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ORIGINAL PAPER

Activation of gibberellin 20-oxidase 2 undermines auxin-dependent root and root hair growth in NaCl-stressed *Arabidopsis* seedlings

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Abstract Although salt stress mainly disturbs plant root growth by affecting the biosynthesis and signaling of phytohormones, such as gibberellin (GA) and auxin, the exact mechanisms of the crosstalk between these two hormones remain to be clarified. Indole-3-acetic acid (IAA) is a biologically active auxin molecule. In this study, we investigated the role of *Arabidopsis* GA20-oxidase 2 (GA20ox2), a final rate-limiting enzyme of active GA biosynthesis, in IAA-directed root growth under NaCl stress. Under the NaCl treatment, seedlings of a loss-of-function *ga20ox2-1* mutant exhibited primary root and root hair elongation, altered GA_4 accumulation, and decreased root Na^+ contents compared with the wild-type, transgenic *GA20ox2*-complementing, and *GA20ox2*-overexpression plant lines. Concurrently, *ga20ox2-1* alleviated the tissue-specific inhibition of NaCl on IAA generation by YUCCAs, IAA transport by PIN1 and PIN2, and IAA accumulation in roots, thereby explaining how NaCl increased *GA20ox2* expression in shoots but disrupted primary root and root hair growth in wild-type seedlings. In addition, a loss-of-function *pin2* mutant impeded *GA20ox2* expression, indicating that GA20ox2 function requires PIN2 activity. Thus, the activation of GA20ox2 retards IAA-directed primary root and root hair growth in response to NaCl stress.

Keywords GA20ox2 · NaCl · IAA · PIN1/2 · Primary root or root hair length

Introduction

High salt stress interferes with the growth and development of plant roots, including primary roots, lateral roots, and root hairs. The plant hormones gibberellin (GA) and auxin are involved in the responses to salt stress (Dinneny 2014). GA mediates plant root growth by altering root cell proliferation and elongation (Kuraishi and Muir 1962; Achard et al. 2009; Ubeda-Tomas et al. 2009; Colebrook et al. 2013), and indole-3-acetic acid (IAA) is the principal biologically active auxin. Auxin regulates root growth in a dose-dependent manner, and physiological concentrations of IAA are capable of promoting plant root growth and development (Pierik and Testerink 2014). Although high salinity controls root growth, the critical mechanisms involved in the functional integration between GA and IAA remain to be elucidated.

The regulation of GA biosynthesis affects plant growth and development (Tanimoto 2012), and the specificities of the biosynthetic enzymes involved in this process are generally understood. Among the more than 130 GA metabolites, only a few, including GA_1, GA_3, GA_4, and GA_7, have biological activities, while the other non-bioactive GAs act as precursors for the bioactive forms or are deactivated metabolites in plants (Yamaguchi et al. 2008; Petricka et al. 2012; Daviere and Achard 2013). The diversity of GA metabolites indicates that the acquisition of bioactive GA requires a series of very complex processes that involve various

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enzymes. Most of the genes encoding GA biosynthetic enzymes have been cloned and characterized. The final rate-limiting enzymes are a set of GA20-oxidases (GA20oxs) that belong to the 2-oxoglutarate-dependent dioxygenase family. The genome of the model plant *Arabidopsis thaliana* L. has five *GA20ox* genes, namely *GA20ox1–5*. Various studies have examined differences in expression patterns and physiological roles in this small gene family. Each of the five members has a spatiotemporal expression profile (Phillips et al. 1995; Garcíamartínez et al. 1997; Rebers 1999; Carrera and Prat 1999) and is considered to play specific roles in regulating physiological or developmental programs (Sakakibara 2005). In vitro studies showed that GA20ox1, GA20ox2, and GA20ox3 catalyze all of the steps in the conversion of the C_{20} intermediate GA_{12} to GA_9, which is the immediate precursor of active GA_4 (Phillips et al. 1995). GA20ox3 functions almost entirely redundantly with GA20ox1 and GA20ox2 in some developmental phenotypes, with the expression patterns of *GA20ox1* and *GA20ox2* genes partially overlapping (Rieu et al. 2008; Plackett et al. 2012). In one study, the different physiological roles of *GA20ox1* and *GA20ox2* were examined by characterizing the phenotypes of the loss-of-function mutants *ga20ox1* and *ga20ox2* (Rieu et al. 2008). *GA20ox1* expression in the loss-of-function *ga20ox2-1* mutant was not increased, whereas *GA20ox2* expression was strongly up-regulated in the leaves and internodes of *ga20ox1* plants (Rieu et al. 2008). GA_4 levels were reduced in *ga20ox2-1* but not in *ga20ox1* (Rieu et al. 2008), indicating that GA20ox2 activity is the main contributor to GA_9 and GA_4 production (Fernando et al. 2014). In contrast to GA20ox1, the activation of GA20ox2 plays an important role in the modification of *Arabidopsis* seedling growth through the MADS-box transcription factor Short Vegetative Phase (Fernando et al. 2014). Thus, the activation of GA20ox2 may be relatively specific, but the molecular mechanisms are mostly unknown. Interestingly, GA's control of root growth occurs particularly in response to salt and drought stresses (Duan et al. 2013; Yu et al. 2013; Colebrook et al. 2013). Although *GA20ox2* expression mainly occurs in the shoot apices of *Arabidopsis* seedlings (Fernando et al. 2014) and the loss of *GA20ox2* function results in a decrease in GA metabolites (Plackett et al. 2012), the specificity of GA20ox2 activity in salt-controlled root responses remains to be clarified.

In addition to GA, auxin/IAA is required for plant root growth. Local IAA levels are vital for plant root morphogenesis (Pierik and Testerink 2014), with the optimum level of IAA generally being determined by the activation of biosynthetic enzymes and transporters. YUCCAs are flavin monooxygenase family members that catalyze a rate-limiting step in IAA biosynthesis (Di et al. 2016). The *Arabidopsis* genome has 10 *YUCCA* genes, namely *YUCCA1–10* (Kasahara 2015). Limited evidence exists, however, for the involvement of IAA biosynthetic enzymes in root growth. In contrast, IAA transporters, especially the plasma membrane IAA polar transporters known as PIN-FORMEDs (PINs), regulate primary root growth and root hair development. The mechanisms of PIN-driven IAA redistribution are generally understood. In roots, PIN1 or PIN2 transports IAA towards the shoot through stele cells or towards the root tip through epidermal cells, respectively (Moubayidin et al. 2010; Willige et al. 2011). The loss-of-function *pin1* mutant, accordingly, impairs shoot tissue differentiation and development (Gälweiler et al. 1999), while the loss-of-function *pin2* mutant causes diminished IAA accumulation in root cells, reducing root hair growth (Ottenschlager et al. 2003). In addition, the root hair-specific over-expression of PIN1 or PIN2 greatly inhibits root hair growth by depleting IAA levels in root hair cells (Sarnowska et al. 2013). The endodermis buffers local IAA levels through IAA transporters, while local IAA maxima define lateral root initiation (Vermeer et al. 2014; Marhavý et al. 2016). Thus, the PIN-driven IAA distribution may be required for primary root, root hair, and lateral root growth. Yet to be determined, however, is how plants integrate the YUCCA-catalyzed IAA generation with the PIN-driven IAA distribution, especially in root growth and development.

