and the concentration of Na^+ in the xylem (Figures 4.7 - 4.9 & Appendices 4.5 – 4.7). These observations are in line with those obtained by Gong *et al.* (2006).

The data in Table 4.1 clearly show that adding Si to the culture solution did not affect the transpiration volume flow, but Na^+ content in the shoot was significantly decreased. These results are in contrast to those of Matoh *et al.* (1986) who suggested that Si decreased Na^+ in the shoot through a reduction of the transpiration volume flow. This distinction might result from difference between rice varieties, Na^+ concentration used and stress durations; however, Matoh *et al.* (1986) did not measure the transpiration volume in their experiment. Instead, the results are consistent with Yeo *et al.* (1999) and Gong *et al.* (2006) who reported that the reduction of Na^+ concentration in shoots of rice seedlings through the addition of Si was independent of the transpiration volume flow. Gong *et al.* (2006) demonstrated that Si deposition in the exodermal and endodermal layers of the seminal root reduced Na^+ uptake in rice seedlings under saline conditions through a reduction of bypass flow across the root. Analysis of data on the uptake of water, PTS and Na^+ by whole shoots of rice seedlings in the present study strongly confirms that Si decreased the Na^+ delivered to the shoots via bypass flow in all genotypes under study (Figure 4.6). The reduction was 63.4, 30.7 and 19.8% for GR4, IR36 and CSR10, respectively, as compared to Si-untreated control plants.

The addition of Si significantly increased K^+/Na^+ ratio (Figure 4.3c), increased K^+ content (Figure 4.4b) and significantly increased net K^+ transport to the shoot of rice under salt stress (Figure 4.5b). These results are in agreement with other reports in barley (Liang 1999), wheat (Saqib *et al.* 2008; Tuna *et al.* 2008) and sugarcane (Ashraf *et al.* 2010) subjected to salinity stress. A possible explanation is that exogenous application of Si stimulates the activities of root plasma membrane H^+-ATPase, thus enhancing the active process of K^+ uptake in the plasma membrane as reported in barley under salt stress (Liang 1999).

In Chapter 3, it was suggested from an analysis of PTS fluorescence in the root and shoot of rice seedlings that PEG might reduce bypass flow more effectively than Si in rice under salt stress. However, Na^+ concentration and the magnitude of bypass flow were not quantified in the previous Chapter. PEG clearly reduced the bypass flow more effectively than Si in all three rice genotypes (Table 4.1 & Appendix 4.4). The Na^+ delivered to the shoots via bypass flow was also less in PEG-treated than Si-treated seedlings in all genotypes (Figure 4.6). These findings are consistent with the hypothesis that PEG acts through reducing bypass flow and confirm the results in the Chapter 3. It is not clear why PEG application causes the reduction in the Na^+ bypass flow more effectively than Si. As stated in the above literature, it is likely that PEG might act as a modifier to the pore size of cell walls through hydrogen bonding and effectively blocked the bypass flow of Na^+, whereas Si might only be deposited within the cell walls. Moreover, PEG might affect both bypass flow and symplastic transport of Na^+ (see Chapter 3, section 3.4).

In the present study, it was shown that shoot Na^+ concentration and the apparent Na^+ in the xylem of rice seedlings under saline stress decreased as RH increased (Tables 4.3, 4.4). The results are in accordance with other reports (Yeo & Flowers 1984b; Lauter & Munns 1987; Asch *et al.* 1999; An *et al.* 2001, 2002; Backhausen *et al*. 2005; Flowers *et al.* 2010). The transpiration volume was not obviously changed when the RH level was increased from 30 to 90% (Table 4.4). This finding is in contrast to the conclusions of Lauter & Munns (1987), Asch *et al*. (1999), An *et al*. (2001) and Backhausen *et al*. (2005) who claimed that increasing RH would increase salt tolerance of plants by a reduction of the transpiration volume, probably because of different salt concentrations, stress durations or the plants used in the studies. For example, in those studies, chickpea

was exposed to 12 and 24 mM NaCl for 35 d (Lauter & Munns 1987); rice was subjected to 30 mM NaCl for 5 months (Asch *et al.* 1999); soybean was treated with 80 mM NaCl for 3 weeks (An *et al.* 2001); potato was subjected to 300 mM NaCl for 3 weeks (Backhausen *et al*. 2005). However, the result is in line with Salim (1989) who reported that an increase in RH from 30 to 90% decreased shoot Na^+ concentration and the rate of Na^+ transport from root to the shoot without affecting the transpiration rate in *Atriplex spongiosa* subjected to salt stress of 100 mM NaCl for 7 d.

The movement of solutes and water across roots can take either a cell-to-cell pathway or bypass flow, and these two parallel pathways can be flexibly switched from one to another depending on the hydraulic and osmotic driving forces on water (Steudle & Frensch 1996; Steudle & Peterson 1998; Steudle 2000a, b, 2001). The present results showed that the flux of water across the roots was significantly decreased when the RH increased from 30 to 90% (Figure 4.10), and changing the flux of water across the roots had changed the extent of bypass flow: the greater the flux of water through the root, the greater the Na^+ uptake and bypass flow (Figure 4.11). These results are in agreement with the notion that bypass flow is more intensively used in the presence of a high transpiration rate (Steudle & Peterson 1998; Steudle 2000a, b). From the data in Table 4.4, it was possible to calculate the Na^+ delivered to the shoots via bypass flow at different RH levels and compare this with the total Na^+ accumulated in the same time: bypass flow delivers 22% of the shoot Na^+ of IR36 at 90% RH, a figure that rose to 32% at 30% RH. Hence, bypass flow of Na^+ in rice after salt treatment was obviously affected by RH levels.

In conclusion, the results in this Chapter clearly showed that the addition of PEG or Si to the culture solution significantly decreased Na^+ uptake to the shoot of rice seedlings under saline conditions through a reduction of bypass flow. PEG proved to be more effective than Si in the reduction of the bypass flow. Increasing RH reduced Na^+ concentration in the shoots by reducing the flux of water across the roots and the magnitude of bypass flow. The percentage of bypass flow and the Na^+ delivered to the shoot via bypass flow were significantly higher in salt-sensitive GR4 than salt-tolerant CSR10, suggesting that bypass flow could be a criterion for screening salt tolerance in rice. The following Chapter investigates this hypothesis in detail.

CHAPTER 5

THE CONTRIBUTION OF BYPASS FLOW TO SODIUM UPTAKE BY RICE (*ORYZA SATIVA* L.) UNDER SALINE CONDITIONS AND ITS APPLICATION AS A NEW SCREENING TECHNIQUE FOR SALINITY RESISTANCE

5.1 INTRODUCTION

A number of traits have been used for screening salt tolerance in rice. Yeo & Flowers (1983) found a strong negative relationship between leaf 3 Na^+ concentrations and survival of seedlings growing in 50 mM NaCl for 6 d and suggested predicting salinity resistance of rice from leaf Na^+ uptake. Yeo, Yeo & Flowers (1988) successfully selected high- and low-Na^+-transporting lines within varieties of rice cvs. IR36 and IR20 by sampling Na^+ concentrations in leaf 3 after exposure to 25-50 mM NaCl for 6 d. Akita & Cabuslay (1990) proposed using leaf area ratio (LAR: leaf area per total dry weight) as a screening criterion for salt tolerance as the authors found that salt-tolerant Pokkali and Nona Bokra had relatively lower LAR than salt-sensitive IR8 and IR50. Yeo *et al.* (1990) evaluated a wide range of physiological characteristics of rice, including shoot Na^+ concentration, leaf-to-leaf compartmentation, tissue tolerance and plant vigour, to indentify the salt resistance in a large number of rice genotypes and found that plant vigour, providing dilution of salt concentrations by growth (Chapter 1, section 1.5.5), was strongly correlated with seedling survival. Aslam *et al.* (1993) also evaluated criteria for the assessment of salt tolerance in rice based on growth parameters (e.g. shoot and root fresh weights, shoot and root lengths and number of tillers) and reported a positive significant relationship between shoot fresh weight at seedling stage and paddy yield of rice grown under saline conditions. They indicated that absolute shoot fresh weight and relative shoot fresh weight (percent of control) could be used as the selection criteria of salt tolerance. Later, Asch *et al.* (2000) reported a positive correlation between K^+/Na^+ ratio of the youngest three leaves after 30 mM NaCl

treatment for 40 d and grain yield of rice at maturity. Their results showed that salt-tolerant IR4630 had the highest K^+/Na^+ ratio and grain yield while salt-sensitive IR31785 had the lowest K^+/Na^+ ratio and grain yield. Consequently, they suggested the use of K^+/Na^+ ratio as a suitable criterion for salt resistance selection. Others have suggested using floret fertility (Khatun *et al.* 1995; Rao *et al.* 2008), whereas Suriya-arunroj *et al.* (2004) proposed the use of relative water content as a useful selection criterion for screening salt tolerance.

To date, although salinity resistance in rice and other crops has been investigated over many years, very few salt resistant varieties have been released (Flowers & Yeo 1995; Rozema & Flowers 2008; Ashraf & Akram 2009) because of (1) the complexity of the traits which includes entry restriction of Na^+ into the root xylem, reducing Na^+ uptake to the shoots, leaf-to-leaf distribution, compartmentation of Na^+ between and within cells, and plant vigour, (2) limited knowledge of the genetics of salinity tolerance and (3) the lack of reliable and reproducible techniques for identifying salt tolerance (Yeo *et al.* 1990; Gregorio & Senadhira 1993; Garcia *et al*. 1995; Xie *et al.* 2000; Koyama *et al*. 2001; Munns & James 2003; Ashraf & Harris 2004; Yadav *et al.* 2008; Mahmood 2009; Pervaiz *et al.* 2009).

Because of the importance of apoplastic transport (bypass flow) of Na^+ in rice, it has been postulated that differences in salt resistance between rice genotypes may be caused by differences in the degree of bypass flow (Yeo 1992). In a previous study (Chapter 4, section 4.3.2.4), it has been shown that the percentage of bypass flow and the Na^+ delivered to the shoots via bypass flow were significantly higher in salt-sensitive GR4 than salt-tolerant CSR10. Consequently, I hypothesised in the present Chapter that if bypass flow causes a difference in salt resistance between rice genotypes, then the magnitude of bypass flow in salt-tolerant lines with low Na^+ concentration will be lower than in salt-sensitive ones with high Na^+ concentration. This Chapter reports the results of my investigation into bypass flow and Na^+ uptake in 10 low- and 10 high-Na^+-transporting recombinant inbred lines of rice IR55178 aimed at evaluating the possibility of using the bypass flow as a new screening technique for salt resistance in rice varieties.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

Rice (*Oryza sativa* L.) IR55178 was chosen for this study since it has been shown to have a good heritability of the traits for Na^+ and K^+ transport (with narrow-sense heritability's of 0.42 and 0.46, respectively; Garcia *et al.* 1997) and has also been evaluated for quantitative trait loci for salt tolerance (Koyama *et al.* 2001). The parents of IR55178 (IR4630 and IR15324) showed extreme differences in Na^+ transport and tissue tolerance; IR4630 had slightly better survival, vigour and leaf-to-leaf distribution than IR15324, but it expressed much lower Na^+ uptake and much better tissue tolerance than IR15324 (Yeo *et al.* 1990). A cross of IR55178 was constructed at the International Rice Research Institute (IRRI), Manila, Philippines, by Dr. D. Senadhira and 118 recombinant inbred lines have been developed (Garcia *et al.* 1997; Koyama *et al.* 2001). Based on a population of 118 recombinant inbred lines, the F_7 generation was evaluated for Na^+ uptake at the University of Sussex and 10 low- and 10 high-Na^+-transporting lines were selected (T.J. Flowers personal communication).

Caryopses of 10 low- and 10 high-Na^+-transporting lines were soaked for 24 h in aerated water before being sown on nylon mesh floated on modified Yoshida culture solution (Chapter 2). The low Na^+-transporting lines used in this study were 10, 52, 108, 111, 124, 137, 139, 142, 153, 178, and the high Na^+-transporting lines were 19, 32, 75, 80, 94, 110, 148, 165, 175 and 179. Uniform 8-day-old seedlings were transplanted into individual black-painted tubes (50 ml) filled with culture solution. Seedlings were held in place with non-absorbent cotton wool and grown in a plant growth cabinet (SANYO MLR-350HT, Osaka, Japan) with day/night temperature of 30/25 °C, photosynthetically active radiation of 250-300 μmol m^{-2} s^{-1} supplied by fluorescent lamp 40W x 15 lamps, photoperiod of 12 h and 30% RH.

For the xylem, phloem sap analysis and survival, seedlings were transplanted into 3l-black-painted boxes, held in place with sponge and grown in the glasshouse with the same conditions as described in Chapter 3.

5.2.2 NaCl and PTS treatment

At 14 d after sowing, the culture solution was replaced with a solution containing 50 mM NaCl and 100 mg/l PTS. Plants were allowed to take up Na^+ and PTS for 96 h, after which the treated solution was replaced by culture solution for a chase period of 48 h to allow transpiration to carry any remaining Na^+ and PTS in the root xylem to the shoot. Shoots were harvested and dried in an oven at 80 °C for 72 h.

5.2.3 Determination of ion concentrations and PTS fluorescence

The whole shoots were weighed and extracted in 10 ml distilled water for 2 h at 90 °C. Na^+ and K^+ in the extracts were measured by atomic absorption spectroscopy (Unicam 919, Cambridge, UK). PTS florescence was analysed at $\lambda_{excitation}$ = 403 nm and $\lambda_{emission}$ = 510 nm using a fluorescence spectrometer (Perkin-Elmer LS-3B; Beaconsfield, Buckinghamshire, UK). Control plants, which had not been treated with PTS, were also extracted to check for autofluorescence using the same excitation/emission wavelengths for PTS. However, this autofluorescence was always negligible as compared to PTS fluorescence in treated plants (Yeo *et al.* 1987, 1999; Yadav *et al.* 1996; and as stated in Chapter 4).

5.2.4 Transpiration and bypass flow

Whole-plant transpiration was measured gravimetrically and corrected for water loss by evaporation during treatment with NaCl and PTS and a subsequent chase period (see Chapter 2, section 2.2.8 for more details). Transpiration of control plants (without added NaCl) was also recorded. Bypass flow and Na^+ delivered to the shoots via bypass flow in each line was calculated as described previously in Chapter 2, sections 2.2.8 and 2.3.5.

5.2.5 Ion and PTS concentrations in xylem sap, leaf blades, leaf sheaths and the quantity of Na^+ in phloem sap

Xylem sap was collected from leaves 2 and 3 at 0, 24, 48, 72 and 96 h after the initiation of salt stress using adults of the xylem-feeding insect *P. spumarius* (see Chapter 4, section 4.2.3 for more details). After the end of treatment at 96 h, leaves 2 and 3 were excised and blades and sheaths were separated. Leaf blades were transferred into EDTA solutions for collecting phloem exudation (see Chapter 2, section 2.2.9 for

more details). Then, the leaf blades and sheaths were dried in an oven at 80 °C for 72 h and subsequently extracted in 5 ml distilled water for 2 h at 90 °C. The excreta of *P. spumarius* and leaf extracts were analysed for Na^+, K^+ and PTS concentrations using atomic absorption spectroscopy and fluorescence spectrometry as mentioned above (Section 5.2.3). The quantity of Na^+ in EDTA solutions was measured by atomic absorption spectroscopy (Unicam 919, Cambridge, UK).

5.2.6 Seedlings survival measurement

Fourteen-day-old rice seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 90 d. Death rate of plants within the lines was recorded every 10 d and the period in which 50% of the individuals died (D_{50}) was calculated (Flowers & Yeo 1981; Yeo & Flowers 1983).

5.2.7 Analysis of data

The experiments were repeated at least twice. The results presented in this Chapter are averages between the repeat experiments by pooling individual data and subjected to statistical analysis by the Mann-Whitney test as the data were not distributed normally about the mean.

5.3 RESULTS

5.3.1 Ion concentrations in shoots

Na^+ concentrations in the shoots were found to be in the range from 0.3-0.5 mmol/gDW in low-Na^+-transporting lines and from 0.4-0.7 mmol/gDW in high-Na^+-transporting lines (Figure 5.1 & Appendix 5.1). There were statistically significant differences in shoot Na^+ concentrations amongst the 20 lines examined (at $P<0.05$; Figure 5.1) with an average shoot Na^+ concentration of high-Na^+-transporting lines 1.4 times higher than that of low-Na^+-transporting lines (at $P< 0.05$; Table 5.1).

K^+ concentrations in shoots were not significantly different between low- and high-Na^+-transporting lines (Figure 5.2, Table 5.1 & Appendix 5.1). In contrast, shoot K^+/Na^+ ratios in low-Na^+-transporting lines were significantly higher than those of high-Na^+-transporting lines (Figure 5.3, Table 5.1 & Appendix 5.1).

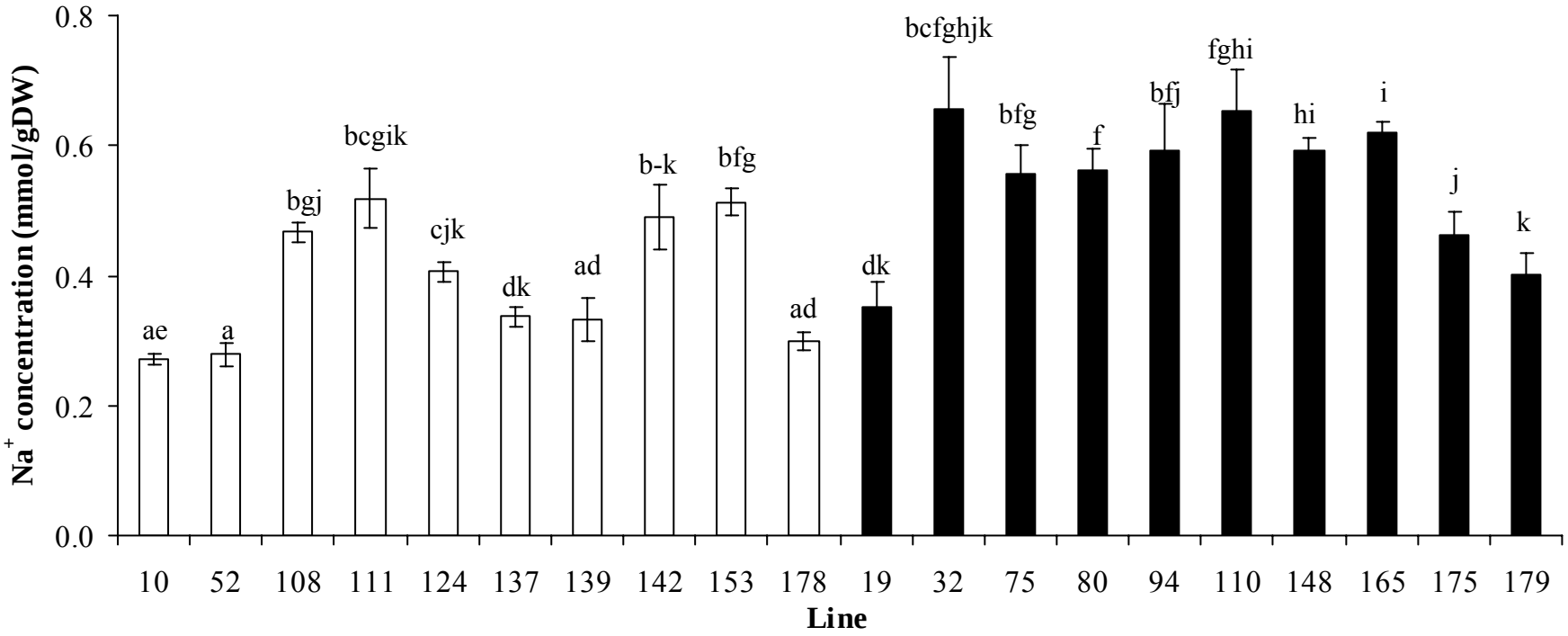

Figure 5.1 Na^+ concentrations in the shoots of 10 low-Na^+-transporting lines (open bars) and 10 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 40).

Table 5.1 Average Na^+, K^+ concentrations and K^+/Na^+ ratio in shoots, transpiration volume, bypass flow, the apparent Na^+ and PTS in the xylem of low- and high-Na^+-transporting lines of seedlings of rice IR55178.

Parameters	Low-Na^+-transporting lines	High-Na^+-transporting lines
Na^+ concentration (mmol/gDW)	0.39±0.01a	0.54±0.02b
K^+ concentration (mmol/gDW)	0.63±0.01a	0.63±0.01a
K^+/Na^+ ratio (on a molar basis)	2.04±0.05a	1.50±0.05b
Transpiration volume (ml)	8.66±0.21a	8.92±0.23a
Transpiration volume (% control)	70.0±1.74a	106±3.14b
Bypass flow (%)	1.37±0.05a	3.29±0.28b
Bypass flow of Na^+ (%)	21.7±0.85a	25.8±0.73b
The apparent Na^+ concentration in the xylem (mM)	4.23±0.26a	7.56±0.83b
The apparent PTS concentration in the xylem (μM)	0.37±0.02a	0.85±0.07b

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Values within a row of a parameter followed by the same letters are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 400).

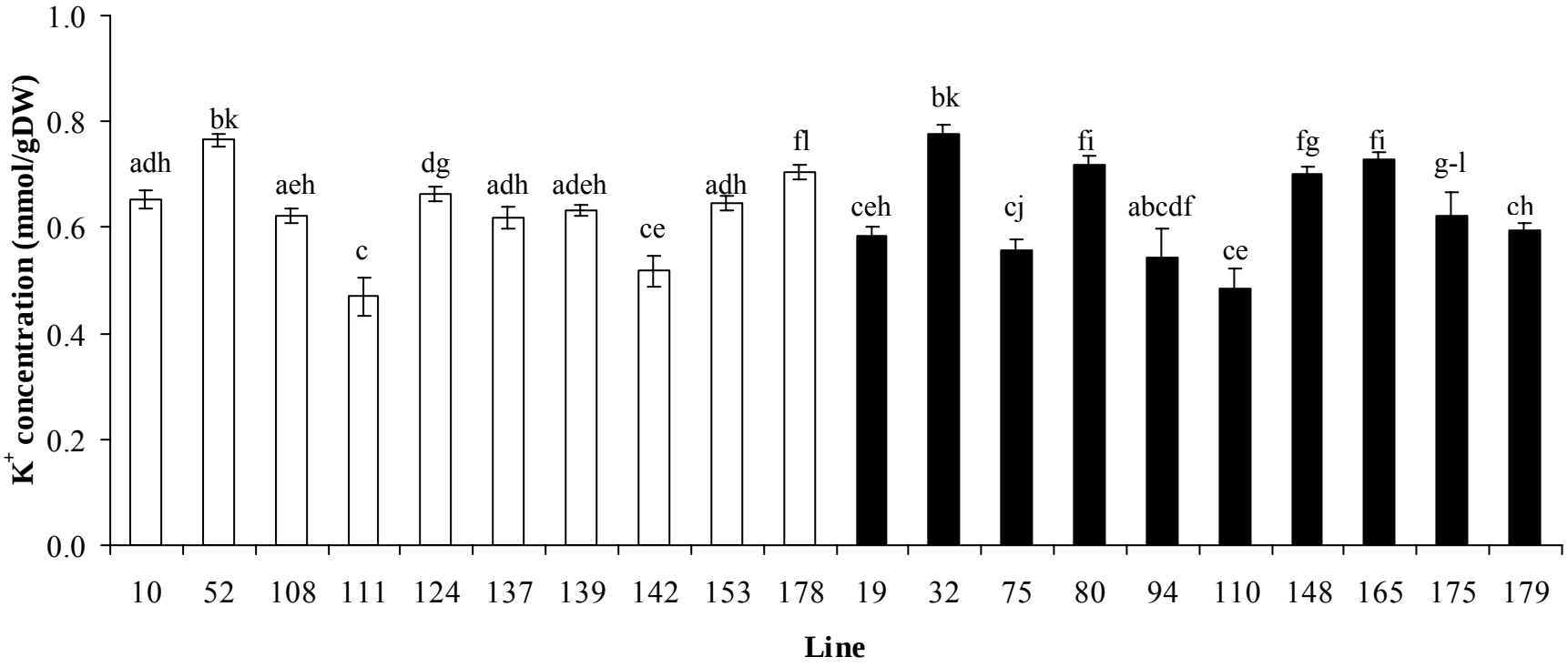

Figure 5.2 K^+ concentrations in the shoots of 10 low-Na^+-transporting lines (open bars) and 10 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 40).

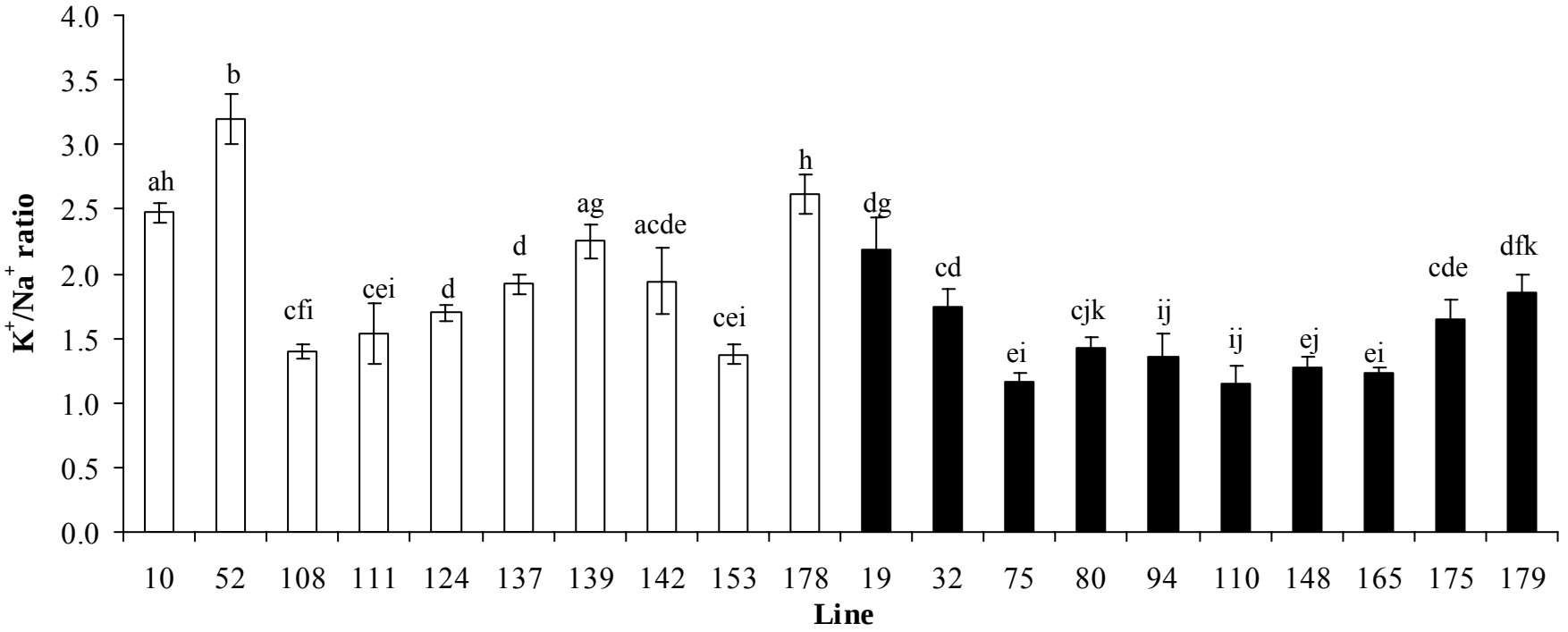

Figure 5.3 K^+/Na^+ ratio on a molar basis in the shoots of 10 low-Na^+-transporting lines (open bars) and 10 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 40).

