

Low Temperature Inhibits Root Growth by Reducing Auxin Accumulation via *ARR1/12*

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Plants exhibit reduced root growth when exposed to low temperature; however, how low temperature modulates root growth remains to be understood. Our study demonstrated that low temperature reduces both meristem size and cell number, repressing the division potential of meristematic cells by reducing auxin accumulation, possibly through the repressed expression of *PIN1/3/7* and auxin biosynthesis-related genes, although the experiments with exogenous auxin application also suggest the involvement of other factor(s). In addition, we verified that *ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1)* and *ARR12* are involved in low temperature-mediated inhibition of root growth by showing that the roots of *arr1-3 arr12-1* seedlings were less sensitive than wild-type roots to low temperature, in terms of changes in root length and meristem cell number. Furthermore, low temperature reduced the levels of *PIN1/3* transcripts and the auxin level to a lesser extent in *arr1-3 arr12-1* roots than in wild-type roots, suggesting that cytokinin signaling is involved in the low-temperature-mediated reduction of auxin accumulation. Taken together, our data suggest that low temperature inhibits root growth by reducing auxin accumulation via *ARR1/12*.

Keywords: Auxin • Low temperature • Root meristem.

Abbreviations: ARR, *ARABIDOPSIS RESPONSE REGULATOR*; GC-MS, gas chromatography–mass spectrometry; GFP, green fluorescent protein; GUS, β -glucuronidase; PIN, *PIN-FORMED*; QC, quiescent center; qPCR, quantitative real-time PCR; YFP, yellow fluorescent protein.

Introduction

The growth and development of plants critically depend on the environmental temperature, with low temperatures limiting plant growth and thus affecting the distribution and yield of crops (Stitt and Hurry 2002, Zhang et al. 2004). Low temperature has a particularly prominent effect in areas where the temperature changes significantly during the transition between warm and cold seasons (Weinig et al. 2014). The complex mechanisms underlying plant adaptation to low temperature

involve extensive remodeling of many physiological and biochemical processes (Thomashow 1999, Zhu et al. 2007, Ghosh and Xu 2014). However, the mechanisms regulating root growth under low temperatures remain unclear.

Root growth depends on the production of cells within the root meristem, as well as cell expansion and differentiation (Beemster et al. 2002). Cell cycle regulators, including the cyclin-dependent kinases (CDKs) and their associated cyclins, control cell proliferation and are involved in the regulation of root growth (Doerner et al. 1996, Beemster et al. 2002, Inze and De Veylder 2006). In addition, environmental factors modulate root growth. For example, cold stress (4°C) reduces root growth in *Arabidopsis thaliana* (Shibasaki et al. 2009). Moreover, cold inhibits cell cycle progression and hence leaf growth in *Zea mays* (maize) (Rymen et al. 2007), implying that cold-repressed root growth could also result from the inhibition of cell division in the root meristem.

Auxin regulates cell division and expansion in the primary root, and plays major roles in the maintenance of cell division potential and patterning of the root meristem (Dharmasiri et al. 2005, Vanneste and Friml 2009). Auxin accumulation in the root apex is mediated by both auxin biosynthesis and transport (Grieneisen et al. 2007, Mano and Nemoto 2012). In particular, optimal auxin accumulation and distribution in the root apex requires *PIN-FORMED (PIN)* proteins, the central rate-limiting components involved in auxin transport (Blilou et al. 2005, Grieneisen et al. 2007, Petrasek and Friml 2009). The polarity and amount of PIN proteins determine the direction and amplitude of auxin transport, and endogenous signals can modulate the activity of PINs (Ruzicka et al. 2009, Fernandez-Marcos et al. 2011, Xu et al. 2013, T.T. Yuan et al. 2013). In *Arabidopsis* roots, at least five PINs (*PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7*) modulate cell to cell auxin transport. Down-regulation or knock-out of these PINs accelerates cell differentiation and reduces the size of the meristematic zone in roots (Blilou et al. 2005, Fernandez-Marcos et al. 2011, Hong et al. 2014). Cold inhibits the gravity response, which depends on auxin signaling (Nadella et al. 2006). Further studies indicated that the effect of cold on the root gravity response results from the inhibition of basipetal auxin transport due to blocking of the intracellular trafficking of *PIN2* and lateral relocalization of *PIN3* (Shibasaki et al. 2009).

Cytokinin also modulates the rate of cell differentiation and the activity of the root meristem, by reducing the expression of *PIN* genes to restrict auxin distribution in the root apex (Dello loio et al. 2007, Ruzicka et al. 2009). The effect of cytokinin on *PIN* genes requires *ARABIDOPSIS RESPONSE REGULATOR 1* (*ARR1*)-mediated up-regulation of *SHORT HYPOCOTYL 2* (*SHY2/IAA3*), an Aux/IAA repressor that functions in auxin signaling (Dello loio et al. 2008). Furthermore, cytokinin signaling also affects the freezing tolerance of Arabidopsis seedlings (Jeon et al. 2010, Jeon and Kim 2013). For example, cold treatment induces the expression of a subset of type-A *ARR* genes, including *ARR7* (Jeon et al. 2010). Other components of cytokinin signaling, including *ARABIDOPSIS HISTIDINE KINASE 2* (*AHK2*), *AHK3*, *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 2* (*AHP2*), *AHP3*, *AHP5* and the type-B *ARR1*, mediate the cold-induced expression of these type-A *ARR* genes (Jeon et al. 2010, Jeon and Kim 2013). The observations that *ahk2 ahk3* and *arr7* mutants exhibit enhanced freezing tolerance and a hypersensitive response to ABA during germination suggest that cytokinin signaling negatively regulates plant tolerance to cold stress by inhibiting ABA responses (Jeon et al. 2010).

In this study, we showed that low temperature reduces meristem size and cell number, a process that involves both auxin and cytokinin. Furthermore, the roots of *arr1-3 arr12-1* seedlings were less sensitive than wild-type roots to low temperature, in terms of reductions in auxin levels. Thus, our data suggest that low temperature inhibits root growth via the *ARR1/12*-mediated reduction in auxin accumulation in roots.

