SAUR53 regulates organ elongation and apical hook development in Arabidopsis

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ABSTRACT

SAUR53 is a member of *SAUR* (*Small Auxin-Up RNA*) gene family of primary auxin responsive genes. In Arabidopsis, *SAUR* gene family is represented by 81 genes including two pseudogenes; however, the functions of most of these genes are not fully characterized yet. In the present study, we show that SAUR53 expresses throughout the plant and localizes to both plasma membrane and the nucleus. Unlike most other *SAUR* genes, expression of *SAUR53* is not induced in response to auxin. Ectopic expression of *SAUR53* results in the elongation of cells and organs, and also interferes with normal apical hook development by accelerating the hook maintenance phase. Moreover, root growth of *SAUR53* over- expression seedlings is significantly insensitive to IAA and 2,4-D, while showing wild-type sensitivity to NAA, suggesting that elevated level of SAUR53 may interfere with normal auxin transport. Collectively, this study indicates that while SAUR53 positively regulates cell and organ elongation, it probably negatively regulates auxin transport in Arabidopsis.

Introduction

Auxin is a major plant hormone, which not only directly controls many aspects of growth and development of plants, but also co-ordinates with other plant hormones to regulate the growth and development.[1-4] Auxin regulates plant growth and developmental processes through direct modulation of the expression of many genes. Of these, expression of three primary auxin responsive gene families, namely *Aux/IAA* (*auxin/indole 3-acetic acid), SAUR* (*small auxin-up RNA*) and *GH3* (*Gretchen Hagen3*) is rapidly induced in response to auxin. Although functions of several of *Aux/IAA* and *GH3* genes have been well characterized in diverse growth and developmental processes,[5-8] functions of most *SAUR* genes are largely elusive. In the model plant Arabidopsis, *SAUR* gene family consists of 81 genes including two pseudogenes.[9] However, probable functions of only a very few *SAUR* genes have been identified so far.[10-15] In addition to Arabidopsis, auxin induced *SAUR* transcripts have also been identified from soybean, corn, mung bean, pea, rice, and radish.[16]

SAUR genes were originally identified from elongating hypocotyl sections of auxin treated soybean,[17] indicating the possible role of *SAUR* genes in auxin induced cell elongation. Consistently, several *SAUR* genes were recently implicated in cell and organ elongation.[11,12,14,15] Additionally, some of the *SAUR* genes have also been shown to regulate the apical hook development, leaf senescence and meristem patterning.[10,13,14,18] It has also been found that at least some of SAUR proteins physically interact with calmodulin (CaM) in calcium dependent manner.[19-22] However, biological significance of this interaction is still inconclusive.

In this study we demonstrate that Arabidopsis *SAUR53* expresses throughout the plant and the encoded protein localizes to both nucleus and plasma membrane. Elevated level of *SAUR53* enhances organ elongation and significantly accelerates apical hook development. Additionally, we demonstrate that ectopic expression of *SAUR53* has a negative effect on polar auxin transport.

Results

SAUR53 expresses throughout the plant and is not induced by auxin

SAUR53 is an intron-less gene that belongs to the group of *SAUR* family. Members of this gene family are known to be transcriptionally induced in response to auxin. It encodes an approximately 16 kD protein, and shares high sequence similarity with SAUR52 (At1g75590) and SAUR69 (At5g10990). To study the tissue specific expression of *SAUR53*, qRT-PCR analysis and histochemical GUS staining of $SAUR53_{pro}$::*GUS* seedlings and adult plants were carried out. qRT-PCR results indicated that *SAUR53* expressed both in seedling and adult stages. In 4-week old adult plants, *SAUR53* expresses in roots, shoots, flowers, young and old leaves (Figure 1A). Histochemical GUS staining of seedlings further revealed that *SAUR53* expression was apparent throughout the cotyledons and upper part of hypocotyl (Supplementary Fig. S1A). In mature plants, *SAUR53* expression was observed in leaf and flower vasculature (Supplementary Fig. S1B and S1C) and also in the silique (Supplementary Fig. S1D).

The expression of most members of SAUR gene family is rapidly up-regulated in response to exogenous application of

Table 1. Primers and primer sequences used in this study.

