Light-dependent gravitropism and negative phototropism of inflorescence stems in a dominant *Aux/IAA* mutant of *Arabidopsis thaliana*, *axr2*

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Abstract Gravitropism and phototropism of the primary inflorescence stems were examined in a dominant *Aux/IAA* mutant of Arabidopsis, *axr2/iaa7*, which did not display either tropism in hypocotyls. *axr2-1* stems completely lacked gravitropism in the dark but slowly regained it in light condition. Though wild-type stems showed positive phototropism, *axr2* stems displayed negative phototropism with the same light fluence-response curve as the wild type (WT). Application of 1-naphthaleneacetic acid-

containing lanolin to the stem tips enhanced the positive phototropism of WT, and reduced the negative phototropism of *axr2*. Decapitation of stems caused a small negative phototropism in WT, but did not affect the negative phototropism of *axr2*. *pgp1 pgp19* double mutants showed no phototropism, while decapitated double mutants exhibited negative phototropism. Expression of auxin-responsive *IAA14/SLR*, *IAA19/MSG2* and *SAUR50* genes was reduced in *axr2* and *pgp1 pgp19* stems relative to that of WT. These suggest that the phototropic response of stem is proportional to the auxin supply from the shoot apex, and that negative phototropism may be a basal response to unilateral blue-light irradiation when the levels of auxin or auxin signaling are reduced to the minimal level in the primary stems. In contrast, all of these treatments reduced or did not affect gravitropism in wild-type or *axr2* stems. Tropic responses of the transgenic lines that expressed axr2-1 protein by the endodermis-specific promoter suggest that AXR2-dependent auxin response in the endodermis plays a more crucial role in gravitropism than in phototropism in stems but no significant roles in either tropism in hypocotyls.

Key words *Arabidopsis thaliana* - dominant *Aux/IAA* mutants - gravitropism - hypocotyl - inflorescence - phototropism

Introduction

Gravitropism and phototropism are fundamental growth responses that allow sessile organisms like plants to adapt to their physical environment, where the downward force of gravity is universal, but where light comes from more different directions. Due to gravitropism, in most cases shoots grow upward and roots grow downward, but light conditions often modulate the magnitude of gravitropism (Kim et al. 2011). Further, it is well known that auxin is involved in the bending response that is the final step in tropic responses (Band et al. 2012; Morita 2010; Sakai and Haga 2012). Thus, Arabidopsis mutants that harbor mutations in auxin transport or signal transduction steps often exhibit obvious defects in root gravitropism. Such mutants include, for example, single mutants for an auxin influx carrier, *auxin resistant 1* (*aux1*; Bennett et al. 1996), an efflux facilitator, *pin-formed 2* (*pin2*; Müller et al., 1998), and double mutants for auxin response factors (arf), *arf7 arf19* (Okushima et al. 2005) and *arf10 arf16* (Wang et al. 2005). A quadruple mutant of auxin F-box receptors, *transport inhibitor 1* (*tir1*) *auxin signaling F box protein 1* (*afb1*) *afb2 afb3* is also compromised in root gravitropism (Dharmasiri et al. 2005). As for auxin/indole-3-acetic acid (Aux/IAA) coreceptor proteins, root gravitropism is also affected by dominant mutations such as *auxin resistant 2* (*axr2*)/*iaa7* (Wilson et al. 1990), *axr3/iaa17* (Rouse et al. 1998), *short hypocotyl 2* (*shy2*)/*iaa3* (Tian and Reed 1999), and *solitary root* (*slr*)/*iaa14* (Fukaki et al. 2002). Among

these auxin-related mutants, only *axr2* shows obvious defects in gravitropism of inflorescence stems. Most agravitropic stem mutants so far identified have lesions in either the formation or movement of statolith (amyloplast), or formation of statolith-containing statocyte (endodermal cells) in inflorescence stems (Morita 2010). Thus, *axr2* appears to be a valuable mutant for studying the role of auxin in stem gravitropism. Since phototropism of Arabidopsis stems has only been examined in the wild type, and phototropin (*phot*; Kagawa et al. 2009) and phytochrome (*phy*; Kumar and Kiss 2006) mutants, in the study we also measured phototropism of *axr2* stems.

axr2 is a dwarf mutant, and has dark green, round, wrinkled leaves with shorter petioles (Wilson et al. 1990). It is agravitropic in the roots and hypocotyls (Timpte et al. 1992). Its inflorescence stems are also short and prostrate, suggesting an agravitropic nature (Wilson et al. 1990). *axr2* cells are shorter than wild-type cells in hypocotyls and stems, and it has been proposed that a reduction in auxin-mediated cell elongation is responsible for the gravitropic defect in roots, hypocotyls and stems (Timpte et al. 1992). However, only one study has examined the tropic responses of *axr2* stems in a quantitative manner (Li 2008), and this found that *axr2* stems did not respond at all to a change of gravity vector. Furthermore, *axr2* completely lacks circumnutation of inflorescence stems, as do other mutants that are defective in shoot gravitropism (Hatakeda et al. 2003). In contrast to the dominant mutant, *axr2-1*, a loss-of-function mutant of *AXR2*, *axr2-5*, appears very similar to the wild type at both seedling and adult

stages, although tropic responses have not been examined (Nagpal et al. 2000). This suggests that *AXR2* acts redundantly with other *Aux/IAA* genes.

Here, we examined phototropism of inflorescence stems as well as gravitropism in *axr2*, and found that defects in stem gravitropism were partially rescued in continuous white-light condition, and that *axr2* stems showed negative phototropism. Because negative phototropism of shoots has been observed only in oat coleoptiles which are exposed to unilateral blue light of a limited range of light fluence (Iino 1988, 2001), we have carried out initial characterization of phototropic responses of *axr2* stems.

It is well known that the endodermal cell layer is critical for tropic responses in shoots including hypocotyls and stems. It functions not only as a statocyte in gravitropism (Fukaki et al. 1998) but also as an important site for the formation of a lateral auxin gradient in gravitropism (Rakusova et al. 2011) and phototropism (Ding et al. 2011). Therefore, we made transgenic lines that harbor *GFP-axr2* fusion cDNA driven by the endodermis-specific promoter of the *SCARECROW* gene (*pSCR*) (Di Laurenzio et al. 1996; Wysocka-Diller et al. 2000). We found that *pSCR:GFP-axr2* phenocopied the agravitropism of *axr2* stems, but that it showed normal phototropism like the wild type. Furthermore, neither gravitropism nor phototropism were compromised in hypocotyls of the transgenic lines.

Materials and methods

Plant materials

Arabidopsis seeds, all of which were Columbia background, were stratified for about a week, sown and grown on agar plates that contained half-strength MS salts (Murashige and Skoog 1962), 1% (w/v) sucrose and 0.9%(w/v) agar for ~10 d under continuous white light, from three 40-W white fluorescent tubes (FL40SSW/37; NEC, Tokyo, Japan; ~70 μmole m^{-2} s^{-1}), at 23°C. The seedlings were then planted in a 1:1 (v/v) mixture of vermiculite and Metromix 350 (Scotts-Sierra, Marysville, OH, USA), and grown thereafter in continuous white-light from two 40-W white fluorescent tubes and one pink fluorescent tube (FL40SBR-A, NEC; ~70 μmole m^{-2} s^{-1}), at 23°C. Seeds of *phot1-5 phot2-1* double mutants were a gift of Prof. K. Shimazaki (Kyushu University), and those of *p-glycoprotein 1-101* (*pgp1-101*) and *pgp19-101* single mutants were a gift from Prof. T. Sakai (Niigata University). Seeds of the other mutants were obtained from the Arabidopsis Biological Resource Center (Ohio State University, USA).

