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Cellular Auxin Homeostasis under High Temperature Is Regulated through a SORTING NEXIN1–Dependent Endosomal Trafficking Pathway[C][W]

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High-temperature-mediated adaptation in plant architecture is linked to the increased synthesis of the phytohormone auxin, which alters cellular auxin homeostasis. The auxin gradient, modulated by cellular auxin homeostasis, plays an important role in regulating the developmental fate of plant organs. Although the signaling mechanism that integrates auxin and high temperature is relatively well understood, the cellular auxin homeostasis mechanism under high temperature is largely unknown. Using the *Arabidopsis thaliana* root as a model, we demonstrate that under high temperature, roots counterbalance the elevated level of intracellular auxin by promoting shootward auxin efflux in a PIN-FORMED 2 (PIN2)-dependent manner. Further analyses revealed that high temperature selectively promotes the retrieval of PIN2 from late endosomes and sorts them to the plasma membrane through an endosomal trafficking pathway dependent on SORTING NEXIN1. Our results demonstrate that recycling endosomal pathway plays an important role in facilitating plants adaptation to increased temperature.

INTRODUCTION

Developmental plasticity of plants is regulated both by endogenous regulators, such as phytohormones, and external environmental factors, including light, temperature, and humidity. Exposure of *Arabidopsis thaliana* to high temperature results in dramatic changes in growth and development, which is primarily linked to the phytohormone auxin (Gray et al., 1998; Franklin et al., 2011; Sun et al., 2012). PHYTOCHROME INTERACTING FACTOR4 (PIF4) has been shown to be the primary regulator of the signaling mechanism that integrates auxin and plant development under elevated temperature. PIF4 plays an instrumental role in activating FLOWERING LOCUS T, which promotes flowering at high temperature under short photoperiods (Kumar et al., 2012). High temperature–induced elongation of the *Arabidopsis* hypocotyl has been shown to be under regulation of PIF4-mediated auxin biosynthesis (Franklin et al., 2011; Sun et al., 2012). PIF4 directly activates the auxin biosynthetic gene *YUCCA8* (*YUC8*) by binding to the G-box-containing promoter region of *YUC8*, thus stimulating auxin biosynthesis under high temperature (Sun et al., 2012). The loss-of-function *yuc8* mutation largely suppresses the long-hypocotyl phenotype of PIF4 overexpression plants and reduces the high temperature–induced hypocotyl elongation, confirming that under high temperature, PIF4 regulates auxin biosynthesis through activating the Indole-3-pyruvic acid (IPA) auxin biosynthesis pathway (Franklin et al., 2011; Sun et al., 2012). Collectively, these results suggest that PIF4-mediated changes in the auxin response may play a crucial role in facilitating plant adaptation to high temperature.

In general, the auxin gradient, which is dependent on the spatial and temporal distribution of auxin, plays an important role in determining the developmental fate of the plant (Vieten et al., 2007). Other endogenous hormones also contribute to regulating the cellular auxin gradient by modulating carrier-driven auxin transport (Rahman, 2013). The majority of studies to date linking auxin and high temperature have elegantly demonstrated that high temperature promotes auxin biosynthesis (Gray et al., 1998; Franklin et al., 2011; Sun et al., 2012), but what remains obscure is how cells respond to this elevated level of auxin and how they maintain the optimal intracellular auxin distribution for growth and development.

Carrier-driven auxin transport plays a central role in intracellular auxin homeostasis and maintaining the optimal auxin gradient (Rosquete et al., 2012). Tissue-specific expression and the cellular localization patterns of auxin influx (e.g., AUXIN [AUX]/LAX) and efflux carriers (e.g., PIN-FORMED [PINs]) further support their functional significance in modulating intracellular auxin transport (Peer et al., 2011). The direction of auxin flow largely depends on the polar localization of PIN proteins. It has been demonstrated that the polar targeting of a subset of PIN proteins (e.g., PIN1 and PIN2) is regulated by various protein trafficking pathways, including

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ADP ribosylation factor GTP-exchanging factor–dependent clathrin-mediated internalization, Rab GTPases, and retromer complex–mediated endosomal pathways (Geldner et al., 2001; Jaillais et al., 2006, 2007; Michniewicz et al., 2007; Kleine-Vehn et al., 2008). The dynamic trafficking process that contributes toward facilitating the polar targeting of the newly synthesized nonpolar PINs to the plasma membrane (Dhonukshe et al., 2008) can be modified following physiological or environmental changes, resulting in the formation of altered intracellular auxin gradient as well as redirection of auxin flow (Paciorek et al., 2005; Laxmi et al., 2008; Shibasaki et al., 2009; Wan et al., 2012). These observations raise an interesting possibility that auxin homeostasis under high temperature may be modulated by intracellular protein trafficking pathways.

Using molecular and cellular approaches and the *Arabidopsis* root as a model, we dissected the cellular auxin homeostasis mechanism under high temperature. Our results reveal that under high temperature, plants adopt a SORTING NEXIN1 (SNX1)–dependent auxin homeostasis mechanism to maintain an optimal auxin gradient in spite of an increase in auxin concentrations, which positively affects root growth and graviperception. We also demonstrate that this SNX1-mediated recycling endosomal pathway plays an important role in facilitating plant adaptation to increased temperature.

RESULTS

High Temperature Alters the Intracellular Auxin Response

In contrast with hypocotyls, where intracellular auxin is suboptimal, the roots have a supraoptimal auxin level that is reflected in their developmental responses to increased auxin levels; all known auxin overproduction mutants exhibit a long hypocotyl and short root phenotype (Zhao, 2010). Consistently, exogenous application of auxin inhibits root growth but stimulates hypocotyl growth (Rahman et al., 2007; Chapman et al., 2012). High temperature has been shown to affect the intracellular auxin response by modulating the cellular auxin level in the shoot (Gray et al., 1998; Franklin et al., 2011; Sun et al., 2012). Although the cellular and developmental responses of hypocotyls linked to high temperature–mediated changes in auxin level have been studied extensively (Koini et al., 2009; Franklin et al., 2011; Greenham et al., 2011), such studies are lacking in roots. To understand the consequences of high temperature in root development, we first monitored the change in the intracellular auxin level using a newly developed sensor DII-VENUS, which is capable of detecting intracellular auxin distribution at high spatio-temporal resolution (Brunoud et al., 2012). Consistent with previous results, we also found that DII-VENUS abundance is directly linked to the intracellular auxin level (see Supplemental Figure 1A online; Band et al., 2012) and responds to minute changes in auxin concentration (see Supplemental Figure 1A online). Comparison of DII-VENUS response at standard (23°C) and high (29°C) temperature conditions revealed a reduced DII-VENUS signal in high temperature–treated roots at all time points examined (Figure 1; see Supplemental Figure 1B online), suggesting that high temperature affects the intracellular auxin response through stimulating the auxin level in root. To obtain another independent

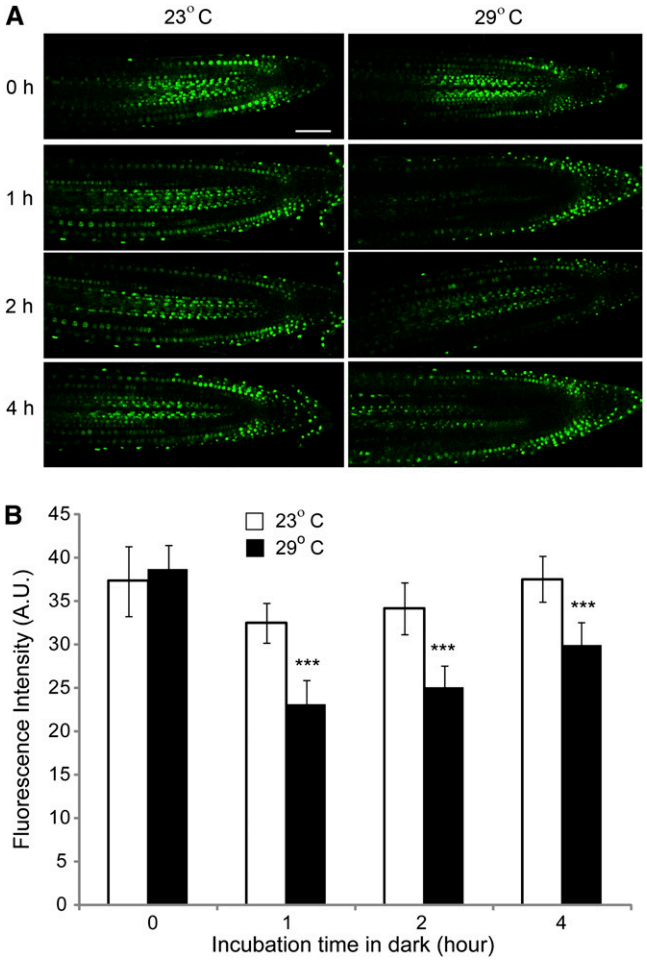

Figure 1. High Temperature Alters the Intracellular Auxin Response.

(A) Time course of high temperature–induced stimulation of the intracellular auxin response. Five-day-old light-grown DII-VENUS seedlings were transferred to new agar plates and subjected to high-temperature treatment (29°C) for the indicated time in darkness and imaged with confocal microscopy using the same settings. Note the reduced DII-VENUS signal in high temperature–treated roots at all time points. Bar = 50 μm.
(B) Quantification of fluorescence intensities. Vertical bars represent mean ± SE of the experimental means from three independent experiments (n = 3), where experimental means were obtained from eight to 10 seedlings per experiment. Compared with the control treatment, the high temperature–induced reduction of DII-VENUS signal (expressed in arbitrary units [A.U.]) was significant at all time points ($P < 0.0001$) as judged by Student's t test.
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line of evidence that high temperature alters the intracellular auxin response, we investigated the effect of high temperature on the widely used auxin reporter *DR5-GUS* (for β-glucuronidase), which contains the auxin-responsive TGTCTC element (Ulmasov et al., 1997). Compared with plants under the standard (control) temperature, more accumulation of GUS was observed in the meristem of 70% of roots grown under high temperature (see Supplemental Figure 2B online), confirming that high temperature effect on DII-VENUS truly represents a change in auxin response.