Importantly, the functions of GA and IAA converge in roots to regulate cell expansion and root growth (Benkova and Hejatko 2009). Like the application of the IAA-transport inhibitor 1-N-naphthylphthalamic acid, the *pin1* mutant attenuates the effect of GA on root growth (Benkova and Hejatko 2009). In addition, NaCl reduces the expression of PIN1 and PIN2 but does not affect local IAA biosynthesis in roots (Liu et al. 2015). A reasonable explanation is that NaCl disturbs the conjunction of IAA generation and its transport, and thus impairs the optimum IAA concentration needed for root growth. Interestingly, the major GA-responsive tissue in roots is the endodermis (Dinneny 2014), which is a gateway for solutes to sense and respond to Na^+ toxicity (Duan et al. 2013; Dinneny 2014). This information raises the question as to whether and how *GA20ox2* is involved in such NaCl-controlled root growth. Thus, in this study, whether and how NaCl modifies *GA20ox2* gene expression, and whether this modification affects PIN-dependent IAA distribution during root growth and development, were studied. The findings suggest that GA20ox2 has an important role in NaCl-controlled primary root and root hair growth through its mediation of IAA generation and transport.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 was used as the wild-type (WT) in the experiments. T-DNA insertion

lines *ga20ox2-1* (At5G51810, GABI-KAT734G06) and *pin2* (At5G57090, SALK_122916.49.40.x) were purchased from the *Arabidopsis* Biological Resource Center, and the respective homozygous mutant plants were confirmed by PCR amplification. PIN2-green fluorescent protein (GFP) and DR5-β-glucuronidase (GUS) seeds (Columbia-0 background) were donated by Jian Xu (Sassi et al. 2012).

All of the seeds were collected and stored under identical conditions. Seeds were surface-sterilized with 0.1% $HgCl_2$ for 5 min, washed five times with distilled water, and sown on Murashige–Skoog (MS) medium (0.6% agar and 3% sucrose). The plates were kept at 4 °C for a 3-day vernalization period and then transferred to a growth chamber for a 3-day germination period. Growth room conditions were as follows: 22 ± 2 °C, a 16-h-light/8-h-dark photoperiod, 65% relative humidity, and a light intensity of approximately 100 µmol m^{-2} s^{-1}. Seedlings were subsequently transferred to fresh MS medium (0.8% agar and 3% sucrose) with or without supplementation. Seedling age (days) was counted from the day of transfer. Plates were placed vertically during seedling growth. In appropriate experiments, seedlings were cut into two sections, roots and shoots. The cutting point was the lower end of the hypocotyls.

Plasmid construction and plant transformation

To construct a *GA20ox2*-complementation line, the promoter fragment and coding sequence of *GA20ox2* were amplified and the resulting product cloned into the pCAMBIA1300 vector. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into *ga20ox2-1* plants by floral infiltration. To construct a *GA20ox2* over-expression vector, full-length *GA20ox2* cDNA was amplified and cloned into the pSUPER1300 vector. The constructs were introduced into *A. tumefaciens* strain GV3101 and transformed into WT plants by floral infiltration. Transformed T_1 plants were selected on hygromycin-containing medium. *GA20ox2* expression in the transgenic lines was detected by reverse-transcription PCR (RT-PCR). Among the *GA20ox2*-complementation plant lines, 3 of the 12 transgenic lines had similar levels of *GA20ox2* mRNA. Among the *GA20ox2* over-expression plant lines, 2 of the 13 transgenic lines had higher levels of *GA20ox2* mRNA. These transgenic lines were selected and used in the following experiments. To generate the *GA20ox2-GUS* construct, the *GA20ox2* promoter fragment was amplified and then cloned into the promoter-less GUS expression vector pCAMBIA1381.

The constructs were introduced into *A. tumefaciens* strain GV3101 and transformed into WT plants by floral infiltration. Transformed T_1 plants were selected on hygromycin-containing medium. *GA20ox2-GUS/pin2, PIN2-GFP/ga20ox2-1*, and *DR5-GUS/ga20ox2-1* lines were obtained by crossing. Pollen was transferred from *GA20ox2-GUS*, PIN2-GFP, and *DR5-GUS* transformed plants to the mature stigmas of *pin2* and *ga20ox2-1* plants. T_1 plants were self-pollinated and grown to form the T_2 generation. T_2 plants were screened on hygromycin-containing medium and identified by RT-PCR. Homozygous T_3 plants were used in the experiments.

Gene expression analysis

Gene expression was analyzed using RT-PCR or qRT-PCR. Based on Duan et al. (2013) and Han et al. (2014), 7-days-old *Arabidopsis* seedlings were used in this work. Total RNA was extracted from 100 mg of roots or shoots grown with or without NaCl using a Plant RNA MIDI kit (Life Feng, Shanghai, China). cDNA was synthesized from the RNA using an oligo (dT)18 primer and Moloney murine leukemia virus reverse transcriptase (Promega, http://www.promega.com). For RT-PCR, the volume of each cDNA sample was adjusted to produce the same signal strength for *Actin2* after 22–24 cycles, and the products were analyzed by electrophoresis on 1.2% agarose gels. qRT-PCR experiments were performed using gene-specific primers and SYBR Premix (Takara, http://www.takara-bio.eu/) on an ABI 7500 real-time PCR system (Bio-Rad, USA).

All of the primers used for vector construction and the gene expression analysis are listed in Fig. S2.

