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SCR Suppressor Mutants: Role in Hypocotyl Gravitropism and Root Growth in *Arabidopsis thaliana*

Archana Sharma [1,*], Zahida Hassan Pervaiz [2] and Joanna Wysocka-Diller [2]

[1] Biology Department, Tuskegee University, Tuskegee, AL 36088, USA
[2] Biological Sciences, Auburn University, Auburn, AL 36849, USA
* Correspondence: asharma@tuskegee.edu; Tel.: +1-334-727-8828

Abstract: The SCARECROW (SCR) transcription factor plays a key role in plant growth and development. However, we know very little about the role of SCR regulated pathways in plant development. Here, we used the homozygous *scr1* mutant *Arabidopsis thaliana* (Wassilewskija ecotype), which had a T-DNA insertion in the *SCR* coding region and lacks a detectable SCR transcript. This *scr1* mutant has a determinate mode of root growth, shoot agravitropism and abnormal internal architecture in all organs examined. To screen for mutants that suppress the *scr1* abnormal phenotypes, we exposed homozygous *scr1* seeds to ethyl methane sulphonate (EMS) mutagen. Upon growth out of these mutagenized seeds, thirteen suppressor mutant-harboring strains were identified. All thirteen suppressor-harboring strains were homozygous for *scr1* and lacked the SCR transcript. Ten *scr* hypocotyl gravitropic suppressor lines showed improved hypocotyl gravitropic response. These ten suppressors fall into six complementation groups suggesting six different gene loci. Similarly, three independent *scr* root length suppressor lines rescued only the root growth phenotype and fell into three complementation groups, suggesting the involvement of three different gene loci. These suppressors might identify novel functions of the SCR gene in plant development.

Keywords: scarecrow; transcription factor; suppressor; hypocotyl gravitropism; root growth

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1. Introduction

Screening of mutants with desired phenotypes followed by identification of the corresponding genes is an important first step in defining key regulatory components of a biological pathway in living organisms [1–4]. Suppressor mutations can hide or suppress the phenotypic effects of other mutation(s). Suppressor screening starts with inactivation (or mutagenesis) of a known gene followed by the identification of second-site mutations (suppressor mutations) that occur at sites distinct from the original mutation. The second site mutation suppresses the original mutant phenotype [5–7]. An important mutagenesis method commonly used in *A. thaliana* mutagenesis is ethyl methane sulfonate (EMS), which preferentially induces guanine to adenine transitions. EMS is one of the most powerful and frequently used chemical mutagens, commonly inducing multiple point mutations in each resultant progeny [8]. This high rate of mutagenesis makes it possible to identify plants with the phenotype of interest while screening relatively few plants [9,10]. Thus, screening of known mutants using mutagenesis can help us understand the functions of novel genes that might interact with the parental mutant gene.

The *SCR* gene is a transcriptional regulator in *A. thaliana* and is essential for the radial patterning of the root, development of endodermis of the shoot, normal shoot gravitropism and indeterminate root growth [11,12]. It has been reported that *scr* mutants of *A. thaliana* exhibited a determinate mode of root growth (short roots), shoot agravitropism (fails to respond to gravity) and abnormal internal architecture in all organs examined [11,13]. Further analysis of the shoot's internal architecture also revealed that both hypocotyl and inflorescence stems had defective radial patterns [11,14]. Another *scr* phenotype is the

absence of a root ground tissue layer reportedly due to the lack of asymmetric cell division, responsible for the development of cortex and endodermis from the cortex/endodermis initials [15,16]. It is also reported that one ground tissue layer was missing in the stems and no stem cell layer contained sedimented amyloplast. According to the "starch-statolith hypothesis" gravity sensing involves sedimentation of amyloplasts (statoliths) in specific cells (statocytes) [17–20]. Studies have suggested that the endodermis of the stem acts as statocyte cells and participates in gravity sensing; therefore, the missing layer could be the endodermis [14]. Although there are several recent studies on the functions of SCR in *Arabidopsis* [21,22], we still do not fully understand its functions in hypocotyl gravity sensing and indeterminate root length growth.

The GRAS proteins, named after the three founding members, GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GA1 (RGA), and SCARECROW (SCR), are a well-defined family of plant-specific transcription factors and currently 33 GRAS genes have been described in *Arabidopsis* [16,23,24]. SCR was the first identified member of the GRAS family [25]. SCR along with SHORTROOT (SHR, also a member of the GRAS family) plays a key role in radial patterning, root meristem maintenance, and endodermal differentiation in *Arabidopsis* [26,27]. The quiescent center (QC), a group of slowly dividing cells located at the center of the root tip, is necessary for the maintenance of "stem" cell phenotype (maintain their undifferentiated state) of the root tip. Both QC and stem cells, together, form the stem niche and both are required for indeterminate growth of the root [28,29]. In addition, other studies have reported the role of QC in root development [30,31]; however, further research is needed to unravel the regulatory mechanism and molecular aspects behind it.

Furthermore, it has been demonstrated that the SCR protein is essential for the repression of initial differentiation in the QC [15,21]. The QC identity is lost in the *scr* mutants, thus resulting in the loss of stem cell activity in initials surrounding the QC that leads to the "short root" phenotype.

Although there have been many studies on the *SCR* gene [27,32–35], there is little information regarding the genes that are controlled by, or that interact with, SCR. We hypothesized that SCR, a transcription factor, works via interaction with target genes and gene products in SCR-regulated pathways. We reasoned that these target genes might be identified by a suppressor mutation strategy that restores SCR-regulated phenotypes in a parental *scr1* mutant line. Therefore, we have used a forward genetic approach of generating and screening the *scr1* suppressors (suppressors of the *scr1* mutant). Homozygous seeds of *scr*1 mutants with a transposon-DNA (T-DNA) insertion in *SCR* coding region were mutagenized with EMS. This T-DNA insertion causes the loss of *SCR* gene function in *scr*1 mutants. The length of T-DNA in *scr1* is approximately 17 kb, and as EMS usually induces point mutations it is highly unlikely that EMS mutagenesis could restore SCR function. Therefore, EMS-treated *scr1* mutant lines that show improved phenotypic traits likely represent second-site mutations. We have identified thirteen suppressors, ten with improved hypocotyl gravitropism (***s****cr* hypocotyl gravitropic **s**uppressors; *shs*) and three with improved root growth (***s****cr* root length suppressors; *srs*). All thirteen suppressors are homozygous for *scr1* and do not carry a functional copy of the *SCR* gene. A detailed phenotypic analysis of these suppressors revealed that both *srs* and *shs* only have rescued root growth and hypocotyl gravitropic responses respectively, while retaining abnormal radial patterns.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Arabidopsis thaliana, Wassilewskija (WS) and Columbia (Col) ecotypes were used as wildtype. The *scr1* mutant used for suppressor screening in this study is the WS ecotype background [25]. For screening and phenotypic characterization of suppressors, seeds were surface sterilized and grown on Murashige and Skoog (MS) agar plates as described by Fukaki et al. [36]. Plants were transferred to pots from MS agar plates and grown under

white light at 23 ± 1 °C in long-day light conditions (16 h light). These plants were used for inflorescence stem gravitropic studies, self-fertilization, and homozygous seed production.

