ISOLATION AND CHARACTERIZATION OF SCARECROW SUPPRESSOR MUTANTS IN *Arabidopsis thaliana*

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ISOLATION AND CHARACTERIZATION OF SCARECROW SUPPRESSOR MUTANTS IN *Arabidopsis thaliana*

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Date of graduation

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THESIS ABSTRACT

ISOLATION AND CHARACTERIZATION OF SCARECROW SUPPRESSOR MUTANTS IN *Arabidopsis thaliana*

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SCARECROW (SCR) is a transcriptional regulator that plays key roles in several developmental processes in a model plant *Arabidopsis thaliana*. SCR orthologs have been identified in agriculturally important plants such as rice and corn. It has also been demonstrated that at least some of the SCR functions have been conserved throughout plant kingdom. In *Arabidopsis*, among its many functions SCR is essential for the maintenance of root meristem, the development of root and shoot endodermis and for normal shoot gravitropism. As a result, *scr* mutants exhibit many phenotypic defects relating to these functions such as short roots, short hypocotyls, small leaves, shoot agravitropism as well as cell layer deletions in both roots and shoots. We have initiated a genetic screen to identify mutants that suppress *scr* mutant phenotype(s). In this *scr* suppressor screen we aim to identify and characterize other components of SCR-regulated pathways. Seeds homozygous for *scr1* were mutagenized with EMS,

germinated and grown to maturity. Seeds were collected in pools from approximately100 plants in each pool. All the seeds from the first 29 pools, representing approximately 1800 independent lines were screened for suppressor phenotypes. Over 200 potential suppressors were isolated. The majority of the putative suppressors had either longer roots or showed some degree of hypocotyl gravitropism. The potential suppressors were grown to maturity and seeds were collected from all the surviving and fertile plants. We collected seeds from 130 of the primary isolates. All of them were retested for suppressor phenotype and those that still had longer roots or gravitropic hypocotyls were tested for the presence of *scr1* allele. Six of the primary isolates have been confirmed as *scr* suppressors. Five of these mutants display hypocotyl gravitropism and only one has a significantly increased root length. Five of the isolated suppressors represent at least two genes involved in hypocotyls gravitropism. Only one of the identified suppressors functions in root meristem maintenance. This suppressor, 24R1, was selected for mapping analysis. 24R1 plants were crossed to *scr3,* which is in a different genetic background. One quarter of the progeny from the cross has long roots indicating that the suppressor is not allele specific. DNAs isolated from individual plants with long root phenotype were used for mapping using molecular markers. Two to three Cleaved Amplified Polymorphic Sequences (CAPS) markers were used for each of the five *Arabidopsis* chromosomes. The only linkage was shown for chromosome 4. The mutation responsible for the suppressor phenotype in 24R1 maps to long arm of chromosome 4 about 21cM away from the closest CAPS marker available. This chromosomal region contains 22 genes that may correspond to 24R1 suppressor.

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INTRODUCTION

Plants are the major autotrophic life forms on earth that play a pivotal role in ecological balance. Plants are sessile therefore, it is essential that they start their postembryonic development in the right location. Embryos of seed plants have the basic body plan set up during embryogenesis. After germination, seedlings grow and develop primarily through the proliferation of stem cells located at both poles of the embryo. All the aerial parts of the plant like leaves, stem, flowers etc., develop from shoot apical meristem (SAM), while the primary root develops from root apical meristem (RAM). The post embryonic organs are generated from SAM and RAM through a regulated program that specifies the timing of cell divisions, the orientation of the plane of cell divisions and the extent of cell expansion (Steeves and Sussex, 1989). This regulated program generates and maintains correct pattern within growing plant organs throughout the life. The program by which the correct pattern is maintained is not clearly defined and much insight is necessary to understand the mechanism by which it is attained.

The basic developmental processes appear to be similar in most the higher plants. Therefore, elucidation of developmental pathways in one plant can lead to an understanding of these processes in other plants. *Arabidopsis thaliana* is a model system for studying plant growth and development. There are many advantages for using *Arabidopsis thaliana* as a plant model organism. Some of them are the availability

of many molecular and genetic tools, such as sequence information, availability of mutants and clones and other resources. In addition to this, *Arabidopsis thaliana* plants are small, have short generation time as well as very few culture requirements. Therefore, large number of plants can be maintained easily in a laboratory.

Isolation of mutants with desired phenotypes and identification of the corresponding genes is an important first step in defining key regulatory components of a pathway. Scarecrow (SCR) gene, the major focus of our laboratory, was initially identified in mutant screen for *Arabidopsis* root mutants (Benfey *et al.,* 1993). *Arabidopsis* wild type (WT) has indeterminate root growth but *scr* mutant seedlings display determinate mode of root growth. The WT root continues to grow throughout plant's life. The *scr* roots grow slower than WT and eventually stop growing at a specific point that is characteristic for each allele; for example: *scr1* roots usually do not grow beyond 2 cm (Sabatini *et al.,* 2003; this thesis). In addition to short root phenotype, *scr* roots also have defects in their internal architecture (Di Laurenzio *et al.*, 1996).

Roots contain three distinguishable regions along their length that represent all the developmental stages. The external and internal morphology of *Arabidopsis* roots shows these three distinct but overlapping zones. At the tip, the meristematic zone is characterized by small cells overlaid by root cap, the elongation zone just above it contains elongating cells and finally the differentiation zone consists of mature fully elongated cells. Within the differentiation zone of *Arabidopsis* primary roots cells are organized in a stereotypical radial pattern (Figure 1A). This pattern consists of single

layers of epidermis, cortex and endodermis surrounding the stele which includes vascular elements (Dolan *et al.,* 1993; Figure 1A).

Root radial pattern is established during embryogenesis and perpetuated by divisions of stem cells called initials, in postembryonic RAM. Fate mapping has shown a distinct clonal relationship between cell layers and their initials (Scheres *et al.,* 1994; Wysocka-Diller and Benfey, 1997). These initials are located within the central portion of the basal root tip within root meristem (Figure 1B). They surround four infrequently dividing cells called the quiescent center (QC). The entire set of initials and QC together work as a construction center for postembryonic root development. All the mature tissues of primary roots are derived from only four types of initials (Figure 1B). They are columella root cap, epidermis/ lateral root cap, cortex/endodermis and vascular initials. The columella initials give rise to columella root cap at the basal tip. The epidermis/lateral root cap initials give rise to both epidermis and lateral root cap. The vascular initials produce all the cells of stele. Cortex/endodermis initials (C/E) give rise to the two ground tissue layers that are affected in *scr* mutants (Figures 1 and 2). C/E initial undergoes two consecutive asymmetric divisions to generate two distinct mature cell layers (Figure 1C). C/E initial first divides anticlinally (transversely) producing another initial and a daughter cell. The daughter cell divides again, this time periclinally (longitudinally) and gives rise to the progenitors of two distinct cell layers. These cell layers are cortex located just inside of epidermis and more internally endodermis (Dolan *et al.,* 1993; Heidstra *et al.,* 2004; Figure 1).

Laser ablation experiments of cells within RAM shed some light on QC and initial functions (Van den Berg *et al.,* 1997). It was shown that if any initial cell is

destroyed, many different cells can replace the destroyed initial cell and take over its function (Van den Berg *et al.,* 1997). However, ablation of any QC cell results in cell differentiation of initials which are in direct contact with those ablated cells (Van den Berg *et al.,* 1997). It was concluded that QC is necessary to maintain initial cells in their undifferentiated state (Van den Berg *et al.,* 1997).

Further support for the role of QC in maintaining initials in their undifferentiate state came from elegant experiments by Sabatini *et al.,* (2003). It was known that older *scr* roots lack meristematic zone resulting in the presence of differentiated cells all the way at the root tip (Wysocka-Diller *et al.,* 2000). Sabatini *et al*., (2003) took advantage of this *scr* phenotype to demonstrate that QC functions in meristem maintenance and this function requires SCR expression. When *scr* mutants express SCR transgene only in QC the roots grow indeterminately and meristematic zone is maintained (Sabatini *et al.,* 2003). This finding suggests that SCR functions in a cell autonomous fashion in the root. SCR expression in QC is necessary for the meristem maintenance by QC cells. However, expression in QC alone cannot bypass the need of SCR in C/E initial for generation of WT radial pattern. This conclusion is further supported by the finding that when SCR is expressed in C/E alone only the radial pattern is restored in *scr* roots but the roots still grow determinately (Sabatini *et al.,* 2003).

In summary, SCR has at least two separate functions in root growth and development. It is essential for indeterminate root growth via its expression in QC and regulation of QC identity. In addition, SCR must also be expressed in C/E initials to regulate the development of two distinct cell layers.

SCR gene has been shown to be involved in processes other than radial patterning of roots and root meristem maintenance. For example, SCR gene is expressed in shoot endodermis where its expression appears to be important for gravitropism.

Plants because they are sessile have mechanisms to reorient their organs depending on environmental conditions or their needs. Directional changes of growth of plant organs called tropisms occur in response to several environmental cues/stimuli. Among these phototropism and gravitropism are of the greatest importance. Gravitropism can be broken down to three consecutive steps: perception of gravity vector by specialized cells, transduction of the resulting signal to the surrounding tissues and coordinated asymmetric growth of the organ. At least three organs of *Arabidopsis* show gravitropism. These organs are hypocotyls, inflorescence stem and roots. Hypocotyls and inflorescence stems grow upwards against gravity thus they show negative gravitropism. Roots those grow downward in the direction of gravity vector show positive gravitropism. Analyses of various agravitropic mutants demonstrate that only some of the molecular mechanisms underlying gravitropism are shared among all of the responsive organs. However, some of the components of gravitropism are clearly organ specific (Tasaka *et al.,* 1999). For example *scr* mutants have agravitropic shoots but gravitropic roots, while, *rhg* mutants show abnormal gravitropism in their roots and hypocotyls but normal gravitropism in inflorescence stems (Fukaki *et al.,* 1997).