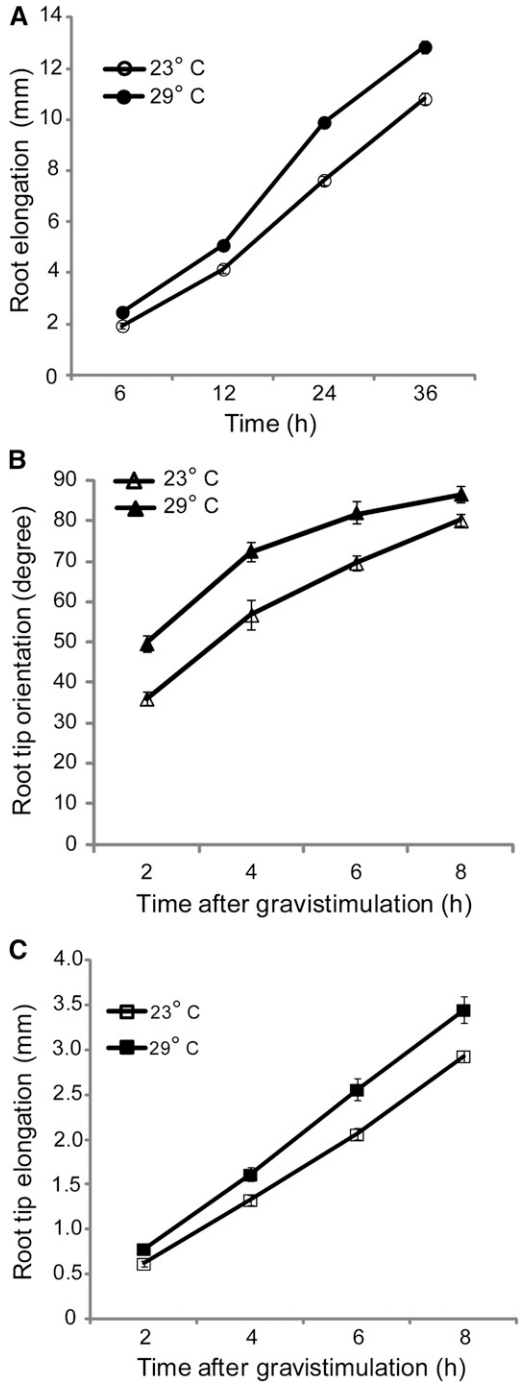

Figure 2. High Temperature Promotes Root Elongation and Enhances the Root Gravity Response.

Five-day-old light-grown wild-type seedlings were transferred to new agar plates and subjected to high-temperature treatment (29°C) for various time lengths under darkness or gravity stimulated by rotating the plates 90°.

(A) Effect of high temperature on root elongation.

(B) and **(C)** Effect of high temperature on gravistimulation. Curvature of root tips **(B)** and root elongation **(C)** plotted against time after reorientation. Data for root tip orientation and elongation were collected for 2, 4, 6, and 8 h. Vertical bars represent mean ± SE of the experimental means from at least five independent experiments ($n = 5$ or more), where experimental means were obtained from eight to 10 seedlings per experiment. Compared with control, high temperature–induced stimulation of root growth and gravity responses were significant at all time points ($P < 0.0001$) as judged by Student's t test.

Collectively, these results strongly suggest that, as in the hypocotyl, high temperature also alters the auxin level in root.

High Temperature Promotes Root Growth and Gravity Response

To understand the significance of increased auxin content in root development under high temperature, we next analyzed the growth and gravity response of roots. The time course of root elongation both in dark and light (illumination from the top) conditions under high temperature indicates that, as in the hypocotyl, high temperature also promotes growth in *Arabidopsis* roots (Figure 2A; see Supplemental Figure 3A online). The stimulation of root elongation by high temperature may result from modifications of either cell elongation or cell division. A cell production assay coupled with expression analysis of the cell cycle marker *CycB1;1-GUS* (Colón-Carmona et al., 1999) revealed that the stimulation of root growth induced by high temperature is largely due to an increase in cell division (see Supplemental Methods1, Supplemental Table 1 and Supplemental Figure 4 online).

The gravity response, which is an auxin-dependent process, was also found to be altered in high temperature–grown seedlings. Roots of the seedlings grown at 29°C responded faster toward gravity compared with the seedling roots grown at 23°C (Figure 2B; see Supplemental Figure 3B online). Consistently, formation of asymmetric auxin response was found to develop faster under high temperature (Figure 3). The asymmetric auxin response was observed in 75% of roots grown at 29°C after 2 h gravity stimulation, compared with 33% of roots grown at 23°C (see Supplemental Table 2 online). During the gravity response window, root elongation was faster in high temperature–grown seedlings (Figure 2C; see Supplemental Figure 3C online). Although a similar trend was observed in both dark- and light-grown seedlings, the magnitude of the response was greater under dark condition (compared with Figure 2 and Supplemental Figure 3 online). These results are in contrast with our current understanding, whereby the gravity response and root growth respond negatively to elevated levels of auxin (Yamamoto and Yamamoto, 1998; Rahman et al., 2007), suggesting that under high temperature, plants adopt an auxin homeostasis mechanism by which the elevated level of auxin acts positively in root growth and gravistimulation.

Carrier-Driven Auxin Transport Plays an Important Role in Root Response to High Temperature

To understand the role of auxin transport in high temperature–mediated root development, we next investigated the root growth response of auxin transport mutants *aux1* (auxin influx carrier mutant) and *eir1/pin2* (auxin efflux carrier mutant) (Bennett et al.,

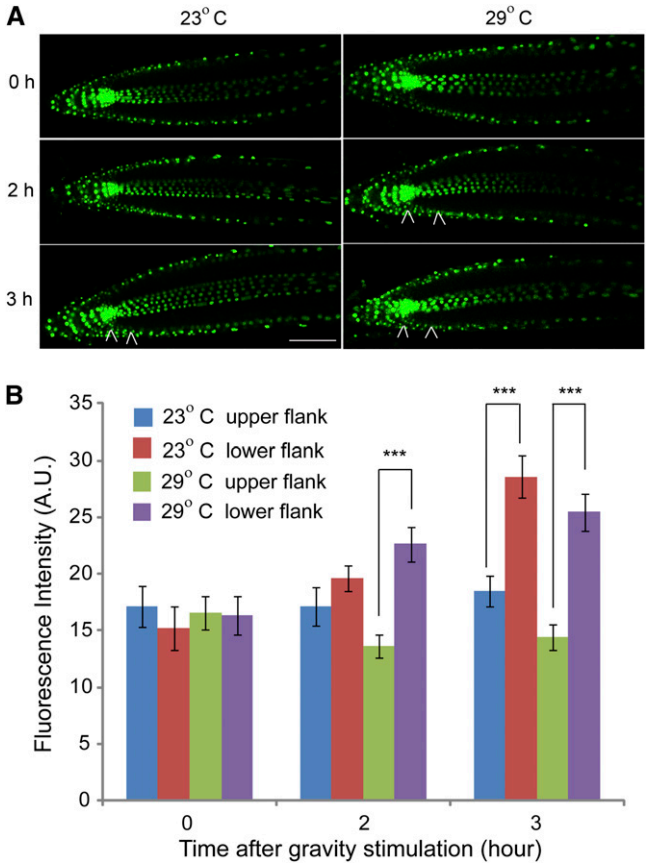

Figure 3. High Temperature Promotes Asymmetric Auxin Response in *Arabidopsis* Root.

(A) Effect of high temperature on auxin redistribution in gravity-stimulated roots. Five-day-old light grown DR5:vYFP transgenic seedlings were transferred to new agar plates and subjected to gravity stimulation for 2 or 3 h at dark condition prior imaging. Controls were grown at 23°C. Arrowheads indicate the DR5:vYFP expression in peripheral layers, which is absent at 0 h. Bar = 50 μm.

(B) Quantification of fluorescence intensities. Asterisks represent statistical significances ($P < 0.0001$) as judged by Student's *t* test. Data represent mean ± SE of the experimental means from three independent experiments ($n = 3$), where experimental means were obtained from eight to 10 seedlings per experiment. A.U., arbitrary units.

1996; Luschnig et al., 1998). Compared with the wild type, both mutants showed considerable resistance to high temperature–induced root elongation (Figure 4A), suggesting that carrier-driven auxin transport plays an important role in roots response to high temperature. The root gravity response is regulated by shootward auxin flow, mediated by the auxin efflux carrier PIN2. The differential shootward auxin transport between the upper and lower flanks of the root, resulting in formation of asymmetric auxin gradient, is an absolute requirement for root gravity response (Muday et al., 2012). Since high temperature promotes the root gravity response and facilitates the formation of an asymmetric auxin gradient (Figures 2B and 3), we investigated the effect of high temperature on shootward auxin transport by a direct auxin transport assay. The transport assay revealed that high temperature promotes shootward auxin transport (Figure 4B). However, the uptake of auxin was unaltered by high temperature (see Supplemental Figure 5A online), suggesting that high temperature modulates the cellular efflux capacity. Consistent with root elongation data, the stimulation of shootward auxin transport by high temperature was absent in *aux1* and *eir1* (Figure 4B), confirming that carrier-driven auxin transport is a regulator of developmental responses mediated by high temperature. To understand the physiological significance of this altered transport activity, we compared the response of roots toward exogenous auxin at normal and high temperatures. Roots grown at high temperature show less sensitivity toward exogenous indole-3-acetic acid (IAA)–induced root growth inhibition (Figure 4C). This result further supports the conclusion that high temperature alters intracellular auxin homeostasis and is consistent with our shootward auxin transport data.

SNX1-Dependent Endosomal Pathway Regulates Intracellular Auxin Homeostasis under High Temperature

PIN1 and PIN2 proteins show distinct membrane targeting patterns, which are dependent on the constitutive cycling of PIN proteins between plasma membrane and endosomal compartments (Geldner et al., 2001). Newly synthesized nonpolar PIN1 and PIN2 in the cortex relocalize themselves toward the rootward domain of the plasma membrane through ADP ribosylation factor GTP-exchanging factors, such as GNOM, and the phosphorylation status of the proteins, which is regulated by the counterbalancing activities of PINOID Kinase and protein phosphatase 2A (Geldner et al., 2001; Michniewicz et al., 2007; Sukumar et al., 2009; Rahman et al., 2010). However, polarization of PIN2 in epidermal and lateral root cap cells is independent of this pathway (Rahman et al., 2010). Recycling and intracellular trafficking of PIN2 require the retromer proteins SNX1 and VACUOLAR PROTEIN SORTING29 (VPS29) (Jaillais et al., 2006, 2007). SNX1 and VPS29, which reside in late endosomes, are thought to be necessary for retrieval of PIN2 from late endosomes and thus prevent it from being internalized into lytic vacuoles (Kleine-Vehn et al., 2008). The targeting of PIN2 has also been shown to be light sensitive; light promotes plasma membrane localization, whereas dark promotes vacuolar targeting, and the respective localizations affect the roots ability to transport auxin (Laxmi et al., 2008). Interestingly, we also observed that plants grown in light and dark conditions respond differentially to high temperature (Figure 2; see Supplemental Figure 3 online), raising the possibility that high temperature may modulate retromer complex-mediated vacuolar targeting. To test this possibility, we investigated the effect of high temperature on vacuolar targeting. Incubation in the dark at 23°C resulted in strong vacuolar accumulation of PIN2 in 77% of roots. The vacuolar targeting of PIN2 was confirmed by colocalization using the vacuolar marker line VAM3 fused to a modified red fluorescent protein (mRFP-VAM3; Sassi et al., 2012). Surprisingly, high-temperature treatment inhibited the vacuolar targeting of PIN2; in 78% of roots, no vacuolar accumulation of PIN2 was observed (Figure 5; see Supplemental Table 3 online). To clarify that the effect of high temperature on PIN2 is not linked to the alteration of *PIN* expression, we compared the expression of *PIN* genes under standard and high temperature conditions and found no significant change (see Supplemental Methods1 and Supplemental Figure 5B