Results

Low temperature represses root growth and meristem development

Low temperature treatment at 4°C dramatically reduces root elongation (Shibasaki et al. 2009). In experiments to examine

the effects of low temperature on root growth, Arabidopsis plants were exposed to a temperature of 16°C, because the roots barely grew at 4°C (data not shown). To eliminate any potential effect of low temperature on germination, the seedlings were grown at 22°C for 3 d, and then transferred to 16°C or kept at 22°C for various numbers of days. The time-course analysis revealed that low temperature significantly inhibited primary root elongation (Fig. 1A, B). To explore the cellular basis for this low temperature-mediated inhibition of root elongation, the root meristem was examined in detail. Our results showed that both the meristem size and the cell number decreased in plants subjected to low temperature treatment (Fig. 1C, D).

Low temperature reduces the division potential of root meristematic cells

A decrease in root meristem size could result from a reduction in stem cell niche activity (Sabatini et al. 2003). Thus, we analyzed the possible involvement of stem cell niche activity in low temperature-mediated inhibition of root elongation using both *QC25::GUS* (Sabatini et al. 2003) and *SCR::H2B-YFP* (Heidstra et al. 2004) marker lines. The *QC25::GUS* is a quiescent center (QC)-expressed promoter trap, and the *SCR::H2B-YFP* can be used to assay *SCR* (*SCARECROW*) promoter activity. β -Glucuronidase (*GUS*) staining or yellow fluorescent protein (*YFP*) fluorescence was similar in the roots of the treated and control seedlings (Fig. 2A, B), indicating that stem cell niche activity is not responsible for the reduced meristematic zone of plant roots subjected to low temperature.

An alternative factor for the decreased root meristem size could be loss of division potential of meristematic cells. Thus, we analyzed the cell division potential by *GUS* staining of seedlings carrying *CYCB1;1::GUS*, a marker for cells undergoing mitosis (Colon-Carmona et al. 1999). Our results showed that *GUS*-stained cells and *GUS* activity were reduced in the root tips of plants grown at 16°C compared with those grown

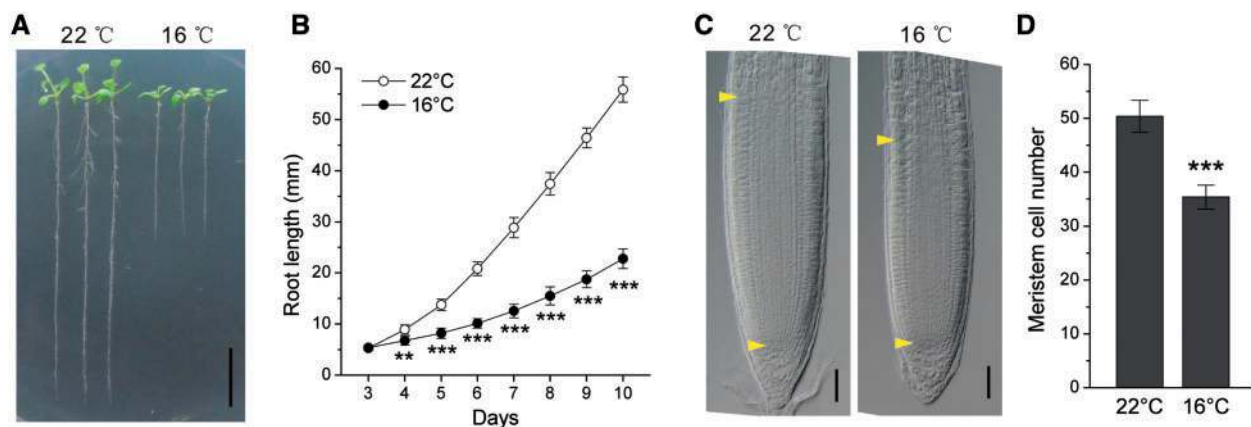


Fig. 1 Low temperature reduces root elongation and meristem cell number. (A) Representative image of 10-day-old wild-type seedlings grown at 22°C (left) and 16°C (right). Scale bars = 1 cm. (B) Root growth kinetics of wild-type seedlings grown at 22 and 16°C. (A, B) Seedlings were grown at 22°C for 3 d, and then transferred to 16°C or kept at 22°C for an additional 7 d. (C) Representative images of 6-day-old wild-type root tips grown at 22°C (left) and 16°C (right). Yellow arrow pairs delineate the meristematic zone. Black bars = 100 μ m. (D) Meristem cell number of 6-day-old wild-type plants. (C, D) Seedlings were grown at 22°C for 3 d, and then transferred to 16°C or kept at 22°C for an additional 3 d. (B, D) Error bars represent the SD, and the asterisks indicate statistically significant differences between the seedlings grown at 22 and 16°C (** $P < 0.01$, *** $P < 0.001$, Student's *t*-test).

