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Jasmonate modulates endocytosis and plasma membrane accumulation of the Arabidopsis PIN2 protein

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Summary

• The subcellular distribution of the PIN-FORMED (PIN) family of auxin transporters plays a critical role in auxin gradient-mediated developmental processes, including lateral root formation and gravitropic growth.

• Here, we report two distinct aspects of CORONATINE INSENSITIVE 1 (COI1)- and AUXIN RESISTANT 1 (AXR1)-dependent methyl jasmonate (MeJA) effects on PIN2 subcellular distribution: at lower concentration (5 μM), MeJA inhibits PIN2 endocytosis, whereas, at higher concentration (50 μM), MeJA reduces PIN2 accumulation in the plasma membrane.

• We show that mutations of *ASA1* (ANTHRANILATE SYNTHASE a1) and the *TIR1/AFBs* (TRANSPORT INHIBITOR RESPONSE 1/AUXIN-SIGNALING F-BOX PROTEINs) auxin receptor genes impair the inhibitory effect of 5 μM MeJA on PIN2 endocytosis, suggesting that a lower concentration of jasmonate inhibits PIN2 endocytosis through interaction with the auxin pathway. In contrast, mutations of *ASA1* and the *TIR1/AFBs* auxin receptor genes enhance, rather than impair, the reduction effect of 50 μM MeJA on the plasma membrane accumulation of PIN2, suggesting that this action of jasmonate is independent of the auxin pathway. In addition to the MeJA effects on PIN2 endocytosis and plasma membrane residence, we also show that MeJA alters lateral auxin redistribution on gravi-stimulation, and therefore impairs the root gravitropic response.

• Our results highlight the importance of jasmonate–auxin interaction in the coordination of plant growth and the adaptation response.

Introduction

The signaling molecule auxin plays a major role in the spatial and temporal coordination of plant growth and development (Tanaka *et al.*, 2006; Vanneste & Friml, 2009). Unique amongst phytohormones, auxin is transported from cell to cell in a directional manner to form an asymmetric distribution in specific tissues, which directs diverse auxin gradient-dependent developmental processes, including embryo patterning, organogenesis and tropisms (Gälweiler *et al.*, 1998; Friml *et al.*, 2002, 2003; Benková *et al.*, 2003; Reinhardt *et al.*, 2003; Blilou *et al.*, 2005; Scarpella *et al.*, 2006). Polar auxin transport (PAT) involves the PIN-FORMED (PIN) family of auxin efflux carriers, the AUX1/LAX family of auxin influx carriers and the ABC-type multidrug resistance p-glycoproteins (MDR/PGPs) (Blakeslee *et al.*, 2007; Titapiwatanakun & Murphy, 2009). PIN proteins exhibit characteristic polar localization

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in the plasma membrane (PM) that determines the direction and rate of intercellular auxin flow (Petrasek *et al.*, 2006; Wisniewska *et al.*, 2006), which contributes to the formation and maintenance of asymmetric auxin distribution. Some of the PIN proteins cycle continuously between the PM and endosomal compartments. This process has been implicated in the control of the subcellular localization of PIN proteins, which, in turn, determines the direction of auxin transport. Emerging evidence indicates that different PIN proteins use distinct trafficking routes (Feraru & Friml, 2008; Kleine-Vehn & Friml, 2008). For example, PIN1 seems to be constitutively recycled from endosomes in a pathway dependent on GNOM (Geldner *et al.*, 2003), a GDP/GTP exchange factor (GEF) for the ADP-ribosylation factor (ARF) GTPases (Steinmann *et al.*, 1999). Trafficking of PIN2, but not PIN1, involves SNX1 (sorting nexin), which is a putative retromer component (Jaillais *et al.*, 2006; Kleine-Vehn *et al.*, 2008). These studies suggest that the intracellular trafficking of PIN proteins is highly regulated by internal and external cues. Indeed, recent elegant reports have indicated that PIN protein trafficking is regulated directly by auxin itself (Paciorek *et al.*, 2005; Pan *et al.*, 2009; Robert *et al.*, 2010) and by membrane sterol composition (Willemsen *et al.*, 2003; Men *et al.*, 2008; Pan *et al.*, 2009). However, the molecular mechanisms of the auxin effect on PIN intracellular trafficking remain controversial. It has been shown that auxin inhibits the endocytosis of PIN2 through the $SCF^{TIR1/AFBs}$-dependent auxin signaling pathway (Pan *et al.*, 2009). However, Robert *et al.* (2010) reported that the auxin effect on PIN internalization does not require the ubiquitin-protein ligase $SCF^{TIR1/AFBs}$-dependent auxin signaling, but is mediated by ABP1 (AUXIN-BINDING PROTEIN1)-dependent nontranscriptional auxin signaling. In addition to auxin, environmental stimuli, such as gravity (Abas *et al.*, 2006) and light (Laxmi *et al.*, 2008), have been shown to modulate subcellular PIN trafficking and auxin distribution.

Significantly, the subcellular localization of PIN proteins appears to be tightly linked to their turnover. For example, PIN2 endocytosis is closely linked to its degradation during the root gravitropic response (Abas *et al.*, 2006). In addition to repressing the endocytosis of PIN proteins, auxin also influences PIN protein stability (Paciorek *et al.*, 2005; Pan *et al.*, 2009). This probably provides a means for PIN accumulation in the PM, and thus an essential feedback regulation of auxin transport by auxin itself. These results led to the proposal that the overall function of PIN constitutive cycling is to enable the rapid changes in PIN polarity in response to developmental and environmental stimuli (Kleine-Vehn *et al.*, 2008; Laxmi *et al.*, 2008; Pan *et al.*, 2009), or to control the levels of PIN proteins in the PM and thus the auxin transport rate (Paciorek *et al.*, 2005; Pan *et al.*, 2009). However, the molecular mechanisms governing the intracellular trafficking and turnover of PIN proteins are still largely unknown.

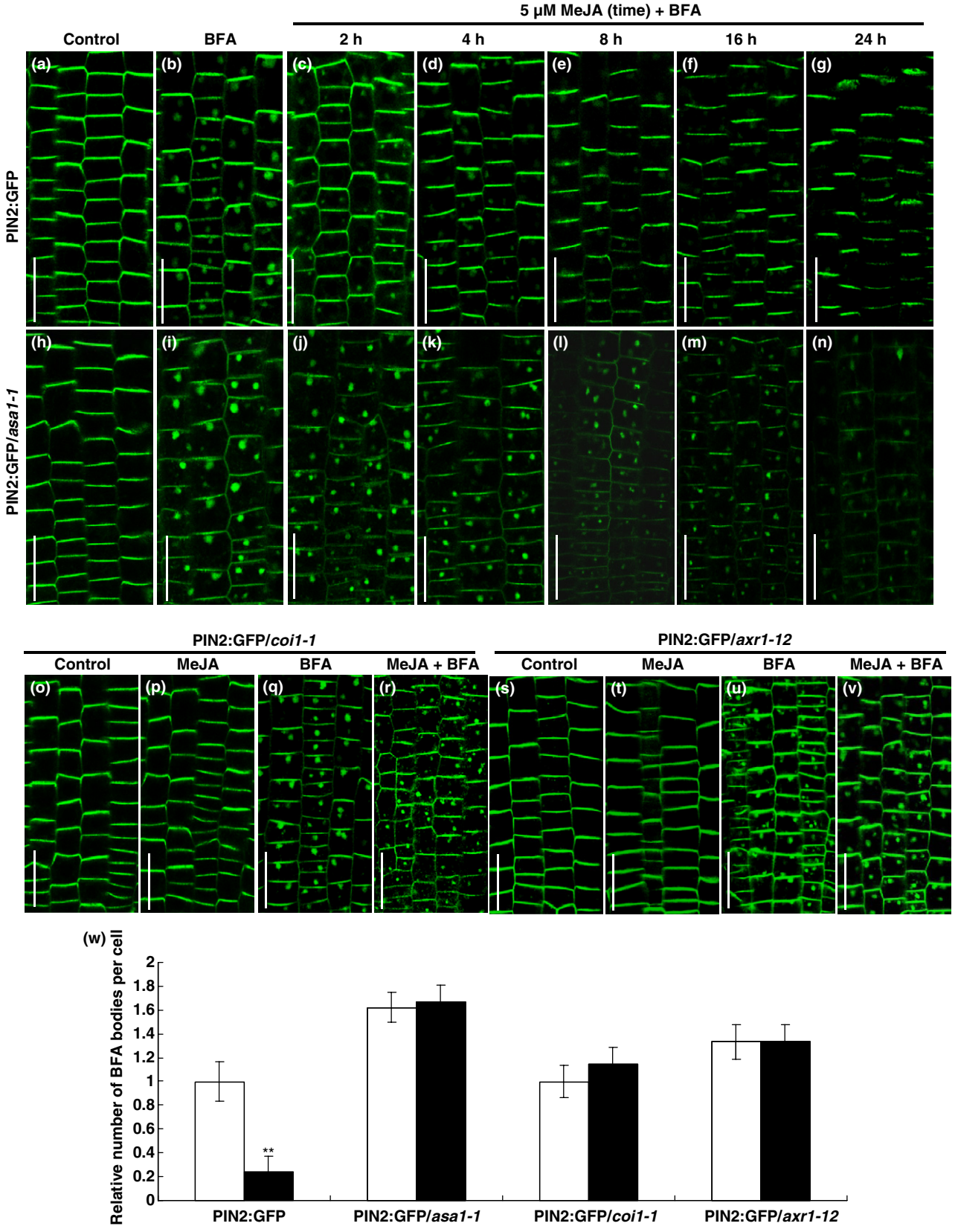
We have reported previously that jasmonate, a stress hormone showing an overall repressive effect on plant growth, regulates lateral root (LR) formation in Arabidopsis through interaction with auxin (Sun *et al.*, 2009). Through the characterization of the *asa1-1* mutant, which is defective in jasmonate-induced LR formation, we have been able to demonstrate that, in addition to promoting auxin biosynthesis through transcriptional activation of the *ASA1 (ANTHRANILATE SYNTHASE a1)* gene, jasmonate negatively regulates auxin transport through the reduction of PIN1 and PIN2 protein levels in the PM. Interestingly, our results also demonstrated that the jasmonate-induced reduction of PM-localized PIN1/2 protein levels may be masked by the function of *ASA1* (i.e. jasmonate-induced auxin biosynthesis). In order to explore the mechanism underlying the jasmonate-induced reduction of PM-localized PIN1/2 protein levels, we investigate here the effects of jasmonate on the intracellular trafficking of the PIN2 protein in Arabidopsis. Our results indicate that jasmonate at lower concentration shows an inhibitory effect on PIN2 endocytosis through ASA1-dependent auxin biosynthesis and $SCF^{TIR1/AFBs}$-dependent auxin signaling; however, jasmonate at higher concentration down-regulates PIN2 protein levels in the PM through a mechanism that is independent of the auxin pathway. We also show that jasmonate-mediated modulation of PIN2 subcellular distribution and abundance affects lateral auxin redistribution during the root gravitropic response.