Gravity perception is a very complex process and many hypotheses have been proposed to explain this process. According to “statocyte-statolith model”, plants contain specialized cells (statocytes – columella root cap cells in roots and endodermal cells in shoots) which are the sites for gravity perception. These cells contain

amyloplasts (statoliths) that sediment in response to the gravity vector. The sedimented amyloplasts are covered with cytoskeleton meshwork and interact with intracellular membrane and/or plasma membrane for signal transduction (Morita *et al.,* 2006). Alternatively, the "gravitational pressure model" proposes that any subtle change in the pressure of cytoplasm by reorientation of the organ is sufficient to generate a perceivable signal (Telewski, 2006). Both models propose that the gravireceptors lie in between plasma membrane and cell wall and are "stimulated" by either amyloplast sedimentation or protoplast pressure (Telewski, 2006).

This gravity perception causes a mechanical stimulus that triggers signal transduction mediated by either Ca^{2+}, calmodulin, inositol 1,4,5-triphosphate and/or pH changes. This signal causes asymmetric distribution of auxin in the organ. Auxin is involved in the asymmetric cell elongation leading to orientation changes of the organ with respect to gravity vector (Fukaki *et al.,* 1998; Silady *et al.,* 2004; Morita and Tasaka, 2004).

Mutations in *SCR* gene result in shoot agravitropism (Fukaki *et al.,* 1998). The agravitropism of hypocotyls and inflorescence stems correlates with morphological defects in these organs. The WT hypocotyl contains one layer of epidermis, two layers of cortex and one layer of endodermis surrounding the stele. In contrast, *scr* hypocotyls have two irregular cell layers between epidermis and stele in place of the original three ground tissue layers (Scheres *et al.,* 1995; Fukaki *et al.,* 1998). Amyloplasts (statoliths) are the starch containing plastids in the cell that are believed to function in gravity perception (Sack, 1997). Large sedimented amyloplasts are normally found in hypocotyl endodermis of WT. In contrast, *scr* hypocotyls contain only some small

amyloplasts in the two irregular ground tissue layers and these amyloplasts do not appear to sediment in response to gravity (Fukaki *et al.,* 1998). The inflorescence stem of WT contains one layer of epidermis, variable number of cortex layers and a single layer of endodermis. WT endodermis is the only cell layer that contains sedimenting amyloplasts (Fukaki *et al.,* 1998). In contrast, *scr* inflorescence stem does not contain any cell layer with sedimented amyloplasts (Fukaki *et al.,* 1998). Axial shoot organs in *scr* are agravitropic (Fukaki *et al*., 1998). The absence of sedimented amyloplasts in *scr* hypocotyls and inflorescence stems suggests that amyloplast sedimentation is essential for gravitropism (Fukaki *et al*., 1998). In roots, sedimenting amyloplasts are located only in central columella cells of the root cap. In *scr* roots, gravitropic responses are similar to those of WT roots (Fukaki *et al.,* 1998). This finding can be explained by the fact that *scr* columella cells contain sedimenting amyloplasts (Fukaki *et al.,* 1998).

SCR gene has been cloned and sequenced and the predicted SCR gene product contains a number of structural motifs found in transcription factors. SCR protein localizes to nuclei and binds to promoters (Pysh, 1999; Helariutta *et al.,* 2000; Cui *et al.*, 2007). SCR belongs to GRAS gene family. Total of 33 genes belong to GRAS family in *Arabidopsis thaliana.* Some of the related genes function in the same pathways as SCR, for example, Shortroot (SHR) (Nakajima *et al.*, 2001; Levesque *et al.,* 2006). *SCR* orthologs were identified in other plants. In peas (*Pisum sativum*), it is referred as *PsSCR* (Sassa *et al.,* 2001), *ZmSCR* in corn (*Zea mays*) (Lim *et al.,* 2000) and as *OsSCR* in rice (*Oryza sativa*) (Kamiya *et al.,* 2003). In addition to sequence homology, similarity of expression patterns of all SCR orthologs suggests the conservation of function. Similar to *AtSCR*, the *PsSCR*, *OsSCR* and *ZmSCR* genes are

expressed in a single cell file of the root, including the meristematic region. In shoots, as in roots, expression of *PsSCR* was similar to *AtSCR* (Sassa *et al.,* 2001). These structural and expression similarities support the hypothesis that *SCR* has a role in radial patterning of both roots and shoots (Sassa *et al.,* 2001). Therefore understanding the functions of SCR in *Arabidopsis* will be applicable to other plants.

Recent findings have shown that SCR together with another gene product SHR may bind to promoters of MAGPIE (MGP), SCL3 and NUTCRACKER (NUC). This SCR protein binding to targets was reduced in *shr* mutants and SHR protein binding to targets was reduced in *scr* mutants suggesting that both were dependent on each other for their effective function (Cui *et al.,* 2007). It has been found that JACKDAW (JKD) and MGP genes, which encode zinc finger proteins, interact with SCR and SHR to regulate root development (Welch *et al.,* 2007). The only genes whose expression may be regulated by SCR are also transcription factors that may interact with SCR by forming regulatory complexes (Welch *et al.,* 2007; Gallagher *et al.,* 2004; Sena *et al.,* 2004). Other SCR targets have not been identified to date.

In order to identify genes involved in SCR-regulated pathways we chose to use a functional approach. We began identification and characterization of other components in the *SCR*-regulated pathways through *scr* suppressor screen. The phenotype of each individual suppressor mutant should point to the specific pathway in which the corresponding gene is involved. Potential suppressor mutants may involve SCR targets that are either activated or inactivated by *SCR* (as *SCR* is a transcriptional regulator). The *scr1* mutants that were used in our suppressor screen were generated by T-DNA insertion within SCR coding region. This insertion leads to the loss of gene function.

Mutagenesis of a large number of *scr1* seeds with ethyl methane sulfonate (EMS) was carried out. EMS usually induces multiple point mutations by inducing C to T changes resulting in C/G to T/A substitution. The T-DNA length in *scr1* is about 17kb so it is highly unlikely that EMS could restore SCR function. Therefore, EMS generated mutants on *scr1* background with any improvement in phenotype would most likely represent second site mutations. These mutations would very likely affect genes that are involved in the same pathway as SCR.

MATERIALS AND METHODS

Plant Materials and Growth conditions:

Two wild type ecotypes of *Arabidopsis thaliana*; Wassilewskija (WS) and Columbia (Col) were used in this study. The *scr-1* mutant is on WS ecotype background (Di Laurenzio *et al.*, 1996) and *scr-3* mutant is on Col background (Fukaki *et al.*, 1996b). For screening and phenotypic characterization experiments, seeds were sterilized and placed on Murashige and Skoog media (Fukaki *et al.,* 1996b). Plates were stratified at 4^{o}C in dark for three days as described by Fukaki *et al.*,(1996b). For seed collection and studies on inflorescence stem gravitropic responses, plants were grown in pots under white light at 23 ± 1^{o}C in long day light conditions (16 hours light).

Mutagenesis:

Mutagenesis was done with ethyl methanesulfonate (EMS) as described by Levin *et al.,* (1998). Seeds of *scr1* were washed with 0.1% Tween-20 for 15 minutes, followed by mutagenesis with 0.2% ethyl methane sulfonate (EMS) for 12 hours. EMS treated seeds were washed with sterile water for 15 minutes, followed by several times with 0.1% Tween-20 for a total of 5.5 hours and sown on soil. First three batches of mutagenized seeds were sown on soil. Next batches of mutagenized seeds were plated on MS agarose plates containing 4.5% sucrose. Plates were stratified by placement in dark at

4°C for three days. Plates were transferred to growth chamber after 3 days and vigorous seedlings were transferred to soil two weeks after germination. Seeds were harvested in pools and each pool contained seeds collected from approximately 100 plants.

Screens for possible *scr1* suppressor mutants:

Primary Screen:

Surface sterilized, mutagenized seeds were placed in rows on MS agar plates containing 4.5% sucrose and were stratified in dark at 4°C for three days (Fukaki *et al.*, 1996b). The plates were placed in vertical position at 23±1°C under continuous light for germination. Plates containing germinated seeds were wrapped in foil and incubated in dark for one day followed by reorientation by 90° for 48 hours. Photos were taken before reorientation and 48 hours after reorientation. Seedlings whose hypocotyls showed response to new gravity vector were transferred to new plates. The original plates without gravitropic seedlings were left in the growth chamber under long day conditions in original vertical orientation for approximately two more weeks. Seedlings with improved phenotypes such as longer roots, longer leaves etc., were transferred to soil allowed to self pollinated, after which seeds were collected for further screening.

Secondary screen:

Progeny of primary screen selected seedlings were juxtaposed with *scr1* and WT on the MS agar plates containing 4.5% sucrose. Plates were subjected to experimental conditions as described in primary screen. Seedling response was compared with WT and

scr1. Seedlings that have shown improvement in phenotypic responses were selected as potential suppressors.

Confirmation of Genotype:

WT, *scr1* and confirmed *scr1* suppressor seeds were placed on MS agar plates containing Kanamycin and 4.5% sucrose as described by Fukaki *et al.*, (1996b).

DNA was extracted from WT, scr1 and potential kanamycin resistant suppressors as described by Edwards *et al.*, (1991; see below "DNA extraction"). The respective DNAs were amplified with SCRF344 5' ACCGTGGTGGTCGGAATGTTATGA, SCRR1956 5'AGTCGCTTGTGTAGCTGCATTTCC and T-DNA right border primer RBF3 5' CCAAACGTAAAACGGCTTGTC.