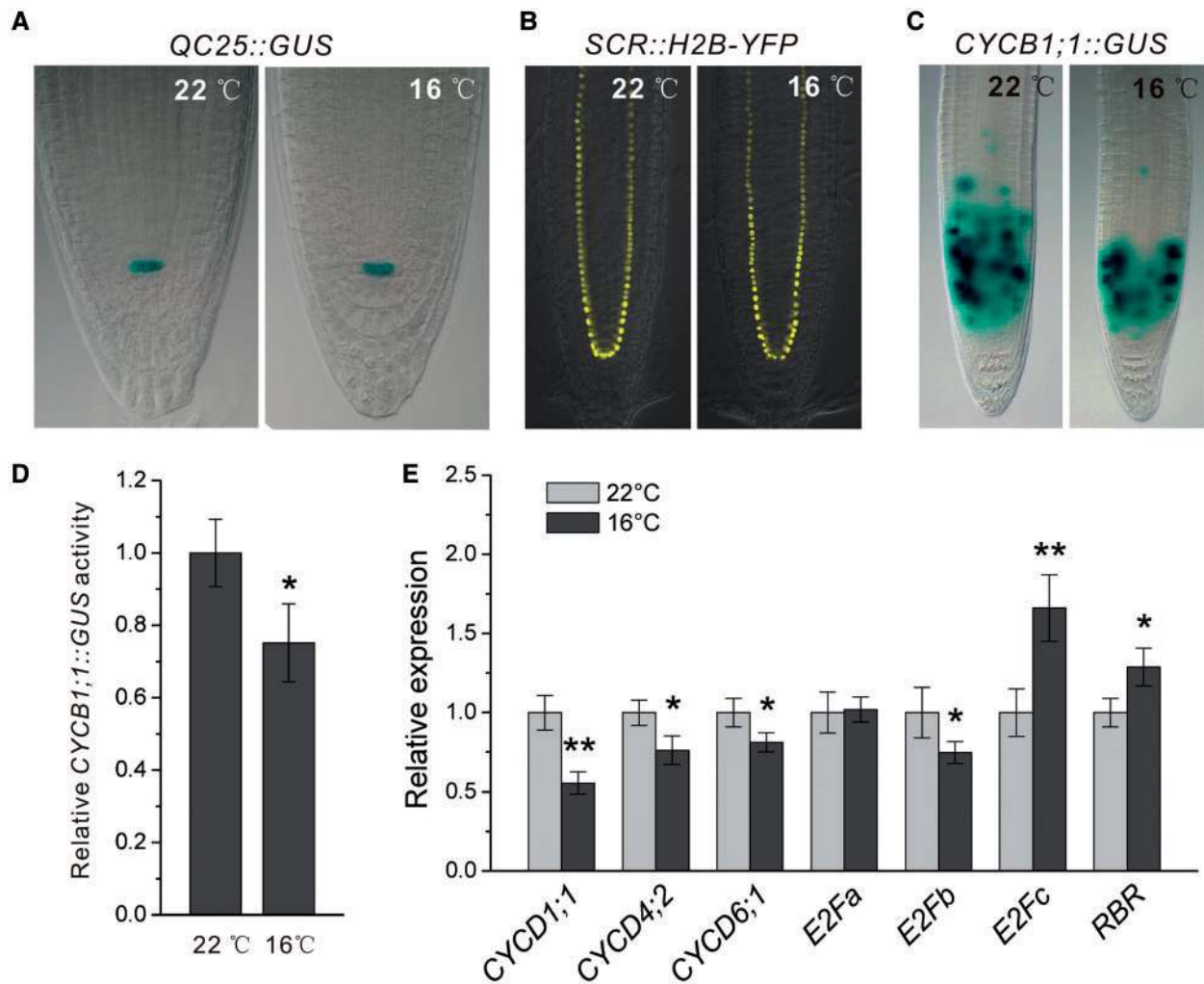


Fig. 2 Low temperature inhibits cell division. Images of GUS-stained 6-day-old (A) *QC25::GUS* and (C) *CYCB1;1::GUS* plants. (B) Fluorescence images of *SCR::H2B-YFP* root tips. At least 12 seedlings were imaged per line for each of the three replicates. (D) Relative activity of *CYCB1;1::GUS* at 22 and 16 °C. The GUS activity at 22 °C is set to 1. (E) The relative expression of cell cycle-related genes in wild-type plants grown at 22 or 16 °C assayed by qPCR. The expression level of the indicated gene at 22 °C is set to 1. (D, E) Error bars represent the SD of three biological replicates, and the asterisks indicate significant differences between the activity of *CYCB1;1::GUS* or expression levels of the indicated genes at 22 and 16 °C (* $P < 0.05$, ** $P < 0.01$, Student's *t*-test). (A–E) Seedlings were grown at 22 °C for 3 d, and then transferred to 16 °C or kept at 22 °C for an additional 3 d.

at 22 °C, revealing that low temperature treatment decreased the division potential of root meristematic cells (Fig. 2C, D).

Then, we examined the effect of low temperature on the expression of a set of cell cycle-related genes, including the D-type cyclins *CYCD1;1*, *CYCD4;2* and *CYCD6;1*, as well as the cell cycle-related transcription factors *E2Fa* and *E2Fb*, all of which promote cell cycle progression and stimulate cell division (De Veylder et al. 2002, Masubelele et al. 2005, Kono et al. 2006, Sozzani et al. 2006, Cruz-Ramirez et al. 2012). Our quantitative real-time PCR (qPCR) analysis revealed that low temperature down-regulated the expression of all of the assayed genes, except for *E2Fa* (Fig. 2E). In contrast, *E2Fc* and *RETINOBLASTOMA-RELATED (RBR)*, which repress cell division (del Pozo et al. 2002, Wildwater et al. 2005), were significantly up-regulated under low temperature (Fig. 2E).

Low temperature affects auxin accumulation in roots

Auxin signaling is required for optimal root growth and maintenance of cell division potential in the root meristem (Benkova et al. 2003, Blilou et al. 2005, Galinha et al. 2007). Thus, we explored whether auxin signaling is involved in low temperature-mediated inhibition of root growth, using the *DR5::GUS* and *IAA2::GUS* marker lines for auxin accumulation and distribution (Ulmasov et al. 1997, Luschnig et al. 1998). The decreased GUS staining in the roots of these two lines indicated that low temperature down-regulated auxin activity (Figs. 3A, B; 6A, B). This was further supported by measurement of IAA contents using gas chromatography–mass spectrometry (GC-MS). Indeed, the auxin level in the roots of wild-type plants grown at 16 °C was significantly lower than in those of plants grown at 22 °C (Fig. 3C). Next, we tested whether the decreased