Measurement of tropic responses

For the gravitropism assay, wild-type plants with a 8 - 12 cm-long primary stem were placed in a horizontal position, and their images were taken at 10 - 30 min interval for 24 h using a digital camera (Nikon D5000). Dim green light was used when necessary, and the temperature was maintained at 23°C. For the phototropism assay, plants were irradiated with unilateral blue light, from blue light-emitting diodes (λ_{max} =

470 ± 30 nm; Stick-B16, Tokyo Rikakikai, Tokyo), and their images were recorded as for the gravitropism assay. Lanolin paste containing 1-naphthaleneacetic acid (NAA) or 1-*N*-naphthylphthalamic acid (NPA) was prepared by mixing anhydrous lanolin (Wako) and 10 mM chemical solution dissolved in ethanol to give a final concentration of 0.5 mM. Growth direction of shoot tips was determined by ImageJ on collected images.

For a quantitative analysis of gravitropism, we compared the maximum bending rate of stems, because, due to circumnutation of the shoots, time-courses of the gravitropic responses in inflorescence stems were too variable for statistical analysis (Yang and Tepper 1996). To determine the maximum bending rate during gravitropism, images were recorded at 10-min interval. Growth angles of the shoot tip during the initial upward bending phase were fitted to a smoothed line with the use of principal curve analysis in R (http://www.r-project.org/). Bending rate was defined as a mean rate for a 30-min period calculated from the smoothed line, and the maximum bending rate was a maximum observed during the initial upward bending phase of the gravitropic response.

For measurements of tropism in hypocotyls, seedlings were grown along an agar surface of vertically-held agar plates in the dark for 2.5 d at 23°C, which were then turned 90° for gravitropism or exposed to unilateral blue light for 24 h for phototropism.

Quantitative reverse transcription- polymerase chain reaction analysis

Total RNA was prepared using a Plant Total RNA Extraction Miniprep

System (Viogene) and a one-cm-long section of the primary inflorescence stem, taken from 0.5 cm below the shoot apex for the wild type, and from just below the shoot apex for *axr2* and *pgp1 pgp19* mutants. Stem sections were also obtained from the primary stems ~ 5 h after decapitation. Total RNA was reverse transcribed with ReverTra Ace reverse-transcriptase (Toyobo) after treatment with RNase-free DNase (Promega). Quantitative real-time polymerase chain reaction (PCR) was performed with a LightCycler 480 II/96 (Roche) using THUNDERBIRD SYBR qPCR Mix (Toyobo). Gene-specific primer pairs used were 5'-CCGCTCTTTCTTTCCAAGC-3' and 5'-CCGGTACCATTGTCACACAC-3' for *ACTIN2*, 5'-GAGCATGGATGGTGTGCCTTAT-3' and 5'-TTCGCAGTTGTCACCATCTT-3' for *IAA19*, 5'-GATGTACACCAGCTACAAGGATC-3' and 5'-GAAATCTATCATCCCTTGTGCTCC-3' for *IAA14* (Jones et al. 2010), 5'-CTCTCAGGAACCCCAAAAACA-3' and 5'-CCCTAAGCTCGAGCATCTCT-3' for *SMALL AUXIU UP RNA 50* (*SAUR50*) (At4g34760), and 5'-ATGCCCGTTATCACCTACGA-3' and 5'-TCCCAGAGCTTGTGAGGAAC-3' for *GH3.5* (At4g27260). *ACTIN2* was used as a reference gene. The comparative $\Delta\Delta C_T$ method was used to evaluate the relative quantities of each amplified product in the samples

Microscopy

Longitudinal sections of stems were obtained by hand sectioning, and their confocal images were taken with a confocal laser scanning microscope

(LSM5 DUO, Leica).

Transgenic lines

The 2.5-kb upstream region of *SCR* start codon was amplified from the Columbia accession by PCR by using the primer pair, 5'-CACCGAGCTTTGCGCTGGTCCGGT-3' and 5'-GCTCTAGAGGAGATTGAAGGGTTGTTGGTCGTG-3', and subcloned into pENTR/D-TOPO (Invitrogen). The vector was digested with HindIII and XbaI, and the 2.4-kb fragment was ligated into HindIII-XbaI site of pGWB6 that contained the cauliflower mosaic virus *35S promoter-GFP* sequence upstream of the attR1 site (Nakagawa et al. 2008), replacing the *35S* promoter with the *SCR* promoter to generate a pGWB6-pSCR destination vector. The coding sequences of *IAA7* or dominantly mutated *iaa7/axr2-1* were amplified using the primer pair, 5'-CACCATGATCGGCCAACTTATGAA-3' and 5'-TCAAGATCTGTTCTTGCAGT-3', from cDNA of the wild-type or *axr2-1* mRNA, were cloned into pENTR/D-TOPO, and then inserted into pGWB6-pSCR by GATEWAY LR reaction (Invitrogen), resulting in *pSCR::GFP-AXR2* or *pSCR::GFP-axr2-1* vectors. The plasmid was introduced into *Agrobacterium tumefaciens* strain pGV3101 by electroporation, which was then used to inoculate wild-type plants by the flower dip method (Clough and Bent 1998). T_1 plants were screened on agar medium containing 20 to 50 μg ml^{-1} kanamycin. Phenotypes were examined in the T_3 plants that were homozygous with respect to the transgene.

Results

Gravitropism of inflorescence stems

The primary stems of *axr2-1* grew almost vertically at bolting in the light condition. The *axr2* plants were then placed horizontally in the dark to examine the gravitropic response of their primary stems. As Li (2008) reported, *axr2* stems showed no gravitropic response (Fig. 1a). Agravitropic mutants of the stem such as *shoot gravitropism 1* (*sgr1*)/*scr* and *sgr7*/*short root* (*shr*) have been shown to lose statocytes (endodermal cell layer) that contain statoliths (amyloplasts) (Fukaki et al. 1998). However, *axr2* had amyloplasts in the endodermal cell layer (Fig. S1), so this cannot account for the absence of gravitropism in *axr2* plants. Next, we measured stem gravitropism in continuous white-light conditions; here, *axr2* stems slowly recovered their gravitroic response and finally bent to an angle of about 45° from the vertical (Fig. 1b). Because *axr2* is a dwarf mutant, gravitropic responsiveness may be related to the ability of the stem to elongate, as has been suggested by Timpte et al. (1992). In fact, during the 18-h period of gravitropic examination, the elongation rate of the stem was significantly faster in the light (0.13 ± 0.08 mm h^{-1}, n = 15) than in the dark (0.047 ± 0.051 mm h^{-1}, n = 14; $P = 0.003$ in *t*-test). The gravitropic response of wild-type stems was also light-dependent (Fig. 1a and b); the maximum bending rate was significantly greater in the light (139 ± 36 degrees h^{-1}, n = 14) than in the dark (82 ± 22 degrees h^{-1}, n = 11; $P < 0.001$ in

t-test; Fig. S2).

We also determined stem gravitropism of three mutants, *endodermal-amyloplast less 1* (*eal1*), *sgr2* (SALK_098981), and *lazy1* of Arabidopsis. *eal1* is a weak allele of *shr* (Morita et al. 2007), and it does not contain amyloplasts in its endodermis (Fujihira et al. 2000). *sgr2* shows abnormal vacuolar morphology in the endodermis, resulting in impaired amyloplast movements (Kato et al. 2002). *lazy1* is likely to have defects at a gravitropic response step between gravity perception and polar auxin transport (Yoshihara et al. 2013). We found that the gravitropism of *eal1* and *sgr2* stems was not recovered by exposure to light (Fig. S3), and that *lazy1* defects were more severe in the light than in the dark (our unpublished data).

Phototropism of inflorescence stem

We next examined the phototropism of the *axr2* primary stem by unilaterally irradiating the plants with blue light for 24 h. Surprisingly *axr2* stems bent away from the blue light (Fig. 2a and d), showing negative phototropism. Wild-type stems displayed positive phototropism, in which growth angles were larger than those of *phot1-5 phot2-1* double mutants (Kinoshita et al. 2001) that had previously been shown to lack stem phototropism (Kagawa et al. 2009). Compared with the time-course of *phot1 phot2* double mutants, *axr2* appeared to develop phototropic curvature later than the wild type. The difference in curvature between *axr2* and *phot1 phot2* became apparent ~10 h after the onset of blue light

exposure, while the difference between the wild type and *phot* double mutant became apparent ~5 h after the onset of the light treatment.