Amplification reactions were performed in PCR Sprint and the reactions were carried out in final volume of 25 µl. The PCR reactions contained 0.2mM of each primer, 1.5-3.0 mM $MgCl_2$ (optimized for each pair of primers), 0.5U Taq polymerase (Promega), 0.2mM each dNTP (Promega) and 50-100ng of DNA template. Amplification was initiated at 94^{o}C for 1 min followed by primer annealing temperatures of 60^{o} for 1 min, followed by extension at 72^{o}C for 2 min for 10 cycles. Later the entire program was repeated for 30 cycles with shorter denaturation time (20 sec) at 94^{o}C, primer annealing at 60^{o}C for 30 sec, 72^{o}C for 2 min and final elongation time at 72^{o}C for 10 min.

Phenotypic Characters:

Hypocotyl gravitropism:

Forty seeds of every confirmed suppressor, WT and *scr1*, were surface sterilized and placed on MS agar plates containing 4.5% sucrose and stratified at 4^{o}C for three days. To induce germination, plates were kept under continuous light. The plates containing newly germinated seedlings were covered with aluminum foil and kept in vertical position for 24 hours. All plates were photographed, foils were replaced and plates were reoriented by 90 degrees. Plates were photographed at 7 different intervals within 48 hours of reorientation. Gravitropic curvature was measured from photographic images (Kodak Image station 440CF). Curvature was calculated as the increment over the initial angle of each individual hypocotyl. Suppressors were categorized based on the curvature's mean and SD values.

Root growth rate and length:

Fifty seeds from each line were surface-sterilized and placed side by side with WT and *scr1* seeds. After 3 days in darkness at 4°C, the plates were placed in growth chamber under long day conditions (16 hours). Root growth was monitored by marking the position of the advancing root tip at 3 day intervals starting 3 days after germination (DAG) until 21 DAG. Suppressors were categorized based on the root length's mean and SD values.

Hypocotyl length:

WT, *scr1* and confirmed suppressor seeds were plated on MS plates containing 4.5% sucrose and incubated at 4^{o}C in dark for 3 days and germination was induced by exposing plates to continuous light. Plates with germinated seeds were divided into two groups. One group of plates was covered with aluminum foil and the other group of plates was left uncovered under the long day light. Hypocotyl length was measured on seven day old seedlings using standard methods.

Inflorescence Gravitropism:

The experiment on inflorescence gravitropism was performed on plants with inflorescence stems that were 6 to 8 cm in length as described by Fukaki *et al*., (1996a). Pots containing suppressor plants, *scr1* and WT plants (one plant/pot) were placed in dark on their sides. This was marked as the beginning of the experiment and was referred to as time T_o. Inflorescence gravitropic response was measured at 0.5, 1.0, 3.0, 12 and 15 hour interval. The curvature was measured from photographic images at each time interval with a protractor.

Histology:

For longitudinal optical sections, roots were cleared as follows. Four to six day old seedlings were incubated in methanol/HCl (2.3 ml methanol, 0.5 HCl and 9.2 ml H_2O) at 55^{o}C for 15 min followed by incubation in a solution containing 7% NaOH and 60% ethanol for 15 min at room temperature. Later, seedlings were incubated serially in different concentrations of ethanol (40% for 10 min followed by 20% for 10 min and

10% ethanol for 5 min) and finally in 5% ethanol: 25% glycerol for 10 min. Cleared seedlings were mounted in 50% glycerol and observed using Nomarski microscopy.

For cross sections of roots, six day old seedlings were embedded in 4% agarose and cross sectioned by hand using double edged razor blades. Fresh agarose embedded sections were processed as described by Brunett *et al.,* (1988) and were viewed under microscopy.

Mapping:

To map mutated gene in 24R1, the 24R1 suppressor (in ecotype WS) was crossed to *scr3* (in ecotype Col). 24R1 (male) pollen was used to pollinate *scr-3* (female) carpels. F_1 seeds were collected and placed on MS agar plates containing kanamycin and 4.5% sucrose. Germinated kanamycin resistant seedlings were later transferred to soil and grown to maturity. Seeds of F_2 population were collected from the self pollinated F_1 plants. F_2 seeds were sown on MS agarose plates containing 4.5% sucrose and seedlings with long roots were selected for DNA extraction. In total, 33 plants were used for mapping.

DNA Extraction:

DNA was extracted from plants as described by Edwards *et al.,* (1991). Extracted DNA was kept in freezer at -20^{o}C for further studies. For DNA extraction, 300µl lysis buffer (10mM Tris-HCl, pH 8; 25mM EDTA, pH 8 and 0.5% SDS) and plant tissue were placed into eppendorf tubes and the tissue was ground with pestle that fits in the eppendorf tubes. To this, another 300µl of the lysis buffer was added. These tubes were

incubated at 55^{o}C for 15 min to 1 hour. After incubation, the tubes were cooled to room temperature and the RNA was digested by adding 3µl RNaseA at 5mg/ml. This mixture was incubated at 37^{o}C for 15 min to 1 hour and later the proteins were precipitated by adding 200µl of 5M ammonium acetate. Tubes were centrifuged at 14000 rpm for 5 min. DNA containing supernatants were transferred to new tubes. DNA was precipitated with an equal amount of isopropanol. Tubes were centrifuged at 14000 X g for 1-5 min. DNA containing pellets were rinsed with 70% ethanol and air dried for at least 15min. Dried pellets were resuspended in 75µl TE (10mM Tris-HCl, pH 8 in 1mM EDTA) and this extracted DNA was quantified by spectrophotometer.

Cleaved Amplified Polymorphic Sequences (CAPS):

For initial and secondary mapping of mutation, CAPS mapping strategy was used as described by Konieczny and Ausubel (1993). CAPS markers are PCR based co-dominant markers. Markers were designed based on the information available in www.arabidopsis.org. Amplification reactions were performed using PCR Sprint and the reactions were carried out in final volume of 25 µl. The PCR reactions contained 0.2mM of each primer, 1.5-3.0 mM $MgCl_2$ (optimized for each pair of primers), 0.5U buffer Taq polymerase (Promega), 0.2mM each dNTP (Promega) and 50-100ng of DNA template. Amplification was initiated at 94^{o}C for 1 min followed by primer specific annealing temperatures (Table 1) for 1 min, followed by extension at 72^{o}C for 0.5 – 2 min (duration for extension temperatures varied according to the PCR product size) for 10 cycles. Later the entire program was repeated for 30 cycles with shorter denaturation time (20 sec) at

94^{o}C, primer annealing for 30 sec, 72^{o}C for 0.5 – 2 min and final elongation time at 72^{o}C for 10 min.

Restriction enzyme digestion and analysis of PCR products:

Restriction enzyme reactions were carried out in tubes containing 20 µl of final volume which contains 4 µl of amplified PCR product, 1 µl of restriction enzyme, 2 µl of 10X buffer and 2 µl of 1X BSA in distilled water. The tubes were incubated at appropriate temperatures for 2 hours and the products were analyzed on agarose gels. The concentrations of the gel varied according to the DNA length and for a final length of 0.2 – 3 kb, 1.5% agarose gel was used while for a DNA length of 0.01 – 0.5 kb, a gel concentration of 2-3% agarose was used (Baumbush *et al.*, 2001; Konieczny and Ausubel, 1993).

RESULTS

1. Mutagenesis and seed collection:

In order to isolate genes involved in SCR regulated pathways we designed and initiated a screen for *scr* suppressor mutants. For this purpose, Arabidopsis *scr1* seeds were mutagenized to introduce second site mutations that would result in suppression of any *scr1* phenotypic defects. Arabidopsis *scr1* seeds were mutagenized with EMS (Levin *et al.*, 1998).

We performed the mutagenesis in batches of 2000 seeds per batch every other week. Following mutagenesis, the first three batches of EMS treated seeds were sown in soil at a density of about 50 seeds per pot. Germinated seedlings were grown to maturity and seeds were collected. This procedure was not efficient because most of plants died before they produced seeds and the surviving plants produced very few seeds. Thus the first three batches of seeds (approximately 6000 seeds) that were sown in soil yielded 21 seed pools representing 40-50 plants per pool. Each pool contained only 150-200 seeds total. These 21 pools represented a maximum of 1000 independent lines.

To enhance seedling survival and to increase seed production from the next batch onwards we plated seeds on MS agar plates and allowed seedlings to grow for 2 weeks before transferring them to soil. Only robust seedlings were transferred to soil and planted at a density of 15 seedlings per pot. Most of the seedlings matured and

produced seeds. Using this more labor intensive technique we generated 30 additional pools, each containing seeds collected from approximately 100 plants. In contrast to the first three batches, these pools contained thousands of seeds per pool. Therefore we generated a total of 51 pools of seeds representing approximately 4000 independent lines.

Primary Screen:

The seeds collected in pools were screened for improved phenotypes such as hypocotyl gravitropism, increased root length, larger leaves etc. All of the seeds from pools 1-29 representing approximately 1800 independent lines were screened using the following procedure. The seeds from each pool were plated on MS plates containing 4.5% sucrose at the density of 50 seeds per plate. Plates were stratified for two days at 4^oC. Then plates were transferred to growth chamber with continuous light for 2-3 days. When most of the seeds on the plate were germinated, the plates were covered with aluminum foil and left in vertical position for 24 hours. All the plates were photographed (T_o), covered with foil again and reoriented by 90^o. Reoriented plates were photographed again after 48 hours (T_1). Images at T_0 and T_1 were compared for gravitropic phenotype. Seedlings with hypocotyl gravitropic responses were transferred to fresh plates, grown for two more weeks in vertical position and transplanted to soil for seed production. All of the remaining hypocotyl agravitropic seedlings left on the original plates were grown under long day conditions (16 hours of light) in the original vertical orientation for additional two weeks. At that time all the seedlings with

improved phenotype such as longer roots or larger leaves were selected as potential suppressors. These seedlings were transferred to soil for seed production.