2.2. Mutagenesis and Mutant Screening

Homozygous seeds of *scr1* were mutagenized with EMS as described by Levin et al. [37], seeds were washed with 0.1% Tween-20 for 15 min, treated with 0.2% EMS (volume/volume in water) for 12 h, rinsed with water eight times, soaked in the last rinse for 15 min, followed by several rinses with 0.1% Tween-20 for a total of 5.5 h. Mutagenized seeds (M0 generation) were plated on MS agarose plates containing 4.5% sucrose, kept in the dark at 4 °C for 3 days to synchronize the seed germination and then transferred to a growth chamber at 20 °C (16 h daylight) for germination. Two weeks after germination vigorous seedlings were transferred to soil, allowed to self-fertilize, and heir seeds were pooled (M1 generation) from 40–100 plants. M1 generation seeds were grown and allowed to self-fertilize, and seeds were collected (M2 generation) as described above. M2 generation seeds were germinated, and the resultant seedlings were used to identify the potential *scr* suppressor harboring plants with improved phenotypic characters such as hypocotyl gravitropic response and longer root length.

To determine hypocotyl gravitropic responses, M2 generation seeds were surface sterilized and plated in rows on 4.5% sucrose MS agar plates and germinated as above for mutagenized seeds. Plates with germinated seedlings were covered with aluminum foil and left in a vertical position for 1 day. These 24 h plates were photographed (0-h photographs), covered again with foil and reoriented by 90 degrees. Plates were photographed again 48 h after reorientation (48-h photographs). These photographic images (Kodak Image Station440CF) were used to compare the gravitropic responses. Seedlings with improved hypocotyl gravitropic responses were transferred to pots. The original plates without improved hypocotyl gravitropic seedlings were left in the growth chamber for approximately two more weeks to identify the potential suppressors with improved root growth. Seedlings with longer roots were transferred to pots. Seedlings in pots were allowed to self-pollinate and eventually seeds were collected for further screening.

2.3. Confirmation of Suppressors' Genotypes

WS, *scr1* and potential *scr1* suppressor seeds were germinated as above. DNA was extracted from seedlings as described previously [38]. The DNA extracted from potential suppressors were amplified by using three primers SCRF344 5′ACCGTGGTGGTCGGAATGTTATGA, SCRR1956 5′AGTCGCTTGTGTAGCTGCATTTCC and T-DNA right border primer RBF 5′ CCAAACGTAAAACGGCTTGTC (Supplementary Figure S1). The polymerase chain reaction (PCR) contained 0.2 mM of each primer, 1.5–3.0 mM $MgCl_2$ (optimized for each pair of primers), 0.5 U Taq polymerase (Promega), 0.2 mM each dNTP (Promega) and ~100 ng of DNA template. Amplification was initiated at 94 °C for 1 min followed by primer annealing temperature of 54 °C for 1 min, followed by extension at 72 °C for 1 min and 30 s for 10 cycles. The entire program was repeated for 24 cycles with shorter denaturation time (30 s) at 94 °C, primer annealing at 54 °C for 30 s, extension at 72 °C for 1 min and 30 s and final elongation at 72 °C for 4 min.

To confirm the continued absence of the SCR transcript, RNA was extracted from the hypocotyl of WS, *scr1* and *scr* hypocotyl gravitropic suppressors according to the RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). cDNAs were synthesized with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™). *SCR* gene-specific primers were used to perform RT-PCR (SCRF836 5′AGGCAGAAGCAAGACGAAG, and SCRR1870 5′CTTCACCGCTTCTCGATGGT). The RT-PCR reaction contained 0.2 mM of each primer, 1.5–3.0 mM $MgCl_2$, 0.5 U Taq polymerase (Promega), 0.2 mM each dNTP (Promega) and 50–100 ng of DNA template. Amplification was initiated at 94 °C for 1 min followed by primer annealing temperature of 54 °C for 1 min, followed by extension at 72 °C for 1 min and 30 s for 10 cycles. The entire program was repeated for 24 cycles with shorter denaturation time (30 s) at 94 °C, primer annealing at 54 °C for 30 s, extension at 72 °C

for 1.5 min, and the final elongation at 72 °C for 4 min. PCR generated fragments were analyzed in agarose gels.

2.4. Phenotype Analysis of Potential Suppressors

Forty seeds of each genotype WS, *scr*1 and each suppressor were grown on the same 4.5% sucrose MS plate, surface-sterilized, germinated and analyzed for hypocotyl gravitropic response as described above. "Imagej 1.48 v" software was used to calculate curvature as the increment over the initial angle for each individual hypocotyl. Curvature values are reported as the mean angle ± standard deviation (SD) and used to categorize the suppressors.

The inflorescence gravitropism of the suppressors was analyzed as described previously [14]. Briefly, pots with WS, *scr1* and each suppressor plant (one genotype/pot) that were grown upright until inflorescence stems reached 6–8 cm length were placed in the dark on their sides (90-degree re-orientation). This was marked as the beginning of the experiment and was referred to as time 0 h. Inflorescence gravitropic response was measured 24 h after reorientation.

Root growth rate and final root lengths were determined for both *shs* and *srs* suppressor plants and compared to both WS and *scr1*. Forty seeds of each genotype were germinated as above. Root growth was analyzed for 15 days after germination (DAG). Kodak Image Station 440CF was used to photograph and analyze root length. Calculated root lengths are reported as mean length ± standard deviation (SD).

Hypocotyl lengths of *shs* plants were determined and compared to WS and *scr1*. Seeds of each *shs* suppressor line, WS and *scr1* were plated as above. Plates with germinating seedlings were covered with aluminum foil and left in vertical position for 7 days. Hypocotyl length of seedlings grown in dark condition for 7 days was measured using "Imagej 1.48 v" software. Hypocotyl lengths were characterized by calculating the mean value ± standard deviation (SD). Unless otherwise stated, we used ANOVA followed by the Fisher's test to determine the statistically significant differences between phenotypes of wild type, *scr1*, suppressors.

For cross sections, hypocotyl, and root fragments of *shs1-10* and *srs1-3*, respectively, were embedded in plastic media as follows. Seedlings grown in dark for 3 days after germination were cut and hypocotyl fragments of *shs1-10* and root fragments of *srs1-3* were selected for further procedure. Samples were fixed at 4 °C overnight in fixative containing 3% glutaraldehyde in 0.05 M phosphate buffer. Thereafter, samples were washed with phosphate buffer and dehydrated in a graded ethanol series. Samples were treated 15–30 min in each of: 10%, 30%, 50%, 75%, 95% and 95% ethanol. JB-4 embedding kit (Sigma-Aldrich) was used for infiltration and embedding in plastic embedding medium. Finally, the embedded tissues were mounted and 2–3 µm thick cross-sections taken via microtome. The cross sections were heat-fixed to slides, stained with 0.05% Toluidine blue O (TBO) in 1% boric acid for 1 min and rinsed with water. Nikon Biophot microscope with a Nikon D-70 digital camera attached was used for examination of cross sections.

To identify the presence and location of amyloplasts in *shs* suppressors the whole mount amyloplast staining was performed as described by Fukaki et al., 1998 [14]. WS, *scr1* and *shs* suppressor seedlings were grown vertically in darkness for 1 day after germination on MS plates without sucrose, as described above. Only those seedlings which were growing in an upright direction were fixed in FAA solution for 24 h at 4 °C. After fixation, seedlings were rinsed with 50% [*v*/*v*] ethanol and stained with IKI solution (2% (*w*/*v*) iodine, 5% (*w*/*v*) potassium iodide and 20% (*w*/*v*) chloral hydrate) for 1 min and mounted with a drop of clearing solution (a mix of 8 gm chloral hydrate, 2 mL water, and 1 mL glycerol). Slides with mounted seedlings were examined and pictures were taken with the use of a Nikon Biophot microscope with a Nikon D-70 digital camera attachment.