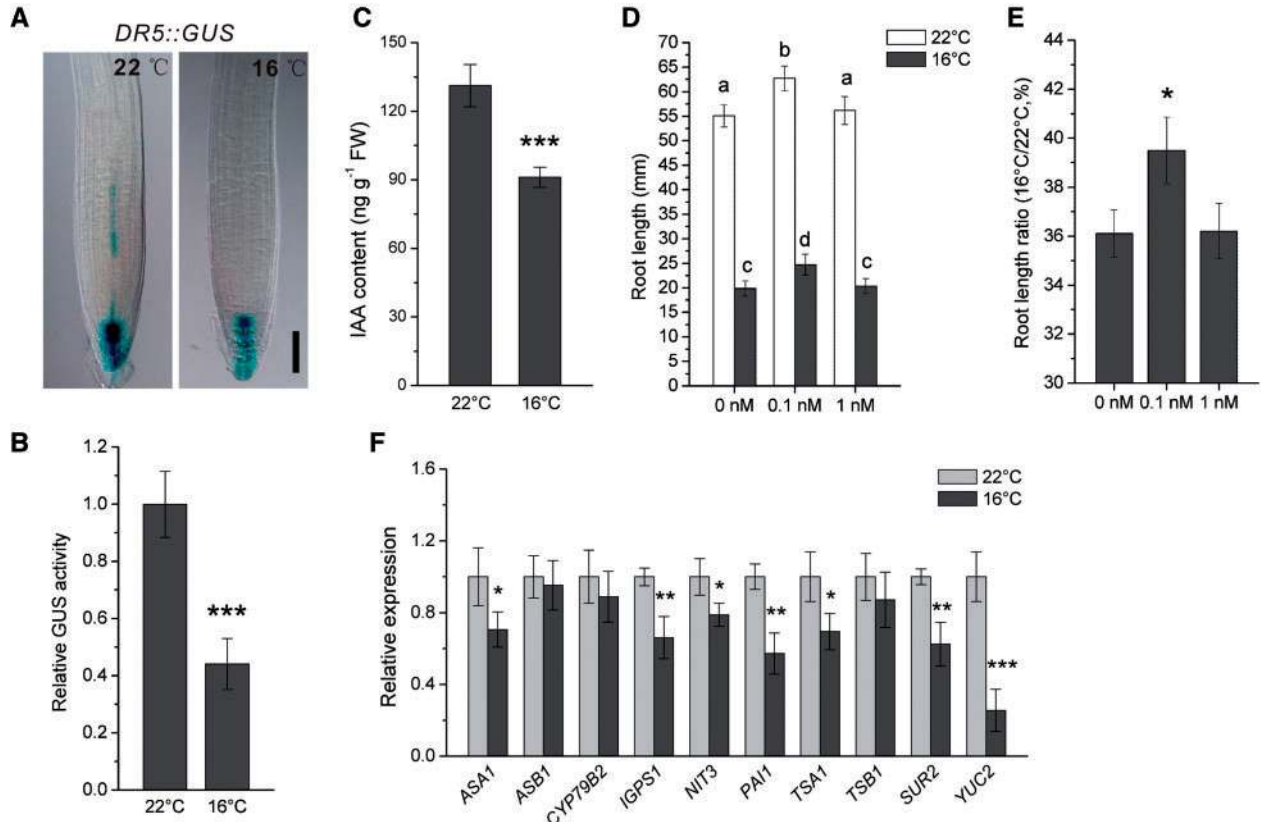


Fig. 3 Low temperature inhibits auxin accumulation in root tips. (A) Images of GUS-stained 6-day-old *DR5::GUS* root tips of plants grown at 22 and 16°C. Scale bars = 100 μm. (B) Relative GUS activity in (A). The GUS activity at 22°C is set to 1. Error bars represent the SD, and the asterisks indicate significant differences between the activity of *DR5::GUS* at 22 and 16°C (***) $P < 0.001$, Student's *t*-test). (C) IAA contents of 6-day-old wild-type plants grown at 22 or 16°C. Error bars represent the SD, and the asterisks indicate significant differences (***) $P < 0.001$, Student's *t*-test). (D) Root length and (E) root length ratio of 10-day-old wild-type plants grown on media containing the indicated concentration of IAA. Error bars represent the SD, and the differences between columns annotated with different letters in (D) or an asterisk in (E) are significant ($P < 0.05$, Student's *t*-test). (D, E) Seedlings were grown on medium without IAA at 22°C for 3 d, and then transferred to media with the indicated concentration of IAA for 7 d at 22 or 16°C. (F) Relative expression of auxin biosynthesis genes in wild-type plants grown at 22 or 16°C assayed by qPCR. The expression level of the indicated gene at 22°C is set to 1. Error bars represent the SD, and the asterisks indicate significant differences between the expression levels of the indicated gene at 22 and 16°C (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test). (A, B, C and F) Seedlings were grown at 22°C for 3 d, and then transferred to 16°C or kept at 22°C for an additional 3 d.

auxin level contributed to low temperature-mediated inhibition of root growth by exogenously applying IAA. Three-day-old seedlings were transferred to media with different concentrations of IAA, and the root length was examined 7 d after transfer. We found that 0.1 nM IAA promoted, but 1 nM IAA did not affect root growth at 22°C as previously reported (Yang et al. 2014), or at 16°C in our experiments (Fig. 3D). However, the root growth was less inhibited by low temperature on the medium with 0.1 nM IAA compared with that without IAA, as revealed by the ratio obtained through comparing the root length grown at 16 and 22°C (Fig. 3E). These results further support the role of auxin in low-temperature-mediated inhibition of root growth.

As the auxin level is determined by the integrated output of auxin biosynthesis and transport, changes in these processes could cause the low-temperature-mediated reduction in auxin level. Thus, we first assayed the expression of auxin biosynthesis-related genes by qPCR. Among the assayed genes, *ASA1*, *IGPS1*, *NIT3*, *PAI1*, *TSA1*, *SUR2* and *YUC2* were down-regulated

by low temperature treatment (Fig. 3F). Secondly, we examined the expression of *PIN* genes using the marker lines *PIN1::PIN1-GFP*, *PIN3::PIN3-GFP* and *PIN7::PIN7-GFP*. The fluorescence intensities for *PIN1*-green fluorescent protein (GFP), *PIN3*-GFP and *PIN7*-GFP were reduced by $58 \pm 8\%$, $27 \pm 9\%$ and $45 \pm 11\%$, respectively, under low temperature stress (Fig. 4A–D). These results were further supported by our qPCR results, which demonstrated that transcription levels for *PIN1/3/7* were significantly reduced in the roots by low temperature (Fig. 4E). These results suggest that both auxin biosynthesis and transport may contribute to the low-temperature-mediated reduction of auxin accumulation in roots. Then, we further verified the role of *PIN1*, *PIN3* and *PIN7* in the regulation of root growth under low temperature by examining root length of *pin1*, *pin3-4*, *pin7-2* and the *pin1 pin3 pin7* triple mutant at both 22 and 16°C. Our results showed that the root growth of *pin1* was less sensitive to low temperature than that of wild-type plants, while *pin3-4* and *pin7-2* exhibited similar sensitivity to wild-type plants, as