The negative phototropism of *axr2* stems occurred at higher light fluence rate than did the positive phototropism of wild-type stems (Fig. 2b): the negative phototropism of *axr2* was first observed at a fluence rate of 5.7 μmole m^{-2} s^{-1}, while the positive phototropism of the wild type was first observed at 1/10 of that rate.

Effects of application of NAA and decapitation

Since the auxin response in *axr2* mutants is thought to be repressed by the dominantly mutated axr2 protein, we examined whether experimentally-induced changes of auxin level could rescue, or would worsen *axr2* defects in stem tropisms. We therefore applied lanolin paste containing a synthetic auxin, NAA, or an auxin transport inhibitor, NPA, to the tip of the primary stem. In some experiments, we also decapitated shoot tips and removed all lateral organs from the stem. Tropic responses were determined ~5 h after the treatments. These treatments generally affected gravitropism negatively although in all cases stem tips finally bent to the vertical. In the wild type, both NAA and NPA application reduced the maximum bending rate in the light condition, and had no effect in the dark (Fig. S2). Decapitation slowed the bending rate in both light and dark conditions. In the case of *axr2*, slow gravitropism in the light disappeared after NAA application, and this was reduced after decapitation (Fig. S4); no gravitropism was observed after any treatments in the dark.

On the other hand, application of NAA markedly promoted the positive phototropism of wild-type stems, and it diminished the negative phototropism of *axr2* stems (Fig. 3a). NPA application did not affect either the positive phototropism of the wild type or the negative phototropism of *axr2* (Fig. S5). Decapitation of the stem counteracted the positive phototropism of the wild type, resulting in a small negative phototropism but did not affect the *axr2* response (Fig. 3b). Decapitated *phot1 phot2* double mutants did not exhibit any phototropisms (Fig. 3b), indicating that negative phototropism was also caused by phototropins.

Since disruption of genes involved in auxin transport probably decreased polar auxin transport along stems, we examined stem phototropism of *pin1-1* (*pin1-1 transparent testa glabra 1* (*ttg1*)) mutants and *pgp1-101 pgp19-101* double mutants. PIN1 is an efflux facilitator of auxin, and the PGP proteins are ATP-binding cassette (ABC) transporters that transport auxin outwardly from cells (Geisler and Murphy 2006). *pin1* lacks lateral organs at the shoot meristem (Gälweiler et al.1998), and *pgp1 pgp19* is dwarf (Noh et al. 2001), like *axr2*. *pin1* stems showed no phototropism (Fig. S6), neither did *pgp1 pgp19* stems. Furthermore, the decapitated *pgp1 pgp19* stems exhibited negative phototropism (Fig. 4).

Gene expression of auxin-responsive genes in inflorescence stem

We determined the expression level of *IAA19*, *IAA14*, *SAUR50* and *GH3.5*/*WESO1* to determine the level of auxin signaling in the primary inflorescence stem (Fig. 5). These genes are known to be auxin responsive

at the seedling stage (Nemhauser et al. 2006). *SAUR50* and *GH3.5* may be induced by phototropic stimulus in etiolated hypocotyls of *Brassica oleracea* (Esmon et al. 2006), and *IAA19* is differentially induced in hypocotyls, with a higher level on the shaded side in phototropism (Saito et al. 2007). In *axr2* and *pgp1 pgp19,* expression of *IAA19*, *IAA14* and *SAUR50* was reduced more than two-fold. With a few exceptions, decapitation of the inflorescence stem reduced the expression level in each genotype. In contrast, expression of *GH3.5* was constant in the three genotypes, and was not affected by decapitation in *axr2*. These results suggest that auxin signaling in the primary stem was suppressed by these mutations, with a further decrease caused by decapitation.

Endodermis-specific expression of axr2-1 protein

We studied the phenotypes of transgenic plants that expressed the dominantly mutated axr2-1 protein driven by an endodermis-specific promoter, *pSCR*, to determine whether *axr2* expression in the endodermis caused defects in stem tropic responses. We made about a dozen transgenic lines, and images of representative lines are shown in Fig. 6a. Many of the lines exhibited a prostrate phenotype when inflorescences grew long (lines #3, #10 and #20), and one of them looked almost like the wild type (#7). Inflorescence stems of the prostrate lines did not respond to either a change of the gravity vector or to unilateral exposure to blue light. We examined the tropic responses of lines #3 and 10 more closely when they were young, when their inflorescences were ~4 cm long. Line #3 was one of

the most affected lines, and line #10 was a less compromised line. Gravitropic characteristics of the #3 stems were almost the same as those of *axr2*; there were no gravitropic responses in the dark, and only slow responses were observed in the light (Fig. 6d). The gravitropic defects in the #10 stems were less severe than in #3 and *axr2;* they did not respond to a change in the gravity vector in the dark, but they bent to the vertical ~10 h after turning 90° in the light (Fig. 6b). In contrast to the agravitropism, stem phototropism of both lines appeared normal, and they showed positive phototropism as found in the wild type (Fig. 6c and e). Furthermore, most of the transgenic lines did not look like *axr2*. Although *axr2* is a dwarf mutant, the internodes of the transgenic lines were not as stunted as those of *axr2*, and their leaves were relatively normal (see #20, Fig. 6a).

We also examined the GFP signal in one of the most affected lines, #3 (Fig. 6f and g). At lower magnification, the GFP signal appeared as a row, which was probably from an endodermal cell layer (Fig. 6f). At higher magnification, the signal was almost entirely restricted to the cell nucleus (Fig. 6g), where Aux/IAA proteins are thought to be located. We found no GFP signal in the least affected line (#7; Fig. 6a) (data not shown).

Finally we determined tropic responses of 2.5-d-old etiolated hypocotyls in #3 and #10 lines of *pSCR:GFP-axr2-1* (Fig. 7). Although *axr2* hypocotyls lost both gravitropic and phototropic responses (see below), hypocotyls of the two transgenic lines exhibited similar responses to the wild type in both gravitropism (Fig. 7a) and phototropism (Fig. 7b), despite the fact that the GFP signal was observed in nuclei of cells which, in both

hypocotyls and roots, were likely to be endodermis cells (Fig. 7c). In fact, hypocotyls of #10 line showed essentially the same responses as those of the wild type. Gravitropism of #3 hypocotyls was not significantly different from that of the wild type ($P > 0.085$ by *t*-test), and their phototropic responses were significantly different from those of the wild type at only three points in time, around t = 8 h ($0.023 < P < 0.043$ by *t*-test). Furthermore, elongation of hypocotyls of *pSCR:GFP-axr2-1* was not significantly compromised, though *axr2* hypocotyls were only about half as long as those of the wild type (Fig. 7d).

The 2.5-d-old *axr2* hypocotyls did not respond at all to either gravity or to unilateral blue-light stimuli (Fig. 7a and b). However, *axr2* hypocotyls were short (Fig. 7d), and did not elongate further in the dark at the time of measurement, and so in order to determine tropic responses of *axr2* hypocotyls, it was necessary to use hypocotyls at a younger stage. Therefore, we next measured the gravitropic and phototropic growth orientation of hypocotyls 2.5 d after germination (Fig. 7e and f). After induction of germination by white-light irradiation for 8 h, seeds on vertically-oriented agar plates were kept in the dark for 2.5 d for measurement of gravitropic growth orientation, or under unilateral blue-light irradiation for measurement of phototropic growth orientation. In the dark, wild-type hypocotyls grew almost directly upwards (0^{o}), while *axr2* hypocotyls grew randomly (Fig. 7e). The distribution of growth angles of *axr2* hypocotyls was not significantly different from a random uniform distribution ($P = 0.350$ by Kolmogorov-Smirnov test), indicating that *axr2*

hypocotyls completely lacked gravitropism. When wild-type seedlings were continuously exposed to unilateral blue-light irradiation for 2.5 d just after germination, their hypocotyls grew almost directly towards the blue light source (~90°), whereas the growth direction of *axr2* hypocotyls was almost random(Fig. 7f). The distribution of the growth angle of *axr2* hypocotyls under blue light was not significantly changed from that observed in the dark ($P = 0.262$ by Kolmogorov-Smirnov test), indicating that *axr2* hypocotyls had also lost phototropism.