2.5. Genetic Analysis of Suppressors

To determine if *shs* and *srs* suppressors are homozygous recessive or dominant and if they represent single gene mutations, backcrosses with *scr1* mutant were performed. Pollen of *scr1* mutant (male) were used to cross the ovary of each suppressor. A Chi square (χ^2) test was performed to accept goodness of fit of observed ratio with expected ratio in the segregated generations.

Complementation tests were performed to determine the number of different loci affected by mutations identified in the screens. Pair-wise crosses of all *shs* and *srs* suppressors were performed. Each *shs* suppressor was crossed with the other nine. Similarly, each *srs* suppressor was crossed with the other two. Chi square (χ^2) test was performed to accept goodness of fit of observed ratio with expected ratio in the segregated generations. Based on the results of these crosses, suppressors complementation groups were determined.

3. Results

3.1. Mutagenesis and Suppressor Screen

The EMS mutagenesis was performed in batches of 2000 *scr1* seeds. The mutagenized seeds (M0 generation) were grown to maturity and allowed to self-pollinate and (M1 generation) seeds were collected in pools. Eventually, 51 pools were generated representing ~3000 independent lines, M1 generation seeds were again grown and allowed to self-pollinate. The resultant seeds represent the M2 generation. M2 generation seeds were analyzed to identify the potential suppressors. The seedlings with improved phenotypes such as long roots and improved hypocotyl gravitropic response were selected as potential suppressors and transferred to soil for M3 generation seed collection.

In the primary screen, 438 seedlings (M2 generation) with suppressor phenotypic characters were selected and transferred to the soil for seed production. Seeds were collected from only 235 of these primary screen candidate suppressors. These 235 seedlings (M3 generation), selected as candidate suppressors, represented 30 pools out of 44 pools screened. To confirm that the phenotype was maintained in the next generation, seeds from these candidate suppressor lines were subjected to secondary screening (M4 generation). After the secondary screening, 38 potential suppressors with improved hypocotyl gravitropism and/or improved root length were identified. Seedlings of these 38 lines were transferred to soil and next generation (M5 generation) seeds were collected. These 38 potential suppressor lines were rescreened for two additional generations to confirm the stability of the potential suppressor phenotypes and only the suppressors with the ability to retain their improved phenotypes for two additional generations were selected as the confirmed suppressor lines. Out of the initial 38 lines, only 13 lines retained their suppressor phenotypes for the next two generations, 10 with improved hypocotyl gravitropism (*shs*) and three with improved root growth (*srs*). These 13 lines were isolated from 11 different pools, potentially representing 11 different loci.

The *SCR* genetic background of the thirteen confirmed suppressors was evaluated by PCR using the three primers (Figure 1A,B). As mentioned earlier, the *scr1* mutant allele has a T-DNA insertion in the *SCR* coding region. To confirm that these suppressors are homozygous for the *scr1* allele and do not carry a WT copy of the *SCR* gene, we designed and used appropriate primers. The primers were designed in such a way that *SCR* forward and reverse primers are positioned on either side of the T-DNA insertion of the *scr1* allele, and third primer was RB-forward positioned at T-DNA right border (Supplementary Figure S1). Wild type does not have T-DNA insertion and generates a 1.6 kb PCR product with SCR F344 and SCR R1954 primers. The *scr1* mutant and suppressors should have a 17 kb T-DNA insertion in the *SCR* gene and therefore should amplify 0.7 kb product with T-DNA RBF and SCR R1954 primers. The PCR analysis confirmed that all 13 predicted suppressors were on *scr1* background, consistent with second-site mutations (Figure 1A,B).

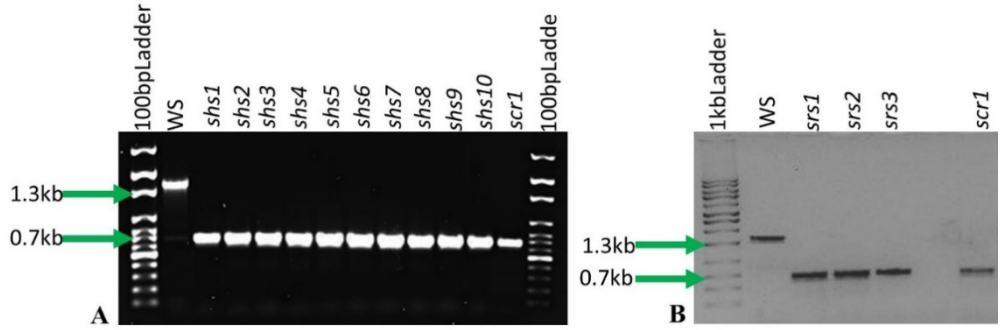

Figure 1. PCR amplification products in wild type, *scr1* and suppressor lines to confirm the presence or absence of T-DNA insertion in the *SCR* gene in (**A**). Lane 1. 100 bp ladder; Lane 2. Wild Type WS (1.6 kb amplification products generated with SCR-F344 and SCR-R1954 primers); Lane 3–12. The *scr* hypocotyl gravitropic suppressors (*shs*), 1 to 10 (0.7 kb amplification products generated with T-DNA RBF and SCR-R1954 primers); Lane 13. *scr1* (0.7 kb amplification products generated with T-DNA RBF and SCR-R1954 primers); Lane 14. 100 bp ladder. SCR-F344 and SCR-R1954 primers cannot amplify the *scr1* allele because of the presence of large T-DNA insertion in the genomic region. Green arrows point to 1.3 kb and 0.7 kb marker bands. (**B**). Lane 1. 1 kb ladder; Lane 2. Wild Type WS (1.6 kb amplification products generated with SCR-F344 and SCR-R1954 primers); Lane 3–5. *scr* root length suppressors (*srs*), 1 to 3 (0.7 kb amplification products generated with T-DNA RBF and SCR-R1954 primers); Lane 6: *scr1* (0.7 kb amplification products generated with T-DNA RBF and SCR-R1954 primers). Green arrows point to 1.3 kb and 0.7 kb marker bands.

To confirm that improved phenotypic responses of the suppressor lines are not due to an SCR reversion, using RT-PCR, we could not identify any SCR transcripts present in our predicted suppressors while wild type SCR strains yielded the expected ~0.9 kb fragment. Taken together, the positive genomic PCR for the T-DNA insertions and the RT-PCR results (absent of any SCR transcript) gives us confidence that all 13 suppressors were in the original *scr1* mutant background (Supplementary Figure S2).