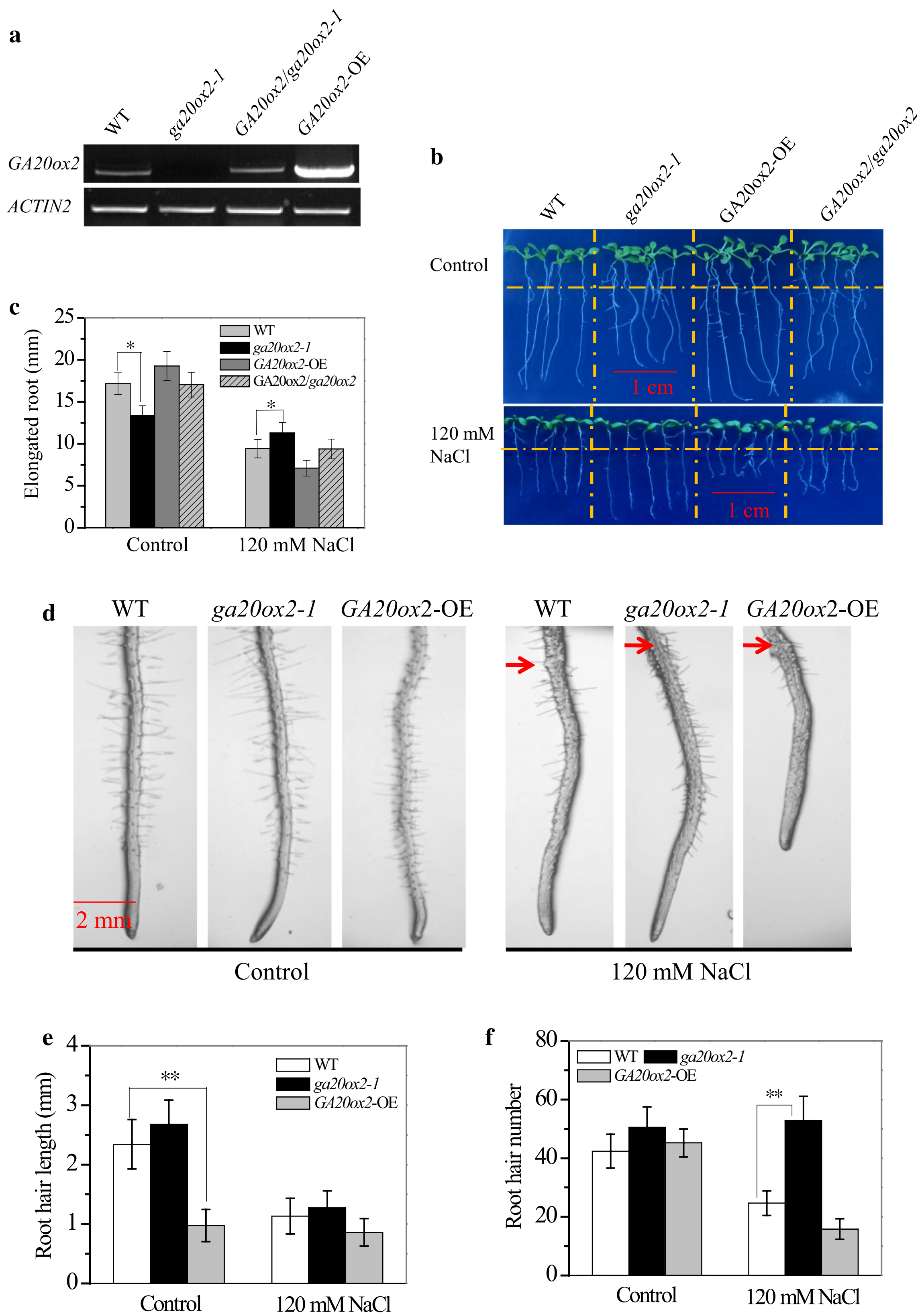
Quantification of primary root and root hair lengths

The lengths of the primary roots of seedlings were measured under a FV1000 microscope (Olympus, Tokyo, Japan). Root hairs were observed as described by Bai et al. (2014). Using a FV1000 microscope and Image J software (http://rsbweb.nih.gov/ij), root hair length and root hair density were determined by measuring the longest root hairs of 10 roots and by counting the number of root hairs within 1 cm of the root tip of each line, respectively.

GUS staining and quantification assay

Histochemical GUS staining was performed according to Han et al. (2014). Seedling roots or shoots were incubated for 6 h in the dark at 37 °C in GUS staining solution (0.1 M sodium phosphate buffer, pH 7.0; 0.05 mM $K_3[Fe(CN)_6]$; 0.05 mM $K_4[Fe(CN)_6]$; 1 mg/ml X-Gluc; and 0.1% Triton X-100) and then maintained for 3 h in 70% ethanol at 65 °C for the removal of chlorophyll. Photographs were obtained using a Stereo-Zoom microscope and a Nikon Coolpix digital camera.

A GUS quantitative assay was performed by homogenizing 20-mg samples in extraction buffer (50 mM Na_3PO_4, pH 7.0; 10 mM β-mercaptoethanol; 1 mM Na_2EDTA; 0.1%



a
WT
ga20ox2-1
GA20ox2/ga20ox2-1
GA20ox2-OE
GA20ox2
ACTIN2
b
WT
ga20ox2-1
GA20ox2-OE
GA20ox2/ga20ox2
Control
120 mM NaCl
1 cm
1 cm
c
Elongated root (mm)
WT
ga20ox2-1
GA20ox2-OE
GA20ox2/ga20ox2
Control
120 mM NaCl
d
WT
ga20ox2-1
GA20ox2-OE
WT
ga20ox2-1
GA20ox2-OE
2 mm
Control
120 mM NaCl
e
Root hair length (mm)
WT
ga20ox2-1
GA20ox2-OE
Control
120 mM NaCl
f
Root hair number
WT
ga20ox2-1
GA20ox2-OE
Control
120 mM NaCl

◂**Fig. 1** Root morphogenesis of *ga20ox2-1* seedlings in response to NaCl treatment. **a** Comparison of *GA20ox2* expression levels among the loss-of-function mutant *ga20ox2-1*, WT, transgenic *GA20ox2*/*ga20ox2-1* complemented line, and transgenic *GA20ox2*-OE over-expression line seedlings. **b, c** Primary root growth status (**b**) and root length statistics (**c**) of WT, *ga20ox2-1, GA20ox2*-OE, and *GA20ox2*/*ga20ox2-1* seedlings in the presence or absence of NaCl. **d**–**f** Root hair growth status (**d**), root hair length (**e**), and root hair number statistics (**f**) of WT, *ga20ox2-1*, and *GA20ox2*-OE seedlings with or without the NaCl treatment. The red arrows in (**d**) indicate the beginning of NaCl treatments. Samples selected randomly from three independent experiments are shown. Values are means ± SDs (** $P < 0.01$, * $P < 0.05$) of 90 seedlings

sodium lauryl sarcosine; and 0.1% Triton X-100). Each extract was centrifuged at 13,000×*g* for 15 min at 4 °C, and the supernatant was used for measurements. Protein concentrations were normalized with Bradford reagent (Bio-Rad). The fluorescence of 4-methylumbelliferyl-β-glucuronide hydrate (Sigma-Aldrich) was measured on a Fluoroskan Ascent FL fluorometer (excitation, 365 nm; emission, 455 nm). Measurements were read every 30 min and fitted to a standard curve. Enzyme activity was calibrated to the 4-methylumbelliferone concentration.

Confocal microscopy

Roots were dissected from 7-days-old WT and *ga20ox2-1* seedlings grown on MS medium with or without NaCl. PIN2-GFP reporter activity was analyzed using a Zeiss LSM710 confocal microscope, with image analyses performed using Zeiss 2011 software (excitation, 488 nm; emission, 500–550 nm), and all pictures were acquired with exactly the same confocal settings. All image analyses were repeated at least three times.