5.3.2 Transpiration, bypass flow, the apparent Na^+ and PTS concentrations in the xylem

Whole-plant transpiration volume during NaCl treatment and a subsequent chase period was not significantly different between low- and high-Na^+-transporting lines (Figure 5.4, Table 5.1 & Appendix 5.1). However, when compared with the transpiration volume of the control plants without added NaCl, the transpiration volume was significantly lower in low-Na^+-transporting lines as compared to high-Na^+-transporting lines (Figure 5.5, Table 5.1 & Appendix 5.1).

Data analysis of the uptake of water, PTS and Na^+ by the whole shoots of seedlings showed that bypass flow observed in low-Na^+-transporting lines was 0.7-2.0%; however, a dramatic increase was noticed in high-Na^+-transporting lines (1.9-5.5%) as shown in Figure 5.6 and Appendix 5.1. The average bypass flow in high-Na^+-transporting lines was 2.4 times greater than that of the low-Na^+-transporting lines (Table 5.1). Lines 10, 52 and 178 showed the lowest percentage of bypass flow, whereas lines 32, 94 and 110 had the highest (Figure 5.6 & Appendix 5.1). There was a positive correlation between bypass flow and shoot Na^+ concentration (significant at $P< 0.05$; Figure 5.7). Using the data of bypass flow, transpiration volume and shoot Na^+ content, it was possible to calculate the Na^+ delivered to the shoots via bypass flow in each line (see Chapter 2, section 2.3.5 for more details): bypass flow delivered an average of 21.7% of the shoot Na^+ in low-Na^+-transporting lines and 25.8% in high-Na^+-transporting lines (Table 5.1 & Appendices 5.1, 5.2).

The apparent Na^+ and PTS concentrations in the xylem of low-Na^+-transporting lines were statistically lower than those of the high-Na^+-transporting lines (Figures 5.8, 5.9 & Table 5.1). Lines 10, 52 and 178 were the lowest apparent Na^+ and PTS concentrations in the xylem, whereas lines 32, 94 and 110 were the highest ones (Figures 5.8, 5.9 & Appendix 5.1). The apparent PTS concentration in the xylem was positively correlated with the apparent Na^+ concentration (significant at $P< 0.05$; Figure 5.10).

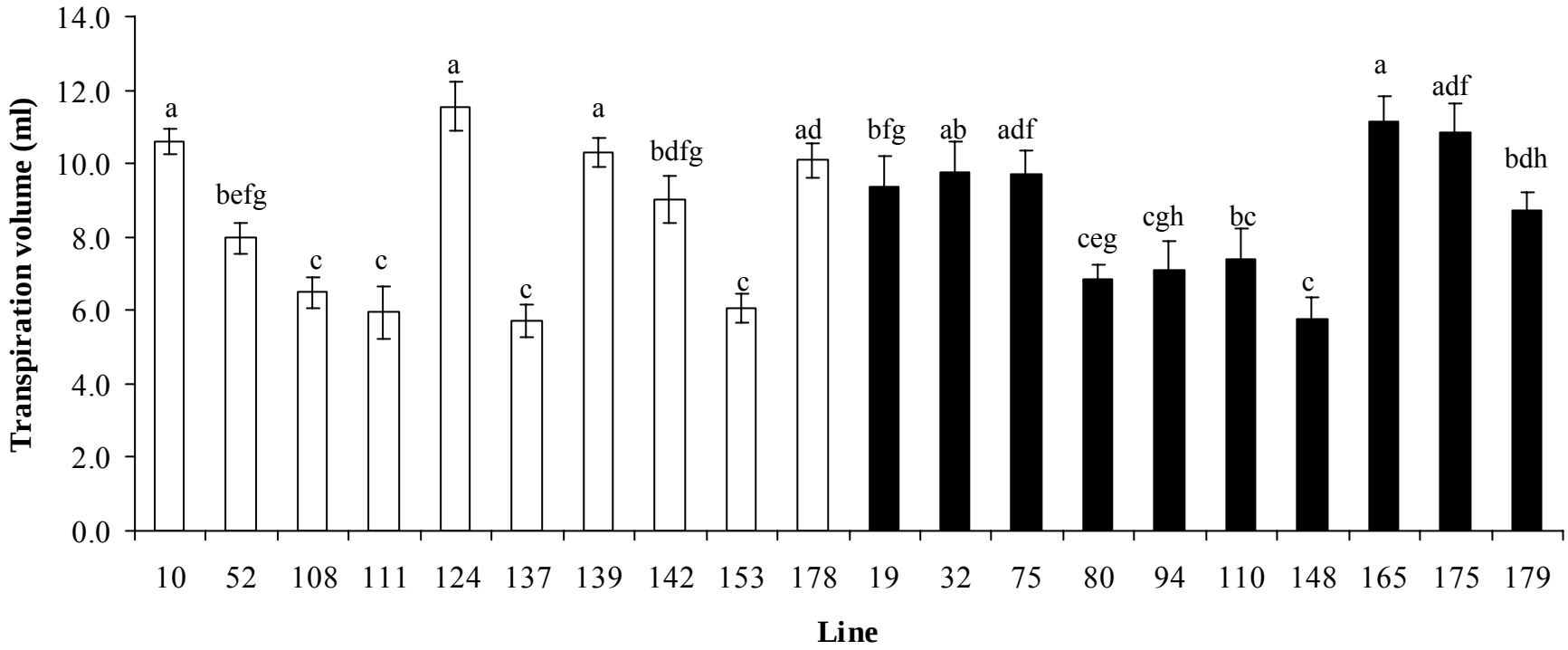

Figure 5.4 Whole-plant transpiration volume of 10 low-Na^+-transporting lines (open bars) and 10 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Transpiration was measured gravimetrically and corrected for water loss by evaporation during NaCl treatment and a subsequent chase period. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 40).

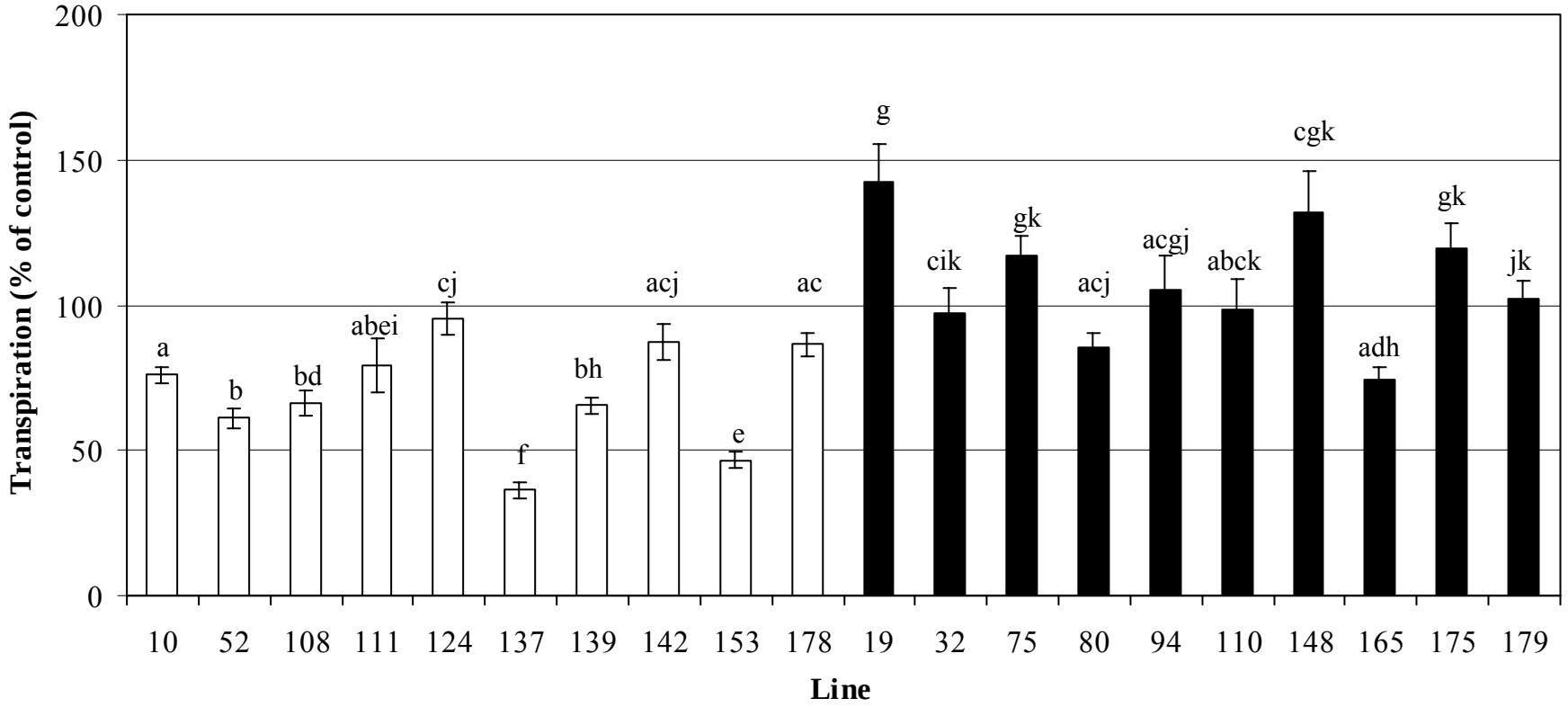

Figure 5.5 Whole-plant transpiration volume of 10 low-Na^+-transporting lines (open bars) and 10 high-Na^+-transporting lines (closed bars) of rice IR55178 treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution as compared to the transpiration of control plants without added NaCl. Transpiration was measured gravimetrically and corrected for water loss by evaporation. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 40).

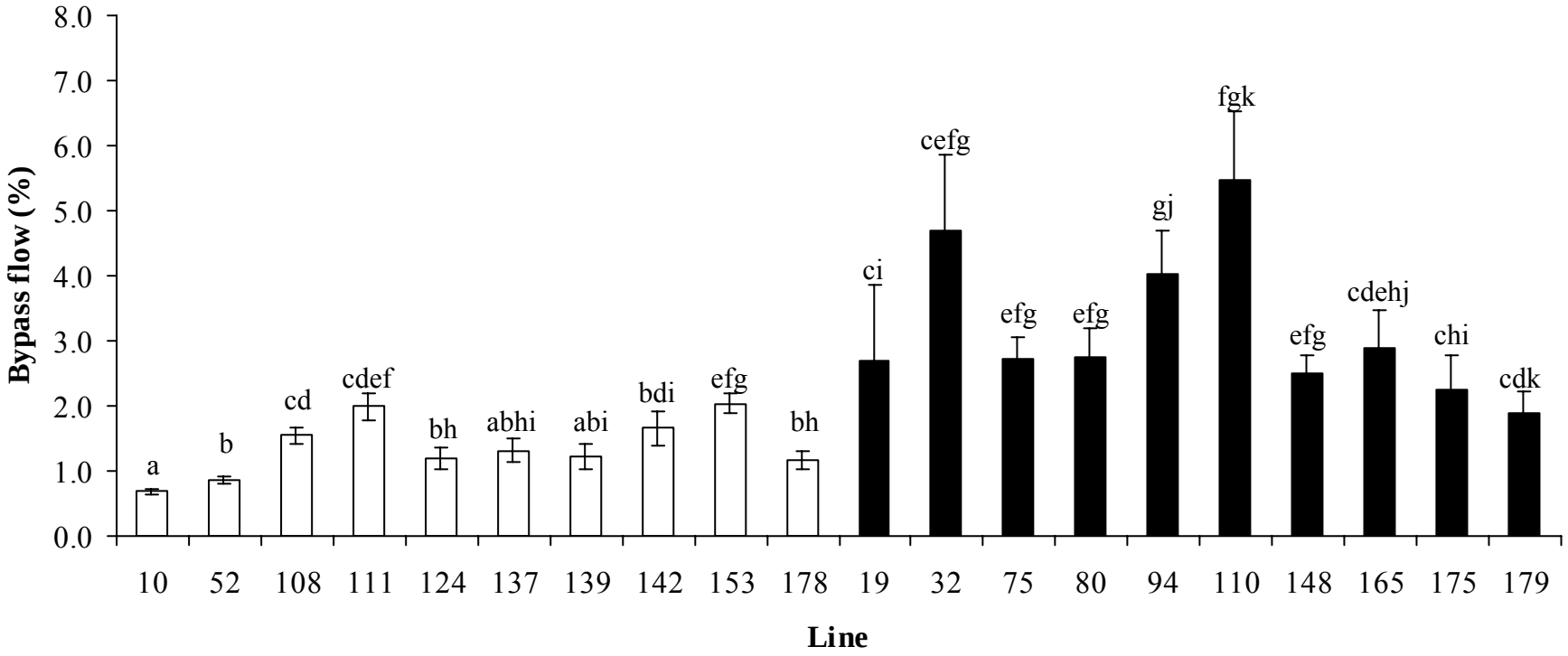

Figure 5.6 The percentage of bypass flow in the shoots of 10 low-Na^+-transporting lines (open bars) and 10 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 40).

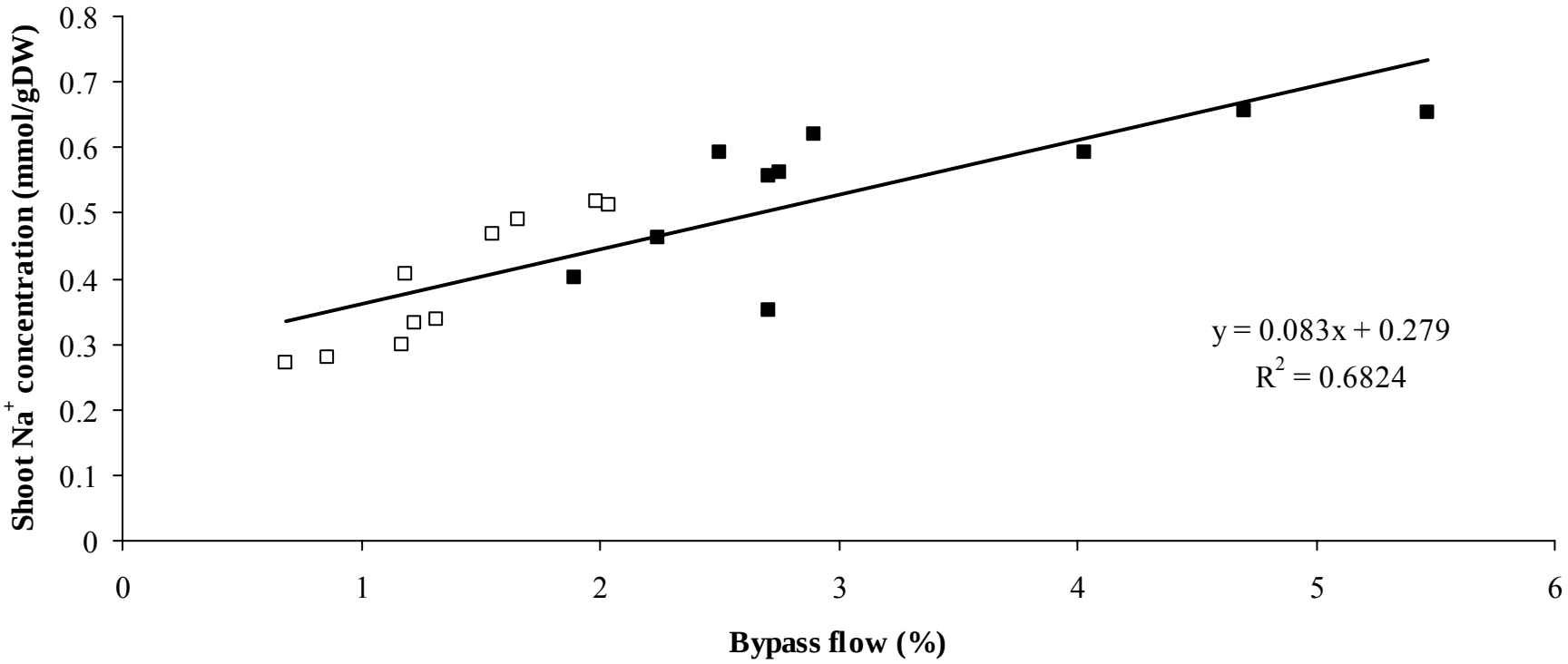

Figure 5.7 The relationship between bypass flow and Na^+ concentration in the shoots of low-Na^+-transporting lines (open symbols) and high-Na^+-transporting lines (closed symbols). Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. All values are means (n = 40).

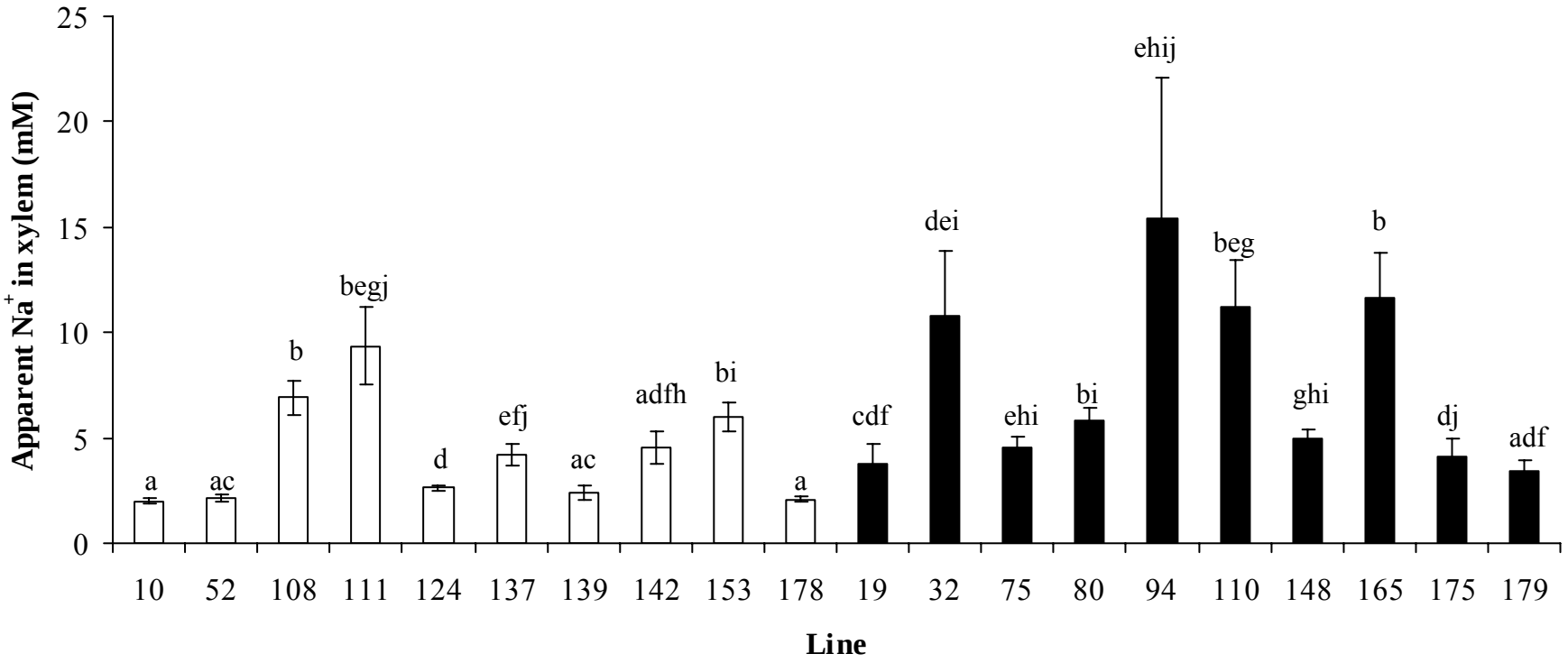

Figure 5.8 The apparent Na^+ concentration in the xylem of 10 low-Na^+-transporting lines (open bars) and 10 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 40).

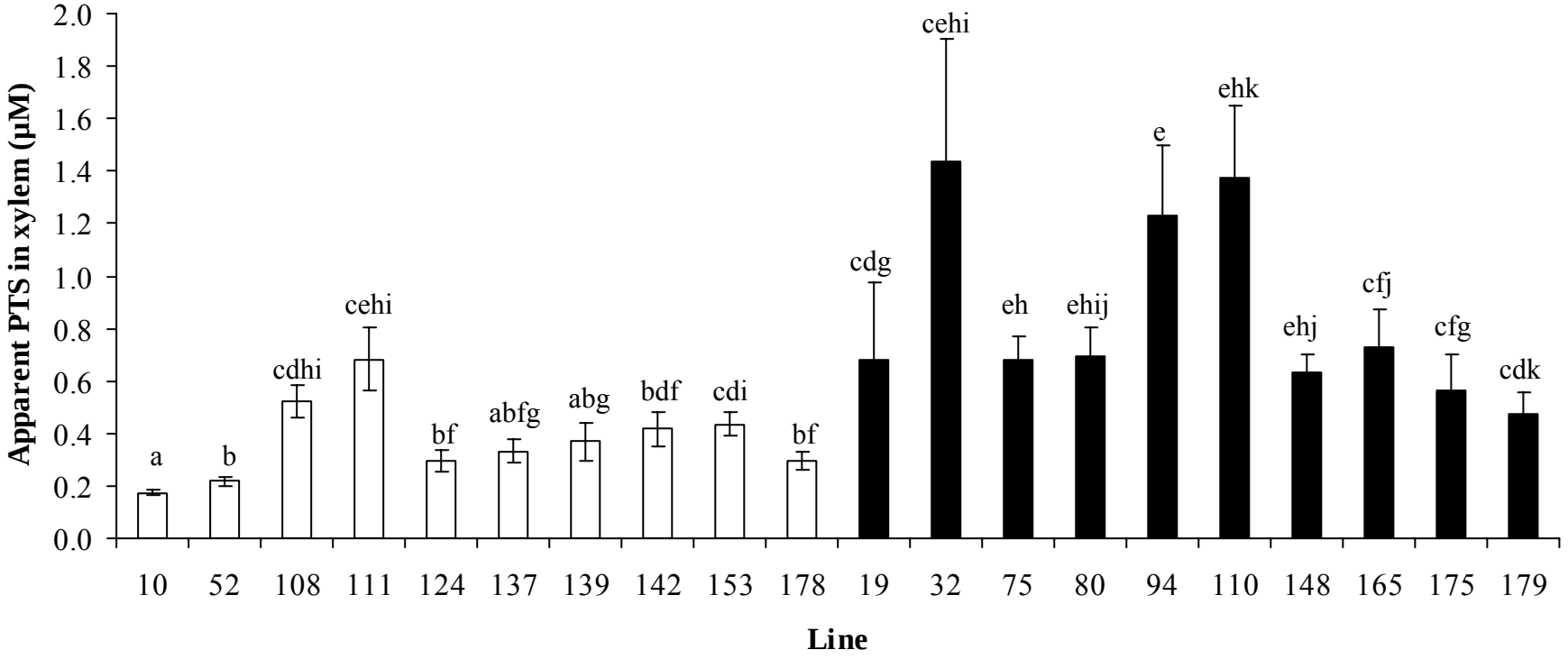

Figure 5.9 The apparent PTS concentration in the xylem of 10 low-Na^+-transporting lines (open bars) and 10 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 40).