Over 250 seedlings with improved phenotype were transferred to soil for seed production. Seeds were obtained from 131 of these potential suppressors (1^o isolates). Each potential suppressor was given a three-part name that indicates its origin. The first part identifies pool number, the second part the plate (indicated by a letter) and the third part indicates the isolate number from that plate. These 131 potential suppressors represent 17 out of 29 pools screened to date (Table 2).

3. Secondary Screen:

Seeds collected from all of the 131 initial (primary) isolates were subjected to secondary screening. In that screen we plated *scr1* seeds, 1^o isolates and WT (wildtype) seeds side by side on the same MS agar plates containing 4.5% sucrose. All 1^o isolates were evaluated for gravitropic responses, root length and leaf size. From this secondary screen we found that 15 of the primary isolates were still potential suppressors. Seedlings from lines 3A2, 3A3, 11A1, 15A1, 16C1, 23C2, 23K1, 24R1, 25G2, 26F1, 26G1, 26G2, 26L1, 28B1 and 28N1 showed enhanced hypocotyl gravitropism and/or had longer roots than *scr1* seedlings grown on the same plate. Next generation of seeds were generated for the 15 lines of potential suppressors identified in the secondary screen. The seeds of that generation were screened as before to establish the stability of the suppressor phenotype. Of the 15 lines only 8 retained their suppressor phenotype over the next two generations. These eight lines represent seven different pools.

Therefore, these lines probably represent at least seven different loci. These remaining potential suppressors were 3A2, 11A1, 15A1, 16C1, 23C2, 23K1, 24R1 and 25G2.

These confirmed phenotypic suppressors were evaluated for the presence of *scr1* allele. The genetic background of these eight lines was confirmed by two tests: the antibiotic resistance of the seedlings and PCR test for the presence and/or absence of WT and mutant *scr1* alleles in their genomes. The *scr1* mutant contains a T-DNA insertion within a coding region. The T-DNA carries a kanamycin resistance gene. Confirmed suppressor *scr1* and WT seeds were plated side by side on MS agarose plates containing kanamycin. All of the 8 lines survived on kanamycin plates, therefore, they all contain a kanamycin resistance gene (Figure 3). In order to confirm that each line of the potential suppressors is homozygous for *scr1* allele and does not carry a WT copy of *SCR* gene we have designed and synthesized several PCR primers to distinguish between different *SCR* alleles (see Figure 4 for diagram of *SCR* gene in *scr1* allele with the PCR primer locations). The *SCR* forward and reverse primers are located on either side of T-DNA insertion of *scr1* allele (Figure 4). We performed PCR analysis on genomic DNAs from all eight potential suppressors, WT and *scr1*. WT DNA yields a single, ~1.6 kb band with the two SCR primers (Figure 5). The *scr1* DNA yields a single, ~ 0.9 kb band only with a combination of SCR reverse primer (SCR R1956) and T-DNA right border primer (Figures 4 and 5). Genomic DNAs of the eight potential suppressors were amplified by using either SCR primers alone or SCR reverse primer and T-DNA right border primer. DNAs from lines: 3A2, 11A1, 23C2, 23K1, 24R1 and 25G2 yield amplification products only with SCR reverse and T-DNA right border primers (Figure 5) indicating homozygous *scr1* genetic background. In contrast,

genomic DNAs from lines 15A1 and 16C1 amplify products only with SCR primers alone, indicating WT SCR genetic background (not shown). The PCR analysis confirmed six of the isolated lines as *scr1* suppressors. Five of the six suppressors (25G2, 3A2, 23C2, 11A1, 23K1) are hypocotyl gravitropic and one suppressor, 24R1, has longer roots.

4. Phenotypic characterization of confirmed suppressors:

a. Gravitropism

Plant axial organs such as roots and stems respond to certain environmental cues with directional growth called tropism. Because stems grow towards light they are phototropic. Germinating seeds may not be exposed to light therefore, in the dark the directional growth of stems and roots can be determined by the orientation of the gravity vector alone. Stems, hypocotyls and inflorescence stems, show negative gravitropism and roots show positive gravitropism. Mutations in many genes can affect gravitropic responses of one, two or all gravitropic organs indicating that gravitropic mechanisms in different organs are not identical. Mutations in *SCR* gene affect only shoot gravitropism because *scr* roots have WT response to gravity.

b. Hypocotyl Gravitropism

Germinating seedlings in the absence of light orient themselves solely based on gravity. It has been shown that *scr* mutants are hypocotyl agravitropic (Fukaki *et al.,* 1996a; Fukaki *et al.,* 1998). However, my preliminary characterization of the gravitropic suppressors of *scr* suggested that there may be some residual hypocotyls

gravitropism in *scr1*. Therefore, we first determined the degree and the time course of gravitropic responses of *scr1* and WT hypocotyls over a 48-hour time period (Table 3 and Figure 6). Next, all of the confirmed suppressors were tested for gravitropic responses and compared to WT and *scr1*. Each suppressor was grown on the same plate with both the WT and *scr1* seedlings. In each case the seeds were germinated in light, the plates were covered with foil and left in original orientation for 24 hours. Plates were photographed after 24-hour period (T_0), reoriented by 90 degrees and photographed at different intervals for the next 48 hours. Response was measured in degrees of curvature by comparing the same seedlings at different time points (Table 3; Figure 6 and Figure 7).

For quantification of gravitropic responses of suppressors we used 40 seedlings of each suppressor for this experiment. Gravitropic response was calculated in degrees and the average values with standard deviations are presented in the Table 3. These data are also represented graphically in Figure 6.

WT hypocotyls showed negative gravitropic response starting as early as three hours after plate reorientation (Figure 6 and Table 3). At that time point WT hypocotyls responded by reorientation of growth by 6 degrees. Four of the suppressors, 3A1, 11A1, 23C2 and 23K1, show significant but lower than WT response after the first three hours. The *scr1* and 24R1 hypocotyls do not show any response at least until 6 hours after reorientation. One of the suppressors, 25G2, has a very similar response to WT hypocotyls at all time points used in this study.

Hypocotyl gravitropic responses of WT and five of the six suppressors were nearly linear for the first 36 hours after reorientation. The response during the last 12

hours examined was slightly lower (Figure 6). The final angles of deflection achieved by WT and 25G2 hypocotyls were in the range of 48-50 degrees after 48-hour exposure to a new gravity vector (Table 3; Figures 6 and 7). The other four suppressors, 3A2, 11A1, 23C2 and 23K1, that had responses significantly better than *scr1* hypocotyls were similar to each other with the final deflection angles ranging from 37 to 43 degrees after 48 hours.

One of the surprising findings was that *scr1* hypocotyls possess some residual gravitropism contrary to the previous reports (Fukaki *et al.*, 1996a; Fukaki *et al.*, 1996b and Fukaki *et al.*, 1998). The response is much slower and represents only approximately 20% of the WT response at the end of the experiment. The *scr1* hypocotyls show clear response to new gravity vector as early as 6 hours after plate reorientation and they slowly continue changing the growth direction for the remainder of the experiment (Table 3 and Figure 6). The final angle of deflection after 48 hours is 11 degrees for *scr1* hypocotyls instead of a 50-degree reorientation by WT. The remaining *scr1* suppressor, 24R1, has the same level of hypocotyl gravitropism as *scr1* (Table 3 and Figure 6).

These results demonstrate that *scr1* retains a low level of hypocotyl gravitropism. The six confirmed *scr1* suppressors fall into at least three categories with respect to hypocotyl gravitropism. One of the suppressors, 24R1, is not involved in hypocotyl gravitropism because it has the same low response as the *scr1* mutant. These data also indicate that lines 3A2, 11A1, 23C2, 23K1 have significantly improved hypocotyl gravitropism over *scr1* but below the WT level. One of the *scr1* suppressors, 25G2, is very similar to WT in its hypocotyl gravitropic responses (Table 3).

b. Inflorescence Stem Gravitropism:

Inflorescence stems of *Arabidopsis thaliana* show negative gravitropism, they grow upwards. When the plants are placed horizontally, turned by 90 degrees by placing pots on their sides in the dark, the inflorescence stems reorient quickly in response to the new gravity vector. WT inflorescence stems can achieve a 90^{o} upward deflection as quickly as two hours after reorientation. It has been shown that *scr* mutants are inflorescence stem agravitropic (Fukaki *et al.,* 1996; Fukaki *et al.,* 1998). To compare the responses of WT, *scr1* and confirmed suppressors we selected plants that all had 6-8 cm long inflorescence stems. The selected plants were placed in the dark on their sides so that the inflorescence stems were reoriented by 90^{o} (Figure 8). Our results were in agreement with previous studies that *scr1* inflorescence stems are agravitropic (Fukaki *et al.,* 1998). WT inflorescences respond quickly and bend 90 degrees within the first three hours of reorientation (Figure 8). Neither *scr1* nor any of the five hypocotyl gravitropic suppressors respond to the new gravity vector within 15 hours of reorientation. Surprisingly, the only suppressor that showed a slight response after 15 hours of reorientation was the long root suppressor, 24R1, that has the same hypocotyl gravitropic responses as *scr1* (Figure 8).

c. Root length:

Arabidopsis plants show indeterminate root growth, thus root growth continues throughout their life. However, *scr1* seedlings have a short root phenotype and their roots stop elongating at an early stage of life (Sabatini *et al.,* 2003; Figure 9 and 10). All of the confirmed suppressors were tested for the type of growth their roots exhibit

during seedling development. On MS agar plates containing 4.5% sucrose, along with WT and *scr1* the suppressor seeds were plated to compare their root lengths side by side on the same plate. After germination root lengths of all seedlings were recorded at three-day intervals. Root lengths were measured first on the third day after germination (DAG) and the last measurement was recorded on the 21st DAG. Root length measurements of suppressors along with WT and *scr1* are presented in Table 4. These data represent averages and standard errors obtained from 50 seedlings of each genotype. These data are also presented graphically in Figure 9.

