Sodium chloride decreases cadmium accumulation and changes the response of metabolites to cadmium stress in halophyte *Carpobrotus rossii*

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Running title: NaCl alters Cd accumulation and metabolites in halophytic Cd-accumulation

Abstract

Background and Aims: Salinity affects the bioavailability of cadmium (Cd) in soils and Cd accumulation in plants, but the associated mechanisms remain unclear. This study aimed to assess the metabolic response to NaCl and Cd and the relationship between metabolites and Cd accumulation in the halophyte *Carpobrotus rossii* (Haw.)*,* which has potential for Cd phytoextraction.

Methods: Plants were grown in nutrient solution with 0-400 mM NaCl in the presence of 5 or 15 µM Cd, with varied or constant solution Cd^{2+} activity. Plant growth and Cd uptake were measured, and the accumulation of peptides, organic and amino acids in plant tissues were assessed.

Key Results: The addition of NaCl to Cd-containing solutions improved plant growth along with 70-87% less shoot Cd accumulation, which had resulted from decreases in Cd root uptake and root-to-shoot translocation irrespective of Cd^{2+} activity in solutions. Moreover, Cd exposure increased the concentration of phytochelatins, which correlated positively with Cd concentrations in plants regardless of NaCl addition. In comparison, Cd inhibited the synthesis of organic acids in shoots and roots in the absence of NaCl, but increased it in shoots in the presence of NaCl. While Cd increased the concentrations of amino acids in plant shoots, the effect of NaCl on the synthesis of amino acids was inconsistent.

Conclusions: Our data provide the first evidence that NaCl decreased Cd shoot accumulation in *C. rossii* by decreasing Cd root uptake and root-to-shoot translocation even under constant Cd^{2+} activity. The present study also supports the important role of peptides and organic acids, particular of phytochelatins, in Cd tolerance and accumulation although the changes of those metabolites was not the main reason for the decreased Cd accumulation.

Key words: amino acids, Cd uptake, Cd translocation, glutathione, organic acids, phytochelatins, phytoremediation, phytoextraction, salinity

INTRODUCTION

Cadmium (Cd) is one of the most serious pollutants in the landscape since it can accumulate readily in plants to levels that are harmful in animal and human diets without being toxic to the plant itself. Besides the origin of the parent material, many anthropogenic activities, such as the application of phosphate fertilizers, sewage biosolids and green wastes to soils, have resulted in the wide-spread addition of considerable Cd to soils (Nicholson *et al.*, 1994). Phytoremediation is a cost-efficient and environmentally friendly way to remove Cd from contaminated soils (Mahar *et al.*, 2016). However, high levels of salinity, another important environmental stress, are associated with high

concentration of bioavailable heavy metals in some areas, such as coastal areas or dry areas of developing countries where waste water is not treated (Ghnaya $et\ al.$, 2007, Lutts and Lefèvre, 2015). In those areas, halophytes (i.e. species which can grow in saline conditions) have been considered as promising candidates for phytoremediation, since most Cd hyperaccumulating species are glycophytes which are unsuited for growth in saline soils (Lutts and Lefèvre, 2015, Wali $et\ al.$, 2015).

It has been widely reported that NaCl, as the most common salt in saline soils, can increase Cd uptake and accumulation in plants when grown in Cd-contaminated soils due to increased total Cd concentrations in soil solution (Smolders $et\ al.$, 1998, Weggler-Beaton $et\ al.$, 2000, Ghallab and Usman, 2007, Ondrasek, 2013). Several possible mechanisms have been described in the literature to explain this greater Cd bioavailability. First, increasing Na^+ concentration increases the chemical activity of Cd^{2+} in soil solution via increased cation exchange on soil colloids or dissolved organic matter (Smolders et al., 1998, Bingham $et\ al.$, 1984). Second, NaCl increases Cd mobilization through the formation of soluble inorganic chloride complexes ($CdCl_n^{2-n}$) and Cd desorption (due to the decreases of positive charges) from charged sites in soil solid phase (Weggler-Beaton et al., 2000). The formation of $CdCl_n^{2-n}$ complexes in soil solution increases Cd uptake, either by direct uptake of the $CdCl_n^{2-n}$ complexes by plants, or by increasing the diffusion of Cd^{2+} around the root uptake sites (Smolders $et\ al.$, 1996, Smolders and McLaughlin, 1996).

NaCl also influences plant growth, antioxidant enzyme activities and the synthesis of metabolites (Muhling and Lauchli, 2003, Amer, 2010, Han $et\ al.$, 2012, Wu $et\ al.$, 2012), which is closely related to Cd toxicity and thus accumulation in plants. To study the specific effect of NaCl on plant growth and biochemical processes, hydroponic culture is widely used to minimise confounding factors of soil-based studies such as changes in soil Cd chemistry, soil pH and soil water potential caused by salt addition. However, hydroponic studies have shown that NaCl reduced the deleterious impact of Cd on plant growth, whereas this is accompanied with a decreased accumulation of Cd in plants (Ghnaya et al., 2007, Lefèvre $et\ al.$, 2009). Unlike in soils, the formation of $CdCl_n^{2-n}$ complexes decreases Cd^{2+} activity in nutrient solution since the free Cd^{2+} ion is the preferred species for plant uptake (Smolders and McLaughlin, 1996) and diffusion would not limit Cd^{2+} uptake by plants in the continuously-aerated nutrient solution (López-Chuken $et\ al.$, 2010). Thus, the addition of NaCl in Cd-containing solutions was shown to decrease Cd concentration in tissues due to the decreased, rather than increased, Cd availability in solutions (Ghnaya et al., 2007, Lefèvre et al., 2009, Han et al., 2012, Mariem $et\ al.$, 2014, Wali et al., 2015). Contrasting results have also been observed. For example, the addition of NaCl increased Cd concentration in salt-sensitive wheat and $Zea\ mays$ (Muhling and Lauchli, 2003, Sepehr and Ghorbanli, 2006), while the response of Cd accumulation to NaCl was concentration-dependent in tobacco and halophyte $Spartina\ alterniflora$ (Chai $et\ al.$, 2013, Zhang $et\ al.$, 2014a). Increasing NaCl addition increased Cd concentration in the tissues of $S.\ alterniflora$ at 1 mM Cd in solution but decreased it at 3 mM Cd (Chai et al., 2013). The addition of NaCl decreased Cd concentration in tobacco at 5 µM Cd but increased it at 50 µM Cd (Zhang et al., 2014a). Furthermore, the impact of NaCl on Cd translocation in plants is not consistent among studies using solution culture. The addition of NaCl decreased Cd translocation in the halophytes $Atriplex\ halimus$ and $Kosteletzkya\ virginica$ (Lefèvre et al., 2009, Han et al., 2012), but increased Cd translocation and shoot accumulation in the halophyte $Sesuvium\ portulacastrum$ (Ghnaya et al., 2007, Mariem et al., 2014, Wali et al., 2015). These apparent discrepancies between studies indicate that, apart from changes in the Cd^{2+} activity in solution, additional mechanisms are involved in the effects of NaCl on Cd uptake and translocation.

It is possible that NaCl changes the complexation of Cd in plant tissues, thereby influencing Cd translocation and accumulation in plants. Only a small fraction of Cd in plants is present as the hydrated ions, while most ions are bound to various ligands, such as organic acids, amino acids and peptides, and those metal complexes provide both solubility and shielding during long-distance transport (Callahan $et\ al.$, 2006, Álvarez-Fernández $et\ al.$, 2014). Moreover, NaCl influences the synthesis of metabolites in plants, which in turn might impact the ligands for Cd complexation. It

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has been reported that NaCl decreased both the concentrations of total organic acids (especially malate and citrate) and amino acids in the tissues of the halophyte *Limonium latifolium* (Gagneul *et al.*, 2007). In the leaves of *K. virginica*, NaCl markedly increased the accumulation of glutathione (GSH), a precursor of phytochelatins (PCs) involved in the vacuolar sequestration of Cd (Han *et al.*, 2013). However, little information is available concerning the metabolic responses to the dual-stress of NaCl and Cd.

Recently, the Australian native halophyte *Carpobrotus rossii* was shown to tolerate a mixture of heavy metals and accumulated Cd (Zhang *et al.*, 2014b). A previous soil study showed that salinity not only increased plant growth, but also enhanced the accumulation of Cd in plant shoots, indicating that *C. rossii* could be a promising candidate in phytoextraction of Cd-polluted soils with high salinity levels (Zhang *et al.*, 2016). To date no information is available regarding the impact of NaCl on plant growth and metabolites in *C. rossii* which may be related to Cd uptake, translocation and hence accumulation under Cd stress.

This study aimed to assess the effect of NaCl on Cd uptake, translocation and accumulation in *C. rossii*; the metabolic response to NaCl and Cd and the relationship between metabolites and Cd accumulation in plants. It showed that NaCl addition improved the growth of *C. rossii* and decreased tissue Cd concentration even when the free Cd^{2+} activity in solutions was kept constant, resulting from the decreased root Cd uptake and Cd root-to-shoot translocation. Although PCs play an important role in Cd accumulation in *C. rossii*, the changes of PCs concentrations in plant tissues could not explain the decreased Cd translocation and accumulation by NaCl.

MATERIALS AND METHODS
Plant growth
Cuttings of *Carpobrotus rossii* (Haw.) Schwantes (Aizoaceae) were initially collected from a rural landfill site (Zhang et al., 2014b), and propagated in a naturally-lit glasshouse with a 14-h photoperiod at 18-25 °C and 45-65% relative humidity in Victoria, Australia. Uniform cuttings with two nodes were taken from ca. 200 mother plants, washed with tap water, and transplanted to 5-L polyethylene pots and grown in a controlled environment growth room with a 14-h photoperiod with a light intensity of 400 µmol m^{-2} s^{-1} at 20 °C during the day and 18 °C during the night; and 50% relative humidity. Each pot contained 5 L of continuously-aerated nutrient solution in the following composition (µM): 200 $MgSO_4$; 10 KH_2PO_4; 600 K_2SO_4; 600 $Ca(NO_3)_2$; 20 FeNaEDTA; 5 H_3BO_3; 1 $MnSO_4$; 0.2 $CuSO_4$; 0.03 Na_2MoO_4; 1 $ZnSO_4$. The solution pH was maintained at ca. 6.0 daily using 1 M KOH. The solutions were replaced every 6 d. After two weeks when the root systems were well developed, and rooted cuttings with similar size were selected for each experiment. Three rooted cuttings were weighed and transferred to each of new 5-L polyethylene pots containing the treatment solutions described under Experiments 1 and 2.

Experiment 1: Interactive effect of NaCl and Cd
To examine the effect of NaCl and Cd on plant growth, the synthesis of metabolites, and Cd uptake and translocation in *C. rossii*, an experiment consisting of six NaCl concentrations (0, 25, 50, 100, 200 and 400 mM), three Cd concentrations (0, 5 and 15 µM) and three replicates was conducted (Supplementary Table S1). The Cd speciation and Cd^{2+} activity in each solution was calculated using the GEOCHEM-PC program (Shaff *et al.*, 2010). To allow plants to gradually acclimate to NaCl, NaCl concentrations were increased by 50 mM per day in the first 4 days and then 100 mM daily until the final treatment concentrations were achieved, and thereafter Cd [as $Cd(NO_3)_2$] was added in the solutions. The plants were then grown under the same culture conditions for 20 days. Each treatment had three pots as replicates and treatments were arranged randomly. The solution pH was buffered with 2 mM MES [2-(N-morpholino) ethanesulphonic acid], and 1.2 ml of 1 M KOH was used to adjust pH to 6.0, which was maintained daily using 1 M KOH. The composition of nutrients in treatment solutions was the same as that of the basal nutrient solution, except that K (final concentration 1210 µM) was added as KOH rather than K_2SO_4. Nutrient solutions were aerated continuously, and renewed every 3 d.

Experiment 2: Effect of NaCl under constant Cd^{2+} activity

The second experiment consisting of three treatments (15 μM Cd; 15 μM Cd + 50 mM NaCl; and 85 μM Cd + 50 mM NaCl) and three replicates was conducted to examine the effects of NaCl on Cd uptake and translocation at a constant Cd concentration of 15 μM and a constant Cd^{2+} activity of 10.9 μM in solutions. The Cd speciation and Cd^{2+} activity in each solution was calculated and is shown in Supplementary Table S1. The rooted cuttings were prepared as outlined previously but from the different batch of cuttings used for Experiment 1, and then grown in the controlled-environment growth room as outlined for Experiment 1 for 10 days.

Ion analysis

After treatment for 10 d or 20 d, plants were harvested and separated into shoots and roots. The roots were immersed in ice-cold 20 mM Na_2-EDTA for 15 min to remove Cd adhering to the root surface. After washing with deionized water, the roots were divided to two parts. The first subsample was frozen in liquid nitrogen and then freeze-dried (Christ, John Morris Scientific, Australia) while root length and surface area of the second subsample were examined using a root scanner at 600 dpi (Epson Perfection 4990 Scanner, model J131B, Epson Inc.) before being oven-dried. Similar to the roots, after washing in deionized water, shoots were divided into two parts: half was freeze-dried and the other half oven-dried. The oven-dried samples were ground and digested using HNO_3 in a microwave digester (Multiwave 3000, Anton Paar), and the concentrations of K, Na, Ca and Cd determined using inductively coupled plasma optical emission spectrometry (ICP-OES) (Perkin Elmer Optima 8000, MA, USA).