Measurement of Na^+ content

Each 7-days-old seedling (incubated with or without 120 mM NaCl for 4 h) was cut into just two parts, including root and shoot. After incubation at 110 °C for 10 min, the segments were dried at 70 °C for 48 h. The dried tissues were incinerated at 550 °C for 6 h. Each aliquot of sample ash was dissolved in 0.5 M HCl solution to determine its Na^+ content by inductively coupled plasma–atomic emission spectrometry (Perkin-Elmer Optima 2100DV, Shelton, CT, USA).

Measurement of the GA_4 content

GA_4 levels were monitored according to Fambrini et al. (2011) and Kurepin et al. (2015). Shoot or root material (0.5–1 g) was ground in liquid nitrogen and transferred to a 4-ml EP tube. After the addition of 1 ml cold 80% (v/v) methanol (first-grade chromatographic quality) and 1% (v/v) acetic acid, each tube was incubated at 4 °C for 12 h. The samples were centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatants were sequentially passed through a column containing C_{18} adsorbent (Qasis MCX 3 cc, 60 mg), evaporated to dryness under vacuum at 30 °C, and then re-suspended in 200 µl 80% (v/v) methanol and 1% (v/v) acetic acid. Aliquots of 100 µl were tested, and three biological replicates were performed. The analysis was performed on an Applied Biosystems MDS SCIEX 4000 QTRAP liquid chromatograph-tandem mass spectrometry system.

Statistical analysis

Differences in various parameters were compared using Student's *t*-test (** $P < 0.01$, * $P < 0.05$). Data from at least three biological replicates were analyzed with similar results.

Results

Root morphogenesis of *ga20ox2-1* seedlings exposed to NaCl stress

To investigate the possible effects of *GA20ox2* expression on the root growth of NaCl-exposed *Arabidopsis* seedlings, we prepared various seeds with altered *GA20ox2* expression levels. The *ga20ox2-1* mutant was identified as a T-DNA knock-out mutant (Fig. 1a). Complementation (*GA20ox2*/*ga20ox2-1*) and over-expression (*GA20ox2*-OE) plants were created using transgenic methods and had mRNA levels of the *GA20ox2* gene equal to, or significantly exceeding, those of WT seedlings (Fig. 1a).

We first compared root elongation in the loss-of-function *ga20ox2-1* mutant with that in WT seedlings in response to various NaCl concentrations. The 120 mM NaCl treatment significantly altered root elongation in *ga20ox2-1* and WT seedlings (Fig. S1). To demonstrate that this alteration resulted from GA20ox2 expression, we compared the effects of 120 mM NaCl on the elongated root of the altered GA20ox2-expression lines. After growth on free MS medium for 7 d post-transfer, the root lengths of *ga20ox2-1* seedlings (13.35 ± 1.18 mm) were shorter than those of the WT (17.17 ± 1.29 mm), *GA20ox2*-OE (19.27 ± 1.73 mm), and *GA20ox2*/*ga20ox2-1* (17.04 ± 1.48 mm) (Fig. 1a, b). Thus, *GA20ox2* expression levels are positively correlated with the elongation of primary roots in *Arabidopsis* seedlings. We expected root lengths of the complementation *GA20ox2*/*ga20ox2-1* lines to be similar to those of the WT. Interestingly, when seedlings were grown on MS medium containing 120 mM NaCl for 7 days, the net elongation of *ga20ox2-1* seedling roots was 11.28 ± 1.25 mm, which was longer than that in the WT (9.42 ± 1.09 mm), *GA20ox2*/*ga20ox2-1* (9.39 ± 1.18 mm), and *GA20ox2*-OE

(7.09 ± 0.94 mm) (Fig. 1a, b). Thus, primary root elongation in *Arabidopsis* seedlings in the presence of NaCl stress was negatively correlated with *GA20ox2* expression levels, a result contrary to that of the blank control. These observations suggest that NaCl can modify GA20ox2-associated root elongation and that the loss-of-function mutant *ga20ox2*'s root growth was insensitive to salt stress in *Arabidopsis* seedlings.

We also examined how NaCl changed the lengths and densities of *ga20ox2-1* root hairs. Under control conditions, no significant differences in the lengths and densities of root hairs were found between the WT and *ga20ox2-1* (Fig. 1d–f), whereas *GA20ox2*-OE root hairs were shorter than those of WT and *ga20ox2-1* seedlings (Fig. 1d–f). Under the 120-mM NaCl treatment, root hairs were longer in *ga20ox2-1* plants (1.27 ± 0.29 mm) than in the WT (1.13 ± 0.30 mm), while the number of elongated root hairs was significantly elevated in *ga20ox2-1* (52.83 ± 8.27 mm) compared with the WT (24.67 ± 4.18 mm). In addition, almost no root hair elongation was found in *GA20ox2*-OE seedling roots (Fig. 1d–f). These results indicate that the activation of *GA20ox2* inhibits the elongation (but not the initiation) of root hairs. We, therefore, hypothesized that the activation of GA20ox2 plays a role in NaCl-modified primary root or root hair growth.

Retardation of active GA accumulation in *ga20ox2-1* roots

Because active GA_4 regulates root elongation and specifically accumulates in the root tips of *Arabidopsis* seedlings (Sarnowska et al. 2013), whether this regulation connected active GA_4 and Na^+ accumulation in *ga20ox2-1* shoots or roots was determined. This information will help clarify the mechanism by which NaCl regulates root growth in *ga20ox2-1* seedlings.

GA_4 levels were monitored by liquid chromatography-mass spectrometry. In the NaCl-free control experiments, GA_4 levels (Fig. 2a) in WT roots [1.19 ± 0.20 ng/g fresh weight (FW)] were greater than those in *ga20ox2-1* (0.58 ± 0.09 ng/g FW) and *GA20ox2*-OE (0.61 ± 0.12 ng/g FW) seedling roots. No significant differences in GA_4 levels were observed between seedling shoots of the WT and *ga20ox2-1*, while GA_4 levels were greater in *GA20ox2*-OE seedling shoots than in WT or *ga20ox2-1* shoots (Fig. 2a). Thus, root elongation under normal conditions may be positively correlated with *GA20ox2* expression and GA_4 accumulation in *Arabidopsis* seedlings. In NaCl-stressed seedlings, GA_4 levels in WT roots (0.74 ± 0.15 ng/g FW) were similar to those in *ga20ox2-1* roots (0.65 ± 0.13 ng/g FW), while GA_4 levels in WT shoots (0.76 ± 0.14 ng/g FW) were significantly depressed compared with those in *ga20ox2-1* shoots (0.91 ± 0.13 ng/g FW) (Fig. 2a). Unexpectedly, GA_4 accumulated in shoots but not in roots, and this accumulation corresponded to root elongation in NaCl-exposed *ga20ox2-1* and WT seedlings. Unlike in the WT and *ga20ox2-1*, NaCl-stressed *GA20ox2*-OE seedlings exhibited increased GA_4 (Fig. 2a) accumulations in roots (1.51 ± 0.18 ng/g FW) as well as in shoots (2.06 ± 0.25 ng/g FW). In response to NaCl stimuli, *GA20ox2*-OE seedlings had excessive GA_4 accumulations. Thus, *GA20ox2* expression may be the basis for the NaCl-modified GA_4 accumulation and distribution observed in *Arabidopsis* seedling shoots and roots.

