5-Aminolevulinic Acid-induced Salt Tolerance in Strawberry: Possible Role of Nitric Oxide on Interception of Salty Ions in Roots

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Research article

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Abstract

Background: 5-Aminolevunic acid (ALA), as a natural non-protein amino acid and the first essential precursor of tetrapyrrole biosynthesis in all living bodies, has been suggested to improve salt tolerance of plants. In the previous work, we reported that ALA induces H_2O_2 accumulation in roots of strawberry, which is involved in up-regulating Na^+ transporter gene expressions to intercept Na^+ in roots with less upward transport. However, the signal route is not clear.

Results: In this study, we propose that nitric oxide (NO) is involved in ALA signaling cascade. Therefore, we applied sodium nitrosylpentacy (SNP, NO donor), Na_2WO_4 (NO biosynthetic inhibitor), and 2, 4-carboxyphenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, NO scavenger) to the culture solution when strawberry (*Fragaria* × *ananassa* Duch. cv. 'Benihoppe') was stressed by 100 mmol L^{-1} NaCl with or without exogenous ALA. The results reveal that salinity greatly impaired plant growth while 10 mg L^{-1} ALA or 10 μM SNP ameliorated the inhibition. When 5 μM Na_2WO_4 or cPTIO was co-treated, the ALA-improved salt tolerance was almost completely eliminated. This suggests that ALA-improved salt tolerance is dependent on NO presence. We found that salinity caused NO, H_2O_2, Na^+ and Cl^- increases in the whole plants, while ALA induced additional increases in roots but significant depressions in leaves. These tissue-specific responses to ALA are important for plant salt tolerance.

Conclusion: We propose that the regulation of ALA in roots is critical, which is mediated through NO and then H_2O_2 signal to up-express genes related with Na^+ and Cl^- transport, selectively retaining Na^+ and Cl^- in roots with less upward transport. The hypothesis can reasonably explain how ALA-treated plants cope with toxic ions under salinity.

Background

Soil salinization is a major problem threatening agricultural production, which seriously inhibits growth and development of crops and results in a decline in yield and quality. Yet, many plants have developed the ability to sense both osmotic stress and ionic stress [1]. It has been found that plants subjected to excess NaCl accumulate high Na^+ concentrations in root tissue within the first 2 min [2], whereas Na^+ efflux from the root tissue starts at 10 min [3]. These results suggest that excess Na^+ is rapidly sensed by plants, which triggers the downstream of salt stress responses [4]. Reactive oxygen species (ROS) can be induced by salt or osmotic stress to be a species of toxic substances on one hand and, which may be linked to cellular signaling to involve biological regulation on the other hand [5]. Na^+ and K^+ transporters play key roles in resistant mechanisms to salt stress [1]. Mechanisms to reduce cytoplasmic Na^+ include restricting Na^+ uptake, increasing Na^+ efflux, and compartmenta-lizing Na^+ in the vacuole [4]. There are three major transporters that are involved in these physiological processes: tonoplast-localized NHX1, segregating Na^+ within the vacuole; plasma membrane-localized SOS1 (Na^+/H^+ antiporters), exporting Na^+ out of the cell; HKT1, removing Na^+ from the xylem sap into the surrounding xylem parenchyma cells

to control long distance root-to-shoot Na^+ partitioning. They are composed of the most important basis for plants to maintain ion homeostasis under salt stress.

5-Aminolevulinic acid (ALA), a new plant growth regulator, is a common precursor of all porphyrin compounds [6]. It has been known to regulate plant growth and development [7, 8], such as callus induction, adventitious root and bud formation [9], stomatal opening [10-12], flower thinning [13], fruit coloration [14-16], and stress tolerance [17-19]. In salt tolerance, ALA can promote seed germination under salt stress [20, 21]. It also improves leaf photosynthesis under salt stress [22]. It has been suggested that ALA alleviates salt stress by increasing antioxidant enzyme activity and reducing membrane injury [23]. However, ALA may also modulate plant salt tolerance through its regulating the metabolism of tetrapyrrole and proline accumulation in *Brassica napus* seedlings [24]. Recently, we demonstrated that ALA improved strawberry salt tolerance by stimulating H_2O_2 level in roots, which in turn regulated the up-expression of genes encoding the ion transmembrane transporters, such as NHX1, SOS1 and HKT1, and then Na^+ is selectively intercepted in the roots with less upward transport and avoiding ion accumulation in shoots [25]. It is a completely new opinion about ALA dealing with plant salt tolerance [26]. However, the signaling route from ALA to H_2O_2 generation is not clear.

It is well known that nitric oxide (NO) as a multifunctional cellular signal, plays an important role in a variety of physiological processes [27]. In animals, NO production is predominantly catalyzed by nitric oxide synthases (NOS) [28]. In plants, nitrate reductase (NR) is the key enzyme for NO production, which catalyzes nitrite reduction to NO using NAD(P)H as co-factor [29]. Up to now, NO has been known to be involved in stomatal movement [30], adventitious root formation [31], flavonoid biosynthesis [32], and alleviation of various abiotic stresses [33], such as drought [34, 35], chilling [36], freezing [37], and salinity [38]. In rice plants, NO induces antioxidant enzyme activity against salt stress [39]. In tomato, NO increases photosynthetic rate and chlorophyll fluorescence parameters under salt stress [40]. Additionally, NO is reported to promote H_2O_2 accumulation in callus of *Populus euphratica*, which improves salt tolerance [41]. Addition of NO can increase the expression of *SOS1* and *NHX1* in *Avicennia marina* under salt stress [42]. These results indicate that both NO and ALA improve salt tolerance of plants by promoting H_2O_2 production and increasing the expression of salt tolerant genes, but the relationship between them is little known. Therefore, the possible role of NO on the salt tolerance of strawberry induced by ALA is studied in present work to understand the possible mechanisms of ALA in alleviating salt injury.

Results

Alleviations of ALA and SNP on plant growth inhibition under salt stress

The dry weights of both shoot and root of strawberry treated with 100 mM NaCl for 9 days were significantly lower than that of the control ($P < 0.01$), where the root dry weight of salt-treated plant was

only 45% of that of the control, while the shoot was 64% of the control ($P < 0.01$) (Fig. 1). This suggests that salt stress inhibited root growth more than leaf. If plants were treated with ALA, the dry weights of both leaves and roots between NaCl + ALA and the control were not significantly different ($P > 0.05$), suggesting that ALA almost completely eliminated growth inhibition caused by salinity. Specifically, the dry weight of roots treated with 10 mg L^{-1}ALA was 79% of the control, while that of shoot was 101%, compared with respective of the control. These indicate that ALA has a strong protective effect on the root growth against salt stress, and it can protect shoot growth avoiding salt injury. Similarly, 10 μM SNP treatment also had alleviating effect on growth inhibition of salt stress, where the dry weight of roots was 70% of the non-salt control, significantly higher than that of NaCl alone ($P < 0.05$). And, the aboveground part of SNP treatment was 94% of the non-salt control ($P > 0.05$). These suggest that exogenous NO also improves salt tolerance of strawberry, in a model similar with ALA. When NO production inhibitor, sodium tungstate Na_2WO_4, or NO scavenger cPTIO, were added with ALA, the effect of ALA to relieve salt inhibition disappeared. The results suggest that the effect of ALA on enhancing salt tolerance of strawberry may depend on the presence of NO.

Effect of ALA and SNP on NO levels in different tissues of strawberry under salt stress

The leaf NO content was generally much higher than that of roots (Fig. 2A). Salt stress induced its doubled increase in both of leaves and roots, and ALA further improved the increase in roots but completely blocked in leaves. Compared with the control, NaCl induced root NO increase by 1.39 times, while ALA induced increase by 3.56 times. However, the leaf NO content in ALA treatment was almost the same with the non-saline control. This exhibits different NO responses to ALA in different tissues. Similarly, the NO content in SNP-treated plants under salt stress was significantly higher than the non-salt control, but lower than that of NaCl alone in leaves ($P < 0.05$). This means that SNP inhibited NO increase in leaves but promoted it in roots under salt stress, a trend similar with ALA. On the contrary, addition with Na_2WO_4 or cPTIO completely inhibited NO increase in strawberry (including leaves and roots), indicating that two inhibitors completely inhibited endogenous NO increase in strawberry plants under salt stress, induced by salt stress or ALA treatment.

When expressions of *NR*, nitrate reductase gene which is responsible for NO generation, were analyzed by qRT-PCR (Fig. 2B), the results showed that salt stress induced it up-expression by 96%, while ALA treatment induced more 4.25-fold compared with NaCl treatment. SNP had no effect on *NR* expression, while Na_2WO_4 completely inhibited the gene expression induced by ALA. Thus, the increase of NO levels in roots induced by ALA might be dependent on the up-regulation of *NR* expression.

To confirm the promotive effect of ALA and NaCl on the NO content in roots of strawberry, the root tips were dyed by the specific fluorescent dyes DAF-FM DA and visualized using LSCM. No obvious NO fluorescence signals can be seen in roots of the control, but it increased greatly when plants were subject to NaCl (Fig. 3). When co-treated with ALA, the NO fluorescence further increased in the stele compared

with NaCl alone. SNP treatment induced the release of NO, filling up the entire root section. However, when Na_2WO_4 or cPTIO was added with ALA, the NO fluorescence signal was negligible again compared with NaCl + ALA treatment. This suggests that two inhibitors eliminated root NO induced by ALA in salt-stressed strawberry.