Materials and Methods

Plant materials and growth conditions

The Arabidopsis mutants and/or transgenic lines described are in the Columbia (Col-0) background. Some of the plant materials used in this study have been described previously: *pPIN1:PIN1-GFP* (Benková *et al.*, 2003), *pPIN2:PIN2-GFP* (Blilou *et al.*, 2005; Xu & Scheres, 2005), *pPIN2:PIN2-GFP/tir1-1* (Pan *et al.*, 2009), *pPIN2:PIN2-GFP/tir1 afb1,2,3* and *pPIN2:PIN2-GFP/axr1-12* (Pan *et al.*, 2009), *DR5rev:GFP* (Benková *et al.*, 2003), *DR5rev:GFP/asa1-1* (Sun *et al.*, 2009), *pAUX1:AUX1-YFP* (Swarup *et al.*, 2004), *proPIN1:GUS* and *proPIN2:GUS* (Sun *et al.*, 2009), inducible *abp1* knockdown line *SS12K* (Tromas *et al.*, 2009). Only seedlings with healthy roots from the population of *tir1 afb1, 2, 3* quadruple mutants were analyzed.

Seeds were surface sterilized for 15 min in 10% bleach, washed four times with sterile water and plated on half-strength Murashige and Skoog (MS) medium. Plants were stratified at 4°C for 2 d in darkness and then transferred to a phytotrone set at 22°C with a 16-h light : 8-h dark photoperiod (light intensity 120 μmol m^{-2} s^{-1}). For methyl jasmonate (MeJA) treatment, seedlings were transferred to a phytotrone with continuous light (light intensity 120 μmol m^{-2} s^{-1}).



Introduction of auxin transport reporters into *asa1-1* or *coi1-1* mutants

To generate *pPIN2:PIN2-GFP* reporter line in the *asa1-1* mutant background, a homozygous *asa1-1* plant was crossed with a transgenic line harboring the *pPIN2:PIN2-GFP* construct (Blilou *et al.*, 2005; Xu & Scheres, 2005) to produce an F2 population. Putative *pPIN2:PIN2-GFP/asa1-1* plants, which were homozygous for the *asa1-1* mutation (Sun *et al.*, 2009) as well as the *pPIN2:PIN2-GFP* construct, were identified in F2 and then re-tested in F3 [i.e. in F3, 100% of seedlings showed no LR formation in the presence of 20 μM MeJA; 100% of seedlings showed uniform green fluorescent protein (GFP) fluorescence]. Similarly, the *pPIN2:PIN2-GFP* reporter was also introduced into the *coi1-1* mutant background (Xie *et al.*, 1998). The *pPIN1:PIN1-GFP* (Benková *et al.*, 2003), *pAUX1:AUX1-YFP* (Swarup *et al.*, 2004), *proPIN1:GUS* and *proPIN2:GUS* (Sun *et al.*, 2009) reporters were introduced into the *asa1-1* mutant background using a similar approach.

Chemical solutions and treatments

All chemicals were purchased from Sigma-Aldrich. MeJA and brefeldin A (BFA) were dissolved in ethanol or dimethyl sulfoxide (DMSO). For MeJA treatment, 5-d-old seedlings were transferred into liquid medium containing the indicated concentrations (5 or 50 μM) of MeJA for different time points; seedlings were then treated (or not) with cycloheximide (CHX) for 30 min and then (or not) with BFA (final concentration 25 μM) for 60 min before confocal laser microscopy analysis.

PIN2 immunolocalization analysis

The PIN2 immunolocalization assays were performed as described previously (Sun *et al.*, 2009). The following antibodies and dilutions were used: anti-PIN2 (1 : 400) and Alexa Fluor 555 secondary (1 : 500; Molecular Probes, Carlsbad, America) antibodies. Fluorescent samples were inspected with a Leica confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

Membrane protein extraction and immunoblot analysis

For immunoblot analysis of PIN2:GFP, 5-d-old *pPIN2:PIN2-GFP* and *pPIN2:PIN2-GFP/asa1-1* seedlings were transferred to liquid medium with or without 50 μM MeJA for 24 h, and seedlings were harvested for membrane protein extraction and immunoblot analysis with anti-GFP antibody (Abcam, Cambridge, United Kingdom). The extraction of membrane protein fractions was performed as described previously (Kleine-Vehn *et al.*, 2008).

Confocal laser microscopy analysis

The GFP and yellow fluorescent protein (YFP) fluorescences were imaged under a Leica confocal laser scanning microscope (Leica Microsystems). To image GFP and YFP, the 488- and 514-nm lines of the argon laser were used for excitation, and emission was detected at 510 and 530 nm, respectively. Images were assembled using Photoshop software (Adobe Systems, San Jose, America). Fluorescence was quantified with the LAS AF Lite program on confocal sections acquired with the same microscope settings (Růžička *et al.*, 2007). Approximately 5–10 images were examined, and at least three independent experiments were carried out.

FM4-64 staining

Four-day-old Col-0 seedlings were transferred into liquid medium (control) or medium containing MeJA (5 or 50 μM) for 16 h, and then incubated with FM4-64 (2 μM, 2 h; Bolte *et al.*, 2004) followed by washout three times. After incubation for 20 or 45 min, fluorescence was observed with a confocal microscope (543 nm excitation, 633 nm emission). All images in a single experiment were captured with the same settings.

Root gravitropism assay

To assess the effect of MeJA on root gravitropism, 5-d-old seedlings of the indicated genotypes were transferred to plates containing different concentrations of MeJA and

Fig. 1 Effect of 5 μM methyl jasmonate (MeJA) on PIN2:GFP endocytosis in the wild-type (WT) (PIN2:GFP), *asa1-1* (PIN2:GFP/*asa1-1*), *coi1-1* (PIN2:GFP/*coi1-1*) and *axr1-12* (PIN2:GFP/*axr1-12*) mutants. (a–n) Five-day-old PIN2:GFP (a–g) and PIN2:GFP/*asa1-1* (h–n) seedlings were pretreated with 5 μM MeJA for the indicated times and then treated with 5 μM MeJA and 25 μM brefeldin A (BFA) for 1 h before laser confocal microscopy analysis. PIN2:GFP fluorescence in the root epidermis cells in the meristematic and elongation zones is shown. Bars, 25 μm. (o–v) Five-day-old PIN2:GFP/*coi1-1* (o–r) and PIN2:GFP/*axr1-12* (s–v) seedlings were pretreated with 5 μM MeJA for 8 h and then treated with 25 μM BFA and 5 μM MeJA for 1 h before laser confocal microscopy analysis. PIN2:GFP in root epidermis cells in the meristematic and elongation zones is shown. Bars, 25 μm. (w) Relative number of BFA bodies per cell. Five-day-old PIN2:GFP, PIN2:GFP/*asa1-1*, PIN2:GFP/*coi1-1* and PIN2:GFP/*axr1-12* seedlings were pretreated with 5 μM MeJA for 8 h and then treated with 25 μM BFA (open bars) and 5 μM MeJA (closed bars) for 1 h before laser confocal microscopy analysis. At least 60 cells were measured for each assay. Error bars represent SD, and Student's *t*-test between MeJA-treated and untreated plants was performed (**, $P < 0.01$).

immediately gravi-stimulated with 90° rotation. After 24 h or the indicated time points, root curvature was measured. Digital images were collected from at least 20 seedlings for each genotype or treatment, and the root tip angles were quantified.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis and β-glucuronidase (GUS) activity analysis

Real-time qRT-PCR analysis and GUS activity analysis for *PIN1* and *PIN2* expression were performed according to our previous report (Sun *et al.*, 2009).