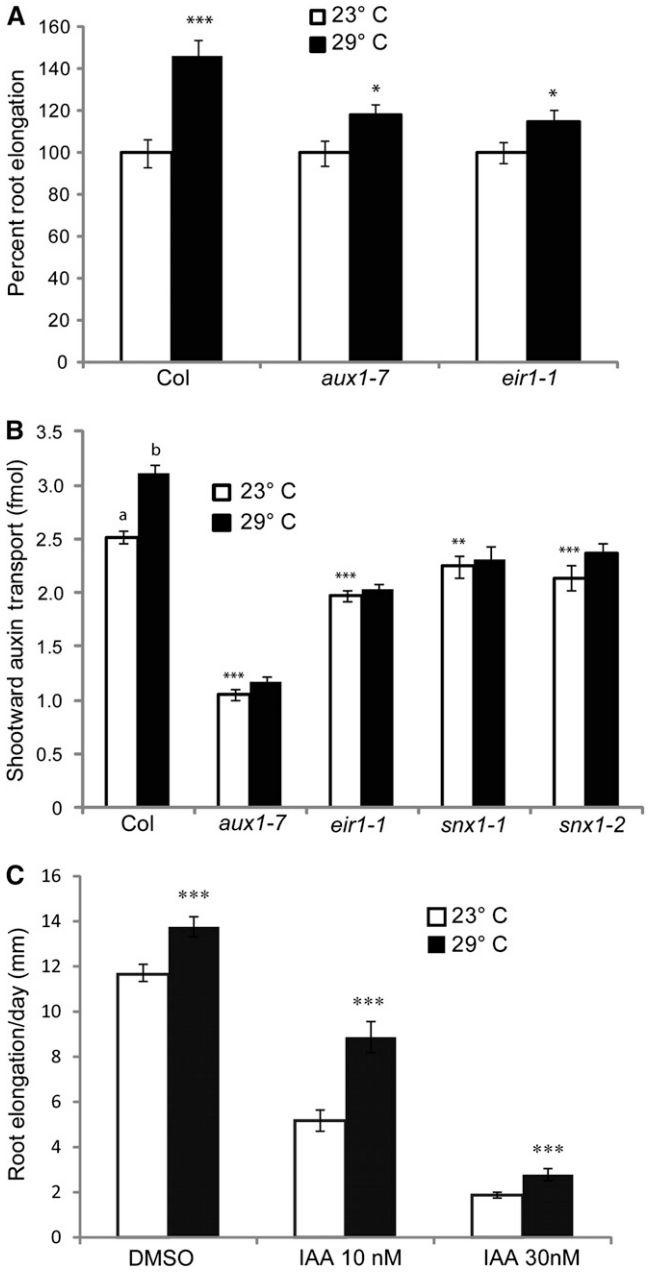

Figure 4. The High-Temperature Response Requires a Functional Auxin Transport System to Promote Root Elongation and Makes the Root Less Sensitive to Exogenous IAA-Induced Root Growth Inhibition.

(A) Effect of high temperature on *aux1-7* and *eir1-1* root elongation. Five-day-old light-grown seedlings were transferred to new agar plates and subjected to high temperature treatment (29°C) under darkness for 12 h. Asterisks represent the statistical significance between treatments (*P < 0.05; ***P < 0.0001). Col, Columbia.

(B) Effect of high temperature on shootward auxin transport. Five-day-old light grown seedlings were transferred to new agar plates and subjected to high-temperature treatment (29°C) under darkness and incubated for 4 h before transport of tritiated IAA over 2 h was measured, as described in Methods. The experiments were conducted using at least three biological replicates. For each biological replicate, three technical replicates were assayed: Col-23°C, *n* = 81; Col-29°C, *n* = 82; *aux1-7*-23°C, *n* = 52; *aux1-7*-29°C, *n* = 51; *eir1-1*-23°C, *n* = 55; *eir1-1*-29°C, *n* = 52; *snx1-1*-23°C, *n* = 32; *snx1-1*-29°C, *n* = 35; *snx1-2*-23°C, *n* = 30; *snx1-2*-29°C, *n* = 37. Asterisks represent the statistical significance between genotypes (**P < 0.001; ***P < 0.0001), and different letters represent the statistical significance between treatment (P < 0.0001). Vertical bars represent mean ± SE of the experimental means from at least three independent experiments (*n* = 3 or more), where experimental means were obtained from 10 to 15 seedlings per experiment.

(C) High temperature confers resistance to exogenous auxin-induced root growth inhibition. Five-day-old light-grown wild-type seedlings were transferred to new agar plates with or without IAA and subjected to high temperature treatment (29°C) for 24 h at dark. After the treatment primary root elongation was analyzed. Compared with control, IAA-induced inhibition of root at high temperature was significantly less at all IAA concentrations (P < 0.0001) as judged by Student's *t* test. Vertical bars represent mean ± SE of the experimental means from three independent experiments (*n* = 3), where experimental means were obtained from eight to 10 seedlings per experiment.

online). These results strongly suggest that high temperature modulates cellular auxin homeostasis by promoting PIN2 targeting to the plasma membrane.

Since vacuolar sorting of PIN2 has been shown to be regulated by SNX1 (Jaillais et al., 2006), we hypothesized that high temperature requires functional SNX1 to promote plasma membrane targeting of PIN2. Two alleles of *snx1* were used to investigate the effect of high temperature on PIN2 targeting. Consistent with our hypothesis, we observed that in darkness PIN2 was targeted to the vacuole in 80% of *snx1* roots grown at either 23 or 29°C (Figure 6; see Supplemental Table 3 online). The loss of SNX1 function also affects PIN2 targeting under continuous light. Previously, it was shown that endocytosed PIN2 colocalizes with SNX1-specific compartments and is recycled to the plasma membrane through these novel endosomal compartments (Jaillais et al., 2006). Consistent with previous results, we also observed that in contrast with darkness, under continuous light, PIN2 accumulated in smaller endosomal compartments (compare Figure 6 see Supplemental Figure 6 online). Compared with the wild type, where ~90% of roots showed no endosomal accumulation of PIN2, in *snx1* mutants, 20 and 50% of roots showed high and weak endosomal accumulation of PIN2, respectively (see Supplemental Table 3 online). In addition, high temperature failed to promote plasma membrane targeting of PIN2 in *snx1* mutants (see Supplemental Figure 6 and Supplemental Table 3 online). Taken together, these results suggest that the SNX1 recycling pathway plays an important role in mediating auxin homeostasis in roots and plant adaptation to high temperature.

To elucidate the specificity of high temperature–mediated PIN2 targeting, we next investigated the intracellular localization of several plasma membrane proteins under darkness. A 4-h incubation in the dark did not alter the intracellular localization of AUX1, PIN1, and another plasma membrane–targeted protein, low temperature-inducible 6b (see Supplemental Figures 7 and 8 online), confirming that high temperature specifically regulates PIN2 targeting via a SNX1-dependent pathway.

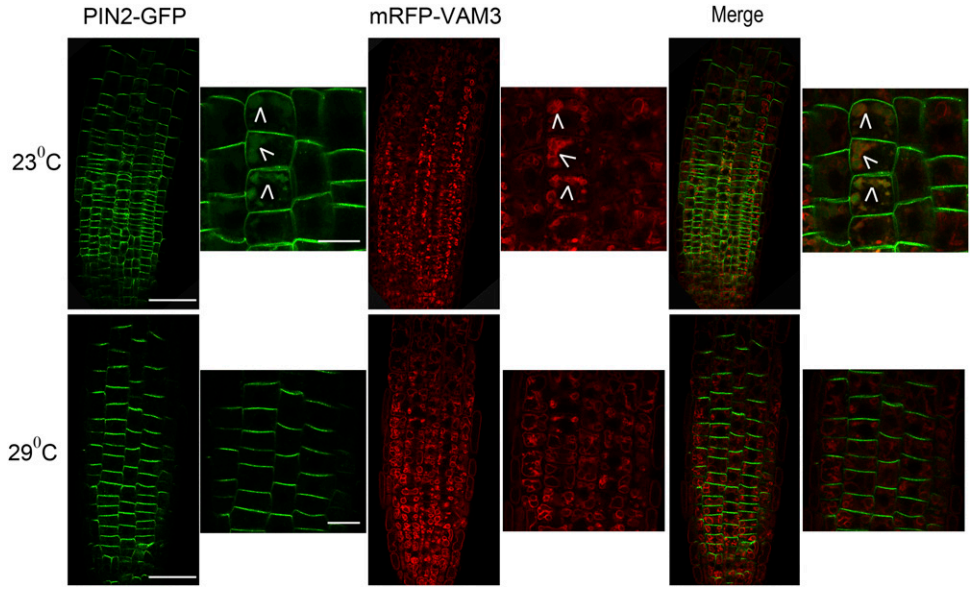

Figure 5. High Temperature Promotes PIN2 Targeting to Plasma Membrane in Roots of *Arabidopsis* Grown under Dark Condition.

Five-day-old light grown seedlings were transferred to new agar plates and subjected to high-temperature treatment (29°C) for 24 h before imaged with confocal microscopy using the same confocal settings and are representative of 15 to 20 seedlings obtained from three independent experiments. Arrowheads indicate vacuolar accumulation of PIN2 at 23°C. Please note that the vacuolar accumulation is absent at high temperature. Right panels are zoomed images of left panels. Bars = 50 and 10 μm.

The *snx1* Mutant Is Resistant to High Temperature–Induced Changes in Root Developmental Processes

To understand the physiological significance of SNX1-mediated PIN2 targeting in high temperature–grown plants, we next investigated root developmental responses and shootward auxin transport in *snx1* loss-of-function mutants. Both *snx1*alleles showed a clear resistance to high temperature as deduced from the lack of promotion of root elongation and gravity response (Figure 7). Consistently we also found that in both alleles, high temperature failed to promote shootward auxin transport (Figure 4B). Collectively, these results suggest that high temperature regulates root development by modulating cellular auxin homeostasis through a SNX1-dependent pathway.