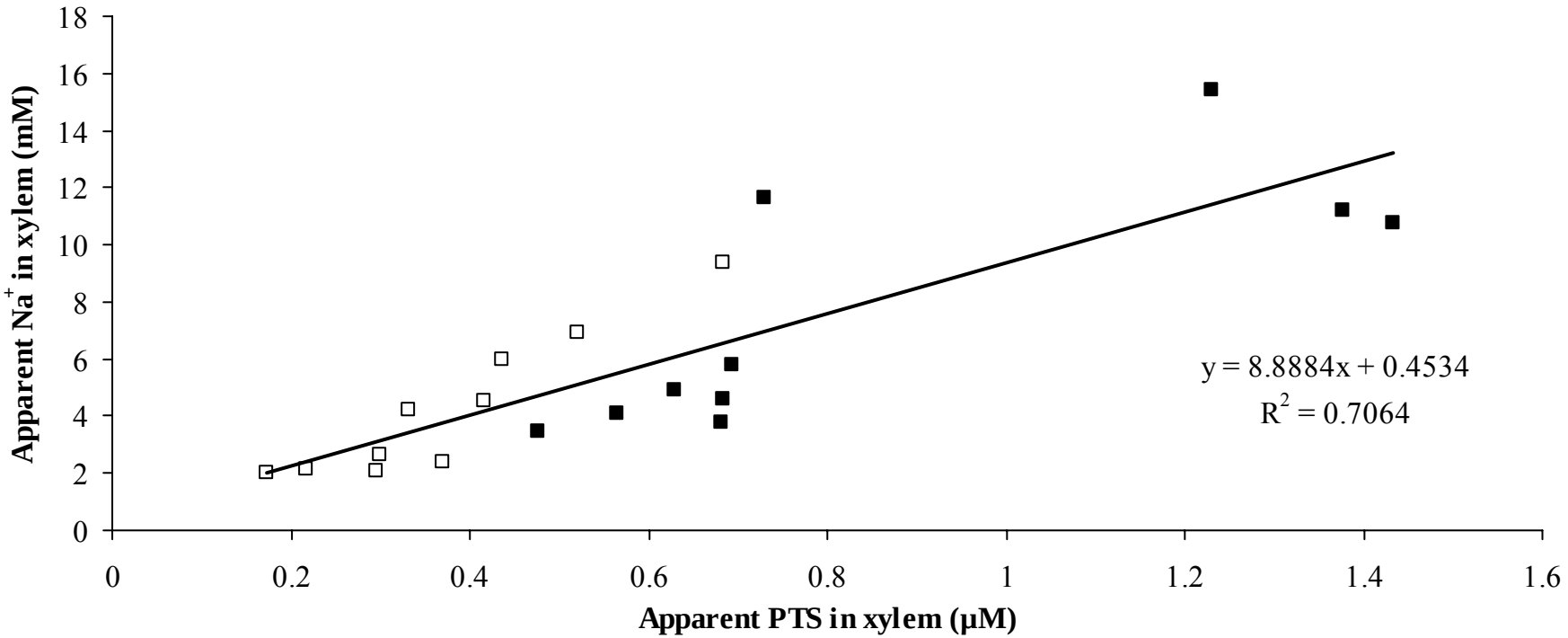

Figure 5.10 The relationship between the apparent PTS concentration and the apparent Na^+ concentration in the xylem of low-Na^+-transporting lines (open symbols) and high-Na^+-transporting lines (closed symbols). Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. All values are means (n = 40).

5.3.3 Concentrations of ions and PTS in the xylem sap, leaf blades and leaf sheaths

Based on the percentage of bypass flow (Figure 5.6), lines 10, 52 and 178 were selected as representatives for the low-Na^+-transporting lines and lines 32, 94 and 110 were representative of the high-Na^+-transporting lines. Those six lines were further studied for the concentrations of Na^+, K^+ and PTS in the xylem sap, phloem sap, leaf blades, leaf sheaths, and for seedling survival.

The results of changes in Na^+, K^+ and PTS concentrations in the xylem sap over 96 h during exposure to 50 mM NaCl and 100 mg/l PTS are shown in Figures 5.11-5.13 and Appendices 5.3-5.8. In general, the concentrations of Na^+ and PTS in the xylem sap of leaves 2 and 3 gradually increased over 96 h after salt stress treatment (Figures 5.11, 5.12). The concentration of K^+ in the xylem sap hardly changed or did not change during salt stress period (Figure 5.13). Statistically, it was found that low- and high-Na^+-transporting lines were not different in the concentrations of Na^+, K^+ and PTS in the xylem sap (Appendices 5.3-5.8). No difference was also found in the xylem K^+/Na^+ ratio (Appendix 5.9).

Na^+, K^+ and PTS concentrations in leaf blades of low-Na^+-transporting lines were not statistically different from those of high-Na^+-transporting lines (Figures 5.14-5.16 & Appendix 5.10), although an average PTS concentration in the blade of leaf 2 of the high transporting lines was 1.8 times higher than that of the low transporting lines (Figure 5.16). In contrast, the concentrations of Na^+ and PTS in the sheath of leaves 2 and 3 of high-Na^+-transporting line 94 were considerably higher than those of low-Na^+-transporting lines (Figures 5.17, 5.18 & Appendix 5.10). Similarly, Na^+ and PTS concentrations in the sheath of leaf 2 of high-Na^+-transporting lines 32 and 110 were higher than those of low-Na^+-transporting lines, although it was statistically different only in PTS concentration of line 110 (Figures 5.17, 5.18). The K^+ concentration in the sheath of leaf 2 of low-Na^+-transporting line 52 was significantly higher than those of high-Na^+-transporting lines, whereas no difference was observed in the sheath of leaf 3 between low- and high- Na^+-transporting lines (Figure 5.19). Na^+ and PTS contents in leaf blades and sheaths showed the same trends shown by the concentrations (Appendices 5.11-5.15).

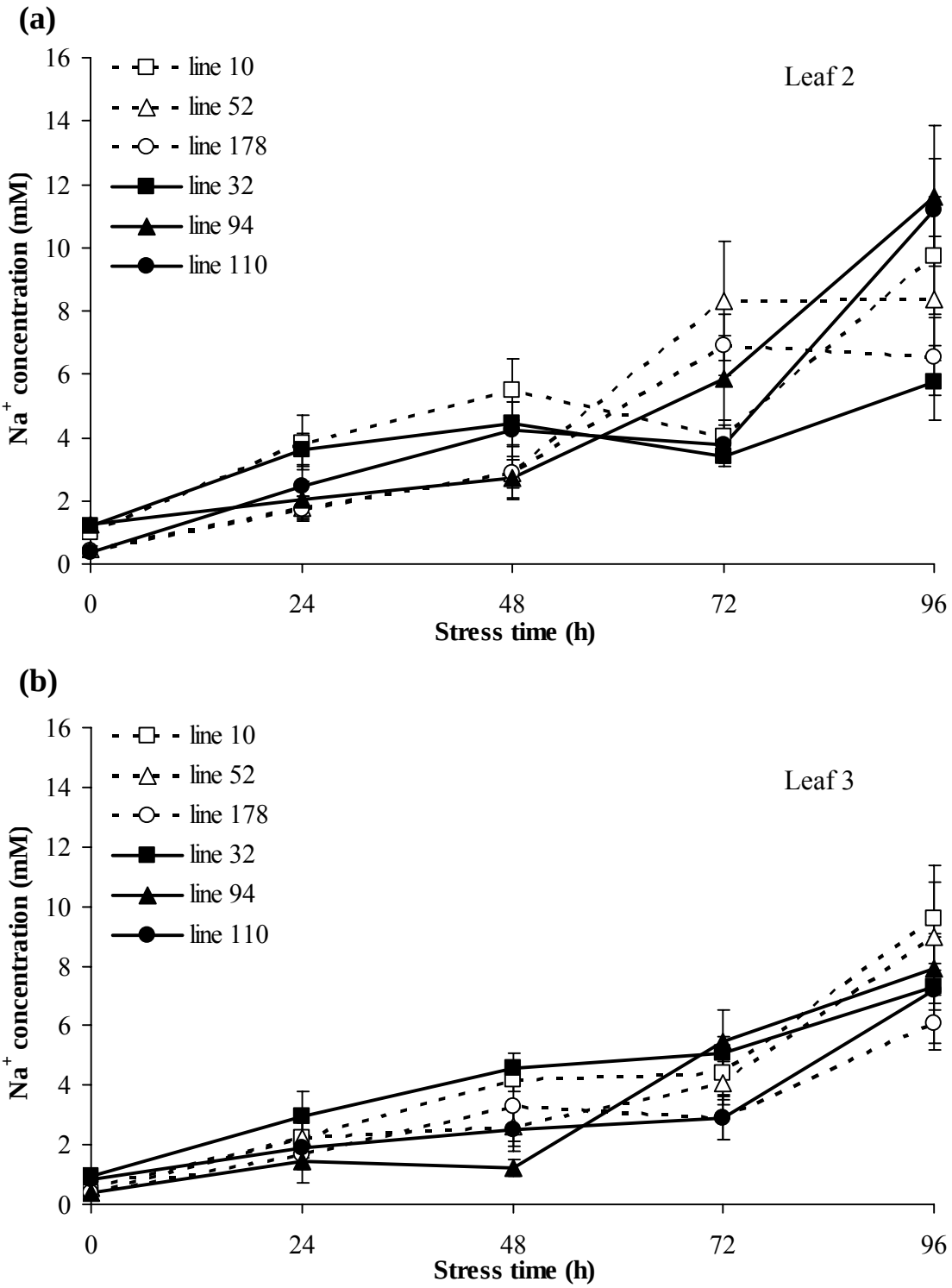

Figure 5.11 Na^+ concentrations in the xylem sap of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (broken graphs) and 3 high-Na^+-transporting lines (solid graphs) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius*. Means and standard errors (n = 3-25).

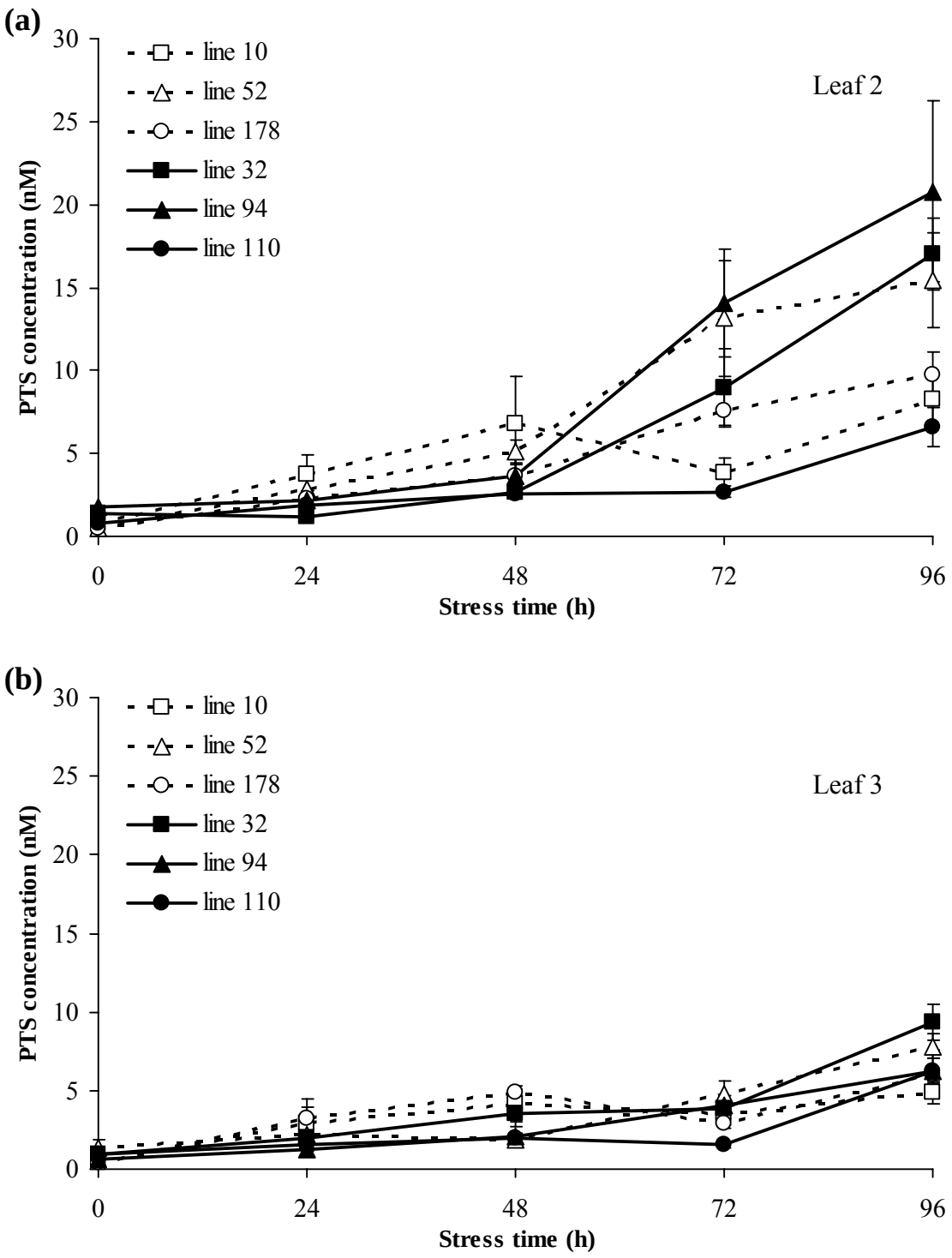

Figure 5.12 PTS concentrations in the xylem sap of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (broken graphs) and 3 high-Na^+-transporting lines (solid graphs) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius*. Means and standard errors (n = 3-25).

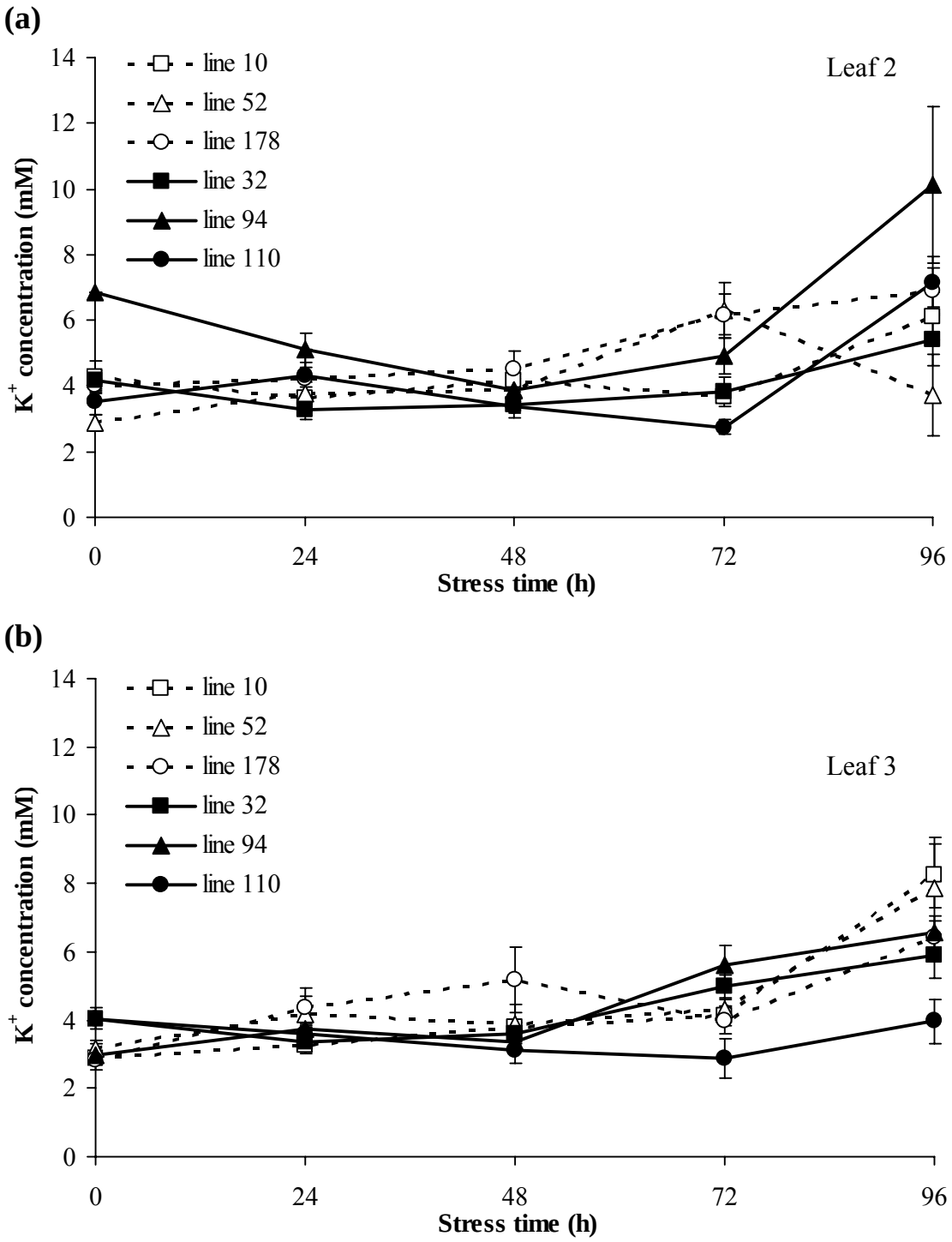

Figure 5.13 K^+ concentrations in the xylem sap of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (broken graphs) and 3 high-Na^+-transporting lines (solid graphs) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius*. Means and standard errors (n = 3-25).

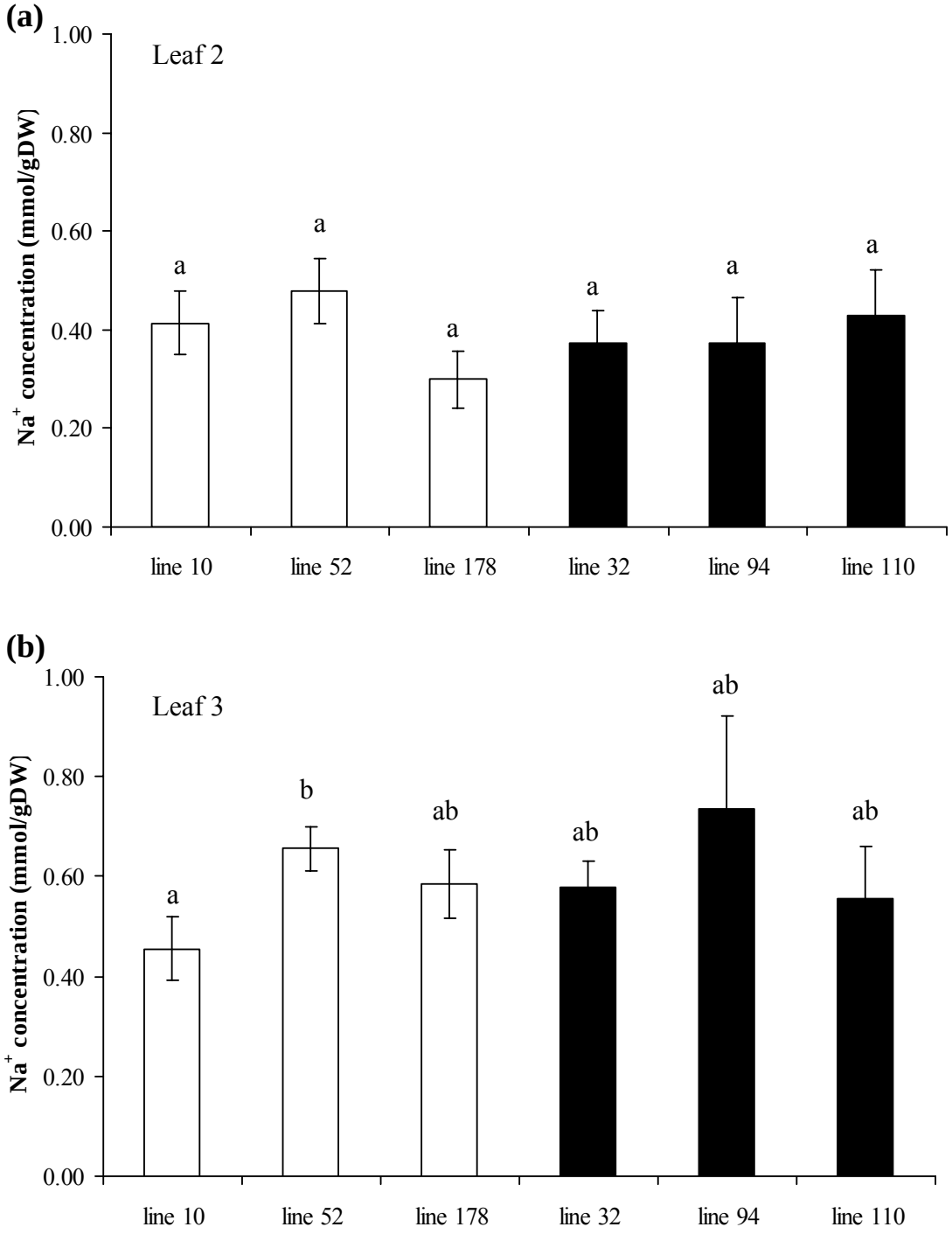

Figure 5.14 Na^+ concentrations in leaf blades of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

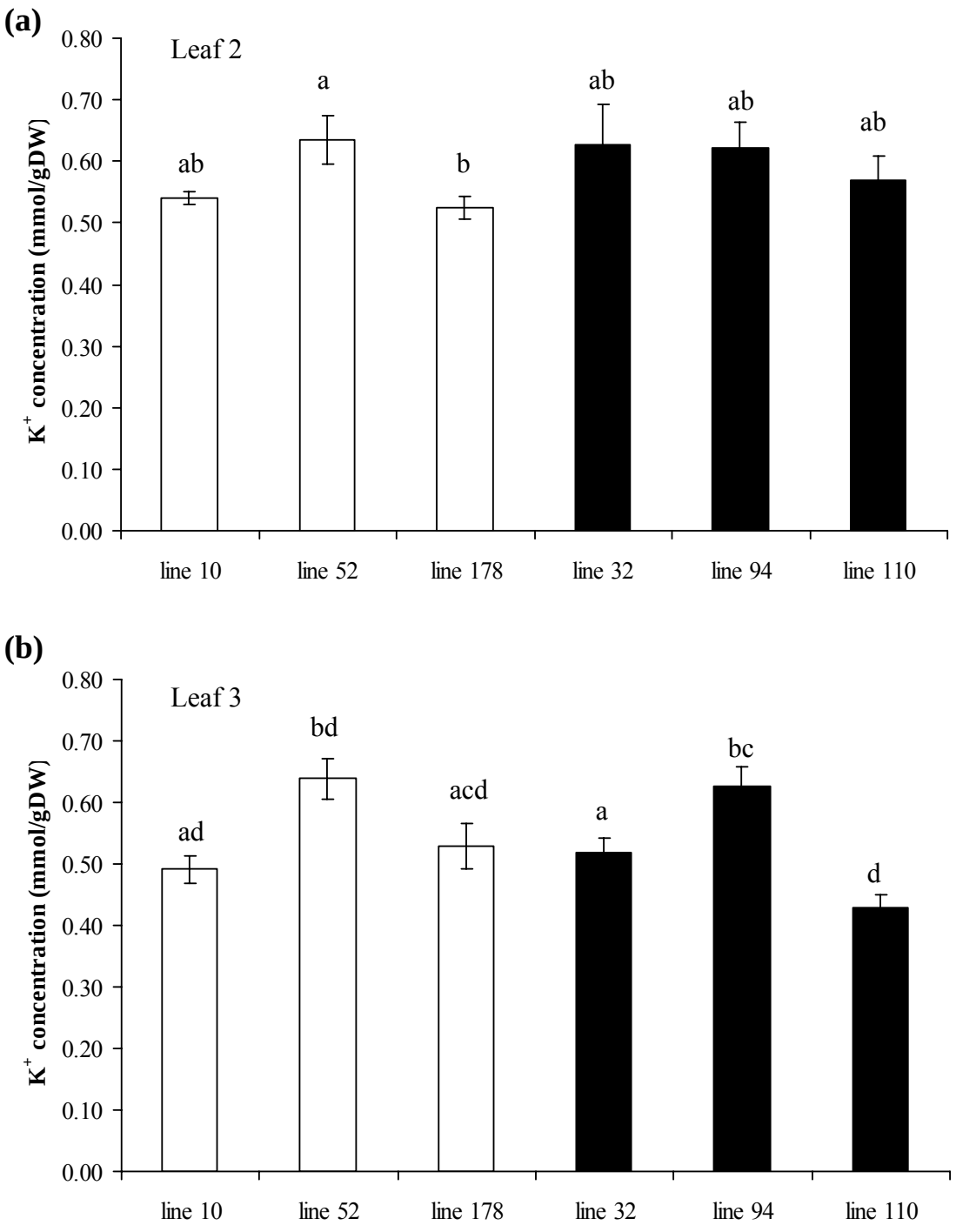

Figure 5.15 K^+ concentrations in leaf blades of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

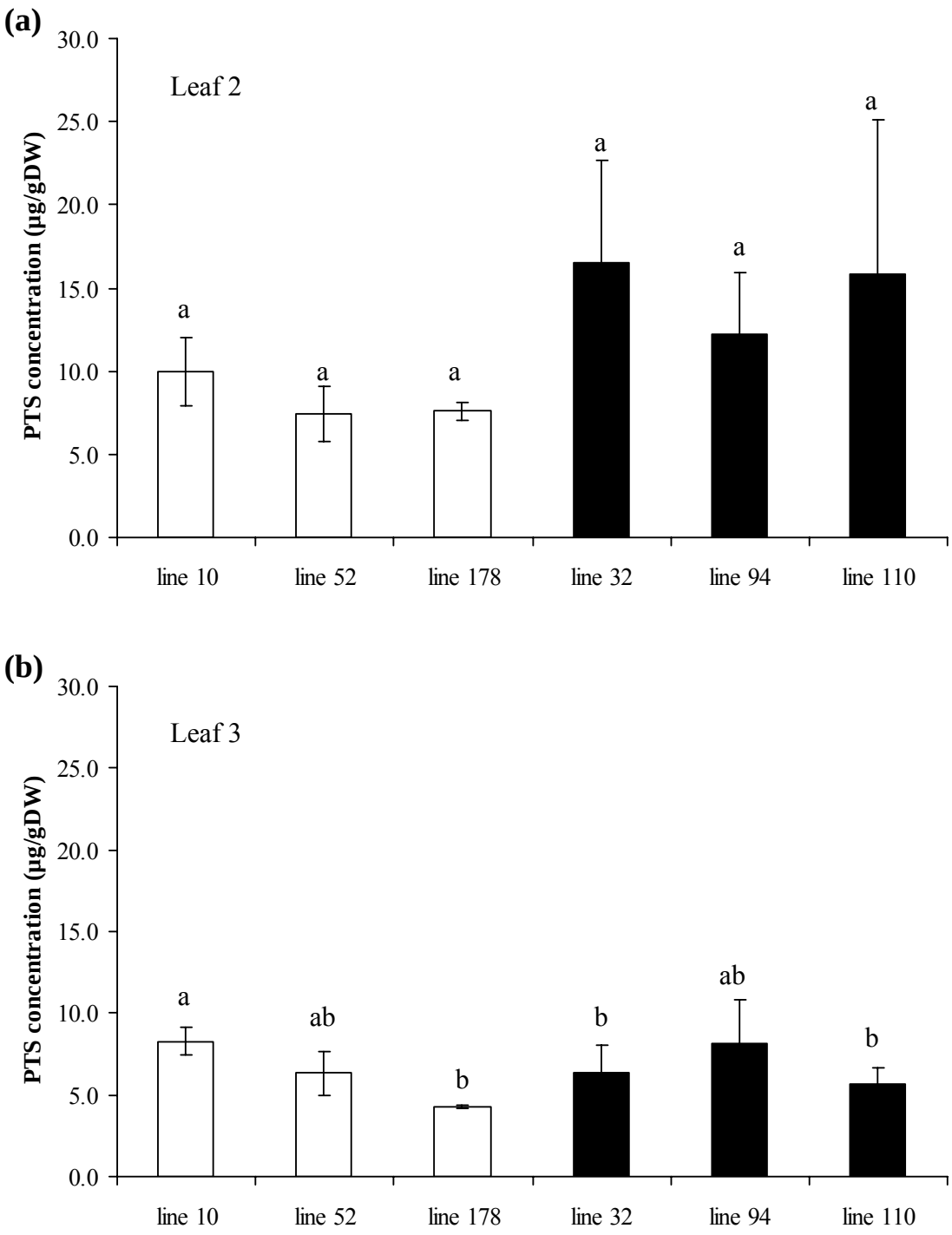

Figure 5.16 PTS concentrations in leaf blades of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