Results

At low concentrations, jasmonate inhibits PIN2:GFP endocytosis in a CORONATINE INSENSITIVE 1 (COI1)- and AUXIN RESISTANT 1 (AXR1)-dependent manner

The subcellular localization of PIN1 and PIN2 is maintained by endocytosis and recycling through vesicle trafficking in a process termed 'constitutive cycling' (Geldner *et al.*, 2001; Muday *et al.*, 2003; Murphy *et al.*, 2005; Shin *et al.*, 2005; Dhonukshe *et al.*, 2007; Kleine-Vehn & Friml, 2008; Pan *et al.*, 2009). It has been shown that auxin inhibits PIN1 and PIN2 endocytosis and promotes their PM localization through the $SCF^{TIR1/AFBs}$ and ABP1-dependent auxin signaling pathways (Paciorek *et al.*, 2005; Pan *et al.*, 2009; Robert *et al.*, 2010). Recently, we have reported that jasmonate treatment leads to a reduction in PIN1 and PIN2 protein levels in the PM (Sun *et al.*, 2009). To investigate whether jasmonate might regulate PIN protein distribution via its effect on vesicle trafficking, we examined the jasmonate-regulated endocytosis of PIN2 using a transgenic line containing a functional PIN2:GFP marker (Blilou *et al.*, 2005; Xu & Scheres, 2005). This PIN2:GFP marker line in the Col-0 background was used as the wild-type (WT) in this study.

It has been shown that, in the presence of the recycling inhibitor BFA, internalized PIN1 and PIN2 accumulate in intracellular compartments termed 'BFA bodies' (Geldner *et al.*, 2001; Paciorek *et al.*, 2005; Pan *et al.*, 2009). Consistent with these observations, the accumulation of PIN2:GFP in BFA bodies was clearly observed after 60 min of BFA treatment (Fig. 1a,b). To investigate the effect of jasmonate on BFA body formation, seedlings were first pretreated with 5 μM MeJA for different times and then with BFA (25 μM) for 1 h to induce BFA body formation. As shown in Fig. 1(c–g), obvious reduction of BFA body formation was clearly observed at 4 h after MeJA pretreatment and, as time went on, the reduction effect became more dramatic, suggesting that a low concentration of MeJA inhibits PIN2:GFP endocytosis. Using the fluorescent dye FM4-64 as an endocytic tracer (Bolte *et al.*, 2004; Ueda *et al.*, 2004), we found that 5 μM MeJA showed little effect on FM4-64 uptake, but 50 μM MeJA inhibited significantly the number of endosomes labelled by FM4-64 (Supporting Information Fig. S1).

To determine whether the observed inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis requires the F-box protein COI1, which has been shown recently to be a jasmonate receptor in Arabidopsis (Yan *et al.*, 2009; Sheard *et al.*, 2010), we compared BFA-induced internalization of PIN2:GFP between WT and *coi1-1*, a loss-of-function mutant of COI1 (Xie *et al.*, 1998). In the absence of MeJA, BFA-induced internalization of PIN2:GFP was similar between *coi1-1* and WT (Fig. 1b,q). However, the inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis was largely abolished in *coi1-1* (Fig. 1r,w). Similarly, the inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis was also attenuated significantly in the *axr1-12* mutant (Fig. 1v,w), which is defective in both jasmonate and auxin signaling (Timpte *et al.*, 1995; Tiryaki & Staswick, 2002). These results indicate that jasmonate inhibition of PIN2:GFP endocytosis strictly depends on the function of COI1 and AXR1.

At low concentrations, jasmonate inhibition of PIN2:GFP endocytosis requires ASA1-dependent auxin biosynthesis and ubiquitin-protein ligase TIR1/AFBs-dependent auxin signaling

Considering that jasmonate induces auxin biosynthesis through transcriptional activation of the *ASA1* gene (Sun *et al.*, 2009) and auxin itself inhibits PIN2 endocytosis (Paciorek *et al.*, 2005; Pan *et al.*, 2009), a plausible explanation for the observed jasmonate inhibition of PIN2:GFP endocytosis is that MeJA inhibits PIN2:GFP endocytosis through the induction of *ASA1*-mediated auxin biosynthesis. To address this, we compared the effect of MeJA on PIN2:GFP endocytosis between WT and *asa1-1*, a mutant defective in jasmonate-induced auxin biosynthesis (Sun *et al.*, 2009). In the absence of MeJA treatment, the PIN2:GFP signal in BFA bodies was higher in *asa1-1* than in WT (Fig. 1b,i,w). The inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis in *asa1-1* (Fig. 1j–n) was dramatically attenuated when compared with that in WT (Fig. 1c–g,w). These results together suggest that endogenous auxin synthesized by ASA1 plays a critical role in the inhibition of basal and BFA-induced PIN2:GFP endocytosis. Indeed, in both the absence and presence of MeJA treatment, exogenous feeding of a low concentration (10 μM) of naphthalene-1-acetic acid (1-NAA) effectively restored the PIN2:GFP endocytosis defect of the *asa1-1* mutant (Fig. S2).

In the context that auxin itself regulates PIN2 endocytosis through $SCF^{TIR1/AFBs}$-dependent auxin signaling

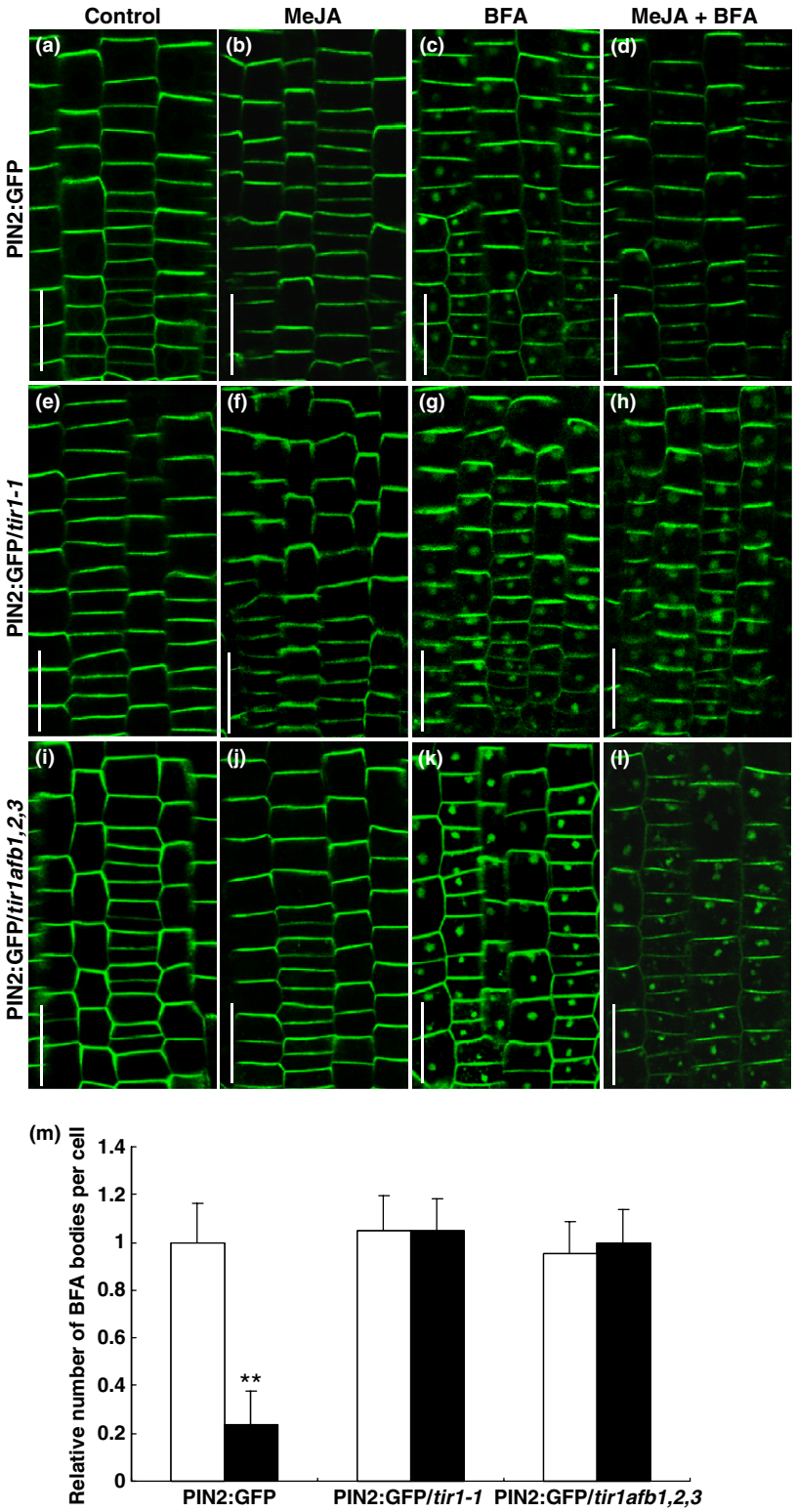


Fig. 2 Effect of 5 μM methyl jasmonate (MeJA) on PIN2:GFP endocytosis in wild-type (WT) (PIN2:GFP), *tir1-1* (PIN2:GFP/*tir1-1*) and the *tir1afb1, 2, 3* quadruple mutant (PIN2:GFP/*tir1afb1, 2, 3*). (a–l) Five-day-old PIN2:GFP (a–d), PIN2:GFP/*tir1-1* (e–h) and PIN2:GFP/*tir1afb1, 2, 3* (i–l) seedlings were pretreated with 5 μM MeJA for 8 h and then treated with 25 μM brefeldin A (BFA) and 5 μM MeJA for 1 h before laser confocal microscopy analysis. PIN2:GFP in the root epidermis cells in the meristematic and elongation zones is shown. Bars, 25 μm. (m) Relative number of BFA bodies per cell as shown in (a)–(l). At least 60 cells were measured for each assay. Error bars represent SD, and Student's *t*-test between MeJA-treated (closed bars) and untreated (open bars) plants was performed (**, $P < 0.01$).