Root length differences between WT and *scr1* can be already seen at the first time point used, 3rd DAG (Table 4). At that point WT root length was 18 mm whereas *scr1* roots were only 3.2 mm. 24R1 is the only one of the six suppressors that has significantly longer roots than *scr1* at 3rd DAG (Table 4 and Figure 9). 24R1 root length at that point was 8.8 mm, clearly shorten than WT. The other five suppressors had roots of similar length to *scr1*. Their root lengths were in the range of 2.9 to 3.6 mm.

Both WT and 24R1 show an indeterminate mode of root growth. The growth rate is nearly linear for these two lines but WT roots grow faster (Table 4 and Figure 9). The other suppressors have a determinate root growth similar to *scr1*. The roots of *scr1* grow very slowly and their lengths do not increase significantly after approximately two weeks after germination. The roots of *scr1* and all of the suppressors except 24R1 do not elongate past 19 mm even three weeks after germination. In contrast, WT roots are already at that length only after three DAG and 24R1 by the ninth DAG (Table 4). The final *scr1* root length was 12% of the WT at the end of the experiment. The final root length of 24R1 was 69% of the WT root length. The root lengths of the other five

suppressors were 11 – 20% of the WT final root length and not significantly longer than *scr1* roots. These data indicate that only one *scr1* suppressor, 24R1 has an indeterminate root growth. However, the root growth rate of 24R1 is below that of WT.

d. Hypocotyl length in *scr1* and *scr1* suppressor mutants:

Hypocotyl is an embryonically generated organ. Cells within the hypocotyl do not divide after germination. Therefore, postembryonic hypocotyl growth is accomplished entirely by cell elongation. Dark grown seedlings have significantly elongated hypocotyls. Hypocotyl elongation of *scr1*, *scr1* suppressors and WT was determined in the light and dark conditions. WT, *scr1* and confirmed suppressor seeds were germinated on MS agar plates in the light. After germination, half of the plates were kept under long-day condition and the other half were wrapped in aluminum foil and kept in the dark for 7 days. Hypocotyl lengths were measured on the seventh DAG. The data are presented in Table 5 and Figure 11.

Light grown seedlings have significantly shorter hypocotyls than dark grown seedlings. The difference ranges from 2.5 to 3 fold (Table 5). Light grown WT seedlings have hypocotyls that are slightly but significantly longer than *scr1* seedlings of the same age (Table 5). In the light all six suppressors have the same hypocotyl phenotype as *scr1*. When grown in the dark, *scr1* and suppressor hypocotyls are at least 2.5 fold longer than when grown in the light (Table 5 and Figure 11). However, all of the mutants have significantly shorter hypocotyls than WT when grown in the dark. There is no significant difference between hypocotyl lengths of *scr1* and any of the suppressors with or without light seven days after germination.

5. Phenotypic characterization of long root suppressor 24R1:

Suppressor 24R1 showed increased root length, hypocotyl agravitropism and slight inflorescence gravitropism. The hypocotyl elongation is also impaired in 24R1.

a. Radial pattern of roots:

WT roots contain single layers of epidermis, cortex and endodermis surrounding the central stele (Dolan *et al.,* 1993). The roots of *scr1* mutants contain only two instead of three layers around stele (Scheres *et al.,* 1995; Di Laurenzio *et al.,* 1996, Wysocka – Diller *et al.,* 2000; Figure 2). We generated cross sections and also examined optical longitudinal sections of WT, *scr1* and 24R1 roots to compare their radial pattern. The results are shown in Figures 12 and 13. The radial pattern of 24R1 suppressor roots is similar to *scr1* (Figures 12 and 13). There is only a single cell layer between epidermis and stele instead of the two layers present in the WT roots (Figures 12 and 13).

6. Initiation of positional cloning of 24R1:

a. Mapping of 24R1:

24R1 is the only long root suppressor identified in our suppressor screen. It has a strong, obvious and reproducible phenotype. The gene mutated in 24R1 suppressor is involved in root meristem maintenance and probably not in the other SCR regulated pathways. Therefore, 24R1 was selected for positional cloning.

In *Arabidopsis*, mapping of mutations is made possible by the availability of many molecular markers. The chromosomal location of a mutant gene can be determined by recombination frequencies of molecular markers with visible markers, such as a mutant phenotype. 24R1 mutation is a suppressor of *scr1* that would most

likely not have a phenotype on WT background. Therefore, to map 24R1 we first crossed it to another *scr* allele that is on different genetic background. 24R1 is on WS (Wassilewskija) background therefore it was crossed to *scr3*, which is on Columbia (Col) background. The *scr1* is kanamycin resistant but *scr3* is sensitive. We used this difference in kanamycin sensitivity for selection of "real" crosses. The 24R1 pollen was used to pollinate *scr3* carpels. Later, F_1 seeds were collected and germinated on kanamycin containing plates. Kanamycin resistant seedlings were transferred to soil and next generation (F_2) seeds were collected for mapping.

F_2 seeds were plated along side *scr1*, *scr3* and both WT ecotypes to determine if 24R1 is allele specific. Approximately a quarter of F2 seedlings had roots significantly longer than either *scr1* or *scr3* seedlings of the same age (not shown). These seedlings with longer roots were selected for DNA extraction to be used in mapping.

We selected CAPS (Cleaved Amplified Polymorphic Sequences) markers that show polymorphism between Col and WS for mapping of 24R1 (Konieczny and Ausubel 1993 and www.arabidopsis.org). The choice of the markers was based on their distribution along the chromosome and also on the gel band pattern after restriction enzyme digestion. A total of 18 markers were selected for initial mapping and tested on WT DNAs. PCR conditions were optimized for each primer set. Only 12 of the markers gave reproducible results on WT DNAs. The markers, their chromosomal positions, restriction enzymes and primer sequences are shown in Table 6 and Figure 14. The 12 sets of primers were used on the DNAs from 33 F2 seedlings exhibiting suppressor phenotype. The data is shown in Table 7a. These data indicate that 24R1 locus is

located on chromosome IV approximately 21cM away from G3883-1.4a and 33cM from CH42 (Table 7a and Figure 15).

b. Initiation of fine mapping of 24R1:

We have tested all available CAPS markers for lower arm of chromosome IV. 8A6-1.3, 5F7R and 1H1L-1.6 were the only ones that show clear polymorphism between Col and WS. We used these three markers to further map 24R1. The results are shown in Table 7b and Figure 15. These results suggest that the locus corresponding to 24R1 mutation is located between G3883-1.4a and 1H1l-1.6.

The only polymorphisms between Col and WS available for this chromosomal region are SNPs (Single Nucleotide Polymorphisms). I designed new CAPS markers based on four of these SNPs as described by Neff *et al.*,(1998). I tested those on the WT DNAs but could not detect clear and reproducible polymorphisms. Currently, we can position the 24R1 locus within a 381kb region located between two closest markers (Figure 15).

DISCUSSION

SCR gene was used as an entry point to identify other genes involved in SCR-regulated developmental pathways. Our hypothesis was that by mutagenizing *scr* mutants, we can generate second site mutations that will result in phenotypic reversions. These *scr* suppressor mutants would represent SCR targets or partners. SCR gene activity is required in different plant organs and at different times. It is reasonable that different sets of genes are involved in different SCR-regulated pathways. We expected that suppressor screen would yield mutants that would lead to identification of the components of specific pathways.

Over 1800 mutagenized independent lines were screened. Primary screen yielded 250 potential suppressors. Six true suppressors were confirmed and characterized from the entire collection of primary isolates. They are 3A2, 11A1, 23C2, 23K1, 24R1 and 25G2. Of these, five suppressors 3A2, 11A1, 23C2, 23K1 and 25G2 showed only hypocotyl gravitropic phenotype. The sixth suppressor, 24R1 exhibited increased root length phenotype and slight inflorescence stem gravitropic response. Plants from different pools would most likely carry different mutations. Because two of the suppressors, 23C2 and 23K1 came from the same pool and have similar phenotype, it is likely that they represent the same mutation. Therefore, our suppressor screen

yielded six suppressors that probably represent five different loci involved in at least two different SCR-regulated pathways.

These six suppressor lines can be categorized into three groups based on their hypocotyl gravitropic response. The suppressor line 25G2 has similar gravitropism to WT, four suppressor lines 11A1, 3A2, 23C2 and 23K1 have improved response but below WT level. The suppressor line 24R1 has hypocotyl gravitropism similar to *scr1*. In addition to identification of hypocotyl gravitropic suppressors it was found that contrary to the previous report (Fukaki *et al.*, 1998), *scr1* itself retains some residual hypocotyl gravitropism. Thus it is evident that hypocotyl gravitropism is partially independent of amyloplast sedimentation, which is entirely absent in *scr* mutants (Fukaki *et al.*, 1998). These findings indicate that hypocotyl gravitropic perception relies on at least two separate mechanisms. From our data it appears that amyloplast sedimentation is responsible for approximately 80% of the gravitropic signal. The other mechanism is responsible for the remaining 20% of the residual gravitropism found in the mutant hypocotyls. It has been postulated that in any mechanism for initial gravitropic stimulation the gravireceptors lie in between the plasmamembrane and the cell wall and would be mechanically stimulated (Guo *et al.*, 2008). In the absence of amyloplast sedimentation, as is the case in *scr1* mutants, these gravireceptors could perceive subtle changes in compression pressure resulting from the reorientation of protoplast in the displaced organs (Telewski, 2006). Our data clearly indicate that at least in hypocotyls, endodermis can perceive gravity even in the absence of sedimenting amyloplasts. It also appears that *scr1* hypocotyls are primarily defective in amyloplast sedimentation and not other aspects of gravitropic responses. However, it is possible that amyloplast

sedimentation contributes even less to the gravity generated signals that would be indicated by *scr1* responses. It has been observed that *scr1* hypocotyls may be defective in cell elongation (Wysocka-Diller unpublished data). Therefore, it is possible that some of the *scr1* hypocotyl agravitropism is related to cell elongation defects and unrelated to gravitropism. The data presented here on hypocotyl elongation in *scr1*, WT and the suppressors clearly indicate that cell elongation defect is not related to *scr1* gravitropic responses (Figure 11). We found that *scr* hypocotyls do not elongate either in the dark or in light as well as WT hypocotyls. Thus, SCR is required for normal hypocotyl cell elongation. Because the gravitropic responses rely on asymmetric cell elongation, one could argue that suppressors respond better to gravity because the cell elongation defect has been restored. Our results do not support this supposition because the most gravitropic suppressor 25G2 shows the same elongation defect as *scr1* (Table 3; Table 5).