3.2. Shoot Gravitropic Responses of Suppressors

In the absence of light, the orientation of an SCR wild-type seedling is based solely on the gravity vector. The preliminary hypocotyl gravitropic response analysis suggested that all ten *scr* hypocotyl gravitropic suppressors (*shs1*, *shs2*, *shs3*, *shs4*, *shs5*, *shs6*, *shs7*, *shs8*, *shs9* and *shs10*) exhibited gravitropic responses above the level of *scr1* but below the WS. It had been reported earlier that *scr* mutants are hypocotyl agravitropic [14,39]. However, our results indicate that *scr1* mutant seedlings harbor some hypocotyl gravitropic responses. Therefore, the scope and the time courses of gravitropic responses of the 10 *shs*, *scr1* and WS were compared simultaneously (Figure 2). For the quantification of hypocotyl gravitropic responses, 35 seedlings of each genotype (*shs1–10*, *scr1* and WS) were used and compared. The mean angles ± standard deviation of hypocotyl gravitropic responses were calculated in degrees and graphically represented (Figure 2). The WS hypocotyls showed negative gravitropic responses by the reorientation of growth by 39.8 ± 11.7 degrees after 12 h. All 10 *shs* suppressor lines showed significant but lower than WS gravitropic responses in the range of 20–28 degrees after 12 h (Figure 2). One suppressor, the *shs1*, exhibited a stronger response than other suppressors and was closest to the WS response at all three time points used in this analysis (Figure 2). Both WS and *shs1* hypocotyls exhibited the final angle of deflection in the range of 53–59 degrees after 48 h of reorientation to a new gravity vector (Figure 2). The other nine suppressors showed hypocotyl gravitropic responses significantly above the level of *scr1* (16.7 ± 11.1 degrees) with the range of 36–47 degrees after 48 h of reorientation (Figure 2). In addition to the improved gravitropic responses of the *shs* lines, these results demonstrate that *scr1* hypocotyls possess some residual gravitropic responses.

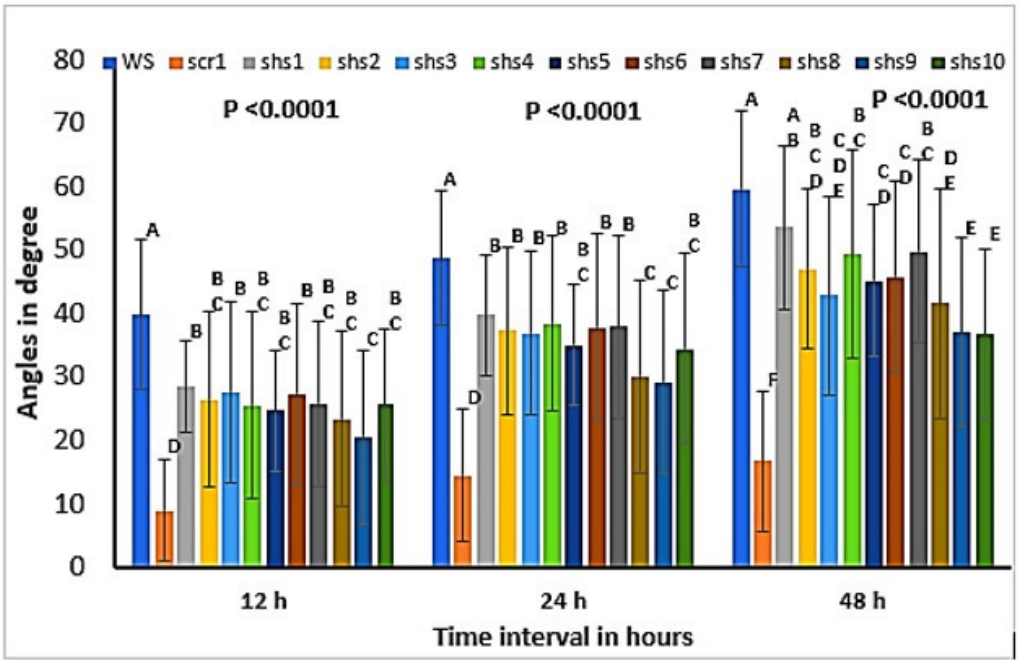

Figure 2. Hypocotyl gravitropic responses of *A. thaliana* wild type (WS), *scr1* and 10 confirmed *scr* hypocotyl gravitropic suppressors (*shs1–10*). Gravitropic responses to new gravity vectors of all the samples were measured in degrees at three time points. The X-axis represents the time interval in hours and the Y-axis represents the angle of deflection of hypocotyls. All 10 *scr* hypocotyl gravitropic suppressors (*shs1–10*) have gravitropic responses above the level of *scr1* but below WS. Error bars represent the means ± standard deviation (SD). The lack of shared letters on the bars indicates a significant difference. To determine the significant differences between wild type and mutant seedlings, we used ANOVA followed by Fisher's test.

The improved root length suppressors (*srs1–srs3*) exhibited the same level of hypocotyl gravitropic response as *scr1* (Figure 3B). Inflorescence gravitropic responses were assessed by placing pots on their sides in the dark. Inflorescence stems of WS plants could turn 90 degrees upward within two hours after reorientation. It was reported that *scr1* mutants exhibited complete inflorescence agravitropism [14,40]. The WS, *scr1* and 13 confirmed suppressors were evaluated for the inflorescence gravitropism. Neither *scr1* nor any of the confirmed *scr1* suppressors (shown only for *shs1* and *srs1*) showed any inflorescence gravitropic responses within 24 h after reorientation (Figure 3C).

3.3. Root Growth of Suppressors

Roots of *A. thaliana* grow continuously throughout their life (indeterminate growth). The *scr1* mutant shows determinate root growth with a short root phenotype at an early stage of life [41,42]. To evaluate if our suppressors were able to rescue the indeterminate root growth phenotype, all the confirmed suppressors (*srs* and *shs*) were assessed for root growth. Root lengths were calculated as mean value ± standard deviation (SD). As expected, the WS showed indeterminate (long roots: lengths mean value ± SD) and *scr1* exhibited determinate root length (short roots: lengths mean value ± SD). Only three suppressors *srs1*, *srs2* and *srs3* exhibited significantly longer roots (lengths mean value ± SD) than *scr1* (Figure 4A,B). However, all three *srs* suppressors still had determinate root growth (lengths mean value ± SD) (Figure 4B). Two of these, *srs1* and *srs2* grew faster and for longer periods of time than *scr1* and achieved their final length at 15 DAG (Figure 4A,B). None of the 10 *shs* showed any significant improvement in their root lengths (Supplementary Figure S3).