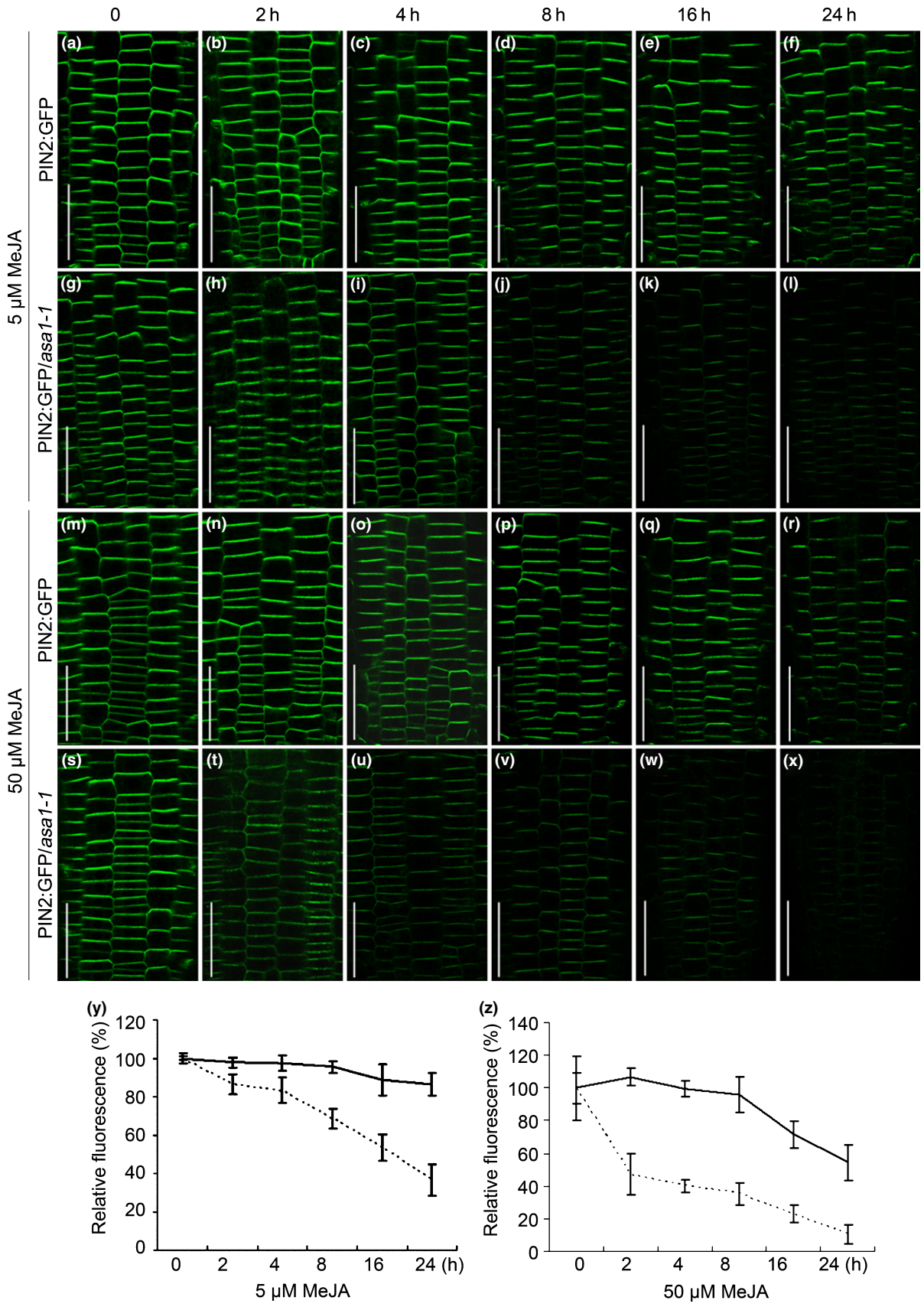


Fig. 3 Reduction effect of methyl jasmonate (MeJA) on plasma membrane (PM)-resident PIN2:GFP in wild-type (WT) (PIN2:GFP) and *asa1-1* (PIN2:GFP/*asa1-1*). (a–l) Five-day-old PIN2:GFP (a–f) and PIN2:GFP/*asa1-1* (g–l) seedlings were treated with 5 μM MeJA for the indicated times before laser confocal microscopy analysis. (m–x) Five-day-old PIN2:GFP (m–r) and PIN2:GFP/*asa1-1* (s–x) seedlings were treated with 50 μM MeJA for the indicated times before laser confocal microscopy analysis. PIN2:GFP in root epidermis cells in the meristematic and elongation zones is shown. It should be noted that the MeJA reduction of PIN2:GFP levels was enhanced by the *asa1-1* mutation. Bars, 50 μm. (y) Quantification of PM-resident PIN2:GFP fluorescence shown in (a)–(l). (z) Quantification of PM-resident PIN2:GFP fluorescence shown in (m)–(x). (y, z) PIN2:GFP, solid line; PIN2:GFP/*asa1-1*, broken line. The error bars in (y) and (z) are ± SD.

(Paciorek *et al.*, 2005; Pan *et al.*, 2009), we next examined the MeJA effect on PIN2:GFP endocytosis in auxin receptor mutants, including *tir1-1* and the *tir1 afb1, 2, 3* quadruple mutants (Dharmasiri *et al.*, 2005; Pan *et al.*, 2009). In line with previous observations (Pan *et al.*, 2009), without MeJA treatment, the PIN2:GFP signal in BFA bodies was higher in the auxin signaling mutants than in WT plants (Fig. 2c,g,k). The inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis was markedly reduced in the *tir1-1* and *tir1 afb1, 2, 3* quadruple mutants when compared with WT (Fig. 2d,h,l,m). These results suggest that the inhibitory effect of jasmonate on PIN2:GFP endocytosis requires $SCF^{TIR1/AFBs}$-dependent auxin signaling.

To determine whether the jasmonate-induced inhibition of PIN endocytosis requires ABP1-mediated auxin signaling, we employed a chemically inducible conditional *ABP1* knockdown line named *SS12K* (Tromas *et al.*, 2009). Under our experimental conditions, we observed obvious root growth inhibition of *SS12K* seedlings on ethanol induction, suggesting an effective knockdown of *ABP1* expression. As shown in Fig. S3, our immunolocalization assays revealed that the effect of 5 μM MeJA on PIN2 endocytosis was largely similar between WT and *SS12K*. These results argue that the inhibitory effect of 5 μM MeJA on PIN2 endocytosis is independent of ABP1.

At high concentrations, jasmonate negatively regulates PM-resident PIN2:GFP abundance in a COI1- and AXR1-dependent manner

We conducted time-course experiments to examine the effect of MeJA on PM-resident PIN2:GFP abundance. At a low concentration (5 μM), MeJA showed a negligible effect on PM-resident PIN2:GFP abundance in WT seedlings (Fig. 3a–f). We then increased the MeJA concentration to 50 μM and found that this concentration of MeJA exerted a reduction effect on the abundance of PM-resident PIN2:GFP. The reduction in PM-resident PIN2:GFP abundance appeared 8 h after MeJA treatment and, as time went on, the reduction effect became more dramatic (Fig. 3m–r). Consistently, as revealed by immunolocalization assays using anti-PIN2 antibodies, 50 μM MeJA exerted similar reduction kinetics on the abundance of PM-resident PIN2 protein (Fig. 4a–f). Given that our qRT-PCR and *PIN2* native promoter-*GUS* assays revealed that MeJA treatments increased the *PIN2* transcripts in WT roots (Figs S4, S5), we excluded the possibility that the observed MeJA reduction of PIN2:GFP abundance in the PM occurs at the transcriptional level. We therefore reasoned that the MeJA-induced reduction of PM-resident PIN2:GFP or PIN2 protein occurs at the post-transcriptional level.

