

# Narciclasine, a potential allelochemical, affects subcellular trafficking of auxin transporter proteins and actin cytoskeleton dynamics in *Arabidopsis* roots

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## Abstract

**Main conclusion** The present study documented the action of a potential allelochemical, narciclasine, on auxin transport in *Arabidopsis* by mainly affecting subcellular trafficking of PIN and AUX1 proteins and through interfering actin cytoskeletal organization.

Narciclasine (NCS), an Amaryllidaceae alkaloid isolated from *Narcissus tazetta* bulbs, has potential allelopathic activity and affects auxin transport. However, little is known about the cellular mechanism of this inhibitory effect of NCS on auxin transport. The present study characterizes the effects of NCS at the cellular level using transgenic *Arabidopsis* plants harboring the promoters of PIN, in combination with PIN-GFP proteins or AUX1-YFP fusions. NCS treatment caused significant reduction in the abundance of PIN and AUX1 proteins at the plasma membrane (PM). Analysis of the subcellular distribution of PIN and AUX1 proteins in roots revealed that NCS induced the intracellular accumulation of auxin transporters, including PIN2, PIN3, PIN4, PIN7 and AUX1.

However, other PM proteins, such as PIP2, BRI1, and low temperature inducible protein 6b (LTI6b), were insensitive to NCS treatment. NCS-induced PIN2 compartments were further defined using endocytic tracer FM 4-64 labeled early endosomes and suggested that this compound affects the endocytosis trafficking of PIN proteins. Furthermore, pharmacological analysis indicated that the brefeldin A (BFA)-insensitive pathway is employed in the cellular effects of NCS on PIN2 trafficking. Although NCS did not alter actin dynamics in vitro, it resulted in the depolymerization of the actin cytoskeleton in vivo. This disruption of actin filaments by NCS subsequently influences the actin-based vesicle motility. Hence, the elucidation of the specific role of NCS is useful for further understanding the mechanisms of allelopathy at the phytohormone levels.

**Keywords** Allelochemical · Auxin · Auxin transport · Actin cytoskeleton

## Abbreviations

ARF	ADP-ribosylation factor family of GTP-binding proteins
AUX1/LAX	AUXIN RESISTANT1/LIKE AUXIN RESISTANT
BFA	Brefeldin A
eEF1A	Elongation factor one alpha
GEF	Guanine nucleotide exchange factor
LatB	Latrunculin B
NCS	Narciclasine
PIN	PIN-FORMED auxin efflux carrier protein
PM	Plasma membrane
TIBA	2,3,5-Triiodobenzoic acid
TryA23	Tyrphostin A23

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## Introduction

Allelopathy is defined as plant to plant, plant to microorganism, and microorganism to microorganism interaction by allelochemicals belonging to secondary metabolites. Allelopathy is believed to be as phenomena occurring in many natural and manipulated ecosystems and plays a role in the evolution of plant communities, exotic plant invasion, and replant failure (Bais et al. 2003; Inderjit and Duke 2003). Many plant species, including crop plants, are capable of producing and releasing allelochemicals into the environment (Bertin et al. 2003). Most of the allelochemicals usually elicit negative effects on themselves and acceptor organisms, with beneficial effects being rare (Weir et al. 2004). In past decades, great efforts have been made to elucidate phytotoxic effects of allelochemicals in acceptor plants. Compared with the vast information on the phytotoxic effects of allelochemicals on many physiological and biochemical reactions, such as photosynthesis, respiration, water utilization, nutrient uptake, and reactive oxygen species generation, etc. (Inderjit and Duke 2003; Blum 2005), far less information is available on their impacts on phytohormone metabolism.

Besides the roles of plant hormones in diverse processes of plant growth and development, its role in plant responses to various abiotic stresses is also important. Some allelochemicals have been recognized to disrupt the distinctive hormonal balance which is required for undisturbed plant growth. Methyl benzoate, a volatile compound emitted from the snapdragon flowers, has allelopathic activity and inhibits *Arabidopsis* root growth via cytokinin and auxin signaling pathways (Horiuchi et al. 2007). Cyanamide, which has been reported as an allelochemical, was shown to alter plant hormone (ethylene and auxin) homeostasis (Soltys et al. 2012). Such variation in phytohormone levels was detected in seedlings treated with allelochemical citral (Graña et al. 2013) or leukamenin E (Ding et al. 2008). Furthermore, it has been also found that flavonoids are involved in plant–plant allelopathy and also affect polar auxin transport in plants (Weston and Mathesius 2013). Taken together, these findings strongly suggest that allelochemicals may also affect plant growth by affecting the phytohormone levels.

Plants of the Amaryllidaceae family produce a wide array of biologically active constituents, such as alkaloids, phenolic compounds, flavonoids, and glycosides (Kornienko and Evidente 2008). As the primary constituents, some of these compounds have been reported to possess various pharmacological and biological properties, including cytotoxicity, antioxidant, antitumor, and anticancer activities (Bastida et al. 2006; Berkov et al. 2008; Zupkó et al. 2009). However, information regarding the

allelopathic activity in the field and in laboratory studies is limited (Lallemand et al. 2009).

Narciclasine (NCS) is a bioactive secondary metabolite (Amaryllidaceae alkaloid) isolated from *Narcissus tazetta* bulbs and also widely exists in the genera *Galanthus*, *Haemanthus*, *Leucojum*, *Pancratium*, *Sprekelia*, *Sternbergia* and *Vallota* (Piozzi et al. 1969). Previous studies showed that NCS possesses antimitotic (Ceriotti 1967), antiviral functions (Gabrielsen et al. 1992) and antitumor activities (Lefranc et al. 2009). In laboratory studies, we have found that NCS exhibits a wide range of inhibitory effects on seed germination and seedling growth in rice, cabbage, *Arabidopsis* and lettuce (Bi et al. 1998; Na et al. 2011a, b; Hu et al. 2014) and on chloroplast development of excised radish cotyledons (Bi et al. 1998). Meanwhile, the NCS phytotoxicity was further demonstrated with its effect on cell cycle activity and modification of cell cycle gene expression (Na et al. 2011a, b). The ability of an allelopathic compound to inhibit the seed germination and/or plant growth is usually defined as its “allelopathic potential”. Our current knowledge of the physiological and biochemical effects of NCS suggests that it may act as a potential allelochemical. Although growth inhibition by NCS has been well documented, the mode of action and the mechanistic details still remain un-deciphered.