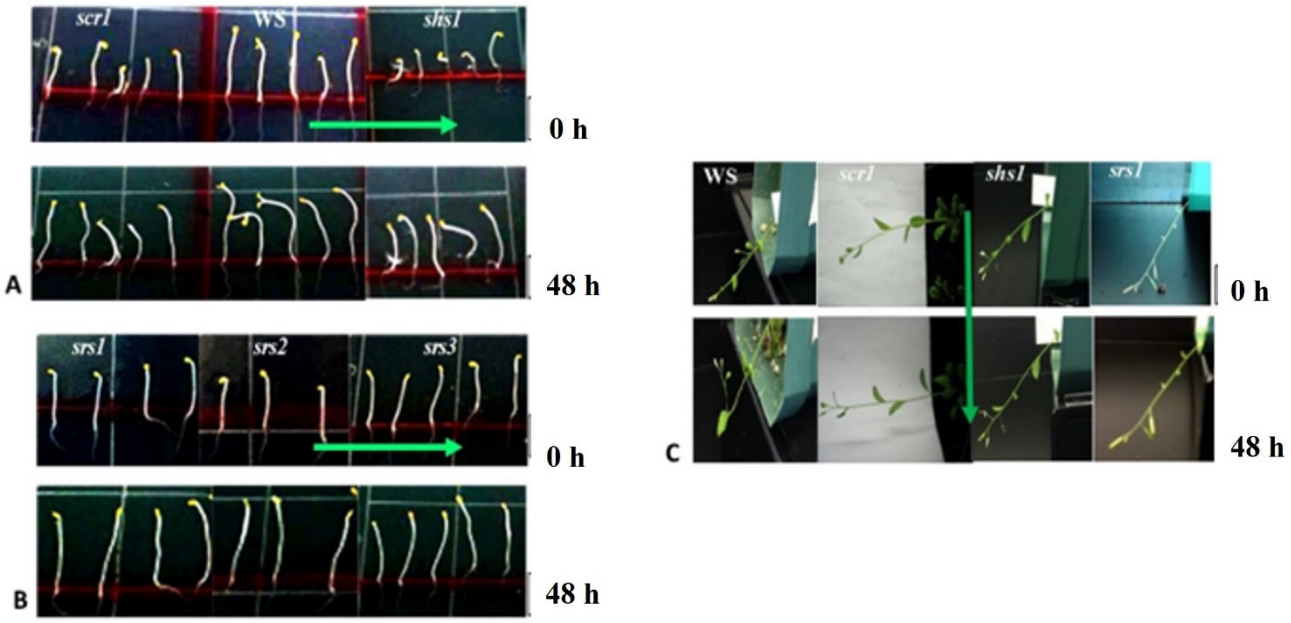

Figure 3. Hypocotyl and Inflorescence gravitropism in *A. thaliana*. (**A**) Hypocotyl gravitropic responses in the dark of the *scr1*, WS and *shs1*. The seedlings of *scr1*, WS and *shs1* (*scr* hypocotyl gravitropic suppressor1) are shown at reorientation (0 h) and 48 h (48 h) after reorientation. Arrows indicate the direction of the gravity vector. (**B**) Hypocotyl gravitropic responses in the dark of *srs1–3*. The seedlings of *srs1–3* are shown at reorientation (0 h) and 48 h (48 h) after reorientation. Arrows indicate the direction of the gravity vector. (**C**) Inflorescence gravitropic response in the dark of the WS, *scr1*, *shs1* and *srs1* (*scr root length suppressor1*). Inflorescences of the WS, *scr1*, *shs1* and *srs1* are shown at reorientation (0 h) and 24 h (24 h) after reorientation in the dark. Arrows indicate the direction of the gravity vector.

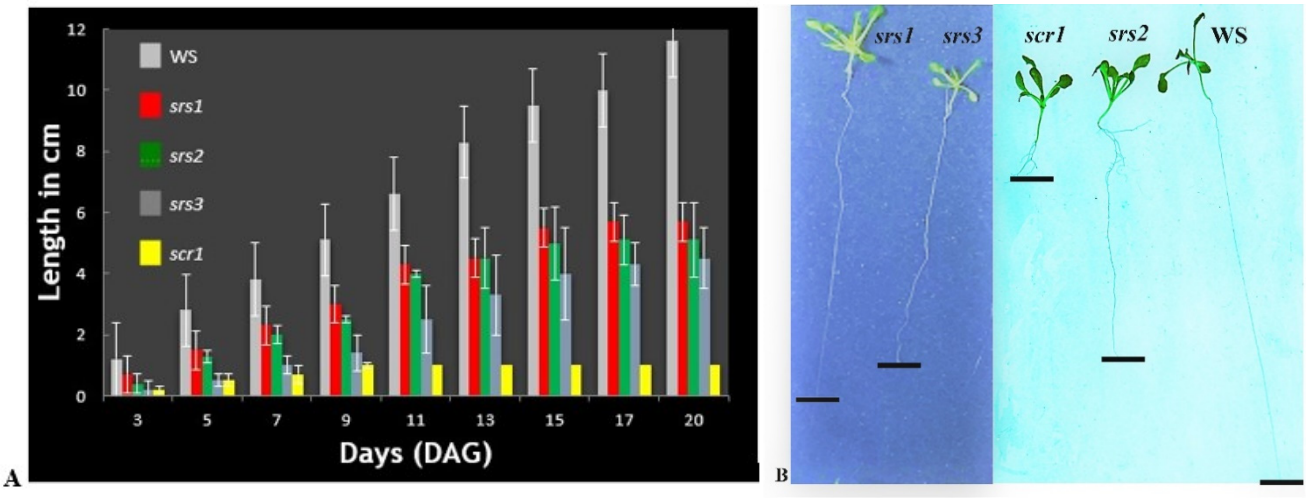

Figure 4. Root growth analysis of *A. thaliana*. (**A**) Root growth of WS, *srs1–3* and *scr1* were measured at different time points (points at the X-axis). Three suppressors *srs1*, *srs2* and *srs3* exhibit significantly longer roots than *scr1* but shorter than WS. Error bars represent means ± standard deviation (SD). (**B**) Root length of *srs1*, *srs3*, *scr1*, *srs2*, and WS at 20 days after germination (DAG).

3.4. Genetic Analysis of Suppressors

All suppressors were backcrossed to *scr1* and the F1 and F2 progeny were analyzed. All the F1 progeny of backcrosses of *shs* and *srs* suppressors with *scr1* mutant were agravitropic

and had short roots, respectively. The χ^2 analysis confirmed that the F2 progeny fit the ratio of 3:1 (Tables 1 and 2), confirming that each suppressor represents a single recessive allele.

Table 1. Backcross of *A. thaliana scr* hypocotyl gravitropic suppressors with *scr1*. F2 generation progenies of *scr1* X *shs suppressors* were analyzed to test the hypocotyl agravitropic/gravitropic ratio. $\chi^2 < 3.84$ is nonsignificant at 0.05 probability level.

F2 Generation of Cross	Sample Size	Hypocotyl Gravitropic	Hypocotyl Agravitropic	Segregation Ratio (Agravi./Gravi.)	$\chi^2 < \chi^2$ 0.95 = 3.841	$p > 0.05$
scr1 male X *shs1* female	167	126	41	3.05:0.95	$\chi^2 = 0.017$	$p > 0.05$
scr1 male X *shs2* female	150	92	58	2.5:1.5	$\chi^2 = 2.4$	$p > 0.05$
scr1 male X *shs3* female	144	113	31	3.13:0.86	$\chi^2 = 0.925$	$p > 0.05$
scr1 male X *shs4* female	156	120	36	3.07:0.92	$\chi^2 = 0.307$	$p > 0.05$
scr1 male X *shs5* female	138	108	30	3.13:0.87	$\chi^2 = 0.782$	$p > 0.05$
scr1 male X *shs6* female	323	249	74	3.08:0.92	$\chi^2 = 0.752$	$p > 0.05$
scr1 male X *shs7* female	281	218	63	3.1:0.9	$\chi^2 = 0.997$	$p > 0.05$
scr1 male X *shs8* female	173	123	50	2.84:1.16	$\chi^2 = 1.404$	$p > 0.05$
scr1 male X *shs9* female	192	149	43	3.1:0.9	$\chi^2 = 0.694$	$p > 0.05$
scr1 male X *shs10* female	313	237	76	3.03:0.97	$\chi^2 = 0.0862$	$p > 0.05$

Table 2. Backcross of *A. thaliana scr root length* suppressors with *scr1*. F2 generation progeny of *scr1* X *srs suppressors* were analyzed to test short root/long root ratio. $\chi^2 < 3.84$ is nonsignificant at 0.05 probability level.