Not surprisingly, the *coi1-1* (Fig. 5c,d,g) and *axr1-12* (Fig. 5e–g) mutations largely impaired the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP abundance, suggesting that jasmonate-induced reduction of the PM-resident PIN2:GFP level requires COI1- and AXR1-mediated jasmonate signaling.

Jasmonate-induced reduction of PIN2:GFP abundance in the PM is enhanced by mutations of *ASA1* and auxin receptor genes *TIR1/AFBs*

As already described, 5 μM MeJA inhibits PIN2:GFP endocytosis through the activation of ASA1-dependent auxin biosynthesis and $SCF^{TIR1/AFBs}$-dependent auxin signaling. We therefore examined the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP abundance in the *asa1-1* mutant. Surprisingly, our time-course experiments revealed that the *asa1-1* mutation dramatically enhanced, rather than impaired, the reduction effect of MeJA on PM-resident PIN2:GFP abundance (Fig. 3m–x). In the WT background, the reduction of PM-resident PIN2:GFP was first observed 8 h after 50 μM MeJA treatment (Fig. 3p), whereas, in the *asa1-1* background, an obvious reduction of PM-resident PIN2:GFP was observed as early as 2 h after 50 μM MeJA treatment (Fig. 3t). Quantification of GFP fluorescence indicated that PM-resident PIN2:GFP reduction by 50 μM MeJA was generally much more severe in *asa1-1* than in WT (Fig. 3z). Similarly, immunolocalization assays revealed that the PM-resident PIN2 reduction by 50 μM MeJA was much more severe in *asa1-1* than in WT (Fig. 4a–l). The application of CHX, which can effectively block *de novo* protein synthesis, did not affect the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP in either WT or *asa1-1* (Fig. S4), providing another line of evidence that the observed effect of MeJA on PM-resident PIN2:GFP occurs at the post-transcriptional level. We also examined the effect of a low concentration of MeJA, and found that 5 μM MeJA led to an obvious reduction in PM-resident PIN2:GFP in *asa1-1*, whereas this concentration of MeJA showed very subtle, if any, reduction effect on

PM-resident PIN2:GFP (Fig. 3a–l,y). Next, we performed protein gel blot assays using anti-GFP antibody probed with total membrane proteins extracted from WT (*PIN2:GFP*) and *asa1-1* (*PIN2:GFP/asa1-1*) seedlings. As shown in Fig. 4m, although 50 μM MeJA had a negligible effect on the abundance of total membrane-bound PIN2:GFP in WT, it substantially reduced the abundance of total membrane-bound PIN2:GFP in *asa1-1* (Fig. 4m,n). These results suggest that a high concentration of MeJA possibly promotes PIN2 protein turnover.

The reduction effect of 50 μM MeJA on PM-resident PIN2:GFP abundance was shown to be similar between *tir1-1* and WT (Fig. 6a–d,g). Considering the existence of functional redundancy between TIR1 and AFB proteins (Dharmasiri *et al.*, 2005), we further examined the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP in the *tir1 afb1, 2, 3* quadruple mutant (Dharmasiri *et al.*, 2005; Pan *et al.*, 2009). Similar to the *asa1-1* mutation, the *tir1 afb1, 2, 3* quadruple mutation also dramatically enhanced the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP (Fig. 6a,b,e–g). Together, our finding that the mutation of *ASA1* and the auxin receptor genes enhances, rather than impairs, the MeJA reduction of PM-resident PIN2:GFP abundance suggests that jasmonate affects PM-resident PIN2:GFP abundance independently of the auxin pathway.

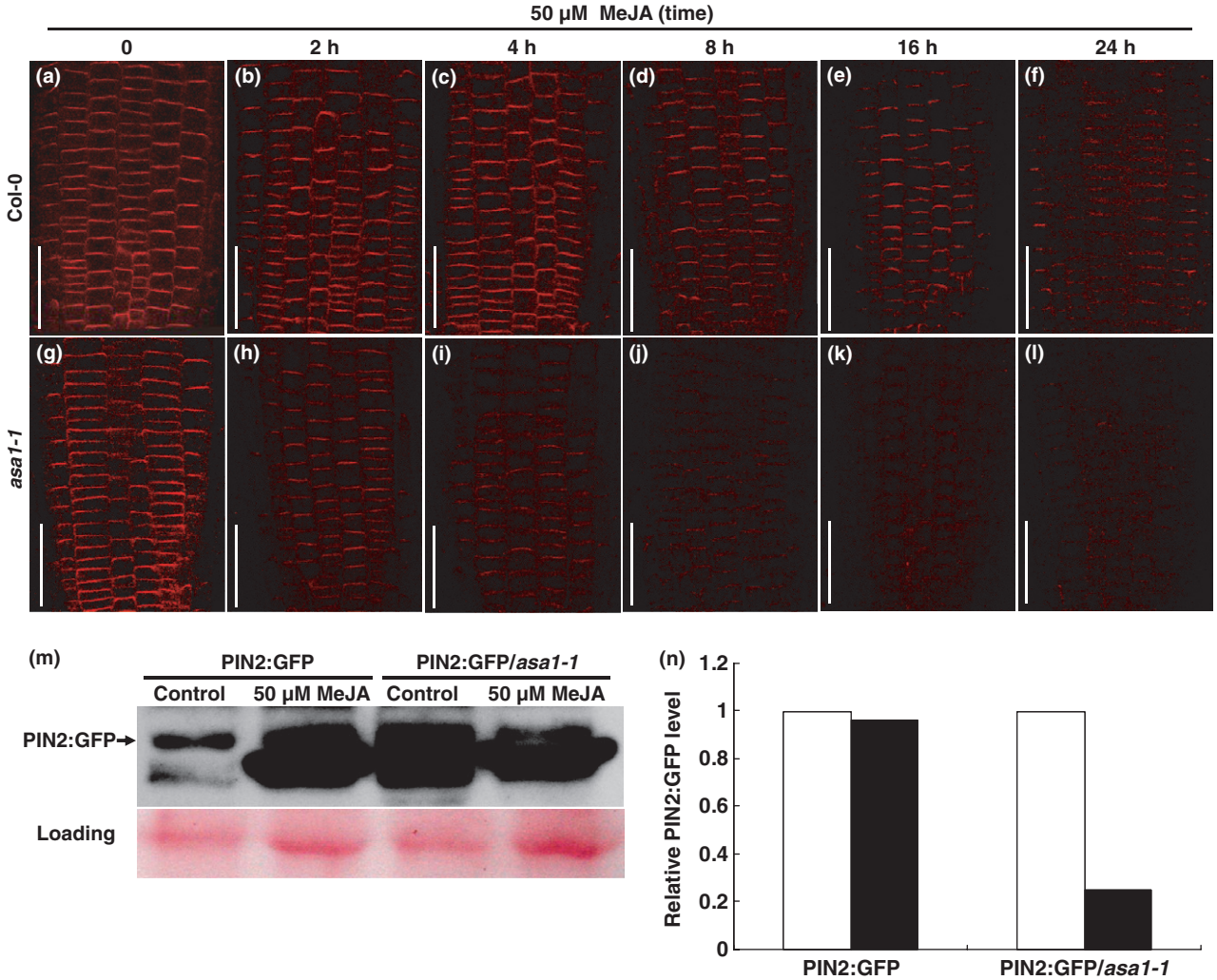


Fig. 4 Effect of 50 μM methyl jasmonate (MeJA) on PIN2 and PIN2:GFP. (a–l) Effect of 50 μM MeJA on plasma membrane (PM)-resident PIN2 revealed by immunolocalization analyses using anti-PIN2 antibody. Five-day-old Col-0 (a–f) and *asa1-1* (g–l) seedlings were treated with 50 μM MeJA for the indicated times before being fixed for immunolocalization assays. Root tips were visualized and photographed with a laser scanning confocal microscope. Bars, 50 μm. (m) Effect of 50 μM MeJA on the abundance of total membrane protein revealed by protein gel blot analysis. WT (PIN2:GFP) and *asa1-1* (PIN2:GFP/*asa1-1*) seedlings were treated with 50 μM MeJA for 24 h before total membrane proteins were extracted. The protein gel blot was probed with anti-green fluorescent protein (anti-GFP) antibody (Abcam). Ponceau-stained membrane is shown as the loading control. (n) Quantification analysis of PIN2:GFP protein level as shown in (m) (control, open bars; 50 μM MeJA, closed bars). Quantification analysis was performed using Image J software.

Jasmonate regulates the subcellular distribution of the auxin efflux carrier PIN1, but not the auxin influx carrier AUX1

To investigate whether jasmonate affects the subcellular distribution of other PIN proteins, we examined the abundance of a functional PIN1:GFP fusion protein (Benková *et al.*, 2003). Similar to the PIN2:GFP case, 50 μM MeJA treatment led to a slight, but significant, reduction in the PM-resident PIN1:GFP signal in WT (Fig. 7a,b,e). Importantly, the reduction effect of 50 μM MeJA on the PM-resident PIN1:GFP signal was substantially enhanced by the *asa1-1* mutation (Fig. 7c–e). *proPIN1:GUS* assays indicated that MeJA increased slightly *PIN1* transcripts in both WT and *asa1-1* (Fig. S6), excluding the possibility that the MeJA reduction of PIN1:GFP abundance occurs at the transcriptional level.