We have isolated five hypocotyl gravitropism suppressors that represent at least two separate mutations because the phenotype of only 25G2 is significantly different than the remaining four which are very similar to each other. In order to establish how many genes these suppressors represent, complementation analysis needs to be performed. To determine the identity of the genes in each complementation group, the genes will have to be mapped and cloned. Until the exact identity of the genes is established we can only speculate on the type of products they might encode. We have already shown that none of these genes is involved in cell elongation. It is possible that some of them are involved in amyloplast sedimentation. To test this possibility the morphology of gravity sensing cells in *scr1* and the suppressors has to be compared. Particular attention should be given to the amyloplast size and distribution. If amyloplast sedimentation is not restored in these

suppressor hypocotyls than the second type of the candidate genes could involve gravireceptor(s) or any part of the signal transduction apparatus. One could imagine that the receptor molecule is more sensitive because of the mutation and the same signal results in a stronger response. The mutation in the suppressor could increase the expression of the receptor protein or enhance the activity of the protein itself. Because the identity of the mechanoreceptors involved in gravitropism is unknown we cannot test these hypotheses until we clone suppressor genes. All of the five hypocotyl gravitropic suppressors are still completely inflorescence stem agravitropic. On the other hand, the sixth *scr1* suppressor, 24R1 that is identical to *scr1* in hypocotyl gravitropic responses shows slight inflorescence stem gravitropism. These findings further support the idea that not all of the components in shoot gravitropic mechanisms are shared between responsive organs. We cannot eliminate the possibility that the hypocotyl gravitropism suppressor genes are not involved in gravitropic responses in other organs. However, it is clear that the mechanisms in stems and hypocotyls are different because we have not identified a single *scr1* suppressor that would be shoot gravitropic in both organs.

In addition to potentially being involved in inflorescence stem gravitropism, 24R1 suppressor is also involved in root development. Previous reports have shown that if *SCR* is expressed only in QC in *scr* mutants, the roots grow indeterminately but WT radial pattern is not restored. Our results indicate that in 24R1 roots the indeterminate growth is restored but not the normal radial pattern. Therefore, the 24R1 suppressor phenotype suggests that the mutated gene functions in QC.

We have determined a rough map position for 24R1. Our results indicate that the gene corresponding to 24R1 mutation is located on the lower arm of chromosome IV.

The region between the two closest markers represents 381 kb and contains 136 genes. Because 24R1 is involved in root meristem maintenance, it is reasonable to assume that this gene is expressed in QC. Without fine mapping, we can narrow down the candidates for 24R1 gene to 74 genes that are expressed in the root. Table 8 lists all of the 74 genes that are expressed in the root as reported by Birnbaum and coworkers (Birnbaum *et al.,* 2003). We can further narrow down the possibilities by identifying the genes that are expressed in the appropriate region of the root. QC is located at the root tip in the middle of the meristematic region. The root expression map was determined by microarray analysis of presorted cell types from different root regions (Birnbaum *et al.,* 2003). The data was presented for 15 different subsets of root cells. One of the data points was for cells within the root tip that are expressing GFP driven by *SCR* promoter and this should be the only data point including QC cells (Birnbaum *et al.,* 2003).

SCR is a transcriptional regulator and it is required for expression of downstream genes in several developmental pathways. The gene mutated in 24R1 suppressor is involved in root meristem maintenance, one of these SCR-regulated pathways. Therefore, the domain of 24R1 expression should overlap with that of SCR. So, it is logical to look at genes which are expressed in the QC, endodermis and in C/E initial cells for the candidate genes for 24R1. Table 8 contains all of the genes that may be expressed within the QC. SCR transcript accumulation in SCR expressing cells within the root tip was used as one of the standards to evaluate the expression of the other genes. In addition, we used the data for transcript levels of PLT1, a QC specific gene, to evaluate the expression of these genes. Based on the expression data and using SCR and PLT1 transcript levels as standards, only 22 genes are expressed in root tip fraction that contains QC cells (Table

9). By examining the structure of the products and any known function we could narrow down the field of the candidate genes even further.

One of the candidate genes is AT4G20270 gene locus, which encodes CLAVATA1-related (CLV1) receptor kinase-like protein called BAM3 (barely any meristem 3; DeYoung *et al.,* 2006). Because the loss of function of BAM3 results in the reduction of shoots meristem size it was suggested that BAM3 is required for stem cell maintenance (DeYoung *et al.,* 2006). It is possible that BAM3 is also involved in regulation of meristem activity and/or size in the RAM. Therefore, if SCR regulates BAM3 expression in the root, specifically in QC than some mutations in BAM3 could lead to the suppressor phenotype of 24R1. These mutations in BAM3 could result in altered level of expression or altered protein activity that would compensate for the absence of its regulator SCR leading to the restoration of meristematic activity in *scr* RAM.

Another candidate gene locus is AT4G20370 that encodes Twin sister of Terminal Flower (TSF), homolog of Flowering locus T (FT) which encodes a protein similar to phosphotidylethanolamine binding protein (PEBP) which is a Raf kinase inhibitor protein (RKIP; Yamaguchi *et al.,* 2005). This RKIP/PEBP family is representative of a new class of regulators of signaling cascades that function to maintain the balance of biological systems (Granovsky *et al.,* 2008). Mutation in the Terminal Flower (TF) results in shorter shoots with terminal flowers (Yamaguchi *et al.,* 2005). It is possible that this gene's expression is regulated by SCR and that its product regulates meristematic activity of RAM by inhibiting kinase activity of other signaling proteins within QC. Therefore, altering AT4G20370's expression or product activity could

suppress *scr* phenotype. For example, if AT420370 is expressed in QC to inhibit proteins that normally stimulate cell differentiation and/or inhibit cell division than increasing its (AT420370) expression or increasing the activity of its product would stimulate cell division and/or inhibit differentiation in neighboring initials.

The other likely candidate is AT4G20380 gene locus, which encodes a transcription factor containing Zinc finger motifs. Zinc finger proteins are known for their abilities to bind specific DNA sequences, proteins and other molecules in transcriptional regulation. It is possible that this Zinc finger protein is regulated by SCR or interacts with SCR and is ultimately responsible for the expression of some downstream genes in this SCR-regulated pathway. In the first case scenario, any change in the expression level of AT4G20380 would lead to the correct control of downstream genes to restore QC identity. This type of mutation would most likely be in the control region of the gene. A second type of mutation in this gene, could affect the activity of the protein itself. This type of mutation could enable the mutant protein to control gene expression of its targets without dependency on binding to SCR for that function.

In addition to our three top choices for the identity of 24R1, potentially any of the 22 genes expressed in QC could represent the mutated gene. The other more likely candidate genes encode either transmembrane proteins of unknown function or proteins that may be involved in transcription (Table 9). Among the 74 genes, AT4G20270 is the best candidate gene for 24R1 because it encodes CLV1-related receptor kinase-like protein BAM3, which is required for similar function in the shoot. Future lines of research should include the expression analysis of candidate genes in *scr* and 24R1 roots by RT-PCR and sequencing of the promoter region belonging to the gene that shows

altered expression level. If the levels of expression of the top candidate genes is unaltered in 24R1 as compared to *scr1* that would suggest that the protein sequence and thus the activity has been altered. Comparing exon sequences of the top candidate genes in 24R1 and *scr1* could be another approach to identify the gene. If neither of these approaches leads to the identification of 24R1 gene fine mapping of the locus has to be employed. We would have to develop new markers for this region.

In summary, we have initiated *scr* suppressor screen and six true suppressor lines were identified. These suppressor lines represent candidate genes for components of at least two SCR-regulated pathways. The majority, five of the six lines, should lead to the identification of at least two components involved in hypocotyls gravitropism. The remaining suppressor, 24R1 represents a gene required for meristem maintenance of RAM. The 24R1 suppressor mutation was mapped to a 381kb region on chromosome IV where there are only 22 genes that are likely candidates for the corresponding gene. Studies on the interaction of this gene with SCR and its function in root development will be the futures lines of research. The other suppressors, those involved in hypocotyl gravitropism will be cloned. Also, the remaining mutagenized pools of seeds representing additional 2200 independent lines will be screened for suppressors. Based on our initial success we could realistically expect to isolate at least five new suppressors. Even only with the suppressors identified to date new grounds can be broken in areas relating to the maintenance of root meristem and hypocotyl gravitropism.

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Table 1: Markers used in the study and their annealing temperatures and annealing times.

Marker	Annealing temperature (^{o}C)	Elongation time (min)
14G4	60.0	0:30
F18B13-50000	59.0	2:00
NCC1	53.0	2:00
ER	59.0	2:00
M429	60.0	0:30
PHYB	54.0	1:30
C6	53.0	2:00
GAPA	59.0	2:00
PUR5	53.0	2:00
ABI3	62.0	1:30
CH42	59.0	2:00
G3883-1.4a	53.0	2:00
5F7R	50.0	1:30
8A6-1.3	55.0	1:30
1H1L-1.6	50.0	2:00
ASA1	54.0	1:30
RPS4NT	51.0	0:30
EG7F2	55.0	1:30

Table 2: Number of seedlings isolated from different pools.