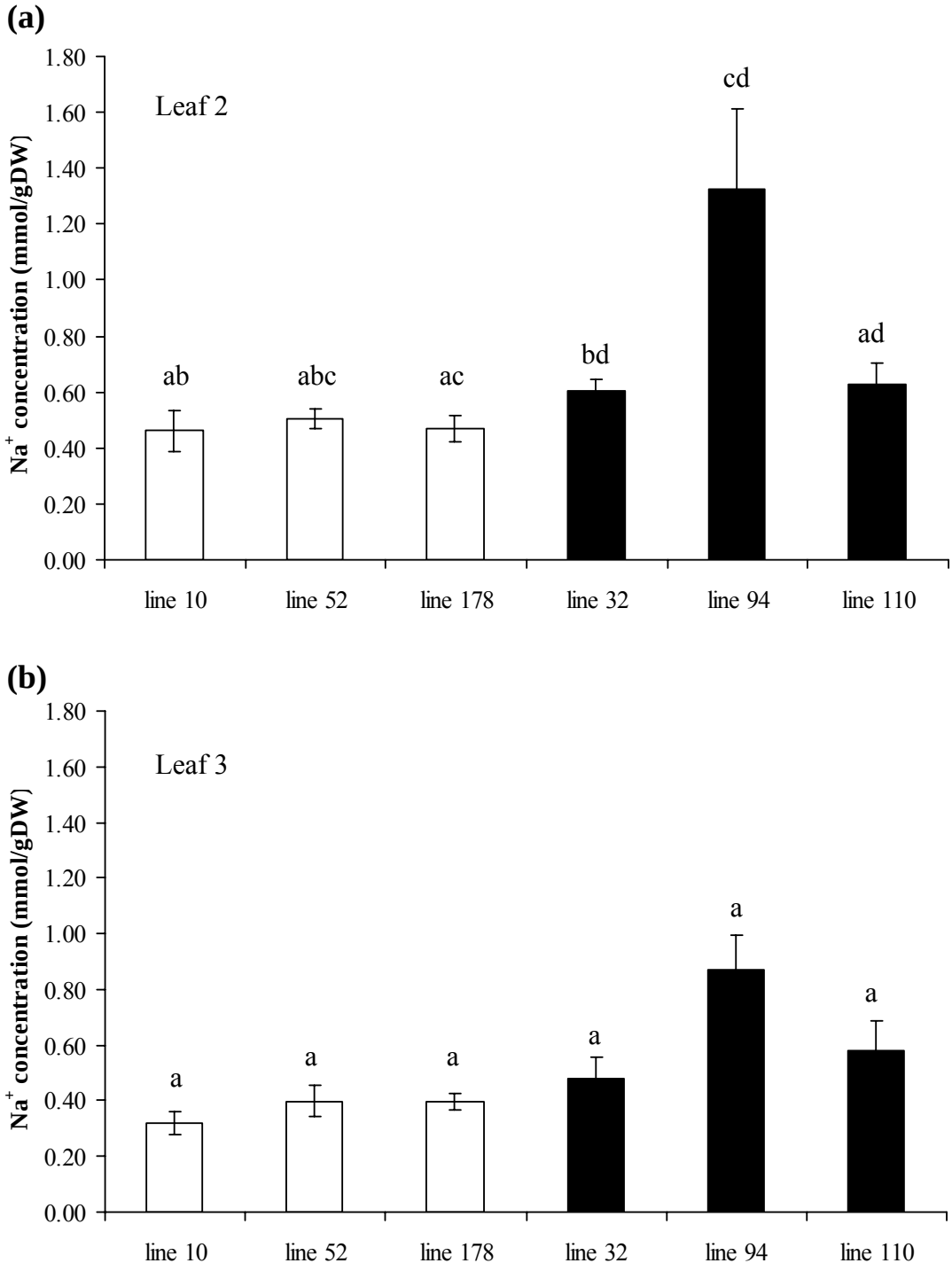

Figure 5.17 Na^+ concentrations in leaf sheaths of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

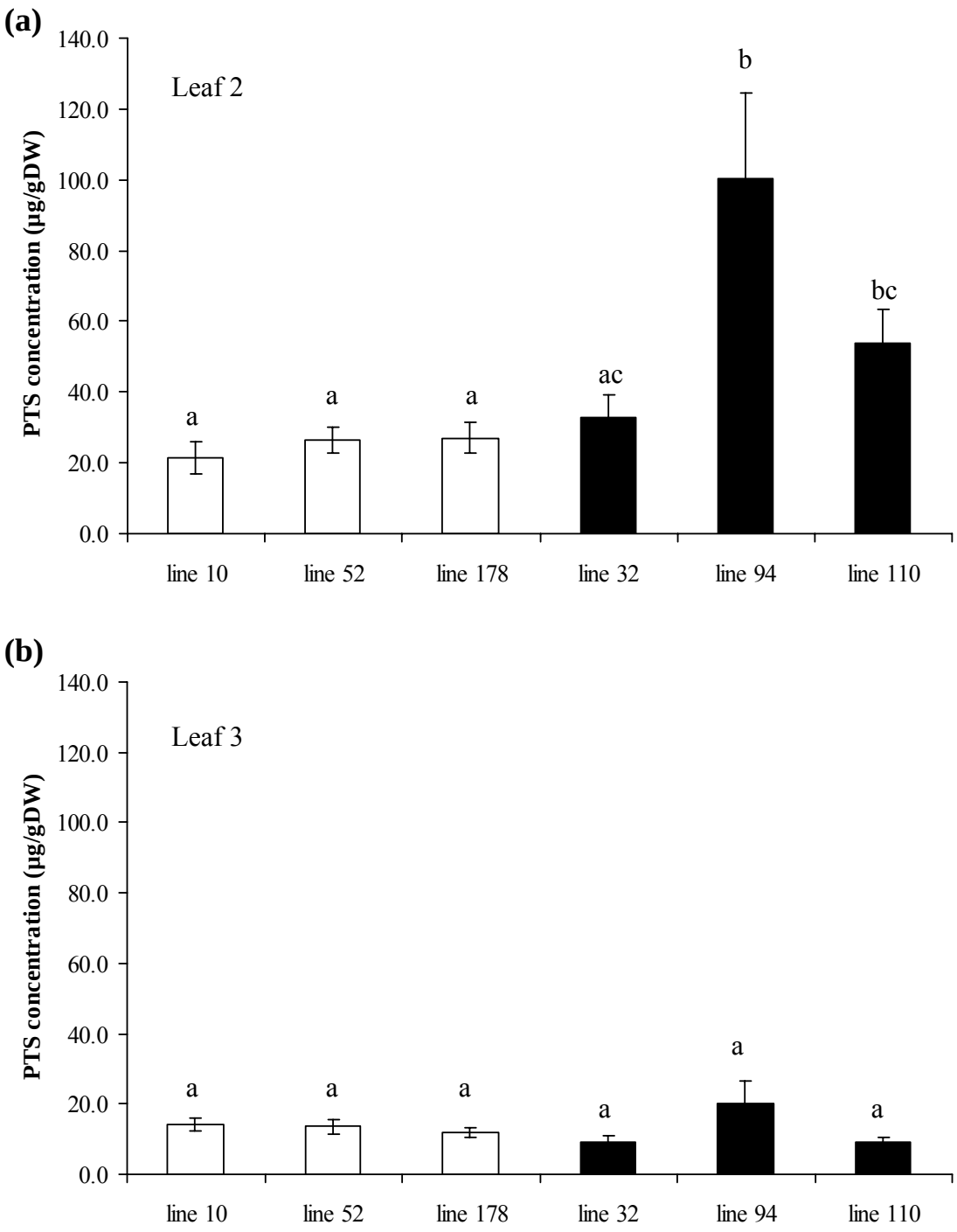

Figure 5.18 PTS concentrations in leaf sheaths of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

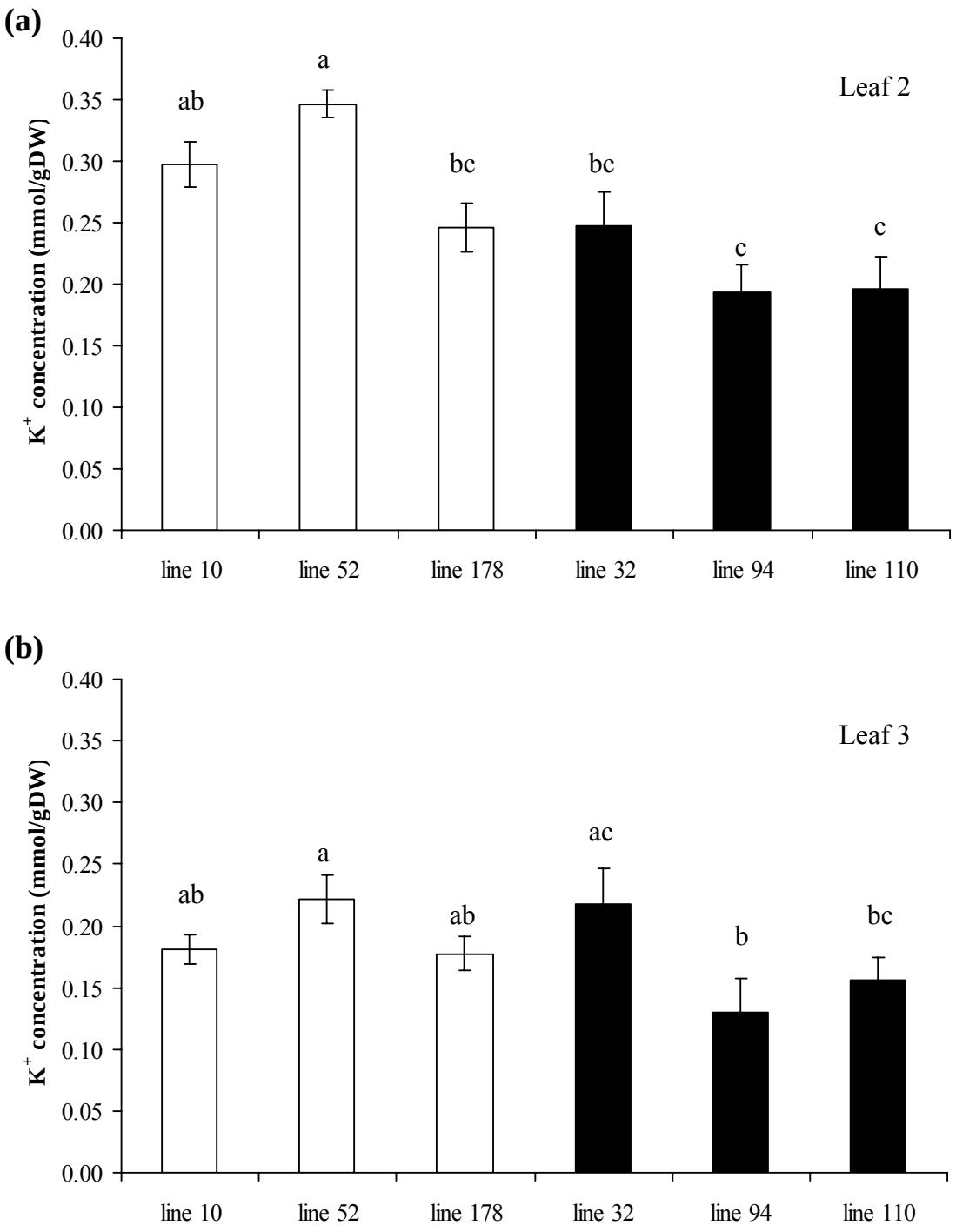

Figure 5.19 K^+ concentrations in leaf sheaths of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

5.3.4 The quantity of Na^+ in phloem sap

The quantity of Na^+ in phloem sap ranged from 40 to 116 μmol in leaf 2, and 10 to 58 μmol in leaf 3 of low- and high-Na^+-transporting lines (Figure 5.20 & Appendix 5.10). These results were negligible as compared to those of *lrt1* (340 μmol for leaf 2 and 144 μmol for leaf 3, see Chapter 2, section 2.3.6), indicating that no significant amount of Na^+ was present in the phloem sap of the six lines of rice IR55178 under study.

5.3.5 Seedling survival

Survival of seedlings in 50 mM NaCl is shown in Figure 5.21. High-Na^+-transporting lines (e.g. line 110) died after 10 d of exposure to salinity while low-Na^+-transporting lines 10, 52 and 178 were unaffected and found to be healthy. After 50 d of salt treatment, lines 94 and 110 had largely died with an average survival of only 12.5%, whereas low-Na^+-transporting lines still had green leaves and mainly survived with an average survival of 98.6% (Figures 5.21, 5.22). High-Na^+-transporting lines 32, 94 and 110 had all died at 80, 70 and 60 d, respectively, whereas low-Na^+-transporting lines 10, 52 and 178 showed little mortality after 60 d and remained alive until 90 d after salt treatment (Figure 5.21). The period in which 50% of the individuals died (defined as D_{50}, Flowers & Yeo 1981; Yeo & Flowers 1983) was 52 d, 37 d and 35 d for high-Na^+-transporting lines 32, 94 and 110, whereas it was 81 d, 76 d and 65 d for low-Na^+-transporting lines 10, 178 and 52, respectively (Figure 5.21).

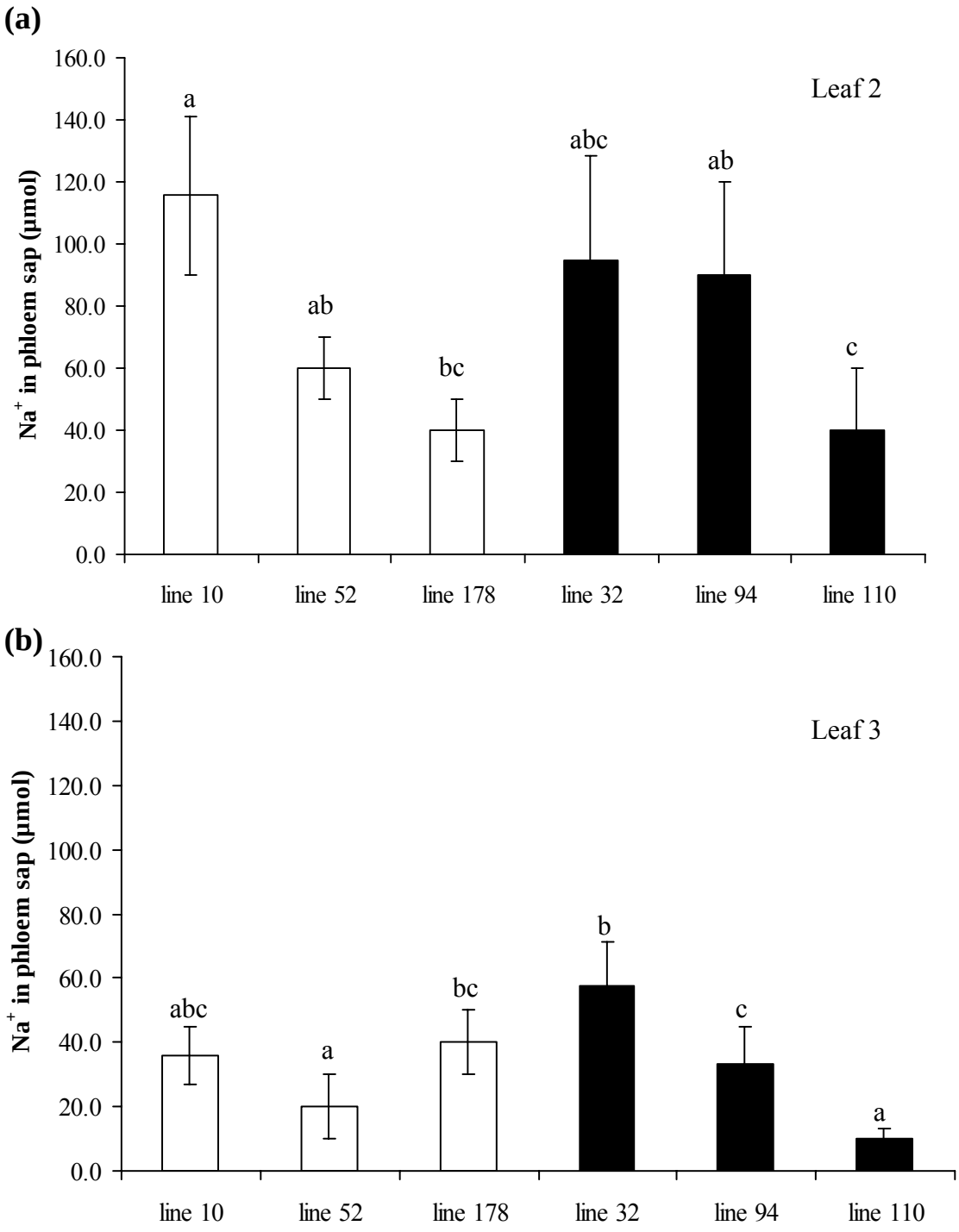

Figure 5.20 The quantity of Na^+ in phloem sap of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. The quantity of Na^+ in the phloem sap was estimated using the EDTA technique. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 20).

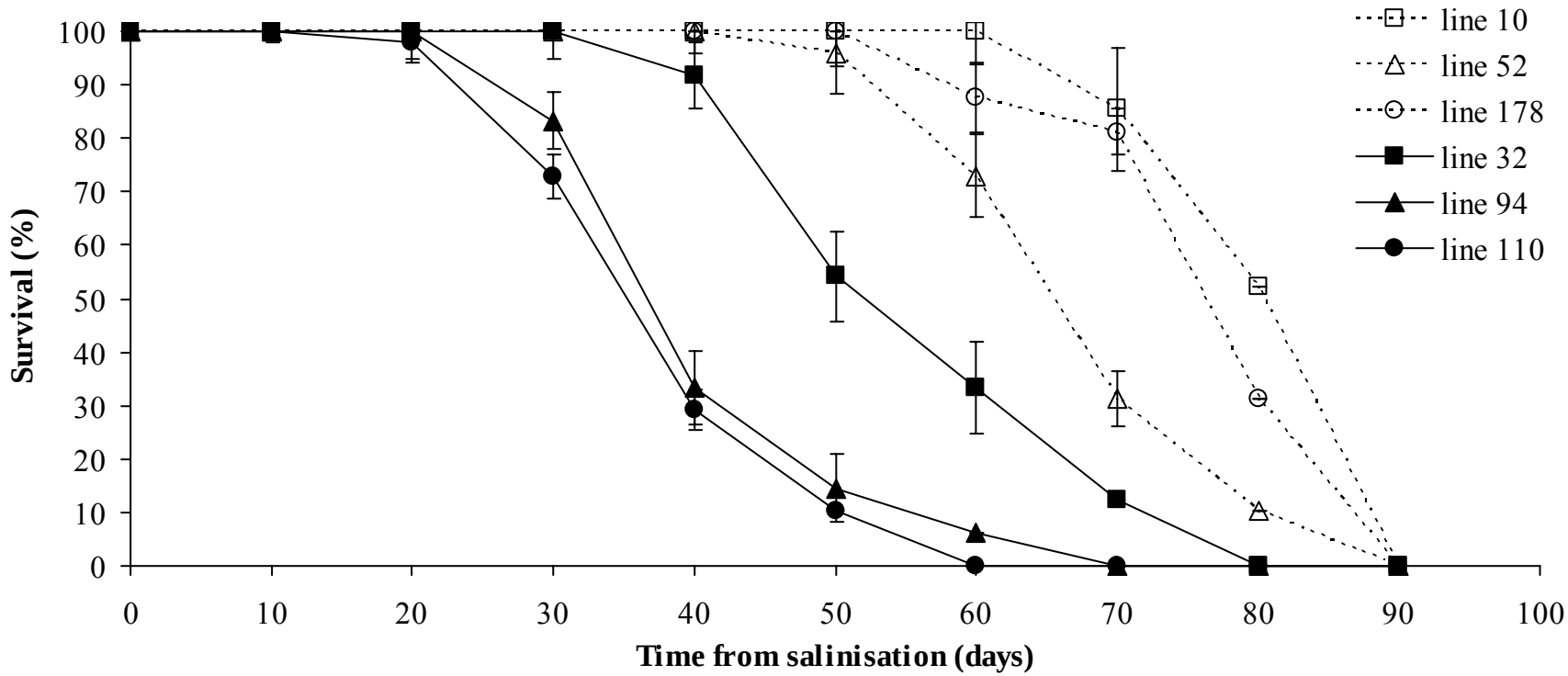

Figure 5.21 Survival of 3 low-Na^+-transporting lines (broken graphs) and 3 high-Na^+-transporting lines (solid graphs) of rice seedlings IR55178 after salinisation with 50 mM NaCl and 100 mg/l PTS at 14-day-old seedlings. Means and standard errors (n = 48).

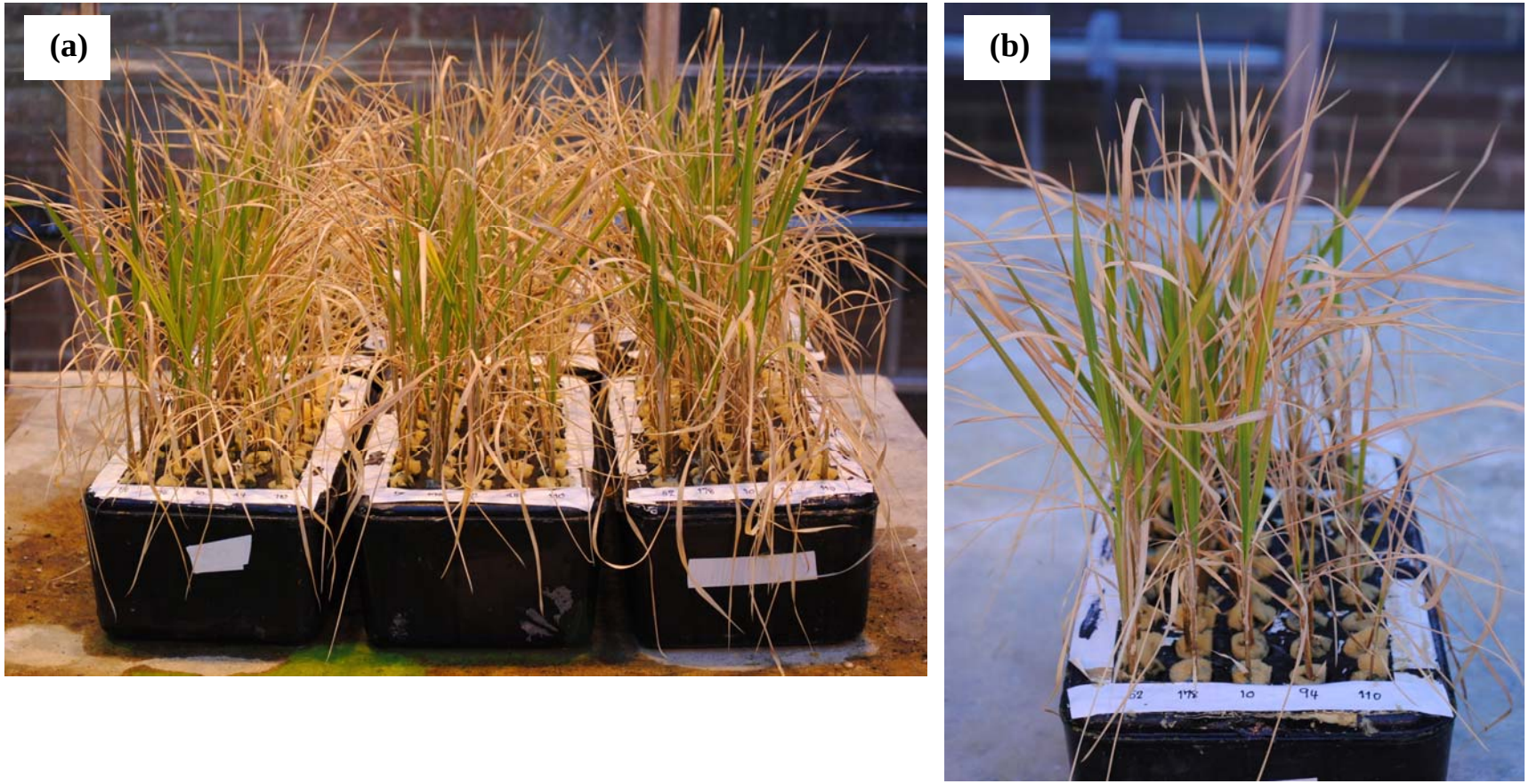

Figure 5.22 Survival of 3 low-Na^+-transporting lines (10, 52 and 178) and 3 high-Na^+-transporting lines (32, 94 and 110) of rice seedlings IR55178 after salinisation with 50 mM NaCl and 100 mg/l PTS at 14-day-old seedlings for 50 d. Overall picture (a) and close up picture (b) showing the youngest leaves of lines 10, 52 and 178 remained green while those of lines 94 and 110 obviously died.