Discussion

Gravitropism is usually examined in the dark to avoid interference with phototropism. Here we determined gravitropism in light conditions, where plants were irradiated with white light mainly from above, and found that gravitropism of inflorescence stems in both the wild type and *axr2* was promoted in the light (Fig. 1). In the case of *axr2*, no stem gravitropism was observed in the dark, as was reported by Li (2008). The light-induced gravitropism of *axr2* stems may be the reason why the primary stems of *axr2* grow vertically just after bolting. The promotive effects of light on gravitropism seem to be instantaneous in the wild-type stems because gravitropism was determined with plants that had been grown in the light just before measurements, and because the gravitropic response of the wild type was completed within 3.5 h (Fig. 1a). Stem gravitropism has been examined in four phytochrome mutants, *phyA*, *phyB*, *phyD* and *phyE*

(Landsberg background) in the dark (Kumar and Kiss 2006). *phyA* and *phyD* exhibit a faster response and the other mutants show a wild-type response, indicating that phyA and phyD are negative regulators of stem gravitropism. On the other hand, blue light perceived by phototropins has been shown to promote auxin signaling via PHYTOCHROME-INTERACTING FACTOR 4/5 and Aux/IAA proteins in hypocotyls (Sun et al. 2013). Obviously further study will be needed to disclose the molecular mechanism of light-induced promotion of stem gravitropism found in the present study.

It is also known that gravitropism of hypocotyls is suppressed by light perceived by phytochromes (Kim et al. 2011) and cryptochromes (Ohgishi et al. 2004). The phytochrome-dependent suppression of gravitropism is caused by loss of amyloplasts in the endodermis of hypocotyls in the light (Kim et al. 2011). On the other hand, we also found that defects in stem gravitropism were not rescued in the light in the case of *eal1* or *sgr2*, in which amyloplasts were not present (Fujihira et al. 2000) or did not move freely in the endodermis (Kato et al. 2002). These results suggest that the light dependence of stem gravitropism is related to amyloplast development or amyloplast function, as is the case in hypocotyls.

In response to continuous irradiation with unilateral blue light, *axr2* primary stems showed negative phototropism while wild-type stems showed positive phototropism (Fig. 2). Kagawa et al. (2009) examined phototropism of the wild-type stems and reported that the wild type showed positive phototropism with a biphasic dose response: phototropism of the

first phase occurred at a fluence rate of as little as 0.02 μmole m^{-2} s^{-1}, and its response was small, while phototropism of the second phase occurred at a higher fluence rate and its response was larger. The dose response of the wild type in our study (Fig. 2b) appears to correspond to the second-phase response, although the experimental conditions were very different between the two studies. In particular, the age of the plants was different: the primary stems were about 8 to 10 cm long in this study, while those of Kagawa et al. (2009) were about one cm long.

Inflorescences of *axr2* showed negative phototropism. Negative phototropism has been reported only when oat coleoptiles are exposed to unilateral pulse irradiation with blue light, of which the light fluence is between 8 and 100 μmoles m^{-2}. Blue light of lower and higher light fluences causes first positive and second positive phototropisms, respectively, and no negative phototropisms are seen except for oat (Iino, 1988, 2001). In fact, instead of negative phototropism, blue light of similar fluences brings about almost no curvature in hypocotyls of Arabidopsis (Steinitz and Poff 1986). Negative phototropic curvature develops later than positive phototropic curvature, suggesting that positive phototropism and negative phototropism are mediated by distinct photosystems (Iino 1988). Interestingly, negative phototropism of *axr2* stems in this study also appears later than positive phototropism of the wild-type stems (Fig. 2a) although continuous light-induced phototropism examined in this study cannot be compared directly with pulse-induced phototropism (Haga and Sakai 2012; Iino 2001).

In *axr2* mutants, auxin signaling is thought to be suppressed because of a dominant mutation in an auxin coreceptor protein, axr2/iaa7. Furthermore, synthesis and transport of auxin may be reduced due to multiple regulatory loops of auxin homeostasis (Benjamin and Scheres 2008). Levels of auxin signaling may be changed in stems by exogenous application of auxin at the shoot tips, or removal of shoot tips, which are thought to be the main source of auxin. Defects in polar auxin transport may also reduce auxin signaling. In order to test the above hypothesis, levels of auxin signaling were estimated by measuring the expression of auxin-responsive genes in both intact and decapitated stems of *axr2* and *pgp1 pgp19* mutants, using quantitative real-time reverse transcription-PCR (Fig. 5). Genes which were analysed included two from a family of auxin coreceptor genes, *IAA14* and *IAA19*, one from a family of *SAUR* genes, *SAUR50*, and one from a family of *GH3* genes, *GH3.5*. Except for *GH3.5*, the expression of these genes was reduced by *axr2* or *pgp1 pgp19* mutations, and in each genotype it was also generally reduced by decapitation. These results appear consistent with the above hypothesis. Although *GH3.5* is known to be auxin responsive in a seedling stage (Nemhauser et al. 2006), in two-week-old plants it is also inducible by salicylic acid, abscisic acid or various biotic and abiotic stresses (Park et al. 2007). Therefore, *GH3.5* does not seem to be a good marker gene for auxin signaling in the stem tissue.

We found that the positive phototropism of the wild-type stems was promoted after application of auxin, and that decapitation changed the

direction of phototropism from a positive to a negative direction. The *pin1* mutants and the *pgp1 pgp19* double mutants showed no phototropic responses in their stems, and the latter double mutants showed negative phototropism when decapitated (Fig. 4). Taken together, these results suggest that inflorescence stems show negative phototropism when the level of auxin signaling is reduced to a minimal level, and that negative phototropism may be a basic response of stems to unilateral blue light. In contrast to the phototropism of inflorescences, the phototropism of hypocotyls is promoted in *pgp1 pgp19* (Noh et al. 2003; Nagashima et al. 2008). The auxin level in the middle portion of *pgp19* hypocotyls is reported to be reduced to ~1/3 that of the wild-type level (Christie et al. 2011). Phototropism of *pgp19* (alias *mdr1*) stems has been also examined: they show a greater magnitude of positive curvature than those of the wild type (Kumar et al., 2011).

Although application of auxin and decapitation have opposite effects on phototropism, both treatments simply diminish the magnitude of response in gravitropism. This suggests that the endogenous level of auxin is limiting to phototropism but optimal for gravitropism, so that both application of NAA to increase the auxin level and decapitation to decrease it reduce the gravitropic bending rate. In pea epicotyls (Britz and Galston 1983) and maize coleoptiles (Iino 1995), application of auxin and decapitation had a greater effect on the phototropic responses than on the gravitropic response.