DISCUSSION

In this work, we provide insight into genetic and cellular mechanisms of cellular auxin homeostasis in roots under high temperature. Plant response to high temperature is linked to increased auxin production, which alters the cellular homeostasis of auxin (Sun et al., 2012). Although increasing intracellular auxin levels by application of exogenous auxin at 23°C results in root growth inhibition (Rahman et al., 2007), high temperature promotes elongation of roots and the root gravity response in spite of increased intracellular auxin levels. This indicates that roots adopt an auxin homeostasis mechanism by which the elevated level of auxin acts positively in root growth and gravistimulation. The direct auxin transport assay revealed that high temperature indeed targets the shootward transport machinery regulated by PIN2 to maintain an optimal intracellular auxin concentration that positively regulates root developmental processes. The increase in shootward auxin transport and faster gravity response observed in high temperature are apparently in contrast with the known role of auxin flux in root gravitropism. Indeed, previous work reported that an increase in shootward auxin transport depletes the auxin maxima at the root tip, which alters the root's ability to form an asymmetric auxin gradient in response to gravity and hence results in a reduced root gravity response (Sukumar et al., 2009; Rahman et al., 2010). This apparent discrepancy can be explained by considering that the increased synthesis of auxin induced by high temperature at the root tip compensates for shootward auxin transport. This may also explain the high temperature–mediated increase in root elongation. Collectively, these results suggest that under high temperature, roots use carrier-driven shootward auxin transport to regulate cellular auxin homeostasis and, thus, the root developmental processes.

Alternatively, high temperature–induced changes in root phenotype might be due to alterations in auxin-independent temperature-responsive pathways. High temperature–induced increases in proton secretion and enzymatic activities involved in cell elongation may function as a major auxin-independent pathway in altering the root developmental processes. Although making a clear distinction between the role of auxin-dependent and -independent temperature-responsive pathways is difficult, here, we provided several lines of experimental evidence supporting the former possibility. First, high temperature has a distinct effect on root elongation of auxin mutants. If the process is independent of the auxin response and mediated by increased proton secretion and enzymatic activities, one would expect to see a wild-type response in

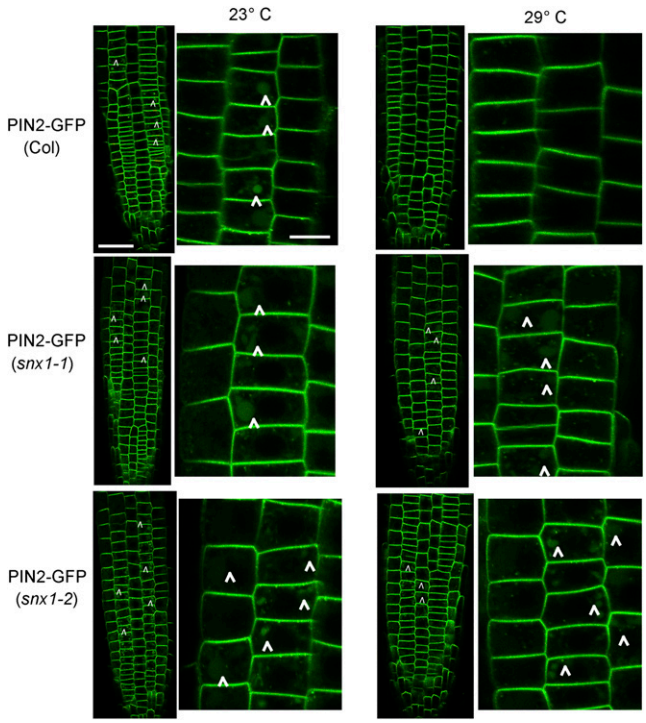

Figure 6. High Temperature Promotes Retrieval of PIN2 from Lytic Vacuole to Plasma Membrane in an SNX1-Dependent Endosomal Trafficking Pathway.

Five-day-old light grown seedlings were transferred to new agar plates and subjected to high-temperature treatment (29°C) under dark condition for 4 h before imaged with confocal microscopy. Images were captured using the same confocal settings and are representative of 15 to 40 seedlings obtained from at least three independent experiments. Arrowheads indicate vacuolar accumulation of PIN2. Please note vacuolar accumulation is absent in high temperature–treated control seedlings, but present in *snx1* mutants. Right panels are zoomed images of left panel. Col, Columbia. Bars = 50 and 10 μm.
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auxin mutants. Both the *aux1* and *eir1* mutants show resistance to a high temperature–induced increase in root elongation. Additionally, the *snx1* mutant, which is not a direct auxin transporter mutant but has perturbed auxin transport due to a defect in intracellular cycling of PIN2, also showed resistance to high temperature, suggesting that the observed physiological changes are linked to intracellular auxin homeostasis. Second, the kinematic analysis revealed that high temperature does not affect cell length to a great extent, an observation that is inconsistent with the idea that high temperature functions through stimulating acid secretion and enzymatic activities involved in cell elongation. Finally, the altered cellular localization of PIN2 in the wild type and *snx1* further supports the notion that high temperature alters auxin homeostasis through specifically targeting the intracellular cycling of PIN2. Taken together, these results strongly suggest that high temperature–mediated changes of root development are linked primarily to an auxin-dependent pathway. However, we do not completely rule out the possibility that an auxin-independent temperature-responsive pathway may contribute partially to alter the root developmental processes. Recent studies have shown that different factors may participate in PIN2 stability, such as the Rho of Plants GTPase 6 (ROP6)/ROP-interactive CRIB motif-containing protein1 signaling pathway (Chen et al., 2012), the plant hormone gibberellic acid (Löfke et al., 2013), or a member of the Major Facilitator Superfamily transporters, Zinc-Induced Facilitator-Like 1 (Remy et al., 2013). Indeed, in the root tip, overexpression of ROP6

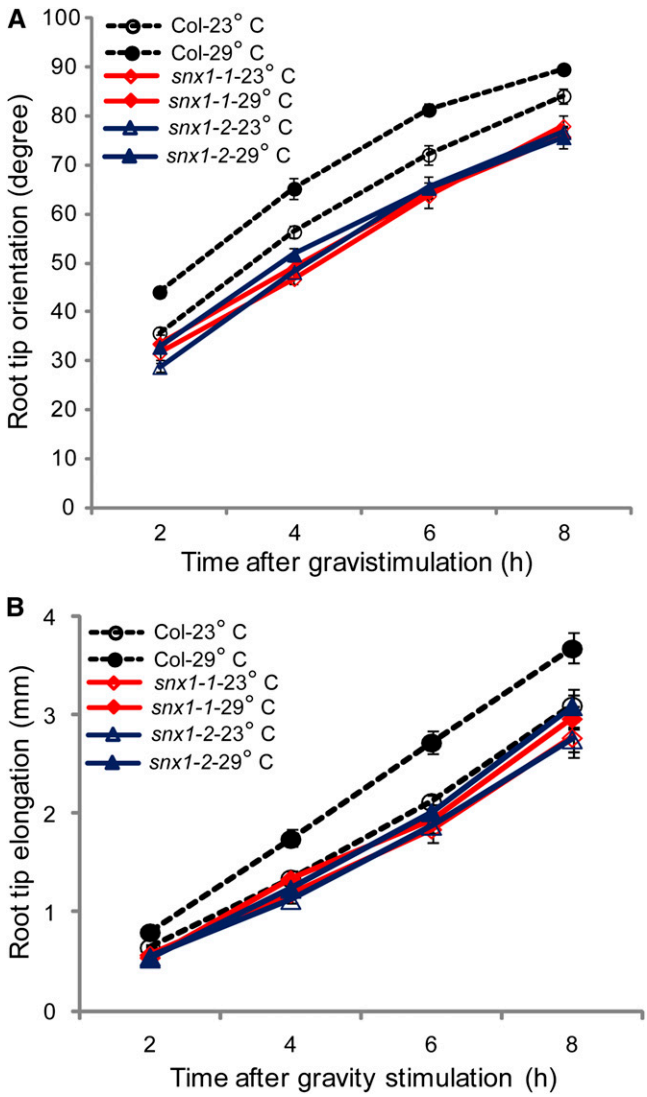

Figure 7. *snx1* Mutants Are Resistant to High Temperature–Induced Stimulation of Root Elongation and Enhancement of Root Gravity Response.

Root elongation and gravity response were measured as described in Figure 2. Col, Columbia.
(A) Curvature of root tips plotted against time after reorientation.
(B) Root elongation. Dotted lines represent the wild-type response. Vertical bars represent mean ± SE of the experimental means from three independent experiments (n = 3), where experimental means were obtained from eight to 10 seedlings per experiment.
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or Zinc-Induced Facilitator-Like1, or high levels of gibberellic acid in the lower part of the root, all lead to increased amounts of PIN2 at the plasma membrane associated with a hypergravitropic response. Therefore, it would be particularly interesting to examine whether high temperature might modulate the activity/expression of these factors as well to alter PIN2 stability.

Molecular and cellular analyses revealed that high temperature enhances shootward auxin transport capability of roots by promoting the efficient targeting of PIN2 to the plasma membrane. We assume that this accumulation of PIN2 to the plasma membrane makes it capable of transporting more auxin out of the cell and, thus, of maintaining the optimal cellular auxin concentration to promote root growth and development. This hypothesis is substantiated by the fact that high temperature promotes shootward auxin transport and makes the roots resistant to exogenous auxin. Further genetic evidence confirmed that the efficient targeting of PIN2 to the plasma membrane induced by high temperature is regulated through a SNX1-dependent pathway, which has been shown explicitly to be involved in the retrieval of PIN2 from late endosomes (Jaillais et al., 2006). Intracellular cycling of PIN2 has also been shown to be a primary target for low-temperature-induced changes in root development (Shibasaki et al., 2009). There is growing evidence that intracellular cycling is a key player in regulating multiple processes, such as plasma membrane protein homeostasis, signaling pathways, and hormone crosstalk, in both animals and plants (Grant and Donaldson, 2009; Reyes et al., 2011), making it a good candidate to function as a central regulator in modulating plant development under various environmental stimuli. Future studies aimed at identifying the specific trafficking pathways that regulate plant development under variable environmental conditions will aid efforts to engineer crops resistant to various abiotic stresses.

METHODS

Plant Materials

All lines are in the Columbia background of *Arabidopsis thaliana*. The PIN2-GFP (for green fluorescent protein) line (Xu and Scheres, 2005) was a gift of B. Scheres (University of Utrecht, The Netherlands), and DR5:venus yellow fluorescent protein (Laskowski et al., 2008) was provided by G. Muday (Wake Forest University, Winston-Salem, NC). DII-VENUS (Brunoud et al., 2012) was a gift from Malcolm Bennett (University of Nottingham, UK). *snx1-1* and *snx1-2* were described earlier (Jaillais et al., 2006). Columbia-0, *eir1-1*, *aux1-7*, and PIN1-GFP were obtained from the ABRC. PIN2-GFP-mRFP-VAM3 (Sassi et al., 2012) was a gift from Teva Vernoux and Jian Xu (Centre National de la Recherche Scientifique and National University of Singapore). *snx1*-PIN2-GFP transgenic lines were generated by crossing, and T3 homozygous lines were used for microscopy observations.