F2 Generation of Cross	Sample Size	Short Root (~1.5 cm)	Long Root (~2.7–5 cm)	Segregation Ratio	$\chi^2 < \chi^2$ 0.95 = 3.841	$p > 0.05$
scr1 male X *srs1* female	96	75	21	3.125:0.875	$\chi^2 = 0.5$	$p > 0.05$
scr1 male X *srs2* female	156	124	32	3.179:0.820	$\chi^2 = 1.66$	$p > 0.05$
scr1 male X *srs3* female	132	104	28	3.151:0.848	$\chi^2 = 1.009$	$p > 0.05$

The complementation test is a method used to determine whether two mutations associated with a similar phenotype are in the same gene (alleles) or are alleles of two different genes involved in the same pathway. The complementation test is appropriate for recessive mutations and, as shown earlier, all the suppressors were single-gene recessive alleles. In the complementation test, the suppressors were crossed with each other. If the mutations of both suppressors are in the same gene, then all F1 progeny should have a suppressor phenotype. However, if suppressors have mutations in two different genes, all F1 progeny should have a parent *scr1* phenotype, and thus the two genes complement each other. Failure to complement suggests that two mutations lie in the same gene and are allelic.

The pair-wise crosses between 10 hypocotyl gravitropic suppressors and between three root growth suppressors were performed. The F1 generation progeny of *shs1*X*shs2*, *shs1*X*shs5*, *shs6*X*shs7* and *shs6*X*shs4* showed hypocotyl gravitropic responses over the level of *scr1* (Supplementary Figure S4). Therefore, *shs1*, *shs2* and *shs5* represented one complementation group. Similarly, *shs4*, *shs6* and *shs7* were in another complementation group. The F1 progeny of all other pair-wise crosses between *shs1*, *shs3*, *shs4*, *shs8*, *shs9* and *shs10* showed hypocotyl agravitropism (same response as *scr1*). The χ^2 analysis confirmed that F2 generation progeny segregation fit 9:7 ratios for agravitropic and gravitropic seedlings, respectively (Table 3). This suggests that *shs1*, *shs3*, *shs4*, *shs8*, *shs9* and *shs10* were all alleles of different genes. Eventually, all ten *scr* hypocotyl gravitropic suppressors were grouped

into six complementation groups that represented six different loci (genes) involved in the hypocotyl gravitropic pathway.

Table 3. Complementation test analysis of *A. thaliana* hypocotyl gravitropic suppressors. F2 generation progenies of *crossed suppressors* were analyzed to test the hypocotyl agravitropic/gravitropic ratio. $\chi^2 < 3.84$ is nonsignificant at 0.05 probability level.

F2 Generation of Cross	Sample Size	Hypocotyl Gravitropic	Hypocotyl Agravitropic	Segregation Ratio (Agravi./Gravi.)	$\chi^2 < \chi^2$ 0.95 = 3.841	$p > 0.05$
shs1 male X *shs3* female	86	57	29	10.6:5.4	$\chi^2 = 3.5$	$p > 0.05$
shs1 male X *shs4* female	133	83	50	9.98:6.02	$\chi^2 = 2.04$	$p > 0.05$
shs1 male X *shs8* female	181	100	81	8.8:7.2	$\chi^2 = 0.073$	$p > 0.05$
shs1 male X *shs9* female	110	66	44	9.2:6.8	$\chi^2 = 0.624$	$p > 0.05$
shs1 male X *shs10* female	250	142	108	9.01:6.99	$\chi^2 = 0.022$	$p > 0.05$
shs3 male X *shs4* female	208	121	86	9.3:6.7	$\chi^2 = 0.312$	$p > 0.05$
shs3 male X *shs8* female	348	203	145	9.33:6.67	$\chi^2 = 0.613$	$p > 0.05$
shs3 male X *shs9* female	410	239	171	9.32:6.68	$\chi^2 = 0.699$	$p > 0.05$
shs3 male X *shs10* female	129	76	53	9.43:6.57	$\chi^2 = 0.108$	$p > 0.05$
shs4 female X *shs8* male	510	287	223	9:7	$\chi^2 = 0.001$	$p > 0.05$
shs4 female X *shs9* male	301	178	123	9.46:6.54	$\chi^2 = 1.018$	$p > 0.05$
shs4 female X *shs10* male	165	100	65	9.7:6.3	$\chi^2 = 1.272$	$p > 0.05$
shs8 male X *shs9* female	258	168	90	10.4:5.6	$\chi^2 = 2.4$	$p > 0.05$
shs8 male X *shs10* female	340	199	141	9.36:6.67	$\chi^2 = 0.717$	$p > 0.05$
shs9 male X *shs10* female	244	134	110	8.78:7.22	$\chi^2 = 0.175$	$p > 0.05$

F1 progeny of pair-wise crosses between *srs1*, *srs2*, and *srs3* showed short root phenotypes. Their F2 generation progeny segregation fit 9:7 ratios for the short root and long root phenotypes, respectively (Table 4). These data suggest that *srs1*, *srs2*, and *srs3* are alleles of different genes. In conclusion, three *scr* root length suppressors were grouped into three complementation groups that represented three different loci (genes) involved in the root growth.

Table 4. Complementation test analysis of *A. thaliana* root length suppressors. F2 generation progeny of *crossed suppressors* was analyzed to test the short root/long root ratio. $\chi^2 < 3.84$ is nonsignificant at 0.05 probability level.

F2 Generation of Cross	Sample Size	Short Root (0.5–2 cm)	Long Root (2.5–6.0 cm)	Segregation Ratio	$\chi^2 < \chi^2$ 0.95 = 3.841	$p > 0.05$
srs1 female X *srs2* male	500	285	215	9.12:6.88	$\chi2 = 0.114$	$p > 0.05$
srs1 male X *srs3* female	410	246	164	9.6:6.4	$\chi2 = 2.34$	$p > 0.05$
srs2 female X *srs3* male	346	205	141	9.48:6.52	$\chi2 = 1.264$	$p > 0.05$

3.5. Phenotypic Characterization of Suppressors

The hypocotyl is the embryonic stem that bears the cotyledons (embryonic leaves) and plumule. From apex to base, the Arabidopsis hypocotyl has approximately twenty cells and after germination, no significant cortical and epidermal divisions occur [43]. However, the hypocotyl may increase more than 10-fold in length with cell elongation being entirely responsible for this postembryonic hypocotyl growth. Numerous factors, such as light, temperature, touch, and plant hormones, have a strong influence on hypocotyl

elongation [44,45]. When grown in the dark, the WT hypocotyls are at least 1.5-fold longer than that of the *scr1* mutants. However, all the suppressors have similar hypocotyl length to the *scr1* mutant (Figure 5A). No significant difference between hypocotyl lengths of *scr1* and any of the suppressors were observed on day seven after germination in the dark.