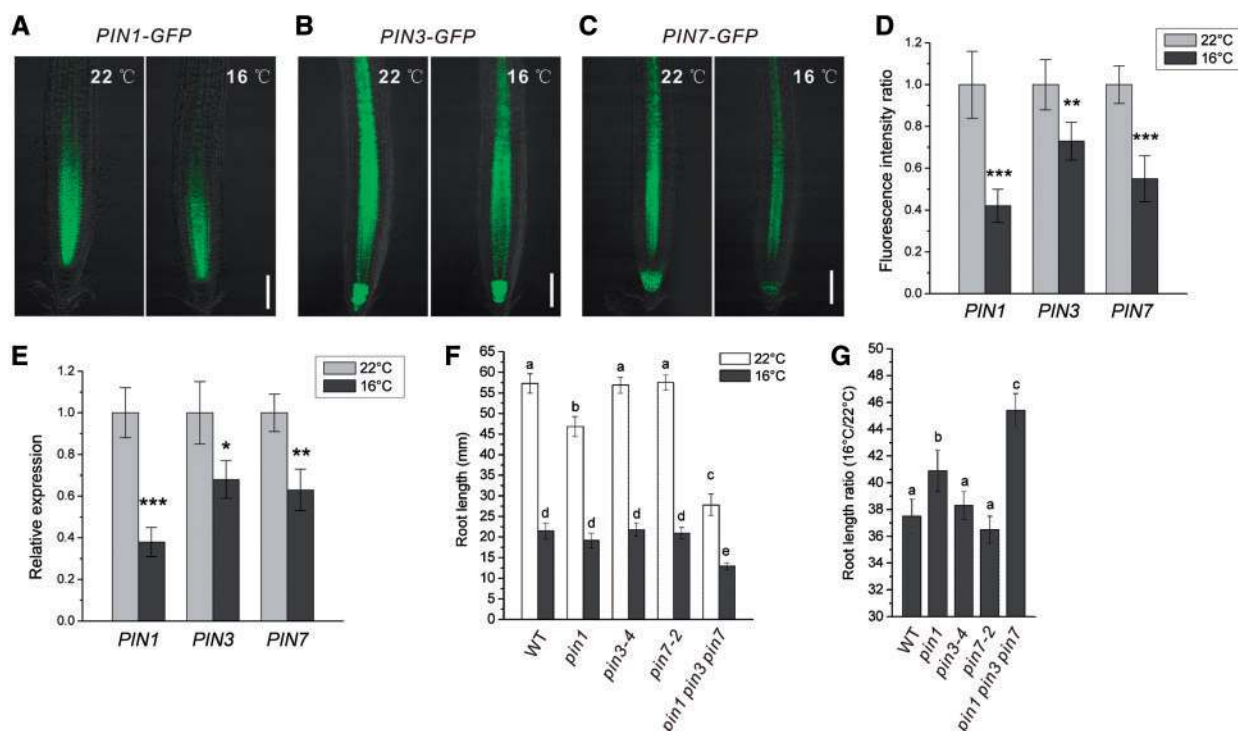


Fig. 4 Low temperature inhibits the expression of *PIN1/3/7*. Fluorescence images of (A) *PIN1::PIN1-GFP*, (B) *PIN3::PIN3-GFP* and (C) *PIN7::PIN7-GFP* root tips at 22°C (left) and 16°C (right). Scale bars = 50 μ m. (D) Quantified fluorescence intensities in (A–C). The fluorescence intensity of the indicated line grown at 22°C is set to 1. At least 12 seedlings were imaged per line for each of the three replicates. (E) Relative expression of *PIN* genes in wild-type plants grown at 22 or 16°C assayed by qPCR. The expression level of the indicated gene at 22°C is set to 1. (D, E) Error bars represent the SD, and the asterisks indicate significant differences at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test). (A–E) Seedlings were grown at 22°C for 3 d, and then transferred to 16°C or kept at 22°C for an additional 3 d. (F) Root length and (G) root length ratio of 10-day-old wild-type plants, *pin1*, *pin3-4*, *pin7-2* and *pin1 pin3 pin7* mutants. Seedlings were grown at 22°C for 3, and then transferred to 16°C or kept at 22°C for 7 d. The ratios indicate the relative root length at 16°C compared with that at 22°C (designated as 100%). Error bars represent the SD, and the differences between columns annotated with different letters are significant ($P < 0.05$, Student's *t*-test).

revealed by the ratio obtained through comparing the root length of corresponding lines grown at 16 and 22°C (Fig. 4F, G). Furthermore, the root growth of the triple mutant *pin1 pin3 pin7* was even less inhibited by low temperature than *pin1* (Fig. 4G).

ARR1 and ARR12 redundantly regulate root growth and meristem development under low temperature

Accelerated cell differentiation can also lead to the reduction of root meristem size and cell number (Dello iolo et al. 2007), and cytokinin promotes cell differentiation via the activity of ARR1 and ARR12 in the transition zone (Dello iolo et al. 2008, Ruzicka et al. 2009, Moubayidin et al. 2010). Thus, we assayed the root length and meristem cell number of *arr* mutants subjected to low temperature stress. The results showed that while the root length and meristem cell number of *arr1-5* and *arr12-1* single mutants were similar to those of wild-type plants, the double mutant *arr1-3 arr12-1* showed increased root length and meristem cell number at both 22 and 16°C (Fig. 5A, C). When we evaluated the sensitivity of root growth to low temperature by comparing the ratio of root length and meristem cell number of corresponding lines grown at 16 and 22°C, we found that while *arr1-5* and *arr12-1* displayed similar phenotypes to the wild type, *arr1-3 arr12-1* was less sensitive to low temperature

than wild-type seedlings (Fig. 5B, D). Further time-course analyses showed that *arr1-3 arr12-1* began to exhibit less sensitivity to low temperature in term of changes in root length at 6 d after germination and meristem cell number at 5 d after germination compared with wild-type plants (Supplementary Fig. S1). These results indicate that ARR1/12 play roles in low-temperature-mediated inhibition of root growth.

ARR1 and ARR12 regulate auxin accumulation under low temperature stress

Since our assays above showed that low temperature stress affects auxin accumulation, we tested whether ARR1/12 function in the regulation of this process. For this purpose, the *arr1-3 arr12-1* mutant was crossed with *IAA2::GUS* plants, and the resulting *arr1-3 arr12-1 IAA2::GUS* line was used to analyze the expression of *IAA2::GUS*. GUS staining and activity analysis showed that *arr1-3 arr12-1* was less sensitive to low temperature, in terms of *IAA2::GUS* expression, than were wild-type plants (Fig. 6A, B), suggesting that ARR1/12 is involved in the low-temperature-triggered reduction of auxin accumulation. This conclusion was further verified by direct measurement of endogenous IAA contents, using GC-MS. The IAA content in *arr1-3 arr12-1* roots was significantly higher than that in wild-type roots at 16°C (Fig. 6C).