Primers	Primer Sequence
SAUR53-F	*CAGAGTTAGTTTGACTTCAAACGT*
SAUR53Sal1-R	*GTCGACCCAGATAGCTTTCTCAGCGA*
SAUR53-PF	*CACCATAATTAGGGGTTGAGATGTATACG*
SAUR53-PR	*CCATCTAGACCCACGTTTGAAGTCAA*

auxin.[11,12,15] Interestingly, under our experimental conditions auxin treatment did not alter the expression of *SAUR53* in Arabidopsis seedlings (Figure 1B and 1C).

SAUR53 *localizes to both nucleus and plasma membrane*

Earlier studies have shown that different SAUR proteins selectively localize to different cellular organelles such as nuclei, cytoplasm and plasma membrane.[11,12,18,20] To study the intracellular localization of SAUR53, GFP tagged SAUR53 was stably expressed in Arabidopsis. Similar to AtSAUR19,[11] SAUR53-GFP was found to be localized to both the nucleus and the plasma membrane in root cells (Figure 2).

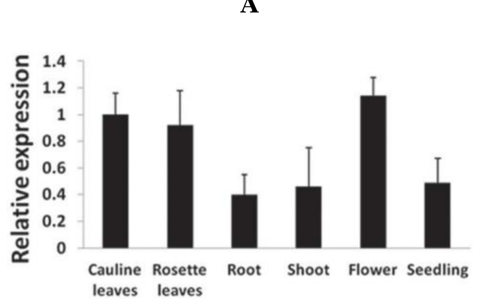
Ectopic expression of SAUR53 *results in organ elongation and defective apical hook development*

SAUR transcripts were originally identified from auxin treated elongating hypocotyls, thus predicted to be involved in auxin dependent cell and organ elongation.[16] Recently, it has been shown that several *SAURs* including *AtSAUR19, AtSAUR41*, and *AtSAUR63* are involved in cell and organ elongation.[11,12,14,15] To study whether *AtSAUR53* is also involved in cell and organ elongation, homozygous *35S::SAUR53-myc (SAUR53-OX)* transgenic lines, and loss-of-function *saur53-1* (Supplementary Fig. S2A-S2C) lines were analyzed for organ morphology.

When grown under continuous light, *SAUR53-OX* seedlings produced significantly longer hypocotyls than in wild-type (Figure 3A, Supplementary Fig. S2D). Average epidermal cell length at the hypocotyl region was also significantly longer in *SAUR53-OX* compared to wild-type (Figure 3B). Additionally, it was observed that *SAUR53-OX* seedlings produced long and wavy primary roots (Supplementary Fig. S2E) and larger cotyledons (Figure 3C, Supplementary Fig. S3F) compared to wild-type seedlings. However, *saur53-1* seedlings did not show any difference in growth and development compared to wild-type seedlings.

To further explore the role of *SAUR53* in growth and development in dark conditions, wild-type, *SAUR53-OX* and *saur53-1* seedlings were germinated and grown in complete dark for 96 hours. In contrast to wild-type seedlings, *SAUR53-OX* lines showed defective skotomorphogenic growth in which cotyledons were open with no apical hooks (Figure 4A). Apical hook development in Arabidopsis proceeds through three successive phases; hook formation (between 30–48 hours), maintenance (between 48–96 hours), and opening (after 96 hours).[23] To pinpoint the defective phase during apical hook development in *SAUR53-OX* lines, hook curvature was measured at various time intervals between 40–96 hours after incubation in dark. As

A



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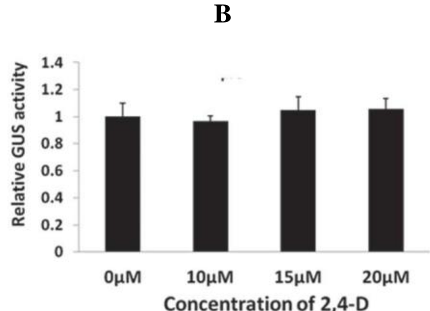

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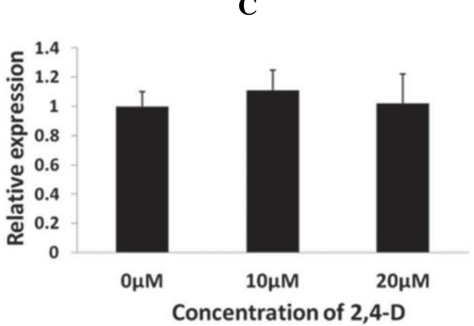

Figure 1. Tissue specific expression and regulation of *SAUR53* induction.