Na^+ levels in various *GA20ox2*-expressing lines were tested after exposure to NaCl for 4 h. Compared with the blank treatment, the exposure of WT seedlings to NaCl increased Na^+ levels in roots and shoots, with similar proportional increases in both. This increase was much greater in *GA20ox2*-OE plants than in WT seedlings (Fig. 2b). Under a NaCl treatment, however, the Na^+ content of *ga20ox2-1* roots was lower than that of WT roots, whereas no significant differences were observed in the contents of *ga20ox2-1* and WT shoots (Fig. 2b). These results suggest that *GA20ox2* expression is involved in Na^+ absorption and distribution in NaCl-stressed *Arabidopsis* seedlings.

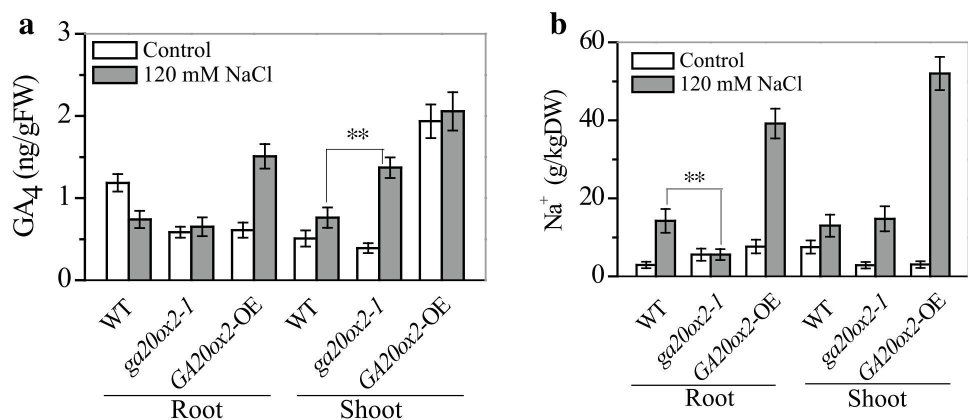


Fig. 2 GA_4 and Na^+ accumulation and distribution in *ga20ox2-1* seedlings exposed to NaCl stress. Levels of **a** GA_4 and **b** Na^+ in root and shoot tissues of WT, *ga20ox2-1*, and *GA20ox2*-OE seedlings with or without the NaCl treatment. Values are means ± SDs (**$P < 0.01$, *$P < 0.05$) of shoots and roots

Induction of *GA20ox2* expression by NaCl

To determine how NaCl affects *GA20ox2* expression, we created transgenic lines containing the *GUS* reporter to trace *GA20ox2* expression. In the absence of NaCl, histochemical staining showed that *GA20ox2* was expressed in various plant tissues, such as cotyledons, microtubules, hypocotyls, sepals (data not shown), and root tips (Fig. 3a), with only very low *GA20ox2* levels detected in leaves (Fig. 3a, b). In contrast, the application of NaCl resulted in a significant increase in *GA20ox2* expression in shoots, including cotyledons and leaves (Fig. 3a, b). The NaCl-stimulated GUS activity levels in shoots and roots were ~ five- and twofold, respectively, greater than those in the control (Fig. 3b). To verify that NaCl-induced *GA20ox2* expression occurs mainly in shoots, we also compared *GA20ox2* transcriptional activity levels in shoots with those in roots using qRT-PCR. Compared with the control, the NaCl treatment increased *GA20ox2* mRNA levels by ~ 25- and ~ 12-fold in shoots and roots, respectively (Fig. 3c). Thus, NaCl predominantly induces *GA20ox2* expression (Fig. 3) and active GA_4 accumulation in shoots (Fig. 2), but inhibits primary root and root hair elongation (Fig. 1). A spatial difference exists between the predominant expression of the *GA20ox2* gene in shoots and NaCl-inhibited primary root or root hair growth in *Arabidopsis* seedlings.

Alteration of IAA homeostasis by NaCl in *ga20ox2-1* roots

We hypothesized that IAA bridges the above-mentioned spatial gap because IAA is required for NaCl-modified root growth in *Arabidopsis* seedlings and stimulates the expression of GA biosynthetic genes in various plants (Wolbang and Ross 2001; Wolbang et al. 2004; Frigerio et al. 2006).

To determine whether NaCl-controlled IAA generation and distribution were modified in *ga20ox2-1* lines, the transcriptional activities of the IAA biosynthetic genes *YUCCA3, YUCCA8*, and *YUCCA9* were investigated. The mRNA levels of the three genes were reduced by NaCl in WT and *ga20ox2-1* seedling roots, but were increased in shoots (Fig. 4a), with this increase being greater in *ga20ox2-1* shoots than in WT shoots (Fig. 4a). Then, the NaCl-triggered IAA distribution was examined by monitoring the expression of the IAA reporter gene *DR5*. In comparison with the control, *DR5-GUS* expression levels were increased in *ga20ox2-1* roots (Fig. 4b). Thus, *ga20ox2-1* ameliorated the inhibition of *YUCCA* gene expression by NaCl in shoot tissues but increased IAA accumulation in root tissues (Fig. 4b).

Alteration of IAA transport by NaCl in *ga20ox2-1* roots

Because IAA transport ensures optimum IAA concentrations for root growth (Dinneny 2014) and PIN1 is involved in GA-mediated root elongation (Benkova and Hejatko 2009), while PIN2 promotes cell division in lateral root primordia (Zhao et al. 2011) by maintaining an adequate IAA level in root cells (Ottenschlager et al. 2003), we hypothesized that *ga20ox2-1* redistributed IAA by modifying the activity levels of PIN1 and PIN2.

How NaCl affected the activity of PIN1 in *ga20ox2-1* seedlings was investigated. In the absence of NaCl, *ga20ox2-1* seedlings displayed increased *PIN1* expression, and mRNA levels of the *PIN1* gene in *ga20ox2-1* roots and shoots were ~ 2.5- and ~ 1.6-fold, respectively, that of the corresponding organs in the WT (Fig. 5a). In NaCl-exposed seedlings, mRNA levels of the *PIN1* gene consistently declined in WT and *ga20ox2-1* roots. In *ga20ox2-1* and WT shoots under NaCl treatment, however, *PIN1* mRNA levels increased, with level in the former being ~ 5.2-fold that of the latter (Fig. 5a). Thus, *ga20ox2-1* may enhance PIN1-dependent IAA transport in shoots relative to the WT in response to NaCl stress.