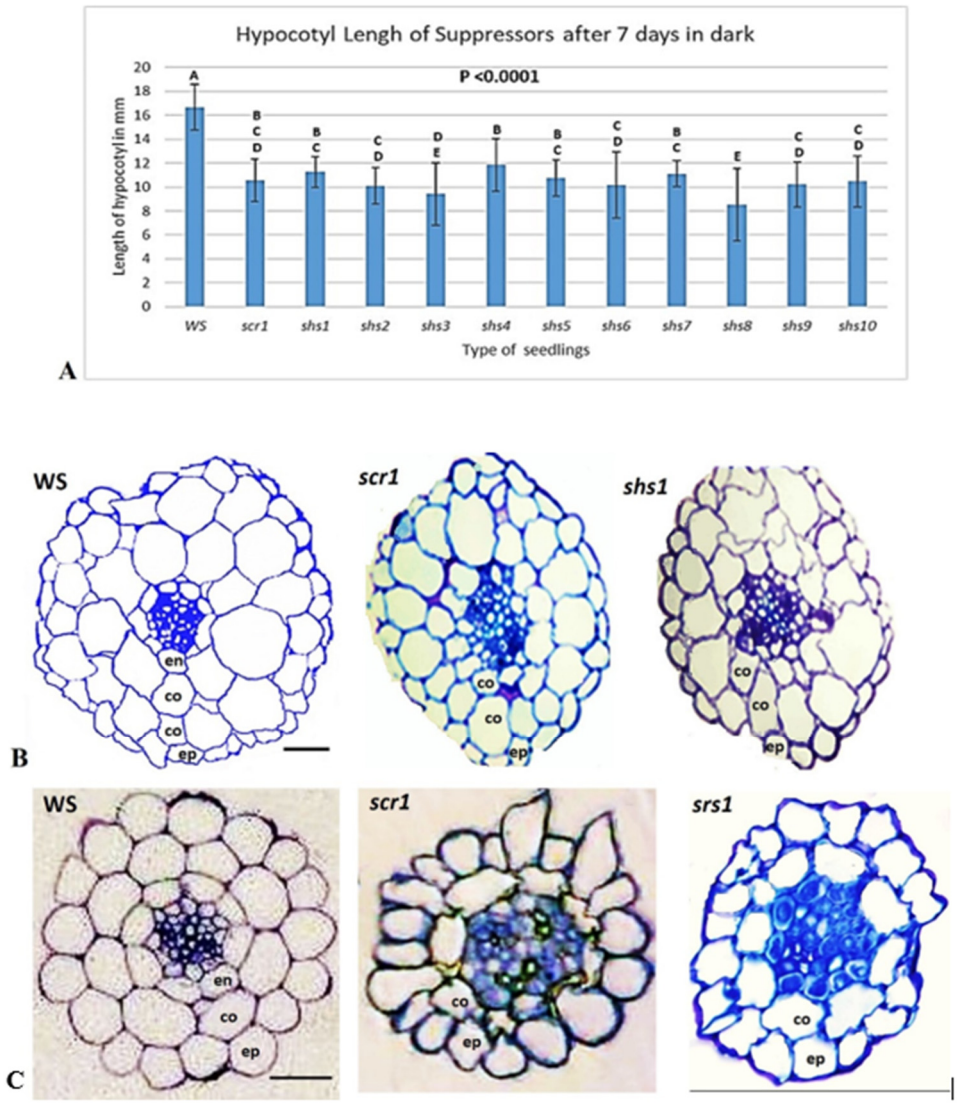

Figure 5. Phenotypic characterization of *A. thaliana* suppressors (**A**) Hypocotyl lengths. Seedlings of hypocotyl gravitropic suppressors *shs1* to *shs10*, along with wildtype (WS) and *scr1* were grown in dark for seven days after germination. Length of hypocotyls was measured in mm. The X-axis represents the type of seedling (genotype), and Y-axis represents the length in mm. Error bars represent the means ± standard deviation (SD). The lack of shared letters on the bars indicates a significant difference. To determine the significant differences between wild type and mutant treatments, we used ANOVA followed by Fisher's test. (**B**) Cross-section of WS, *scr1*, and *shs1* hypocotyls. Seedlings were grown in dark for three days after germination. WS has endodermis followed by two ground tissue layers (two cortex), and epidermis. The endodermal layer is missing in both *scr1* and *shs1*. Scale bar, 50 µm. (**C**) Cross-section of WS, *scr1*, and *srs1* roots. WS has endodermis followed by one ground tissue layer (cortex), and epidermis. The abnormal radial pattern displayed by *scr1* and *srs1* roots is shown. en, endodermis; co, cortex; ep, epidermis. Scale bar, 50 µm.

In *A. thaliana* both the root and shoot exhibit an invariable radial pattern of cell arrangement. In roots, the stele that includes the vascular system is surrounded by the single layers of endodermis, cortex, and epidermis. There has been report of the *scr* of

A. thaliana exhibiting abnormal internal architecture (one missing ground tissue layer) in all organs examined [11]. To determine the radial pattern of all the suppressors, the cross-sections of the hypocotyl of WT, *scr1,* and all ten *shs* and cross-sections of the root of WT, *scr1* and all three *srs* were generated and observed under a microscope (Figure 5B,C). The radial patterns of the hypocotyl of all ten *shs* were similar to *scr1* mutant (Figure 5B, shown only for *shs1*). Similarly, radial patterns of the root of all three *srs* were similar to the *scr1* mutant root shown in Figure 5C, (shown only for *srs1*). The suppressors have not rescued the radial pattern defects observed in *scr1* mutants.

To gain a better understanding of the gravity-sensing mechanism in plant shoots and to confirm the most favored hypothesis of this complex mechanism—the "starch-statolith hypothesis", which postulates that gravity sensing involves sedimentation of amyloplasts (statoliths) in specific cells (statocytes) [18,46–48]—whole-mount amyloplast staining was performed. It has been reported that in *scr* of *A. thaliana* no hypocotyl layer contains sedimented amyloplast [14]. The results show that all *shs* suppressors resemble *scr1* for the presence of sedimented amyloplast in hypocotyl layers rather than the WS (Figure 6, shown only for *shs1*). As shown in Figure 6, when seedlings were grown on MS agar plate without sucrose and were stained with IKI solution the presence of sedimented amyloplast was detected only in the WS endodermal layer while in *scr1* and *shs* suppressors no sedimented amyloplasts could be detected.

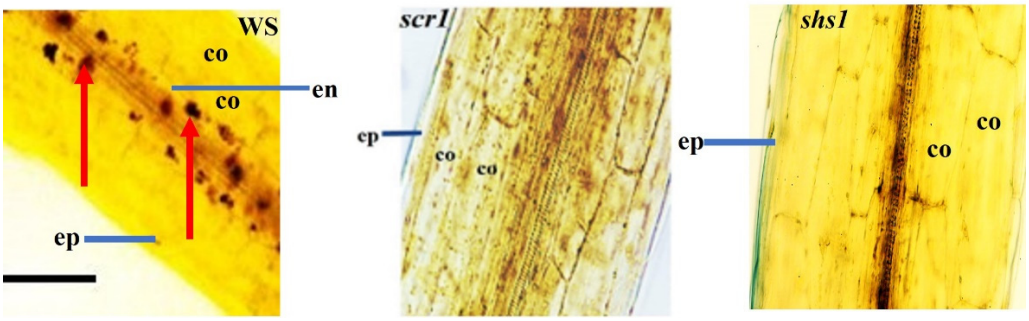

Figure 6. Analysis of Amyloplast sedimentation in hypocotyl of *A. thalian suppressors.* Whole- mount staining of starch in amyloplasts of hypocotyl of WS, *scr1* and *shs1* was performed. The red arrow indicates the sedimented amyloplasts in response to gravity. en, endodermis; co, cortex; ep, epidermis. Scale bar, 50 μm.

4. Discussion

We used EMS mutagenesis on *scr 1* mutant *A. thaliana* to screen for suppressor mutations to identify other genes involved in the SCR-regulated developmental pathways. Thousands of *scr1* mutant seeds were mutagenized with EMS and ~3000 independent lines were screened to identify potential *scr1* suppressor lines. After two rounds of screening, thirteen suppressor lines were selected as confirmed true *scr1* suppressors. Ten suppressors rescued only hypocotyl agravitropic phenotype and were named *scr* hypocotyl gravitropic suppressors *(shs1–10).* The other three suppressors exhibited only longer root phenotype and were named as *scr* root length suppressors *(srs1–3).* These suppressors might find application in the study of developmental pathways in plants.