Growth Conditions

Surface-sterilized seeds were placed in round, 9-cm Petri plates on modified Hoagland medium (Baskin and Wilson, 1997) containing 1% (w/v) Suc and 1% (w/v) agar (Difco Bacto agar; BD Laboratories). Two days after stratification at 4°C in the dark, plates were transferred to a growth chamber (NK System; LH-70CCFL-CT) at 23°C under continuous white light (at an irradiance of ~100 μmol m^{-2} s^{-1}). The seedlings were grown vertically for 5 d. For high-temperature treatment, 5-d-old seedlings were transferred to a 29°C growth chamber (NK System; LH-1-120.S) and incubated for various time lengths under dark or continuous white light (illumination from the top) at an irradiance of ~100 μmol m^{-2} s^{-1}.

Chemicals

[^{3}H]-IAA (20 Ci $mmol^{-1}$) was purchased from American Radiolabeled Chemicals. Other chemicals were from Wako Pure Chemical Industries.

Gravitropism Assay

Root tip reorientation was assayed as described earlier (Rahman et al., 2001). In brief, 5-d-old vertically grown seedlings were transferred to new square plates and allowed to remain vertical under dark or light condition for 12 h at 29 or 23°C before being rotated by 90°. To measure the curvature of roots and elongation, photographs of plates were taken at specific time points after reorientation using a digital camera (Canon; Power Shot A 640) and analyzed by an image analyzing software Image J (http://rsb.info.nih.gov/ij/).

Live-Cell Imaging

To image GFP or YFP, the transferred seedlings were incubated at 29 or 23°C under dark or continuous light for various times and mounted in liquid growth medium on a cover glass for observation on a Nikon laser scanning microscope (Eclipse Ti equipped with Nikon C2 Si laser scanning unit) and imaged with a ×40 water immersion objective. To observe *DR5*:vYFP during gravitropism, seedlings were mounted 0, 2, and 3 h following rotation of the plate and imaged with a ×20 objective. Images were processed with Adobe Photoshop CS4 only for adjustment of contrast and brightness.

The vacuolar/endosomal accumulation in PIN2-GFP and *snx1* PIN2-GFP was quantified in the 500-μm area of the root tip. The number of vacuolar/endosomal bodies were counted for each root and categorized according to the size of the vacuole.

Fluorescence intensities were measured by drawing a region of interest (ROI) in the images obtained from the live-cell imaging using Image J software. For DII-VENUS, a ROI was drawn comprising the cells in the central tissue and fluorescence intensity was measured for that integrated area (see Supplemental Figure 2A online). To measure the differential response of auxin signal during gravity response, ROIs were drawn in upper and lower flanks of gravity stimulated roots, as shown in Supplemental Figure 2A online, and fluorescence intensities were quantified.

Auxin Transport Assay

Five-day-old vertically grown *Arabidopsis* seedlings were transferred to agar plate and incubated at 23 or at 29°C under dark condition for 4 h. For measuring shootward auxin transport, seedlings were transferred to 2% agar plates containing 3-(N-morpholino)-propanesulfonic acid buffer solution (5 mM 3-(N-morpholino)-propanesulfonic acid, 5 mM KNO_3, 2 mM $CaNO_3$, 2 mM $MgSO_4$, and 1 mM KH_2PO_4, pH 6.7). A donor drop was prepared by mixing 5 μM [^{3}H]-IAA (3.7 MBq mL^{-1}) in 1.5% agar containing MES buffer solution (5 mM MES, 5 mM KNO_3, 2 mM $CaNO_3$, 2 mM $MgSO_4$, and 1 mM KH_2PO_4, pH 5.7). The donor drop was placed on the edge of the root tip. Plates were then incubated vertically under dark and nearly saturating humidity for 2 h. To measure auxin transport, 5-mm root segments away from the apical 2 mm were carefully cut and soaked for overnight in 4 mL of liquid scintillation fluid (Ultima Gold; Perkin-Elmer), and the radioactivity was measured with a scintillation counter (model LS6500, Beckman ACSII; USA Instruments).

Statistical Analysis

Results are expressed as the means ± SE from appropriate number of experiments as described in the figure legends. A two-tailed Student's *t* test was used to analyze statistical significance.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *PIN1* (At1g73590), *PIN2* (At5g57090), *PIN3* (At1g70940), *PIN4* (At2g01420), *SNX1* (At5g06140), *AUX1* (At2g38120), *LTI6b* (At3g05890), and *VAM3* (At5g46860).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Effect of Auxin and High Temperature on DII-Venus Abundance.

Supplemental Figure 2. Effect of High Temperature on Auxin-Responsive Reporter Line *DR5-GUS*.

Supplemental Figure 3. Effect of High Temperature on Root Elongation and Root Gravity Response under Light Condition.

Supplemental Figure 4. Effect of High Temperature on Expression of a Reporter of M-Phase CyclB1;1-GUS in *Arabidopsis* Primary Root Tip.

Supplemental Figure 5. Effect of High Temperature on Auxin Uptake and *PIN* Gene Expression.

Supplemental Figure 6. Loss of SNX1 Results in Endosomal Targeting of PIN2 in Light Condition.

Supplemental Figure 7. Effect of High Temperature on Intracellular Localization of Auxin Influx Carrier AUX1.

Supplemental Figure 8. Effect of High Temperature on Intracellular Localization of Auxin Efflux Carrier PIN1 and a Plasma Membrane Protein LTI6b.

Supplemental Table 1. Effect of High Temperature on Cell Length and Cell Production in the *Arabidopsis* Primary Root.

Supplemental Table 2. Percent Roots Forming Auxin Gradient after Gravity Stimulation.

Supplemental Table 3. Vacuolar and Endosomal Accumulation Patterns of PIN2 in Wild-Type and *snx1* under Optimal and High Temperature.

Supplemental Methods 1. Plant Materials and Methods Used in Supplemental Figures and Tables.

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AUTHOR CONTRIBUTIONS

A.R. designed the study. K.S., T.H, and T.N. carried out the experiments. T.G. and Y.K. provided materials and discussed the study with A.R. All authors analyzed and discussed the data. A.R., T.G., and K.S. wrote the article.