Metabolite Analysis

The freeze-dried samples were ground into a fine powder using a mill mixer (MM400, Retch Germany) and were extracted for organic acids, amino acids and peptides. The extraction method was modified according to Cao *et al.* (2015) and Liu and Rochfort (2013). Briefly, 20 mg of sample was mixed with 1 mL cold (4°C) aqueous solution of 100 mM dithiothreitol (DTT, Sigma-Aldrich) in a 2-mL polypropylene centrifuge tube. The suspension was vortexed for 4 min, and then sonicated (Digital benchtop ultrasonic cleaners, Australia) for 15 min, and vortexed again for 4 min. The extracts were then centrifuged at $13000 \times g$ (Microfuge 18 Centrifuge, USA) for 10 min, and 0.6 mL of the supernatant transferred to a 1.5-mL amber vial for direct LC-MS analysis or diluted appropriately.

Stock solutions of organic acids, amino acids and peptides were separately prepared at 100 μg mL^{-1} and dissolved in deionized water for organic acids or in 100 mM DTT for amino acids and peptides. A series of 7 dilutions (ranging from 0.1 to 10 μg mL^{-1}) were prepared according to the preliminary tests to span the linear range.

The concentrations of organic acids, amino acids and peptides were determined using high performance liquid chromatography mass spectrometry (HPLC-MS, Agilent 6460 Triple Quadrupole LC/MS). HPLC separation was achieved using a Synergi Hydro-RP column (250 × 4.6 mm, 4 μm, Phenomenex) for amino acids and peptides, or a Rezex ROA-Organic acid column (150 × 4.6 mm, Phenomenex) for organic acids, on an Agilent 1290 Infinity system (Agilent, Walbronn), including degasser, binary pump, temperature-controlled autosampler (maintained at 10°C) and column compartment (maintained at 20°C). The mobile phase for the measurement of organic acids was 0.5% formic acid, while that for amino acids, GSH and PCs was composed of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). An isocratic elution at a flow rate of 0.5 mL min^{-1} was adopted for organic acid analysis (total run time 15 min). For measurement of amino acids, GSH and PCs, a gradient elution was applied, which started with 5% B, increased to 35% B at 13 min, and further increased to 80% B at 18 min before column equilibration with 5% B; the total run time was 26 min with a flow rate of 0.5 mL min^{-1}. The injection volume was 5 μL for all analyses. The product ions used for quantification and detailed MS parameters are shown in Supplementary Table S2.

Statistical analysis
For Exp. 1, the effects of NaCl and Cd concentration in solution on plant biomass, tissue concentrations of K, Na, Ca and Cd, shoot Cd content, Cd translocation and metabolites were examined using a two-way analysis of variance, and significant ($P \leq 0.05$) differences between means were identified by LSD using GenStat v.11 (VSN international). Correlation and principal component analyses were performed among the concentrations of Cd, nutrients and metabolites in plant tissues using the SPSS version 16.0 software package. For Exp. 2, a one-way analysis of variance was performed on plant growth, Cd concentration in tissues, and metabolites in response to Cd^{2+} activity in bulk solutions. Significant ($P \leq 0.05$) differences between means were identified by LSD using GenStat v. 11.

RESULTS
Experiment 1: Plant growth
In the absence of Cd, all plants appeared healthy without any deficiency or toxicity symptoms throughout the experimental period, except for plants grown in 400 mM NaCl which were visibly smaller than those in other treatments. Overall, increasing NaCl concentration from 0 to 100 mM in nutrient solution did not significantly affect plant growth. However, the inhibited growth induced by the high NaCl was evident in the harvested shoot dry weight which was 16% less in the 400 mM NaCl treatment than in the 0-200 mM NaCl treatments (Fig. 1a). In comparison, roots were more sensitive to high NaCl than shoots, with 17% and 49% lower root biomass when plants were grown in 200 and 400 mM NaCl, respectively (Fig. 1b). A similar trend was observed for the relative growth rate (RGR), which was decreased by 14% at 200 mM NaCl and 27% at 400 mM NaCl ($P<0.001$) (Fig. 1c). Furthermore, 400 mM NaCl decreased the root length per plant by 65% compared to other NaCl treatments, but plants grown in 400 mM NaCl had 25% larger root diameter (Figs. 1d and Supplementary Fig. S1).

The presence of Cd alone without NaCl severely reduced plant growth (Fig. 1). After 20 days of growth, plants exposed to Cd only had 82% (at 5 µM Cd) and 57% (at 15 µM Cd) shoot dry weight compared to control plants. Root dry weight and RGR showed almost the same pattern as for shoot dry weight. The addition of Cd in solutions only influenced the root length and root diameter at 15 µM Cd, with 36% lower root length but 32% greater root diameter compared with the control plants (Figs. 1d and Supplementary Fig. S1).

The response of plant growth to the combination of NaCl and Cd was concentration-dependent (Fig. 1). For example, increasing NaCl concentration from 0 to 200 mM in the solution containing 5 µM Cd had no influence on plant growth, except for the 25 and 50 mM NaCl where the root biomass was increased by 18%. In contrast, 400 mM NaCl reduced shoot biomass, root biomass, RGR, and root length at 5 µM Cd by 13%, 50%, 16% and 60%, respectively. However, the addition of NaCl greatly improved plant growth at 15 µM Cd. Specifically, plant growth increased by up to 50% (shoot biomass), 135% (root biomass), 82% (RGR) and 160% (root length) with the addition of 25-200 mM NaCl, compared to the plants exposed to 15 µM Cd only. To lesser extent, 400 mM NaCl also increased shoot dry weight and RGR by 26% and 30%, respectively.

Experiment 1: The concentrations of Na, K and Ca in shoots
The concentrations of K and Ca in the shoot of control plants were the highest among all the plants (Fig. S2) and above the critical values for plant growth. Similar trends were observed for other cations, including Mg, Fe and Zn (data not shown). Increasing NaCl concentration in the nutrient solution generally increased shoot Na concentration but decreased K concentration with the effect being more from 0 to 50 mM than from 50 mM to 400 mM (Fig. S2a). The addition of Cd slightly but significantly increased shoots Na concentration at 0-50 mM NaCl, while it did not influence the Na concentration at NaCl $\geq$100 mM. In comparison, Cd addition increased shoot K concentration except that 15 µM Cd decreased the K concentration at 0 NaCl (Fig. S2b). In the absence of Cd, increasing solution NaCl from 0 to 50 mM did not affect shoot Ca concentration, but further

increasing NaCl decreased the Ca concentration by 15-45%. The addition of Cd decreased shoot Ca concentration at 25-200 mM NaCl with the effect being greater at 15 µM Cd (Fig. S2c).

Experiment 1: Plant uptake and tissue Cd concentration
The concentration of Cd in the tissues of plants growing without Cd was below the detection limit (0.03 µg g^{-1} dry weight) and is therefore not presented. The shoot Cd concentrations ranged between 40 and 668 µg g^{-1}, with the highest concentrations found in the plants subjected to Cd alone, with Cd levels 2.4-fold higher at 15 µM Cd than that at 5 µM Cd (Fig. 2a). Increasing NaCl concentration decreased shoot Cd concentration with decreases being more from 0 to 25 mM than from 25 to 400 mM at both Cd levels. For example, increasing NaCl from 0 to 25 mM decreased shoot Cd concentration by 45% and 70% at 5 and 15 µM Cd, respectively. In comparison, the NaCl and Cd treatments did not affect the root Cd concentration, except for the 400 mM NaCl treatment where the root Cd concentration decreased by 65% and 41% at 5 and 15 µM Cd, respectively (Fig. 2b). As a result, the translocation factor (i.e. the shoot-to-root concentration ratio) showed the same pattern as for shoot Cd concentration (Fig. 2c).

Accordingly, the addition of NaCl decreased the shoot Cd content taken up by plants at both Cd levels (Fig. 3a). Specifically, increasing solution NaCl concentration from 0 to 25 mM decreased shoot Cd amount by 43% at 5 µM Cd and 56% at 15 µM Cd, while 400 mM NaCl decreased it by 87% at both 5 and 15 µM Cd.

Similarly, at 15 µM Cd, Cd uptake per unit root length was decreased by 73% when NaCl concentration in solution increased from 0 to 25 mM, and was kept constant in 25 to 400 mM NaCl treatments (Fig. 3b). In comparison, Cd uptake per unit root length was not affected by 0-200 mM NaCl but decreased by 63% at 400 mM NaCl at 5 µM Cd.

Experiment 1: The relationship of Cd^{2+} activity in bulk solution and Cd uptake and translocation
Increasing NaCl concentration in solution exponentially decreased Cd^{2+} activity in solutions at both Cd levels with the decrease being less at 5 µM Cd (Fig. S3a). Irrespective of solution composition, increasing Cd^{2+} activity linearly increased shoot Cd concentration (R^2=0.98) and Cd translocation factor (R^2=0.96) (Fig. S3b and S3c). The Cd^{2+} activity curve-linearly correlated with root Cd concentration and total Cd uptake in plants (Fig. S3b and d), even when the data from the NaCl-free treatment were excluded due to the extremely high Cd^{2+} activity (data not shown).

NaCl and Cd alter metabolites and inorganic elements in plant tissues
A total of 26 metabolites were identified using LC-MS and were classified as amino acids, organic acids and peptides (Figs 4 and 5, and Supplementary Table S3). In both shoots and roots of control and treated plants, proline, malate, citrate, succinate, GSH, lysine, glutamate, leucine and isoleucine were major metabolites. In addition, glutamine and tryptophan were also major metabolites in shoots, while glycine, aspartate, and alanine were major metabolites in roots. However, PCs became major metabolites in both shoots and roots in all Cd-treated plants.

Principal component analysis (PCA) showed that in both shoots and roots, different NaCl treatments (0-400 mM) were separated along the component 1 axis, although the samples treated with 25-100 mM NaCl were clustered closely (Fig. 6a and 6b). This separation of shoots could be mainly explained by the decreases in aspartate, glutamate, serine, asparagine, alanine, K, Ca, Mg and S on the positive axis side, and increases in proline, citrate, malate, succinate and Na with increasing NaCl addition on the negative side of component 1 axis (Figs. 4-6 and Supplementary Table S3). Similarly, differences among the NaCl treatments in roots mainly involved GSH, malate, succinate, threonine, valine, glutamate, glutamine, serine, methionine, tyrosine, cysteine, lysine, Ca, Mg and K on the positive side of component 1 axis, and proline, Na, Zn on the negative side of component 1 axis.

The Cd-treated plants were clearly discriminated from the non-Cd control plants along the positive axis of component 2, with no significant separation between plants treated with 5 and 15 µM Cd (Fig. 6a and 6b). This separation of shoots could be mainly explained by increases in PCs,

methionine, glutamine, leucine, cysteine, phenylalanine, valine, tyrosine and Cd on the positive axis side, and decreases in GSH, Fe and Zn by Cd addition on the negative axis side (Figs 4-6 and Supplementary Table S3). However, the differences in roots between non-Cd and Cd treated plants only involved PCs and Cd on the positive axis side.

When Cd was absent in the hydroponic solutions, increasing NaCl concentration from 0 to 400 mM decreased GSH concentrations in both shoots (by 72%) and roots (by 55%). Similarly, 20-d exposure of Cd in the absence of NaCl decreased GSH concentrations by 60% in shoots and 27% in roots, with no difference between the 5 and 15 μM Cd treatments. When Cd was present, increasing NaCl concentration decreased GSH concentration by up to 3.4-fold in the shoot and 1.7-fold in the root with little difference between the 5 and 15 μM Cd treatments (Fig. 4).

Unlike the effect on GSH, increasing Cd addition from 5 to 15 μM increased the concentrations of PCs by 2.4-fold for the shoot and 1.6-fold for the root, although no PCs were detected in the no-Cd control (Fig. 4). When Cd was present, increasing NaCl addition from 0 to 400 mM decreased PCs concentrations by 13 fold in shoots and 3 fold in roots with the decrease in shoots mainly occurred when NaCl addition increased from 0 to 25 mM. Moreover, the impact of NaCl on concentrations of PCs in shoots was greater at 15 than at 5 μM Cd. Furthermore, the average impact of NaCl on the concentration of PC3 was the greatest and PC4 was the smallest.

When Cd was not present in solutions, increasing NaCl concentration from 0 to 25 mM decreased but from 100 to 400 mM increased the concentrations of citrate, malate and succinate in shoots with the effect being the greatest on citrate (Fig. 5). In comparison, increasing NaCl in solutions without Cd generally decreased the concentrations of these organic acids in roots. When no NaCl was added, Cd exposure lowered the concentrations of organic acids in both shoots and roots (by 50-120% except for the citrate in shoot) (Fig. 5). However, the addition of NaCl to the Cd-containing solutions increased the concentrations of organic acids by up to 4-fold in shoots but not in roots.