We evaluated a role of the endodermis in gravitropism and

phototropism of inflorescence stems by examining transgenic plants that expressed axr2 protein by endodermis-specific *SCR* promoter. Based on *AXR2* promoter-*β-GLUCURONIDASE* (*GUS*) signals, *AXR2* is expressed all over in the shoot and in the root apical meristems in the seedling stage (Tian et al. 2002). Microarray analyses show that *AXR2* is ranked at the 91st percentile for expression intensity in inflorescence stems (Hanada et al. 2013). *AXR2* is also ranked at the 89th percentile when RNA is prepared from endodermal and quiescent center cells of roots, and ranked at the 88th percentile when RNA is isolated from whole root cells (Iyer-Pascuzzi et al. 2011). Taken together, *AXR2* is likely to be expressed in the stem endodermis. Our finding that expression of axr2 in the endodermis causes agravitropism in stems, but that it does not affect phototropism (Fig. 6b - e) confirms the previous conclusion that the endodermis has a crucial role only in gravitropism in inflorescence stems (Fukaki et al. 1996, 1998). Expression of axr2 protein in other cell layers of the stem may be necessary to mimic phototropic defects of *axr2*. We previously made transgenic lines, where axr2 protein was driven by the promoter of another *Aux/IAA* gene, *MSG2/IAA19*. These lines looked more like *msg2* mutants than *axr2* mutants (Muto et al. 2007). These results together with the present results suggest that the expression pattern of *AXR2* is a major determinant of the *axr2* phenotype.

In contrast to inflorescence stems, hypocotyls of *pSCR:GFP-axr2-1* were normal with respect to elongation, gravitropism and phototropism (Fig. 7a, b and d), although GFP fluorescence was likely to be observed in

endodermal cells (Fig. 7c). This clearly indicates that expression of *axr2* in the endodermis is not sufficient to affect these growth responses in hypocotyls, and that molecular mechanisms of these responses are different between inflorescence stems and hypocotyls. In fact, a few mutants show different gravitropic responses between hypocotyls and stems: In *pgp19* the gravitropic response is reduced in stems, but it is normal in hypocotyls (Kumar et al. 2011), while malfunction of a syntaxin, SGR3/ARABIDOPSIS VACUOLAR MORPHOLOGY 3 (AtVAM3), in the endodermis causes reduced gravitropism in stems, but does not affect gravitropism in hypocotyls (Yano et al. 2003).

Recently another dominantly-mutated Aux/IAA protein, bodenlos (bdl)/iaa12, was expressed by the use of a *SCR* promoter (Roychoudhry et al. 2013). *pSCR:bdl* plants look like the wild type except for a larger gravitropic setpoint angle of lateral shoots: their lateral branches grow to a more horizontal direction than those of the wild type though growth angle of the primary stems is not affected. The less compromised phenotype of *pSCR:bdl* compared to *pSCR:GFP-axr2* in the present study may be due to a larger dissociation constant between auxin and the BDL-TIR1 complex (270 nM) than that between auxin and the AXR2-TIR1 complex (17 nM) (Calderon Villalobos et al. 2012).

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Figure legends

Fig. 1 Gravitropic responses of the *axr2-1* inflorescence stems. **a, b** Time-course of gravitropic response of wild-type (circles) and *axr2-1* (triangles) stems in the dark (a) and under white-light conditions (b). Two independent measurements are shown for each genotype. **c, d** Representative images of *axr2* before (left panels) and 18 h after (right panels) application of gravitational stimuli in the dark (c) and under white-light (d) conditions. Bar = 2 cm.

Fig. 2 Phototropic responses of the *axr2-1* inflorescence stems. **a** Time-course of phototropic response of wild-type (circles), *axr2* (triangles), and *phot1-5 phot2-1* double mutants (grey circles). Growth angle of 0^{o} indicates vertically upward growth, and positive and negative values mean positive and negative phototropism, respectively. Plants were irradiated with blue light of 57 μmole m^{-2} s^{-1}. Mean and SD of 3 measurements are shown. **b** Dose-response of phototropism of wild-type (circles) and*axr2* (triangles) stems. Each point indicates a single measurement observed after blue-light irradiation for 24 h. Growth angles before light exposure were -7.8 ± 11.4^{o} (n = 20) and 1.5 ± 17.5^{o} (n = 18) for the wild type and *axr2*, respectively. c and d Representative images of the wild type (left) and *axr2* (right) before (c) and after (d) unilateral irradiation with blue light (57 μmole m^{-2} s^{-1}) for 24 h from the left side. Bar = 2 cm.

Fig. 3　Effects of application of NAA and decapitation on phototropism of inflorescence stems of the wild type and *axr2-1*. **a**　NAA application. Data of the wild type (circles) and *axr2* (triangles) are shown with those of mock treatment (grey symbols). **b**　Data of the decapitated wild type (circles) and *axr2* (triangles) are show with those of decapitated *phot1-5 phot2-1* double mutants (grey circles). Unilateral irradiation with blue light (57 μmole m^{-2} s^{-1}) was started ~5 h after application of lanolin paste containing 0.5 mM NAA to apical 1-cm-long region of stem, or after decapitation and removal of all the lateral organs, and the time course of the phototropic response was determined for 24 h thereafter. Each point represents mean and SD of 3 - 4 independent measurements. For more details, see the legend to Fig. 2.

Fig. 4　Phototropic responses of the inflorescence stems of the *pgp1-101 pgp19-101* double mutants. **a**　Time-course of phototropic response of intact (circles) and decapitated (triangles) stems induced by unilateral irradiation with blue light (57 μmole m^{-2} s^{-1}). Each point represents mean and SD of 4 measurements. **b, c**　Representative images before (b) and 24 h after (c) the light exposure (57 μmolem^{-2} s^{-1}) from the left side. Circles and triangles indicate intact and decapitated primary stems, respectively. Bar = 2 cm.

Fig. 5　Expression of auxin-responsive genes in the primary stem of *axr2-1* and *pgp1-101 pgp19-109*. One-cm-long sections of the primary stems were

obtained from the intact stems or stems which had been decapitated for ~ 5 h before sectioning. Quantitative real-time RT-PCR was employed for measurements of expression of *IAA19*, *IAA14*, *SAUR50* and *GH3.5*, and their expression is shown relative to that of the wild type. Data are reported as means ± SD of three independent samples. Student *t*-test was conducted between the intact stem samples of the wild type and each mutant, or between the intact and decapitated stem samples in each genotype. + and ‡ denote $P < 0.05$ and $0.05 < P < 0.1$, respectively.

Fig. 6 Tropic responses of inflorescence stems of a few transgenic lines harboring *pSCR:GFP-axr2-1*. **a** Approximately 6 -week-old plants of T_3 generation which were homozygous with respect to the transgene; the size of pot is 5.5 cm square. **b, d** Gravitropic responses of #10 (b) and #3 (d) stems in the dark (triangles) and light (circles) conditions. **c, e** Phototropic responses of #10 (c) and #3 (e) stems (for conditions, see legend to Fig. 2a). Data represent mean and SD of 3 - 5 measurements (**b – e**). **f, g** Confocal images of the longitudinal sections of #3 stems with a lower (f) and a higher (g) magnification. Images of the green and red channel, and a merged image of the two colors and bright-field image are shown from left to right. Brightness and contrast were adjusted in merged images to more clearly show the structure of stems. ep, epidermis. Bar = 50 μm in (**f**) and 10 μm in (**g**).

Fig. 7 Tropic responses of etiolated hypocotyls of transgenic lines

harboring *pSCR:GFP-axr2-1*. **a, b** Time-course of gravitropic (a) and phototropic (b) responses of hypocotyls of the wild type (open circles), *axr2-1* (closed circles), and #10 (open triangles) and #3 (closed triangles) lines of *pSCR:GFP-axr2-1*. The 2.5-d-old etiolated seedlings were subjected to a change of the gravity vector of 90° (a) or to unilateral irradiation with blue light (5.7 μmole m^2 s^{-1}) (b). Data represent mean and SD of 6 - 10 hypocotyls. Because the growth angle of *axr2-1* hypocotyls was almost random, two hypocotyls which were growing almost upward at time 0 were chosen for measurements. In b, mean values are shown with a 20-min interval, but SD's are shown in every 3 time points to avoid graphical overlapping. **c** Merged images of green fluorescent and bright-field images. The 2.5-d-old etiolated hypocotyl (top) and root (bottom) of #3 line of *pSCR:GFP-axr2-1* were examined. Bar = 100 μm. **d** Hypocotyl length of 2.5-d-old etiolated seedlings. Data represent mean and the standard error of the mean of 4 measurements, in which 6 - 11 hypocotyls were examined. **e, f** Gravitropic (e) and phototropic (f) growth orientation of *axr2-1* hypocotyls. After induction of germination by white-light irradiation for 8 h, wild-type (solid bars) and *axr2-1* (open bars) seeds were grown on vertically-oriented agar plates for 2.5 d in the dark (e) or under unilateral irradiation with blue light (0.57 μmole m^2 s^{-1}) (f), and the growth angle of hypocotyls was determined thereafter. The 0° corresponds to vertically upward growth, and 90° corresponds to the direction of unilateral blue-light irradiation. Growth angle was determined in 47 - 60 hypocotyls and 67 - 104 hypocotyls for the wild type and *axr2-1*, respectively.