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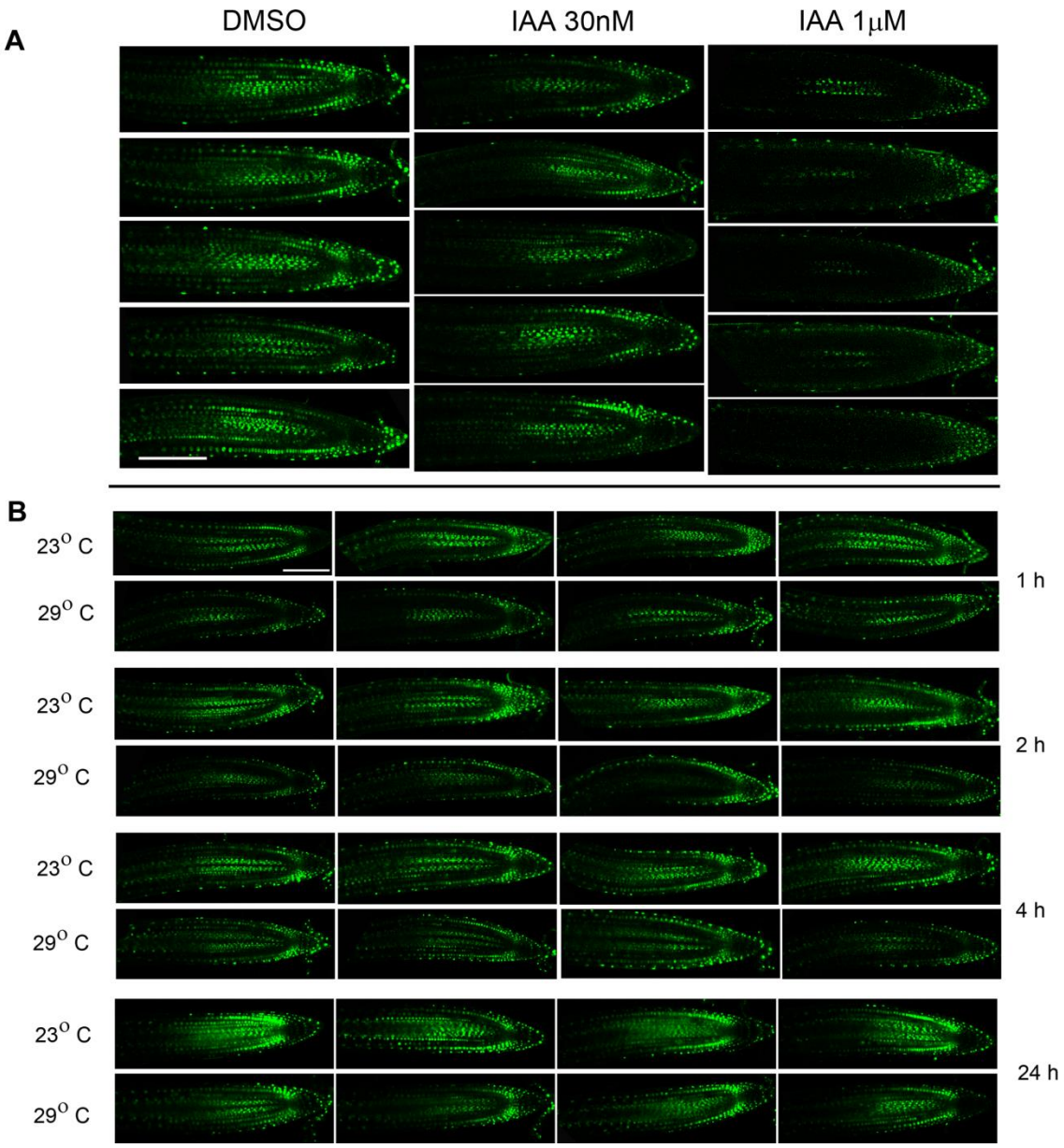
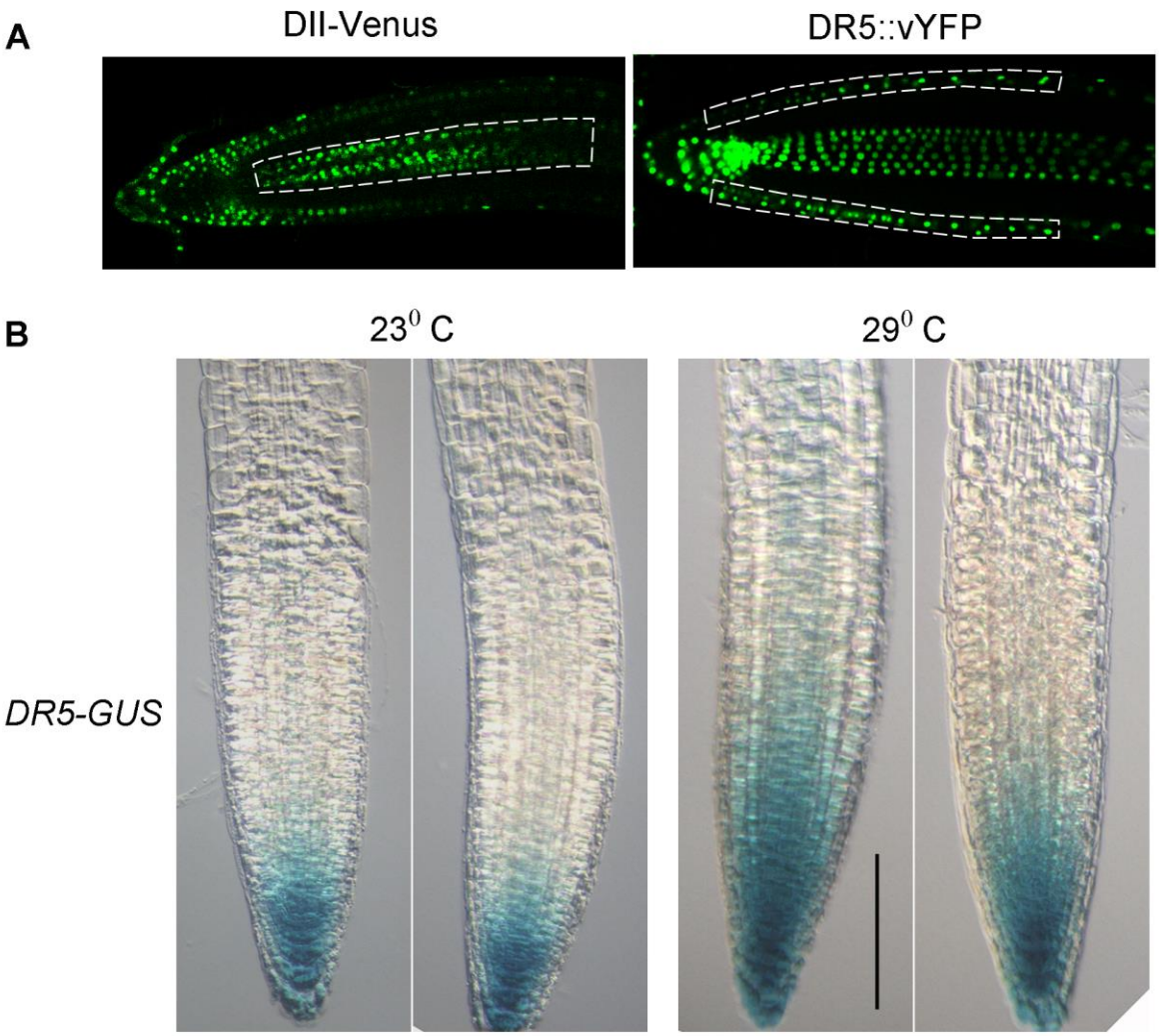
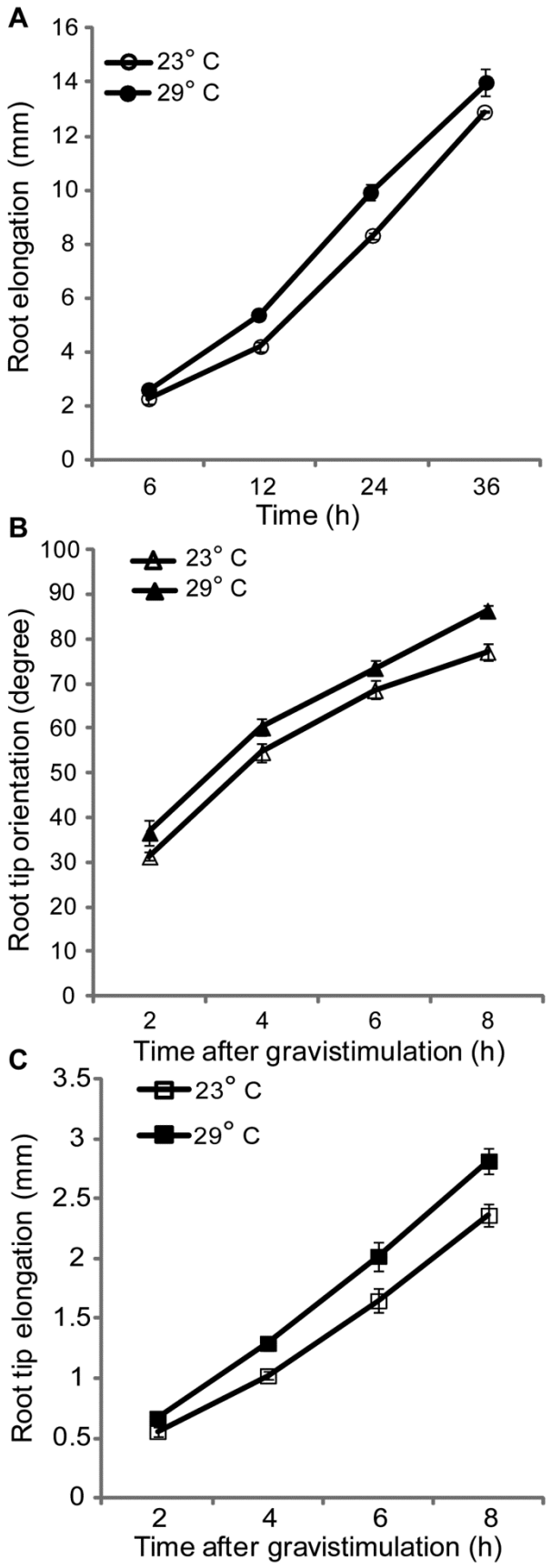


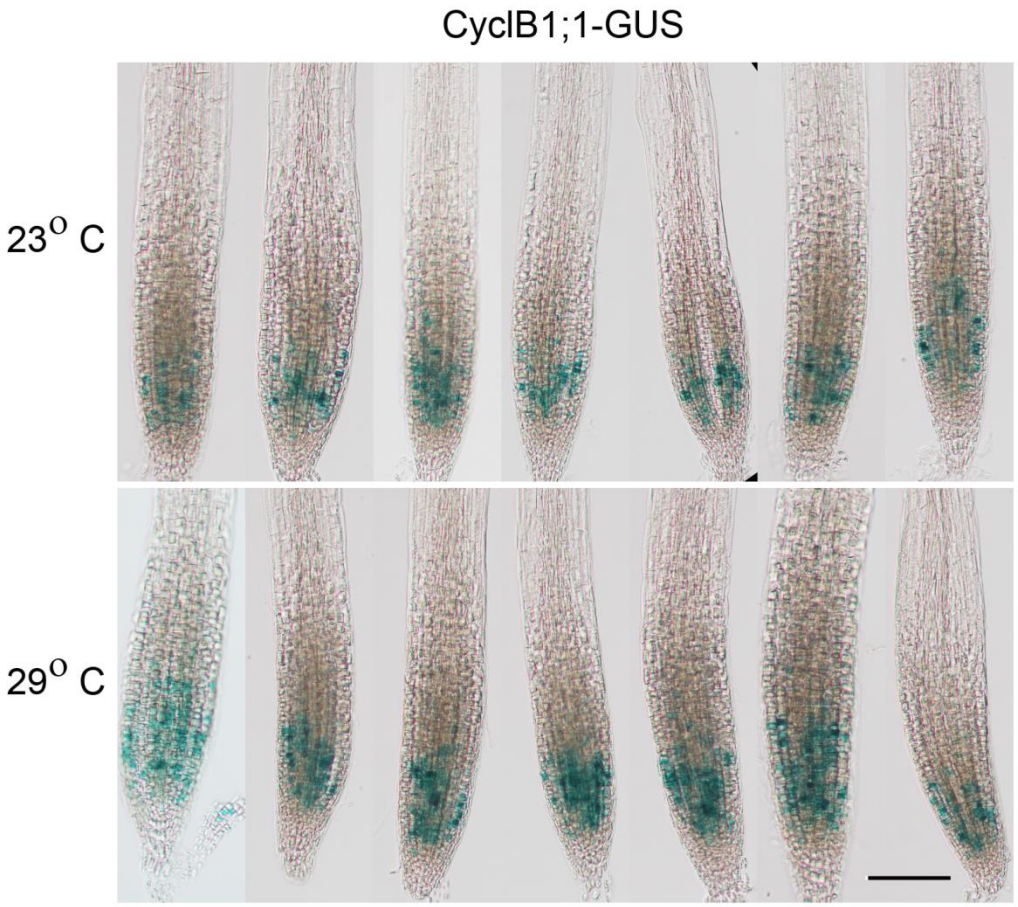
Supplemental Figure 1. (A) DII-Venus abundance is directly related to intracellular auxin concentration. (B) Time course of DII-VENUS response to optimal and high temperatures. Five-day-old light grown DII-VENUS seedlings were transferred to new agar plates and subjected to high temperature treatment (29° C) for 4 h at dark and imaged with confocal microscopy using same settings. Decrease in signal is proportional to auxin concentration (A) and reduced DII-VENUS signal was observed in high temperature treated roots at all-time point (B). Images are representative of 15-20 seedlings obtained from three independent experiments. Bars represent 100 μm.



Supplemental Figure 2. (A) Fluorescence intensity measurement. Representative images of DII-VENUS and *DR5v:YFP* showing the region of interests (ROIs), where the fluorescence intensities were measured**. (B) Effect of high temperature on auxin responsive reporter line *DR5-GUS*.** High temperature elevates auxin maximum at root tip. Five-day-old light grown *DR5-GUS* seedlings were transferred to new agar plates and subjected to high temperature treatment (29° C) for 6 h under dark. After high temperature incubation at dark, seedlings were stained in a buffer containing 1mM X-gluc for 1 h at 37° C, and cleared for photography. Two representative images are shown out of 18-20 seedlings imaged in three independent experiments. Bar represents 100μm.