Effect of ALA and NO on the H_2O_2 content in different tissues of strawberry under salt stress

Strawberry leaves contained more H_2O_2 than roots (Fig. 4). Salt stress induced H_2O_2 increase significantly in both of leaves and roots. The treatment with ALA or SNP completely eliminated the H_2O_2 increase induced by salt stress in leaves but further promoted it in roots. This means that ALA and NO share a similar effect on H_2O_2 levels, although the H_2O_2 responses to ALA or SNP treatment under salinity are different between leaves and roots. Co-treatment of ALA with Na_2WO_4 or cPTIO greatly depressed the endogenous H_2O_2 content in the strawberry stressed by NaCl, which in the leaves was even significantly lower than NaCl treatment alone. Therefore, it can be deduced that H_2O_2 accumulation induced by NaCl either with or without ALA may be dependent on the presence of NO, which probably acts on the upstream of H_2O_2 signal route and participates in the induction of salt tolerance by ALA.

Effect of ALA and SNP on antioxidant enzyme activity of strawberry under salt stress

The SOD activity in leaves was much higher than that in roots, while the POD activity in roots was much higher than that in leaves, and the CAT activity in leaves was comparable with that in roots (Fig. 5). After NaCl stress, all three antioxidant enzymes activity increased significantly in both leaves and roots, and ALA or SNP treatment further promoted the increases. These suggest that ALA and NO possess similar ability to promote the activities of the antioxidant enzymes in strawberry under salt stress. When Na_2WO_4 or cPTIO was co-treated with ALA, the increases of enzyme activities induced by ALA were all eliminated. This means that the promotion of ALA is dependent on NO presence. If NO generation was inhibited or scavenged, ALA did not induce increases of the antioxidant enzyme activities. It is worth noting that the effects of ALA on antioxidant enzyme activities in leaves and roots of strawberry are consistent, without tissue specificity.

Effect of ALA and SNP on the ion content in different tissues of strawberry under salt stress

The Na^+ content in strawberry was rather low under non-saline condition, which increased more than 17-fold and 6-fold, respectively in leaves and roots after 100 mM NaCl treatment for 9 days (Fig. 6A). When

ALA was co-treated, the leaf Na^+ content was decreased to 40% of NaCl alone, but 11% more accumulated in the roots ($P < 0.05$). It suggests that ALA has ability to induce more Na^+ interception in roots with less transport upward to leaves. When Na_2WO_4 and cPTIO was co-treated with ALA, the leaf Na^+ content was 58%-64% higher than that without inhibitors, but in roots, the Na^+ interception induced by ALA was eliminated. These mean that ALA-induced Na^+ retention in roots is dependent on NO presence. When NO generation was blocked or eliminated, the root Na^+ retention was greatly impaired. Nevertheless, effects of exogenous SNP treatment on Na^+ distribution was some different from ALA. The Na^+ content in leaves and roots of SNP-treated plants was only 46% and 66% of NaCl treatment alone, respectively. Thus, SNP induced almost half decrease of total Na^+ content in strawberry under salt stress, instead of Na^+ retention in roots. The effect of SNP was not completely consistent with ALA.

Similar responses were found when the Cl^- content was measured. It increased to 2.7-fold and 2.2-fold of the control, respectively in leaves and roots under salt stress. If ALA was co-treated with NaCl, the Cl^- content in two tissues was 65% and 154% of NaCl treatment alone, respectively. Here, we found ALA treatment induced Cl^- retention in roots with much less transport upward (Fig. 6B), quite similar with Na^+. When co-treated with Na_2WO_4 and cPTIO, the ALA-induced Cl^- retention in roots was eliminated. Thus, the effect of ALA on Cl^- retention may be also dependent on NO presence. Interestingly, the effect of SNP treatment was not the same with ALA. The leaf Cl^- content of SNP treatment was 69% of NaCl alone, similar with ALA treatment, whose content of Cl^- in roots was also 70% of NaCl alone. Thus, the total Cl^- content in SNP-treated plants was about 70% of NaCl alone. No Cl^- interception in roots was found in SNP-treated plants, although the salt tolerance was significantly improved. Declination of salt uptake may be an important mechanism of salt tolerance induced by SNP.

Effects of ALA and SNP on gene expressions related with ion transporters

To understand molecular mechanisms underlying the role of ALA and NO in salt tolerance, we analyzed the expressions of genes related to Na^+ and Cl^- transport. Four *SOS* expressions in strawberry roots tended to increase after NaCl treatment for 48 h, however, only *SOS1* and *SOS2* were significant at $P = 0.05$ (Fig. 7). ALA treatment greatly induced all four *SOS* gene expressions under salt stress, and SNP also up-regulated most of *SOSs* but not *SOS4* expression. When Na_2WO_4 or cPTIO was co-treated with ALA, the up-regulation of gene expressions (including *SOS1*, *SOS2* and *SOS4*) by ALA was almost completely eliminated, suggesting these three gene up-expressions by ALA were dependent on NO presence. However, the up-expression of *SOS3* by ALA might not be dependent on NO, because the gene expression in Na_2WO_4 treatment was still 3.3 times as high as NaCl alone, and cPTIO did not inhibit the effect of ALA on *SOS3* expression. Thus, there should be other routes besides NO signal when ALA induced *SOS3* expression. The fact that ALA rather than SNP induced *SOS4* up-expression and two inhibitors of NO eliminated ALA's effect suggests that NO may be one of essential inductive factors for *SOS4* expression.

The responses of *NHX1*and *HKT1* expressions to different treatments were quite consistent with each other (Fig. 7B). It seems that salt stress tended to up-regulate these gene expressions, however, the effect was not significant at 48 h after salt stress. ALA and SNP dramatically induced the gene expressions, while Na_2WO_4 or cPTIO completely eliminated the promotion of ALA. These results suggest that ALA-induced gene expressions in strawberry roots was dependent on NO presence.

CLCs is a family of genes coding chloride channel proteins, mediating Cl^- uptake, transport and compartmentation. In strawberry genome, we found four genes highly homologous with *Arabidopsis* and analyzed their expressions by qRT-PCR. Salt stress up-regulated all *CLC* expressions in strawberry roots ($P < 0.05$) and ALA further up-regulated the increases under salt stress (Fig. 7C). SNP also up-regulated the expressions of *CLC-d*, *CLC-f* and *CLC-g* but not *CLC-a*. Co-treatment with Na_2WO_4 or cPTIO eliminated the promotion of ALA on *CLC-a*, *CLC-d* and *CLC-f* but not *CLC-g*. It seems that ALA treatment is sufficient to up-regulate *CLC-a* expression, where NO is essential but not enough. Therefore, only SNP addition was not enough to induce *CLC-a* up-expression, while Na_2WO_4 or cPTIO completely eliminated the effect of ALA. Additionally, ALA was sufficient to up-regulate *CLC-d* and *CLC-f* expression, where NO was both sufficient and essential. The responses of *CLC-g* expression were different from the formers. Its expression in ALA + Na_2WO_4 treatment was much higher than ALA itself, implying that inhibition of NO generation did not block the gene expression induced by ALA. Similarly, the gene expression in ALA + cPTIO treatment was comparable with ALA only. Thus, the up-regulation of *CLC-g* expression by ALA cannot be eliminated by NO inhibitors, which suggests that both ALA and NO induced the gene expression, but ALA-induced regulation did not depend on NO presence. ALA-induced up-expression of *CLC-g* may be mediated through the other routes.

Discussion

The beneficial effect that ALA improves plant salt tolerance has been received much attention in recent years [7, 24], but the regulatory mechanisms remain unclear. In most of previous studies, the increases of antioxidant enzyme activities are considered as the main mechanisms for ALA to improve salt tolerance [8, 23, 43, 44, 45]. Additionally, leaf pigments and photosynthesis [19, 46], proline accumulation [47], especially ion homeostasis [48] are also considered important. A great amount of salt ions is absorbed by plant roots, transported to shoots and accumulated in leaves, which is the lethal reason for salt injury and plant death. How to cope with harmful ions to maintain ion homeostasis under salt stress should be the key for plant salt tolerance. No clear relationship between ALA-induced salt tolerance and ion distribution has been known [49] until a recent report of our group. We found that ALA induced H_2O_2 accumulation in strawberry roots to selectively retain Na^+ in the underground with less accumulation in leaves under salt stress. We estimated Na^+ and K^+ levels in different tissues and xylem sap with several methods. All results turned out that NaCl stress significantly increased the Na^+ content in both leaves and roots with higher Na^+/K^+, and ALA further improved the root Na^+ content but depressed it in leaves [25]. Thus, ALA-induced Na^+/K^+ ratio increased greatly in roots but decreased in leaves. In xylem sap, ALA induced 33% decrease of Na^+ concentration but K^+ concentration was not different from the NaCl treatment without

ALA. This means that ALA induces Na^+ selective retention in roots (rather than K^+ selective transport upward) with less transport upward to shoots. The root intercepted Na^+ may be extruded by plasmolemma-located Na^+/H^+ antiporter (encoded by *SOS1*) into soil solution, or sequestrated into vacuoles by tonoplast-located Na^+/H^+ antiporter (encoded by *NHX1*) to store as cheap osmotic solutes. Additionally, *HKT1*, coding a Na^+-selective transporter was also induced up-expression by ALA, which is responsible for Na^+ unloading from xylem vessels to parenchyma cells in roots to reduce ion concentrations in xylem sap [1], or Na^+ removal from xylem sap to phloem through plasmodesmata via symplastic diffusion, then downward to roots, and avoiding too much Na^+ accumulation in shoots [50]. Thus, the findings, different from the other previous reports, open a new insight for us to understand the mechanisms of ALA in inducing salt tolerance, especially ion homeostasis strategy [26].