For amino acids, the addition of NaCl from 0 to 400 mM in no-Cd solutions generally decreased the concentrations of cysteine, glutamic acid, glutamine, serine and threonine in roots and alanine, asparagine, aspartic acid, glutamic acid and glutamine in shoots (Supplementary Table S3). Moreover, 400 mM NaCl decreased the concentrations of leucine, lysine, methionine, phenylalanine, tyrosine, valine and glycine in roots but increased the concentrations of leucine, methionine, phenylalanine and tyrosine in shoots. The exposure of Cd alone had less impact on the concentrations of amino acids in roots, but increased the concentrations of asparagine, cysteine, glutamine, leucine, methionine, serine and threonine in shoots. However, increasing NaCl in the Cd-containing solutions decreased the concentrations of asparagine, cysteine, serine and threonine in plant shoots, especially at 5 μM Cd.

Experiment 1: Metabolites and Cd correlation
The concentration of Cd in shoots correlated positively with GSH, PC2, PC3, PC4, asparagine, aspartic acid, glutamic acid, glutamine, serine and threonine ($P<0.05$-0.01); but negatively with citrate, malate and succinate ($P<0.01$) (Supplementary Table S4). In comparison, Cd concentration in roots positively correlated with peptides, organic acids (except for citrate), and most amino acids ($P<0.05$-0.01); but negatively with proline and asparagine ($P<0.01$). Overall, Cd concentration had stronger correlations with PCs in shoots than in roots.

Experiment 2: The effect of NaCl at constant Cd^{2+} activity
The effect of 50 mM NaCl at 15 μM Cd on plant growth and Cd accumulation was confirmed in Experiment 2, with 23% higher dry weight but 76% less Cd concentration in shoots (Fig. 7a and 7b). Interestingly, increasing Cd^{2+} activity in solution at 50 mM NaCl to 10.9 μM (85 μM total Cd), which has the same Cd^{2+} activity as solutions containing 15 μM Cd alone, did not alter the effect of NaCl on Cd concentration in tissues, although the impact of NaCl on plant dry weight became smaller. Specifically, the addition of 50 mM NaCl doubled root biomass but had little impact on shoot biomass when compared to solutions with the same Cd^{2+} activity. Meanwhile, increasing

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NaCl from 0 to 50 mM did not affect root Cd concentration, but decreased shoot Cd concentration by 70%, irrespective of Cd^{2+} activity in treatment solution (Fig. 7b). The shoot Cd amount and Cd translocation factor from root to shoot showed the same pattern as for shoot Cd concentration (Fig. S4).

Similarly, the effect of 50 mM NaCl on the concentrations of metabolites in plants (except for the peptides in roots) was consistent in both experiments, irrespective of Cd^{2+} activity in solutions (Fig. 7c, d). Specifically, 50 mM NaCl decreased the concentrations of GSH (30%) and PCs (80%) in shoots, but doubled or even tripled the concentrations of citrate, malate and succinate in shoots, regardless of Cd^{2+} activity in solution. However, 50 mM NaCl had no significant effect on the concentrations of PCs in roots when the Cd^{2+} activity was same. In comparison, NaCl addition or Cd^{2+} activity variation did not affect the concentrations of organic acids in roots.

DISCUSSION

The effect of NaCl on Cd uptake, translocation and accumulation

The result showed that the addition of NaCl improved the growth of *C. rossii* and decreased tissue Cd concentration when the free Cd^{2+} activity in solutions was varied (Figs 1 and 2). The improved plant growth by NaCl addition in the presence of Cd was not due to the beneficial effect of NaCl on the halophyte growth, since the addition of NaCl did not increase the biomass or the relative growth rate of *C. rossii* in the absence of Cd. Furthermore, the decreased Cd concentration was not the result of a 'dilution' effect as NaCl addition also decreased the total amount of Cd accumulated per shoot (Fig. 3a). Similar results have also been reported in the halophytes *A. halimus* (Lefèvre *et al.*, 2010), *K. virginica* (Han et al., 2013, Han et al., 2012), and the glycophyte tobacco (Zhang *et al.*, 2013).

This study, for the first time, showed that NaCl actually decreased shoot Cd accumulation in plants when the Cd^{2+} activity in solutions was kept constant (Figs 7b and S4a). This is in contrast to previous studies using Swiss chard (Smolders and McLaughlin, 1996), *Arabidopsis thaliana* and *Solanum nigrum* (Xu *et al.*, 2010). The reduced Cd accumulation in *C. rossii* shoots by NaCl can be mainly attributed to the decreased Cd uptake by roots and a decreased efficiency of Cd translocation from roots to shoots (Figs 2, 3 and S4).

A possible explanation for the decreased Cd uptake by roots could be that NaCl decreased Cd^{2+} activity in bulk solutions, due to the formation of $CdCl_n^{2-n}$ species. Although Smolders and McLaughlin (1996) speculated that $CdCl_n^{2-n}$ species might be taken up directly by plants or dissociated in solution to alleviate the depletion of Cd around the plant root, the contribution of $CdCl_n^{2-n}$ species to Cd accumulation in the plant was much smaller than that of Cd^{2+}. This explanation is supported by the positive relationship between Cd^{2+} activity in solutions and shoot Cd concentration, and total Cd uptake (Fig. S3). However, the decreased Cd uptake by roots at the same Cd^{2+} activity in solutions (Fig. S4c) indicated that NaCl directly influences Cd uptake by the roots of *C. rossii*.

The decreased Cd uptake by the roots might be attributed to the decreased activity of Cd^{2+} on the plasma membranes by the addition of NaCl, although the Cd^{2+} activity in solutions was kept constant. According to Kopittke *et al.* (2011) and Wang *et al.* (2011), increased concentrations of cations (such as Na^+) would reduce the negativity of the electrical potential at the surface of plasma membrane, and thus decrease the activities of metal cations (such as Cd^{2+}) at the membrane surface. The Cd^{2+} activity at the outer surface of the plasma membrane of root cells would be more relevant to the metal uptake and toxicity in plants than the Cd^{2+} activity in bulk solutions (Kinraide and Wang, 2010, Wang et al., 2011). In addition, NaCl might reduce root Cd uptake by competing for the cation channels and/or transporters. For example, Mei *et al.* (2014) found that NaCl decreased Cd uptake in the root of *Amaranthus mangostanus* because of the competition between Na^+ and Cd^{2+} for Ca^{2+} channels.

The greater negative effect of NaCl on Cd translocation than on Cd uptake suggests that the decrease in Cd accumulation in the shoot induced by NaCl (Figs 3 and S4) was more attributed to the decreased Cd translocation from root to shoot in *C. rossii*. The addition of NaCl might decrease

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Cd translocation by influencing the speciation and/or distribution of Cd inside root cells, which is also supported by the improved root growth without affecting root Cd concentration (Figs 1d and 2b). Our previous study showed that 70% of Cd in the root of *C. rossii* was bound to S ligands such as GSH and PCs (Cheng *et al.*, 2016). Because of the high-affinity for thiolates in particular, PC-Cd complexes are formed in the cytosol of root cells and subsequently transported into the vacuoles that are the major sites of metal sequestration, delaying and/or preventing Cd transport to shoots (Clemens, 2006; Sadi *et al.*, 2008).

To understand if this decreased Cd translocation by NaCl addition was due to a change in the synthesis of PCs and thus Cd speciation in plant tissues, the response of metabolites to Cd and NaCl stresses was examined. Our present study confirmed the dominant role of PCs in Cd tolerance and accumulation in *C. rossii* by the positive relationship between the concentrations of PCs and Cd in plant shoots (Table S4). Similar results were also observed in many other plant species (Vázquez *et al.*, 2006). However, NaCl addition did not influence or even decreased the concentrations of thiol groups in plant roots under Cd stress (Figs 4 and 7c), different from the previous finding in *A. thaliana* (Xu *et al.* 2010). Accordingly, the molar ratio of PC-SH:Cd in the roots decreased from 5:1 to 2:1 with the increasing NaCl concentration in solution when the Cd^{2+} activity was varied but did not change when the Cd^{2+} activity was constant. It indicated that the decreased Cd translocation by NaCl was not due to the changes of PCs concentration in the roots.

However, the molar ratio of PC-SH:Cd in the roots was greater than the expected value of 2:1 stoichiometry for Cd-SH complexes (Rauser, 1999), implying that PCs were over-saturated in the tissues of *C. rossii* and not the limiting factor for the formation of Cd-SH complexes. Similarly, the molar ratio of COOH:Cd was higher than 7:1 in the roots of *C. rossii*. Indeed, owing to the over-saturation of those ligands, and the varied distribution of Cd and ligands within the cells, the concentrations of organic ligands could not reflect the real complexation of Cd (Salt *et al.*, 1997; Zhang *et al.*, 2010). Therefore, the addition of NaCl might influence Cd speciation by other mechanisms, rather than the synthesis of those ligands.

This study also showed that NaCl enhanced the tolerance of *C. rossii* to Cd. Indeed, in the presence of ≤100 mM NaCl, the Cd concentration in the shoot of *C. rossii* was greater than the shoot Cd concentrations in the previously reported halophytes, including *Spartina alterniflora*, *Kosteletzkya virginica* and *Atriplex halimus*, even when the solution contained up to 1000 μM Cd (Lefèvre *et al.*, 2009; Chai *et al.*, 2013; Han *et al.*, 2013). Furthermore, 20-d growth of *C. rossii* produced high biomass and accumulated 200 mg Cd per plant shoot in the presence of 200 mM NaCl at 15 μM Cd. This is tripled that observed in the halophyte *Sesuvium portulacastrum* at 25 μM Cd, although the shoot Cd concentration in *Sesuvium portulacastrum* (Wali *et al.*, 2015) was slightly higher than the concentration in *C. rossii* in our study. Therefore, *C, rossii* is a promising candidate for the Cd phytoextraction in Cd-contaminated sites with high salinity.

The effect of NaCl and Cd on the synthesis of metabolites
The increased concentration of PCs with increasing Cd stress (Fig. 5) confirmed that the synthesis of PCs is specifically caused by exposure to toxic metals or metalloids such as Cd, As, Hg or Pb (Sneller *et al.*, 1999). This was further supported by the result that the concentrations of PCs were highly related to the Cd concentrations in plant tissues irrespective of NaCl addition (Table S4). As expected, the concentrations of GSH in tissues were decreased by Cd alone treatment, probably due to the accelerated biosynthesis of PCs from GSH. In addition, NaCl decreased GSH in plants, consistent with the findings in NaCl-tolerant and NaCl-sensitive pea (Hernández *et al.*, 2000) and *A. thaliana* (Xu *et al.*, 2010), but different from that observed in *K. virginica* (Han *et al.*, 2013).

The addition of NaCl decreased the Cd-induced synthesis of PCs in both roots and shoots (Figs 5 and 8c), which might be partly due to the negative effect of NaCl on the synthesis of GSH. In addition, the concentrations of PCs in the roots correlated more closely with the Cd^{2+} activity in solutions than with Cd concentration in the roots, indicating that the influence of NaCl on the concentrations of PCs had resulted from its effect on the Cd^{2+} activity in solutions. This is consistent with the previous finding that the activity of phytochelatin synthase was governed by the

availability of metal ions (Loeffler *et al.*, 1989). Unlike the effect on root PCs, NaCl decreased the concentration of PCs in shoots, irrespective of Cd^{2+} activity in solutions, which was the consequence of decreased Cd concentration in shoots and thus the decreased degree of Cd-imposed stress rather than the direct effect of NaCl (Stolt *et al.*, 2003, Sun *et al.*, 2006).

Although the role of organic acids in Cd tolerance and accumulation is limited to vacuolar sequestration and Cd transportation in xylem (Verbruggen et al., 2009, Cheng et al., 2016), contrasting responses of organic acids to Cd stress have been reported in different studies (Boominathan and Doran, 2003, Wójcik *et al.*, 2006, Iori *et al.*, 2012, Fernández *et al.*, 2014). In the present study, Cd exposure decreased the levels of organic acids in the absence of NaCl (Fig. 6), which could be due to the high metabolic costs that cells must bear to sustain the defense systems against Cd oxidative attack (López-Bucio *et al.*, 2000a, Iori et al., 2012), since citrate, malate and succinate are key intermediates of the Krebs cycle.

However, the response of organic acids to both NaCl and Cd differed in tissues of *C. rossii* (Figs 6 and 8d). The concentrations of organic acids were correlated positively with Cd concentration in the root but negatively with that in the shoot, indicating the more important role of organic acids in Cd accumulation in roots than in shoots. In the presence of Cd, NaCl had little influence on the concentrations of organic acids in the roots, which may due to the little impact of NaCl on root Cd concentration. In contrast, NaCl increased the concentrations of organic acids in shoots, which was likely the consequence of healthy growth due to the less Cd oxidative attack with NaCl addition (López-Bucio *et al.*, 2000a; López-Bucio *et al.*, 2000b).

The Cd exposure had little influence on the synthesis of amino acids in roots, but increased the concentrations of most amino acids in the shoots (Table S3), suggesting that amino acids play a role in Cd tolerance in plant shoots (Rai, 2002). For example, Cd stress increased the concentration of proline in shoots, which is a marker to abiotic stresses (Sharma and Dietz, 2006). An increase in shoot asparagine and glutamine, which serve as major nitrogen transport and storage compounds in plants, might participate in the Cd detoxification processes (Gaufichon *et al.*, 2010; Xie *et al.*, 2014). The increased concentration of asparagine and aspartate may act as ligands to complex Cd in cells (Khan *et al.*, 2016). The increased cysteine concentration might be due to the Cd-induced oxidative stress and the need for the synthesis of GSH and PCs (Khan *et al.*, 2016).