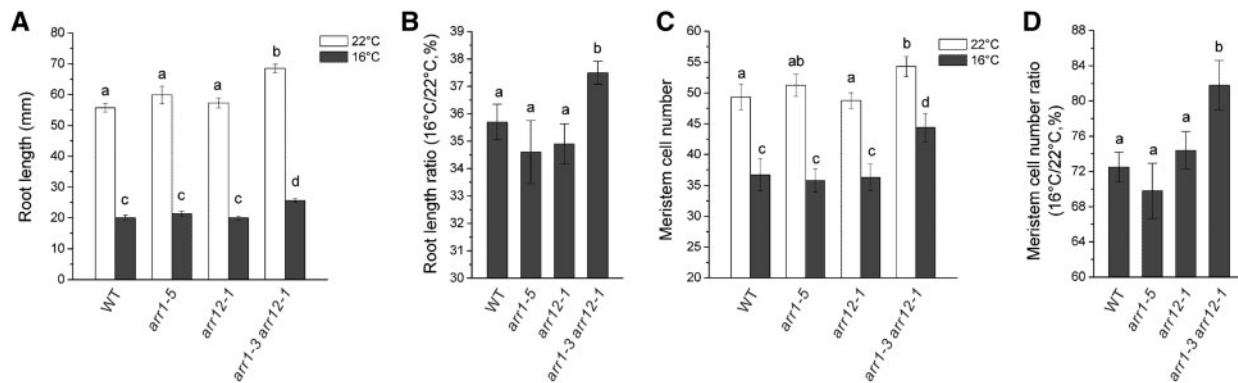


Fig. 5 ARR1 and ARR12 are required for the inhibition of root elongation and meristem cell number at low temperature. (A) Root length and (B) root length ratio of 10-day-old wild-type plants, and *arr1-5*, *arr12-1* and *arr1-3 arr12-1* mutants. Seedlings were grown at 22°C for 3 d, and then transferred to 16°C or kept at 22°C for 7 d. (C) Meristem cell number and (D) meristem cell number ratio of 6-day-old wild-type, *arr1-5*, *arr12-1* and *arr1-3 arr12-1* plants. Seedlings were grown at 22°C for 3 d, and then transferred to 16°C or kept at 22°C for an additional 3 d. The ratios indicate the relative root length or meristem cell number at 16°C compared with that at 22°C (designated as 100%). Error bars represent the SD, and the differences between columns annotated with different letters are significant ($P < 0.05$, Student's *t*-test).

The higher auxin accumulation in *arr1-3 arr12-1* at 16°C could result from the different responses of auxin biosynthesis and transport in *arr1-3 arr12-1* and wild-type plants to low temperature. Thus, we analyzed the expression of auxin biosynthesis-related genes in both *arr1-3 arr12-1* and wild-type roots subjected to low temperature treatment. However, the expression of all assayed genes in *arr1-3 arr12-1*, except for *ASA1* and *YUC2*, was similar to that in wild-type plants (Fig. 6D). Next, we examined the expression of *PIN* genes in *arr1-3 arr12-1* and wild-type roots at 22 and 16°C. While the repression of *PIN7* expression by low temperature was similar in *arr1-3 arr12-1* and wild-type roots, the expression of *PIN1* and *PIN3* was only reduced to $67.6 \pm 1.8\%$ and $92.7 \pm 10.5\%$, respectively, in *arr1-3 arr12-1* by low temperature, compared with $43.5 \pm 7.9\%$ and $68.1 \pm 8.8\%$ in wild-type roots (Fig. 6E), indicating that in the *arr1-3 arr12-1* mutant, low temperature caused less inhibition of *PIN1/3* expression. This difference in low-temperature-mediated repression of *PIN1/3* expression in the roots of the wild type and *arr1-3 arr12-1* may affect auxin accumulation in these two lines.

Discussion

Plants usually undergo stress-induced morphogenic responses, such as reductions in root elongation and leaf expansion, when subjected to low temperature (Rymen et al. 2007, Shibasaki et al. 2009). Previous reports also indicated that plants subjected to 4°C conditions showed a disturbance of auxin transport in the plant gravitropism response (Nadella et al. 2006, Shibasaki et al. 2009). However, little is known about the mechanisms underlying low-temperature-triggered inhibition of root development. In this study, our data suggest that low temperature inhibits root growth by reducing auxin accumulation, which may involve the regulation of both auxin transport and biosynthesis. In addition, ARR1 and ARR12 are shown to function in this process.

Many reports have demonstrated that normal plant development requires cell cycle-related genes, which are regulated by

various factors (Breyne et al. 2002, Vandepoele et al. 2002, Dhondt et al. 2010). In this study, a panel of cell cycle-related genes was analyzed for their expression in response to low temperature. Among them, low temperature repressed *CYCD1;1*, *CYCD4;2*, *CYCD6;1* and *E2Fb*, positive regulators of cell cycle progression. The decreased expression of these genes may be due to the reduction of auxin accumulation in root tips. Indeed, a previous report indicated that auxin induces *CYCD6;1* expression (Cruz-Ramirez et al. 2012). Low temperature also enhances the expression of *E2Fc* and *RBR*, negative regulators of cell proliferation. Similarly, maize leaves exposed to cold nights (25°C/4°C, day/night) also showed up-regulation of cell cycle inhibitors and down-regulation of positive regulators of cell division (Rymen et al. 2007). In contrast, a previous report showed that long-term exposure of Arabidopsis seedlings to 10°C up-regulates *CYCB1;1* mRNA levels (Lee et al. 2009). This may be due to the different temperatures and durations used to treat plants, and the different parts of plants sampled in the experiments.

Auxin accumulation mediated by auxin biosynthesis and auxin transport is important for the onset of cell division in root meristems (Trehin et al. 1998, Petrasek et al. 2002, Blilou et al. 2005). Auxin is synthesized through tryptophan-dependent and independent pathways, under the control of many endogenous signals and environmental factors (Mano and Nemoto 2012, Sun et al. 2012, Gao et al. 2014). Auxin flow towards the root tip is maintained by the action of basally localized *PIN1*, *PIN3* and *PIN7* in the cells of the stele (Blilou et al. 2005). Also, many factors, including endogenous signals and environmental stimuli, regulate root meristem development by controlling the expression of *PIN* genes and thereby altering auxin transport (Fernandez-Marcos et al. 2011, Xu et al. 2013, H.M. Yuan et al. 2013, T.T. Yuan et al. 2013). Our study revealed that not only the expression of auxin biosynthesis-related genes but also the expression of *PIN* genes decreased under low temperature, suggesting that attenuated auxin transport may function synergistically with the reduced auxin biosynthesis to block auxin accumulation in