To determine whether jasmonate may specifically affect the PIN family proteins, we examined the effect of MeJA on the AUX1:YFP marker, which contains the auxin influx carrier AUX1 fused with YFP (Swarup *et al.*, 2004). Notably, 50 μM MeJA treatment did not cause a significant change in the overall abundance of the AUX1:YFP signal in WT and the *asa1-1* mutant (Fig. S7). The contrasting effects of MeJA on the intracellular behavior of the auxin efflux carriers (PIN1 and PIN2) and auxin influx carrier (AUX1) are in agreement with previous studies suggesting that AUX1 and PIN1 are internalized through distinct endocytic pathways (Kleine-Vehn *et al.*, 2006). Collectively, our data suggest that jasmonate may selectively regulate PIN-type auxin efflux carriers, but not the auxin influx carrier AUX1.

Jasmonate-mediated reduction of the root gravitropic response is enhanced significantly by the *asa1-1* mutation

In order to explore the physiological relevance of the jasmonate effect on PIN proteins, we examined the root gravitropic response of WT and *asa1-1* in the presence of exogenous MeJA. Consistent with the previous finding that the intracellular trafficking and turnover of PIN2 are involved in root gravitropism (Abas *et al.*, 2006), MeJA treatment led to an obvious reduction in the root gravitropic response in WT seedlings (Fig. 8), and the MeJA reduction of the root gravitropic response was largely abolished in the *coi1-2* mutant (Fig. 8), which harbors a weak mutation of the *COI1* gene (Xu *et al.*, 2002). Significantly, the MeJA-induced reduction in the root gravitropic response was much more severe in *asa1-1* than in WT (Fig. 8). Therefore, *asa1-1* is a jasmonate response mutant in the root gravitropic response. These results support the hypothesis that the jasmonate regulation of PIN2 endocytosis and PM residence is involved in the jasmonate-mediated agravitropic response, and jasmonate-induced *ASA1*-dependent auxin biosynthesis plays an important role in this process.

Jasmonate impairs gravity-induced lateral auxin redistribution in root tips

We next explored *in planta* the link between the effects of jasmonate on PIN2 subcellular distribution and auxin transport during the root gravitropic response. In gravity-responding roots, PIN2 has been shown to function in the redistribution of auxin to rapidly establish a lateral auxin gradient after gravi-stimulation (Geldner *et al.*, 2001; Ottenschläger *et al.*, 2003; Paciorek *et al.*, 2005; Abas *et al.*, 2006; Pan *et al.*, 2009). In this context, we propose that the MeJA interference in PIN2 endocytosis and abundance may affect the gravity-induced lateral auxin redistribution. To test this proposal, we used the auxin-responsive reporter *DR5rev:GFP* to monitor the lateral auxin redistribution pattern in gravi-stimulated WT and *asa1-1* root tips. Without MeJA treatment, gravi-stimulation led to asymmetric *DR5rev:GFP* accumulation at the lower side of WT and *asa1-1* roots (Fig. 9c,d). Consistent with the perturbation effect of MeJA on PIN2 abundance in the PM, MeJA treatment impaired significantly the asymmetric *DR5rev:GFP* accumulation pattern formation in WT and *asa1-1*, and the impairment was much more severe in the latter (Fig. 9e,f). Together, these results may explain the root gravitropic defects of WT and *asa1-1* mutant seedlings in the presence of exogenous MeJA.

Discussion

In response to internal or external cues, plant development displays exceptional plasticity that involves the dynamic, asymmetric distribution of the phytohormone auxin (Vanneste & Friml, 2009). It has been proposed that the polar subcellular localization of PIN proteins determines the strict directionality of PAT flow, which contributes to the establishment and maintenance of auxin gradients (Petrasek *et al.*, 2006; Wisniewska *et al.*, 2006). An important mechanism of auxin-regulating cellular behavior is the modulation of the subcellular translocation of PIN proteins, which perform constitutive internalization and recycling between the PM and endosomal compartments (Paciorek *et al.*, 2005; Pan *et al.*, 2009). Previous investigations have indicated that biologically active auxins, such as the naturally occurring indole-3-acetic acid (IAA) and its synthetic analogues 1-NAA and 2,4-dichlorophenoxyacetic acid (2,4-D), can efficiently inhibit the internalization of several PIN proteins (Paciorek *et al.*, 2005; Pan *et al.*, 2009). By contrast, the biologically inactive naphthalene-2-acetic acid (2-NAA) and several other hormones, including abscisic acid, brassinosteroids, cytokinins, ethylene and gibberellins, have no detectable effect on PIN protein trafficking

(Paciorek *et al.*, 2005). We have demonstrated recently that jasmonate, a stress hormone showing extensive crosstalk with auxin, down-regulates PIN1 and PIN2 protein levels in the PM (Sun *et al.*, 2009), suggesting a possible role of this hormone in PIN protein trafficking. We describe here two distinct aspects of jasmonate action on PIN2:GFP. First, at a low concentration (5 μM), MeJA inhibits PIN2:GFP endocytosis (Fig. 1). Second, at a high concentration (50 μM), MeJA reduces PM-resident PIN2:GFP abundance (Figs 3, 4). The fact that *coi1-1* and *axr1-12* diminished both the inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis (Fig. 1) and the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP abundance (Fig. 5) suggests that the two aspects of MeJA action require the function of COI1 and AXR1, central regulators of jasmonate signaling.

To explore whether the above-described MeJA effects on PIN2:GFP are jasmonate specific, or are simply achieved through the auxin pathway, we employed the *asa1-1* and *tir1 afb1, 2, 3* quadruple mutants, which are defective in jasmonate-induced auxin biosynthesis (Sun *et al.*, 2009) and auxin perception (Dharmasiri *et al.*, 2005), respectively. The *asa1-1* and *tir1 afb1, 2, 3* quadruple mutations impaired the inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis (Figs 1, 2), supporting a scenario in which jasmonate promotes *ASA1*-dependent auxin biosynthesis, which, in turn, inhibits PIN2:GFP endocytosis through $SCF^{TIR1/AFBs}$-dependent auxin signaling. Indeed, the impaired inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis in *asa1-1* could readily be restored by exogenous 1-NAA (Fig. S2). These results led us to the conclusion that the inhibitory effect of 5 μM MeJA on PIN2:GFP endocytosis is achieved through interaction with the auxin pathway, and that ASA1-dependent auxin biosynthesis and $SCF^{TIR1/AFBs}$-dependent auxin signaling are required in this process.

Given that 5 μM MeJA inhibits PIN2:GFP endocytosis through activation of ASA1-dependent auxin biosynthesis and $SCF^{TIR1/AFBs}$-dependent auxin signaling, it is reasonable to speculate that the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP abundance might be achieved through a similar mechanism. If this is the case, we would expect that mutations of *ASA1* and *TIR1/AFBs* could impair the reduction effect of 50 μM MeJA on PM-resident