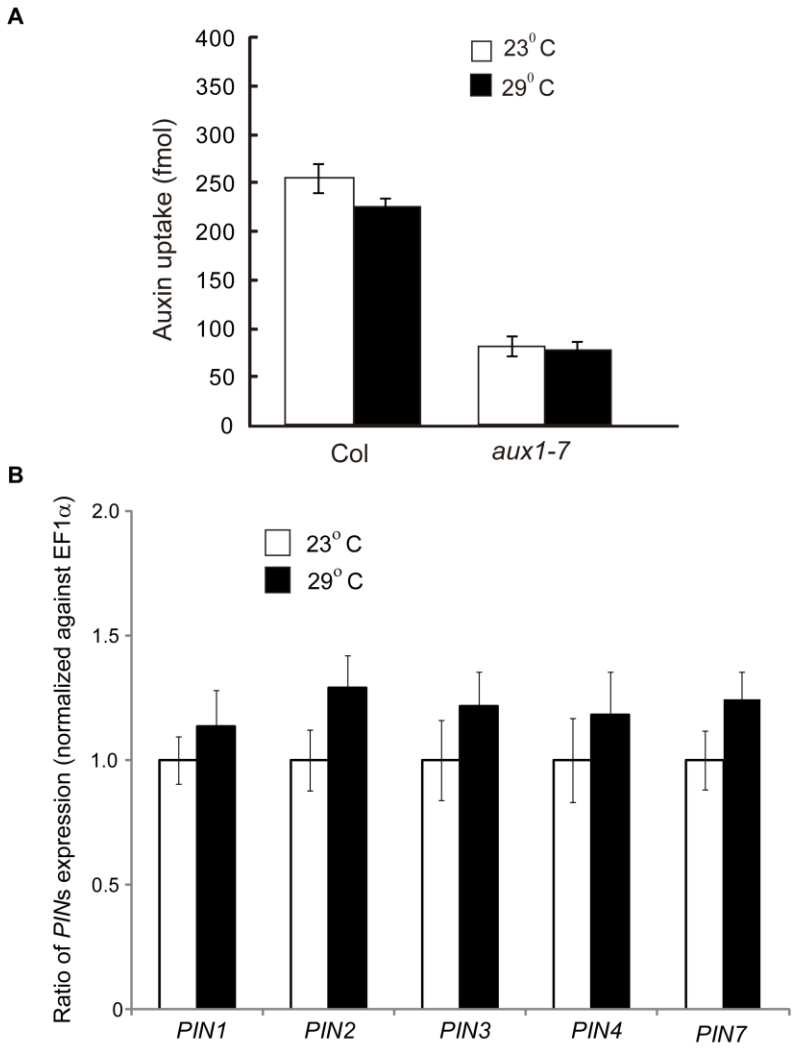


Supplemental Figure 3. Effect of High temperature on root elongation and root gravity response under light condition.
Time course of high temperature induced stimulation of root elongation. A) Five-day-old light grown seedlings were transferred to new agar plates and subjected to high temperature treatment (29° C) for various time lengths under continuous light condition. Effect of high temperature on Arabidopsis root gravity response and elongation (B-C). Five-day-old light grown seedlings were transferred to new agar plates and subjected to gravity stimulation by rotating the plates 90°. Data for root tip orientation and elongation were collected for 2 h, 4 h, 6 h and 8 h. (B) Curvature of root tips plotted against time after reorientation. (C) Root elongation. Vertical bars represent mean ± S.E. of the experimental means from at least four independent experiments (n = 4 or more), where experimental means were obtained from 8-10 seedlings per experiment. Compared with control, high temperature induced stimulation of root growth and gravity responses were significant at all-time points ($P < 0.001$) as judged by Student's t-test.

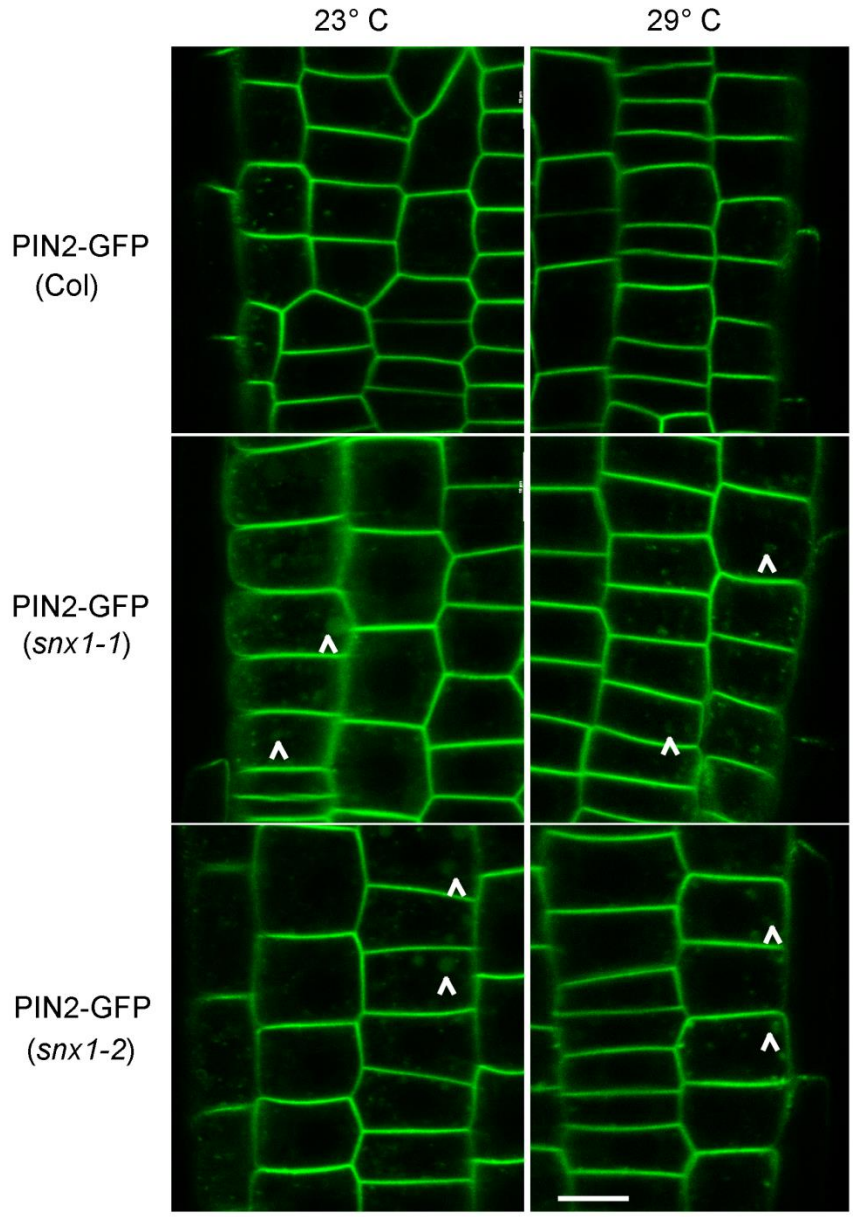


Supplemental Figure 4. Effect of high temperature on expression of a reporter of M-phase CyclB1;1-GUS in Arabidopsis primary root tip.

Five-day-old light grown CyclB1;1-GUS seedlings were transferred to new agar plates and subjected to high temperature treatment (29° C) for 24 h at dark. After high temperature incubation at dark, seedlings were stained in GUS staining buffer for 3 h at 37° C, and cleared for photography. Images are representative of at least 20 seedlings obtained from three independent experiments. Bar represents 100 μm.

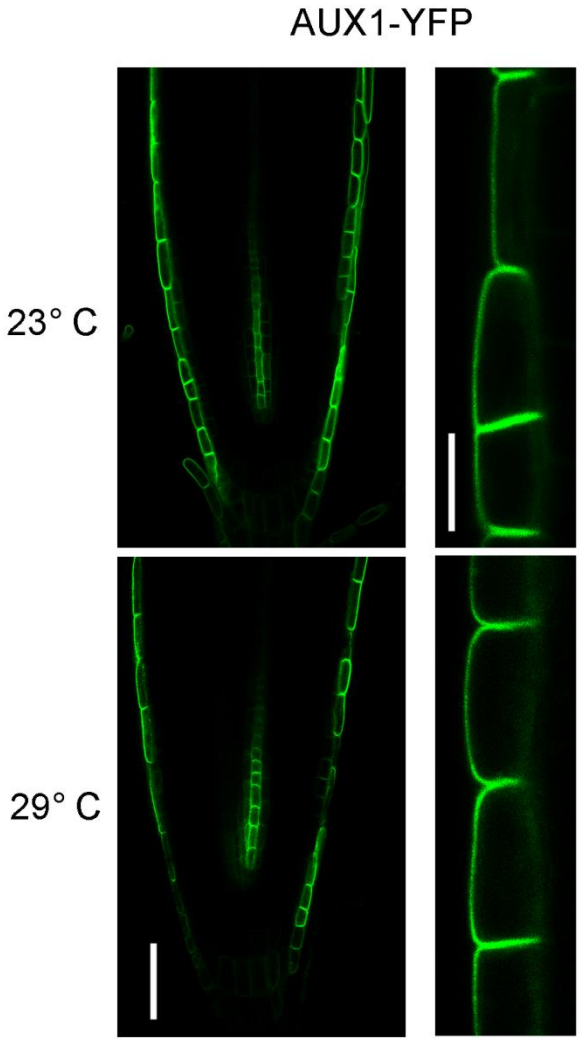


Supplemental Figure 5. (A) High temperature does not alter root auxin uptake capacity. Five-day-old light grown seedlings were transferred to new agar plates and subjected to high temperature treatment (29° C) for 12 h under dark condition and IAA uptake in root tip was measured for 1 h at room temperature immediately after the high temperature treatment. Six-eight root tips of 3 mm in length were incubated with 100 nM ^{3}H IAA for 1 h. After the incubation, root tips were washed and the radioactivity was counted. The experiments were conducted using three biological replicates.For each biological replicate, three technical replicates were assayed. (n=55-65). Vertical bars represent mean ± S.E. of the experimental means from at least three independent experiments (n = 3), where experimental means were obtained from 8-10 seedlings per experiment **(B) High temperature does not alter the expression of *PIN* genes.** Five-day-old light grown seedlings were transferred to new agar plates and subjected to high temperature treatment (29° C) under dark condition for 24 h. *PIN*s expression was analyzed in the root of Arabidopsis seedlings using qRT-PCR.