SAUR53 expresses during seedling and adult stages (1A). qRT-PCR was performed with cDNA prepared from total RNA isolated from 7-day-old seedlings and various organs of 4-week-old plants.Auxin does not induce the expression of either $SAUR53_{pro}$*::GUS* expression (1B) or endogenous *SAUR53* gene (1C). Seven-day-old $SAUR53_{pro}$*::GUS* transgenic seedlings were either mock treated or treated with different concentrations of 2,4-D for one hour. Following treatment, seedlings were used for quantitative MUG assay (n = 3 biological repeats). For qRT-PCR analysis, 7-day-old Col-0 seedlings were either mock treated or treated with 10µM 2,4-D for one hour and total RNA was isolated. Expression of SAUR53 was determined by qRT-PCR with *UBA* gene as an endogenous control. Each data point represents mean of the data set and bars represent standard deviation (n = 3)

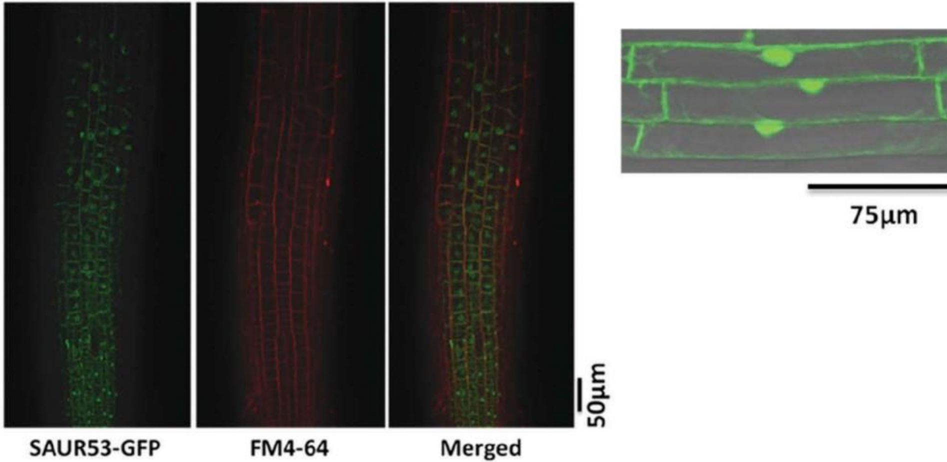

Figure 2. Intracellular localization of SAUR53 in root cells.

SAUR53-GFP localizes to both plasma membrane and nucleus. Green fluorescence of SAUR53-GFP, and red fluorescence of plasma membrane tracker dye FM4-64 were superimposed to produce merged image. Magnified view of SAUR53-GFP localization in primary root cells (right panel).