In present work, we once again observed that ALA treatment decreased the leaf Na^+ content but significantly increased the retention in the roots of strawberry (Fig. 6A). It is quite agreed with our previous findings [25]. Furthermore, we also found Cl^-, like Na^+, was preferably retained in roots with less accumulation in leaves after ALA treatment (Fig. 6B). In a transgenic canola (*Brassica napus*) which can over-produce endogenous ALA, the leaf Cl^- content is strictly limited to a very low level, even seedlings were stressed by 450 mM NaCl [51]. Additionally, in the previous study when the ion levels of xylem sap was measured, it was also found that ALA significantly depressed $[Cl^-]_{xylem}$ under salt stress, which were 2.39, 6.20 and 3.50 μmol L^{-1} in the control, NaCl stress and NaCl + ALA, respectively. Thus, ALA induces plants to intercept both Na^+ and Cl^- in roots to decrease the toxic ion contents in the shoots. The opinion is agreed with Hanin et al. [52], who pointed out that plants have evolved mainly two types of tolerance mechanisms to cope with salt stress, one is limiting the entry of salt by the roots, and the second is controlling its concentration and distribution. Comparatively, the other responses in ALA-treated plants, such as higher levels of leaf chlorophylls and photosynthetic capacity may be secondary mechanisms for plants salt tolerance [46, 47]. Additionally, we reported that ALA increased the K^+ content in both leaves and roots, but the K^+ concentration in xylem sap of strawberry was not improved by ALA treatment under salt stress [25]. Thus, K^+ level is not the most critical when ALA improves salt tolerance of strawberry.

In the previous report [25], the effect of ALA-induced root Na^+ retention was ascribed to the role of H_2O_2 on up-expressions of Na^+ transporter genes. H_2O_2 is known as a reaction oxygen species (ROS), as well as an important cellular signal. However, in most studies about ALA, H_2O_2 was considered as ROS rather than a cellular signal [23]. Never attention had been payed to different responses of H_2O_2 between shoots and roots [7] until Wu et al., who found that salt stress induced H_2O_2 increase in both leaves and roots, and ALA induced more H_2O_2 increase in roots but depressed in leaves [25]. The tissue-specific response is confirmed recently [53] and observed in present work once again (Fig. 4), which may be an important characteristic for ALA to induce plant stress tolerance. In another report, it was found that ALA decreased the $O_2^{\bullet-}$ production rates in both leaves and roots strawberry under salt stress, however, there was no

H_2O_2 information available [56]. In present work, we do not only validate the results of Wu et al. [25], but reveal that SNP, a NO donor also improves H_2O_2 increase in roots of strawberry, while Na_2WO_4 or cPTIO inhibit the H_2O_2 increase induced by ALA (Fig. 4). This means that ALA-induced H_2O_2 increase in roots of strawberry is dependent on NO presence. In another word, NO may be a signal located at the upstream of H_2O_2, involved in ALA-induced plant salt tolerance. However, in cucumber roots, ALA did not induce H_2O_2 increase [54]. The reason for this difference needs further clarification.

It is the first time to show NO involved in the signal route that ALA improves salt tolerance of strawberry (Fig. 1). NO is an important gaseous signal molecule involved in regulation of plant response to salt stress [33, 38]. In higher plants, nitrate reductase (*NR*) is the key enzyme for NO production [29, 55]. Since the activity is dependent on molybdenum, Na_2WO_4 is often used as competitive inhibitor [57]. In pakchoi [58] or barley [59], ALA has been found to induce *NR* up-expression and enzyme activity. In present work, we observed that NaCl and ALA significantly induced *NR* up-expression in roots of strawberry, while Na_2WO_4 completely eliminated the expression induced by ALA (Fig.2B). Thus, the inhibition of *NR* expression by Na_2WO_4 is responsible for the block of NO generation in strawberry under salt stress. It is interesting to notice that ALA induces NO accumulation in roots of strawberry but without effect in leaves (Fig. 2A), suggesting that NO generation induced by ALA is also tissue-specific, similar with H_2O_2 (Fig. 4). We used DAF-FM DA, a highly specific NO fluorescent probe to stain root tips of strawberry, the visual results are agreed with measurements with spectrophotometer (Fig. 3). Thus, both salinity and ALA greatly induce NO accumulation in roots of strawberry. However, the reason for ALA to induce NO accumulation only in roots not in leaves is not clear now. Yet, our unpublished data shows that carbon monoxide (CO), released by catalysis of heme oxygenase may be involved in the ALA-NO-H_2O_2 signaling route in ALA regulating salt tolerance of strawberry. We have found that hematin, a CO donor can induce both increases of NO and H_2O_2 levels in the roots of strawberry under salt stress, while hemoglobin, a CO scavenger can eliminate the root Na^+ interception induced by ALA. ALA is the key precursor of heme biosynthesis. Significant higher levels of endogenous heme have been reported in the *Yhem1* transgenic Arabidopsis [60] or the exogenous ALA treated pakchoi seedlings [21]. In mouse macrophage cell lines, exogenous ALA enhances the heme oxygenase gene (*HO-1*) expression, which catalyzes the rate-limiting step in the oxidative degradation of heme to free iron, biliverdin and CO [61]. Whether the similar mechanism occurs in higher plants is not known, but we can deduce that the signals of ALA in improving salt tolerance may exist in its conversion into porphyrin compounds and their metabolites, such as CO and NO.

However, SNP is an exogenous substance, which may generate cyanide and ferricyanide ions beside NO in aqueous solution [62]. One might argue that CN^- or $Fe(CN)_6^{3-}$ instead of NO is the key active factor in salt tolerance improvement induced by SNP. Fortunately, in an independent experiment, we observed that 5 μM cPTIO completely eliminated the effect of SNP on promotion of salt tolerance of strawberry, while treatments with 10 - 100 μM $K_4Fe(CN)_6$ did not exhibit significant effect on salt tolerance (Fig. 1S). Therefore, the promotion of SNP on salt tolerance can only be attributed to the effect of NO.

NO has been reported to induce antioxidant enzyme activity under salt stress [39, 63]. In present work, we found that salt stress, ALA and SNP all induced increases of antioxidant enzyme activity in strawberry, but eliminated by Na_2WO_4 or cPTIO (Fig. 5). These mean that ALA-induced antioxidant enzyme activity is dependent on NO presence. However, no tissue-specific responses of antioxidant enzyme activity to ALA are found here. Thus, the response of H_2O_2 accumulation to ALA is different from the antioxidant enzyme activity. In fact, the activities of all three enzymes measured in the study changed coincidently between leaves and roots after treatments. We can also see that leaves possess higher activity of SOD but lower POD than roots, and the CAT activities are comparable between roots and leaves. However, the biological meaning of differences is not clear. Correlation analysis shows that the root H_2O_2 content was significantly positive correlated with the antioxidant enzyme activities in roots, where $r_{SOD} = 0.898^{*}$, $r_{POD} = 0.944^{**}$, $r_{CAT} = 0.936^{**}$, respectively. However, the leaf H_2O_2 content does not correlate with three enzyme activities ($P > 0.05$). It seems that the relations between H_2O_2 and antioxidant enzyme activities in the leaves are more complex than that in the roots, which needs study further.

It is known that the most important genes related with Na^+ transport are *NHX1*, *HKT1*, and *SOSs* in *Arabidopsis* [64, 65]. In the previous report, Wu et al. proposed that ALA-induced H_2O_2 was necessary for up-expression of these genes (including *FaNHX1, FaHKT1* and *FaSOS1*) when strawberry was subject to salt stress [25]. In present study, both ALA and SNP enhanced expressions of *FaSOSs* (Fig. 6A), *FaNHX1* and *FaHKT1* (Fig. 6B) significantly in strawberry roots, while Na_2WO_4 or cPTIO eliminated the effect induced by ALA. Thus, we propose that NO is another necessary component in the signal cascade of ALA-induced salt tolerance of strawberry, at the up-stream of H_2O_2, responsible for up-regulation of Na^+ transporter gene expressions. In *Jatropha curcas*, NO has been suggested to decrease harmful ion accumulation under salt stress [38]. ALA-induced salt tolerance may accord with the similar signal route.