Fig. S1　Longitudinal sections of the primary stem of the wild type (left) and *axr2-1* (right) stained for amyloplasts.　Stem segments about 1-cm-long were fixed with 3% paraformaldehyde in phosphate-buffered saline overnight, and embedded in 3% agarose.　About 60-μm-thick longitudinal sections were prepared with a vibrating blade microtome (VT1200S, Leica), and stained with 5% I_2-KI solution for a few min.　en, endodermis.

Fig. S2　Effects of decapitation (a) or application of NAA or NPA (b) on maximum bending rate of gravitropism in wild-type inflorescence stems in the dark or under light conditions.　Plants were placed in a horizontal position ~5 h after decapitation or after application of lanolin paste containing 0.5 mM NAA or NPA to apical 1-cm-long portions of stem, and the time course of the gravitropic response determined for 18 h thereafter in the dark or under light conditions.　Each data represents mean and SD of 3 - 14 measurements.

Fig. S3　Time-course of the gravitropic response of *eal1* and *sgr2* (SALK_098981) inflorescence stems under different light conditions.　a *eal1* was placed in the dark (closed symbols) and white-light conditions (open symbols) after changing the position of plants by ~90°.　**b**　*sgr2* was examined in the same way as in **a**.　Two independent measurements are shown for each genotype (circles and triangles).

Fig. S4　Effects of application of NAA or decapitation on gravitropism of inflorescence stems of *axr2-1* in white-light conditions.　a　Gravitropic response was determined with 0.5 mM NAA (triangles) or mock treatment (circles).　b　Gravitropic response was determined after decapitation and removal of all the lateral organs (triangles).　Circles show response of intact inflorescences.　Each point represents mean and SD of 3 - 14 measurements.　For more details, see the legend to Fig. 3.

Fig. S5　Effects of application of NPA on phototropism of inflorescence stems of the wild type (circles) and *axr2-1* (triangles).　Lanolin paste containing 0.5 mM NPA was applied to stems.　Each point represents mean and SD of 7 measurements.　Data of the wild type (grey circles) and *axr2* (grey triangles) with mock treatment are the same as those in Fig. 3a.　For more details, see legend to Fig. 3.

Fig. S6　Phototropic responses of inflorescence stems of *pin1-1* induced by unilateral irradiation with blue light (57 μmole m^{-2} s^{-1}).　Each point represents mean and SD of 4 measurements.

Fig. 1

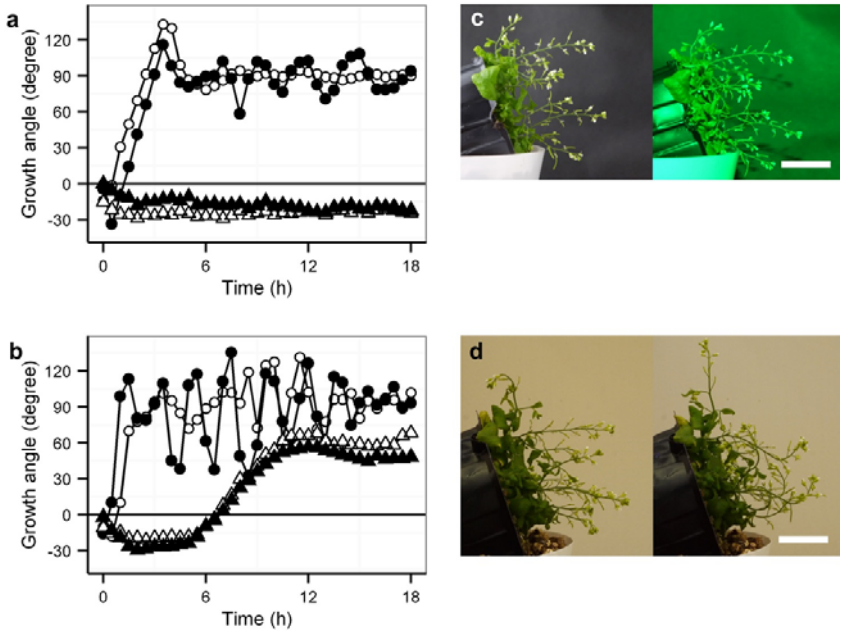
a
Growth angle (degree)
120
90
60
30
0
-30
0
6
12
18
Time (h)
b
Growth angle (degree)
120
90
60
30
0
-30
0
6
12
18
Time (h)
c
d

Fig. 2

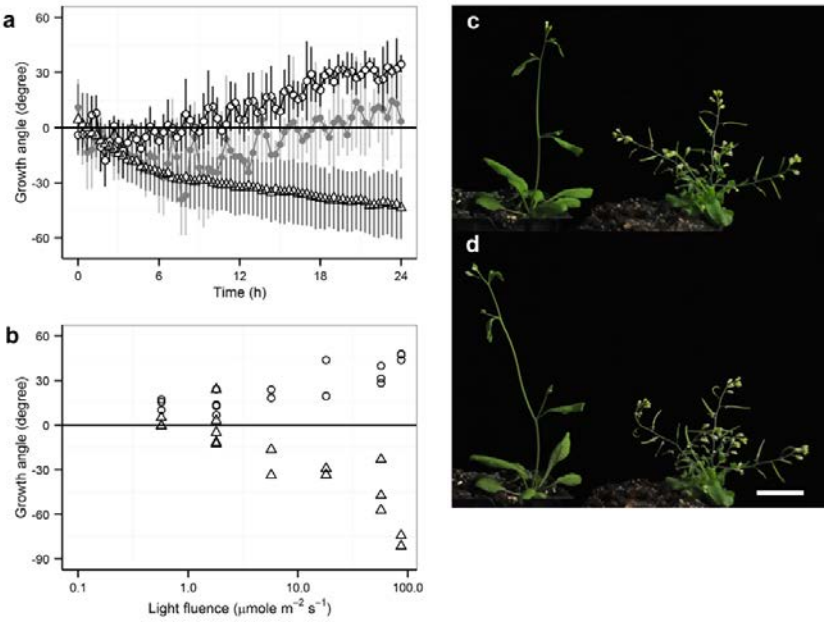
a
Growth angle (degree)
60
30
0
-30
-60
0
6
12
18
24
Time (h)
b
Growth angle (degree)
60
30
0
-30
-60
-90
0.1
1.0
10.0
100.0
Light fluence (μmole m⁻² s⁻¹)
c
d