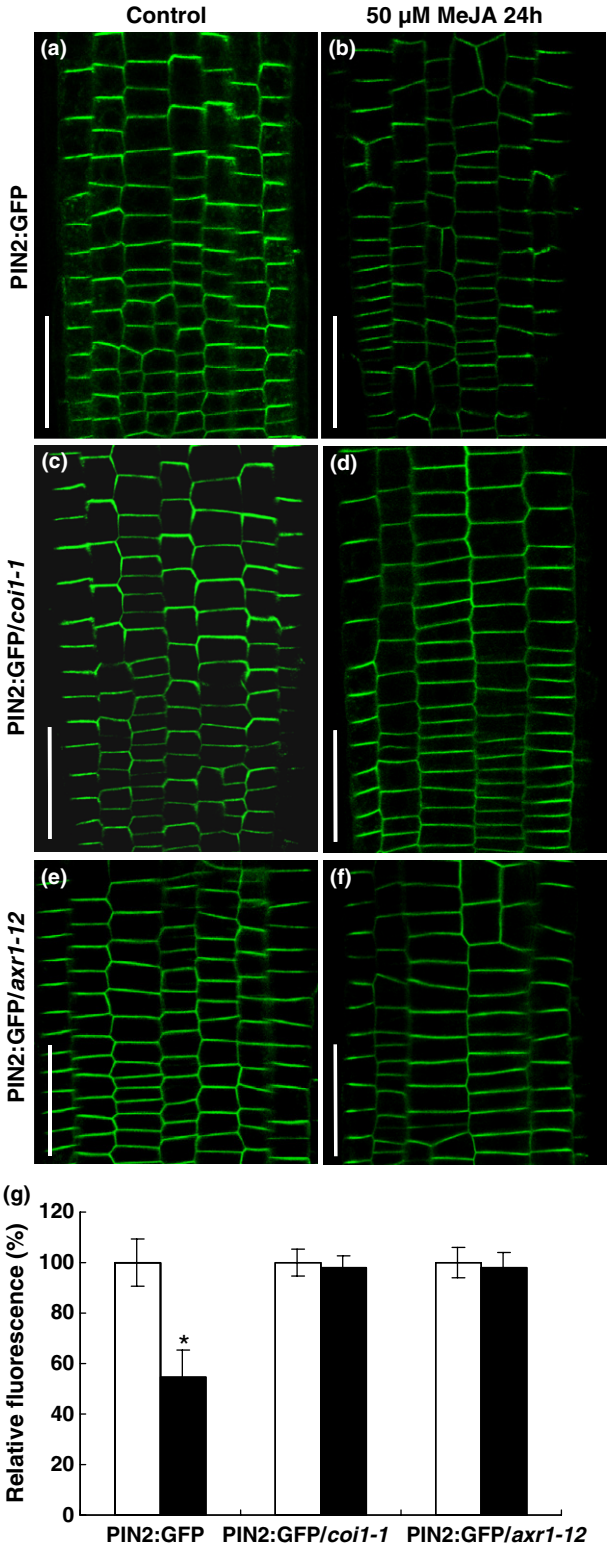


Fig. 5 Reduction effect of 50 μM methyl jasmonate (MeJA) on plasma membrane (PM)-resident PIN2:GFP abundance in the wild-type (WT) (PIN2:GFP), *coi1-1* (PIN2:GFP/*coi1-1*) and *axr1-12* (PIN2:GFP/*axr1-12*). (a–f) Five-day-old PIN2:GFP (a, b), PIN2:GFP/*coi1-1* (c, d) and PIN2:GFP/*axr1-12* (e, f) seedlings were treated with 50 μM MeJA for 24 h before laser confocal microscopy analysis. PIN2:GFP in the root epidermis cells in the meristematic and elongation zones is shown. Bars, 50 μm. (g) Quantification of PM-located green fluorescent protein (GFP) fluorescence shown in (a)–(f). Asterisk denotes Student's *t*-test significance between MeJA-treated (closed bars) and untreated (open bars) plants: *, $P < 0.01$. The error bars in (g) are ± SD.

PIN2:GFP abundance. Surprisingly, however, the *asa1-1* and *tir1 afb1, 2, 3* mutations enhanced, rather than impaired, the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP abundance (Figs 3, 4, 6), suggesting that the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP is achieved independently of the auxin pathway. Taking into consideration that auxin itself inhibits the endocytosis of PIN proteins and maintains their PM localization (Paciorek *et al.*, 2005; Pan *et al.*, 2009), our observation that the *asa1-1* and *tir1 afb1, 2, 3* mutations enhanced the reduction effect of 50 μM MeJA on PM-resident PIN2:GFP abundance reveals that 50 μM MeJA could counteract the effect of auxin on the PM localization of PIN2:GFP: that is, although auxin promotes the PM accumulation of PIN2:GFP, 50 μM MeJA reduces it.

In addition to the MeJA effects on the endocytosis and PM residence of PIN2:GFP, we found that MeJA application alters the normal lateral auxin redistribution pattern on gravi-stimulation, and therefore impairs the root gravitropic response in WT (Figs 8, 9). Again, MeJA impairment of root gravitropism is much more severe in the *asa1-1* mutant than in WT (Fig. 8), suggesting that *ASA1*-dependent auxin biosynthesis is involved in the jasmonate-mediated modulation of root gravitropism. Collectively, our results not only highlight the importance of *ASA1* in mediating jasmonate-induced auxin biosynthesis, but also reveal a repressive role of jasmonate on auxin transport through interference with the intracellular trafficking and abundance of PIN2 proteins. The two aspects of jasmonate action may represent a fine-tuned regulation of the appropriate auxin gradient pattern in the root meristem that is critical for the root gravitropic response. Considering that *ASA1*-dependent auxin biosynthesis is also essential for jasmonate-induced LR formation (Sun *et al.*, 2009), our results are in agreement with a recent proposal that both LR formation and root gravitropism are co-regulated by a single auxin transport route (Lucas *et al.*, 2007). In the context that jasmonate plays an important role in the plant stress response, the jasmonate-mediated fine-tuned regulation of LR formation and root gravitropism may have adaptive significance under conditions in which jasmonate levels are elevated.

Of interest is our observation that the jasmonate regulation of subcellular PIN2:GFP distribution is tightly linked to the protein abundance of PIN2:GFP, raising an interesting question of how jasmonate exerts its effect on PIN2

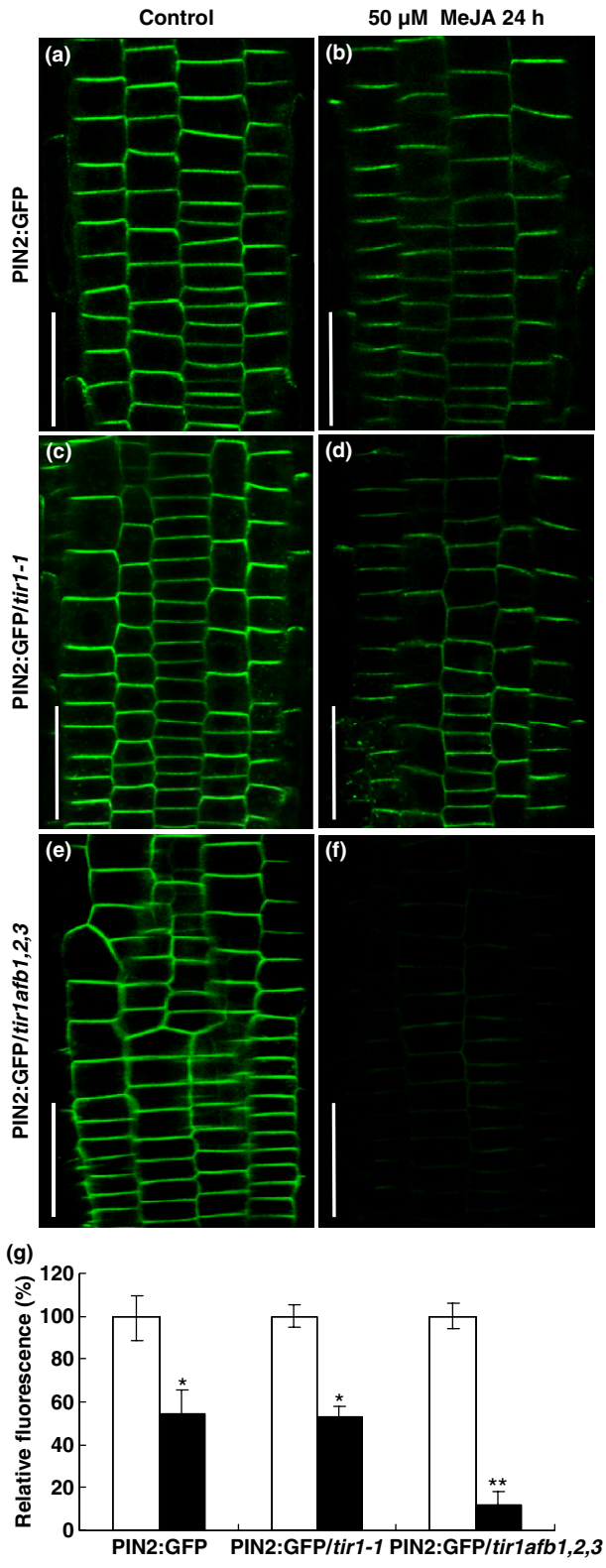


Fig. 6 Reduction effect of 50 μM methyl jasmonate (MeJA) on plasma membrane (PM)-resident PIN2:GFP abundance in the wild-type (WT) (PIN2:GFP), *tir1-1* and the *tir1 afb1, 2, 3* quadruple mutant. (a–f) Five-day-old PIN2:GFP (a, b), PIN2:GFP/*tir1-1* (PIN2:GFP/*tir1-1*) (c, d) and PIN2:GFP/*tir1afb1, 2, 3* (e, f) seedlings were treated with mock (control) or MeJA (50 μM) for 24 h before laser confocal microscopy analysis. PIN2:GFP in the root epidermis cells in the meristematic and elongation zones is shown. Bars, 50 μm. (g) Quantification of PM-located green fluorescent protein (GFP) fluorescence shown in (a)–(f). Asterisks denote Student's *t*-test significance between MeJA-treated (closed bars) and untreated (open bars) plants: *, $P < 0.05$; **, $P < 0.01$. The error bars are ± SD.