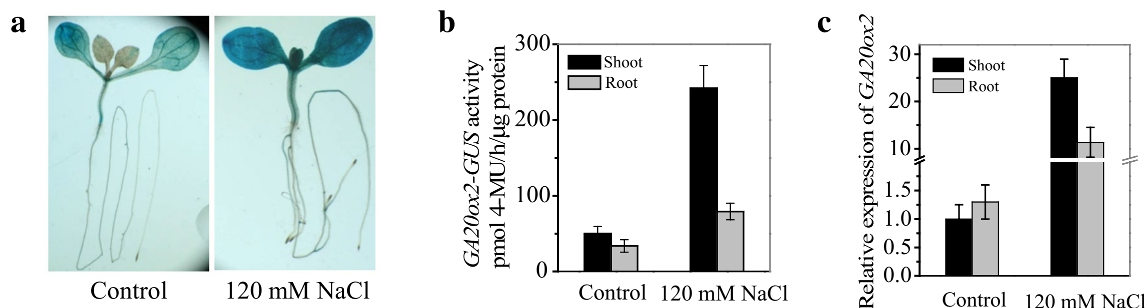


Fig. 3 Characterization of *GA20ox2* expression under the NaCl treatment. **a** Variations in *GA20ox2* expression in 7-days-old seedlings after transfer and growth on MS medium with or without 120 mM NaCl. **b** Activation of the *GA20ox2* promoter-GUS construct in shoot and root tissues of transgenic WT plants grown on MS with or without 120 mM NaCl for 7 days. **c** *GA20ox2* gene expression levels monitored by quantitative real-time PCR in shoots and roots with or without NaCl treatment

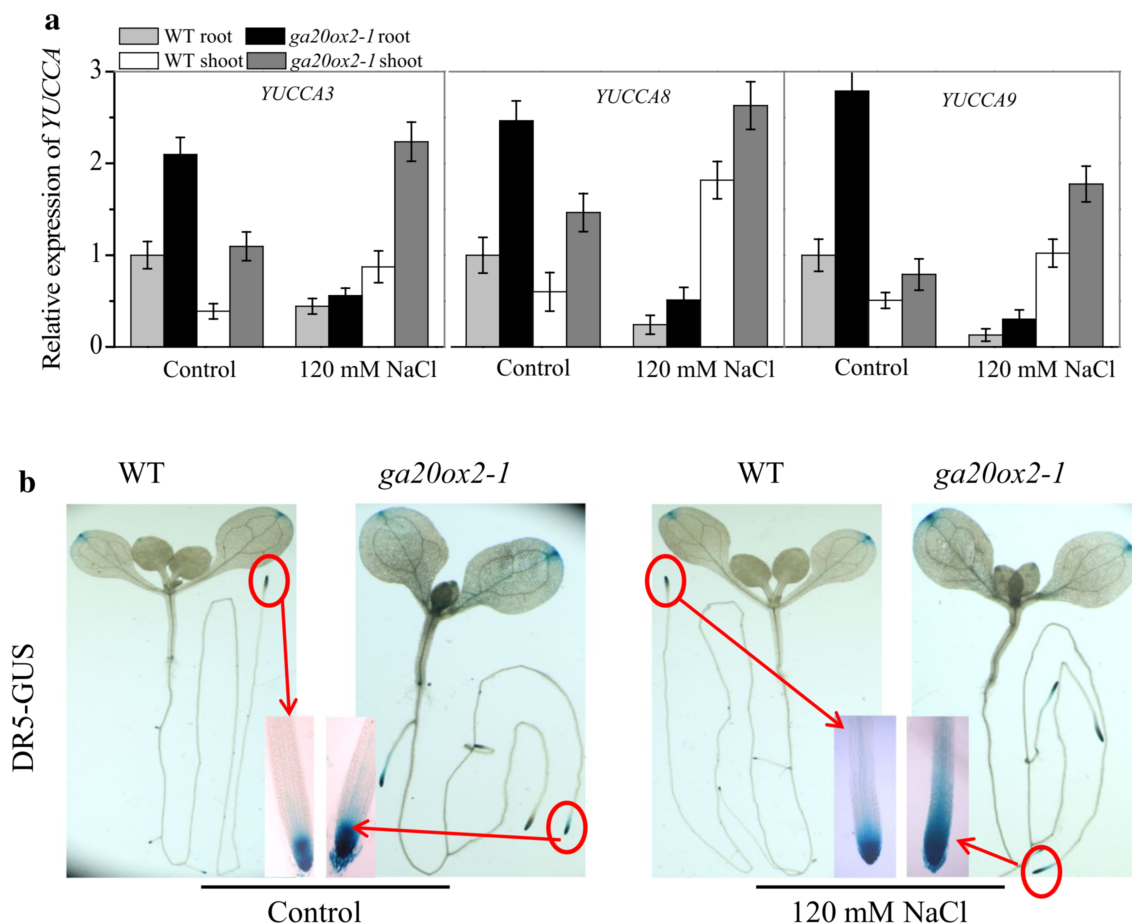


Fig. 4 NaCl-mediated IAA generation and distribution in *ga20ox2-1* seedlings. **a** Expression levels of *YUCCA3, YUCCA8*, and *YUCCA9* genes monitored by RT-qPCR in shoots and roots of *ga20ox2-1* and WT seedlings with or without 120 mM NaCl for 7 days. **b** DR5–GUS-marked IAA levels in shoot and root tissues of *ga20ox2-1* and WT seedlings with or without 120 mM NaCl for 7 days. Red circles and arrows indicate DR5-GUS expression in root tips

These parallel experiments demonstrated that *ga20ox2-1* under control conditions depressed *PIN2* transcription levels in roots and increased them in shoots compared with the WT (Fig. 5b). After the NaCl treatment, the WT exhibited an enhanced *PIN2* expression level in shoots but not in roots. Unlike the WT, *ga20ox2-1* significantly increased *PIN2*'s expression in the roots of NaCl-exposed seedlings, which displayed *PIN2* mRNA levels that were ~4.6-fold that of the control. However, *PIN2* expression was not significantly modified in *ga20ox2-1* shoots (Fig. 5b). Thus, *ga20ox2-1* enhanced PIN2-dependent IAA transport in roots compared with the WT.

Next, GFP-tagged PIN2 protein was monitored in root tissues. In NaCl-exposed WT seedling roots, the fluorescence intensity of the GFP protein was decreased compared with that in the blank treatment (Fig. 5c, d). In contrast to the WT, *ga20ox2-1* increased GFP fluorescence intensity under an NaCl treatment (Fig. 5c, d). The protein level data further implied that *ga20ox2-1* relieved the inhibition of NaCl on PIN2 transport activity, thereby facilitating IAA redistribution in roots.