The plant hormone auxin is an important regulator of plant developmental processes, including embryogenesis, organogenesis, tissues patterning and growth responses to environmental stimuli. Auxin is synthesized in young aerial tissues and is then transported to other parts of the plant by an intercellular transport system (Grieneisen et al. 2007; Titapiwatanakun and Murphy 2009). This unique polar transport relies on an array of membrane transporters including influx carriers AUX1/LAX, efflux carriers PIN-FORMED (PIN) and ATP-binding cassette subfamily B (ABCB) (Titapiwatanakun and Murphy 2009). PIN auxin efflux carriers display polar localization at the plasma membrane (PM) and undergo constitutive recycling between the PM and endosomal compartments (Geldner et al. 2001; Paciorek et al. 2005). This recycling seems to be under the control of guanine-nucleotide exchange factor for ADP-ribosylation factor GTPase (ARF-GEF), which is sensitive to brefeldin A (BFA), a known inhibitor of secretion and subcellular trafficking (Geldner et al. 2001, 2003; Kleine-Vehn et al. 2008). It has also been confirmed that the subcellular dynamics of auxin transport carriers is dependent on the actin cytoskeleton (Geldner et al. 2001; Kleine-Vehn et al. 2006). In this way, their polarity can be modulated rapidly in response to developmental or external cues, thus redirecting auxin wherever is needed.

Our preliminary studies indicated that *Arabidopsis* may be used as an excellent model to investigate the mode of action of NCS mostly due to its convenience in cytological

observation and extensive genomic and molecular information (Na et al. 2011a, b). Recently, our research demonstrated that the phytotoxic effects of NCS in *Arabidopsis* roots involve not only the disruption of auxin signaling pathway (Hu et al. 2012), but also the potent inhibitory activity against auxin transport (Na et al. 2011b). However, the underlying cellular mechanism of this effect of NCS is unclear. Here, we demonstrate that the inhibitory effects of NCS on auxin transport in *Arabidopsis* is mainly due to its role in affecting the subcellular trafficking of PIN proteins and AUX1 auxin influx carrier via a target that differs from that of the vesicle trafficking inhibitor, BFA. NCS also interferes with the actin cytoskeletal organization, providing an explanation for its effect on vesicle trafficking. Analysis of the phytotoxic effects of this active compound will be useful for better understanding the mechanism of action of allelochemicals.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used in all experiments unless otherwise stated. Seeds of Col-0 and  $\beta$ -*tubulin6-GFP* (Bannigan et al. 2006) were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH, USA). Seeds of the transgenic lines GFP-ARA7 (Jaillais et al. 2006), PIN1-GFP (Benková et al. 2003), PIN2-GFP (Xu and Scheres 2005), PIN3-GFP (Friml et al. 2002), PIN7-GFP (Blakeslee et al. 2007), BRI1-GFP (Geldner et al. 2007), and AUX1-YFP (Swarup et al. 2004) were provided by the Ministry of Education Key Laboratory of Cell Activities and Stress Adaptations (Lanzhou University, Lanzhou, Gansu, China). Seeds of YFP-SYP22 (Robert et al. 2008) and VHA-a1-GFP (Dettmer et al. 2006) were a gift from Prof. Glenn R. Hicks at University of California (Riverside, CA, USA). Seeds of EGFP-LTI6b (Kurup et al. 2005) and NAG-GFP (Essl et al. 1999) were kindly provided by Prof. Abidur Rahman (Iwate University, Japan). Seeds of PIP2A-GFP (Cutler et al. 2000) were a gift from Prof. Tom Beeckman at Gent University. Seeds of the transgenic line GFP-ABD2 (Wang et al. 2008) were kindly provided by Dr. Jinxing Lin (Institute of Botany, Chinese Academy of Sciences, China). All seeds were surface sterilized with 70 % ethanol containing 0.05 % Triton X-100 for 30 s and 15 % sodium hypochlorite for 15 min, and rinsed five times with sterilized water before sowing on agar plates containing 0.5× MS medium (pH 5.7), 1 % (w/v) sucrose, 0.8 % (w/v) agar. After stratification on 0.5× MS medium for 2–4 days at 4 °C, seedlings were grown vertically in a climate chamber at 23 °C under a light intensity of 200  $\mu\text{M m}^{-2} \text{s}^{-1}$  conditions (16/8 h light cycle).

### Chemical treatments

NCS was isolated and purified from *Narcissus tazetta* L. bulbs according to Bi et al. (1998). The purity and identity of NCS were verified by LC–MS analysis. For long-term chemical treatments, seedlings were grown vertically on 0.5× solid MS medium containing chemicals for 7 days. For short-term treatments, 5-day-old light-grown seedlings were transferred from agar plates into 24-well cell culture plates containing 0.5× liquid MS medium with or without the indicated concentration of chemicals and incubated for indicated times. Chemical stocks were 25 mM BFA (Sigma-Aldrich, St. Louis, MO, USA), 10 mM cycloheximide (CHX; Sigma-Aldrich), 20 mM latrunculin B (LatB; Sigma-Aldrich), 1 mM naphthalene1-acetic acid (NAA; Sigma-Aldrich), 1 mM NCS, 1 mM 2,3,5-triiodobenzoic acid (TIBA; Sigma-Aldrich) and 30 mM tyrphostin A23 (TryA23; Santa Cruz Biotechnology, Dallas, TX, USA) in 100 % DMSO. Equal volumes of solvents (DMSO) were used in control experiments. For FM 4-64 staining, seedlings were incubated for 10 min with 4  $\mu\text{M}$  FM 4-64 (Invitrogen, Carlsbad, CA, USA) from a 10 mM stock in water.

### Microscopy

Confocal images were captured with a confocal laser-scanning microscope (Olympus, FV1000, Tokyo, Japan) equipped with a 40× or 60× (water immersion) objective, a HeNe laser (543 nm) and an argon laser (488 nm, 514 nm). Emission filters set for GFP, YFP, and FM 4-64 was at 505–530, 490–510, and 560 nm, respectively. The fluorescence intensity of the membrane or cytosolic fluorescent signals was quantified with NIH Image software (Image J, version 1.43), and the quotients of values between the inside and the plasma membrane were calculated. All images within a single experiment were captured with the same gain and exposure settings.

### In vitro actin assay

Rabbit skeletal muscle actin (>99 % purity), pyrenylated rabbit muscle actin and recombinant human gelsolin were purchased from Cytoskeleton Inc. (Denver, CO, USA) and the assay was carried out according to the manufacturer's instructions.

### In vitro actin polymerization assay

Polymerization assays were performed as described by Mullin and Machesky (2000). 4  $\mu\text{M}$  rabbit skeletal muscle actin monomers (5 % pyrenylated) in G-buffer (2 mM Tris–HCl, pH 8, 0.5 mM DTT, 0.2 mM  $\text{CaCl}_2$  and 0.2 mM

ATP) were mixed with NCS, LatB or DMSO, then polymerization was induced by the addition of 10× KME (500 mM KCl, 10 mM MgSO<sub>4</sub>, 10 mM EGTA and 100 mM imidazole, pH 6.5). Pyrene fluorescence (excitation at 365 nm, emission at 407 nm) was followed using a fluorescence spectrofluorometer (Photon Technologies International, Edison, NJ, USA). Measurement started 10 s after addition of 10× KME buffer.