It is worth noting that NaCl greatly influence the concentrations of amino acids in whole plants, which could be related to osmotic adjustment (Cuin and Shabala, 2007). Furthermore, the closer relationship of the concentrations of amino acids with Na than with Cd in plant tissues from the principle component analysis and linear correlation analysis indicates that the concentrations of amino acids were more influenced by NaCl than Cd when both NaCl and Cd were present. A similar observation has also been reported previously in the red alga *Pterocladiella capillacea* (Schmidt *et al.*, 2016). Taking together, amino acids play an important role in metabolism and tolerance to the stresses but have little effect on Cd accumulation.

CONCLUSION

This study investigated how NaCl affects Cd accumulation and the concentrations of associated metabolites in halophyte *C. rossii*, and showed that NaCl addition greatly improved plant Cd tolerance by decreasing Cd root uptake and root-to-shoot translocation. While PCs play an important role in Cd tolerance and accumulation in this halophyte, organic acids and amino acids were more likely involved in protective biological metabolisms against Cd and/or NaCl stresses rather than affecting Cd accumulation. Furthermore, the changes in metabolite concentrations in the plants could not explain the decreased Cd translocation by NaCl addition. This is the first study, to our knowledge, showing that NaCl decreased Cd accumulation in plants even when the solution Cd^{2+} activities was kept constant, and suggested that the concentration of organic ligands would not reflect Cd speciation in plant tissues. Overall, *C. rossii* is a promising candidate for Cd phytoextraction in Cd-polluted saline soils and estuarine environments. Further work is required to understand the mechanisms associated with this decreased Cd accumulation in the plants grown in saline environments.

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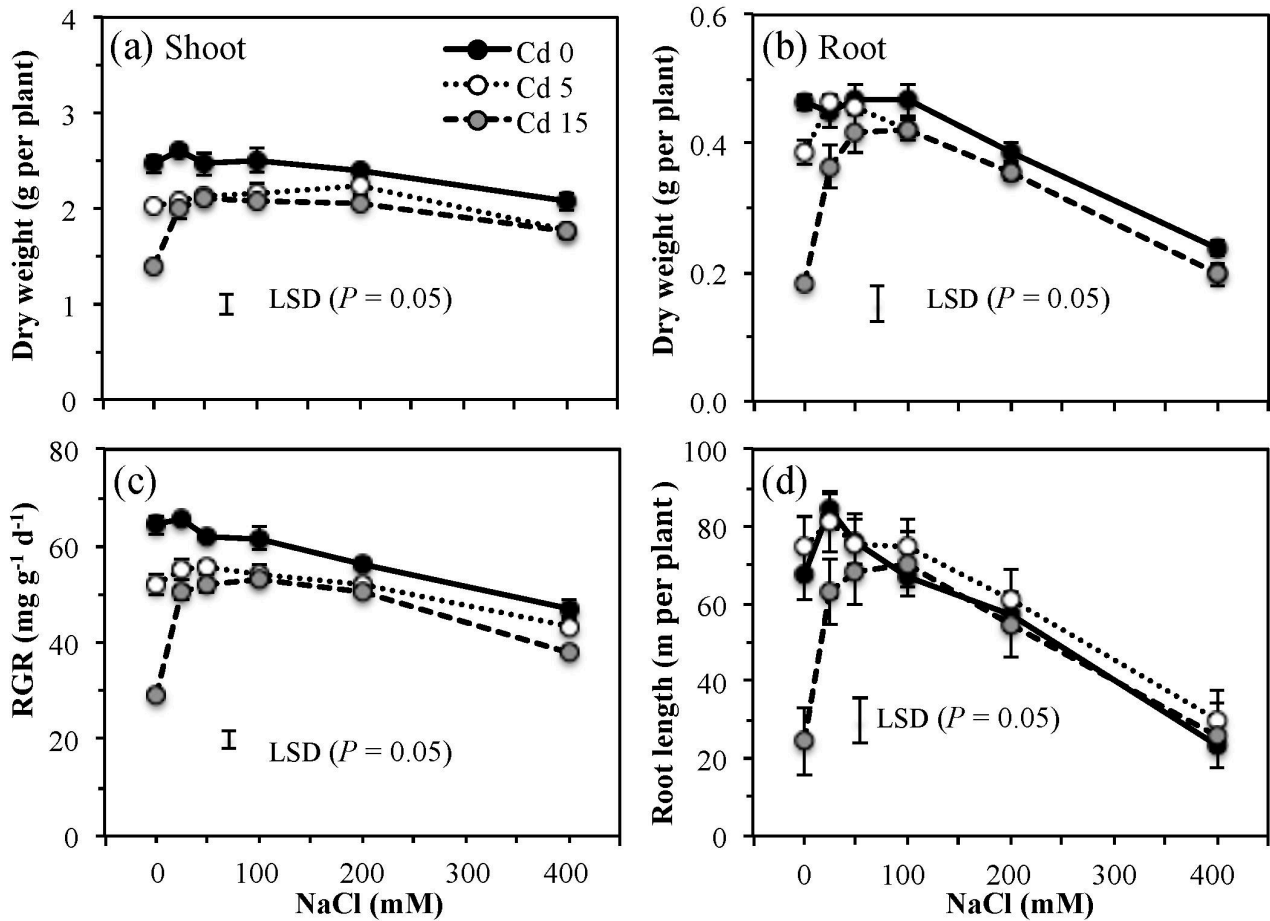
Figure 1. Dry weights of shoots (a) and roots (b), relative growth rate (RGR) (c) and root length (d) of *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (0, 5 and 15 μM) and NaCl (0-400 mM). Error bars represent ± standard errors (n = 3). The main effects of NaCl and Cd and their interactions are highly significant (*P*<0.001). For each panel, the LSD (*P*=0.05) bar is for the Cd × NaCl interaction (Exp. 1).
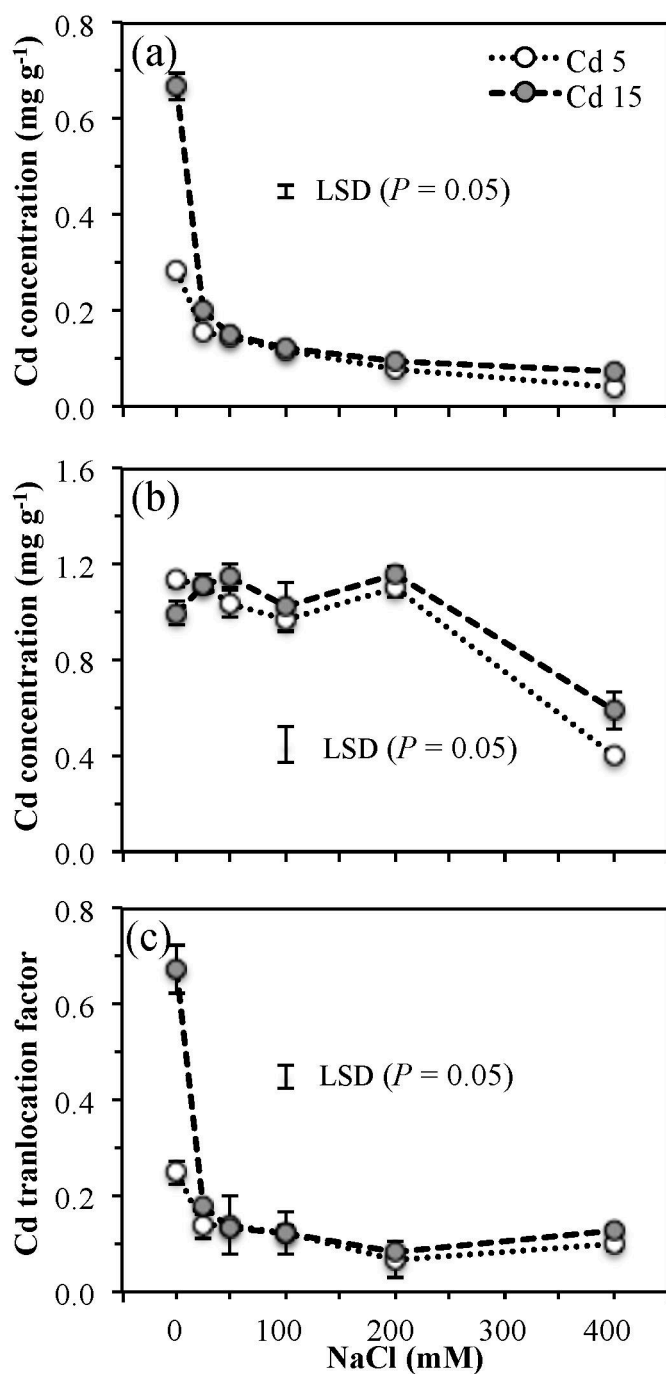
Figure 2. Concentrations of Cd in shoots (a) and roots (b) and Cd translocation factor (c) of *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (5 and 15 μM) and NaCl (0-400 mM). Error bars represent ± standard errors (n = 3). The main effects of NaCl and Cd and their interactions are highly significant ($P<0.001$). For each panel, the vertical LSD ($P=0.05$) bar is for the Cd × NaCl interaction. The concentration of Cd in the tissues of plants growing in solutions without Cd was below the detection limit (0.03 μg g^{-1} dry weight) (Exp. 1).

Figure 3. The content of Cd in shoot (a) and Cd uptake per root length (b) of *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (5 and 15 μM) and NaCl (0-400 mM). Error bars represent ± standard errors (n = 3). The main effects of NaCl and Cd and their interactions are highly significant ($P<0.001$). For each panel, the vertical LSD ($P=0.05$) bar is for the Cd × NaCl interaction (Exp. 1).
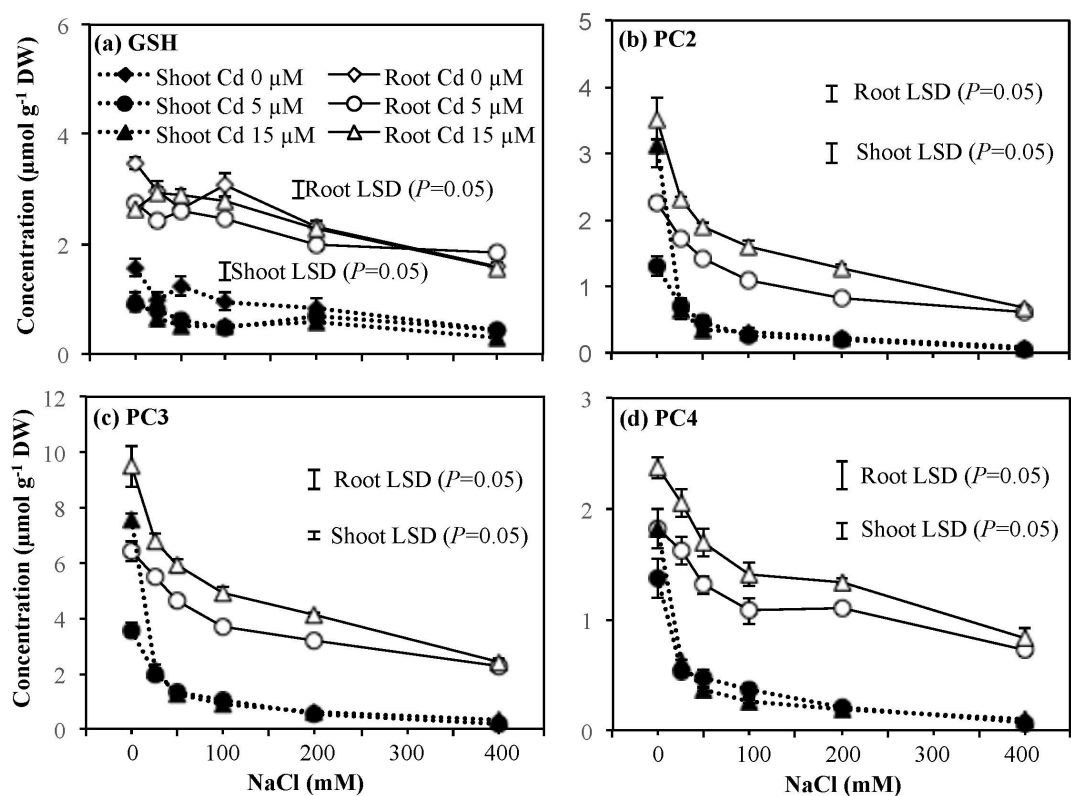
Figure 4. Concentrations of glutathione (GSH) (a), phytochelatin 2 (PC2) (b), phytochelatin 3 (PC3) (c), and phytochelatin 4 (PC4) (d) in *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (0, 5 and 15 µM) and NaCl (0-400 mM). Error bars represent ± standard errors (n = 3). For each panel, the vertical LSD (*P*=0.05) bar is for the Cd × NaCl interaction for each tissue. The concentration of PC2, PC3 and PC4 in the tissues of plants growing in solutions without Cd was below the detection limit (0.1 µg mL^{-1} dry weight) (Exp. 1).