To provide further evidence for the interaction of *GA20ox2* and *PIN2* in NaCl-controlled root growth, whether *PIN2* activation affects *GA20ox2* expression was examined. In contrast to the notable elevation of *GA20ox2* mRNA levels in WT seedlings, especially in shoots, under NaCl treatment (Figs. 3c, 5e), the transcriptional activity of *GA20ox2* in loss-of-function mutant *pin2* seedlings (including shoots and roots) was almost zero in the absence or presence of NaCl (Fig. 5e). Furthermore, histochemical staining was used to determine GUS-tagged *GA20ox2* levels. In marked contrast to the control, NaCl deepened the blue staining of WT seedlings (especially in shoots; Fig. 5f); however, almost no blue staining was apparent in *pin2* seedlings (including shoots and roots) regardless of whether a salt treatment was applied (Fig. 5f). These low *GA20ox2* levels were consistent with the decline in *GA20ox2* transcriptional activity. Thus,

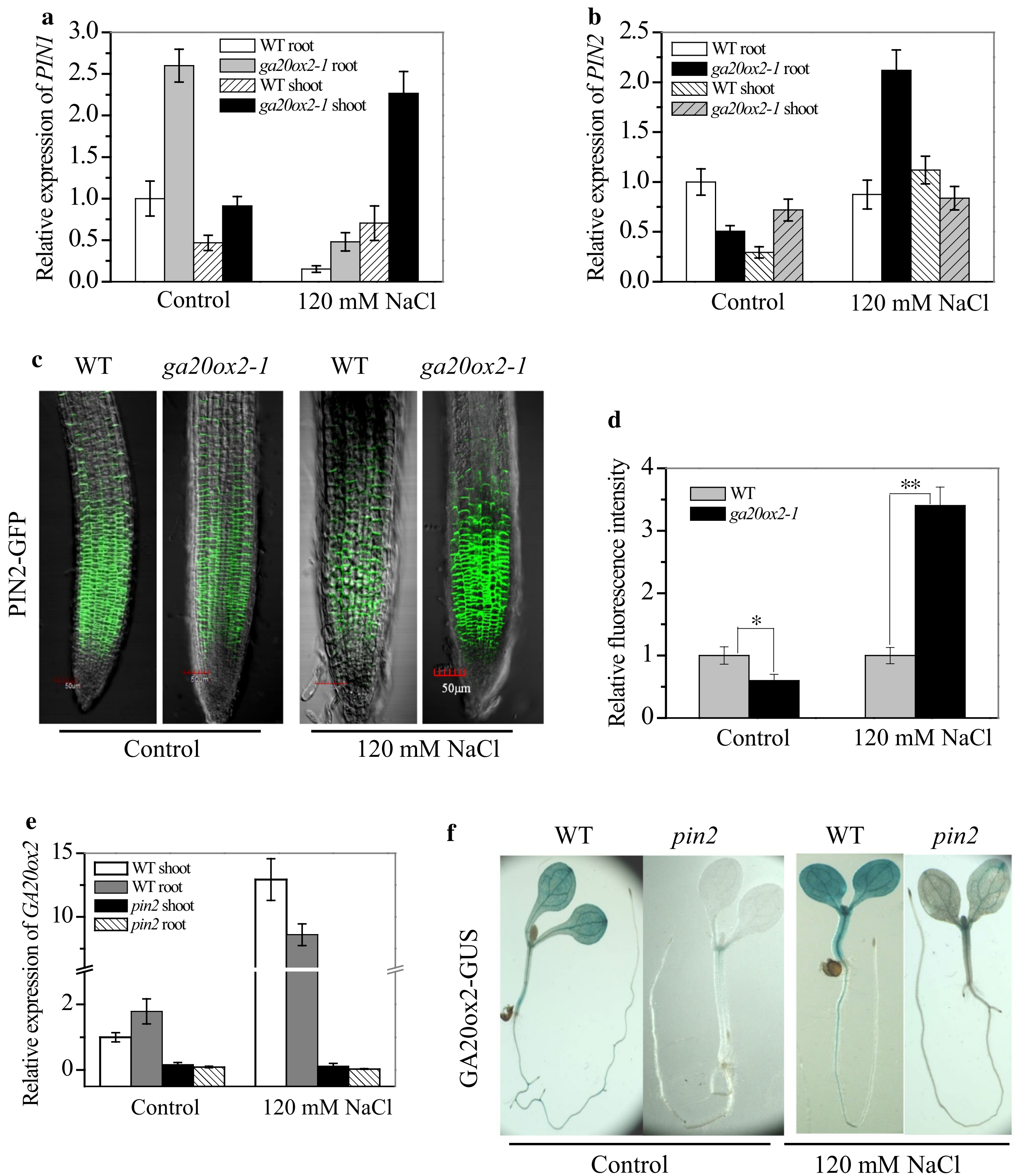


Fig. 5 Control of PIN1 and PIN2 by NaCl in *ga20ox2-1* seedling roots. **a, b** Expression levels of *PIN1* (**a**) and *PIN2* (**b**) detected by RT-qPCR in shoots and roots of *ga20ox2-1* and wild-type (WT) seedlings with or without 120 mM NaCl for 7 days. **c, d** GFP fluorescence (**c**) and statistics of relative fluorescence intensity (**d**) of PIN2-GFP in root tissues of *ga20ox2-1* and WT seedlings with or without 120 mM NaCl for 7 days. **e, f** Expression levels of the *GA20ox2* gene (**e**) and *GA20ox2* promoter-GUS activity (**f**) in *pin2* mutant seedlings with or without 120 mM NaCl for 7 days

GA20ox2 histochemical staining and *GA20ox2* transcriptional activity levels demonstrated that PIN2 regulates *GA20ox2* activity.

Discussion

Active GAs often have limited effects on root growth and development that are dependent on IAA transport and accumulation (Niu et al. 2013). To understand the mechanism by which the biosynthesis factor GA20ox2 mediates salt-controlled plant root growth and development, in this study the relationship between *GA20ox2* expression and IAA generation and distribution during NaCl-inhibited root growth in *Arabidopsis* seedlings were assessed. The results indicate a specific role for *GA20ox2* in NaCl-controlled root growth.

GA20ox2 expression may be involved in NaCl-controlled root growth of *Arabidopsis* seedlings because *GA20ox2* expression times, levels, and tissue localization may satisfy root growth requirements for young *Arabidopsis* seedlings. The *GA20ox2* mRNA levels, among the five family members, are greatest in 3- and 7-days-old *Arabidopsis* seedlings, especially in roots (Rieu et al. 2008). When the IAA-permeable analog 1-naphthalene acetic acid was applied to mediate root elongation, the greatest expression of the *GA20ox2* gene was observed in 6-days-old *Arabidopsis* seedlings (Wolbang and Ross 2001). In the present study, root length gradually increased as *GA20ox2* expression increased in 7-days-old *ga20ox2-1*, WT, and transgenic *GA20ox2*-OE plants under NaCl-free conditions (Fig. 1). Thus, root elongation was positively correlated with *GA20ox2* expression levels in young *Arabidopsis* seedlings. Applications of NaCl, however, transformed this positive correlation into a negative one (Fig. 1). This sharp contrast is a strong indication that *GA20ox2* expression may act as a signaling element in NaCl-inhibited root growth of *Arabidopsis* seedlings, or, alternatively, that NaCl inhibits primary root growth by increasing *GA20ox2* expression (Fig. 3).