In *SOS* family of *Arabidopsis*, six genes have been identified. All of them consist of an *SOS* signaling route responsible for Na^+ transport [66]. AtSOS1 is the Na^+/H^+ antiporter located in plasma membrane. AtSOS2 interacts with AtSOS3 to form SOS2/SOS3 complex, in turn phosphorylating and activating AtSOS1 [67]. AtSOS4 is a pyridoxal kinase (PLase) involved in the biosynthesis of pyridoxal-5-phophate (PLP), regulating Na^+, K^+ channel or transporter. Thus, *sos4* mutant is hypersensitive to KCl and NaCl [68]. In strawberry, we find out four genes of *FaSOS* family, where *FaSOS1* and *FaSOS2* can be coincidently up-regulated by ALA and SNP under salt stress, but the effect is eliminated by Na_2WO_4 or cPTIO (Fig. 7A). These suggest that ALA as well as its induced NO is sufficient and necessary for the gene expressions. For *FaSOS3*, ALA can induce its up-expression, but cPTIO cannot eliminate the effect. This may imply that NO is sufficient but not necessary. There may be other regulatory branches. For *FaSOS4*, ALA rather than SNP can up-regulate its expression, and two inhibitors eliminate the promotion. This may suggest that NO is necessary but not sufficient. Obviously, the regulatory mechanisms of *FaSOSs* are more complex than we have known. Beside NO, other factors such as ABI2, 14-3-3 [66], WRKY40 [67], ethylene signals [69] may also be involved in regulation of SOS signal route. Furthermore, SOS2 and SOS3 complex can interact with CIPK and CBL proteins [4], which is an important node linking H_2O_2 and salt stress [70].

From our results here, it seems that the synergy of gene up-expressions by ALA under salt stress causes Na^+ extruded from the cytosol out of cells, where NO signaling is necessary in strawberry roots.

CLCs are a gene family coding important anion channels or transporters, widely distributed on the membranes of prokaryotic and eukaryotic cells to mediate Cl^- or NO_3^- transport [71]. In *Arabidopsis*, there are seven members of *CLCs*, including *AtCLC-a ~ AtCLC-g* [72]. The function and subcellular location of AtCLCs have been identified. For example, AtCLC-a as well as the highly homologues AtCLC-b, is responsible for NO_3^-/H^+ or Cl^-/H^+ transport across tonoplast [73]. AtCLC-c is also located on tonoplast, mainly in guard cells, may be involved in stomatal regulation and beneficial for plant salt tolerance [74]. AtCLC-d is localized to the membrane of Golgi bodies. AtCLCe is targeted to the thylakoid membrane in chloroplast and related to photosynthesis activity. AtCLC-f is localized on the Golgi apparatus and mainly responsible for Cl^- transport [75] while AtCLC-g mainly distributed in leaf mesophyll and phloem cells [76]. Based on the sequences of *Arabidopsis*, we obtained part of the respective homologs in strawberry. Analysis with qRT-PCR shows that the *FaCLC* expressions were significantly induced by salinity, while doubled or redoubled by ALA (Fig. 6C). Similarly, SNP also induced these gene up-expressions, suggesting that ALA-induced NO is involved in Cl^- compartmentation and transport in strawberry. Nevertheless, NR inhibitor Na_2WO_4 or NO scavenger cPTIO eliminated the up-expression of *FaCLC-a*, *FaCLC-d* and *FaCLC-f*, implying that the gene expressions induced by ALA may be dependent on NO presence. However, the expression of *FaCLC-g* induced by ALA cannot be blocked by the inhibitors, suggesting its regulation maybe be specific. Anyway, it is interesting to notice that all the gene up-expressions induced by ALA and NO seem to be beneficial for Cl^- subcellular compartmentation. It can also be used to reasonably explain the possible mechanisms for ALA to promote Cl^- retention in roots (Fig. 5B) and relieve salt injury of strawberries (Fig. 1) when they are subject to salt stress.

Conclusion

Our results here have verified the previous finding [25] that ALA induces Na^+ preferable retention in roots of strawberry to avoid excess accumulation in leaves. Also, Cl^- is preferably retained in roots with less transport to the aboveground after ALA treatment (Fig. 6). Thus, ALA-induced retention of Na^+ and Cl^- in roots may be one of the most important mechanisms for plants to cope with toxic ions. In the signaling cascade of ALA regulation, we find that ALA can induce NO and then H_2O_2 accumulation in roots of strawberry, where NO functions at the up-stream of H_2O_2 signaling, involved in up-regulating gene expressions for ion homeostasis under salt stress. We modified the hypothesis (Fig. 8) from the previous [25]. We believe that it is one of the most attractive hypotheses about ALA regulating salt tolerance up to now. More detailed interactions at different levels need to be elucidated in the future.

Methods

Plant material and treatments

Experiments were conducted in Nanjing Agricultural University. Strawberry (*Fragaria* × *ananassa* Duch. cv. 'Benihoppe') plants with uniform size were transplanted to a disposable plastic cup filled with mixture medium (peat: vermiculite: perlite = 4: 2: 1, v: v: v, about 2/3 attached to the top of cup), with one plant in each cup. Plants were watered with 1/2 Hoagland nutrient solution every three days. When the 6^{th} leaf fully expanded, the plants were divided into six groups for following treatments, with 15 plants in one group. (1) Control, each plant was poured with 200 mL 1/2 Hoagland solution. (2) NaCl, poured with 200 mL 1/2 Hoagland solution containing 100 mmol L^{-1} NaCl. (3) NaCl + ALA, poured with 200 mL 1/2 Hoagland solution containing 100 mmol L^{-1} NaCl and 10 mg L^{-1} ALA. (4) NaCl + SNP, poured with 200 mL 1/2 Hoagland solution containing 100 mmol L^{-1} NaCl and 10 μM sodium nitrosylpentacy (SNP, a donor of NO). (5) NaCl + ALA + Na_2WO_4, poured with 200 mL 1/2 Hoagland solution containing 100 mmol L^{-1} NaCl, 10 mg L^{-1} ALA and 5 μM Na_2WO_4, an inhibitor of nitrate reductase, which is responsible for NO generation, (6) NaCl + ALA + cPTIO, poured with 200 mL 1/2 Hoagland solution containing 100 mmol L^{-1} NaCl, 10 mg L^{-1} ALA and 5 μM 2,4-carboxyphenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, a specific scavenger of NO). Three days later, the control was poured with the same amount of 1/2 Hoagland solution, and the others were poured with NaCl solution without the other chemicals. Treatments were arranged in a completely randomized design with three replications. Five plants consisted of a block. Part of plants were harvested on the 2^{nd} day for gene expression analysis, part harvested on the 6^{th} day for physiological analysis, and the remains harvested on the 9^{th} day for growth and salty ion analysis.

Plant growth

Plant growth was measured in terms of dry weight of strawberry. Three plants were collected in a treatment, washed clear and divided into roots and shoots, oven dried at 105℃ for 10 min firstly, and then 70℃ for 72 h and weighed with a digital balance.

Measurement of NO levels

Endogenous NO content of strawberry after 6-days treatment was determined using the Greiss method with slight modifications [77]. Leaves and roots (0.5 g) were ground in a mortar in 5 mL of 40 mM cool HEPES buffer (pH 7.2) and little quartz sand. The homogenates were centrifuged at 8000 × *g* for 10 min at 4℃. One milliliter supernatant was mixed with 1 mL Greiss reagent, incubated at room temperature for 30 min. Absorbance was determined at 540 nm. NO content was calculated according to a standard curve of $NaNO_2$.

Fluorescent imaging of NO in roots

NO in roots was visualized using the highly specific NO fluorescent probe 3-amino, 4-aminomethyl-2', 7'-difluorofluorescein diacetate (DAF-FM DA), according to the method described by Corpas et al. [78]. The slices of strawberry roots were incubated with 20 μM DAF-FM DA at 25℃ under darkness for 2 h, then washed clear using phosphate buffer solution (pH 7.4) to discard excess fluorophore. DAF-FM DA fluorescence was visualized using a Zessi Laser Scanning Confocal Microscope (LSCM) with 480 nm excitation and 535 nm emission filters. Ten individual roots of each treatment were observed, and fluorescence intensities from about 50 cells were averaged for each treatment.

Determination of H_2O_2 content

H_2O_2 content of strawberry after 6-days treatment was determined according to the method of Liu et al. [79] with slight modifications. Leaves and roots (0.2 g) were ground in a mortar containing 5 mL of cool acetone and a little quartz sand. After 8000 × *g* centrifugation for 10 min, 1 ml of the supernatant was taken to mix with 3 ml mixture of CCl_4:$CHCl_3$ (3:1, V/V) and 5 ml distilled water to extract hydrophobic pigment. After 4000 × *g* centrifugation for 1 min, the water phase was taken for next reaction. In a reaction mixture, 1 mL of the supernatant, 1 mL of 5% titanium sulfate and 2 mL of strong ammonia were added in turn. After precipitate formed, the mixture was centrifuged at 8000 × *g* for 10 min to discard the supernatant. Then, 5 mL of sulfuric acid was added to dissolve the precipitation. Absorbance at 450 nm was determined with a spectrophotometer.

Assay of antioxidant enzymes

Fresh tissues (0.2 g) were ground in a mortar and pestle in 8 mL of 50 mM cool phosphate buffer (pH 7.8), containing 1% (w/v) polyvinyl pyrrolidone (PVP). The homogenates were centrifuged at 12000 × *g* for 15 min at 4℃. The supernatants were used for assays of enzyme activity [80]. Superoxide dismutase activity was determined according to Zhang et al. [81]. Peroxidase and catalase activities were determined according to Chance & Maehly [82].