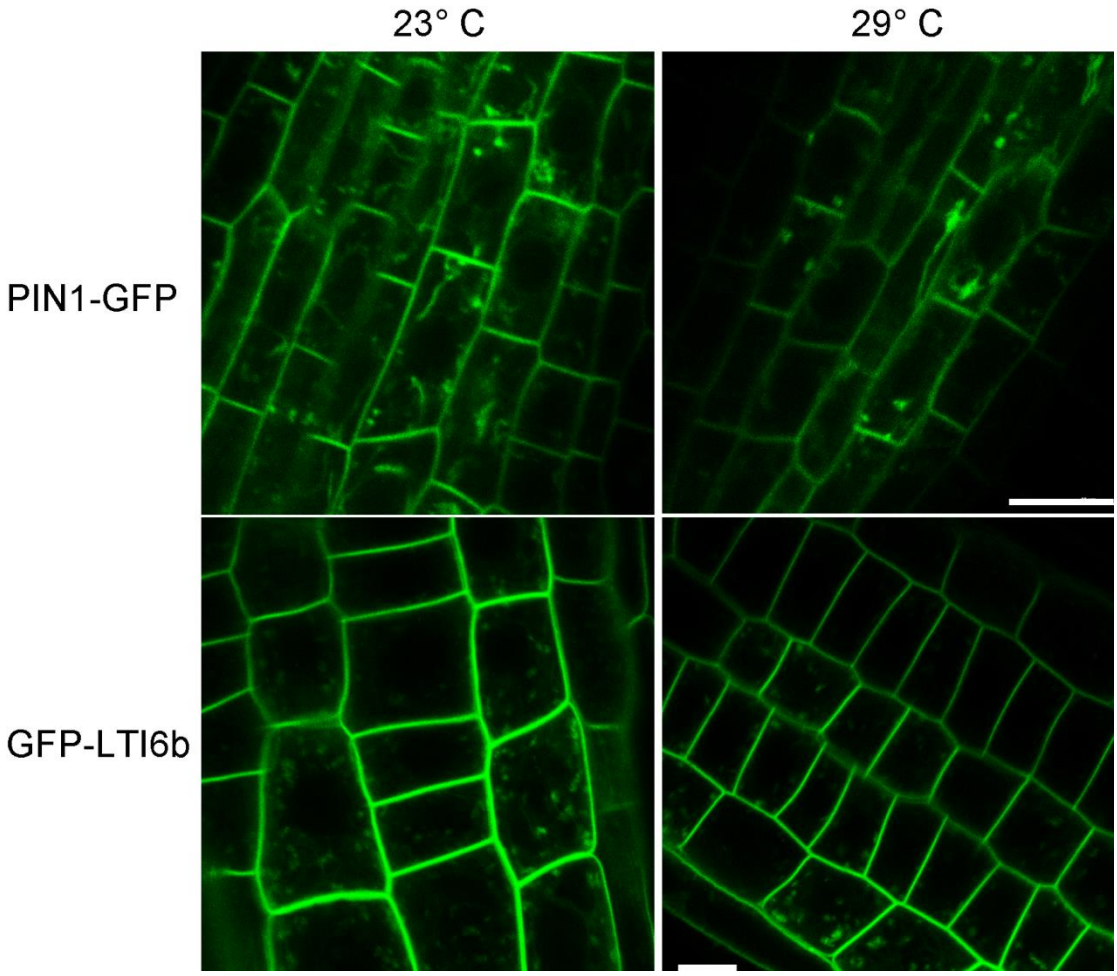


Supplemental Figure 6. Loss of SNX1 results in endosomal targeting of PIN2 in light condition.

Five-day-old light grown seedlings were transferred to new agar plates, subjected to high temperature treatment (29° C) under light condition for 4 h and imaged with confocal microscopy. Images were captured using the same confocal settings and are representative of 25-30 seedlings obtained from at least four independent experiments. Arrowheads indicate endosomal accumulation of PIN2. Please note endosomal accumulation is present both in low and high temperature treated *snx1* seedlings, but absent in wild-type seedlings. Scale bar represents 10 μm.



Supplemental Figure 7. Effect of high temperature on intracellular localization of auxin influx carrier AUX1.
Five-day-old light grown AUX1-YFP seedlings were transferred to new agar plates and subjected to high temperature treatment (29° C) for 4 h at dark before imaged with confocal microscopy. Images were captured using the same confocal settings and are representative of 15-20 seedlings obtained from at least three independent experiments. Right panels are zoomed images of left panels. Scale bars represent 50 μm and 10 μm respectively.



Supplemental Figure 8. Effect of high temperature on intracellular localization of auxin efflux carrier PIN1 and a plasma membrane protein LTI6b.
Five-day-old light grown seedlings were transferred to new agar plates and subjected to high temperature treatment (29° C) under dark condition for 4 h before imaged with confocal microscopy. Images were captured using the same confocal settings and are representative of 15-20 seedlings obtained from at least three independent experiments. Scale bars represent 50 μm and 10 μm respectively.

Supplemental Table 1

Effect of high temperature on cell length and cell production in the Arabidopsis primary root

Treatment	Elongation rate (mm day^{-1})	Cell length (μm)	Cell production rate Cells day^{-1}
23° C	8.98 ± 0.16 (100)	146.5 ± 1.19 (100)	61.3 ± 2.1 (100)
29° C	12.02 ± 0.19 (134)***	155.2 ± 0.84 (106)***	77.4 ± 0.5 (126)***

*** Compared with control, high temperature induced stimulation of root elongation, cell length and cell production rate were significantly high ($P < 0.0001$) as judged by Student's *t*-test.

Supplemental Table 2

Percent roots forming auxin gradient after gravity stimulation

	0hr	2hr	3hr
23° C	0% (0 out of 12)	33% (4 out of 12)	75% (9 out of 12)
29° C	0% (0 out of 12)	74% (11 out of 15)	88% (11 out of 13)

Supplemental Table 3

Vacuolar and endosomal accumulation patterns of PIN2 in wild-type and *snx1* under optimal and high temperature

	No accumulation	Weak accumulation	High accumulation
Dark condition (Vacuolar accumulation)			
Col PIN2-GFP -23° C	5% (2 out of 39)	18% (7 out of 39)	77% (30 out of 39)
Col PIN2-GFP -29° C	78% (34 out of 44)	20% (9 out of 44)	2% (1 out of 44)
*snx1-1*PIN2-GFP-23° C	0% (0 out of 17)	18% (3 out of 17)	82% (14 out of 17)
*snx1-1*PIN2-GFP-29° C	15% (3 out of 20)	10% (2 out of 20)	75% (15 out of 20)
*snx1-2*PIN2-GFP-23° C	0% (0 out of 17)	12% (2 out of 17)	88% (15 out of 17)
*snx1-2*PIN2-GFP-29° C	0% (0 out of 17)	23% (3 out of 17)	77% (14 out of 17)
Light condition (endosomal accumulation)			
Col PIN2-GFP -23° C	82% (23 out of 28)	18% (5 out of 28)	0% (0 out of 28)
Col PIN2-GFP -29° C	92% (22 out of 24)	8% (2 out of 24)	0% (0 out of 24)
*snx1-1*PIN2-GFP-23° C	15% (2 out of 13)	47% (5 out of 13)	38% (6 out of 13)
*snx1-1*PIN2-GFP-29° C	23% (3 out of 13)	54% (7 out of 13)	23% (3 out of 13)
*snx1-2*PIN2-GFP-23° C	38.5% (5 out of 13)	38.5% (5 out of 13)	23% (3 out of 13)
*snx1-2*PIN2-GFP-29° C	43% (6 out of 14)	43% (6 out of 14)	14% (2 out of 14)

Supplemental Methods

Plant materials

Arabidopsis expressing CycB1;1-GUS (Col-0 background) (Supplemental Figure 4) was provided by Adan Colon-Carmona (University of Massachusetts, Boston); EGFP-LTI6b (C24 background) (Kurup et al., 2005) (Supplemental Figure 8) was provided by G. Muday (Wake Forest University, Winston-Salem, USA); AUX1-YFP (Col-0 background) (Supplemental Figure 7)(Swarup et al., 2004) was a gift from Malcolm Bennett(University of Nottingham, UK).

Cell production rate assay

The cell production assay (Supplemental Table 1) was performed as described earlier (Rahman et al., 2007). In brief, 5-day-old light grown seedlings were transferred to new agar plates and subjected to high temperature or control temperature treatments in darkness for 24h. The length of 10 mature cortical cells was measured from each root, eight roots used per treatment. The cell production rate (cells day^{-1}) was calculated by taking the ratio of root elongation rate (mm day^{-1}) and average of cell length (μm).

GUS staining

GUS staining (Supplemental Figures 2, 4) was performed as described earlier (Rahman et al., 2007). In brief, 5-day-old seedlings were transferred to new agar plates and grown vertically at 29° C or 23° C in darkness as indicated. Seedlings were then transferred to GUS staining buffer (100mM sodium phosphate, pH7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100) containing 1 mM X-gluc and incubated at 37° C in the dark as indicated. The cells were cleared as described earlier (Rahman et al., 2007). The roots were imaged with a light microscope (Nikon, Diaphot, Japan, www.nikon.co.jp) equipped with a digital camera control unit (DIGITAL SIGHT (DS-L2); Nikon, Japan, www.nikon.co.jp).

Auxin uptake assay

Seedlings were grown and incubated for 12 hrs at 23° C or 29° C as described in main text and auxin uptake was performed as described earlier (Rahman et al., 2001). After the incubation, root tips of 3 mm in length were excised and placed on nylon mesh (1 cm^2) with 250-μm openings. The nylon mesh containing 10 root tips was transferred to a 2.6 cm Petri dish on a piece of filter paper of 1.2 cm^2 (Advantec No. 2) wetted with 75 μl of the MES buffer solution supplemented with 100 nM [^{3}H] IAA (3.7 MBq ml^{-1}) and incubated for 1 h under nearly saturating humidity. After incubation, the root tips were carefully transferred to a 2.6 cm Petri dish containing 3 mL of buffer without labelled compound and washed for 2 minutes with gentle shaking, three times. The root tips were then soaked for overnight in 5 ml liquid scintillation fluid (ACSII, Amersham Biosciences, USA), and the radioactivity was measured with a scintillation counter.

Gene Expression Analysis

5-day-old vertically grown Arabidopsis seedlings were transferred to agar plate and incubated at 23° C or at 29° C for 24 h under darkness. RNA was extracted from the root tissue using PlantRNA extraction kit (Qiagen, USA) and tested for quality and quantity. Each RNA concentration was normalized with RNase free water. 1μg RNA was applied to synthesize single strand cDNA using Rever Tra Ace qPCR RT master mix (Toyobo, Japan). Quantitative PCR reactions were performed using the Takara TP-850 thermal cycler (Takara Bio, Japan) and SYBR® Premix Ex Taq qPCR kit (Takara Bio, Japan).The reaction was performed as per manufacturer's instruction. For quantification of PINs expression, we used the $2^{-\Delta\Delta CT}$ (cycle threshold) method. Data were obtained from three biological replicates..

Primer sequences used to analyze *PIN* expression are as follows:

PIN1	Left primer	ATCTTCACATGTTTGTGTGG
	Right Primer	TCGTCTTTGTTACCGAAACT
PIN2	Left primer	AGATGCCAACGATAATGAGT
	Right Primer	AGTAATCACCTGAACGATGG
PIN3	Left primer	AGATCTGACCAAGGTGCTAA
	Right Primer	CCTAGACCTGTCTTGGATTG
PIN4	Left primer	ACTTCAACCCAAAATCATTG
	Right Primer	GTGGGATGCACATTGTACT
PIN7	Left primer	AGTTGATAATGGAGCCAATG
	Right Primer	TTATGAGTTTCCTCCACACC

Supplemental References

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