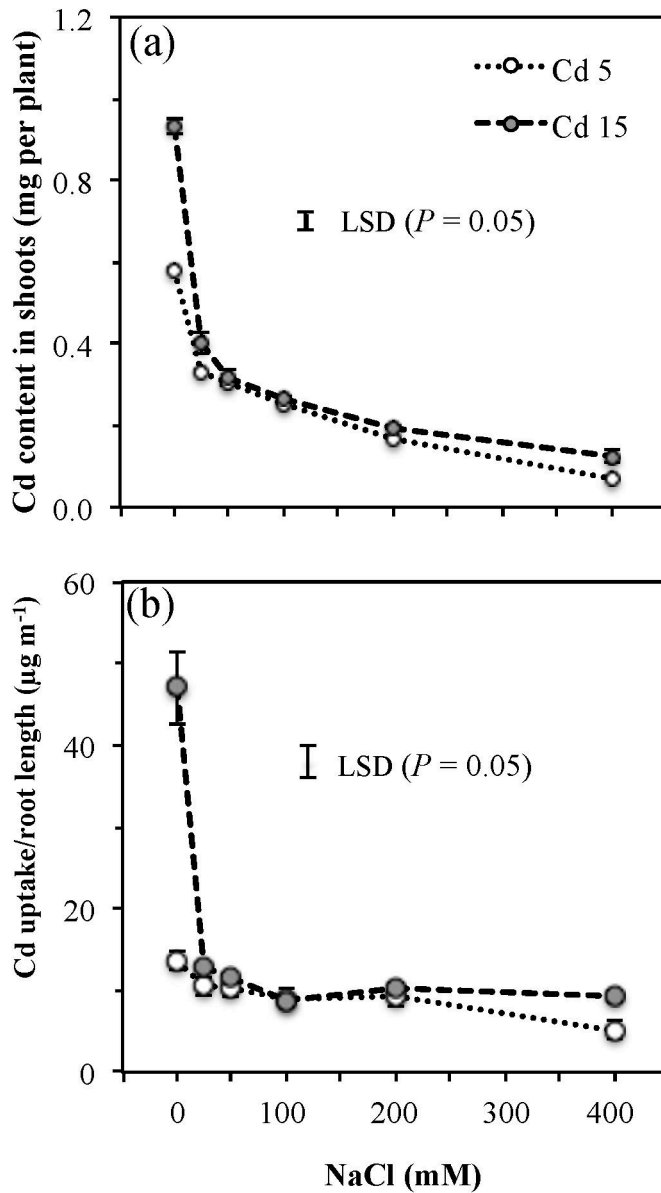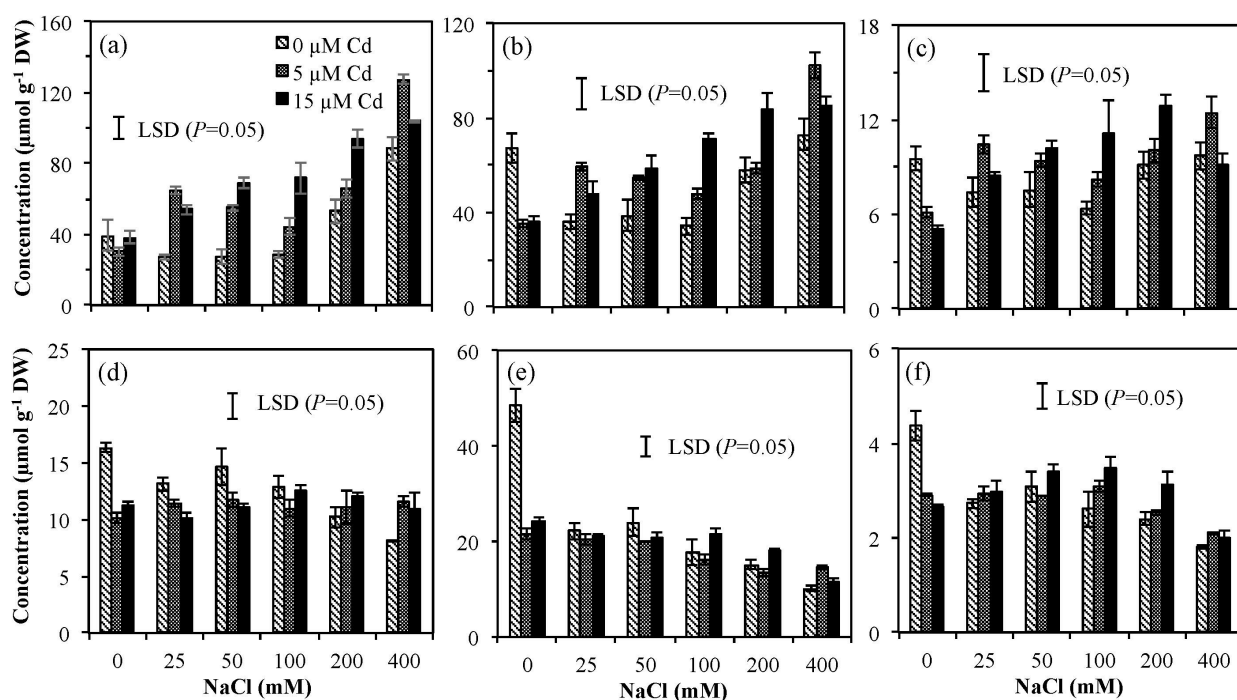**Figure 5.** Concentrations of citrate (a, d), malate (b, e) and succinate (c, f) in shoots (a, b, c) and roots (d, e, f) of *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (0, 5 and 15 µM) and NaCl. Error bars represent ± standard errors (n = 3). For each panel, the vertical LSD (*P*=0.05) bar is for the Cd × NaCl interaction (Exp. 1).

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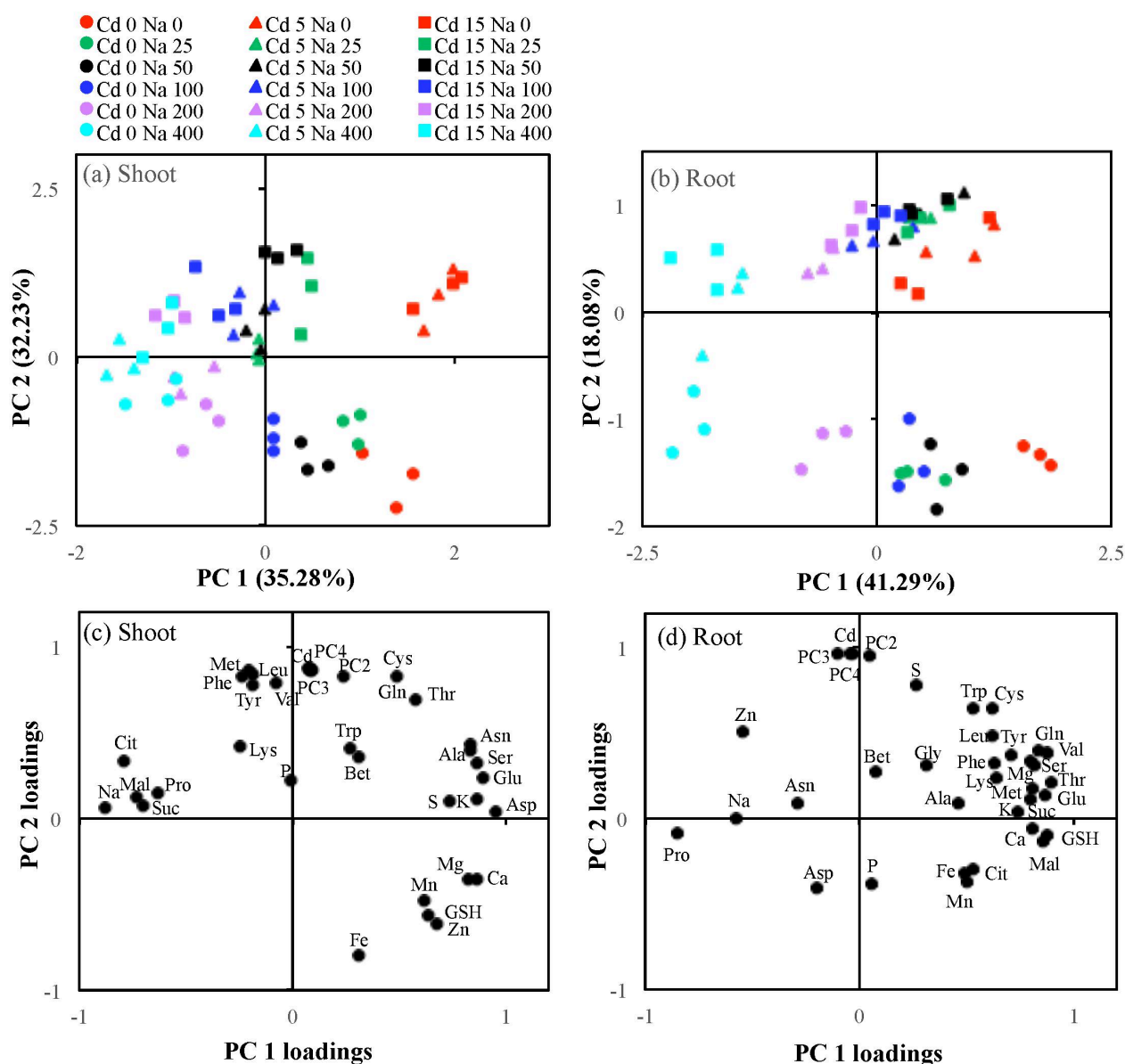
Figure 6. The scores plots (a and b) and corresponding PC1 and PC2 loading plots (c and d) from the principle component analysis of metabolites and mineral nutrients of shoot (a, c) and root (b, d) of *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (0, 5 and 15 μM) and NaCl (0-400 mM). For each principal component, loadings are indexed with the corresponding metabolite names. Alanine, Ala; Asparagine, Asn; Aspartic acid, Asp; Betaine, Bet; Cysteine, Cys; Glutamic acid, Glu; Glutamine, Gln; Glycine, Gly; Glutathione, GSH; Leucine, Leu; Lysine, Lys; Methionine, Met; Phytochelatin 2, PC2; Phytochelatin 3, PC3; Phytochelatin 4, PC4; Phenylalanine, Phe; Proline, Pro; Serine, Ser; Threonine, Thr; Tryptophan, Trp; Tyrosine, Tyr; Valine, Val; Citrate, Cit; Malate, Mal; Succinate, Suc (Exp. 1).

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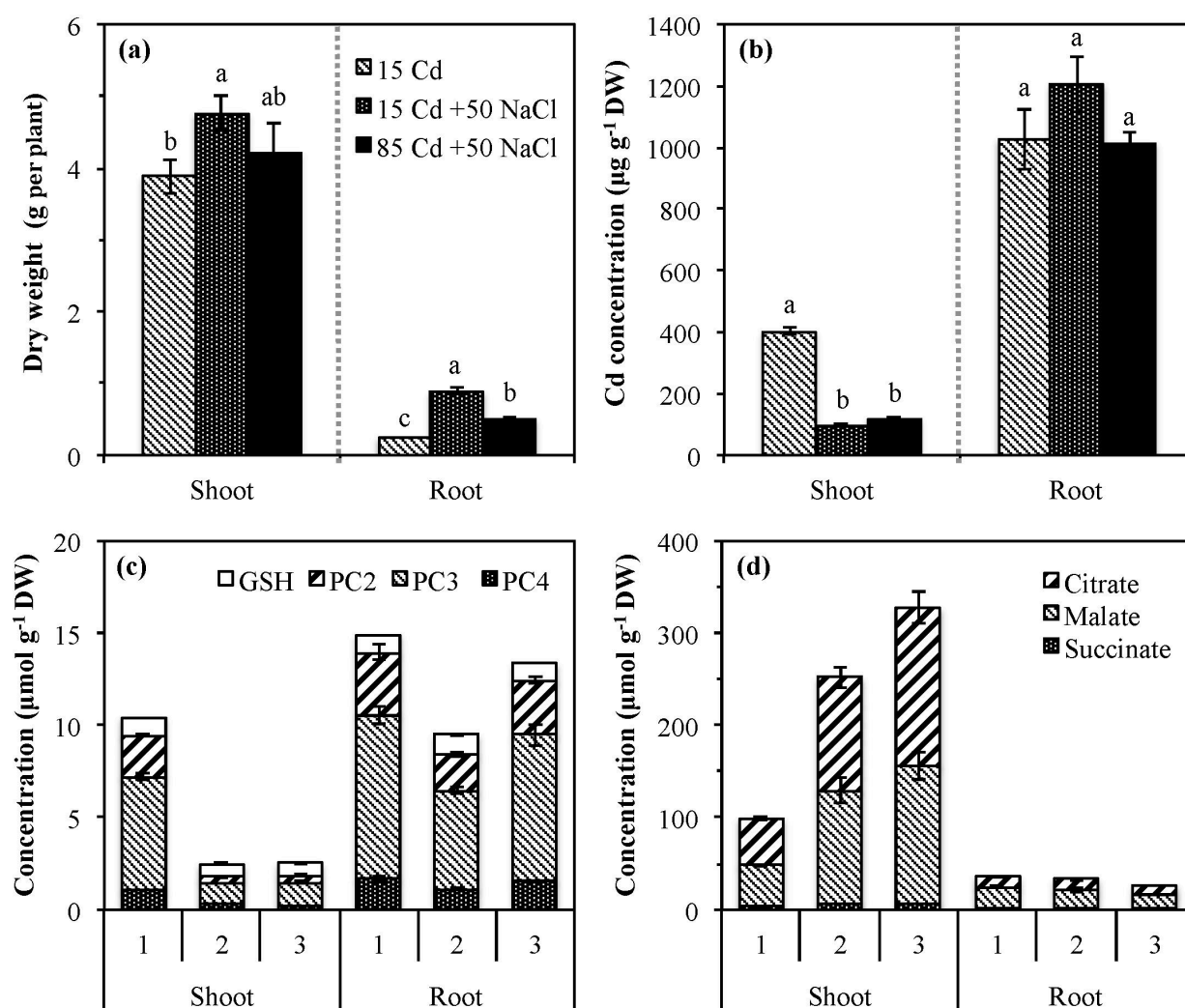
Figure 7. Dry weight (a), and concentrations of Cd (b), peptides (c) and organic acids (d) in tissues of *Carpobrotus rossii* exposed to treatments with constant Cd^{2+} activity in bulk solutions for 10 days. GSH, glutathione; PC2, phytochelatin 2; PC3, phytochelatin 3; PC4, phytochelatin 4. The treatments in (c) and (d) are: 1, 15 μM Cd; 2, 15 μM Cd + 50 mM NaCl; 3, 85 μM Cd + 50 mM NaCl. Error bars represent ± standard errors (n = 3) (Exp. 2).