Determination of Na^+ and Cl^-

About 0.2 g of dried powders of leaves and roots were put in a digestion tube containing 5 mL of HNO_3 for digestion in a micro-wave digestion system. The solution was transferred to a 50 mL volumetric flask and dilute with distilled water. The Na^+ concentrations were determined by an inductively coupled plasma optical emission spectrometer (ICP-OES 2100).

Determination of Cl^- content was referred to Lei et al. [83]. About 0.2 g tissue powder was added to 20 mL of distilled water, shaken in the boiling water bath for 1 h and cooled. The solution was diluted to 50 mL with deionized water. Appropriate amount of Cl^- test solution was taken in a 25 mL volumetric flask,

mixed with 2 ml of HNO_3 solution (concentrated HNO_3: deionized water = 3:1), 2 mL of acetone, 1 mL of 5 g L^{-1} $AgNO_3$. The reaction mixture was finally dilute to 25 mL with deionized water. The absorbance was measured at 335 nm after placing it in the dark for 10 min.

RNA extraction and qRT-PCR

The roots of strawberry 48 h after treatment were used for gene expression analysis. Total RNA was extracted with CTAB method according to TransScriptÒ One-Stpe gDNA Removal instructions. RNA was reversely transcribed to produce cDNA using First-Strand cDNA synthesis kit (Transgen Biotech), and the resulting cDNA mixture was used as templates for subsequent PCRs. Real-time quantitative PCR was performed according to the manufacturer's instructions using the SYBR® Premix Ex TaqTM (Tli RNaseH Plus, TaKaRa, RR420A). Relative gene expression of mRNA was calculated using the $2^{-\Delta\Delta CT}$ method [84]. All qRT-PCR primers were listed in Table 1 with *Actin* as the internal control. Three biological replicates were prepared for each sample.

Table 1
Primers of RT-PCR for strawberry genes related with salt stress

Primer name	Forward primer	Reverse primer
Actin	TGGGTTTGCTGGAGATGAT	CAGTTAGGAGAACTGGGTGC
CLC-a	ACGAACTGGCAACTTCAAACA	ATGGCAATACCAAGCATACGG
CLC-d	TCGCCGTGATCTTGTAACCTGT	TCCACTCTTGCACCATCCCATT
CLC-f	GCGAACCCGAGGTCTATT	GGTTGTCTGCCACGCTTA
CLC-g	GGGGCTGCTTCTTTACTTGG	AAACATCTGCCGTGGTCTTG
HKT1	GTTTGTGGCGTCATTGTT	GTGGGTCTTCCTTCATCTTT
NHX1	CTTTCTTGGGTTATTTGGCTTT	TAATGATGAGTTGGGTGTTTGA
SOS1	AGGCTATCAGCAGCTAAGAGGC	ACAGGTTCACCGTCCACATCAT
SOS2	GAGTCTCAAGGTCCATACACG	ATCAGCAAGTCGTCGTTCTA
SOS3	GCGTTTCTGAGTCCTATCTTC	TTCTAGCATTGCCTTATCTTG
SOS4	AAGCTGTATGTCCCTCCAGAGC	TGGTCCTGCAGCGTGAAGAATA
NR	ATCACTCCCGTTTACCAA	ATTCTCCACCACATACCAA

Statistical analysis

Each experiment was repeated at least three times. Values in figures were expressed as means ± se. The statistical significance of the data was analyzed using a univariate analysis of variance ($P < 0.05$) (one-

way ANOVA; version 20.0 IBM SPSS Inc. Chicago. IL).

Abbreviations

ALA: 5-aminolveulinic acid; CLC: chloride channel; CO: carbon monoxide; cPTIO: 2, 4-carboxy -phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA: 4-amino-5-methylamino-2,7-difluorofluorescein diacetate; HKT1: high-affinity potassium transporter 1; LSCM: laser scanning confocal microscope; NAD(P)H: nicotinamide adenine dinucleotide (2'-phosphate); NHX1: Na^+/H^+ antiporter 1; NO: nitric oxide; NOS: nitric oxide synthases; ROS: reactive oxygen species; NR: nitrate reductase; qRT-PCR: quantitative real-time polymerase chain reaction; SNP: sodium nitrosylpentacy; SOS: salt overly sensitive.

Declarations

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Authors’ contributions

LJW and YYA designed the research and wrote the article. RQC and QT repaired the materials and provided experimental assistance. SSH and HY carried out the research. All authors interpreted the data and approved the manuscript.

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Availability of data and materials

All data generated and analysed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The strawberry cultivar used in the experiment is widely planted globally. This article does not contain any studies with human participants or animals and did not involve any endangered or protected species.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

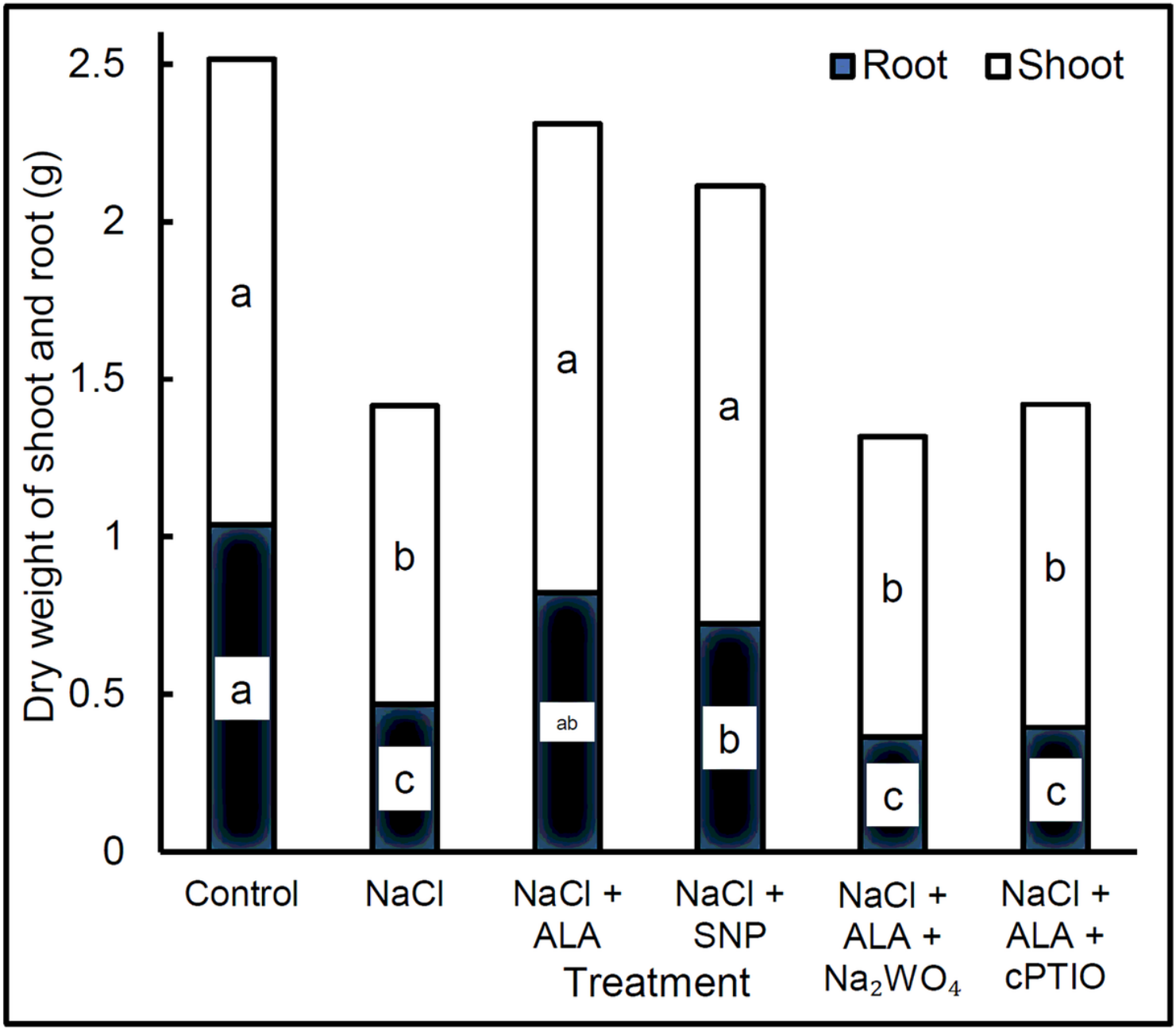

Figure 1

Comparison of the dry weights of roots and leaves of strawberry cultured for 9 days with the following solutions: Control, 100 mM NaCl, 10 mg L-1 ALA + 100 mM NaCl, 10 μM SNP + 100 mM NaCl, 10 mg L-1 ALA + 5 μM Na2WO4 + 100 mM NaCl, 10 mg L-1 ALA + 5 μM cPTIO + 100 mM NaCl. The same small letters in the bars of each tissues represent no significant differences at P = 0.05 level.