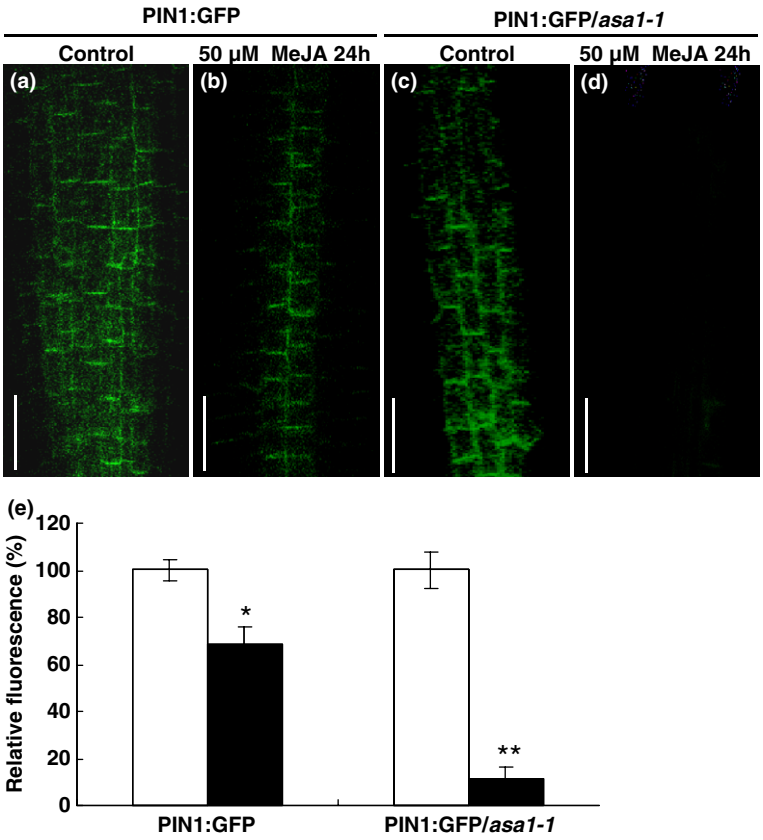


Fig. 7 Effect of 50 μM methyl jasmonate (MeJA) on plasma membrane (PM)-resident PIN1:GFP in wild-type (WT) and *asa1-1* roots. (a–d) Five-day-old PIN1:GFP (a, b) and PIN1:GFP/*asa1-1* (c, d) seedlings were treated with mock (control) or 50 μM MeJA for 24 h before laser confocal microscopy analysis. PIN1:GFP in the central provascular cells in the meristematic and elongation zones is shown. Bars, 20 μm. (e) Quantification of PM-located green fluorescent protein (GFP) fluorescence shown in (a)–(d). Asterisks denote Student's *t*-test significance between MeJA-treated (closed bars) and untreated (open bars) plants: *, $P < 0.05$; **, $P < 0.01$. The error bars in (e) are ± SD.

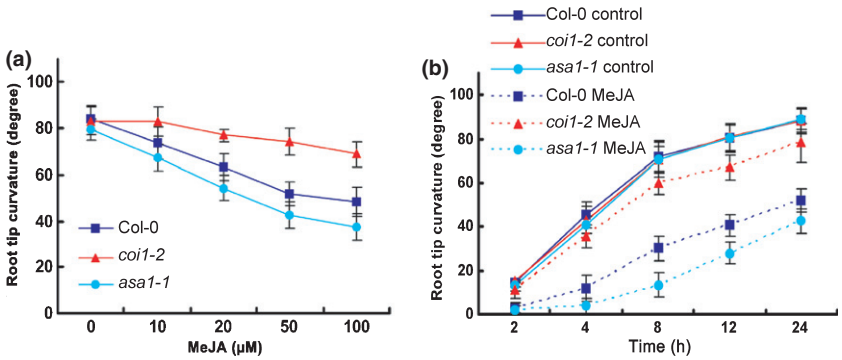


Fig. 8 Methyl jasmonate (MeJA) effect on the root gravitropic response in Col-0, *coi1-2* and *asa1-1*. (a) Five-day-old wild-type (WT) (Col-0), *coi1-2* and *asa1-1* seedlings were transferred to plates containing different concentrations of MeJA. Immediately after transfer, plates were reoriented by 90°. After 24 h of growth, root tip curvatures were measured. Values represent the average and SD of 20 seedlings. The experiments were repeated at least three times with similar results. (b) Five-day-old WT (Col-0), *coi1-2* and *asa1-1* seedlings were transferred to plates with or without 50 μM MeJA. Immediately after transfer, plates were reoriented by 90°. At different time points after gravitropic reorientation, the root tip curvatures were measured. Values represent the average and ± SD of 20 seedlings. The experiments were repeated at least three times, yielding similar results.

turnover. It has been shown that, on gravi-stimulation, differential degradation of PIN2 at the upper and lower sides of the root involves both the ubiquitin-proteasome pathway (Abas *et al.*, 2006) and lytic vacuoles (Kleine-Vehn *et al.*, 2008; Laxmi *et al.*, 2008). The molecular mechanism governing jasmonate regulation of PIN protein turnover

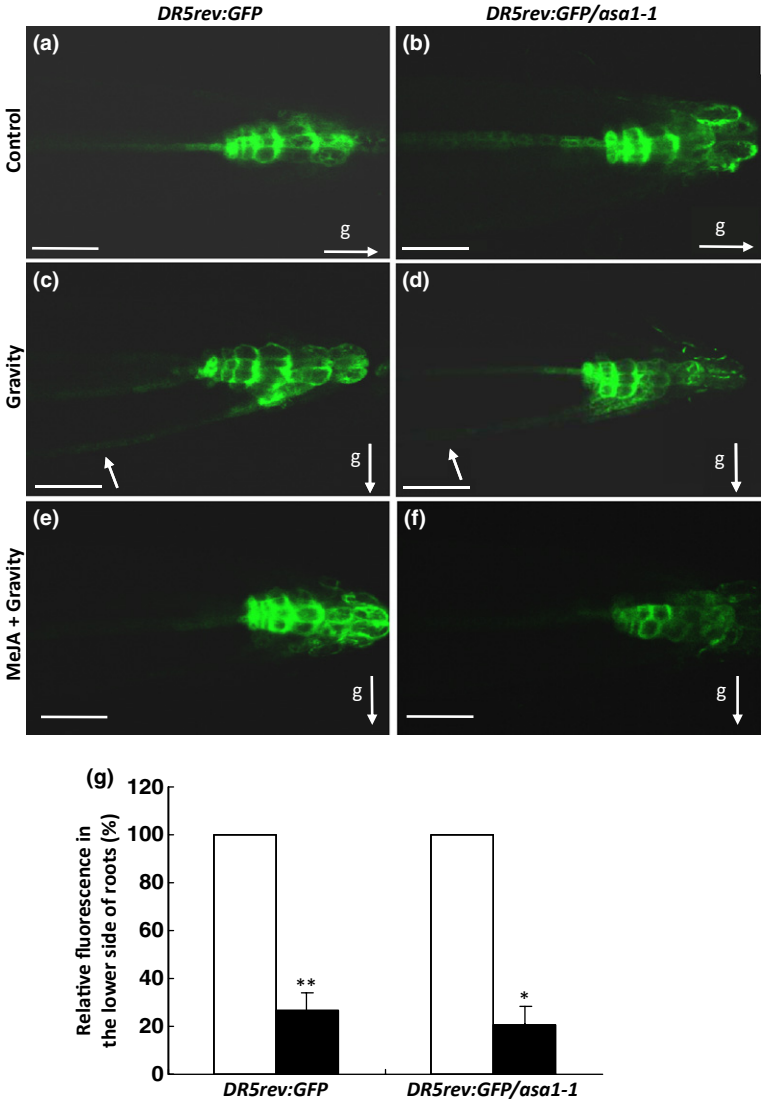


Fig. 9 Jasmonate effect on gravity-induced lateral auxin redistribution in root tips. Five-day-old *DR5rev:GFP* (a, c, e) and *DR5rev:GFP/asa1-1* (b, d, f) seedlings were transferred to vertical plates with or without methyl jasmonate (MeJA) (50 μM). Twenty-four hours after transfer, seedlings were gravi-simulated by 90° for 6 h before laser confocal microscopy analysis. Arrows indicate that, 6 h after gravi-stimulation, there was asymmetric accumulation of *DR5rev:GFP* in the lower side of the root. Representative images from one replicate are shown; the experiments were repeated three times, yielding similar results. 'g' represents the direction of gravity. Bars, 50 μm. (g) Quantification of green fluorescent protein (GFP) fluorescence distribution in the lower side of the roots by image analysis of confocal sections for (c)–(f). Open bars, gravity; closed bars, MeJA + gravity. Asterisks denote Student's t-test significance between gravity-treated and MeJA + gravity-treated plants: *, $P < 0.05$; **, $P < 0.01$. The error bars in (g) are ± SD.

remains an important question to be answered in future studies.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Effect of 5 and 50 μM methyl jasmonate (MeJA) on FM4-64 uptake in the wild-type (WT).

Fig. S2 Effect of 5 μM methyl jasmonate (MeJA) on PIN2:GFP endocytosis in the wild-type (WT) (PIN2:GFP) and *asa1-1* (PIN2:GFP/*asa1-1*) in the presence of exogenous naphthalene-1-acetic acid (1-NAA).

Fig. S3 Effect of 5 μM methyl jasmonate (MeJA) on brefeldin A (BFA)-induced PIN2 endocytosis in the wild-type (WT) and *abp1* (*SS12K*).

Fig. S4 Effect of cycloheximide (CHX) on jasmonate-induced reduction of PIN2:GFP abundance in the plasma membrane (PM) in the wild-type (WT) and *asa1-1*.

Fig. S5 Effect of methyl jasmonate (MeJA) on *PIN2* transcripts in Col-0 and *asa1-1* roots revealed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Fig. S6 Methyl jasmonate (MeJA)-regulated *proPIN1:GUS* and *proPIN2:GUS* expression in the wild-type (WT) and *asa1-1*.

Fig. S7 Effect of 50 μM methyl jasmonate (MeJA) on plasma membrane (PM)-resident AUX1:YFP in the wild-type (WT) and *asa1-1*.

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