The regulation of *GA20ox2* expression and active GA levels are involved in NaCl-inhibited primary root or root hair growth. NaCl-triggered *GA20ox2* expression (Fig. 3) appeared to correspond to the decreased root elongation (Fig. 1) and GA_4 accumulation (Fig. 2a) of WT seedlings. In contrast, the reduction in *GA20ox2* expression in *ga20ox2-1* seedlings depressed NaCl-increased GA_4 accumulation (Fig. 2a) and thus ameliorated NaCl-inhibited root elongation (Fig. 1). Thus, NaCl-induced *GA20ox2* expression was negatively correlated with primary root growth in *Arabidopsis* seedlings. The number and elongation of root hairs, however, may be associated with GA20ox2-dependent GA_4 levels because increased GA_4 accumulation reduces the number of root hairs in *Arabidopsis* seedlings (Jiang and Fu 2008), while *GA20ox2* expression promotes the production of GA_4 and its precursor, GA_9 (Phillips et al. 1995; Fernando et al. 2014). In our study, NaCl-induced *GA20ox2* expression (Fig. 3) was negatively correlated with GA_4 accumulation (Fig. 2a) and root hair density (Fig. 1). *GA20ox2* expression inhibits the elongation of root hairs but not their initiation (Fig. 1), which explains the decline in root hair numbers. Thus, NaCl inhibits root hair elongation by elevating *GA20ox2* expression and GA_4 accumulation.

GA20ox2 expression may mediate Na^+ uptake and distribution during the NaCl-inhibited primary root or root hair growth of *Arabidopsis* seedlings. The root endodermis, which executes very early adaptive responses through ions or channel proteins (Vermeer et al. 2014), acts as a gateway for solutes to sense and respond to Na^+ toxicity (Duan et al. 2013; Dinneny 2014). Interestingly, the root endodermis is the major GA-responsive tissue (Dinneny 2014). The suppression of *GA20ox2* expression (Fig. 1) and GA_4 accumulation (Fig. 2) in the *ga20ox2-1* mutant accordingly decreased Na^+ absorption and accumulation in root tissues (Fig. 2b). As a consequence, the tolerance of *ga20ox2-1* root or root hair growth to NaCl increased (Fig. 1). Thus, *GA20ox2* expression can alter the sensitivity of *Arabidopsis* seedling root or root hair growth to NaCl stress.

The mechanisms underlying GA20ox2's regulation of NaCl-stressed root growth involve IAA generation and distribution. In addition to 1-naphthalene acetic acid-facilitated *GA20ox2* expression during *Arabidopsis* seedling root elongation (Wolbang and Ross 2001), GA's promotion of root growth (Benkova and Hejatko 2009; Eilon et al. 2013) depends on IAA generation (Benkova and Hejatko 2009). GA alone does not elongate roots if IAA generation is disrupted by decapitation at the shoot apices of *Arabidopsis* seedlings (Benkova and Hejatko 2009). A similar mechanism appears to have operated in this study. Compared with the WT, the loss of *GA20ox2* expression in the *ga20ox2-1* mutant was inimical to root growth (Fig. 1), possibly because of the decrease in *YUCCA* expression (Fig. 4) and IAA accumulation in roots (Fig. 5). Under NaCl treatment, in contrast, the amelioration of root elongation in *ga20ox2-1* seedlings (Fig. 1) was inconsistent with the activation of YUCCAs in shoots (Fig. 4) as well as IAA accumulation in roots (Fig. 5). Thus, IAA generation plays an important role in the involvement of GA20ox2 in NaCl-controlled root growth.

The influence of NaCl on *ga20ox2-1* root growth may result from the regulation of IAA transport and redistribution because local IAA levels in root cells direct plant root morphogenesis (Pierik and Testerink 2014), and optimized IAA distribution is required for GA-mediated root growth and development (Niu et al. 2013). IAA redistribution is managed by PIN family members, and the collaboration between PIN1 and PIN2 recycles IAA from shoots to roots (Dinneny 2014). Considering that shoot apex-derived IAA promotes root growth by integrating PIN1 activity with

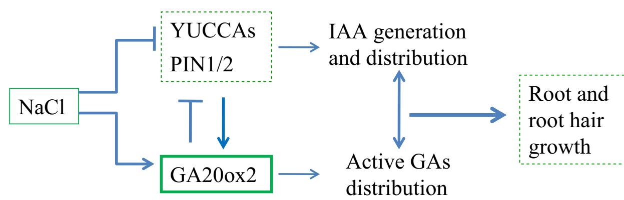


Fig. 6 Schematic diagram of GA20ox2 involvement in NaCl-controlled IAA distribution and root morphology of *Arabidopsis* seedlings

GA-response signaling (Fu and Harberd 2003) and that PIN1 is expressed predominantly in shoot tissues (Gälweiler et al. 1999), *ga20ox2-1* may alleviate PIN1 activity in shoot tissues (Fig. 4a) and IAA accumulation in roots (Fig. 4b), maintaining in turn root and root hair growth (Fig. 1) under NaCl-stress conditions. This maintenance also requires PIN2 activity (Figs. 4a, 5) because IAA-induced primary root or root hair growth depends on *PIN2* expression (Gou et al. 2010). Furthermore, because GA can control PIN turnover during the root elongation of *Arabidopsis* seedlings (Moubayidin et al. 2010; Willige et al. 2011) and *ga20ox2-1* reduced GA accumulation in seedling roots in our study (Fig. 2), *ga20ox2-1* seedlings accordingly ameliorated PIN2 activity (Figs. 4, 5) in NaCl-stressed seedling roots. A similar scenario was described in transgenic GA-deficient *Populus* lines. In particular, a GA deficiency in transgenic *Populus* roots facilitated the formation of new lateral root primordia by amending PIN9 activity and IAA accumulation in root tissues (Gou et al. 2010).

In addition to the *ga20ox2-1*-induced alteration in PIN2 activity, the loss-of-function *pin2* mutant decreased *GA20ox2* expression with or without the NaCl treatment (Fig. 5). Thus, NaCl reduces root meristem growth by inactivating PINs (Liu et al. 2015). These observations suggest that crosstalk occurs between GA20ox2 and PIN2 during NaCl control of root growth and development, increasing the complexity of GA-regulated root growth (Niu et al. 2013).

The expression of *GA20ox2* facilitates NaCl's inhibition of primary root or root hair growth, with this facilitation being dependent on YUCCA-catalyzed IAA generation and PIN1/2-driven IAA redistribution in *Arabidopsis* seedlings. The relationships among these factors are outlined in Fig. 6.

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