POOL No.	No. of Seedlings Isolated
3	2
11	1
15	1
16	4
17	1
18	1
19	1
20	5
21	1
22	19
23	25
24	14
25	17
26	13
27	17
28	4
29	5

Table 3: Hypocotyl gravitropic responses of WT, *scr1* and confirmed *scr1* suppressor seedlings. Angles of deflection were measured at seven time intervals starting 3 hours after reorientation.

Time Interval (Hrs)	**3A2**	**11A1**	**25G2**	**23C2**	**23K1**	**24R1**	***scr1***	**WT**
3	3.6 ± 0.7	3.2 ± 0.7	5.0 ± 0.5	4.2 ± 1.1	3.5 ± 0.9	0	0	6.0 ± 0.7
6	6.8 ± 0.7	4.3 ± 0.6	7.6 ± 1.2	7.3 ± 1.5	6.1 ± 1.6	1.1 ± 1.3	1.0 ± 1.3	9.2 ± 1.9
9	9.6 ± 0.8	8.0 ± 1.4	11.4 ± 1.7	10.7 ± 2.1	9.2 ± 1.9	2.5 ± 1.4	2.3 ± 1.6	12.3 ± 2.2
12	15.0 ± 1.1	12.4 ± 0.9	16.3 ± 1.3	14.0 ± 1.3	11.5 ± 1.3	5.7 ± 1.9	6.8 ± 1.6	17.9 ± 1.9
24	24.6 ± 1.1	25.6 ± 1.3	28.4 ± 1.4	26.8 ± 2.1	23.4 ± 1.8	9.1 ± 1.5	8.6 ± 1.5	29.5 ± 2.6
36	33.2 ± 1.4	34.3 ± 1.4	40.2 ± 3.3	35.9 ± 2.3	30.5 ± 2.8	10.5 ± 1.6	10.3 ± 1.6	45.2 ± 3.1
48	39.4 ± 1.8	41.0 ± 1.7	48.0 ± 2.7	43.2 ± 1.6	37.1 ± 4.0	11.6 ± 1.8	11.0 ± 2.4	50.2 ± 1.9

Table 4: Average root lengths of WT, *scr1* and confirmed *scr1* suppressors measured at 3 day intervals starting 3 days after germination. Root lengths were measured in mm.

Interval	**WT**	***scr1***	**24R1**	**11A1**	**3A2**	**25G2**	**23K1**	**23C2**
3rd day	18.0 ± 2.3	3.2 ± 0.6	8.8 ± 1.6	3.1 ± 0.9	3.6 ± 1.2	2.9 ± 0.7	2.9 ± 0.7	3.6 ± 0.7
6th day	33.8 ± 4.0	7.1 ± 0.7	14.8 ± 2.7	6.6 ± 0.7	7.0 ± 1.1	6.9 ± 0.6	6.7 ± 0.7	7.4 ± 1.1
9th day	61.8 ± 2.8	10.2 ± 0.9	20.2 ± 2.3	10.1 ± 1.0	13.1 ± 1.3	12.2 ± 1.0	9.9 ± 0.7	10.9 ± 1.4
12th day	83.1 ± 4.0	13.7 ± 0.7	42.6 ± 2.9	12.2 ± 1.5	14.3 ± 1.3	13.5 ± 1.1	11.9 ± 0.3	12.3 ± 1.4
15th day	107.2 ± 2.3	15.7 ± 0.8	63.2 ± 3.4	14.5 ± 1.3	15.7 ± 1.6	15.2 ± 0.9	13.4 ± 0.7	14.4 ± 1.2
18th day	127.4 ± 4.3	16.7 ± 0.9	83.2 ± 3.5	17.6 ± 1.0	16.9 ± 0.7	16.6 ± 1.0	14.8 ± 0.4	15.7 ± 1.1
21st day	138.4 ± 4.4	16.8 ± 0.9	95.3 ± 6.0	18.5 ± 0.7	17.2 ± 0.8	16.9 ± 0.9	15.2 ± 0.4	16.3 ± 0.7

Table 5: Hypocotyl lengths of WT, *scr1* and confirmed suppressors at 7 DAG when grown in light, under long day conditions. “Dark” seedlings were germinated as “light” seedlings after which they were moved to a chamber with continuous light for two days and then grown in the dark for additional 3 days. Hypocotyl lengths were measured in mm.

	Light	**Dark**
WT	5.1 ± 0.3	15.2 ± 1.0
scr1	3.7 ± 0.4	9.3 ± 1.8
24R1	4.1 ± 0.5	11.2 ± 0.7
23C2	4.3 ± 0.3	12.1 ± 1.0
23K1	4.1 ± 0.4	10.3 ± 1.4
11A1	4 ± 0.1	10.8 ± 1.0
3A2	3.9 ± 0.6	9.9 ± 0.6
25G2	4.1 ± 0.4	10.9 ± 1.4

Table 6: Markers used in our study and their sequence and the restriction enzymes

Marker Name	Restriction enzyme	Position (bp or cM)	Forward Primer/Reverse Primer
14G4	SCRFI		CGTCACCGTTGCCACTTCCGCC/CACATATATGGCCACAACTCTCTGTAG
F18B13-50000	RSAI		GATAAGTGATTACATCACCAGTTCC/CCCCACCACTAGCATATGCTCA
NCC1	RSAI	4106613	AAGTTATAAGGCATTAGAATCATAATC/GTCCTATCTCTACGATGTGGATG
ER	DDEI	11219636	GAGTTTATTCTGTGCCAAGTCCCTG/CTAATGTAGTGATCTGCGAGGTAATC
M429	SCRFI	73.19 cM	GGCAGTTATTATGAATGTCTGCATG/TGGTAACATGTTGGCTCTATAATTG
PHYb	XHOI	34.46cM	CAATCCTATGAAGAATGGCG/ATAAACCATTAGCCCACGTG
GAPA	DDEI	9796456	TGTGCTCAACCAAACTTAGCC/CACCGTGATCTAAGGAGAGCAAG
PUR5	RSAI	20398432	AAACCTTTCACTCCTCCTTTTTC/GATGTAGACCTTGCTGAAAA
ABI3	HINFI	8999565	GGGCCTCCGGCTTTTGTCCGCTCGG/CCACGTCAGCAGGTGGTACCAGATC
CH42	CLAI	10201965	CATCTTCTTCTGCAATCTGGG/CAGTGGATCTTTCCTCAGACG
G3883-1.4a		10612866	TGTTTCAGAGTAGCCAATTC/CATCCATCAAACAAACTCC
8A6-1.3	TAQ1	11009990	AACAATAGGAGGTGCAGAGT/CCAAGAAGAGAAAACGGAGA
5F7R	NLA IV	11028100	CATACCGTATGATGGAAC/GAATGGTGTAACCAAACTC
ASA-1a	BCLI	18.35cM	CCTCTAGCCTGAATAACAGAAC/CTTACTCCTGTTCTTGCTTAC
EG7F2	XBAI	24644037	GCATAGAATTTGACGATAACGAGC/GATCTGTGTAGGACTACGAGAC
RPS4NT	XHOI	18339331	TCTCTGTATCCCTCTCTCAG/TCAACAGGCATAACGTACTT

PAI	DDEI	2409487	GATCCTAAGGTATTGATATGATG/GGTACAATTGATCTTCACTATAG
M246	MAEIII	1130867	GCTTGAACTCCTCCTCCTTC/TGAAGAGCTATCCGAGATGG
C6	DDEI	2086875	ATGAAAGACATCACAGATCC/GCCTACCATCAATAAACC
DHS	BSAI	18537942	AGAGAGAATGAGAAATGGAGG/CAAGTGACCTGAAGAGTATCG
1H1L-1.6	DDEI	10993508	CTAGAGCTTGAAAGTTGATG/TTGAGTCCTTCTTGTCTG

Table 7a: CAPS Markers used in our study and the recombination frequencies obtained in the first step of mapping the 24R1 suppressor.

Marker	Colombia chromosomes/Total Chromosomes	Recombination Frequency (%)
Chromosome I		
14G4	37/70	53
F18B13-50000	33/56	59
NCC1	24/50	48
Chromosome II		
ER	43/68	63
M429	25/62	40
Chromosome III		
C6	28/58	48
GAPA	42/64	66
PUR5	32/56	57
ABI3	21/58	36
Chromosome IV		
CH42	20/60	33
G3883-1.4a	11/52	21
Chromosome V		
ASA-1a	28/48	58
EG7F2	38/58	66
RPS4NT	48/70	69

Table 7b: Recombination frequencies with CAPS markers on Chromosome IV alone used in the second step of 24R1 mapping analysis

Marker	Colombia Chromosomes/Total Chromosomes	Recombination Frequencies
8A6-1.3	10/32	34
5F7R	21/54	39
1H1L-1.6	37/104	36

Table 8: Genes located between G3883-1.4a and 1H1L-1.6 markers and their expression level in cortex and endodermis of root (Birnbaum *et al.*, 2003). (Expression levels below 50 are indicated by "-"; 50-99 by "±"; 100-299 by "+"; 300-499 by "++"; 500-699 by "+++"; above 700 by "++++"). Markers used for cell sorting in Birnbaum study Endodermis: *pSCARECROW::GFP*; Cortex + Endodermis: *J0571*.