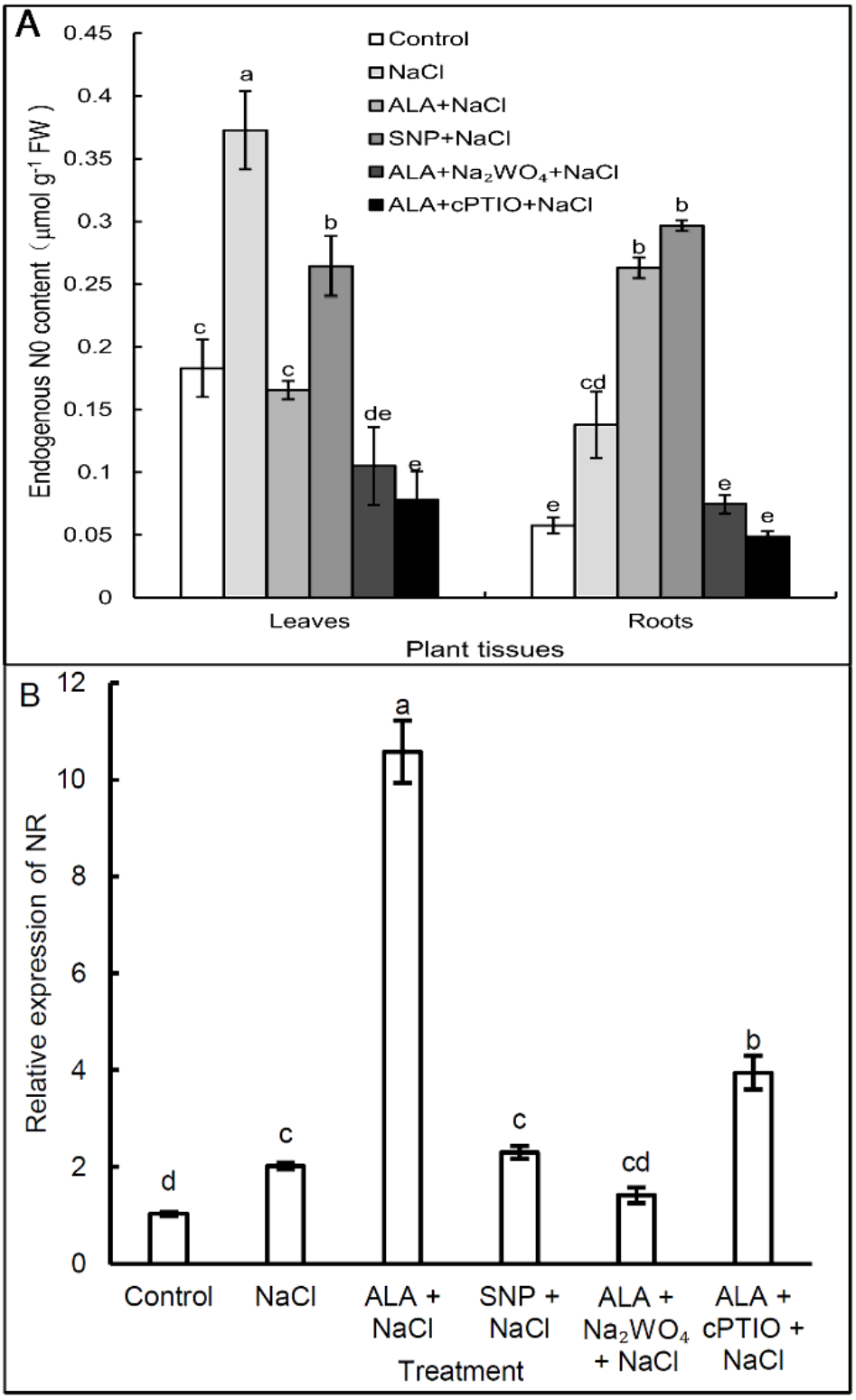

Figure 2

Effect of ALA and SNP on the endogenous NO content (A) and nitrate reductase gene (NR) expression (B) in roots of strawberry under salt stress. Plants were cultured for 6 days under the following solutions: Control, 100 mM NaCl, 10 mg L-1 ALA + 100 mM NaCl, 10 μM SNP + 100 mM NaCl, 10 mg L-1 ALA + 5μM Na2WO4 + 100 mM NaCl, 10 mg L-1 ALA + 5μM cPTIO + 100 mM NaCl. The same small letters above bars represent no significant differences at P = 0.05 level.

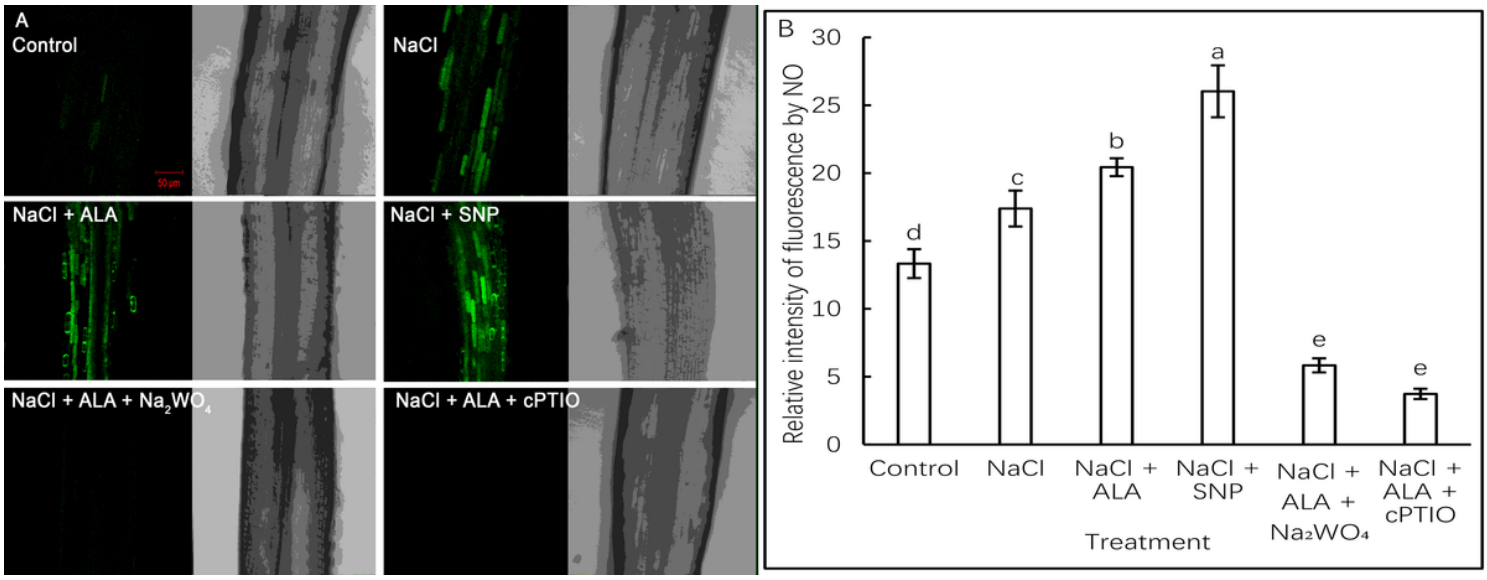

Figure 3

The green fluorescence intensity in the roots of strawberry, stained by DAF-FM DA, a highly specific NO fluorescent probe. The plants were cultured for 6 days with the following solutions: 1/2 Hoagland, 100 mM NaCl, 10 mg L-1 ALA + 100 mM NaCl, 10 μM SNP + 100 mM NaCl, 10 mg L-1 ALA + 5 μM Na2WO4 + 100 mM NaCl, 10 mg L-1 ALA + 5 μM cPTIO + 100 mM NaCl. A: fluorescence images, B: comparisons of fluorescent intensity digitalized by Zeiss confocal software. The different small letters above bars represent significant differences at P = 0.05 level.