All suppressors were shown to lack the functional copy of the SCR gene or any detectable level of SCR transcripts. These results confirm that all suppressors identify second-site mutations and not *scr1* revertants. The backcross results indicate that each suppressor represents a recessive allele of a single locus. Furthermore, the complementation tests established that ten of the *shs* suppressors fall into six complementation groups and three *srs* suppressors fall into three complementation groups. Thus, the suppressors correspond to six loci involved in the hypocotyl gravitropic pathway, and three loci participated in the root meristem maintenance pathway.

In addition to the identification of *shs* suppressors, we also determined that *scr1* itself is not completely agravitropic and retains some residual hypocotyl gravitropic response (Figure 2). All three *srs* suppressors showed gravitropic responses similar to *scr1* mutants (Figure 3B), thus indicating that these three loci are functionally unrelated to gravitropism.

Several genes that play key roles in gravitropism have been identified with the help of mutational analysis [20,49]. Characterizations of these genes have suggested that root, hypocotyl, and inflorescence stem do not share identical pathways for their gravitropic responses [50,51]. Mutation in the *SCR* gene is only responsible for the shoot (hypocotyl and inflorescence stem) agravitropism because *scr* roots have normal (wildtype) root gravitropism in response to the gravity vector [14,52]. All thirteen of the suppressors exhibited complete inflorescence agravitropism similar to the *scr1* (Figure 3C). As expected, the roots of all thirteen suppressors have WT positive gravitropic responses. These observations support the theory that root, hypocotyl and inflorescence stem did not have identical pathways or molecular mechanisms for their gravitropic responses [53–56]. It is also possible that hypocotyl suppressor genes only work in the hypocotyl gravitropic pathway and are not involved in the gravitropic pathway of other organs.

Three *scr* root length suppressors showed improvement in their root growth. The root length of all three root suppressors is greater than *scr* mutants but below the level of wildtype plants (Figure 4A). However, all the root growth suppressors still had determinate root growth. All ten hypocotyl gravitropic suppressors did not show any significant improvement in their root growth (Supplementary Figure S3). The only phenotype rescued by the *srs* is longer root length; therefore, it is highly likely that root suppressor genes engage in root development. The indeterminate type of root growth phenotype requires that the *SCR* gene be expressed in the quiescent center (QC). Recent studies performed on *A. thaliana* suggest that SCR maintains the stem cell niche in the root by enhancing the expression of genes that control telomere integrity [57]. It has also been reported that when the *SCR* gene is only expressed in QC of *scr* mutant roots, only indeterminate root growth was rescued; however, plants still exhibited an abnormal radial pattern [21,42]. All three *srs* mutants showed the abnormal radial pattern as in *scr1*, suggesting that *srs* genes function in QC and are possibly involved in meristem maintenance.

The orientation of germinating seedlings in the absence of light is mostly dictated by the hypocotyl gravitropism. Therefore, hypocotyl gravitropism is an essential phenotype for plant survival and seedling growth following germination. It is noteworthy that we identified six different genes involved in the hypocotyl gravitropic pathway suggesting that there may be at least one alternative pathway to achieve this essential phenotype to ensure seedling survival.

Since numbers of hypocotyl cells are fixed at an embryonic stage, hypocotyl length depends upon cell elongation. Mutant *scr1* plants have much shorter hypocotyls than the WS (Figure 5A), suggesting that *scr1* hypocotyl may be defective in cell elongation. When considering that asymmetric cell elongation is essential for the gravitropic response, it could be speculated that the weak gravitropic response of *scr1* mutant hypocotyls is due to impaired cell elongation. To address this hypothesis, the lengths of the hypocotyls of all suppressors, grown in the dark were examined. The hypocotyl lengths of all the suppressors were very similar to *scr1* but significantly shorter than WS (Figure 5A). These results suggest that hypocotyl length and thus normal cell elongation might not be an essential factor for the gravitropic response. These data also suggest that *SCR* was essential for normal hypocotyl cell elongation, and that none of the suppressor genes participate in hypocotyl cell elongation.

We have shown that all six *shs* rescued the gravitropic responses but neither the radial pattern of the hypocotyl architecture nor amyloplast sedimentation in any endodermal layers was restored (Figures 5B and 6). Amyloplast sedimentation is entirely absent in *scr1* mutants; however, it was observed that *scr1* itself retains some residual gravitropic response. These findings support the view that the presence of normal statocyte cells and/or amyloplast sedimentation are not necessary for gravitropic sensing and hypocotyl

gravitropic perception relies on at least two separate mechanisms [17,19,58,59]. It has been postulated that the gravireceptors lie in between the plasma membrane and the cell wall and would be mechanically stimulated for the initial sensing of gravity vector in any mechanism [42,58]. According to the "gravitational pressure model", the reorientation of the organ can cause a subtle change in cytoplasmic pressure, and is sufficient to generate a perceivable signal [60,61]. The gravireceptors could perceive subtle changes in compression pressure resulting from the reorientation of protoplast in the displaced organs [62]. As is the case of *scr1* mutants and hypocotyl gravitropic suppressors, perhaps changes in pressure of cytoplasm are responsible for gravity perception. The stronger hypocotyl gravitropic responses of suppressors, even though they have not rescued the amyloplast sedimentation and WT architecture, could be because the gravireceptors have an enhanced sensitivity to the detection of any subtle changes in the cytoplasmic pressure and gravity vector [63,64]. Furthermore, it can be speculated that since SCR is a transcription factor and is required for the expression of downstream genes in several developmental pathways [65], it could be responsible for turning "on" or "off" other genes involved in shoot gravitropic pathways. It could be that some of the suppressors have a mutation in regulatory regions regulated by the SCR. The mutation may eliminate the need for SCR to turn it "on" or "off." Another possibility is that the gene products need to interact directly with the SCR protein to be activated, and that the mutation produces a constitutively active product. Until the identities of mutated genes are revealed, we can only speculate on the type of product they encode and their involvement in gravity sensing. Finally, these data indicate that hypocotyls can perceive gravity even in the absence of the endodermal layer and amyloplast sedimentation. This also suggests that *scr1* mutants might be primarily defective in gravity sensing mechanisms and no other aspects of gravitropic responses.

5. Conclusions

Thirteen confirmed suppressors have been identified by *scr* suppressor screening of *A. thaliana*. Ten hypocotyl gravitropic suppressors that represent six different genes involved in hypocotyl gravitropic pathway and three root length suppressors that represent three different genes involved in root development pathway were identified. None of the suppressors exhibited both a hypocotyl gravitropic response and a long root phenotype. No suppressor with improved inflorescence gravitropism was identified. Each of these suppressors represents a recessive allele of a single locus. Hypocotyl gravitropic suppressors rescued only the hypocotyl gravitropic phenotypes, while they retain abnormal radial patterns and do not exhibit the amyloplast sedimentation. These results indicate that in hypocotyl there is at least one alternative gravity sensing pathway that does not involve the endodermal layer and/or amyloplast sedimentation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb13040041/s1, Figure S1. Schematic of SCR gene in scr1 allele with the position of T-DNA and PCR primers presented, Figure S2. RT-PCR analysis of suppressors, Figure S3. Root growth of hypocotyl gravitropic suppressors (shs), Figure S4. Complementation test analysis.

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