### In vitro actin depolymerization assay

10 μM actin monomers (5 % pyrenylated) were polymerized by the addition of 10× KME (pH 8.0) in the presence of 0.1 μM human recombinant plasma gelsolin and aged for 2 h. Actin filaments were diluted to 1 μM with 1× KME containing 25 μM NCS or DMSO. Fluorescence measurement started 12 s after the dilution and was performed at room temperature using a fluorescence spectrofluorometer with excitation set at 365 nm and emission detected at 407 nm.

### In vitro actin bundling assay

4 μM F-actin was incubated with 25 μM NCS or DMSO for 1 h at pH 7.0 then spun with 13,500 g for 30 min. Supernatant and pellet were separated and loaded on 10 % SDS-PAGE gel and visualized with Commassie Brilliant Blue staining. Densitometric quantification of the signals was done with NIH Image software (Image J, version 1.43).

## Results

### NCS affects the expression of PIN and AUX1 proteins

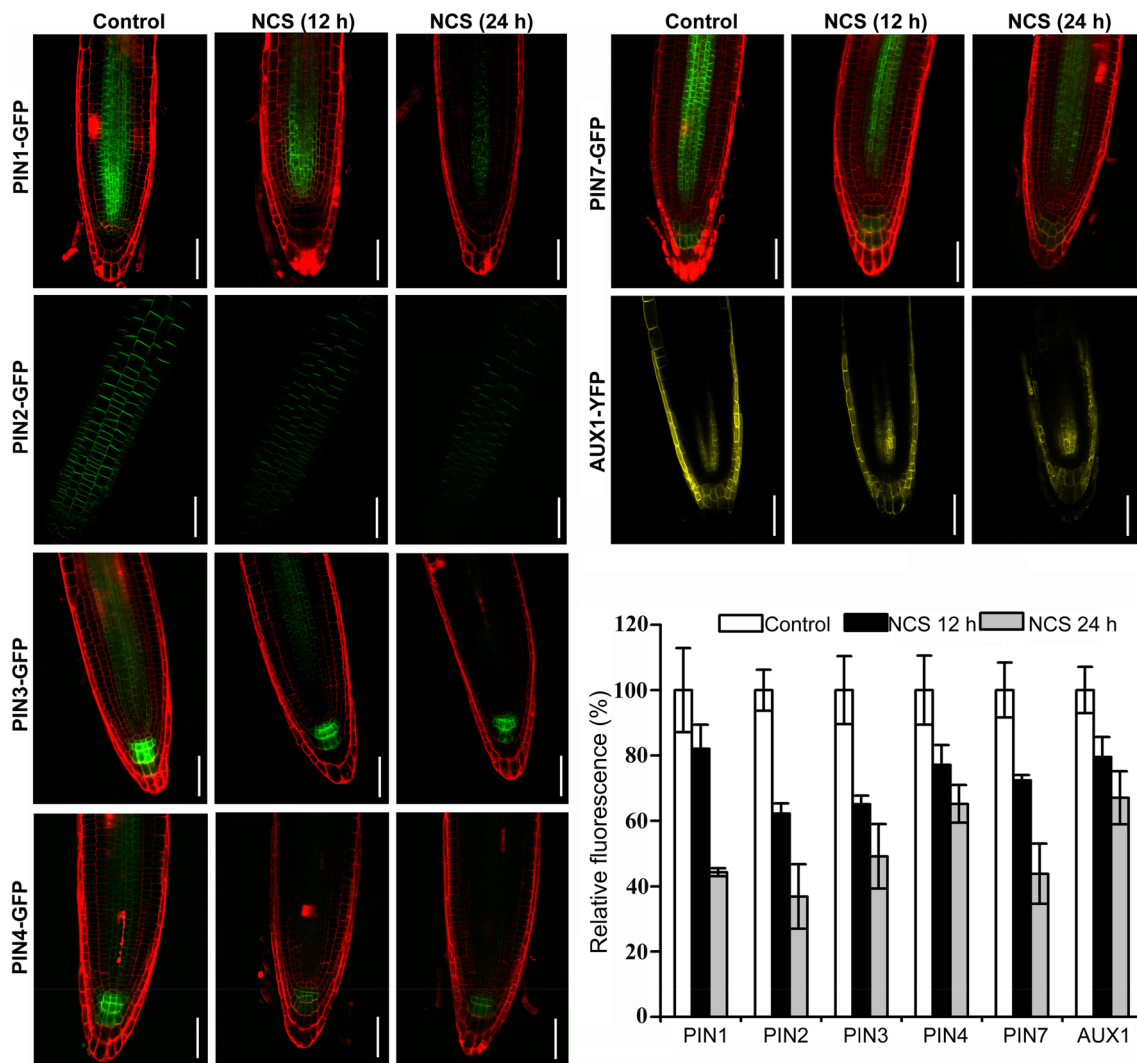
The natural compound NCS displays broad range of inhibition of plant growth observed in our previous works; however, the root growth in *N. tazetta* seedlings was less sensitive and no inhibitory effect was observed when exposed to NCS from 1 to 100 μM (Supplementary Fig. S1). This suggests that NCS may have an allelopathic potential. The use of *Arabidopsis* as a good system to characterize the effects of this potential allelochemical can help us fully understand the mode of action in planta. Evidence strongly supports a significant inhibitory effect of NCS on polar auxin transport in *Arabidopsis* root (Na et al. 2011b). Polar auxin transport has been considered to be predominantly regulated by the PIN family of auxin efflux carriers. Polarized distribution of PIN proteins at the PM determines the direction and rate of intercellular auxin flow (Wisniewska et al. 2006). Therefore, the hypothesis that

NCS may affect the polar localization or abundance of PIN proteins at the PM was tested using transgenic plants containing the promoters of PINs in combination with PIN-GFP protein fusions. 5 μM NCS substantially reduced the levels of PIN1, PIN2, PIN3, PIN4, or PIN7 at the PM compared with the controls (DMSO-treated) when seedlings (5-day-old) expressing GFP markers were treated for 12 or 24 h (Fig. 1). NCS treatment also resulted in significant reduction of AUX1 (auxin influx carrier) levels at the PM (Fig. 1). These results indicate that NCS inhibits the expression of PIN and AUX1 proteins at the PM.

Very interestingly, the reduction in protein levels of PIN2 and AUX1 is not consistent with the changes in transcriptional levels of *PIN2* and *AUX1* in *Arabidopsis* roots after NCS treatment (Na et al. 2011b). Quantitative reverse transcription-PCR analysis showed that the expression of *PIN2* was significantly up-regulated after 12 or 24 h of 5 μM NCS treatment, whereas *AUX1* gene expression was not inhibited by NCS treatment at 12 or 24 h (Na et al. 2011b), implying that the reduction of PIN and AUX1 protein levels at the PM in NCS-treated plants is not only associated with their transcriptions, but also is achieved probably via the alternate use of other pathways.