Supplementary Information

Sodium chloride decreases cadmium accumulation and changes the response of metabolites to cadmium stress in halophyte *Carpobrotus rossii*

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Table S1. The composition of nutrient solutions used for the experiments. Only the concentrations of NaCl and Cd are shown, with basal nutrients added at the concentrations listed in the *Materials and Methods*.

	Experiment 1																		Experiment 2		
NaCl (mM)	-			25			50			100			200			400			-	50	50
Cd (µM)	-	5	15	-	5	15	-	5	15	-	5	15	-	5	15	-	5	15	15	15	85
Cd^{2+} activity (µM)	-	3.3	10.9	-	1.1	3.2	-	0.6	1.9	-	0.3	1.0	-	0.2	0.5	-	0.1	0.2	10.9	1.9	10.9
Na^+/Cl^- activity (mM)	-	-	-		20.9			39.6			73.9			136.2			249.5		-	39.6	39.6
Cd speciation (%)																					
Cd^{2+}	-		94.2	-		41.1	-		28.6	-		18.5	-		10.6	-		5.0	94.2	28.6	28.8
$CdSO_4^0$	-		3.3	-		0.7	-		0.3	-		0.1	-		0.1	-		-	3.3	0.3	0.3
$CdCl^+$	-		-	-		56.8	-		70.0	-		80.1	-		88.8	-		94.7	-	70.0	70.3
$CdNO_3^+$	-		0.2	-		0.1	-		-	-		-	-		-	-		-	0.2	-	-
$CdEDTA^{2-}$	-		2.3	-		1.3	-		1.0	-		0.7	-		0.5	-		0.3	2.3	1.0	0.6
Na/Cl speciation (%)																					
Na^+ or Cl^-		99.9*			98.17			96.8			94.5			90.9			85.4		99.9*	96.8	96.8
NaCl		-			1.79			3.2			5.5			9.1			14.6		-	3.2	3.2

The speciation of Cd in the bulk nutrient solution was calculated using GEOCHEM-PC.

-, no addition or not applicable, or < 0.1.

*, the speciation of Na^+, no Cl^- in solution.

Table S2. Multiple reaction monitoring (MRM) parameters for metabolite quantification by LC-MS/MS.

Compound name	Precursor ion	Product ion	Fragmentor	Collision energy	Cell accelerator voltage	Polarity
Amino acids						
Alanine	90	44	51	5	7	Positive
Arginine	175	70	80	25	7	Positive
Asparagine	133	116	68	5	7	Positive
Aspartic acid	134	88	68	5	7	Positive
Betaine	118	59	68	29	7	Positive
Cysteine	122	76	68	20	7	Positive
Glutamic acid	148	102	68	22	7	Positive
Glutamine	147	130	68	5	7	Positive
Leucine/isoleucine	132	86	64	5	7	Positive
Lysine	147	84	68	19	7	Positive
Methionine	150	133	68	5	7	Positive
Phenylalanine	166	120	64	9	7	Positive
Proline	116	70	84	13	7	Positive
Serine	106	60	68	5	7	Positive
Threonine	120	74	68	5	7	Positive
Tryptophan	205	188	68	1	7	Positive
Tyrosine	182	91	60	5	7	Positive
Valine	118	72	68	5	7	Positive
Peptides						
GSH	309	179	68	12	7	Positive
PC2	541	337	135	23	7	Positive
PC3	773	233	135	40	7	Positive
PC4	1005	233	135	50	7	Positive
Organic acids						
Citric acid	191	111	70	15	7	Negative
Malic acid	133	115	70	15	7	Negative
Succinic acid	117	73	70	12	7	Negative

Table S3. Concentrations of amino acids (µmol g^{-1} DW) in shoots (Part A) and roots (Part B) of *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd and NaCl. Data are means ± standard errors (n = 3) (Exp.1).

Part A

Shoot		NaCl (mM)						LSD (P=0.05)	Significance level		
		0	25	50	100	200	400		Cd	NaCl	Cd×NaCl
Alanine	0 µM Cd	0.75 ± 0.10	0.67 ± 0.00	0.38 ± 0.01	0.41 ± 0.07	0.33 ± 0.02	0.31 ± 0.03				
	5 µM Cd	1.42 ± 0.05	0.36 ± 0.02	0.47 ± 0.07	0.43 ± 0.05	0.35 ± 0.00	0.28 ± 0.04	0.24	***	***	***
	15 µM Cd	1.37 ± 0.28	0.81 ± 0.10	0.81 ± 0.07	0.47 ± 0.05	0.43 ± 0.06	0.41 ± 0.02				
Asparagine	0 µM Cd	0.74 ± 0.06	0.89 ± 0.04	0.54 ± 0.10	0.42 ± 0.08	0.31 ± 0.03	0.13 ± 0.02				
	5 µM Cd	2.49 ± 0.14	0.62 ± 0.06	0.71 ± 0.11	0.53 ± 0.07	0.24 ± 0.03	0.17 ± 0.00	0.29	***	***	***
	15 µM Cd	3.56 ± 0.12	2.08 ± 0.26	1.50 ± 0.20	0.59 ± 0.05	0.41 ± 0.03	0.32 ± 0.04				
Aspartic acid	0 µM Cd	0.95 ± 0.10	0.41 ± 0.04	0.30 ± 0.04	0.19 ± 0.02	0.13 ± 0.01	0.06 ± 0.00				
	5 µM Cd	1.00 ± 0.08	0.18 ± 0.02	0.18 ± 0.03	0.13 ± 0.02	0.11 ± 0.01	0.06 ± 0.01	0.14	***	***	**
	15 µM Cd	1.25 ± 0.12	0.49 ± 0.08	0.34 ± 0.06	0.16 ± 0.02	0.12 ± 0.02	0.06 ± 0.01				
Betaine	0 µM Cd	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01				
	5 µM Cd	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.05 ± 0.02	0.03 ± 0.00	0.03 ± 0.00	0.02	***	n.s.	n.s.
	15 µM Cd	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.00				
Cysteine	0 µM Cd	0.03 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.01				
	5 µM Cd	0.25 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.14 ± 0.01	0.05 ± 0.01	0.03 ± 0.00	0.04	***	***	***
	15 µM Cd	0.31 ± 0.01	0.17 ± 0.05	0.19 ± 0.01	0.13 ± 0.01	0.10 ± 0.02	0.05 ± 0.01				
Glutamic acid	0 µM Cd	1.89 ± 0.14	1.54 ± 0.04	1.43 ± 0.03	1.35 ± 0.04	1.17 ± 0.05	0.88 ± 0.03				
	5 µM Cd	2.80 ± 0.36	1.44 ± 0.03	1.44 ± 0.02	1.27 ± 0.01	1.10 ± 0.09	0.78 ± 0.08	0.40	*	***	*
	15 µM Cd	2.57 ± 0.34	1.71 ± 0.13	1.62 ± 0.10	1.44 ± 0.09	1.30 ± 0.12	0.90 ± 0.12				
Glutamine	0 µM Cd	3.28 ± 0.17	5.03 ± 0.40	3.36 ± 0.15	4.45 ± 0.33	4.58 ± 0.44	7.39 ± 0.83				
	5 µM Cd	10.6 ± 3.5	5.54 ± 0.48	6.08 ± 0.43	12.80 ± 0.80	4.12 ± 0.05	5.43 ± 0.00	3.04	***	***	***
	15 µM Cd	12.1 ± 0.1	7.52 ± 1.16	16.5 ± 0.4	10.47 ± 1.82	8.39 ± 0.75	6.62 ± 0.69				
Leucine/iso leucine	0 µM Cd	1.22 ± 0.12	2.04 ± 0.34	1.27 ± 0.10	1.86 ± 0.15	2.65 ± 0.73	3.92 ± 0.31				
	5 µM Cd	3.25 ± 0.95	2.38 ± 0.26	2.81 ± 0.37	7.46 ± 0.81	1.80 ± 0.01	3.92 ± 0.66	2.92	***	n.s.	**
	15 µM Cd	5.54 ± 0.18	6.75 ± 3.36	9.46 ± 0.39	5.60 ± 1.21	4.90 ± 0.70	5.75 ± 1.48				
Lysine	0 µM Cd	5.02 ± 1.35	1.02 ± 0.00	9.78 ± 0.47	6.00 ± 0.79	5.44 ± 0.79	4.51 ± 0.46				

	5 µM Cd	5.41 ± 0.30	6.04 ± 0.71	11.1 ± 1.7	5.99 ± 0.17	7.06 ± 0.71	6.02 ± 0.79	4.13	***	***	***
	15 µM Cd	4.96 ± 0.48	4.50 ± 0.08	37.7 ± 5.1	9.80 ± 1.32	8.95 ± 0.80	9.09 ± 0.60				
Methionine	0 µM Cd	0.76 ± 0.07	1.38 ± 0.22	0.87 ± 0.01	1.24 ± 0.07	1.71 ± 0.36	2.81 ± 0.35				
	5 µM Cd	3.74 ± 0.45	1.84 ± 0.16	2.01 ± 0.16	3.63 ± 0.45	1.70 ± 0.25	2.80 ± 0.54	1.09	***	**	***
	15 µM Cd	2.57 ± 0.18	2.30 ± 0.46	5.18 ± 0.14	3.58 ± 0.66	2.85 ± 0.21	3.12 ± 0.85				
Phenylalanine	0 µM Cd	0.99 ± 0.36	2.51 ± 0.31	1.50 ± 0.09	2.23 ± 0.19	2.36 ± 0.28	4.90 ± 0.64				
	5 µM Cd	5.04 ± 1.86	2.91 ± 0.32	4.94 ± 1.65	5.78 ± 2.09	2.72 ± 0.59	4.11 ± 0.92	3.79	***	n.s.	n.s.
	15 µM Cd	3.93 ± 1.25	7.27 ± 3.48	9.54 ± 0.18	6.27 ± 1.33	5.17 ± 0.53	5.84 ± 1.76				
Proline	0 µM Cd	97 ± 11	105 ± 8	110 ± 3	127 ± 19	252 ± 16	602 ± 41				
	5 µM Cd	118 ± 20	97 ± 5	108 ± 6	134 ± 10	268 ± 12	514 ± 32	53.6	***	***	**
	15 µM Cd	257 ± 17	158 ± 16	152 ± 5	149 ± 10	295 ± 23	594 ± 30				
Serine	0 µM Cd	0.09 ± 0.01	0.17 ± 0.01	0.13 ± 0.01	0.09 ± 0.03	0.04 ± 0.01	0.03 ± 0.00				
	5 µM Cd	0.52 ± 0.11	0.07 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.02 ± 0.00	0.09	***	***	***
	15 µM Cd	0.43 ± 0.01	0.22 ± 0.02	0.21 ± 0.05	0.08 ± 0.01	0.06 ± 0.00	0.03 ± 0.01				
Threonine	0 µM Cd	0.33 ± 0.05	0.47 ± 0.04	0.23 ± 0.02	0.21 ± 0.00	0.24 ± 0.04	0.26 ± 0.03				
	5 µM Cd	0.71 ± 0.05	0.26 ± 0.01	0.35 ± 0.07	0.42 ± 0.01	0.22 ± 0.04	0.25 ± 0.04	0.16	***	***	***
	15 µM Cd	0.83 ± 0.14	0.56 ± 0.11	0.61 ± 0.04	0.41 ± 0.05	0.36 ± 0.03	0.38 ± 0.02				
Tryptophan	0 µM Cd	1.57 ± 0.11	2.42 ± 0.34	1.80 ± 0.32	2.09 ± 0.40	1.34 ± 0.24	1.56 ± 0.33				
	5 µM Cd	2.11 ± 0.65	1.24 ± 0.04	2.12 ± 0.46	2.34 ± 0.72	1.07 ± 0.17	1.13 ± 0.25	1.29	n.s.	*	n.s.
	15 µM Cd	1.53 ± 0.31	2.76 ± 1.09	3.46 ± 0.15	2.25 ± 0.38	1.71 ± 0.16	1.91 ± 0.60				
Tyrosine	0 µM Cd	0.71 ± 0.29	2.88 ± 0.99	1.43 ± 0.29	2.59 ± 0.93	2.25 ± 0.61	3.98 ± 0.63				
	5 µM Cd	4.25 ± 1.69	2.27 ± 0.25	4.08 ± 1.44	4.61 ± 1.68	2.05 ± 0.38	3.37 ± 0.90	3.35	***	n.s.	n.s.
	15 µM Cd	3.65 ± 1.11	5.99 ± 2.90	7.43 ± 0.38	4.88 ± 0.99	3.99 ± 0.37	4.77 ± 1.47				
Valine	0 µM Cd	0.07 ± 0.01	0.23 ± 0.08	0.10 ± 0.02	0.21 ± 0.08	0.18 ± 0.04	0.22 ± 0.07				
	5 µM Cd	0.36 ± 0.15	0.18 ± 0.00	0.33 ± 0.11	0.37 ± 0.13	0.16 ± 0.04	0.19 ± 0.02	0.25	***	n.s.	n.s.
	15 µM Cd	0.33 ± 0.10	0.49 ± 0.22	0.57 ± 0.01	0.40 ± 0.05	0.30 ± 0.02	0.25 ± 0.07				