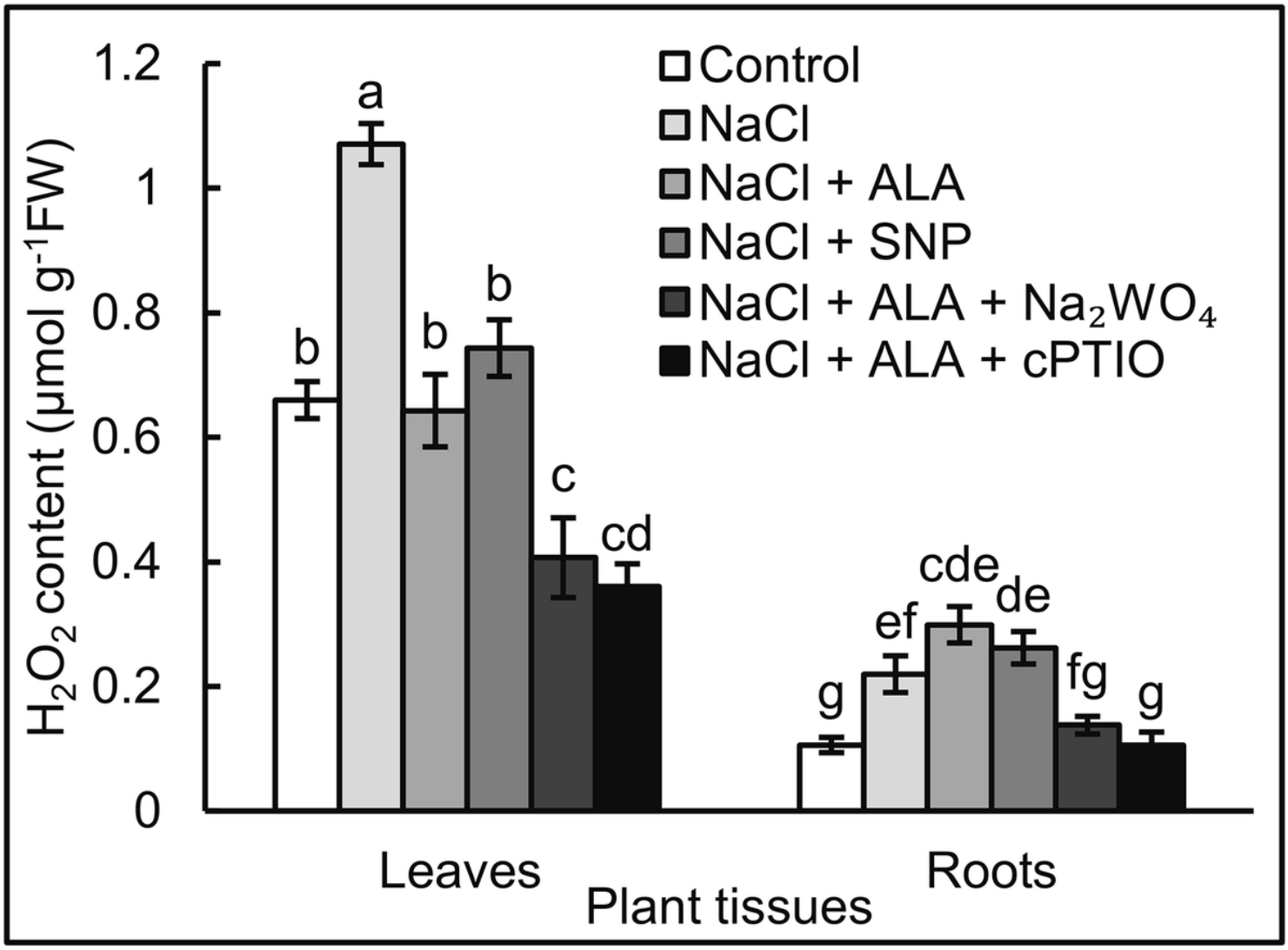

Figure 4

Effect of ALA and SNP on the endogenous H2O2 content in the leaves and roots of strawberry. Plants were cultured for 6 days under the following solutions: Control, 100 mM NaCl, 10 mg L-1 ALA + 100 mM NaCl, 10 μM SNP + 100 mM NaCl, 10 mg L-1 ALA + 5μM Na2WO4 + 100 mM NaCl, 10 mg L-1 ALA + 5μM cPTIO + 100 mM NaCl. The same small letters above bars represent no significant differences in each tissue at P = 0.05 level.

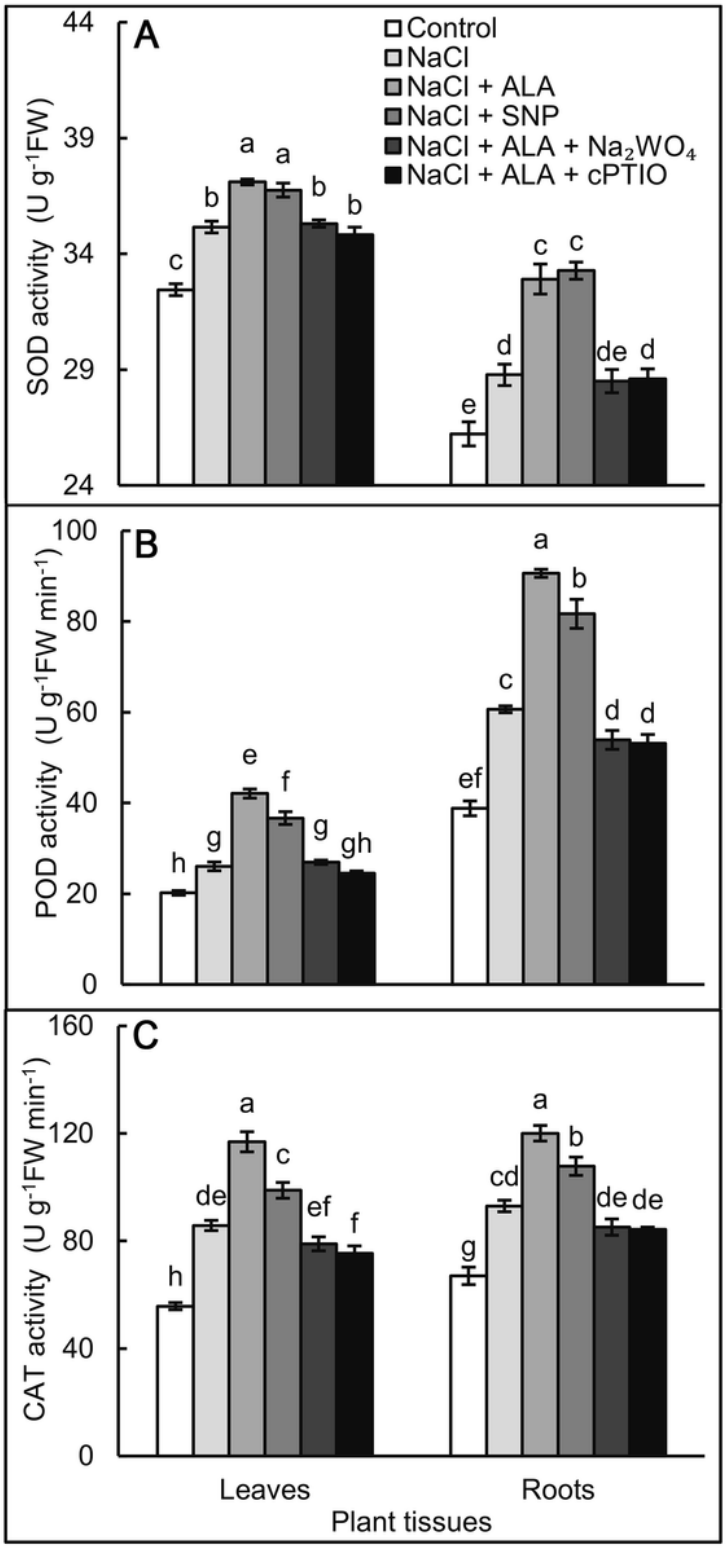

Figure 5

Effect of ALA and NO on the activities of SOD (A), POD (B) and CAT (C) in the leaves and roots of strawberry. The plants were cultured for 6 days under the following solutions: Control, 100 mM NaCl, 10 mg L-1 ALA + 100 mM NaCl, 10 μM SNP + 100 mM NaCl, 10 mg L-1 ALA + 5μM Na2WO4 + 100 mM NaCl, 10 mg L-1 ALA + 5μM cPTIO + 100 mM NaCl. The same small letters in each enzyme represent no significant differences at P = 0.05 level.