### NCS-induced subcellular agglomerations of PIN2 and AUX1 proteins

Auxin transport inhibitors are widely reported to affect gravitropism. Some of these compounds also interfere with the subcellular localization and intracellular trafficking of PIN proteins (Robert et al. 2008; Nishimura et al. 2012). It has been shown that NCS also inhibited the root gravitropic response (Na et al. 2011b). Because PIN2 proteins has been proven to play important roles in controlling gravitropism in *Arabidopsis* seedlings, the intracellular distribution of PIN2 proteins was investigated in *Arabidopsis* roots at NCS concentrations that inhibit auxin transport (Na et al. 2011b). Results showed that NCS caused the accumulation of PIN2-GFP in intracellular agglomerations (termed NCS bodies) as early as 2 h after treatment with 0.5 μM (Supplementary Fig. S2, arrow). Dose-dependent increase in the amount of NCS bodies were detected at 5 μM and 10 μM NCS (Supplementary Fig. S2b, c). In the time-course experiment, the internalization of PIN2-GFP was aggravated after 30 min (Fig. 2) or longer time of 25 μM NCS treatment (Supplementary Fig. S2). Large intracellular aggregates were found at 6 h after 25 μM NCS treatment (Supplementary Fig. S2h). To observe consistent effects, the concentration of 25 μM NCS was used for further experiments. Similarly, NCS also induced the intracellular accumulation of PIN3-GFP, PIN4-GFP, PIN7-GFP, or AUX1-YFP after incubation for 30 min or 2 h (Fig. 2). However, the effects of NCS on intracellular trafficking in



**Fig. 1** Effects of NCS on the abundance and localization of PIN and AUX1 proteins in *Arabidopsis* roots. 5-day-old seedlings harboring indicated markers were transferred to medium with DMSO (*control*) or 5  $\mu$ M NCS for indicated times (control images were taken at 24 h). The cell walls of roots were visualized by staining with 10  $\mu$ g/ml

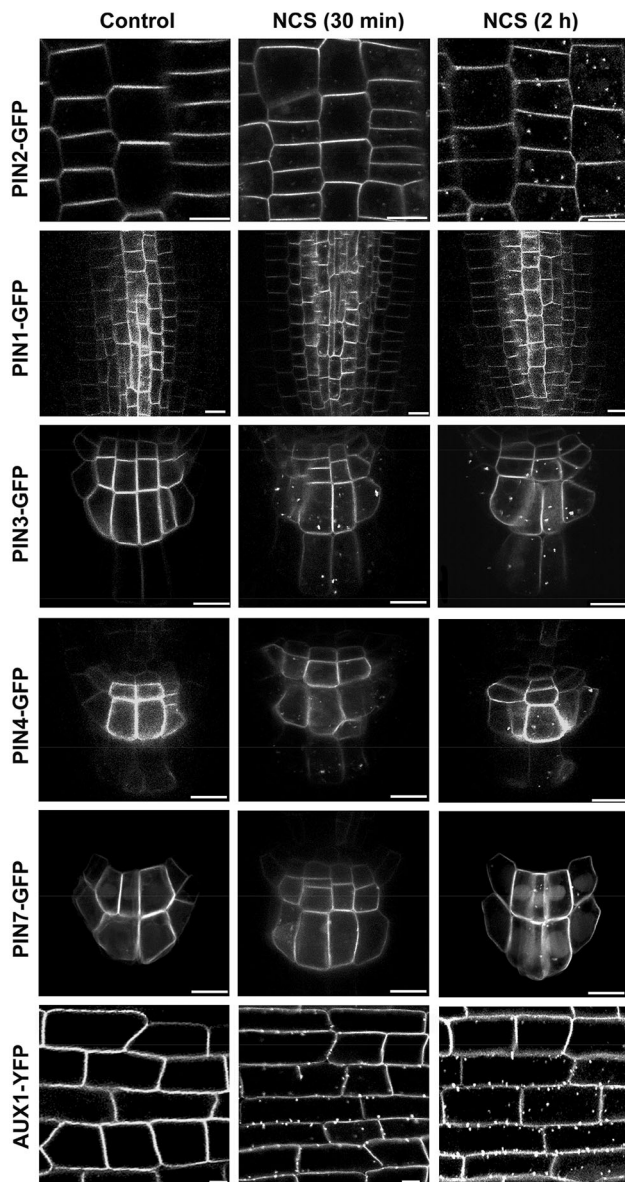
propidium iodide. Images shown are representative of at least three independent experiments. Bars 50  $\mu$ m. Quantification of GFP/YFP fluorescence by image analysis of confocal sections as described above. Data shown are the mean  $\pm$  SD for 20 seedlings and are representatives of at least three independent experiments

roots could be reversed when NCS was washed out (data not shown). Overall, these results indicated that NCS acts in the regulation of intracellular trafficking of PIN and AUX1 proteins in a dose-dependent and reversible manner.

**NCS affects the subcellular trafficking of PIN2 through endocytosis**

PIN proteins are targeted to the PM through several secretory pathways including constitutive endocytosis from the PM (Geldner et al. 2001, 2003; Dhonukshe et al. 2007). To determine whether the NCS-induced intracellular accumulation is dissociated from the PM via endocytosis, we used PIN2-GFP as a marker and investigated the co-localization of PIN2-GFP with FM 4-64 after 2 h of NCS