5.4 DISCUSSION

Rice is classified as a salt-sensitive species which is ineffective in controlling the influx of Na^+ across the roots, leading to the rapid accumulation of toxic concentrations in the shoots (Chapter 1, section 1.1). However, there are variations in salinity resistance both within and between genotypes of rice (Flowers & Yeo 1981; Yeo & Flowers 1986; Yeo *et al.* 1988) and these variations have been believed to be influenced by bypass flow (Yeo 1992). The present work has shown that there were significant differences in the percentage of bypass flow between recombinant inbred lines of rice IR55178 in which the high-Na^+-transporting lines showed a greater magnitude (an average of 2.4 times) of bypass flow than that of the low-Na^+-transporting lines (Figure 5.6 & Table 5.1). The average shoot Na^+ concentration for high-Na^+-transporting lines was 1.4 times higher than that of low-Na^+-transporting lines (Table 5.1). These findings are in line with the view that the difference in Na^+ uptake in rice is a result of the difference in bypass flow (Yeo 1992) and compatible with the work of Yadav *et al.* (1996) who reported that the high-Na^+-transporting line of rice cv. IR36 took up more PTS (a bypass flow tracer) than the low-Na^+-transporting line.

Data analysis of the uptake of water, PTS and Na^+ by whole shoots of rice showed that the apparent Na^+ and PTS concentrations in the xylem of high-Na^+-transporting lines were statistically higher than those of low-Na^+-transporting lines by 1.8 and 2.3 times, respectively (Figures 5.8, 5.9 & Table 5.1), indicating that significantly greater amounts of Na^+ and PTS were transported in the xylem to the shoots of high- than of low Na^+-transporting lines. However, Na^+, K^+ and PTS concentrations in the xylem sap obtained by a direct sampling from leaves 2 and 3 using the xylem-feeding insect *P. spumarius* were not statistically different between low- and high-Na^+-transporting lines (Figures 5.11-5.13 & Appendices 5.3-5.8). Also, no differences in the concentrations of Na^+, K^+ and PTS in leaf blades were noticed between low- and high-Na^+-transporting lines (Figures 5.14-5.16). The results are consistent with the work of Roshandel (2005, 2007) who reported that despite a higher Na^+ concentration in the shoots of salt-sensitive rice IR15324 by 1.5 times in comparison to salt-tolerant rice IR4630 after salinisation with 50 mM NaCl for 96 h, the average of Na^+, K^+ and PTS concentrations in the xylem sap, quantified by *P. spumarius*, of leaf 3 of both varieties was similar. Roshandel (2005,

2007) speculated that the difference in Na^+ uptake between IR15324 and IR4630 was a consequence of a difference in Na^+ retranslocation in the phloem between those two varieties. In the present study, an analysis of Na^+ in phloem sap estimated using the EDTA technique (King & Zeevaart 1974; Berthomieu *et al*. 2003) showed no significant difference in Na^+ between low- and high-Na^+-transporting lines and the quantity of Na^+ collected from the phloem sap was negligible in all six lines examined (Figure 5.20). The results are compatible with Yeo & Flowers (1982) who demonstrated, by using radio-labelled $^{22}Na^+$, that Na^+ retranslocation through the phloem in rice was insignificant, either within the plant or to the culture solution. Also, the results in Chapters 2 and 4 showed that no significant quantities of Na^+ were found in phloem sap of rice genotypes Oochikara, Nipponbare, *lrt2*, Taichung 65, *crl1*, CSR10, IR36 and GR4. Although a considerable amount of Na^+ was found in the phloem of *lrt1* (Chapter 2, section 2.3.7), the result is in line with the view that salt-sensitive variety, with high shoot Na^+ concentration, cannot keep the Na^+ out of its phloem (Flowers *et al.* 1986; Munns *et al.* 1986, 1988, 2002; Munns & Tester 2008; Singh & Flowers in press).

The similarity of Na^+ concentrations in the xylem of low- and high-Na^+-transporting lines is also consistent with the recent report in barley (Shabala *et al.* 2010). It was found that Na^+ concentrations in the xylem sap collected by Scholander pressure bomb of the cutting stems of salt-tolerant genotypes cvs. CM72 and Numar were as high as those of salt-sensitive genotypes cvs. Gairdner and ZUG403 when exposed to 320 mM NaCl for 14 d. In contrast, the xylem K^+/Na^+ ratio was about 70% in tolerant varieties, and 20% in sensitive varieties as compared to the control plants. As a result, the authors pointed out that minimising entry of Na^+ to the xylem was not essential for conferring salt tolerance in barley, but it depended on the abilities to maintain high K^+/Na^+ ratio in the xylem and efficiently sequester Na^+ in leaves. In the present study, although the xylem K^+/Na^+ ratio was not different between low- and high-Na^+-transporting lines (Appendix 5.9), the K^+/Na^+ ratio in the shoot was found to be significantly higher in low-Na^+-transporting lines than that of high-Na^+-transporting lines (Figure 5.3 & Table 5.1).

The fact that low- and high-Na^+-transporting lines were similar in concentrations of Na^+ and PTS in the xylem sap as well as in leaf blades, despite obvious difference in whole-

shoot Na^+ and PTS concentrations, leads to a hypothesis that there would be substantial concentrations of Na^+ and PTS in the leaf sheaths of high-Na^+-transporting lines. Obviously, the results shown in Figures 5.17-5.18 are consistent with this hypothesis; Na^+ and PTS concentrations in leaf sheaths of high-Na^+-transporting lines were higher than those of low-Na^+-transporting lines. Similarly, Na^+ contents in the leaf sheaths of high-Na^+-transporting lines were higher than those of low-Na^+-transporting lines (Appendix 5.13), making it unlikely that the difference in Na^+ concentrations in leaf sheaths was due to differences in the dilution by growth, but indeed it was a consequence of differences in Na^+ uptake by the different rice lines. The results obtained are in line with the studies in salt-sensitive durum wheat and salt-tolerant bread wheat under salinity stress as reported by Munns and co-workers (Munns *et al.* 2000; James, Davenport & Munns 2006). In their experiments, they found that Na^+ concentrations in leaf blades of durum wheat line 149 subjected to 150 mM NaCl for 10 d were as low as salt-tolerant bread wheat cv. Janz, whereas shoot Na^+ concentrations in durum wheat line 149 were higher than bread wheat (Husain, von Caemmerer & Munns 2004; James *et al.* 2006). Subsequent study indicated that Na^+ was removed from the xylem and retained in the leaf sheaths of durum wheat line 149 and the mechanism was mediated by Na^+ exclusion gene, *Nax1*, on the long arm of chromosome 2A (Lindsay *et al.* 2004; Davenport *et al.* 2005; James *et al.* 2006). In sorghum (*Sorghum bicolor* L.), it was reported that Na^+ concentrations in leaf sheaths were higher than in leaf blades in both salt sensitive cv. CSF18 and salt tolerant cv. CSF20 when exposed to 100 mM NaCl for 4, 6 or 8 d, and the leaf sheaths of salt-sensitive variety contained more Na^+ concentrations than salt-tolerant one (de Lacerda *et al.* 2003).

Preferential retention of Na^+ in the leaf sheath of rice has previously been reported by Yeo & Flowers (1982). They found that in rice IR2153 treated with 50 mM NaCl for 40 d, leaf sheaths contained higher concentrations of Na^+ than leaf blades. Likewise, Matsushita & Matoh (1991) found that the shoot base region, including the mesocotyl and leaf sheaths, of rice cv. Kinmaze growing hydroponically in 10, 25 or 50 mM NaCl for 7 d accumulated more Na^+ than leaf blades. It was also seen in rice cv. Nipponbare subjected to 257 mM NaCl for 2 weeks in soil (Mitsuya *et al.* 2002). Gong *et al.* (2006) also reported similar results in rice cv. IR36 growing in 50 mM NaCl for 2 d. In general, the ability to exclude Na^+ from photosynthetic tissues by compartmentalisation Na^+ in leaf sheaths rather than leaf blades could be a crucial mechanism for preventing salinity

damage (Yeo & Flowers 1982; Yeo *et al.* 1990; Munns 2005; Davenport *et al.* 2005; Gong *et al.* 2006; James *et al.* 2006). The mechanism by which Na^+ was sequestered in leaf sheaths of rice is unknown; however, it has been speculated that *Nax1* gene may be present in this species (James *et al.* 2006).

The spatial distribution of ionic concentrations (e.g. Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}, NO_3^-, SO_4^{2-}) in the leaf sheath is well documented in sorghum (Bernstein *et al.* 1995), wheat (Hu & Schmidhalter 1998; Hu *et al.* 2005), barley (Delane *et al.* 1982; Fricke & Flowers 1998) and maize (Meiri, Silk & Läuchli 1992; Neves-Piestun & Bernstein 2005). Studies on a millimetre scale of sorghum (Bernstein *et al.* 1995), wheat (Hu & Schmidhalter 1998; Hu *et al.* 2005) and maize (Neves-Piestun & Bernstein 2005) subjected to saline conditions revealed that Na^+ concentration in the leaf sheath increased sharply from the leaf base reaching a maximum concentration at approximately 15 mm from the leaf base, gradually decreased with a minimum at about 30-40 mm, and then increased toward the end of the leaf sheath. Four-cm-long segments of the leaf sheath of rice cv. Nipponbare exposed to salinity also showed that Na^+ content was the highest in the middle part of the sheath (Mitsuya *et al.* 2002). It is likely that an accumulation of Na^+ and Cl^- in the leaf sheaths of monocotyledonous plants exposed to saline conditions is beneficial for osmotic adjustment, if the ions are compartmentalised appropriately (Yeo *et al.* 1991; Fricke 2004a; Fricke *et al.* 2004, 2006). For example, Delane *et al.* (1982) reported that the deposition of Na^+, K^+ and Cl^- in the growing zone of barley leaf, 0-20 mm from the base, contributed approximately 55% to osmotic pressure under salinity stress at 120 and 180 mM NaCl for 5 d. Yeo *et al.* (1991) reported that Na^+ contents in the elongating region (10 mm from the base) of leaf 4 of rice IR2153 increased substantially when exposed to 50 mM NaCl for 2 d. The authors stated that an increase in Na^+ uptake contributed to osmotic adjustment in the growing region because turgor pressure in cells of this region was not changed when the culture solution was replaced by the culture solution plus 50 mM NaCl. In sorghum grown in 100 mM NaCl, Bernstein *et al.* (1995) reported that the highest peak of concentration and deposition rate of Na^+ was at 15 mm from the base, which is a region of maximum elongation rate. In barley subjected to 75 mM NaCl for 4-6 d, Fricke & Peters (2002) showed that epidermal cells in the elongation zone responded to a decrease in external water potential, induced by salt stress, by an increase in the deposition of solutes (e.g. Na^+ and Cl^-; Hu *et al.* 2005) to maintain cell osmolality and

turgor. However, at high external NaCl (120 mM), the solute deposition rate was decreased, probably because it already reached its maximum at 75 mM NaCl (Fricke & Peters 2002) or because high salinity limited the translocation of water and solutes to growing cells by reducing the numbers of veins (Hu *et al.* 2005). Consequently, one of the explanations for the rapid cessation in leaf growth of plants under salinity is an inadequate supply of mineral nutrients to elongating cells in the growth zone (Bernstein *et al.* 1995; Hu & Schmidhalter 1998; Fricke 2002, 2004a; Fricke & Peters 2002; Hu *et al.* 2005; Neves-Piestun & Bernstein 2005). Recently, water permeability and K^+ uptake in growing leaf cells of barley have been studied by Fricke and co-workers (Volkov *et al.* 2007, 2009; Wei *et al.* 2007; Boscari *et al.* 2009). An available technique of single-cell-sap extraction with the silicone-oil-filled glass microcapillary, together with picolitre osmometry and X-ray microanalysis (Fricke, Leigh & Tomos 1994a, b; Fricke *et al.* 1994, 1995, 1996, 2006; Tomos *et al.* 1994; Fricke 1997, 2004a, b; Fricke & Peters 2002; Volkov *et al.* 2007; Knipfer & Fricke 2010) would be useful for the assessment of solute distribution in rice.

Generally, the leaf sheaths of rice function as a mechanical support to the plant and serve as a temporary storage site for carbohydrate before heading (Yoshida 1981; Chen & Wang 2008). The rice leaf sheath is a significant proportion of the total leaf, approximately 40-60% of the total leaf dry weight (Appendix 5.15), making it a good site for Na^+ retention. However, once Na^+ concentrations saturate the storage capacity of the sheaths, Na^+ transported to the shoots would be accumulated in leaf blades, subsequently inducing leaf injury and eventually reducing plant survival. It is obvious in the present study that after exposure to 50 mM NaCl for 50 d, high-Na^+-transporting lines 94 and 110 died with an average survival of only 12.5%, whereas low-Na^+-transporting lines 10, 52 and 178 remained healthy with a high survival percentage of 98.6% (Figures 5.21, 5.22). It is noteworthy that lines with lower shoot Na^+ concentration and lower percentage of bypass flow had greater values of D_{50} and there was a highly significant negative correlation between shoot Na^+ concentration, the percentage of bypass flow and seedling survival: the lower shoot Na^+ concentration and bypass flow, the greater survival of seedlings (Figure 5.23).

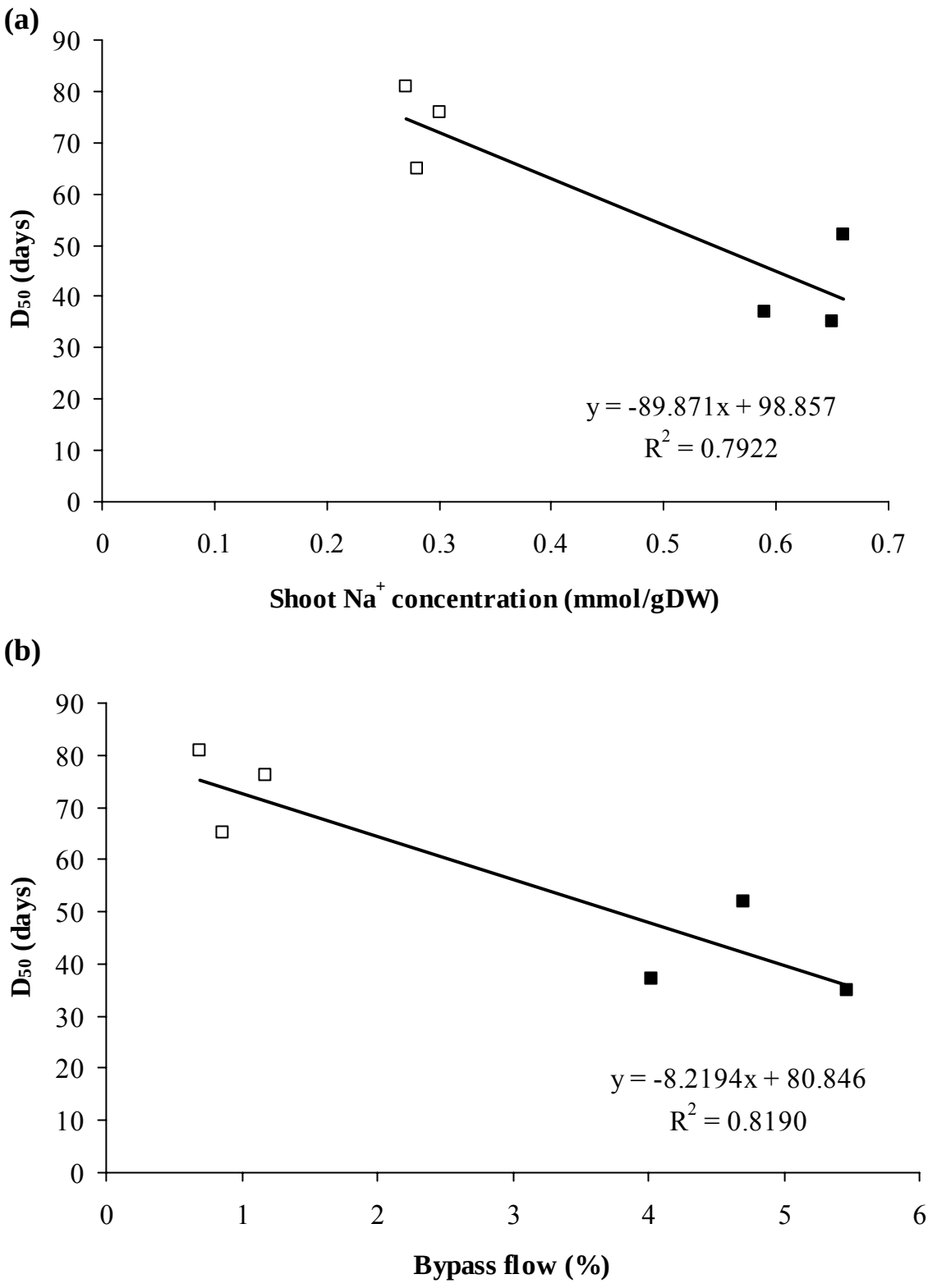

Figure 5.23 The relationships between seedling survival (D_{50}) and shoot Na^+ concentration (a) and bypass flow (b) of low-Na^+-transporting lines 10, 52 and 178 (open symbols) and high-Na^+-transporting lines 32, 94 and 110 (closed symbols). Data are from Figures 5.1, 5.6 and 5.21.

Salinity resistance in rice is a complex issue, which includes restriction of entry of Na^+ into the root xylem, reducing Na^+ uptake to the shoots, leaf-to-leaf distribution, compartmentation of Na^+ between and within cells and plant vigour (Chapter 1, section 1.5). Since the transport of solutes from the root to the shoot is related to the transpirational volume flow (Chapter 1, section 1.5.2), any reduction in transpiration could also reduce the uptake of Na^+. In the present study, it was found that the transpiration volumes in low-Na^+-transporting lines were significantly lower than those of high-Na^+-transporting lines (Figure 5.5 & Table 5.1), indicating the ability to control transpiration volume during salinity stress of low-Na^+-transporting lines reduced net Na^+ uptake to the shoots (Figure 5.1 & Table 5.1). Similar results were also reported by Moradi & Ismail (2007) who found that salt-tolerant rice IR632 and IR651 reduced Na^+ accumulation in the shoots by maintaining lower transpiration rates as compared to salt-sensitive rice IR29 in response to salinity stress of 100 mM NaCl for 14 d.

Bypass flow has been accepted to be a significant pathway for Na^+ leakage into rice plants under saline conditions (Chapter 1, section 1.3.2). A highly positive correlation between bypass flow and shoot Na^+ concentration in Figure 5.7 confirms that a substantial amount of Na^+ in the shoots was taken up via this pathway. The results in a previous study (Chapter 4, section 4.3.3.2) showed that approximately 30% of the total Na^+ in the shoots of rice cv. IR36 growing hydroponically in 50 mM NaCl arrived via bypass flow. In the present study, it was also found that bypass flow delivered an average of 22 and 26% of the shoot Na^+ in low- and high-Na^+-transporting lines, respectively (Table 5.1). Obviously, high-Na^+-transporting lines had significantly greater Na^+ bypass flow than low-Na^+-transporting lines (Table 5.1). Such a result supports the earlier suggestion (Yeo 1992) that bypass flow contributes to the variability of Na^+ uptake in rice and adds more evidence to support the view that bypass flow could be considered as a screening criterion for salinity resistance in rice.

A number of traits have been used for screening salt tolerance in rice such as leaf Na^+ concentration (Yeo & Flowers 1983; Yeo *et al.* 1988, 1990), leaf area per total dry weight (Akita & Cabuslay 1990), leaf-to-leaf distribution, tissue tolerance and plant vigour (Yeo *et al.* 1990), shoot fresh weight (Aslam *et al.* 1993), floret fertility (Khatun *et al.* 1995; Rao *et al.* 2008), K^+/Na^+ ratio (Asch *et al.* 2000) and relative water content

(Suriya-arunroj *et al.* 2004), but very few salt-resistant varieties have been released (Flowers & Yeo 1995; Rozema & Flowers 2008). One of the main reasons for this is the lack of reliable and reproducible techniques for identifying salt tolerance in rice and the others are the complexity of the trait and limited knowledge of the genetics of salinity tolerance (Yeo *et al.* 1990; Gregorio & Senadhira 1993; Garcia *et al*. 1995; Xie *et al.* 2000; Koyama *et al*. 2001; Yadav *et al.* 2008). The results in the present study in which individual lines with low Na^+ transport possessed low bypass flow and lines with high Na^+ transport showed high bypass flow (Figures 5.1, 5.6, 5.7), together with the findings outlined earlier in Chapter 4 that the bypass flow was significantly higher in salt-sensitive GR4 than salt-tolerant CSR10 confirm the reproducibility of differences in bypass flow in varieties of rice differing in salt resistance. A high correlation found between the percentage of bypass flow and survival of seedlings (D_{50}) after prolonged treatment with 50 mM NaCl (Figure 5.23) also indicates the strength of the relationship.

In conclusion, this Chapter has shown that bypass flow contributes to differences in degree of salinity resistance in rice. High-Na^+-transporting lines with high shoot Na^+ concentrations showed a high magnitude of bypass flow, and low-Na^+-transporting lines with low shoot Na^+ concentrations possessed low bypass flow. With the reproducibility and validity of the technique, bypass flow could be used as a new criterion for screening salt resistance in rice.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

It is well known that soil salinity is an important agricultural problem limiting crop productivity worldwide. Of the cereal crops, rice is the most salt-sensitive because it is ineffective in controlling the influx of Na^+ across the roots, leading to the rapid accumulation of toxic Na^+ concentrations in the shoots. Studies have shown that bypass flow is a significant pathway for Na^+ uptake in rice under saline conditions; however, the precise site of entry is not yet known (Chapter 1, section 1.3.2). The purpose of this thesis was to produce a better understanding of bypass flow of Na^+ in rice in order to limit such flow and to increase salinity resistance in this species and guarantee food supply for future generations as rice is the staple food for one third of world population and will remain so in the future (Sengupta & Majumder 2010; Singh & Flowers in press).

Previous work (Peterson *et al.* 1981; Enstone & Peterson 1998; Ranathunge *et al.* 2005a) has considered that the emergence of lateral roots from the main root interrupted bypass flow barriers (e.g. Casparian bands and suberin lamellae) and might provide the site for bypass flow of water and solutes into the xylem. For this reason, I hypothesised that if bypass flow of Na^+ in rice originates at the site where lateral roots emerge from the main root, then bypass flow and Na^+ concentration will be higher in seedlings with numerous lateral roots than ones with fewer laterals. My investigations into the correlation between the lateral root emergence and bypass flow using lateral rootless mutants (*lrt1*, *lrt2*), a crown rootless mutant (*crl1*) and their wild types (Oochikara, Nipponbare and Taichung 65, respectively) and seedlings of rice cv. IR36 in which the number of lateral and crown roots had been reduced by physical cutting and 2,4-D treatment revealed that although the number of lateral roots was reduced by 99-100% in *lrt1* and *lrt2*, by 77% in *crl1* and by 60% in IR36 (Chapter 2, section 2.3.1), the magnitude of bypass flow in rice seedlings was not decreased; in contrast, it was significantly increased by 136% for *lrt1*, 264% for *lrt2*, 163% for *crl1* and 75-265% for IR36 as compared to their wild types and control plants when exposed to 50 mM NaCl and 100 mg/l PTS for 72 h (Chapter 2, section 2.3.5). Similarly, the delivery of Na^+ to

the shoots via bypass flow in the rice mutants was shown to be increased by an average of 70% compared with the wild types (Chapter 2, section 2.3.5). These findings are not consistent with the hypothesis and strongly indicated that bypass flow in rice did not originate at the sites of lateral root emergence. So, where is the start of the path of bypass flow in rice? The results of a detailed investigation are reported in Chapter 3.

North & Nobel (1996) found that PTS was observed in the xylem of lateral roots of *Opuntia ficus-indica*. Enstone & Peterson (1998) reported that the bypass flow tracer berberine moved into the xylem of the main root of maize through the xylem of the lateral roots. Another bypass flow tracer, Fe^{2+}, reached the stele of nodal roots of common reed via the vascular tissues of the lateral roots (Soukup *et al.* 2002). Accordingly, I hypothesised that if bypass flow in rice occurs through the lateral roots, then the bypass flow tracer PTS will be seen in the xylem of lateral roots of plants that had been treated with PTS. To test this hypothesis, the uptake of PTS in the roots and shoots of rice seedlings exposed to PTS solution (50 g/l for 30 min) was followed by using epifluorescence microscopy, CLSM and fluorescence spectrometry. The results showed that PTS was observed in the stele of lateral roots both with the epifluorescence microscope and CLSM (Chapter 3, section 3.3.2). The PTS fluorescence in the stele of the lateral roots was weaker when the seedlings were transferred into distilled water for a chase period of 1 and 3 h, while its concentration in the shoots increased with increasing the chase periods (Chapter 3, section 3.3.3), indicating that this fluorescence tracer was transported from the xylem of lateral roots to the shoots. An investigation carried out on the anatomy of lateral roots using Cryo-SEM and epifluorescence microscopy of sections stained with berberine-aniline blue (for Casparian bands) and Fluorol Yellow 088 (for suberin lamellae) revealed that the exodermal and sclerenchymatous layers were absent in the lateral roots (Chapter 3, section 3.3.4). As a result, it can be concluded that the lack of the bypass flow barrier (an exodermis) in the lateral roots of rice allowed the entry of PTS into the stele and transport to the shoot via the transpiration stream. These results are consistent with the hypothesis and support the concept of the role of the lateral roots in bypass flow in rice under saline conditions.

The overall results suggest that bypass flow in rice would be decreased if an exodermis was present in the lateral roots, meaning that salinity resistance in rice would be increased if breeding programme or genetic manipulation induced the formation of an

exodermis in the lateral roots of rice. To the best of my knowledge, rice genes that involve in the exodermal formation have not been reported. Studies have indicated that *lrt1* and *lrt2* genes are involved in lateral root formation in rice; however, the precise mechanisms underlying lateral root development in rice have not been documented; *lrt2* gene has been found to be on chromosome 2, whereas *lrt1* gene is currently being investigated (Wang *et al.* 2006). Once the molecular mechanisms of lateral root development in rice are fully understood, I believe that it would be feasible to induce the exodermal formation in the lateral roots using genetic engineering and thus reducing the bypass flow. The lack of an exodermis in the lateral roots correlated with an increase in PTS fluorescence in the stele confirms the earlier suggestion that the magnitude of bypass flow in rice depends on the anatomical and morphological development of the roots (Chapter 2, section 2.1).