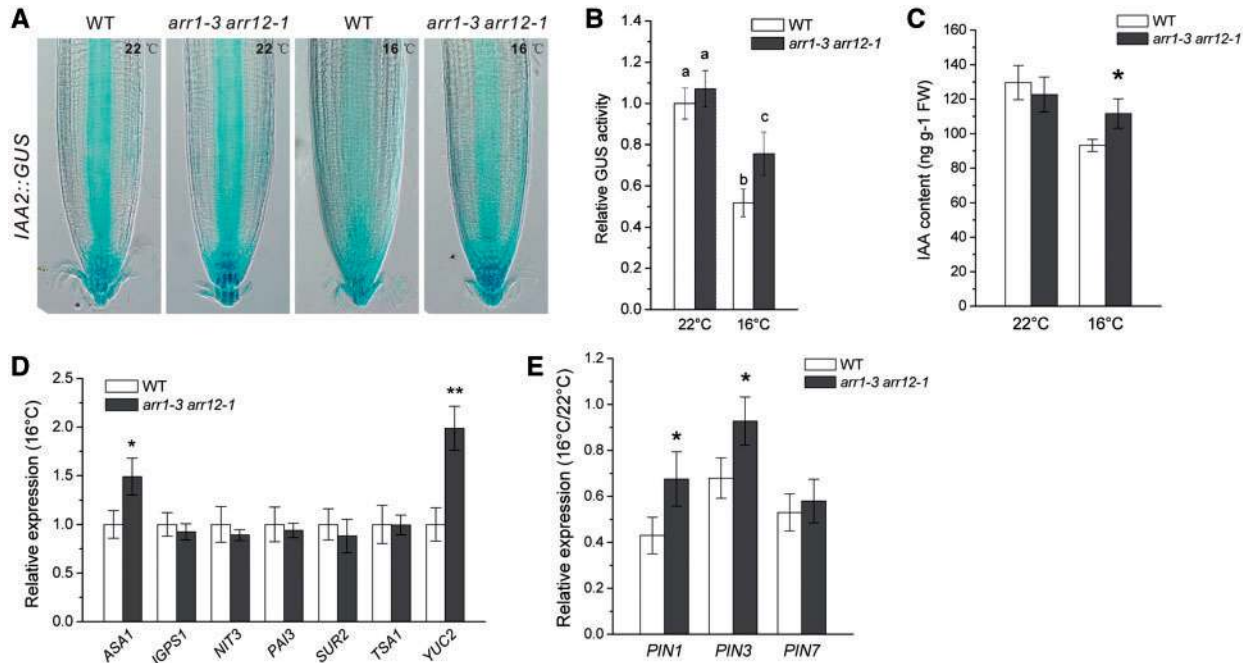


Fig. 6 ARR1 and ARR12 regulate the accumulation of auxin in root tips at low temperature. (A) Images of GUS-stained *IAA2::GUS* in 6-day-old wild-type or *arr1-3 arr12-1* root tips grown at 22 or 16°C. (B) Relative GUS activity in (A). The GUS activity in the wild-type background at 22°C is set to 1. Error bars represent the SD, and the differences between columns annotated with different letters are significant ($P < 0.05$, Student's *t*-test). (C) IAA contents of 6-day-old wild-type and *arr1-3 arr12-1* root tips grown at 22 or 16°C. Error bars represent the SD, and the asterisks indicate significant differences between wild-type and *arr1-3 arr12-1* plants grown at 16°C. (D) Relative expression of auxin biosynthesis genes in wild-type and *arr1-3 arr12-1* plants grown at 16°C assayed by qPCR. The expression level of the indicated gene in wild-type plants is set to 1. (E) Relative expression of *PIN* genes in the roots of wild-type and *arr1-3 arr12-1* seedlings assayed by qPCR. The ratios between the expression levels at 16°C and at 22°C in the wild type or *arr1-3 arr12-1* are shown. (D, E) Error bars represent the SD, and the asterisks indicate significant differences between wild-type and *arr1-3 arr12-1* plants at * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test). (A–E) Seedlings were grown at 22°C for 3 d, and then transferred to 16°C or kept at 22°C for an additional 3 d.

low-temperature-treated roots. We also noted that exogenous auxin did not completely recover the low-temperature-mediated inhibition of primary root growth, suggesting the involvement of other factor(s) in this process.

A previous study indicated that 4°C treatment represses the root gravity response which depends on the asymmetric basipetal auxin transport mediated by PIN2 and PIN3, through a post-transcriptional mechanism (Shibasaki et al. 2009). This study by Shibasaki et al. also shows that 4°C treatment inhibits root growth, but the mechanism underlying this cold-mediated inhibition of root growth has not been further explored. Our present study showed that low temperature (16°C) inhibited the expression of *PIN1/3/7* at the transcriptional level and verified the involvement of *PIN* genes in the regulation of root growth under low temperature by examining the root length of *pin1* and *pin1 pin3 pin7* mutants. Under normal temperature (22°C), the mutants of *PIN1/3/7* exhibit different degrees of defects in root meristem development (Blilou et al. 2005). The *pin1* mutant displays a reduction in root length and root meristem size, whereas *pin3* and *pin7* mutants do not. Moreover, the *pin1 pin3* and *pin1 pin7* double mutants show additive defects in root length and root meristem size compared with *pin1* (Blilou et al. 2005), suggesting that PIN1 plays a major role in mediating auxin flow towards the root tip, and PIN3/7 play additional roles. In our study, the roots of *pin1* and

pin1 pin3 pin7 showed less sensitivity to low temperature treatment, while *pin3-4* and *pin7-2* single mutants did not, suggesting the different level of contribution of PIN1, PIN3 and PIN7 to low temperature-mediated inhibition of root growth.