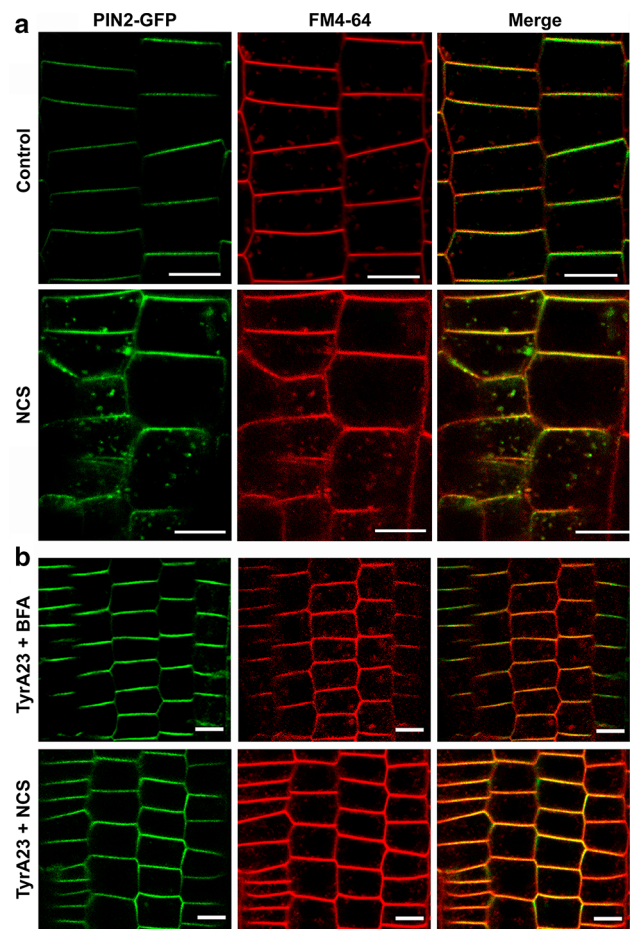
treatment. FM 4-64 is a widely used endocytic tracer in living eukaryotic cells and mainly stains early endosomes within 30 min (Bolte et al. 2004). NCS did not block the accumulation of FM 4-64 into endosomal compartments or NCS bodies (Fig. 3a). Furthermore, NCS-induced intracellular compartments of PIN2-GFP partially co-localized with the membrane-selective endocytic tracer FM 4-64 (Fig. 3a), suggesting that PIN2 proteins agglomerated in NCS bodies originate from the PM through endocytosis. In addition, the NCS body formation was monitored in the presence of cycloheximide (CHX), an inhibitor of protein biosynthesis (Paciorek et al. 2005). The results showed that intracellular PIN2 accumulation caused by NCS was not prevented by application of 50  $\mu$ M CHX for 30 or 90 min (Supplementary Fig. S3), indicating that PIN2-GFP



**Fig. 2** NCS induces internalization of PIN and AUX1 proteins at the PM. 5-day-old transgenic *Arabidopsis* seedlings were incubated in medium containing DMSO (*control*) or 25  $\mu$ M NCS for indicated times. Compared with the control, intracellular bodies are apparent in seedlings expressing PIN2-GFP, PIN3-GFP, PIN4-GFP, PIN7-GFP, or AUX1-YFP after NCS treatment. The images were captured using same confocal settings and are representatives of 25 roots obtained from three independent experiments. Scale bars 10  $\mu$ m

aggregated in NCS bodies is independent of de novo protein synthesis.

Previous studies showed that PIN proteins are cargos of endocytic mechanism involving the vesicle coat protein clathrin (Ortiz-Zapater et al. 2006; Dhonukshe et al. 2007). The study by Robert et al. (2008) further suggested that auxin inhibits the BFA-induced internalization of PIN proteins through clathrin-mediated endocytosis. In order to examine whether the NCS-induced intracellular



**Fig. 3** NCS impacts endocytosis. **a** Co-localization of PIN2-GFP (*green*) with FM 4-64 (*red*) at the PM and endosomes. Seedlings expressing PIN2-GFP were stained with FM 4-64 for 10 min, and then exposed to DMSO (*control*) or 25  $\mu$ M NCS for 2 h. **b** Internalization of PIN2-GFP into BFA bodies or NCS bodies was inhibited by TyrA23. Plants were pretreated with 45  $\mu$ M TyrA23 for 30 min, followed by co-incubation with TyrA23 and 25  $\mu$ M BFA (*upper panels*) or 25  $\mu$ M NCS (*lower panels*) for 2 h. Scale bars 10  $\mu$ m

accumulation is achieved through the clathrin-mediated process, seedlings expressing PIN2-GFP were treated with TyrA23 (a well-known inhibitor of clathrin-mediated processes) in the presence of NCS or BFA (Ortiz-Zapater et al. 2006; Dhonukshe et al. 2007). Consistent with previous reports (Ortiz-Zapater et al. 2006; Dhonukshe et al. 2007), the internalization of PIN2 caused by the vesicle trafficking inhibitor BFA was blocked by TyrA23 (Fig. 3b). Similarly, in the NCS-treated plants, TyrA23 prevented the accumulation of PIN2 in NCS bodies (Fig. 3b), suggesting that NCS affects the downstream pathway of a TyrA23-sensitive clathrin-mediated endocytosis.

To get more insights into the effects of NCS on intracellular trafficking, the NCS-induced intracellular accumulation of PIN2-GFP was analyzed in presence of BFA. With BFA treatment, plasma membrane/endosome

recycling PIN proteins are accumulated in intracellular compartments called BFA bodies (Geldner et al. 2001, 2003). Geldner et al. (2003) described BFA-induced PIN2 accumulation in BFA bodies as a result of endocytosis (Fig. 4a). When PIN2-GFP seedlings (5-day-old) were pretreated with 25  $\mu$ M NCS and followed by 25  $\mu$ M NCS and 50  $\mu$ M BFA treatment for 2 h, the accumulation of PIN2-GFP in BFA bodies was not inhibited. Instead, the PIN2-GFP internalization was co-induced by NCS and BFA (Fig. 4b). This result indicates that the NCS bodies are not sensitive to BFA treatment. It was reported that auxin transport inhibitor TIBA can interfere with ARF-GEF-dependent vesicle trafficking and PM localization of PIN proteins (Geldner et al. 2001; Kleine-Vehn et al. 2006). We found that application of TIBA inhibited the internalization of PIN2-GFP into BFA bodies (Fig. 4c). PIN2-labelled BFA bodies recycled back to the PM when seedlings were transferred from BFA to DMSO for 2 h (Fig. 4d). Similarly, intracellular PIN2-GFP accumulation was also recovered from BFA bodies to the PM when BFA was removed even in the presence of NCS (Fig. 4e), suggesting that the NCS-induced intracellular accumulation is not due to the inhibition of protein trafficking from endomembranes to the PM. However, PIN2 remained in intracellular compartments when BFA was washed out in the presence of TIBA (Fig. 4f). These findings suggested

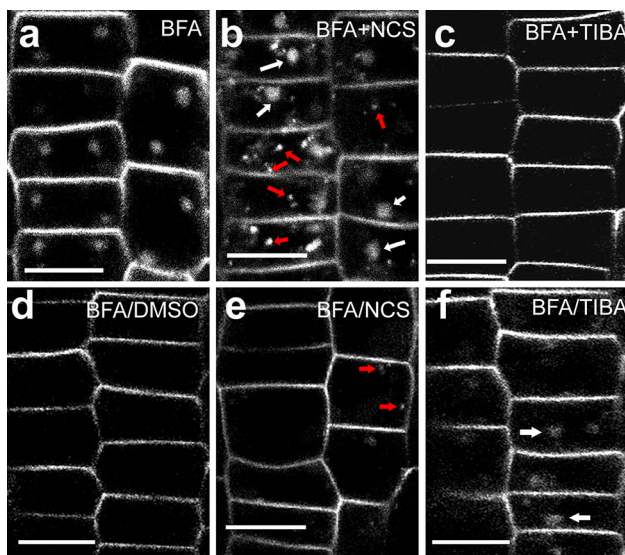
that NCS affects the intracellular trafficking of PIN2 proteins via a BFA-insensitive pathway.