Part B

Root		NaCl (mM)						LSD	Cd	NaCl	Cd ×
		0	25	50	100	200	400	(P=0.05)			NaCl
Alanine	0 μM Cd	1.67 ± 0.09	1.52 ± 0.28	1.3 ± 0.01	1.52 ± 0.18	1.55 ± 0.21	1.26 ± 0.12				
	5 μM Cd	1.44 ± 0.10	1.23 ± 0.10	1.51 ± 0.25	1.64 ± 0.10	1.45 ± 0.28	1.05 ± 0.10	0.50	n.s.	*	n.s.
	15 μM Cd	1.46 ± 0.21	1.44 ± 0.22	1.76 ± 0.09	1.69 ± 0.23	1.43 ± 0.18	1.12 ± 0.06				
Asparagine	0 μM Cd	1.23 ± 0.08	0.90 ± 0.09	1.23 ± 0.15	1.12 ± 0.29	0.84 ± 0.07	1.35 ± 0.11				
	5 μM Cd	1.21 ± 0.10	1.19 ± 0.06	1.29 ± 0.10	1.08 ± 0.09	0.89 ± 0.02	1.67 ± 0.13	0.36	n.s.	***	n.s.
	15 μM Cd	0.69 ± 0.15	1.14 ± 0.10	1.07 ± 0.05	1.06 ± 0.10	1.11 ± 0.18	1.62 ± 0.16				
Aspartic acid	0 μM Cd	2.45 ± 0.43	2.72 ± 0.40	2.73 ± 0.43	2.49 ± 0.24	1.25 ± 0.22	26.6 ± 0.37				
	5 μM Cd	1.44 ± 0.21	2.27 ± 0.38	1.95 ± 0.38	1.24 ± 0.14	1.83 ± 0.47	1.31 ± 0.07	0.87	***	***	***
	15 μM Cd	2.84 ± 0.17	1.81 ± 0.10	1.44 ± 0.14	1.35 ± 0.30	1.70 ± 0.38	1.05 ± 0.07				
Betaine	0 μM Cd	0.27 ± 0.04	0.28 ± 0.06	0.25 ± 0.05	0.27 ± 0.04	0.25 ± 0.02	0.23 ± 0.01				
	5 μM Cd	0.26 ± 0.02	0.29 ± 0.04	0.27 ± 0.02	0.24 ± 0.03	0.23 ± 0.03	0.27 ±0.02	0.10	*	n.s.	n.s.
	15 μM Cd	0.40 ± 0.08	0.29 ± 0.01	0.30 ± 0.02	0.27 ± 0.04	0.29 ± 0.01	0.33 ± 0.01				
Cysteine	0 μM Cd	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00				
	5 μM Cd	0.17 ± 0.03	0.17 ± 0.01	0.17 ± 0.03	0.12 ± 0.02	0.06 ± 0.00	0.01 ± 0.00	0.04	***	***	***
	15 μM Cd	0.04 ± 0.00	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.02	0.04 ± 0.01	0.02 ± 0.00				
Glutamic acid	0 μM Cd	3.23 ± 0.09	2.91 ± 0.08	2.65 ± 0.09	2.50 ± 0.18	2.09 ± 0.09	1.65 ± 0.07				
	5 μM Cd	2.83 ± 0.13	2.66 ± 0.06	2.87 ± 0.13	2.57 ± 0.13	2.01 ± 0.17	1.82 ± 0.17	0.66	n.s.	***	n.s.
	15 μM Cd	3.33 ± 0.71	3.30 ± 0.21	3.22 ± 0.30	2.76 ± 0.31	2.09 ± 0.10	1.55 ± 0.12				
Glutamine	0 μM Cd	0.94 ± 0.01	0.45 ± 0.05	0.56 ± 0.04	0.49 ± 0.05	0.36 ± 0.04	0.30 ± 0.01				
	5 μM Cd	0.94 ± 0.14	0.68 ± 0.02	0.83 ± 0.13	0.58 ± 0.07	0.47 ± 0.04	0.38 ± 0.01	0.21	**	***	n.s.
	15 μM Cd	0.91 ± 0.20	0.69 ± 0.02	0.72 ± 0.02	0.58 ± 0.02	0.56 ± 0.04	0.37 ± 0.03				
Leucine/isoleucine	0 μM Cd	1.91 ± 0.14	1.41 ± 0.14	1.87 ± 0.11	1.97 ± 0.22	1.58 ± 0.19	1.06 ± 0.08				
	5 μM Cd	1.88 ± 0.22	1.93 ± 0.01	2.32 ± 0.30	2.02 ± 0.17	1.89 ± 0.08	1.41 ± 0.20	0.51	**	***	n.s.
	15 μM Cd	1.78 ± 0.34	1.92 ± 0.11	2.15 ± 0.16	2.11 ± 0.12	2.25 ± 0.11	1.48 ± 0.16				
Lysine	0 μM Cd	4.25 ± 0.41	3.31 ± 0.26	3.13 ± 0.24	3.04 ± 0.32	2.10 ± 0.14	2.24 ± 0.16				
	5 μM Cd	4.06 ± 0.39	3.67 ± 0.30	3.23 ± 0.06	2.71 ± 0.49	2.41 ± 0.21	2.96 ± 0.23	0.88	**	***	n.s.
	15 μM Cd	5.34 ± 0.22	4.33 ± 0.35	3.59 ± 0.24	3.07 ± 0.22	3.14 ± 0.61	2.50 ± 0.12				
Methionine	0 μM Cd	0.77 ± 0.05	0.48 ± 0.10	0.69 ± 0.07	0.55 ± 0.04	0.49 ± 0.08	0.21 ± 0.04				
	5 μM Cd	0.61 ± 0.07	0.60 ± 0.05	0.74 ± 0.14	0.63 ± 0.09	0.56 ± 0.06	0.34 ± 0.03	0.20	n.s.	***	n.s.

	15 μM Cd	0.48 ± 0.10	0.53 ± 0.03	0.61 ± 0.02	0.55 ± 0.05	0.64 ± 0.06	0.28 ± 0.05			
Phenylalanine	0 μM Cd	0.75 ± 0.03	0.52 ± 0.09	0.74 ± 0.06	0.64 ± 0.03	0.59 ± 0.07	0.38 ± 0.04			
	5 μM Cd	0.67 ± 0.08	0.67 ± 0.04	0.87 ± 0.13	0.76 ± 0.10	0.69 ± 0.07	0.46 ± 0.09	0.21	n.s.	***	n.s.
	15 μM Cd	0.57 ± 0.11	0.62 ± 0.05	0.78 ± 0.05	0.71 ± 0.06	0.80 ± 0.04	0.50 ± 0.08			
Proline	0 μM Cd	624 ± 47	567 ± 10	615 ± 36	679 ± 42	915 ± 58	1785 ± 178			
	5 μM Cd	385 ± 18	519 ± 73	574 ± 16	683 ± 87	908 ± 21	1643 ± 32	193	**	***	n.s.
	15 μM Cd	646 ± 15	598 ± 37	634 ± 61	801 ± 76	1083 ± 96	1695 ± 78			
Serine	0 μM Cd	0.55 ± 0.04	0.48 ± 0.04	0.47 ± 0.02	0.42 ± 0.04	0.34 ± 0.01	0.31 ± 0.03			
	5 μM Cd	0.54 ± 0.04	0.55 ± 0.04	0.55 ± 0.05	0.44 ± 0.04	0.36 ± 0.02	0.34 ± 0.03	0.11	*	***	n.s.
	15 μM Cd	0.54 ± 0.07	0.61 ± 0.05	0.58 ± 0.04	0.49 ± 0.03	0.40 ± 0.04	0.32 ± 0.02			
Threonine	0 μM Cd	0.77 ± 0.02	0.61 ± 0.01	0.60 ± 0.04	0.59 ± 0.06	0.41 ± 0.02	0.35 ± 0.02			
	5 μM Cd	0.66 ± 0.05	0.64 ± 0.01	0.64 ± 0.04	0.53 ± 0.05	0.47 ± 0.02	0.41 ± 0.05	0.11	n.s.	***	n.s.
	15 μM Cd	0.62 ± 0.07	0.69 ± 0.05	0.67 ± 0.04	0.57 ± 0.00	0.52 ± 0.05	0.42 ± 0.01			
Tryptophan	0 μM Cd	0.21 ± 0.01	0.21 ± 0.02	0.24 ± 0.03	0.24 ± 0.04	0.20 ± 0.02	0.19 ± 0.01			
	5 μM Cd	0.34 ± 0.05	0.43 ± 0.04	0.34 ± 0.03	0.32 ± 0.02	0.28 ± 0.02	0.15 ± 0.01	0.08	***	***	*
	15 μM Cd	0.32 ± 0.05	0.34 ± 0.03	0.35 ± 0.03	0.33 ± 0.00	0.31 ± 0.03	0.19 ± 0.03			
Tyrosine	0 μM Cd	0.65 ± 0.04	0.49 ± 0.07	0.66 ± 0.06	0.62 ± 0.03	0.53 ± 0.05	0.36 ± 0.04			
	5 μM Cd	0.64 ± 0.07	0.62 ± 0.05	0.76 ± 0.11	0.69 ± 0.05	0.60 ± 0.03	0.40 ± 0.04	0.16	n.s.	***	n.s.
	15 μM Cd	0.59 ± 0.11	0.61 ± 0.04	0.68 ± 0.03	0.65 ± 0.01	0.71 ± 0.02	0.44 ± 0.03			
Valine	0 μM Cd	0.45 ± 0.03	0.31 ± 0.02	0.36 ± 0.04	0.33 ± 0.08	0.21 ± 0.03	0.09 ± 0.01			
	5 μM Cd	0.52 ± 0.05	0.52 ± 0.04	0.54 ± 0.08	0.42 ± 0.05	0.25 ± 0.03	0.12 ± 0.02	0.12	***	***	n.s.
	15 μM Cd	0.46 ± 0.06	0.51 ± 0.02	0.52 ± 0.01	0.45 ± 0.03	0.33 ± 0.02	0.14 ± 0.02			

*, **, *** and n.s. indicate $P<0.05$, $P<0.01$, $P<0.001$ and $P>0.05$, respectively.