The results in Chapters 2 and 3 do not contradict each other. It was shown in Chapter 3 that the sites of bypass flow in rice occur through the lateral roots due to their lacking of an exodermis. Consequently, bypass flow would be higher in seedlings with numerous lateral roots than ones with fewer laterals. Unexpectedly, the results of bypass flow in *lrt1*, *lrt2* and *crl1* showed an increase in the percentage of bypass flow as compared to their wild types, although the mutants had a significantly reduced number of laterals (Chapter 2, sections 2.3.1 and 2.3.5). The significant increase in the bypass flow of the mutants could be explained by a reduction in the deposition of suberin lamellae on exodermal and endodermal walls of the seminal roots of the mutants (Chapter 2, sections 2.3.1 and 2.3.2). However, any reduction of suberin formation in the exodermis and endodermis would inevitably facilitate bypass flow in the mutants, and this effect was more pronounced than the reduction of bypass flow due to lack of lateral roots. It is also interesting to note that the mutants *lrt1*, *lrt2* and *crl1* exhibited several morphological and anatomical changes. For example, *lrt1* had more central conducting vessels compared with Oochikara; *lrt2* had a longer seminal root and more central conducting vessels than Nipponbare, whereas *crl1* had larger diameter of xylem vessel than Taichung 65 (Chapter 2, sections 2.3.1 and 2.3.2); these changes might enhance the percentage of bypass flow in the mutants.

Flowers and co-workers (Yeo *et al.* 1987; Yadav *et al.* 1996; Garcia *et al.* 1997; Gong *et al.* 2006) have established that substantial amount of Na^+ enters the rice plant through

the bypass flow. In the present study, I was also able to demonstrate a positive correlation between bypass flow and Na^+ uptake to the shoots of rice (Chapter 2, section 2.3.5; Chapter 5, section 5.3.2), confirming that bypass flow is a significant component for Na^+ transport in rice under saline conditions. It has also been reported that the addition of Si to the culture solution reduced Na^+ concentration in the shoots of rice subjected to salinity stress (Matoh *et al.* 1986; Yeo *et al.* 1999; Gong *et al.* 2006). X-ray microanalysis showed that Si was deposited in the exodermal and endodermal layers of the roots and effectively blocked the bypass flow (Gong *et al.* 2006). In the same way, shoot Na^+ concentration of rice seedlings subjected to salinity was decreased when PEG was added to the culture solution (Yeo & Flowers 1984b; Ochiai & Matoh 2004), but the mechanism by which PEG reduced Na^+ uptake is not fully understood. As indicated in Chapter 3, pre-treatment of seedlings with PEG (10 g/l) or Si (3 mM) for 7 d before exposure to PTS solution (50 g/l for 30 min) reduced the intensity of PTS fluorescence in the xylem of lateral roots and significantly decreased the concentration of PTS in the shoots (Chapter 3, section 3.3.5), indicating the mechanism of PEG reducing Na^+ uptake would be similar to that of Si in reducing the bypass flow. Interestingly, it was noticed that the reductions of PTS fluorescence in the lateral root and PTS concentration in the shoot were more pronounced with PEG than Si treatments (Chapter 3, section 3.3.5), suggesting that PEG might reduce bypass flow more effectively than Si. This suggestion led to my work for Chapter 4.

Three rice genotypes of CSR10 (salt tolerance), IR36 (intermediate tolerance) and GR4 (salt sensitive) were selected in order to evaluate the effectiveness of PEG and Si in reducing the bypass flow and Na^+ uptake to the shoots of rice subjected to salt stress of 50 mM NaCl for 96 h (Chapter 4). The xylem-feeding insect *Philaenus spumarius* L. was also used to collect Na^+ and PTS in the xylem sap from leaves 2 and 3 of rice seedlings. The results showed that the addition of PEG or Si to the culture solution significantly decreased shoot Na^+ concentration, shoot Na^+ content and net Na^+ transport from the root to the shoot of rice under saline condition (Chapter 4, sections 4.3.2.2, 4.3.2.3 and 4.3.2.5). As I expected, PEG was found to reduce bypass flow more than Si in all three rice genotypes under investigation (Chapter 4, section 4.3.2.4). Furthermore, Na^+ delivered to the shoots via bypass flow was less in PEG-treated seedlings than that of Si-treated ones (Chapter 4, section 4.3.2.4). Although it is not clear why PEG application causes the reduction in the Na^+ bypass flow more effectively

than Si, I believe from the literature that PEG might reduce the pore size of the cell walls through a hydrogen bonding and effectively block the bypass flow of Na^+ (Yeo & Flowers 1984b; Ochiai & Matoh 2004), whereas Si might only be deposited within the cell walls (Gong *et al.* 2006).

Increasing RH levels have been shown to reduce Na^+ transport to the shoot of rice and other plants subjected to salinity (Chapter 4, section 4.1); however, the mechanism by which RH reduced Na^+ uptake is unclear. Salim (1989) reported no change in the transpiration volume of *Atriplex spongiosa* during increasing RH, whereas others reported a reduction of the transpirational volume flow in chickpea, soybean and potato (Lauter & Munns 1987; An *et al*. 2001; Backhausen *et al*. 2005). A model of water and solute transport in plants, involving cell-to-cell and apoplastic pathways has been discussed over many years (Weatherley 1965; Slatyer 1967; Steudle & Frensch 1996; Steudle & Peterson 1998; Fricke 2000, 2002; Steudle 2000a, b, 2001). Those pathways of water movement can be switched from one to another depending on the flux and influenced by environmental factors such as drought, salinity, temperature, low oxygen and heavy metals. As a consequence, I hypothesised that increasing the RH levels would reduce the uptake of Na^+ by changing the proportion of water flowing through the cell-to-cell pathway and the bypass flow of the root. To elucidate this hypothesis, the rice seedlings cv. IR36 were salinised with 50 mM NaCl and 100 mg/l PTS for 96 h with different RH levels of 30, 50, 70 and 90% and shoot Na^+ concentration, bypass flow and transpiration volume were compared. The results revealed that shoot Na^+ concentrations and bypass flow significantly decreased when RH increased from 30 to 90%, whereas the transpiration volume was unaffected (Chapter 4, sections 4.3.3.1 and 4.3.3.2). However, it was shown that the flux of water across the roots (expressed as the transpiration volume per unit of root dry weight per unit of time) was statistically decreased when RH increased from 30 to 90% and this flux of water was correlated with shoot Na^+ concentration and bypass flow: the greater the flux of water across the roots, the greater shoot Na^+ concentration and bypass flow (Chapter 4, section 4.3.3.2). Therefore, my results revealed that a change in RH levels had a significant effect on the flux of solution across the root and this was correlated with a change in the magnitude of the bypass flow.

Salinity resistance in rice has been investigated for many years; however, very few salt-tolerant varieties have been released (Flowers & Yeo 1995; Rozema & Flowers 2008). One of the reasons for this is the lack of reliable and reproducible techniques for identifying salt tolerance (Xie *et al.* 2000; Yadav *et al.* 2008). As discussed above, bypass flow is considered as an important pathway for Na^+ uptake in rice under saline conditions. Keeping this in view, the aim of my work in Chapter 5 was to evaluate the possibility of using bypass flow as a new screening technique for salt resistance in rice. Therefore, 10 low- and 10 high-Na^+-transporting recombinant inbred lines of rice IR55178 were treated with 50 mM NaCl and 100 mg/l PTS for 96 h; shoot Na^+ concentration and bypass flow were determined. The results showed that the average shoot Na^+ concentration for high-Na^+-transporting lines was 1.4 times significantly higher than that of low-Na^+-transporting lines (Chapter 5, section 5.3.1) and the average bypass flow for high-Na^+-transporting lines was 2.4 times greater than that of low-Na^+-transporting lines (Chapter 5, section 5.3.2). It was shown that individual lines with low Na^+ transport possessed low magnitudes of bypass flow, whereas lines with high Na^+ transport showed a high degree of bypass flow (Chapter 5, section 5.3.2). The percentage of bypass flow was positively correlated with the concentration of Na^+ in the shoot of rice (Chapter 5, section 5.3.2). Furthermore, a highly significant negative correlation was found between the percentage of bypass flow and survival of seedlings after prolonged treatment with salinity (Chapter 5, section 5.4) and this indicated that bypass flow could be used as a new criterion for screening salt resistance in rice.

In conclusion, the results obtained in this thesis showed that the path of bypass flow in rice was not at the sites of lateral root emergence, but it occurred through the xylem of the lateral roots. Cryo-SEM and epifluorescence microscopy revealed that the bypass flow barrier, an exodermis, is absent in the lateral roots of rice. The addition of PEG and Si to the culture solution reduced Na^+ uptake to the shoot of rice under saline conditions through the reduction of bypass flow, but PEG proved to be more effective than Si. Increasing RH levels reduced Na^+ concentration in the shoots of rice by reducing the flux of water across the roots and the bypass flow. With its reproducibility and validity, bypass flow could be used as a new criterion for rapid screening salt resistance in rice varieties.

To shed more light on salt tolerance in rice, I believe that future work should be focused on the compartmentation of Na^+ between leaf cells. To the best of my knowledge, there has only been one study that investigated the intercellular compartmentation of Na^+ in the leaf of rice subjected to salinity (Yeo & Flowers 1984a). However, their results were presented as Na^+/K^+ ratio due to the problems of standardisation of X-ray microanalysis during that time. There is also no report on the ion distribution in cells of the leaf sheath in rice, although studies have indicated that Na^+ was accumulated in the leaf sheath more than leaf blade (Chapter 1, section 1.5.3). An available technique of single-cell-sap extraction with the silicone-oil-filled glass microcapillary, together with picolitre osmometry and X-ray microanalysis (Fricke *et al.* 1994a, b, c, 1995, 1996, 2006; Fricke 1997, 2004a, b; Fricke & Peters 2002; Knipfer & Fricke 2010) would be useful for the assessment of solute distribution in rice. In addition, with regard to climate change, it is also interesting to study the effect of temperature, radiation, flooding and drought on the magnitude of bypass flow of Na^+ in rice under combining conditions of salinity with such stresses. These studies will increase our understanding of salinity resistance and guarantee food supply for the world population as rice is the staple food for both present and future generations.

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APPENDICES

Appendix 2.1

Modified Yoshida culture solution

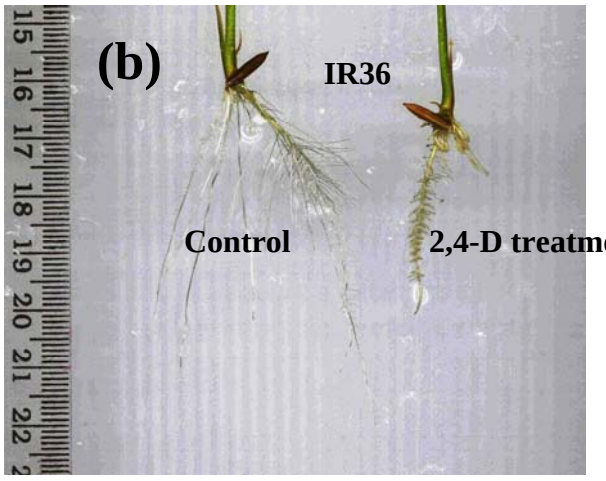
1.25 ml of stocks 1, 2, 4 and 5 were added to 1 litre of deionised water and the pH was adjusted to 4.5 using 1 N HNO_3. Then 1.25 ml of stocks 3, 6 and 7 were added to the solution and the pH was readjusted to 4.5 to produce the final culture solution.

Stock no.	Reagent	g/l	Concentration in stock (mM)	Concentration in culture (mM)
1	Ammonium nitrate (NH_4NO_4)	91.4	1131	1.4
2	di-Potassium sulphate (K_2SO_4)	71.4	410	0.51
3	Potassium di-hydrogen orthophosphate (KH_2PO_4)	46.2	339	0.42
	di-Potassium hydrogen orthophosphate (K_2HPO_4)	8.6	49	0.06
4	Calcium chloride ($CaCl_2.6H_2O$)	175	799	1.0
5	Magnesium sulphate ($MgSO_4.7H_2O$)	324	1315	1.6
6	Manganese chloride ($MnCl_2.4H_2O$)	1.5	7.6	0.01
	Ammonium molybdate $(NH_4)_6.Mo_7O_{24}.4H_2O$	0.074	0.06	0.00008
	Boric acid (H_3BO_3)	0.93	15.04	0.02
	Zinc sulphate ($ZnSO_4.7H_2O$)	0.035	0.12	0.0002
	Copper (II) sulphate ($CuSO_4.5H_2O$)	0.03	0.12	0.0002
7	Ethylenediaminetetra-acetic acid ferric monosodium salt (FeNaEDTA)	10.5	28.6	0.04

Recipe is modified from Yoshida S., Forno D.A., Cock J.H. & Gomez K.A. (1972) *Laboratory manual for physiological studies of rice.* International Rice Research Institute, Manila.

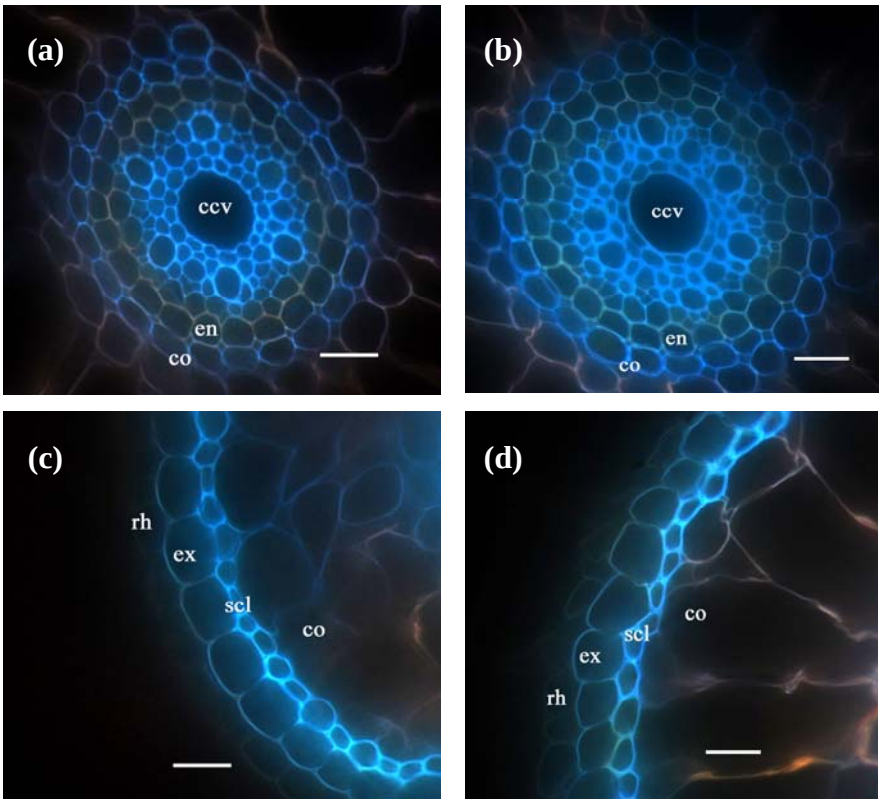
Appendix 2.2

Root systems of 25-day-old rice seedlings of Oochikara, *lrt1*, Nipponbare and *lrt2* (a), and 17-day-old seedlings of IR36 (b).



Appendix 2.3

Root anatomy of rice Taichung 65 and *crl1*



Freehand cross-sections of seminal roots of rice Taichung 65 (a, c) and *crl1* (b, d). Sections taken at a distance of 45 – 55 mm from the root tip of 20-day-old seedlings were stained for 1 h with 0.01% (w/v) Fluorol Yellow 088 to detect suberin lamellae and observed with an epifluorescence microscope (excitation filter G 365, chromatic beam splitter FT 395, barrier filter LP 420; Zeiss Axiophot, Oberkochen, Germany). Scale bars = 20 µm. ccv, central conducting vessel; co, cortex; en, endodermis; ex, exodermis; rh, rhizodermis; scl, sclerenchyma.

Appendix 2.4

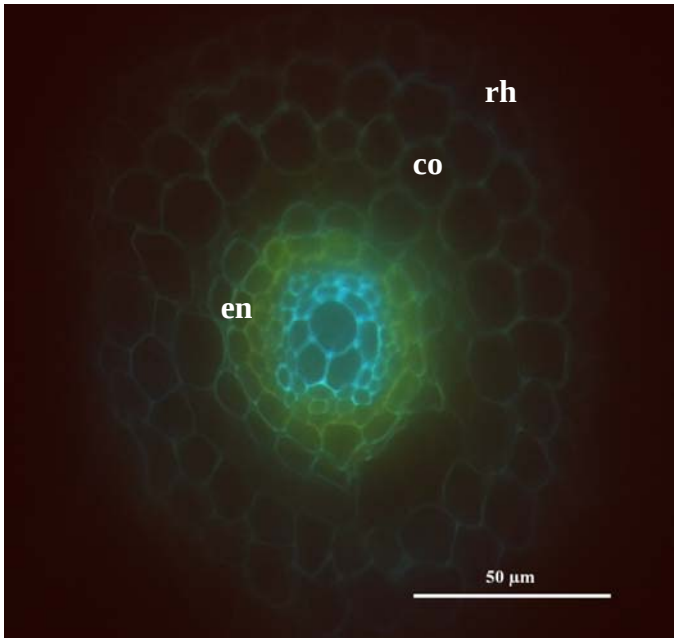
Average Na^+, K^+ concentrations and K^+/Na^+ ratio in shoots, and the quantity of Na^+ in phloem sap in leaves of rice seedlings

	Oochikara	*lrt1*	Nipponbare	*lrt2*	Taichung65	*crl1*	IR36					
							Control 1	Lateral root cut	Control 2	2-4-D treatment	Control 3	Crown root cut
Shoot Na^+ concentration (mmol/gDW)	0.22±0.01	0.17±0.01	0.22±0.01	0.17±0.01	0.21±0.02	0.17±0.02	0.72±0.05	1.13±0.07	0.67±0.05	1.16±0.12	0.60±0.03	0.62±0.05
Shoot K^+ concentration (mmol/gDW)	1.22±0.05	1.33±0.03	0.96±0.06	1.09±0.05	0.93±0.02	0.79±0.02	0.80±0.02	0.82±0.01	1.03±0.02	1.09±0.05	0.77±0.02	0.73±0.01
Shoot K^+/Na^+ ratio (a molar basis)	6.16±0.37	10.1±0.75	5.33±0.53	7.85±0.58	5.44±0.43	6.48±0.90	1.18±0.06	0.76±0.03	1.84±0.13	1.32±0.14	1.36±0.08	1.36±0.12
Na^+ in phloem sap of leaf 2 (μmol)	53.0±16.0	340±33.6	46.3±9.85	65.5±8.69	ND	ND	29.5±4.23	ND	ND	ND	ND	ND
Na^+ in phloem sap of leaf 3 (μmol)	21.8±10.5	144±35.6	40.9±10.7	42.2±6.18	63.6±17.8	52.0±16.7	27.2±3.39	ND	ND	ND	ND	ND
Na^+ in phloem sap of leaf 4 (μmol)	6.69±2.75	36.0±5.23	8.32±3.49	8.04±2.48	14.6±7.22	12.2±6.06	18.7±1.68	ND	ND	ND	ND	ND

Seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 72 h and harvested after a chase period of 48 h in culture solution. The quantity of Na^+ in phloem sap of leaves 2, 3 and 4 was collected in EDTA solutions. Means and standard errors (n = 10 -105). ND = not determined.

Appendix 3.1

Lateral root anatomy of rice cv. IR36



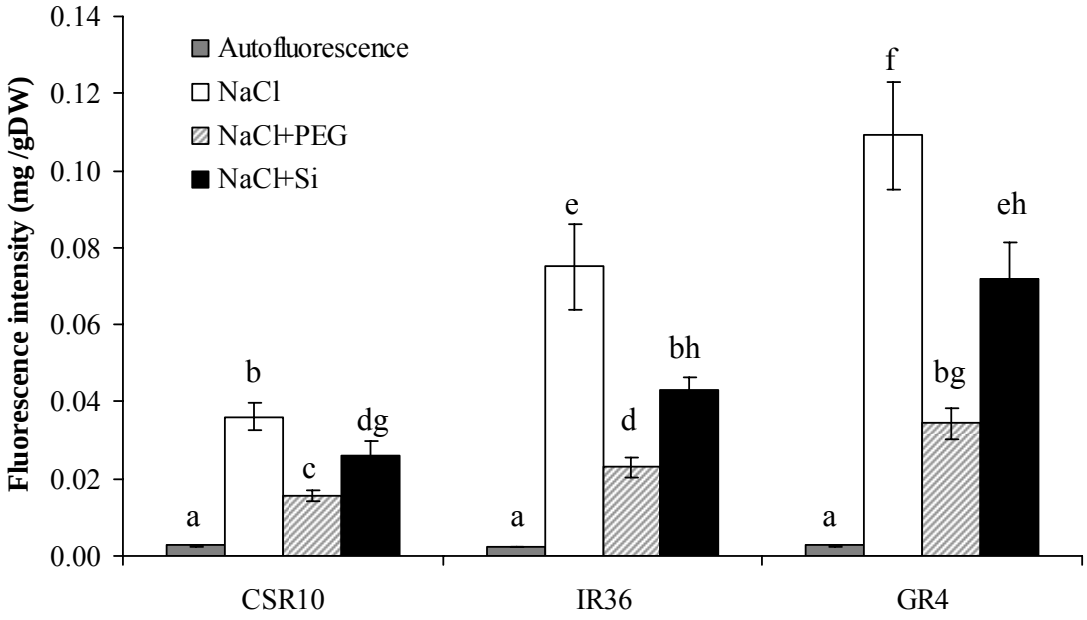
Freehand cross-sections of the lateral root of 14-day-old rice cv. IR36 in the region 45-55 mm from the root tip of the seminal root. The sections were stained for 1 h with 0.01% (w/v) Fluorol Yellow 088 to detect suberin lamellae and observed with an epifluorescence microscope using an ultraviolet filter set (excitation filter G 365, chromatic beam splitter FT 395, barrier filter LP 420; Zeiss Axiophot, Oberkochen, Germany). rh, rhizodermis; co, cortex; en, endodermis.

Appendix 4.1

The xylem-feeding insect *Philaenus spumarius* L.

The xylem-feeding insect *P. spumarius* L. (a) and *P. spumarius* caged in Eppendorf tubes whilst feeding on rice leaves (b). Figure (a) taken by Dr. P. Scott of the University of Sussex.

Appendix 4.2



The fluorescence intensity in the shoots of PTS-untreated seedlings (autofluorescence) of rice CSR10, IR36 and GR4 at excitation/emission wavelength used to estimate PTS compared with PTS fluorescence in the shoots when treated with 50 mM NaCl and 100 mg/l PTS (NaCl), 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500 (NaCl+PEG), or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si (NaCl+Si) for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 20).

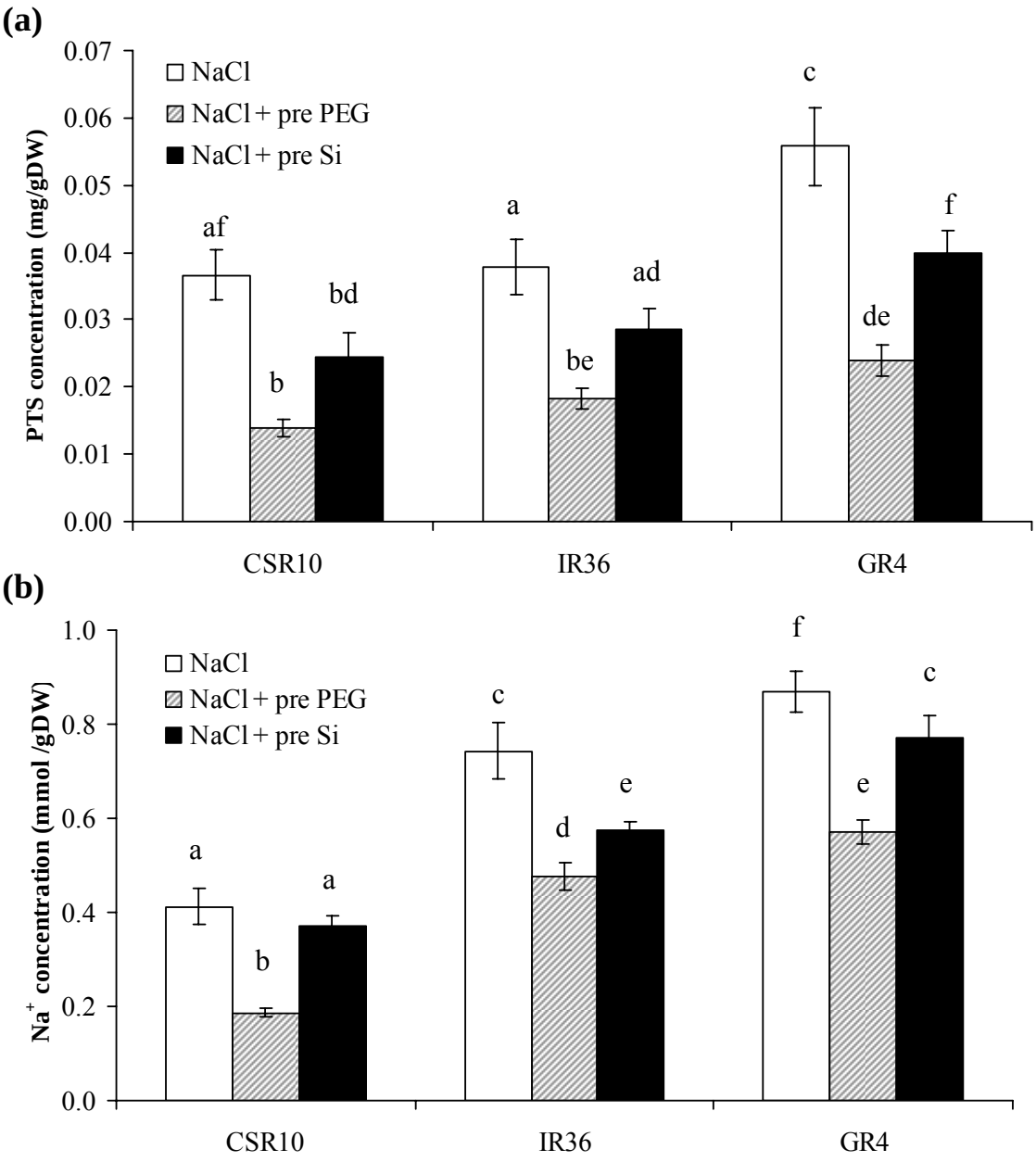
Appendix 4.3

Average shoot, root dry weight and Na^+, K^+ uptake in rice seedlings cvs. CSR10, IR36 and GR4

	CSR10			IR36			GR4		
	NaCl	NaCl+PEG	NaCl+Si	NaCl	NaCl+PEG	NaCl+Si	NaCl	NaCl+PEG	NaCl+Si
Shoot dry weight (mg)	63.8±1.66	63.3±2.31	68.7±2.70	65.0±1.73	65.6±1.77	69.3±1.83	64.9±2.90	64.2±3.17	68.3±3.24
Root dry weight (mg)	16.3±0.69	17.3±0.73	15.3±0.71	22.7±0.76	22.5±0.69	20.0±0.69	15.7±0.86	16.1±0.90	15.0±0.86
Na^+ concentration (mmol/gDW)	0.11±0.01	0.07±0.01	0.08±0.01	0.35±0.02	0.28±0.01	0.25±0.02	0.35±0.03	0.23±0.01	0.22±0.01
K^+ concentration (mmol/gDW)	0.99±0.02	1.06±0.01	0.92±0.01	1.00±0.02	1.09±0.01	0.98±0.01	0.88±0.03	0.94±0.02	0.88±0.02
K^+/Na^+ ratio (on a molar basis)	11.0±1.3	16.9±1.8	13.9±1.2	3.02±0.15	4.01±0.13	4.25±0.23	2.77±0.24	4.39±0.25	4.49±0.41
Na^+ content (µmol)	6.99±0.69	4.73±0.48	5.33±0.49	22.4±1.12	18.3±0.91	16.9±0.98	22.8±1.87	14.6±1.14	14.8±1.14
K^+ content (µmol)	62.7±1.80	66.9±2.18	63.2±2.28	65.1±2.09	71.5±2.07	67.9±1.57	56.1±1.84	60.3±3.10	60.4±3.16
Net Na^+ transport (µmol/g RDW/h)	2.94±0.25	1.84±0.14	2.25±0.15	6.91±0.33	5.65±0.19	5.98±0.39	10.2±0.77	6.32±0.35	6.81±0.37
Net K^+ transport (µmol/g RDW/h)	15.5±0.63	16.0±0.41	16.3±0.46	13.8±0.47	16.0±0.44	16.8±0.39	15.0±0.63	16.0±0.60	17.2±0.86
Na^+ bypass flow (µmol)	5.89±1.17	3.47±0.32	4.72±0.91	17.5±1.28	8.28±0.61	12.1±0.52	21.4±2.16	6.15±0.51	7.83±1.14

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS (NaCl), 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500 (NaCl+PEG), or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si (NaCl+Si) for 96 h and harvested after a chase period of 48 h in culture solution. Means and standard errors (n = 7-40).