### NCS selectively interferes with endomembrane-traffic pathways

In order to examine whether NCS has a general effect on the PM protein turnover, we tested the effects of this compound on other PM proteins including brassinosteroids receptor BRI1 (Geldner et al. 2007), water channel protein PIP2A (Cutler et al. 2000), and low temperature inducible protein 6b fused to GFP (GFP-LTI6b) (Kurup et al. 2005) in root cells. We found that NCS did not induce the intracellular accumulation of all these membrane proteins (Supplementary Fig. S4). These results demonstrated that the effect of NCS on the trafficking of membrane proteins has pronounced proteins specificity. Furthermore, the effect of NCS on the endomembrane trafficking was also examined using multiple endomembrane markers. ARA7 is a member of the Rab5 family of Rab GTPase (Ueda et al. 2001) and GFP-ARA7 was used as an endosomal marker (Jaillais et al. 2006). NCS did not affect the GFP-ARA7-labeled endosomes. Similarly, NCS also had no effects on other endosomal compartment markers, such as VHA-a1-GFP (early endosome/trans-Golgi network, TGN) (Dettmer et al. 2006), YFP-SYP22 (prevacuole compartment) (Robert et al. 2008), and the Golgi marker NAG-GFP (Essl et al. 1999) (Supplementary Fig. S5). This finding further confirmed that NCS selectively interferes with endosomal compartments.

### Effects of NCS on the cytoskeleton

The cytoskeleton determines cellular shape and provides guidance for vesicle trafficking in all eukaryotic cells. Intracellular accumulation of PIN proteins has been widely reported upon depolymerization of the actin cytoskeleton by inhibitor treatments (Geldner et al. 2003; Dhonukshe et al. 2008a, b). On the other hand, the apical location of AUX1 proteins appears to be sensitive to actin interference (Kleine-Vehn et al. 2006). Thus, the effects of NCS on the actin cytoskeleton were tested. We first determined whether NCS affects actin structure in vivo by visualizing a GFP-tagged actin-binding domain from fimbrin (GFP-ABD2) (Wang et al. 2008) after 2 or 6 h treatments. Compared to the control showing fine filament of the actin network, NCS treatment for 2 h disrupted the organization of actin cytoskeleton by reducing its bundling (Fig. 5a, arrows). Severe depolymerization of the actin cytoskeleton was observed after 6 h of NCS treatment. The effect of NCS on actin dynamics was further examined using in vitro assays. However, the in vitro polymerization (Supplementary Fig. S6a) and depolymerization of actin



**Fig. 4** NCS does not affect ARF-GEF-dependent subcellular vesicle trafficking of PIN2 protein. Seedlings expressing PIN2-GFP was incubated with 50  $\mu$ M BFA for 2 h (a), 25  $\mu$ M NCS and 50  $\mu$ M BFA for 2 h following a pretreatment with 25  $\mu$ M NCS for 30 min (b), 25  $\mu$ M TIBA and 50  $\mu$ M BFA for 2 h following a pre-treatment with 25  $\mu$ M TIBA for 30 min (c), DMSO for 2 h after 90 min with 50  $\mu$ M BFA (d), 25  $\mu$ M NCS for 2 h following 90 min with 50  $\mu$ M BFA (e), or 25  $\mu$ M TIBA for 2 h after 90 min with 50  $\mu$ M BFA (f). *White arrows* represent the BFA bodies and *red arrows* for NCS bodies. *Scale bars* 10  $\mu$ m

filaments (Supplementary Fig. S6b) were not altered by NCS treatment. Furthermore, NCS did not affect filament bundling *in vitro* (Supplementary Fig. S6c). In contrast, LatB, an established actin cytoskeleton de-stabilizer, had the expected actin-destabilizing effect *in vivo* or *in vitro* assay (Supplementary Fig. S6a, d). These data suggest that NCS dose not directly interfere with the cellular actin cytoskeleton.

Microtubules have been illustrated to be instrumental for both plant and animal polar vesicle transport during cytokinesis (Van Goietsenoven et al. 2012). Therefore, we investigated the effect of NCS on microtubule organization using a tubulin-GFP transgenic line (Bannigan et al. 2006). As shown in Fig. 5b, no visible changes in microtubule

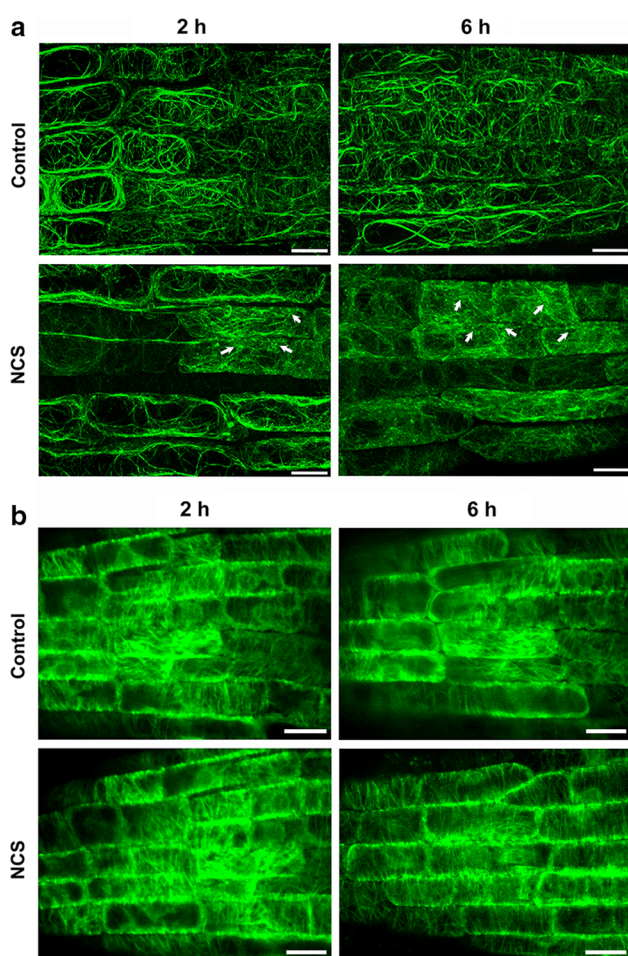
organization were detected after 2 or 6 h of NCS treatments.

Actin cytoskeleton and vesicle motility are interconnected processes. Dhonukshe et al. (2008a, b) reported that stabilizing actin cytoskeleton by TIBA slows down the endosomal motility both in plant and non-plant systems. Therefore, the effect of NCS on endosomal motility was examined using endosomal marker ARA7-GFP. The results showed that NCS blocked the movement of endosomal marker ARA7 (Fig. 6d–f) in contrast with the control (Fig. 6a–c), suggesting that NCS impairs the vesicle motility.