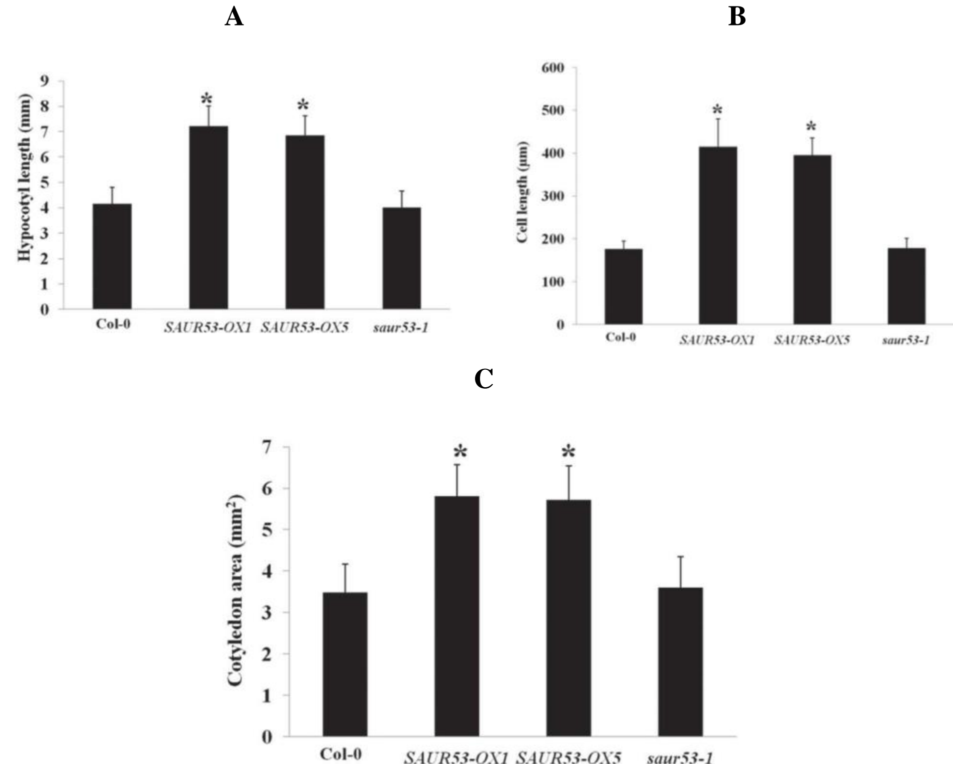

Figure 3. Effect of *SAUR53* overexpression on different organs.

SAUR53-OX seedlings produce significantly longer hypocotyls (3A) and longer epidermal cells than wild type or *saur53-1* (3B). *SAUR53-OX* seedlings produce larger cotyledons than wild type or *saur53-1* (3C). Seven-day-old seedlings were imaged using NIKON SMZ1500 stereomicroscope. Measurements were taken using the imagJ software. Each data point represents mean of the data set and bars represent standard deviation (n = 10)

shown in Figure 4B, *SAUR53-OX* lines were not defective in hook formation, but rather defective in hook maintenance and therefore showed accelerated hook opening in dark conditions.

Elevated levels of SAUR53 *may interfere with normal auxin transport*

Earlier attempts to discover the functions of *SAUR* genes in plant growth and development reveal that ectopic expression or the stabilization of the some of SAUR proteins interferes with normal auxin transport and auxin responses.[10-12,18] To analyze whether ectopic expression of *SAUR53* may also interfere with normal auxin responses, primary root growth inhibition on IAA (Indole-3-acetic acid) and synthetic auxin 2,4-D (2,4-dichlorophenoxy acetic acid) was studied. As shown in Figure 5A and 5B, compared to wild type, primary root growth of *SAUR53-OX* was found to be insensitive to both IAA and 2,4-D. To further test whether *SAUR53-OX*