Appendix 4.4



The effect of pre-treatment of PEG and Si on concentrations of PTS (a) and Na^+ (b) in the shoots of rice CSR10, IR36 and GR4. Seven-day-old seedlings were pre-treated with 10 g/l PEG 1500 or 3 mM Si for 7 d. Then 14-day-old seedlings were subjected to 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 30).

Appendix 4.5

Concentrations of Na^+, K^+ and PTS in the xylem sap of leaves 2 and 3 of rice cv. CSR10

Stress time		Na^+ concentration (mM)		K^+ concentration (mM)		PTS concentration (µM)		Number of samples (n)	
		Leaf 2	Leaf 3	Leaf 2	Leaf 3	Leaf 2	Leaf 3	Leaf 2	Leaf 3
24	NaCl	5.57±2.31a	1.82±0.37b	4.86±0.95ac	2.84±0.21b	0.09±0.03a	0.02±0.002bc	10	10
	NaCl+PEG	4.77±0.73a	1.39±0.12b	4.66±0.64ac	3.72±0.24c	0.06±0.02a	0.02±0.001b	9	11
	NaCl+Si	4.92±0.17a	1.69±0.17b	5.33±0.56a	3.62±0.63abc	0.07±0.01a	0.02±0.012c	6	6
48	NaCl	5.83±1.93ab	4.22±1.13ab	6.16±1.23abc	4.46±0.42b	0.13±0.02a	0.06±0.009bc	7	12
	NaCl+PEG	5.34±0.65a	2.39±0.30b	6.62±1.49abc	4.26±0.42b	0.07±0.02ab	0.05±0.007b	8	10
	NaCl+Si	5.62±0.78a	3.18±0.51b	6.86±1.12a	3.15±0.24c	0.08±0.01bc	0.08±0.007c	7	9
72	NaCl	4.17±0.58a	3.77±0.82ab	4.89±0.27a	4.24±0.38a	0.09±0.01a	0.08±0.017ab	13	9
	NaCl+PEG	3.15±0.68ab	2.74±0.49b	5.38±0.59a	4.18±0.53a	0.05±0.01bc	0.04±0.003c	7	8
	NaCl+Si	3.91±0.91ab	2.94±0.52ab	5.32±1.29a	4.03±0.40a	0.08±0.02abc	0.06±0.008bc	6	10
96	NaCl	8.74±1.83a	5.07±0.85abc	5.26±0.40a	3.83±0.13b	0.17±0.04a	0.13±0.021ab	8	8
	NaCl+PEG	3.79±0.87bc	3.63±0.69c	4.12±0.45ab	6.56±1.47ab	0.08±0.01bc	0.06±0.007c	6	8
	NaCl+Si	5.82±0.53b	4.10±0.55bc	5.81±0.87a	4.35±0.47ab	0.11±0.01a	0.08±0.004c	6	14

Fourteen-day-old rice seedlings were treated with 50 mM NaCl and 100 mg/l PTS, 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500, or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si for 96 h. The xylem sap was collected using the xylem-feeding insect *Philaenus spumarius* at 24, 48, 72 and 96 h after salt stress. Statistical comparison was made among leaves and treatments (2x3). Values followed by the same letters are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n= 6-14).

Appendix 4.6

Concentrations of Na^+, K^+ and PTS in the xylem sap of leaves 2 and 3 of rice cv. IR36

Stress time		Na^+ concentration (mM)		K^+ concentration (mM)		PTS concentration (µM)		Number of samples (n)	
		Leaf 2	Leaf 3	Leaf 2	Leaf 3	Leaf 2	Leaf 3	Leaf 2	Leaf 3
24	NaCl	7.31±1.55a	5.02±0.71ac	6.03±0.98ac	5.45±0.70ac	0.18±0.04ac	0.11±0.027ac	8	6
	NaCl+PEG	4.56±0.77abc	2.85±0.41b	6.04±1.76ac	6.44±0.63a	0.11±0.01a	0.05±0.014b	7	7
	NaCl+Si	6.07±0.89ac	4.00±0.46bc	2.64±0.38bc	3.93±0.49c	0.15±0.03ac	0.16±0.019c	6	7
48	NaCl	9.72±1.58a	13.6±2.68a	4.23±0.51a	5.16±0.82a	0.24±0.05ab	0.17±0.036abc	8	5
	NaCl+PEG	7.29±1.29a	7.69±2.03a	4.64±0.88a	5.55±0.85a	0.14±0.03ac	0.09±0.019c	7	6
	NaCl+Si	8.82±1.42a	12.8±3.23a	4.32±0.36a	6.29±1.05a	0.21±0.04b	0.17±0.039abc	6	8
72	NaCl	18.6±2.17a	16.5±3.48ac	6.86±0.90ab	7.33±0.49b	0.42±0.16ab	0.25±0.038a	6	8
	NaCl+PEG	12.8±3.84ab	9.05±2.88bc	7.32±1.68abc	6.28±1.26ac	0.20±0.06ab	0.13±0.021b	6	6
	NaCl+Si	8.16±2.73b	7.59±1.58bc	5.20±1.89ac	4.54±0.45c	0.26±0.11ab	0.13±0.019b	6	9
96	NaCl	35.9±5.73a	18.9±3.08c	9.38±0.63a	7.56±0.20ac	1.20±0.23a	0.28±0.035b	6	8
	NaCl+PEG	9.69±1.81b	6.86±2.06b	6.52±0.74bc	5.20±0.59b	0.18±0.06bc	0.13±0.051c	6	6
	NaCl+Si	7.41±2.36b	9.83±3.68b	5.20±1.21b	5.02±1.17b	0.28±0.09b	0.14±0.018c	6	10

Fourteen-day-old rice seedlings were treated with 50 mM NaCl and 100 mg/l PTS, 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500, or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si for 96 h. The xylem sap was collected using the xylem-feeding insect *Philaenus spumarius* at 24, 48, 72 and 96 h after salt stress. Statistical comparison was made among leaves and treatments (2x3). Values followed by the same letters are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n= 6-10).

Appendix 4.7

Concentrations of Na^+, K^+ and PTS in the xylem sap of leaves 2 and 3 of rice cv. GR4

Stress time		Na^+ concentration (mM)		K^+ concentration (mM)		PTS concentration (µM)		Number of samples (n)	
		Leaf 2	Leaf 3	Leaf 2	Leaf 3	Leaf 2	Leaf 3	Leaf 2	Leaf 3
24	NaCl	5.68±1.14a	1.42±0.12b	4.93±0.24a	2.75±0.22c	0.05±0.01ad	0.03±0.005b	9	9
	NaCl+PEG	3.70±1.20ac	1.39±0.09b	4.61±0.87ace	1.63±0.12d	0.04±0.01ab	0.02±0.002c	5	10
	NaCl+Si	6.12±0.96a	2.22±0.16c	7.42±0.70b	3.71±0.23e	0.09±0.02a	0.04±0.007bd	5	8
48	NaCl	12.0±1.10a	7.86±3.51ab	7.20±0.79a	3.43±0.54bc	0.15±0.03ab	0.10±0.063ab	6	5
	NaCl+PEG	5.44±0.88b	2.46±0.32c	4.51±0.58ab	2.93±0.14c	0.09±0.02ac	0.05±0.012c	7	7
	NaCl+Si	6.26±0.18b	2.65±0.36c	5.33±0.37a	2.84±0.27c	0.16±0.07b	0.11±0.016ac	6	8
72	NaCl	14.0±2.51a	11.0±2.27a	5.30±0.54a	6.40±0.71a	0.65±0.08a	0.10±0.010b	5	7
	NaCl+PEG	7.76±2.28a	2.65±0.55b	3.59±0.48b	3.41±0.41b	0.16±0.03bc	0.09±0.023c	7	6
	NaCl+Si	9.38±2.77a	1.84±0.25b	3.95±0.74ab	2.60±0.22b	0.20±0.03b	0.14±0.015bc	7	7
96	NaCl	13.4±3.60ac	21.5±4.65c	2.60±1.06ab	8.70±1.68b	0.84±0.10a	0.69±0.120a	6	7
	NaCl+PEG	4.50±0.53b	5.66±1.80ab	3.52±0.81a	5.31±0.61ab	0.28±0.05bc	0.17±0.023c	7	6
	NaCl+Si	6.02±0.92b	10.0±2.58ab	4.06±1.17a	4.45±0.72a	0.31±0.02b	0.21±0.016c	8	12

Fourteen-day-old rice seedlings were treated with 50 mM NaCl and 100 mg/l PTS, 50 mM NaCl and 100 mg/l PTS plus 10 g/l PEG 1500, or 50 mM NaCl and 100 mg/l PTS plus 3 mM Si for 96 h. The xylem sap was collected using the xylem-feeding insect *Philaenus spumarius* at 24, 48, 72 and 96 h after salt stress. Statistical comparison was made among leaves and treatments (2x3). Values followed by the same letters are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n= 5-12).

Appendix 5.1

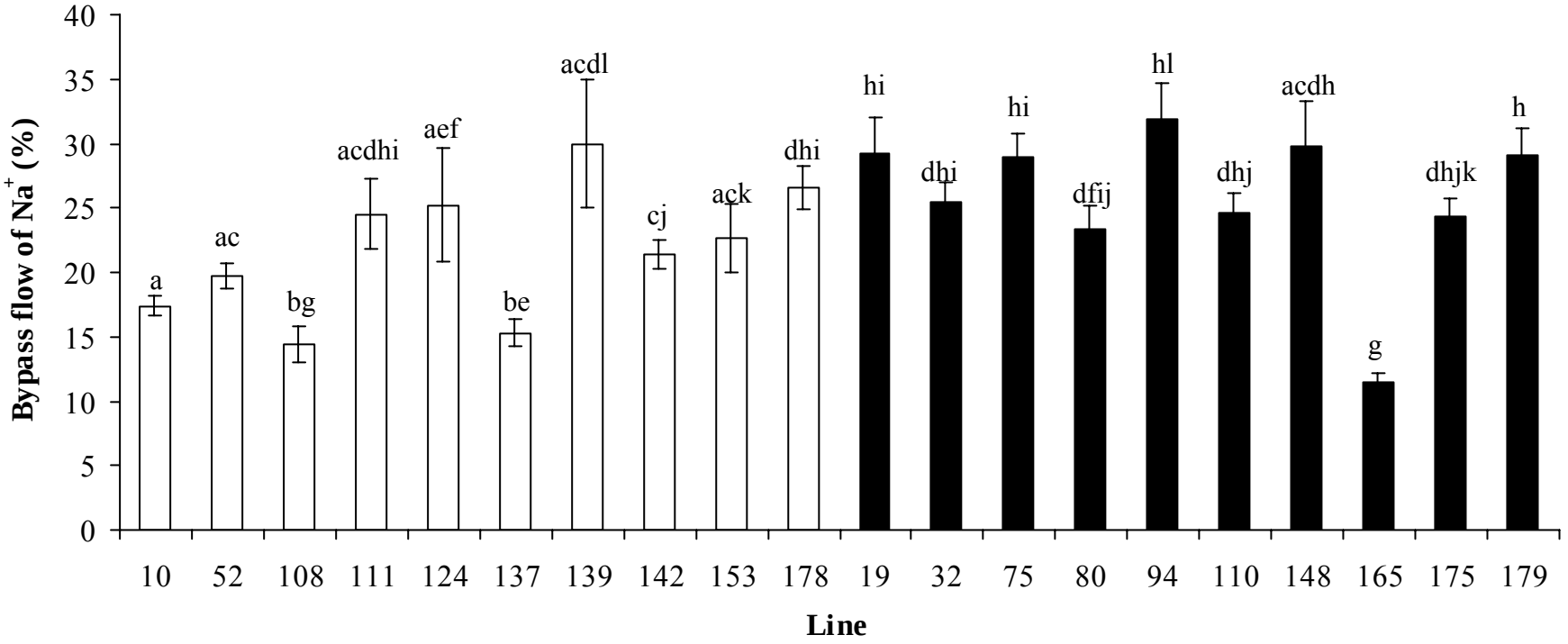
Average Na^+, K^+ uptake, transpiration and bypass flow of rice IR55178

lines	Na^+ concentration (mmol/gDW)	K^+ concentration (mmol/gDW)	K^+/Na^+ ratio (on a molar basis)	Transpiration (ml)	Transpiration (% control)	Bypass flow (%)	Apparent Na^+ in xylem (mM)	Apparent PTS in xylem (μM)	Na^+ bypass flow (%)
10	0.27±0.01	0.65±0.02	2.47±0.08	10.6±0.36	76.0±2.61	0.69±0.04	2.01±0.10	0.17±0.01	17.4±0.81
52	0.28±0.02	0.77±0.01	3.20±0.20	7.98±0.42	61.0±3.19	0.86±0.06	2.14±0.14	0.22±0.01	19.7±1.01
108	0.47±0.02	0.62±0.01	1.39±0.05	6.48±0.42	66.0±4.29	1.55±0.12	6.91±0.82	0.52±0.06	14.4±1.42
111	0.52±0.05	0.47±0.04	1.54±0.24	5.95±0.70	79.0±9.31	1.99±0.20	9.37±1.85	0.68±0.12	24.5±2.76
124	0.41±0.01	0.66±0.01	1.70±0.06	11.6±0.69	95.5±5.69	1.19±0.16	2.63±0.15	0.30±0.04	25.2±4.36
137	0.34±0.02	0.62±0.02	1.92±0.08	5.71±0.44	36.3±2.80	1.31±0.18	4.21±0.52	0.33±0.04	15.3±1.01
139	0.33±0.03	0.63±0.01	2.25±0.13	10.3±0.41	65.4±2.60	1.23±0.18	2.41±0.52	0.37±0.07	30.0±4.99
142	0.49±0.05	0.52±0.03	1.94±0.26	9.01±0.63	87.5±6.13	1.65±0.25	4.52±0.78	0.42±0.06	21.3±1.11
153	0.51±0.02	0.65±0.01	1.37±0.08	6.07±0.38	46.7±2.95	2.04±0.15	5.98±0.68	0.44±0.05	22.7±2.67
178	0.30±0.02	0.70±0.01	2.62±0.15	10.1±0.46	86.5±3.96	1.17±0.13	2.09±0.13	0.29±0.03	26.6±1.69
19	0.35±0.04	0.58±0.02	2.18±0.25	9.36±0.86	142±13.1	2.70±1.16	3.78±0.92	0.68±0.29	29.3±2.77
32	0.66±0.08	0.78±0.02	1.74±0.15	9.78±0.84	97.3±8.36	4.70±1.15	10.8±3.16	1.43±0.47	25.4±1.62
75	0.56±0.04	0.56±0.02	1.16±0.08	9.73±0.60	117±7.26	2.71±0.34	4.58±0.50	0.68±0.09	29.0±1.75
80	0.56±0.03	0.72±0.02	1.43±0.08	6.86±0.38	85.4±4.74	2.75±0.44	5.79±0.62	0.69±0.11	23.3±1.85
94	0.59±0.07	0.54±0.05	1.36±0.18	7.11±0.78	105±11.5	4.03±0.65	15.4±6.67	1.23±0.26	31.8±2.91
110	0.65±0.06	0.48±0.04	1.15±0.14	7.41±0.80	98.2±10.6	5.47±1.08	11.2±2.27	1.38±0.27	24.7±1.45
148	0.59±0.02	0.70±0.01	1.27±0.08	5.75±0.63	132±14.4	2.50±0.29	4.94±0.45	0.63±0.07	29.8±3.51
165	0.62±0.02	0.73±0.01	1.23±0.05	11.1±0.69	74.3±4.57	2.90±0.57	11.6±2.15	0.73±0.14	11.4±0.70
175	0.46±0.04	0.62±0.05	1.64±0.16	10.8±0.79	120±8.69	2.25±0.52	4.08±0.84	0.57±0.13	24.3±1.41
179	0.40±0.03	0.59±0.01	1.85±0.14	8.72±0.51	102±6.04	1.89±0.33	3.45±0.46	0.48±0.08	29.1±2.06

Fourteen-day-old seedlings of 10 low-Na^+-transporting lines (white rows) and 10 high-Na^+-transporting lines (gray rows) of rice IR55178 were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Means and standard errors (n = 40).

Appendix 5.2

Bypass flow of Na^+



The bypass flow of Na^+ compared with total Na^+ accumulated in the shoots of 10 low- Na^+-transporting lines (white bars) and 10 high-Na^+-transporting lines (black bars) of rice seedlings IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h and harvested after a chase period of 48 h in culture solution. Bars with the same letters are not significantly different at $P < 0.05$ according to the Mann-Whitney test. Means and standard errors (n = 40).

Appendix 5.3

Na^+ concentration in the xylem sap of leaf 2 of low-Na^+-transporting lines (10, 52 and 178) and high-Na^+-transporting lines (32, 94 and 110) of rice IR55178.

Lines	Na^+ concentration (mM)					Number of sample (n)				
	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
10	0.97±0.07a	3.84±0.84a	5.51±0.96a	4.04±0.51a	9.74±1.85ac	5	15	14	11	8
52	0.47±0.10b	1.79±0.37b	2.85±0.43bc	8.29±1.88ab	8.38±1.97abc	10	18	20	15	10
178	0.44±0.04b	1.75±0.39b	2.90±0.81b	6.92±0.97b	6.56±1.24ab	7	18	16	18	17
32	1.21±0.06ac	3.63±0.52a	4.43±0.67ac	3.41±0.21a	5.74±1.18b	6	23	19	11	19
94	1.25±0.01c	2.05±0.56ab	2.71±0.68ab	5.88±1.31ab	11.6±2.21c	3	9	6	12	8
110	0.34±0.06b	2.47±0.69ab	4.21±0.94ab	3.75±0.66a	11.2±1.60c	7	15	15	13	13

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius* at 0, 24, 48, 72 and 96 h after salt stress. Values followed by the same letters in the same column are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 3-23).

Appendix 5.4

Na^+ concentration in the xylem sap of leaf 3 of low-Na^+-transporting lines (10, 52 and 178) and high-Na^+-transporting lines (32, 94 and 110) of rice IR55178.

Lines	Na^+ concentration (mM)					Number of sample (n)				
	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
10	0.37±0.03a	2.21±0.50ab	4.19±0.38a	4.43±0.91a	9.61±1.77a	8	20	25	9	11
52	0.53±0.05b	2.23±0.58ab	2.62±0.66b	4.07±0.74ab	8.98±1.85a	12	24	19	18	14
178	0.39±0.05ab	1.65±0.50ac	3.30±1.18bc	2.91±0.76b	6.08±0.92a	15	16	14	15	23
32	0.96±0.17c	2.97±0.83b	4.59±0.51a	5.06±0.59a	7.29±0.77a	6	13	19	14	10
94	0.37±0.05a	1.45±0.74c	1.22±0.27c	5.49±1.06a	7.90±1.16a	3	16	22	18	11
110	0.82±0.04c	1.88±0.34ab	2.50±0.73bc	2.88±0.72b	7.20±1.78a	6	17	18	13	13

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius* at 0, 24, 48, 72 and 96 h after salt stress. Values followed by the same letters in the same column are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 3-25).

Appendix 5.5

PTS concentration in the xylem sap of leaf 2 of low-Na^+-transporting lines (10, 52 and 178) and high-Na^+-transporting lines (32, 94 and 110) of rice IR55178.

Lines	PTS concentration (nM)					Number of sample (n)				
	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
10	0.76±0.08a	3.72±1.16a	6.74±2.90ab	3.82±0.94ac	8.24±1.60ac	5	15	14	11	8
52	0.46±0.10ab	2.87±0.75a	5.13±0.68a	13.2±3.49b	15.4±2.82ab	10	18	20	15	10
178	0.47±0.06b	2.25±0.54bc	3.65±1.04ab	7.60±0.91b	9.77±1.31a	7	18	16	18	17
32	1.41±0.21c	1.18±0.11b	2.61±0.39b	8.96±2.36ab	17.0±2.16b	6	23	19	11	19
94	1.77±0.01c	2.18±0.55ac	3.65±0.70ab	14.1±3.23b	20.8±5.47b	3	9	6	12	8
110	0.76±0.02a	1.82±0.30ac	2.55±0.33b	2.70±0.32c	6.57±1.17c	7	15	15	13	13

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius* at 0, 24, 48, 72 and 96 h after salt stress. Values followed by the same letters in the same column are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 3-23).

Appendix 5.6

PTS concentration in the xylem sap of leaf 3 of low-Na^+-transporting lines (10, 52 and 178) and high-Na^+-transporting lines (32, 94 and 110) of rice IR55178.

Lines	PTS concentration (nM)					Number of sample (n)				
	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
10	0.37±0.02a	2.88±1.09a	4.12±1.15ac	3.57±0.77a	4.85±0.72a	8	20	25	9	11
52	1.32±0.56acd	2.18±0.50b	1.90±0.37b	4.78±0.85a	7.81±0.77b	12	24	19	18	14
178	0.41±0.05a	3.19±1.24ab	4.87±1.78ab	2.92±0.34a	5.97±0.50a	15	16	14	15	23
32	0.98±0.01b	1.97±0.54b	3.49±0.78c	3.86±0.46a	9.30±1.14b	6	13	19	14	10
94	0.65±0.04c	1.23±0.08b	2.07±0.19ab	4.08±0.58a	6.19±1.34ab	3	16	22	18	11
110	0.91±0.15d	1.52±0.28b	1.95±0.10a	1.51±0.12b	6.21±0.80ab	6	17	18	13	13

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius* at 0, 24, 48, 72 and 96 h after salt stress. Values followed by the same letters in the same column are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 3-25).

Appendix 5.7

K^+ concentration in the xylem sap of leaf 2 of low-Na^+-transporting lines (10, 52 and 178) and high-Na^+-transporting lines (32, 94 and 110) of rice IR55178.

Lines	K^+ concentration (mM)					Number of sample (n)				
	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
10	4.26±0.50a	3.60±0.38abcd	4.19±0.39ac	3.66±0.16ac	6.13±0.71ab	5	15	14	11	8
52	2.89±0.24b	3.77±0.37ac	3.88±0.35abc	6.30±0.85ab	3.73±1.24ab	10	18	20	15	10
178	4.04±0.20a	4.24±0.48ab	4.52±0.55a	6.17±0.63b	6.89±0.71a	7	18	16	18	17
32	4.18±0.28a	3.27±0.30cd	3.43±0.26bc	3.82±0.44c	5.41±0.81b	6	23	19	11	19
94	6.83±0.01c	5.10±0.52b	3.88±0.49abc	4.93±0.54ab	10.1±2.37a	3	9	6	12	8
110	3.52±0.37ab	4.33±0.80d	3.38±0.36b	2.750.24±d	7.16±0.77a	7	15	15	13	13

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius* at 0, 24, 48, 72 and 96 h after salt stress. Values followed by the same letters in the same column are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 3-23).