Table S4. Bivariate linear correlations between the concentrations of Cd and metabolites in shoots (n = 36) and roots (n = 36) of *Carpobrotus rossii* grown in treatments for 20 days. The measured metabolites not significantly correlated with Cd are not presented. Pearson's correlation coefficients and a 2-tailed test of significance at the 0.05 (*) and 0.01 (**) levels were applied.

| Shoots		Roots	
Metabolite	Correlation (r)	Metabolite	Correlation (r)
PC3	0.990**	Tryptophan	0.687**
PC2	0.958**	Valine	0.675**
PC4	0.922**	Succinic acid	0.656**
Aspartic acid	0.905**	GSH	0.644**
Asparagine	0.900**	Threonine	0.617**
Cysteine	0.827**	Tyrosine	0.608**
Alanine	0.764**	PC4	0.603**
Glutamic acid	0.750**	Methionine	0.591**
Serine	0.738**	Malic acid	0.575**
Threonine	0.730**	Cysteine	0.543**
GSH	0.644**	Leucine	0.537**
Betaine	0.338*	Serine	0.530**
Glutamine	0.334*	PC3	0.530**
Succinic acid	-0.691**	Glutamic acid	0.524**
Malic acid	-0.626**	Phenylalanine	0.494**
Citric acid	-0.586**	Glutamine	0.476**
		PC2	0.470**
		Alanine	0.382*
		Aspartic acid	0.344*
		Proline	-0.793**
		Asparagine	-0.635**
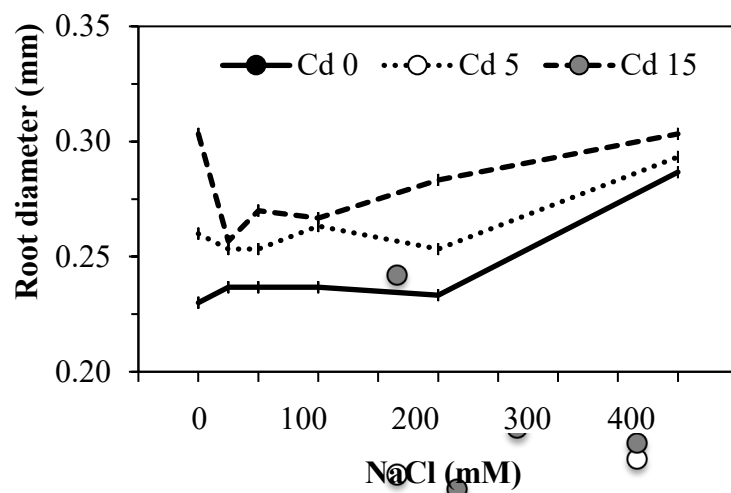
Figure S1. Root average diameter of *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (0, 5 and 15 μM) and NaCl. Error bars represent the standard errors (n = 3) (Exp.1).
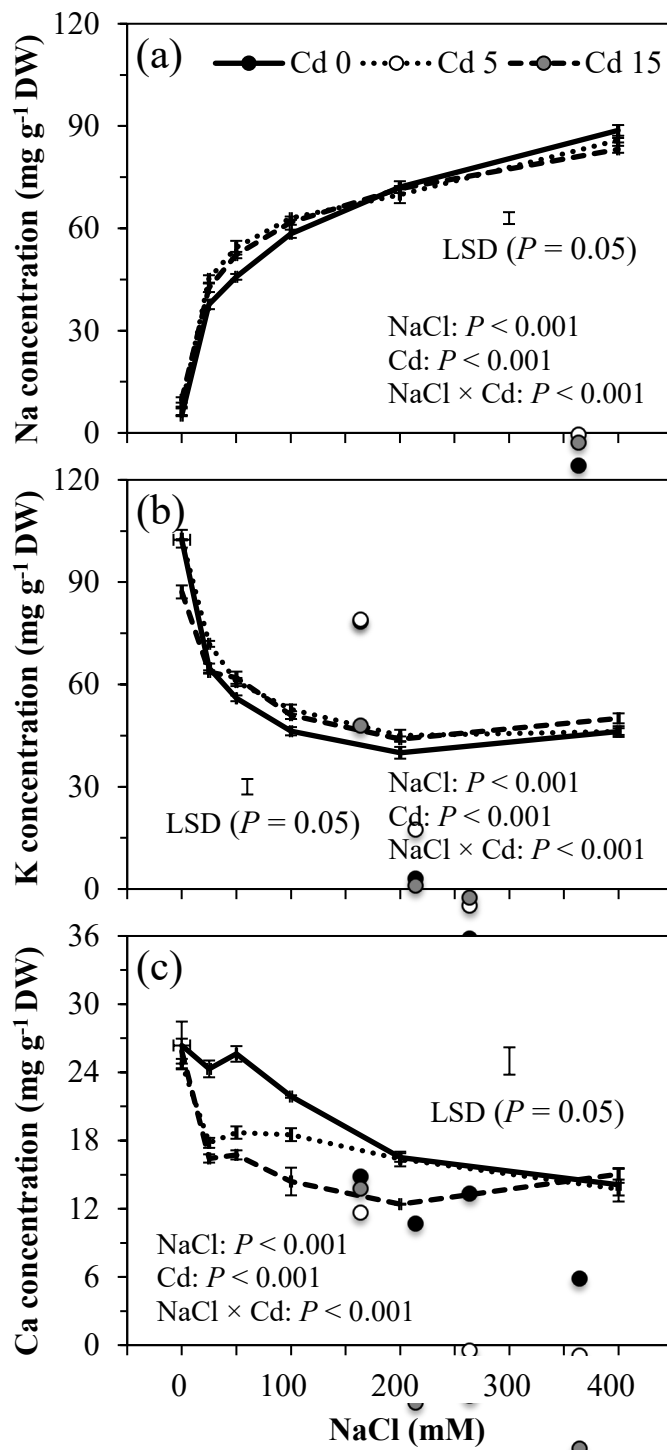
Figure S2. Concentrations of Na (a), K (b) and Ca (c) in shoots of *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (0, 5 and 15 μM) and NaCl. Error bars represent the standard errors (n = 3). For each panel, the vertical LSD (*P*=0.05) bar is for the Cd × NaCl interaction (Exp.1).
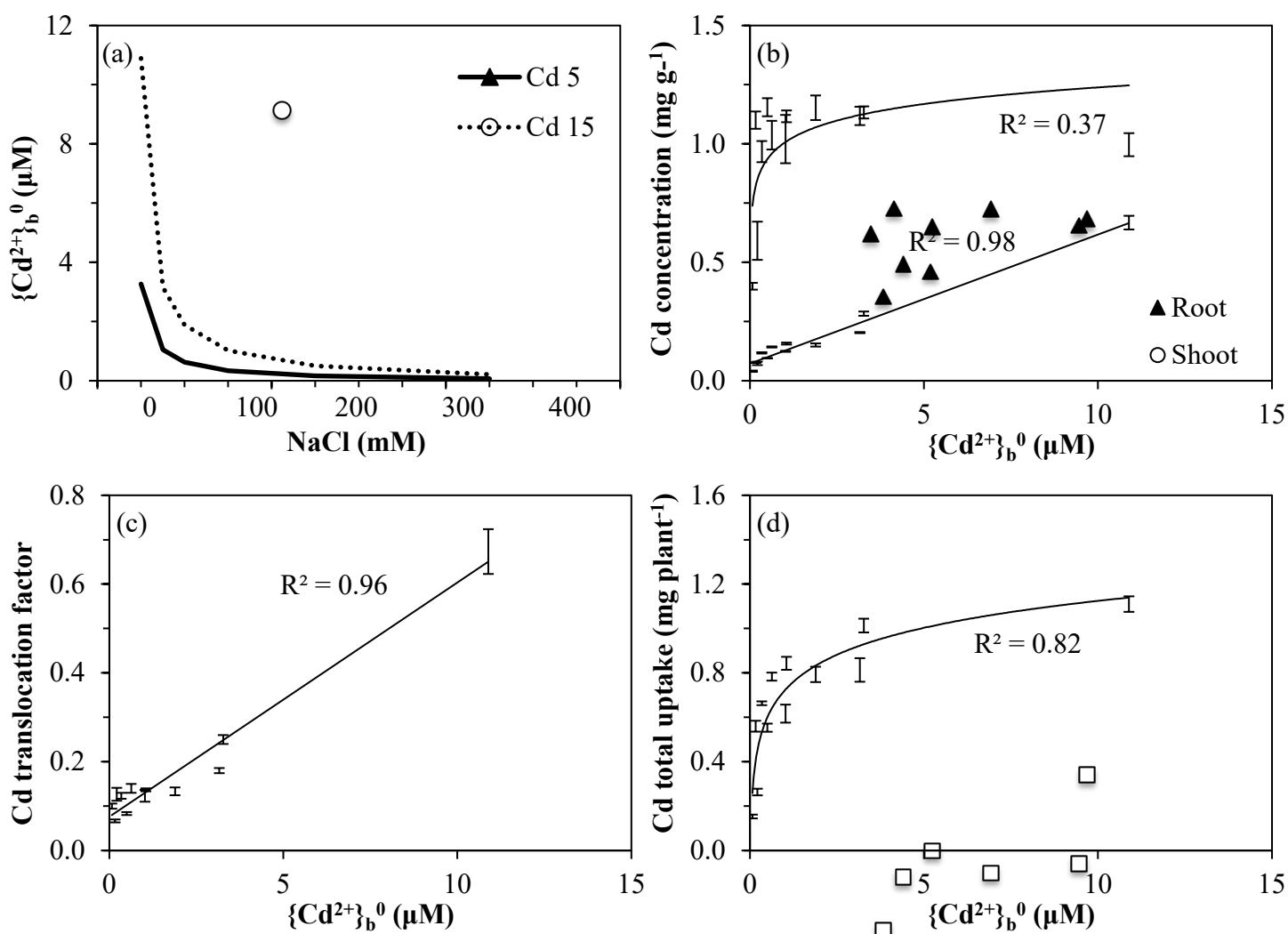
Figure S3. Effect of NaCl on the activity of Cd^{2+} in bulk solutions $\{Cd^{2+}\}_b^0$ (a), and the relationship of Cd^{2+} activity in the bulk solution $\{Cd^{2+}\}_b^0$ and Cd concentration in tissues (b), Cd translocation from root to shoot (c) and total Cd amount (d) in *Carpobrotus rossii* grown for 20 days in solutions with different concentrations of Cd (5 and 15 μM) and NaCl. Error bars represent the standard error (n = 3) (Exp.1).
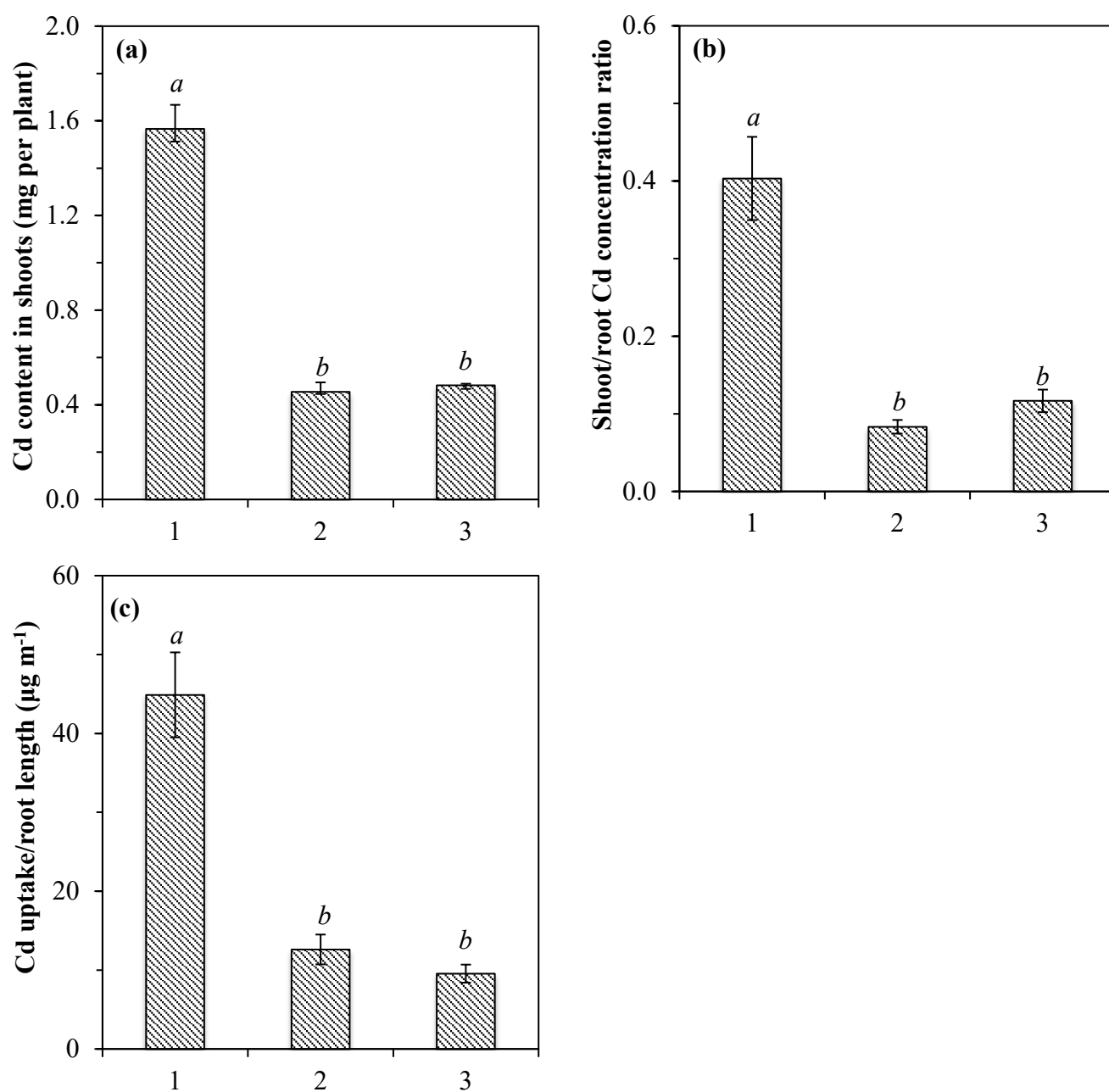
Figure S4. Shoot Cd content (a), shoot-to-root Cd concentration ratio (b) and Cd uptake per root length (c) of *Carpobrotus rossii* exposed to treatments with same Cd^{2+} activity in bulk solutions for 10 days. The treatments are: 1, 15 μM Cd; 2, 15 μM Cd + 50 mM NaCl; 3, 85 μM Cd + 50 mM NaCl. Error bars represent the standard errors (n = 3) (Exp.2).