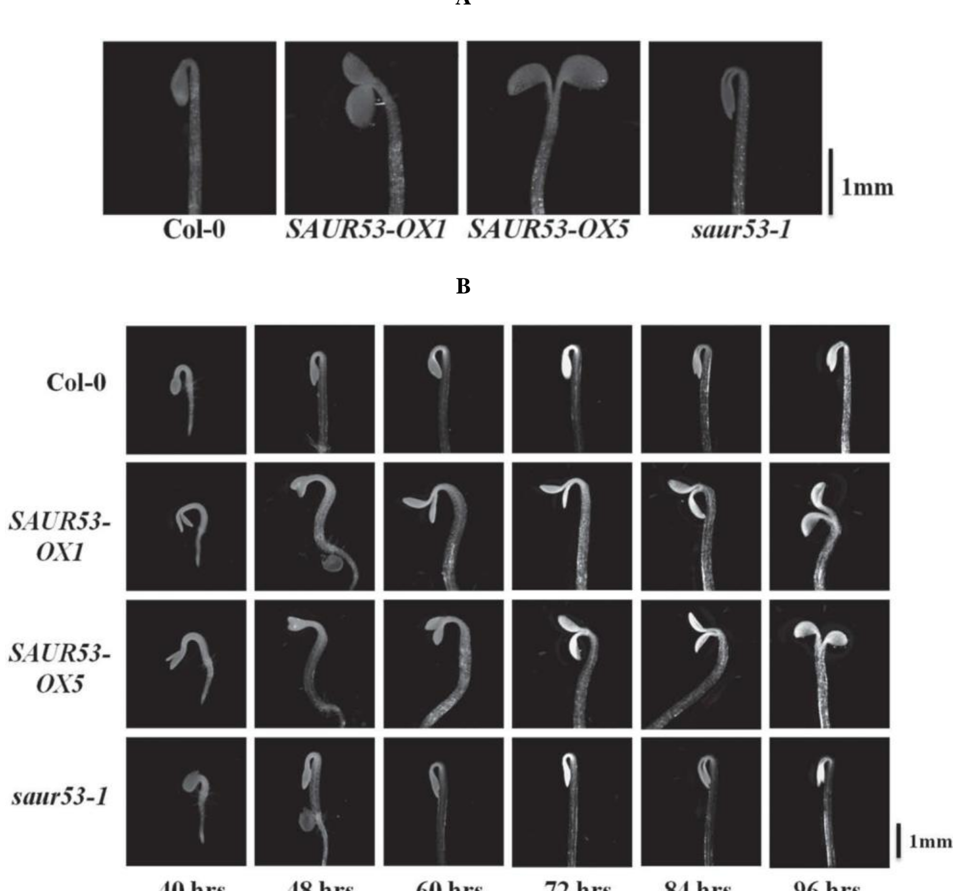

Figure 4. Regulation of apical hook development by *SAUR53*.

Four-day-old *SAUR53-OX* seedlings have opened the apical hook, while *saur53-1* has slightly closed apical hook compared to wild type (4A). *SAUR53-OX* lines are defective in hook maintenance phase (4B). Seeds were plated on solid ATS media and were incubated under complete dark at 21°C for different time intervals. Images were acquired using NIKON SMZ 1500 stereomicroscope.

seedlings are either defective in auxin response or auxin transport, root growth inhibition on the synthetic auxin NAA (Naphthalene acetic acid) was carried out. NAA is a lipophilic molecule and freely diffuses into cells, and does not need auxin influx carrier to enter into the cells. Unlike on 2,4-D or IAA, root growth of *SAUR53-OX* seedlings was found to be sensitive to NAA (Figure 5C), suggesting that the auxin insensitivity observed in *SAUR53-OX* seedlings most probably results from defects in auxin transport.

Discussion

Auxin is a vital phytohormone required for proper growth and development of plants throughout their life cycle. Plants respond to auxin by modulating the expression of a number of genes that belong to three primary auxin response gene families *Aux/IAA, GH3* and *SAUR*. Arabidopsis genome contains 20 *GH3*, 28 *Aux/IAA*[16] and 81 *SAUR* genes.[9] Unlike members of *GH3* and *Aux/IAA* gene families, functions of *SAUR* genes in plant growth and development are largely unknown. So far, none of the forward genetic approaches has captured any *SAUR* mutants to assign the gene function. This might be largely due to functional redundancy among *SAUR* genes. Thus, most of the studies characterizing *SAUR* genes have been done using reverse genetic approaches.[10-12,14,15,18]

Several studies have shown that expression of most of the *SAUR* genes is readily induced by exogenous application of auxin, indicating that these *SAUR* genes are auxin responsive.[11,12,14,24] However, *SAUR53* was found to be distinct from them as its expression was neither induced nor repressed by auxin (Figure 1B and 1C). Moreover, promoter analysis using AGRIS (http://agris-knowledgebase.org) tool also indicated that *SAUR53* lacks auxin responsive elements in its promoter, while most of known *SAUR* genes contain such elements (http://agris-knowledgebase.org). In the present study, we also observed that SAUR53-GFP localizes to both nucleus and the plasma membrane (Figure 2). Previous studies with *SAUR63* have shown that larger C-terminal fusions such as GFP and GUS enhance the stability of SAUR63 resulting in predominately membrane localization while smaller fusion of HA results in both membrane and cytoplasmic localization.[12] Thus, it is possible that stability of SAUR53 may also play a role on plasmamembrane localization of SAUR53. Similar to SAUR63, SAUR53 also does not contain a putative membrane localization signal and therefore, may be interacting with another membrane localized protein.