The interplay between auxin and cytokinin has a major role in meristem development (Dello loio et al. 2008). Cytokinin signaling depends on the core transcription factors ARR1 and ARR12, which redundantly control the cell differentiation rate by regulating *PIN1/3/7* expression, and thereby auxin transport (Dello loio et al. 2007, Dello loio et al. 2008, Moubayidin et al. 2010). Our study showed that ARR1 and ARR12 also affect low temperature-mediated inhibition of root growth by regulating the expression of *PIN1/3*. However, *arr1-3 arr12-1* mutant and wild-type plants subjected to low temperature show similar reductions of *PIN7* mRNA levels. These data suggest that ARR1/12 function in this process by modulating only *PIN1/3*. In addition, low temperature treatment decreased the expression of many auxin biosynthesis-related genes in wild-type plants, implying the possible involvement of auxin synthesis in low-temperature-mediated inhibition of root growth. However, most of these genes (except *ASA1* and *YUC2*) were not affected in *arr1-3 arr12-1* by low temperature. Based on these data, we could not exclude the possibility that these two genes, *ASA1* and *YUC2*, were involved in changes in the IAA level in *arr1-3 arr12-1* at 16°C, because Cheng et al. (2006)

reported that *YUC2* overexpression can increase the auxin level in transgenic lines. We also examined the root growth of another cytokinin signaling mutant *ahp1-1 ahp2-1 ahp3* (Hutchison et al. 2006). Similar to *arr1-3 arr12-1*, the *ahp1-1 ahp2-1 ahp3* mutant showed less sensitivity to low temperature in terms of changes in root growth compared with wild-type plants (Supplementary Fig. S2), further supporting the involvement of cytokinin signaling in the regulation of root growth under low temperature.

In summary, we showed that low temperature inhibits root growth partially through the ARR1/12-mediated reduction of auxin accumulation in roots. Our study advances knowledge of how root growth is regulated by the plant in response to low temperature, and may be helpful in understanding plant adaptations to low temperature environments.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type. The plant materials used in this study have been described previously: *DR5::GUS* (Ulmasov et al. 1997); *IAA2::GUS* (Luschnig et al. 1998); *PIN1::PIN1-GFP* (Friml et al. 2002a); *PIN3::PIN3-GFP* (Friml et al. 2002b); *PIN7::PIN7-GFP* (Friml et al. 2003); *SCR::H2B-YFP* (Heidstra et al. 2004); *QC25::GUS* (Sabatini et al. 2003); *CYCB1;1::GUS* (Colon-Carmona et al. 1999); and *pin1 pin3 pin7* (Blilou et al. 2005). Seeds of *arr1-5* (N664392), *arr12-1* (N6978) and *arr1-3 arr12-1* (N6981) were obtained from the European Arabidopsis Stock Centre. Seeds of *pin1* (SALK_047613), *pin3-4* (SALK_038609) and *pin7-2* (SALK_044687) were obtained from the Arabidopsis Biological Resource Center. The mutants and transgenic lines were confirmed by PCR. The Arabidopsis seeds were surface sterilized with 5% bleach for 5 min, washed three times with sterile water, and then plated on agar medium containing half-strength Murashige and Skoog (MS) medium supplemented with 1% agar and 1% sucrose, pH 5.8. Plants were stratified at 4°C for 3 d in the dark and then transferred to a phytotron. Seedlings were grown vertically at 22°C and 100 μmol m⁻² s⁻¹ illumination under 16 h light/8 h dark conditions for 3 d, and then transferred to 16°C or kept at 22°C for the indicated number of days.

Measurement of root length and meristem cell number

Three-day-old seedlings were treated with different temperatures (16 or 22°C) for the indicated number of days, and then their root tips were aligned. Digital images of seedlings were captured after 3 or 7 d. The seedlings were mounted with clearing solution (50 g of chloral hydrate, 15 ml of water and 10 ml of glycerol) on glass slides, and the root tips were examined using differential interference contrast (DIC) optics (Olympus BX64) and photographed using a charge-coupled device (CCD) camera (Olympus dp72). The number of root meristem cells was defined by counting the number of cells in a file extending from the initial cell adjacent to the QC to the first elongated cell in the cortex layer (Dello loio et al. 2007). Changes in root length were quantified using ImageJ software. Error bars were obtained based on the measurement of >30 seedlings per treatment. At least three replicates were performed for each treatment. The analysis of the *pin1* mutant phenotype was carried out according to the method described previously (H.M. Yuan et al. 2013).

GUS staining

GUS staining was carried out according to the method described previously (Hu et al. 2010). Briefly, the seedlings were incubated in GUS staining solution [50 mM sodium phosphate, pH 7.0, 0.2% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide and 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid), Sigma-Aldrich] at 37°C in the dark. GUS staining was performed for 1 h for *DR5::GUS*, 15 min for *IAA2::GUS*, 12 h for *CYCB1;1::GUS*

and 12 h for *QC25::GUS*. The root tips were mounted with clearing solution on glass slides, examined using a DIC microscope (Olympus BX64), and photographed with an Olympus dp72 CCD. Each treatment was performed in triplicate. The MUG assays were carried out according to the method described previously (T.T. Yuan et al. 2013). The experiments were repeated three times.

Quantitative real-time PCR

Three-day-old seedlings were treated with different temperatures (16 or 22°C) for 3 d. The root tips were collected for total RNA isolation using Trizol reagent (Invitrogen), according to the manufacturer's instructions. After treatment with RQ1 RNase-free DNase I (Promega), first-strand cDNA synthesis was carried out using Superscript II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. The qPCR analysis was performed on a Bio-Rad CFX96 apparatus using SYBR Green (Invitrogen). The PCR protocol comprised an initial 3 min incubation at 95°C for complete denaturation, followed by 40 cycles of denaturation for 20 s at 95°C, annealing for 20 s at 58°C and extension for 30 s at 72°C. *PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PDF2; AT1G13320)*, which is constitutively expressed in the tissues examined, was selected as an internal control (Hong et al. 2010). All experiments were performed in biological and technical triplicates. The genes analyzed and the corresponding specific primers are listed in Supplementary Table S1 or were described previously (Yan et al. 2013).

Confocal microscopy

An Olympus FV1000 ASW confocal scanning microscope was used. Three-day-old seedlings were treated with different temperatures (16 or 22°C) for 3 d, and then the root tips of GFP/YFP lines were mounted onto microscope slides for observation. At least 15 seedlings were analyzed per treatment. The signal intensity was measured using Photoshop CS5, and error bars were obtained based on measurements of >12 seedlings per treatment. The ratio of fluorescence intensity was obtained by comparing the fluorescence intensities of plants grown at 22 and 16°C. Each treatment was performed in triplicate.

Measurement of endogenous IAA

The amount of endogenous, free IAA was measured as described previously (Hong et al. 2014). Briefly, 0.1 g of root tips (FW) of plants grown at 22 and 16°C was sampled. Endogenous IAA was extracted, purified, methylated by a stream of diazomethane gas, resuspended in 100 μl of ethyl acetate, and then analyzed by GC-MS-SIMS (GC-MS-QP2010 Plus equipped with a HP-5MS column; Shimadzu). The experiments were repeated three times.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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