Fig. 3

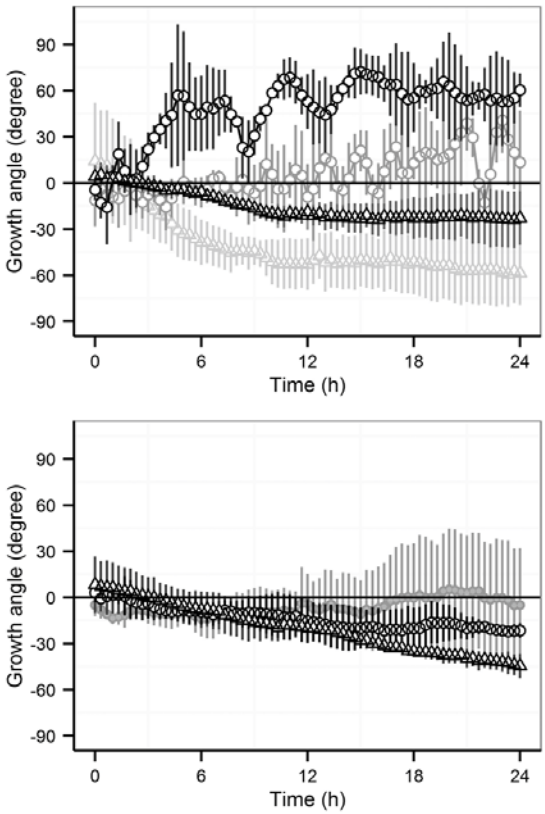
Growth angle (degree)
90
60
30
0
-30
-60
-90
0
6
12
18
24
Time (h)
Growth angle (degree)
90
60
30
0
-30
-60
-90
0
6
12
18
24
Time (h)

Fig. 4

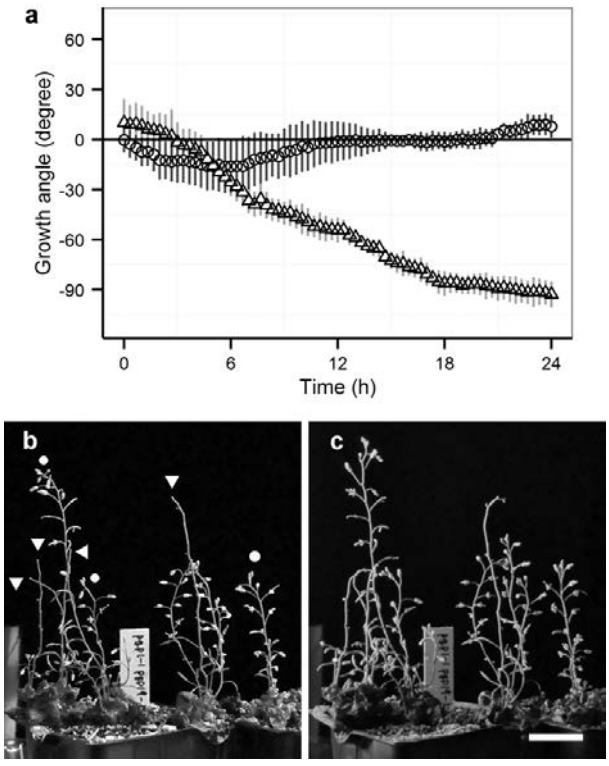
a
Growth angle (degree)
60
30
0
-30
-60
-90
0
6
12
18
24
Time (h)
b
c

Fig. 5

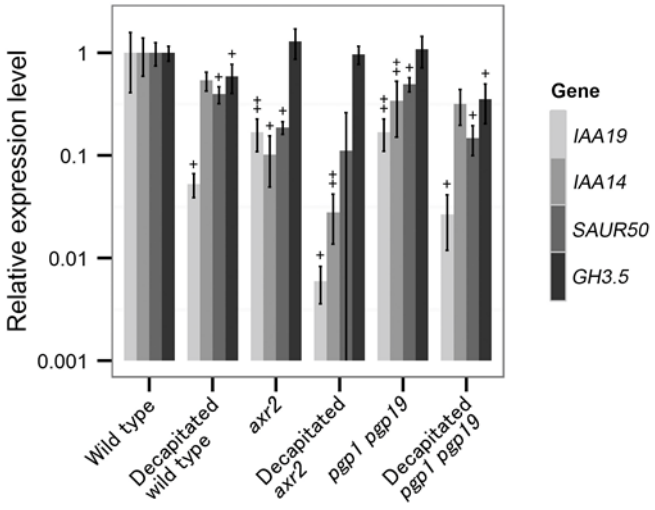
Relative expression level
1
0.1
0.01
0.001
Wild type
Decapitated wild type
axr2
Decapitated axr2
pgp1 pgp19
Decapitated pgp1 pgp19
Gene
IAA19
IAA14
SAUR50
GH3.5

Fig. 6

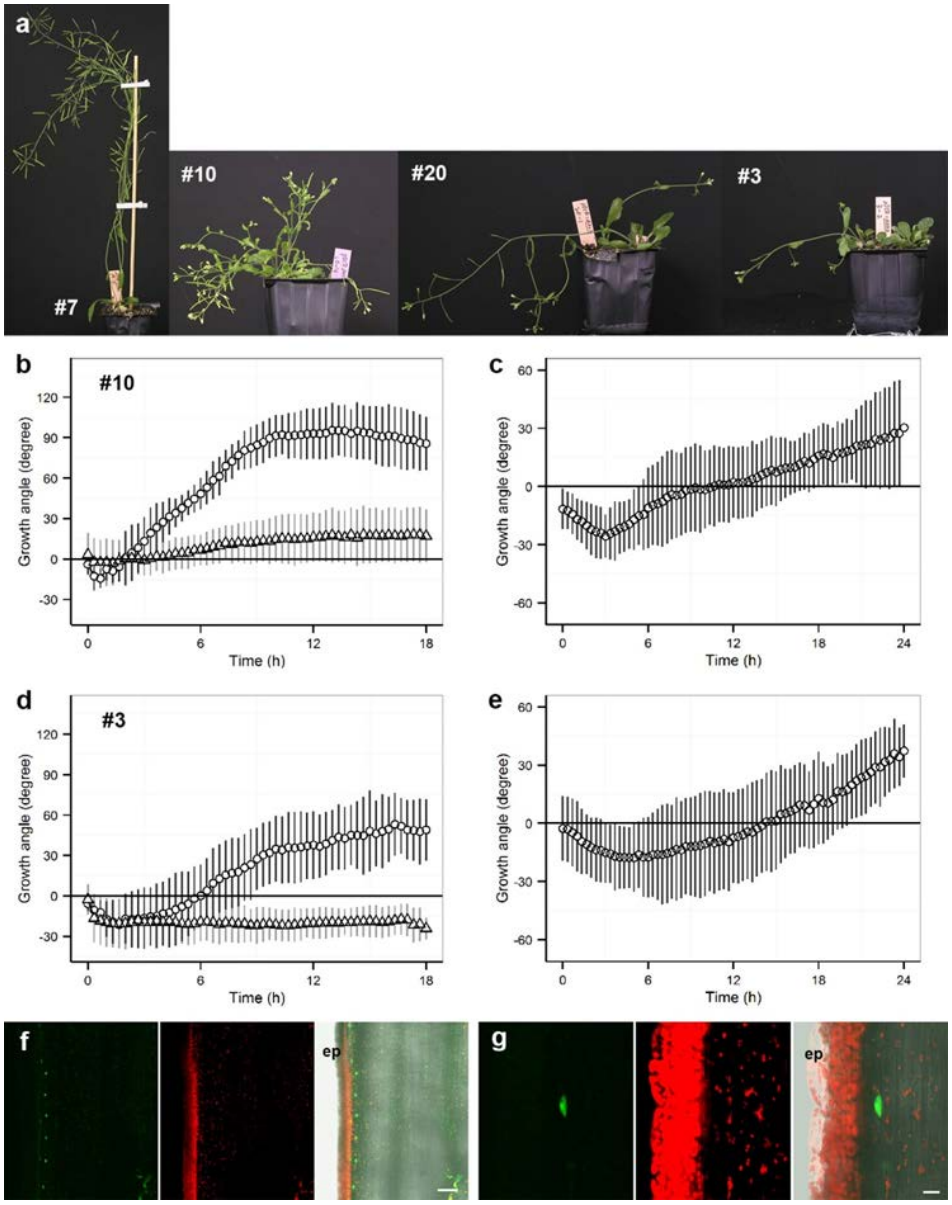
a
#7
#10
#20
#3
b
#10
Growth angle (degree)
Time (h)
c
Growth angle (degree)
Time (h)
d
#3
Growth angle (degree)
Time (h)
e
Growth angle (degree)
Time (h)
f
ep
g
ep