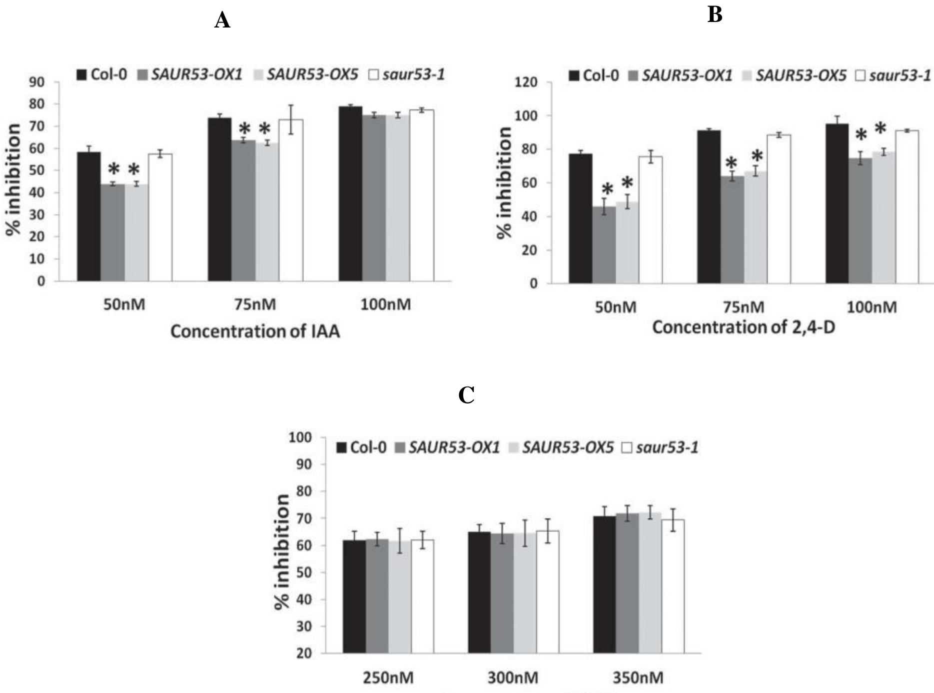

Figure 5. Root growth inhibition of *SAUR53-OX* lines on different auxins.

Primary root growth of *SAUR53-OX* seedlings was insensitive to IAA (5A) and 2,4-D (5B) but sensitive to NAA (5C). Four-day-old seedlings were transferred to media containing either mock treatment or indicated concentrations of different auxin. After incubation, primary root length was me asured and percentage inhibition was calculated. Error bars indicate standard percentage error of mean of percentage inhibition (n = 15).

Our data indicate that *SAUR53* may function as a positive regulator of organ elongation. When grown under continuous light, seedlings of *SAUR53-OX* lines produce longer hypocotyl and primary root, and broader cotyledons than wild type seedlings grown under similar conditions (Figure 3A-3C and Supplementary Fig. S2D-F). Organ elongation may either be due to increased cell division or increased cell elongation/ expansion. In *SAUR53-OX* lines, increased cell elongation appears to be the primary reason for organ elongation (Figure 3B). Recently it was shown that SAUR19 promotes cell expansion by indirectly promoting the activity of plasma membrane (PM) H^+ ATPase activity.[25] It was established that SAUR19 physically interacts with PP2C-D subfamily of phosphatases, which are the negative regulators of PM H^+ ATPase and inhibits their phosphatase activity. It is possible that SAUR53 might also promote cell expansion through a similar mechanism, however, further studies are needed to verify similarities and dissimilarities between mode of action between SAUR19 and SAUR53.

Etiolated seedlings develop apical hook because of differential cell elongation and division within hook region. Differential cell elongation along the hook region is maintained through cross-talk between several plant hormones including auxin and ethylene.[23] Since *SAUR53-OX* seedlings are defective in apical hook development in the dark (Figure 4A and 4B), it is possible that SAUR53 might function in auxin and/or ethylene signaling and transport. Very recently it was demonstrated that a group of three SAUR proteins (SAUR76-78) negatively regulates ethylene sensitivity in Arabidopsis by physically interacting with subfamily II ethylene receptors, EIN4 and ETR2.[15] Therefore, it is possible that the apical hook defect observed in *SAUR53-OX* lines may have been caused by the reduced ethylene sensitivity. However, further genetic and biochemical experiments are necessary to confirm this possibility.

Root growth inhibition responses of *SAUR53-OX* seedlings convey very interesting possibilities. *SAUR53-OX* seedlings were highly insensitive to both IAA and 2,4-D (Figure 5A, B), while retaining wild-type sensitivity against NAA (Figure 5C). IAA and 2,4-D require functional AUX1 (AUXIN RESISTANT 1) influx carrier protein to enter the cell, while NAA, which is a lipophilic compound, enters into cell without the assistance of carrier protein.[26] Therefore, some mutants that are defective in auxin influx through AUX1 are less sensitive to both IAA and 2,4-D but show wild-type sensitivity to NAA.[26,27] As SAUR53 is localized to the plasma membrane, and *SAUR53-OX* transgenic seedlings show defective root growth sensitivity characteristic of defective auxin influx, it can be suggested that *SAUR53* may be involved in the regulation of polar auxin transport.