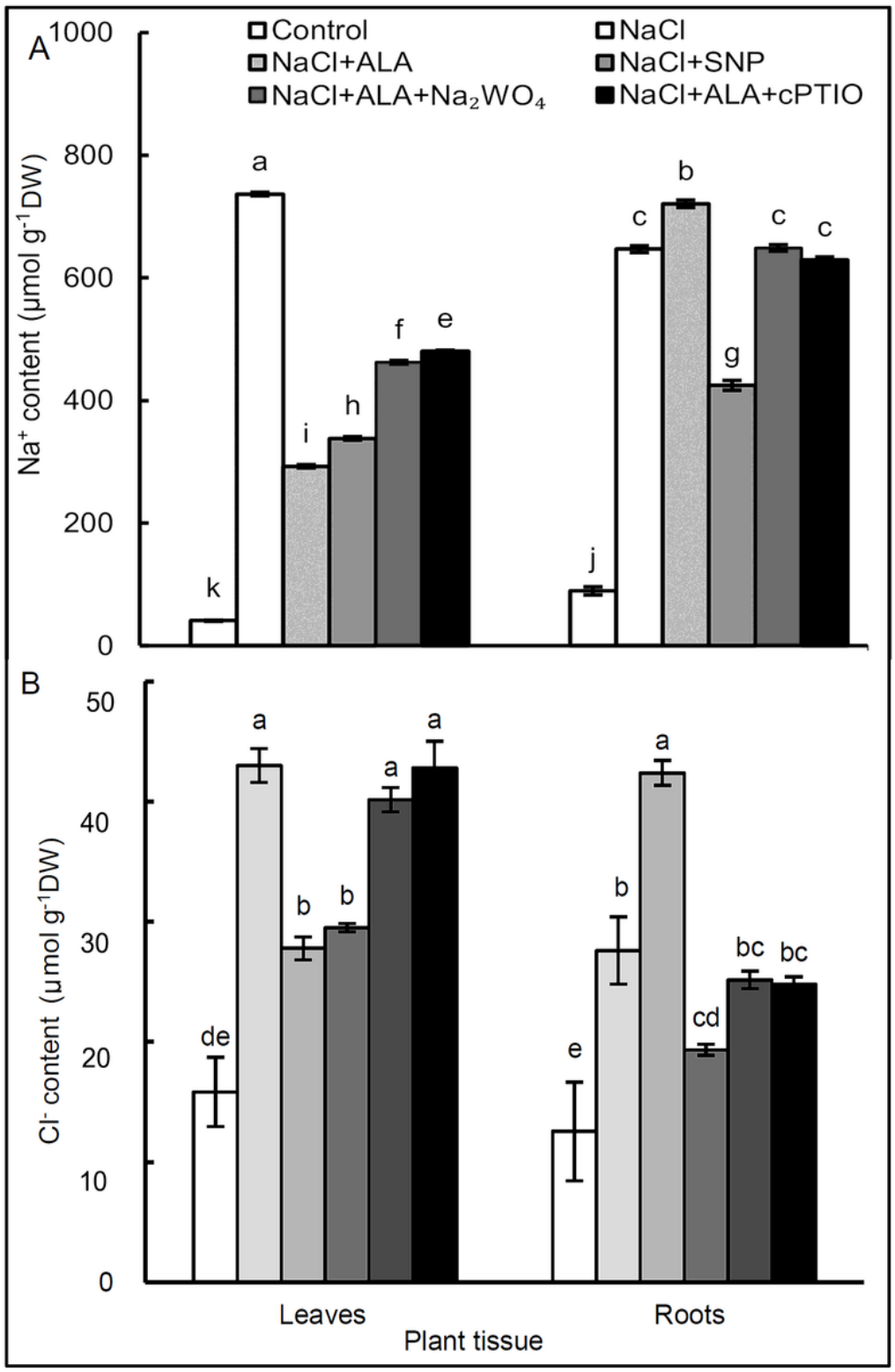

Figure 6

The content of Na+ (A) and Cl- (B) in strawberry plants after 9-days treatment in the following solutions: Control, NaCl, ALA + NaCl, SNP + NaCl, ALA + Na2WO4 + NaCl, ALA + cPTIO + NaCl. The same small letters in each ion represent no significant differences at P = 0.05 level.

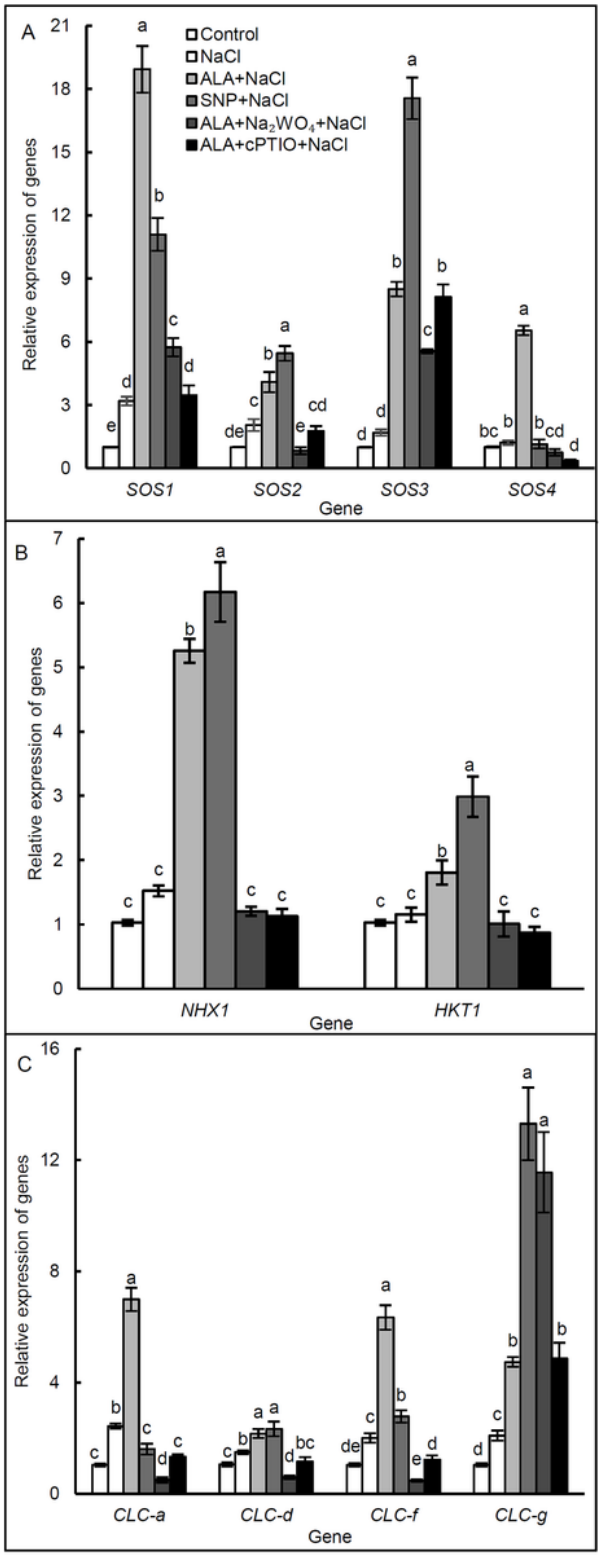

Figure 7

Expressions of genes related with Na+ and Cl- transport in roots of strawberry under salt stress, where A is SOS family genes, B includes NHX1 and HKT1 gene, C is CLC family genes. Root samples were taken at 48 h after treatments. The data are means of three replications and the same letters in each gene represent no significant difference at P = 0.05.

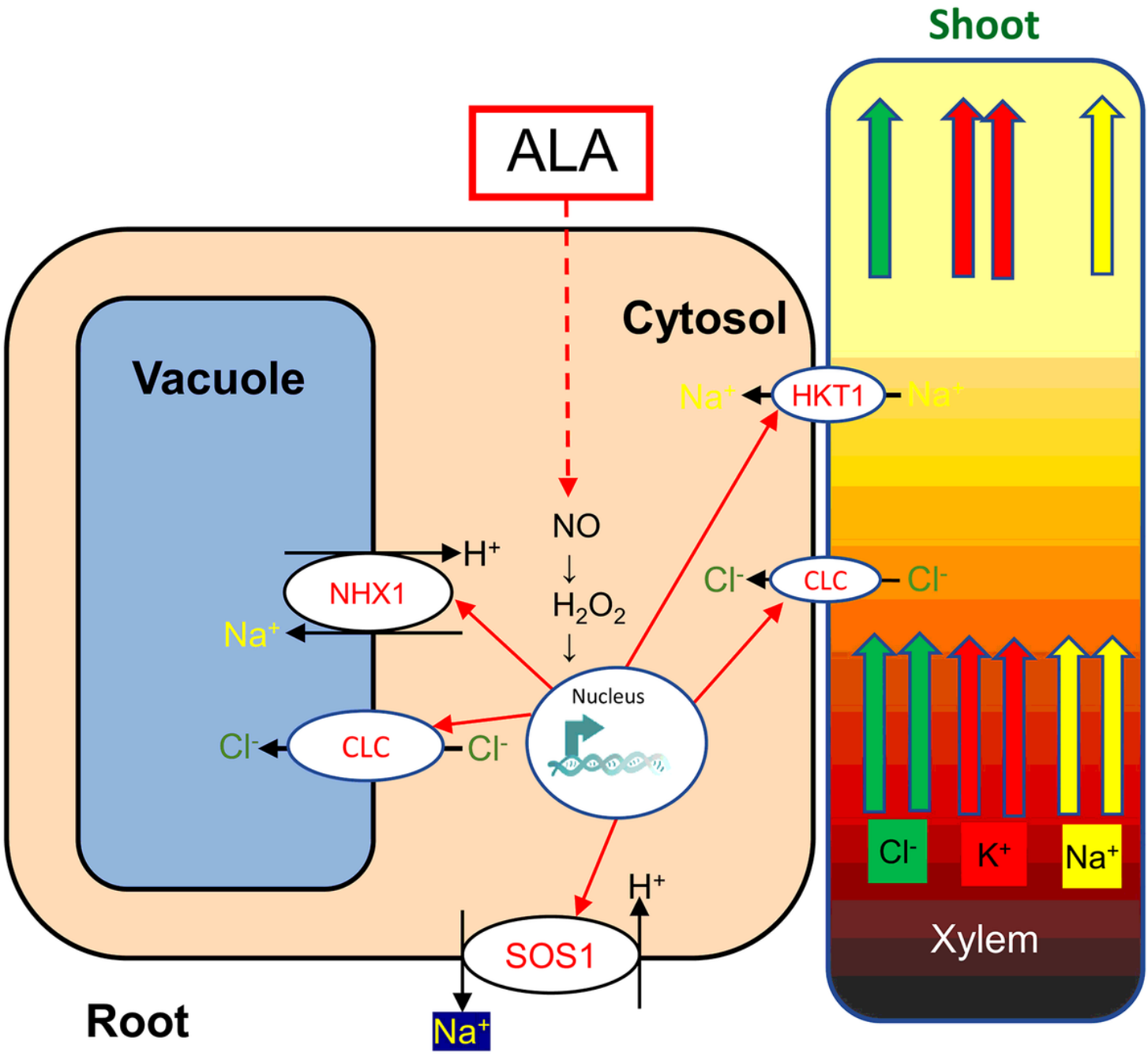

Figure 8

A new hypothesis for ALA to induce salt tolerance in strawberry, modified from Wu et al [25]

Supplementary Files

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