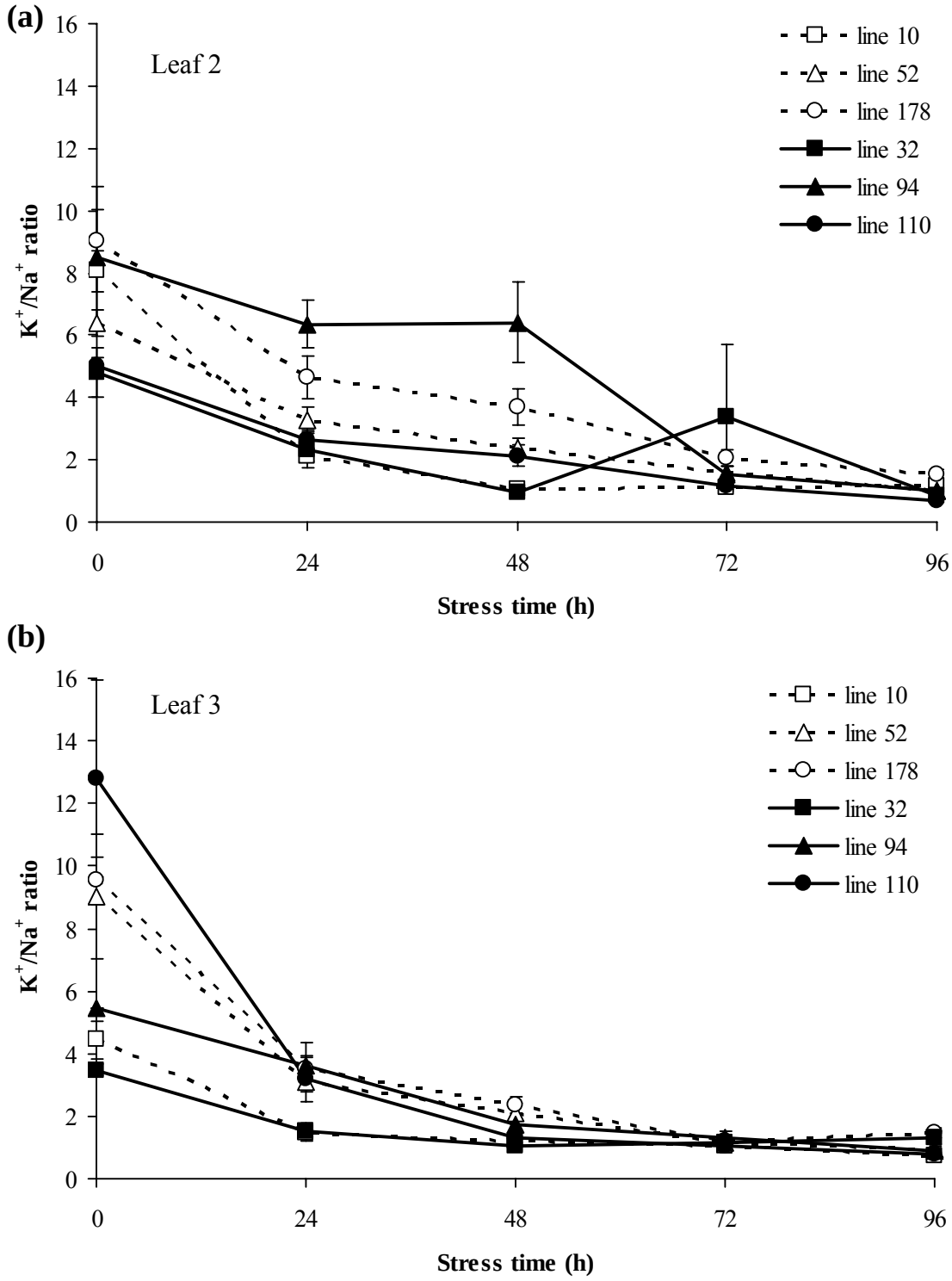
Appendix 5.8

K^+ concentration in the xylem sap of leaf 3 of low-Na^+-transporting lines (10, 52 and 178) and high-Na^+-transporting lines (32, 94 and 110) of rice IR55178.

Lines	K^+ concentration (mM)					Number of sample (n)				
	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
10	2.86±0.14a	3.28±0.27a	3.78±0.13a	4.13±0.28ab	8.24±0.93a	8	20	25	9	11
52	3.18±0.14a	4.19±0.51b	3.89±0.59b	4.32±0.29ab	7.87±1.49a	12	24	19	18	14
178	2.82±0.14a	4.36±0.59b	5.18±0.94ac	3.99±0.40a	6.43±0.48a	15	16	14	15	23
32	4.03±0.31b	3.36±0.28ab	3.60±0.22ab	4.99±0.34b	5.88±0.63a	6	13	19	14	10
94	2.96±0.44ab	3.75±0.14b	3.37±0.25bc	5.62±0.57ab	6.59±0.45a	3	16	22	18	11
110	4.05±0.18b	3.59±0.23b	3.11±0.39d	2.88±0.57c	3.96±0.66b	6	17	18	13	13

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius* at 0, 24, 48, 72 and 96 h after salt stress. Values followed by the same letters in the same column are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 3-25).

Appendix 5.9



K^+/Na^+ ratio on a molar basis in the xylem sap of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (broken graphs) and 3 high-Na^+-transporting lines (solid graphs) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS. The xylem sap was collected using the xylem feeding insect *Philaenus spumarius*. Means and standard errors (n = 3-25).

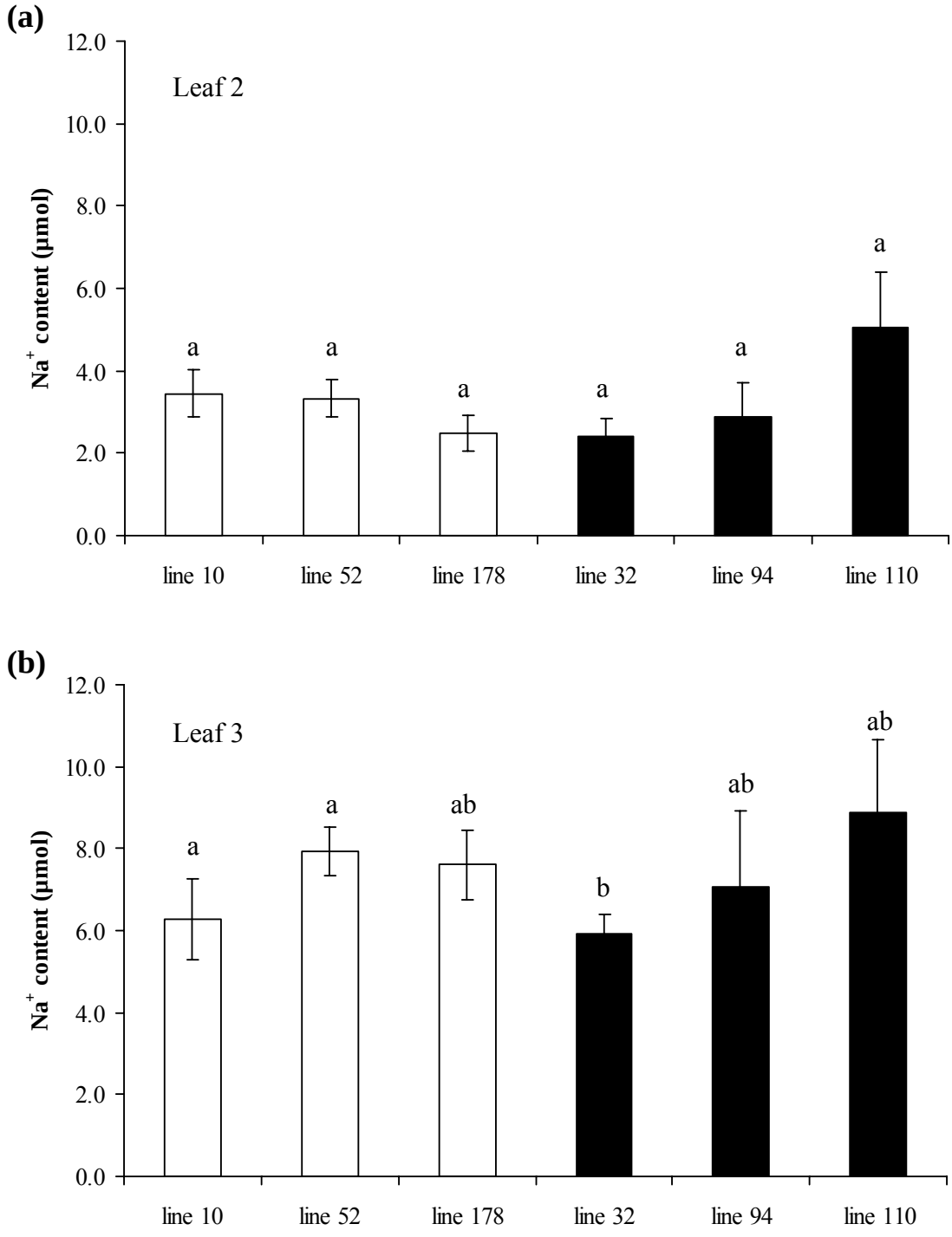
Appendix 5.10

Average Na^+, K^+ and PTS concentrations, and the quantity of Na^+ in phloem sap in leaves 2 and 3 of rice IR55178

	Line 10	Line 52	Line 178	Line 32	Line 94	Line 110
Na^+ concentration in L2 blade (mmol/gDW)	0.41±0.06	0.48±0.07	0.30±0.06	0.37±0.06	0.37±0.09	0.43±0.09
Na^+ concentration in L3 blade (mmol/gDW)	0.46±0.06	0.66±0.04	0.58±0.07	0.58±0.05	0.73±0.19	0.56±0.11
K^+ concentration in L2 blade (mmol/gDW)	0.54±0.01	0.63±0.04	0.53±0.02	0.63±0.07	0.62±0.04	0.57±0.04
K^+ concentration in L3 blade (mmol/gDW)	0.49±0.02	0.64±0.03	0.53±0.04	0.52±0.03	0.63±0.03	0.43±0.02
PTS concentration in L2 blade (µg/gDW)	9.99±2.06	7.45±1.67	7.58±0.58	16.5±6.18	12.3±3.64	15.8±9.34
PTS concentration in L3 blade (µg/gDW)	8.29±0.83	6.32±1.35	4.29±0.12	6.36±1.73	8.14±2.69	5.63±1.05
Na^+ concentration in L2 sheath (mmol/gDW)	0.46±0.07	0.50±0.04	0.47±0.05	0.61±0.04	1.33±0.29	0.63±0.07
Na^+ concentration in L3 sheath (mmol/gDW)	0.32±0.04	0.40±0.06	0.40±0.03	0.48±0.08	0.87±0.13	0.58±0.11
PTS concentration in L2 sheath (µg/gDW)	21.3±4.61	26.6±3.74	27.0±4.42	32.9±6.15	100±24.1	54.0±9.48
PTS concentration in L3 sheath (µg/gDW)	14.2±1.83	13.6±2.05	11.9±1.42	8.98±2.08	20.3±6.14	9.01±1.46
K^+ concentration in L2 sheath (mmol/gDW)	0.30±0.02	0.35±0.01	0.25±0.02	0.25±0.03	0.19±0.02	0.20±0.03
K^+ concentration in L3 sheath (mmol/gDW)	0.18±0.01	0.22±0.02	0.18±0.01	0.22±0.03	0.13±0.03	0.16±0.02
Na^+ in phloem sap of L2 (µmol)	116±25.6	60.0±10.0	40.0±10.0	94.09±33.6	90.0±30.0	40.0±20.0
Na^+ in phloem sap of L3 (µmol)	35.9±8.82	20.0±10.0	40.0±10.0	57.6±13.8	33.5±11.4	10.0±3.00

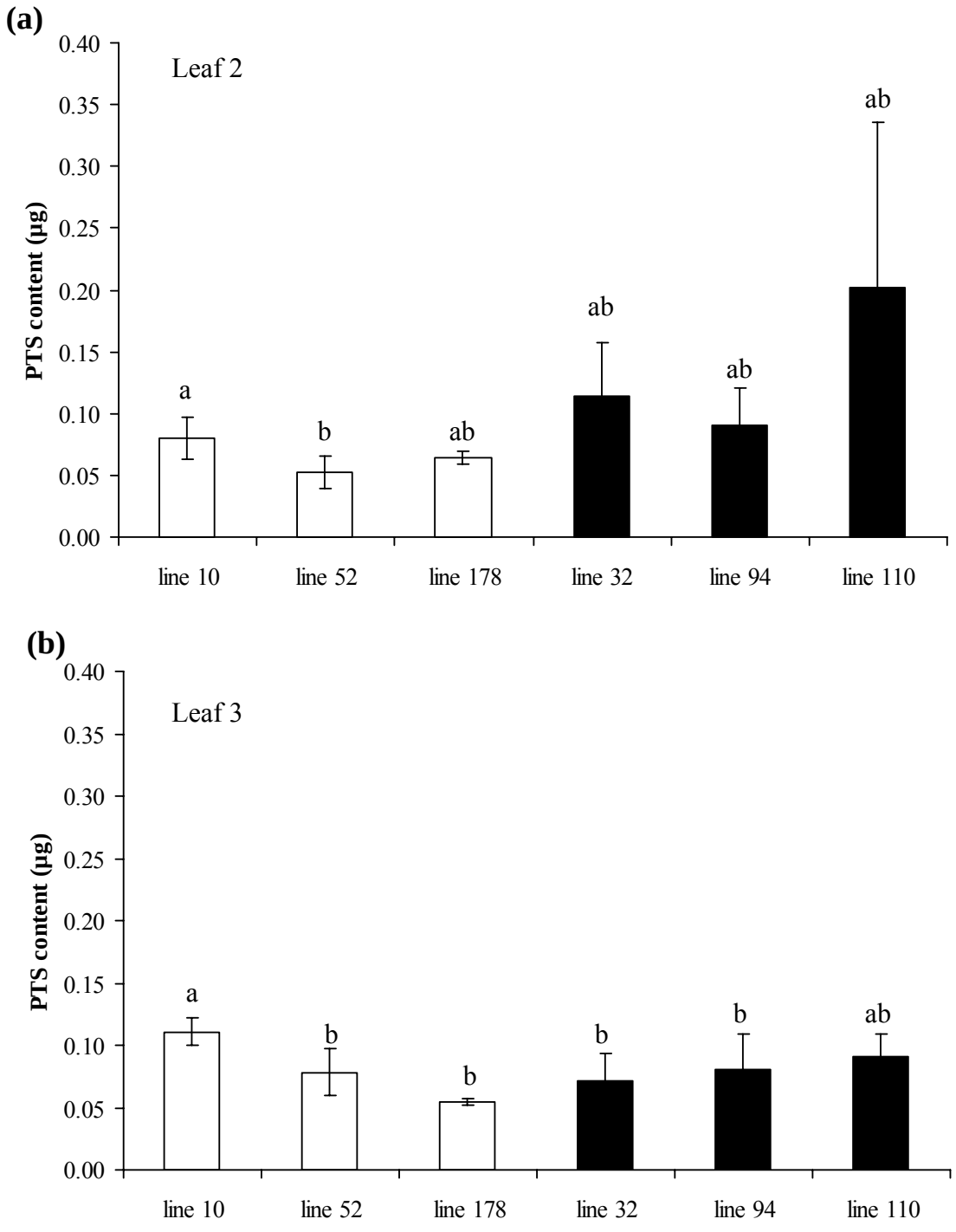
Fourteen-day-old seedlings of rice IR55178 were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Means and standard errors (n = 10-20).

Appendix 5.11



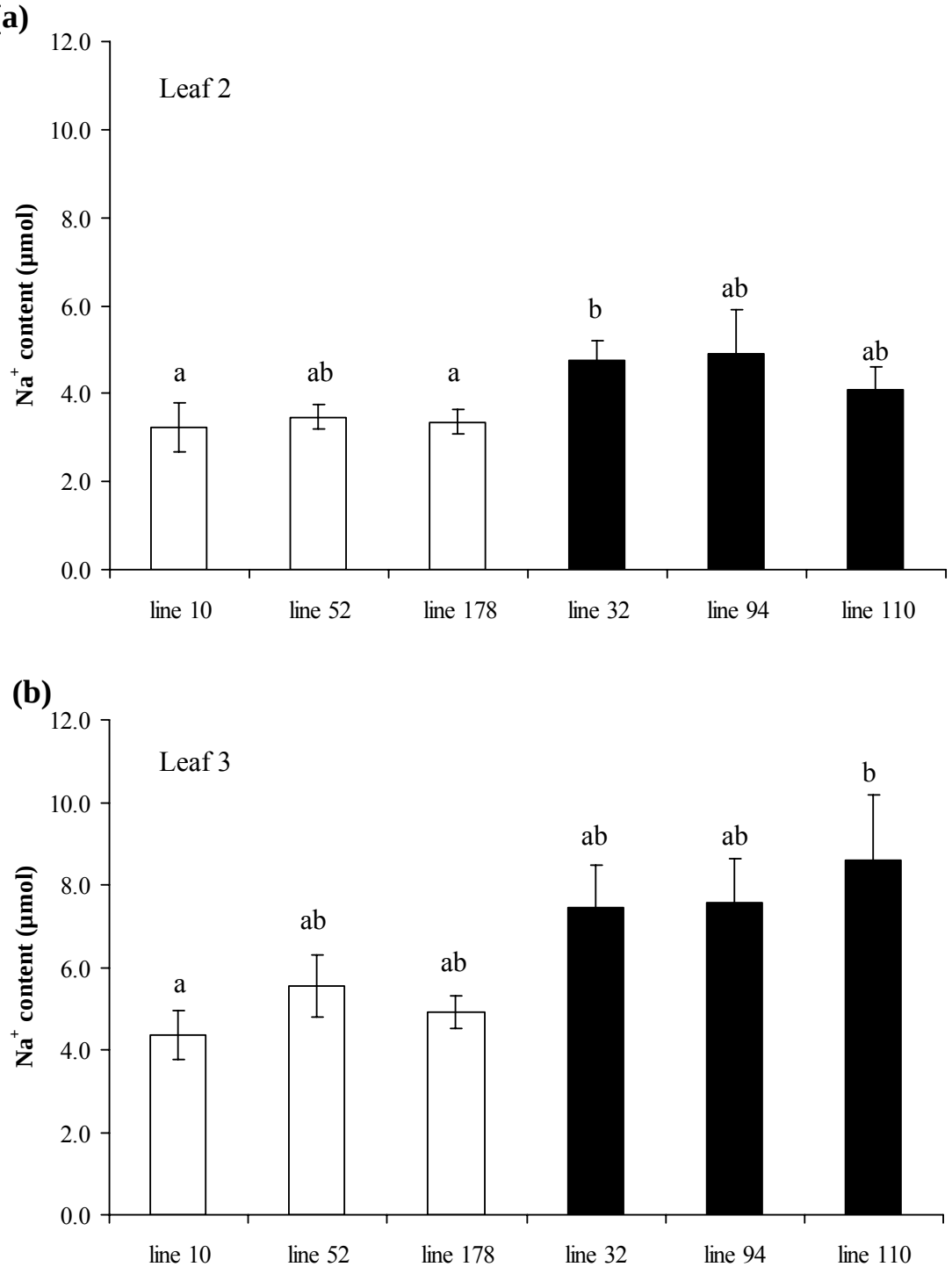
Na^+ contents in leaf blades of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

Appendix 5.12



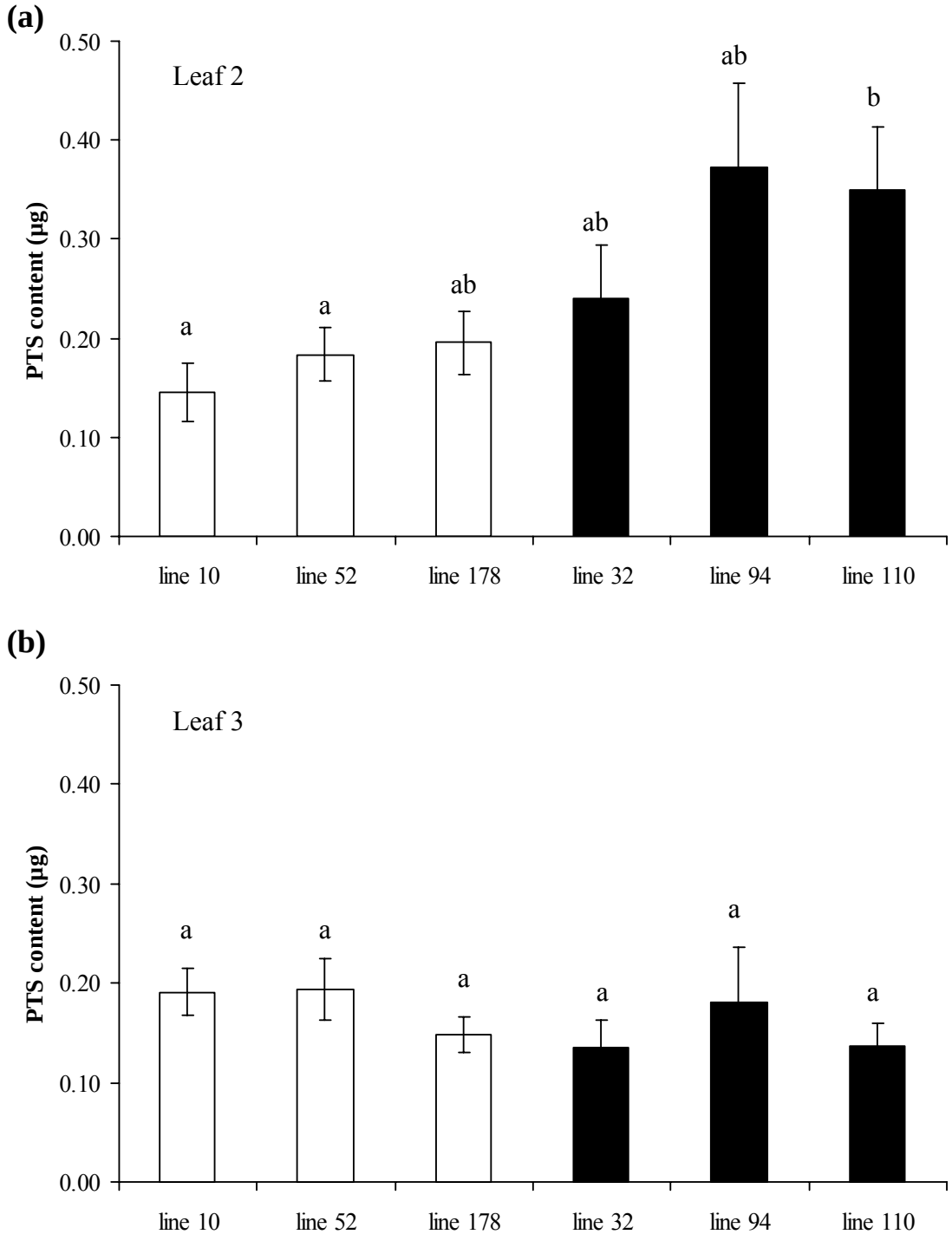
PTS contents in leaf blades of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

Appendix 5.13



Na^+ contents in leaf sheaths of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

Appendix 5.14



PTS contents in leaf sheaths of leaves 2 (a) and 3 (b) of 3 low-Na^+-transporting lines (open bars) and 3 high-Na^+-transporting lines (closed bars) of rice IR55178. Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Bars with the same letters are not significantly different at P <0.05 according to the Mann-Whitney test. Means and standard errors (n = 10).

Appendix 5.15

Average leaf Na^+ and PTS content and dry weight of leaves 2 and 3 of rice IR55178

	Line 10	Line 52	Line 178	Line 32	Line 94	Line 110
Shoot Na^+ content (µmol)	20.4±0.7a	15.9±0.8b	19.7±0.9a	38.9±3.0c	25.8±1.7d	33.9±2.0c
Na^+ content in L2 sheath (µmol)	3.2±0.6a	3.5±0.3ab	3.4±0.3a	4.8±0.4b	4.9±1.0ab	4.1±0.5ab
Na^+ content in L2 blade (µmol)	3.5±0.6a	3.3±0.5a	2.5±0.4a	2.4±0.4a	2.9±0.8a	5.0±1.4a
Na^+ content in L3 sheath (µmol)	4.4±0.6a	5.6±0.7ab	4.9±0.4ab	7.4±1.0ab	7.6±1.1ab	8.6±1.6b
Na^+ content in L3 blade (µmol)	6.3±1.0a	7.9±0.6a	7.6±0.8ab	5.9±0.5b	7.1±1.9ab	8.9±1.8ab
Total Na^+ content in leaf sheath (µmol)	7.6±0.6a	9.0±0.7ac	8.3±0.4a	12.2±0.9b	12.5±1.3b	12.7±1.7bc
Total Na^+ in leaf sheath /shoot Na^+ content (%)	37.2	56.9	42.0	31.4	48.4	37.5
PTS content in L2 sheath (µg)	0.15±0.03 a	0.18±0.03 a	0.20±0.03 ab	0.24±0.05 ab	0.37±0.09 ab	0.35±0.06 b
PTS content in L2 blade (µg)	0.08±0.02 a	0.05±0.01 b	0.06±0.01 ab	0.11±0.04 ab	0.09±0.03 ab	0.20±0.13 ab
PTS content in L3 sheath (µg)	0.19±0.02 a	0.19±0.03 a	0.15±0.02 a	0.14±0.03 a	0.18±0.06 a	0.14±0.02 a
PTS content in L3 blade (µg)	0.11±0.01 a	0.08±0.02 b	0.05±0.00 b	0.07±0.02 b	0.08±0.03 b	0.09±0.02 ab
L2 sheath DW (mg)	7.0±0.3a	6.9±0.2a	7.4±0.3a	7.8±0.5a	3.9±0.2a	6.7±0.5a
L2 blade DW (mg)	8.0±0.3ac	6.9±0.3b	8.4±0.3a	6.6±0.3b	7.1±0.3bc	10.9±0.5d
Total L2 DW (mg)	15.0±0.4ac	13.7±0.4b	15.8±0.5ae	14.3±0.5bc	11.0±0.4d	17.6±0.7e
L2 sheath DW/L2DW (%)	46.6	50.1	46.7	54.3	35.2	37.9
L3 sheath DW (mg)	13.5±0.5ab	14.3±0.8a	12.3±0.3b	16.3±1.1a	8.7±0.2c	15.1±0.6a
L3 blade DW (mg)	13.5±0.4a	12.1±0.6ab	13.1±0.3a	10.4±0.5bc	9.3±0.3c	15.8±0.3d
Total L3 DW (mg)	27.0±0.8a	26.4±1.0a	25.4±0.4a	26.7±1.2a	18.1±0.5b	31.0±0.7c
L3 sheath DW/L3DW (%)	50.1	54.1	48.4	61.0	48.3	48.9

Fourteen-day-old seedlings were treated with 50 mM NaCl and 100 mg/l PTS for 96 h. Values followed by the same letters within a row are not significantly different at $P<0.05$ according to the Mann-Whitney test. Means and standard errors (n = 10-40).