Under our experimental conditions, the loss-of-function mutant *saur53-1* did not differ significantly from wild type. This result was not surprising, as it has been shown previously that most of the SAUR members show functional redundancy owing to their sequence similarity.[11,12] Multiple protein sequence analysis of SAUR53, SAUR52 and SAUR69 shows that SAUR53 protein sequence is approximately 82% and 80% similar to SAUR52 and SAUR69 respectively. It is possible that these three proteins may function redundantly, thus, creating a triple knock-out line with these gene combinations would

probably show phenotypic differences from that of wild type. Hence, future studies with higher order mutants of *SAUR52, SAUR53* and *SAUR69* might shed light onto the functions of these proteins in auxin mediated plant growth and development.

Materials and methods

Plant material and growth conditions

Wild type and mutant seeds used in this study were in Columbia (Col-0) background. Seeds of *saur53-1* (*SALK_056069C*) allele carrying a T-DNA insertion in the exon were obtained from ABRC. For root growth inhibition assay, 4-day-old seedlings were transferred onto ATS agar[28] plates containing mock treatment or appropriate concentrations of different auxins, and were incubated vertically for another 3–4 days at 21°C depending on the experiment. To study the apical hook development, surface sterilized and stratified seeds were plated on ATS agar plates. Seedlings were grown vertically in complete dark at 21°C for designated times, and images were acquired using Nikon SMZ 1500 stereomicroscope.

Vector construction and plant transformation

To prepare *SAUR53-OX* (CaMV*35S$_{pro}$::SAUR53-myc*) construct, full length *SAUR53* (At1g19840) coding sequence without stop codon was amplified from Col-0 genomic DNA using primer combination of *SAUR53-F* and *SAUR53Sal1-R*. Table 1 Amplified fragment was cloned into modified *pROKII* binary vector containing 5X *myc* tag. To prepare *SAUR53$_{Pro}$::GUS* construct, approximately 2.2 kb region upstream of transcriptional start codon of *SAUR53* was amplified from Col-0 genomic DNA using the primer combination of *SAUR53-PF* and *SAUR53-PR*, (Table 1) and was directionally cloned into *pENTR/D-TOPO* vector as per manufacturer's instructions. Upon homologous recombination in the presence of LR clonase enzyme mixture (Invitrogen, Carlsbad, CA), fragment was introduced into *pHGWS7* destination binary vector.

To prepare *SAUR53-GFP* (*CaMV35S$_{pro}$:: SAUR53-GFP*) construct, full length *SAUR53* coding region without stop codon was amplified from Col-0 genomic DNA, and directionally cloned into *pENTR/D-TOPO*. Through LR clonase reaction, *SAUR53* was shuttled into *pB7WG2.0* binary vector. Binary vectors were shuttled to Agrobacterium strain GV3101 and subsequently transgenic plants were prepared by transforming wild type Col-0 plants by floral dip method.[29]

SDS-PAGE and western blotting

Proteins were separated using 12.5% SDS-PAGE (sodium dodecyl sulphate- polyacrylamide gel electrophoresis) and transferred onto polyvinylidene fluoride (PVDF, Bio-rad) membrane. Western blotting was done using 1:10,000 ratio of anti-myc (Covance, Princeton, NJ) primary antibody followed by 1:10,000 ratio of specific secondary antibody. Bands were detected by enhanced chemiluminescence (ECL) plus kit as per manufacturer's instructions (Thermo Fisher, Austin, TX).

Histochemical GUS staining and quantitative MUG assay

Histochemical GUS staining and quantitative MUG assays were performed following the protocol described elsewhere[30]

Quantitative RT-PCR

Seedlings and tissues from which RNA to be isolated were frozen and ground in liquid nitrogen. Total RNA was extracted using TRI reagent following the commercial protocol (Sigma-Aldrich Inc, MO). cDNA was synthesized using approximately 3 µg of total RNA using oligo dT primers and reverse transcriptase (NEB), following manufacturer's instructions. qRT-PCR analysis was performed following the protocol described elsewhere.[30] *Ubiquitin Associated gene* (*UBA*) was used as the standard for qRT-PCR analysis.

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