## Discussion

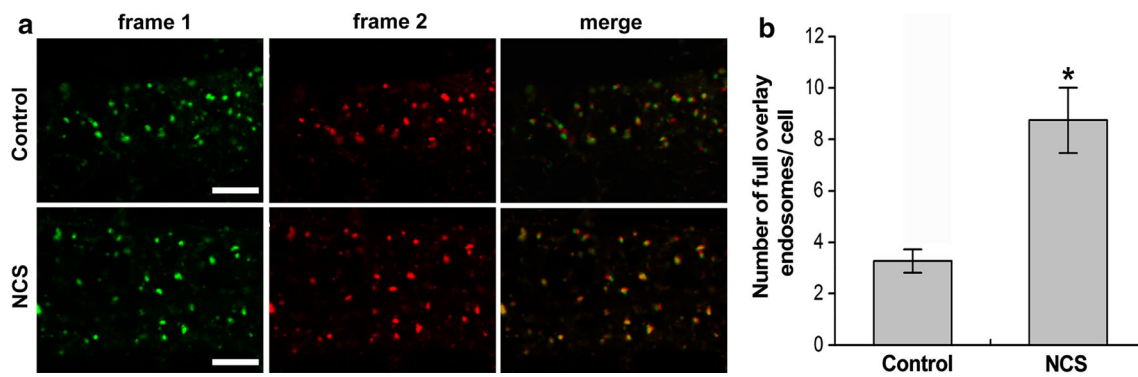
There are some reports indicating that allelochemicals affect the metabolism and responses of phytohormones such as auxin, cytokinin, and ethylene (Ding et al. 2008; Soltys et al. 2012; Graña et al. 2013); however, little information is available about the mechanism by which allelochemicals disrupt phytohormone homeostasis. We first reported that the natural compound NCS has allelopathic potential and inhibits auxin action including auxin signaling and transport (Bi et al. 1998, 2003; Na et al. 2011a, b; Hu et al. 2012, 2014). In the present study, we showed that short-term NCS exposure resulted in the inhibition of subcellular trafficking of PIN and AUX1 proteins and accumulation of intracellular agglomerations (NCS bodies) (Fig. 2 and Supplementary Fig. S2). This observation further supports the conclusion that NCS induces inhibition of gravitropic response and auxin transport in *Arabidopsis* roots (Na et al. 2011b).

The PIN auxin efflux carriers are crucial for auxin transport. The polar subcellular localization of PIN proteins at the PM determines the direction of the polar auxin transport (Wisniewska et al. 2006). PIN polarization at the PM is highly dynamic and regulated at several levels, such as PIN transcription, protein stability, and intracellular trafficking (Geldner et al. 2001; Paciorek et al. 2005). We found that NCS application dramatically reduces the abundance of PIN proteins and AUX1 auxin influx carrier at the PM (Fig. 1). These results are in agreement with our previous findings that NCS inhibits the polar auxin transport in *Arabidopsis* roots (Na et al. 2011b). Normally, the equilibrium of the cell surface and intracellular pools of PINs and AUX1 is shifted in favor of the PM pool, so the intracellular auxin transporters are difficult to visualize (Paciorek et al. 2005). However, NCS treatments caused internalization of PIN2-GFP into intracellular compartments at 0.5  $\mu\text{M}$  or higher concentrations required to affect auxin transport (Fig. 2 and Supplementary Fig. S2). Similar phenotypes were observed in PIN3-GFP, PIN4-GFP,



**Fig. 5** Effects of NCS on the cytoskeleton structure. **a** Effect of NCS on actin cytoskeleton structure. 5-day-old GFP-ABD2 transgenic seedlings were subjected to DMSO (*control*) or 25  $\mu\text{M}$  NCS treatment for 2 h or 6 h. *Arrows* indicate the small aggregations of GFP-ABD2. *Bars* 10  $\mu\text{m}$ . **b** Effect of NCS on microtubule cytoskeleton structure. Five-day-old TUB6-GFP transgenic seedlings were incubated in medium containing DMSO or 25  $\mu\text{M}$  NCS for 2 or 6 h. *Bars* 10  $\mu\text{m}$ . Images were captured using same confocal settings and are representatives of 20 roots obtained from at least three independent experiments. Images are projections of 10–12 optical sections





**Fig. 6** Effect of NCS on endosomal movement. **a** Seedlings of GFP-ARA7 were incubated in DMSO (*control*) or 25  $\mu$ M NCS for 2 h. Movies were captured for 30 s with 6 s time lapse between frames. Merged images were composed by superimposing frame 1 (*green*) and frame 2 (*red*). Images are representatives of 20 roots obtained from three independent experiments. Scale bars 10  $\mu$ m. **b** Number of

GFP-ARA7-labeled endosomes full overlay of frame 1 (*green*) on frame 2 (*red*) per cell. Error bars represent standard deviation ( $*P < 0.05$ ). Data points are the mean of 80 cells from 20 seedlings per experiment, and similar results were obtained in three independent experiments

PIN7-GFP, or AUX1-YFP seedlings treated with 25  $\mu$ M NCS (Fig. 2), suggesting that this compound affects intracellular trafficking of PIN and AUX1 proteins. This finding could explain the reduction of PIN and AUX1 levels at the PM is not only mainly due to the effect of NCS on their transcription (Na et al. 2011b).

Interestingly, NCS does not display a general effect on membrane proteins since other membrane-localized proteins, such as PIN1, PIP2A, BRI1, and LTI6b, were not affected (Fig. 2 and Supplementary Fig. S4). Seedlings expressing PIN1-GFP treated with NCS for 2 h did not form intracellular PIN1 agglomerations (Fig. 2). This result is against the reduction of PIN1 protein levels after 12 h of NCS treatment (Fig. 1). The specificity of the NCS effects is similar to a previous report indicating that a small molecule endosidin1 selectively disrupts the trafficking of the specific PM proteins, such as BRI1, AUX1, and PIN2A (Robert et al. 2008). According to these studies, the differential effects of NCS on PIN and other PM proteins might be explained by the fact that the recycling of PIN proteins and other PM proteins is achieved through different trafficking pathways. For instance, the recycling of PIN1 is dependent on ARF-GEF GNOM, whereas PIN2 takes advantage of additional ARF-GEFs pathways (Geldner et al. 2003).