Fig. 7

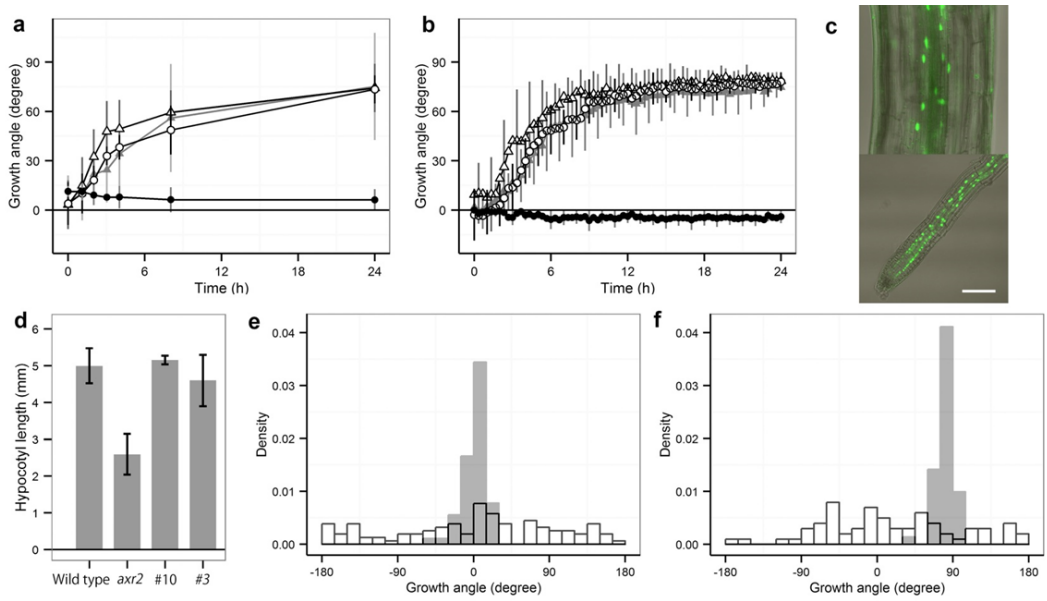
a
b
c
Growth angle (degree)
Time (h)
d
Hypocotyl length (mm)
Wild type
axr2
#10
#3
e
f
Density
Growth angle (degree)

Fig. S1

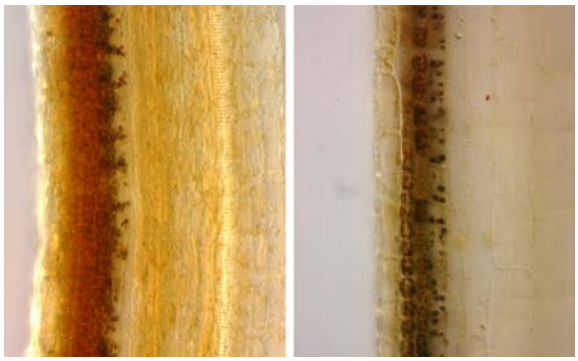

Fig. S2

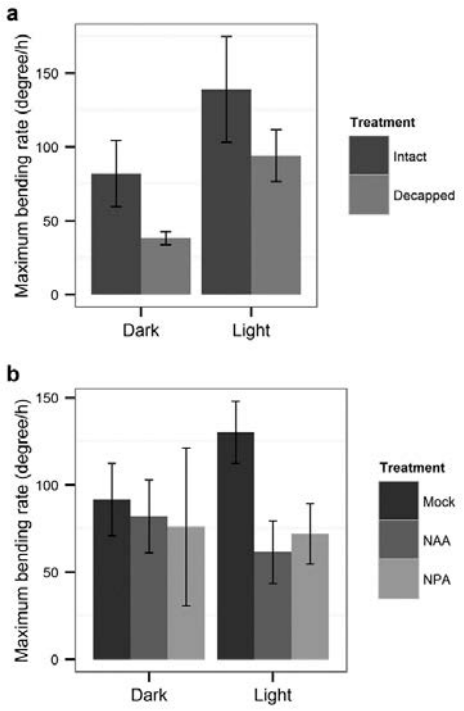
a
Maximum bending rate (degree/h)
150
100
50
0
Dark
Light
Treatment
Intact
Decapped
b
Maximum bending rate (degree/h)
150
100
50
0
Dark
Light
Treatment
Mock
NAA
NPA

Fig. S3

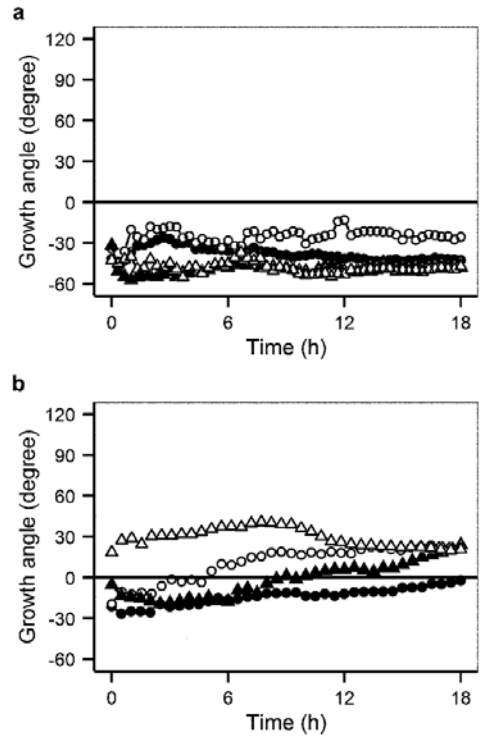
a
Growth angle (degree)
120
90
60
30
0
-30
-60
0
6
12
18
Time (h)
b
Growth angle (degree)
120
90
60
30
0
-30
-60
0
6
12
18
Time (h)

Fig. S4

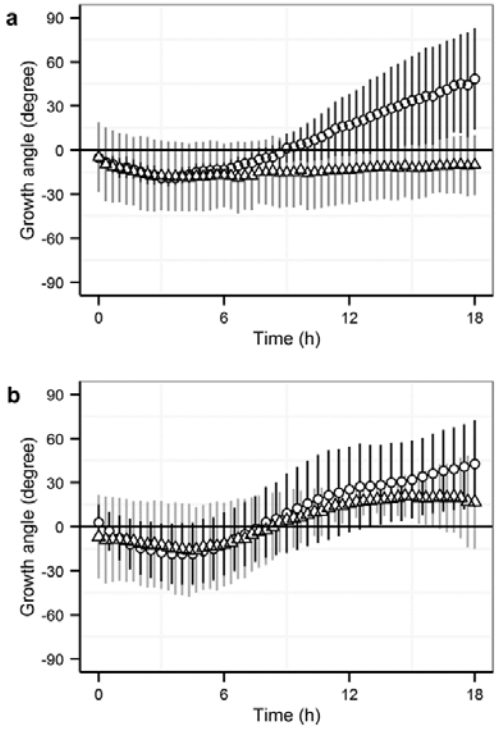
a
b
Growth angle (degree)
90
60
30
0
-30
-60
-90
0
6
12
18
Time (h)

Fig. S5

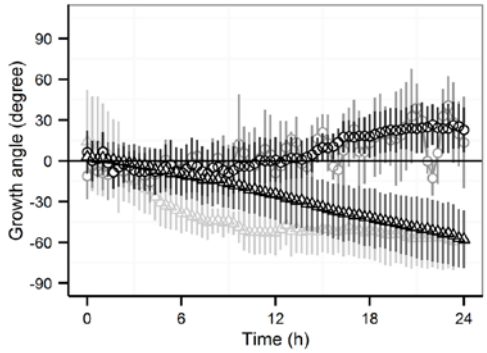
Growth angle (degree)
90
60
30
0
-30
-60
-90
0
6
12
18
24
Time (h)

Fig. S6