The dye FM 4-64 is widely used to study endocytosis trafficking in plant cells. Upon incorporation into PM, the dye molecules are internalized into the cell via endocytosis, from where they are either recycled back to the membrane or transported to different endosomes (Bolte et al. 2004). We found that NCS-induced intracellular accumulation of PIN2-GFP partially co-localized with the endocytic tracer FM 4-64 in early endosomes (Fig. 3a), indicating that NCS affects the endocytosis trafficking of PIN proteins. This finding also suggests that the PIN2 protein recycling from

the PM mainly contributes to the formation of NCS bodies. It has also been proposed that the constitutive endocytosis of PIN proteins is dependent on the coat protein clathrin (Dhonukshe et al. 2007). Our pharmacological analysis through TyrA23 (Fig. 3b) in NCS-treated plants showed that NCS affects clathrin-mediated endocytosis trafficking of PIN proteins. These observations further emphasize the functional importance of clathrin-dependent endocytic recycling that is required for establishing the polar PIN localization (Dhonukshe et al. 2008a, b). The crucial role of GNOM in recycling PM proteins was widely reported in studies using the ARF-GEF inhibitor BFA (Geldner et al. 2003; Kleine-Vehn et al. 2008; Naramoto et al. 2014). BFA inhibits the endocytic recycling of PIN proteins and induces the accumulation of PIN proteins in the GNOM-positive intracellular compartments, suggesting that the BFA-sensitive ARF-GEF is functionally associated with the polarized distribution of PIN proteins at the PM (Geldner et al. 2003; Kleine-Vehn et al. 2008). NCS induced the intracellular accumulation of PIN2-GFP in structures that were distinct in appearance from BFA bodies, because PIN2-GFP internalization was co-induced by NCS and BFA (Fig. 4b). Whereas in BFA-treated cells PIN2-GFP did not accumulate in BFA bodies in the presence of TIBA (Fig. 4c), which is consistent with a previous report that TIBA could inhibit the BFA-sensitive ARF-GEF (Kleine-Vehn et al. 2006), PIN2-GFP in BFA bodies was recovered to the PM when PIN2-GFP seedlings were transferred to medium containing NCS (Fig. 4e). This finding suggests that NCS does not affect the BFA-sensitive ARF-GEF pathway and that NCS has a different mode of action relative to that of BFA. Taken together, these results showed that NCS may interfere with the endocytosis trafficking of PIN and AUX1 proteins involved in the BFA-insensitive ARF-GEF pathway, which consequently results

in decreased levels of the auxin transporters in PM and reduces the efficiency of polar auxin transport.

To address the mechanism of NCS effect on PIN proteins trafficking, we explored whether NCS treatment could result in the formation of endomembrane compartment using seedlings expressing GFP-ARA7 (endosomal compartments), VHA-a1-GFP (early endosome/TGN), YFP-SYP22 (pre-vacuole compartment), and NAG-GFP (Golgi marker). The appearance of ARA7-labelled compartments was not obviously different compared with the control (Supplementary Fig. S5). In addition, NCS also has no effects on Golgi markers, endosome markers, or pre-vacuole markers (Supplementary Fig. S5). These results suggested that NCS appears to display more compartment specificity in its action than BFA.

Actin and some of the actin-binding protein (ABP) families are highly conserved between all eukaryotes (Hussey et al. 2006). In general, the assembly, disassembly, and organization of the actin cytoskeleton are regulated by actin-binding proteins including the actin-depolymerizing or polymerizing factor, and some actin-associated regulatory proteins (Hussey et al. 2006). In this study, we also found that NCS treatment disrupted actin filaments in *Arabidopsis* roots, and induced small aggregations of GFP-fimbrin (Fig. 5a), which is a characteristic feature of actin stabilization. This result is similar to the effects observed in mammalian cells that NCS modifies actin cytoskeleton organization in prostate cancer and glioblastoma cells through modulating actin related protein RhoA activity (Lefranc et al. 2009). Recent data obtained in brain cancer cells indicated that NCS targets eukaryotic elongation factor one alpha (eEF1A), resulting in marked actin cytoskeleton disorganization (Lefranc et al. 2009; Van Goietsenoven et al. 2012). NCS interferes with the actin dynamics in both mammalian and plant cells, indicating that its target is highly conserved. eEF1A proteins are abundant and evolutionarily conserved in eukaryotic cells and play a critical role in actin cytoskeleton organization and functions (Rahman et al. 2007). The four genes that encode eEF1A in *Arabidopsis* are designated A1, A2, A3, and A4 (Axelos et al. 1989). Thus, eEF1A could be the direct target of NCS in allelopathic effects on other plants. In addition, the effect of NCS on actin cytoskeleton seems to be indirect, since actin filament polymerization or depolymerization in vitro is unaffected by NCS treatment (Supplementary Fig. S6). Therefore, we hypothesized that NCS functions by either activating or repressing an actin-associated factor which has a strong impact on cellular actin cytoskeleton organization.

Polar localization and intracellular dynamics of PIN and AUX1 proteins require actin filaments but not microtubules (Friml et al. 2002; Kleine-Vehn et al. 2006). It has been

shown that actin drugs selectively affect the subcellular trafficking of auxin transporters. For example, LatB and cytochalasin D cause the enlargement of endosomal compartments including those containing PIN2 (Rahman et al. 2007), PIN3 (Friml et al. 2002), and AUX1 (Kleine-Vehn et al. 2006), but hardly influence PIN1 endocytosis (Rahman et al. 2007). However, LatB treatments could disrupt the polar distribution of PIN1 in protophloem cells of *Arabidopsis* roots (Kleine-Vehn et al. 2006). Similar patterns were also observed in PIN-GFP and AUX1-YFP with NCS treatments, suggesting that the effects of NCS on subcellular trafficking of PINs and AUX1 might be the consequence of disruption of actin cytoskeleton. Multiple studies indicated that the vesicular motility relies mainly on the actin cytoskeleton in plants (Kim et al. 2005). Some auxin transport inhibitors, such as TIBA and 2-(1-pyr-enoyl) benzoic acid (PBA), primarily act to stabilize the actin cytoskeleton, resulting in inhibition of vesicle trafficking and auxin transport (Dhonukshe et al. 2008a, b). Indeed, the movement of endosomal marker ARA7 was inhibited in NCS-treated roots, which demonstrates that NCS slows down the endosomal and vesicle motility (Fig. 6). This is consistent with the effects of NCS on actin dynamics. These observations further prove that the actin-dependent constitutive recycling of auxin transporters may play a more fundamental role in auxin polar transport. Identification of the NCS target may shed lights into the mechanism of NCS action and its effects on cellular trafficking pathways. Further characterization of mutants with altered sensitivity to NCS will provide a potential genetic route to identifying the molecular target(s). Although we observed the effects of NCS on auxin transport and subcellular trafficking of PIN and AUX1 proteins in laboratory studies, whether this compound in natural ecosystems also affects the growth of other species (target plant) when growing close to *Narcissus*, remains unclear. In fact, the concentrations of NCS used in the present study did not mimic the concentrations in natural ecosystems, because the content of this compound released into soil is largely unknown and it could be investigated in the future.

**Author contribution statement** YB, YH, and XN conceived and designed the experiments. YH, XN, JL, LY, JY, XL, JW, and LP performed the experiments. YH and XN analyzed the data. YB, YH, and XN wrote the manuscript. All authors read and made better the manuscript.

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