Gene ID	Cortex-endodermis	Endodermis	Gene ID	Cortex-Endodermis	Endodermis
AT4G19470	-	-	AT4G19980	-	-
AT4G19480	-	-	AT4G19985	-	-
AT4G19490	-	-	AT4G19990	-	-
AT4G19500	-	-	AT4G20000	-	-
AT4G19510	-	-	AT4G20010	-	-
AT4G19520	-	-	AT4G20020	-	-
AT4G19530	-	-	AT4G20030	-	-
AT4G19540	-	-	AT4G20050	-	-
AT4G19550	-	-	AT4G20060	-	-
AT4G19570	-	-	AT4G20070	-	-
AT4G19580	-	-	AT4G20080	±	+
AT4G19600	-	-	AT4G20090	±	±
AT4G19610	-	-	AT4G20100	±	±
AT4G19620	-	-	AT4G20110	±	±
AT4G19640	-	-	AT4G20120	-	-
AT4G19650	-	-	AT4G20140	±	±
AT4G19660	-	-	AT4G20150	-	-
AT4G19670	-	-	AT4G20160	±	±
AT4G19680	-	-	AT4G20170	±	-
AT4G19690	-	-	AT4G20200	-	-
AT4G19710	-	-	AT4G20210	±	±
AT4G19720	-	-	AT4G20220	-	±
AT4G19740	-	-	AT4G20230	-	-
AT4G19750	-	-	AT4G20240	±	+
AT4G19780	-	-	AT4G20250	±	±
AT4G19790	-	-	AT4G20260	±	±
AT4G19810	-	-	AT4G20270	±	±
AT4G19820	-	-	AT4G20280	+	+
AT4G19830	-	-	AT4G20290	±	±
AT4G19840	-	-	AT4G20300	±	±
AT4G19850	-	-	AT4G20310	+	+
AT4G19860	-	-	AT4G20320	+	+
AT4G19870	-	-	AT4G20330	++	+
AT4G19880	-	-	AT4G20350	++	++
AT4G19900	-	-	AT4G20370	+	++
AT4G19920	-	-	AT4G20380	+++	+++
AT4G19940	-	-	AT4G20390	++++	++++

Table 9: Genes expressed in the endodermis and their functions.

Gene Locus	Function
AT4G20080	Phosphoribosylanthranilate transferase activity
AT4G20090	Membrane-associated salt-inducible-like protein
AT4G20100	putative protein probable membrane protein
AT4G20140	leucine rich repeat-like protein
AT4G20160	Glu-rich protein mature-parasite-infected, erythrocyte surface antigen
AT4G20170	putative protein gene F4P9.34 chromosome II BAC F4P9
AT4G20210	cadinene synthase like protein (+)-delta-cadinene synthase isozyme XC14
AT4G20220	hypothetical protein
AT4G20240	cytochrome p450 like protein
AT4G20250	hypothetical protein
AT4G20260	endomembrane-associated protein; supported by full-length cDNA
AT4G20270	CLV1-related receptor kinase like protein
AT4G20280	putative protein transcription initiation factor IID beta chain, supported by cDNA
AT4G20290	putative protein
AT4G20300	putative protein hypothetical protein; supported by full-length cDNA
AT4G20310	putative protein, conserved hypothetical protein involved in proteolysis has metalloendopeptidase activity
AT4G20320	CTP synthase like protein CTP synthase
AT4G20330	putative protein transcription initiation factor IIE, beta subunit; supported by cDNA
AT4G20350	putative protein hypothetical protein-*Caenorhabditis elegans*; supported by cDNA
AT4G20370	TWIN SISTER OF FT (TSF) terminal flower1, *Arabidopsis thaliana*; supported by cDNA
AT4G20380	zinc-finger protein Lsd1; supported by full-length cDNA; involved in transcription factor activity

AT4G20390	putative protein predicted proteins, *Arabidopsis thaliana*;supported by full-length cDNA

Figure 1. Schematic representation of *Arabidopsis* primary root and its meristem A . Cross section of the differentiated region of primary root. B. Longitudinal section of the primary root tip showing the organization and location of different (initials) stem cells within the root meristem and its spacial relation to different tissue types. C. The pattern of asymmetric cell divisions of cortex/endodermis initial cell that leads to formation of two distinct cell files in the root: cortex and endodermis. (from Wysocka-Diller and Benfey, 1997).

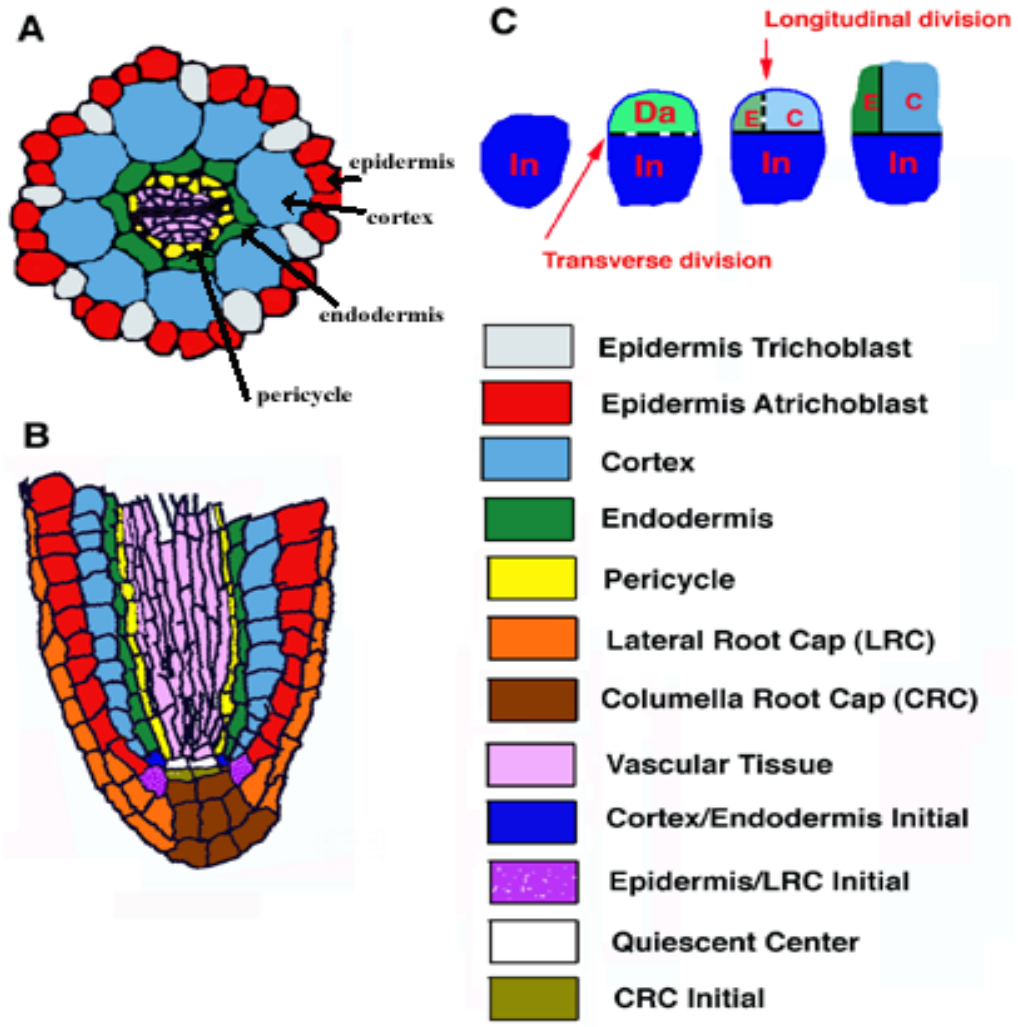

Figure 2: Comparison of WT, *scr* and *shr* root radial pattern in the cross sections through differentiated region of the primary root. (From Wysocka-Diller and Benfey, 1997).

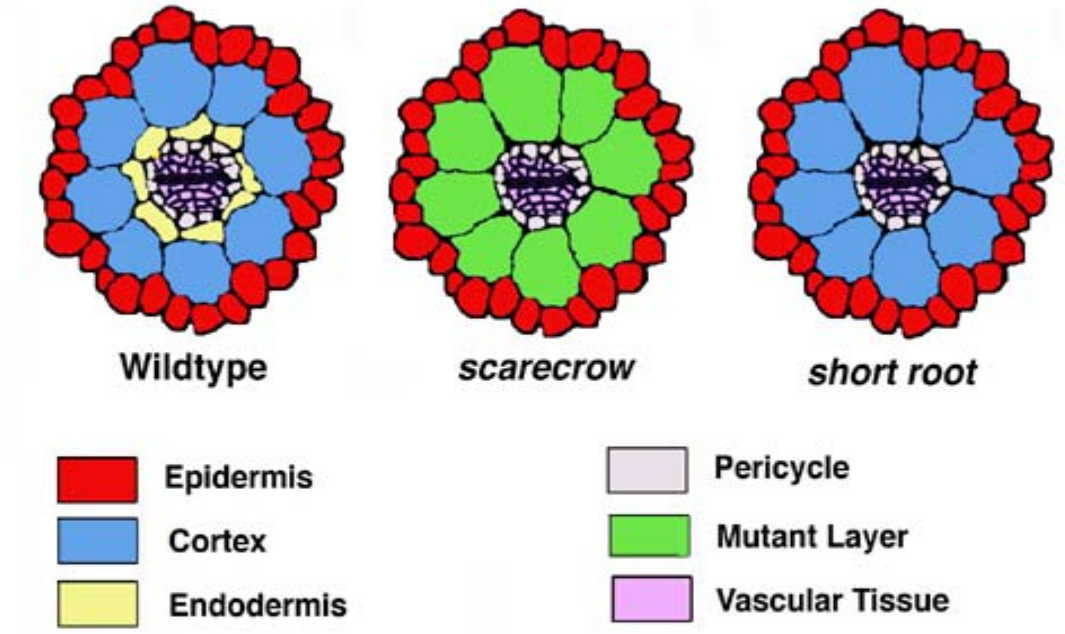

Figure 3: Two-week-old WT, *scr1* and 24R1 suppressor seedlings on MS agar plate containing antibiotic kanamycin.

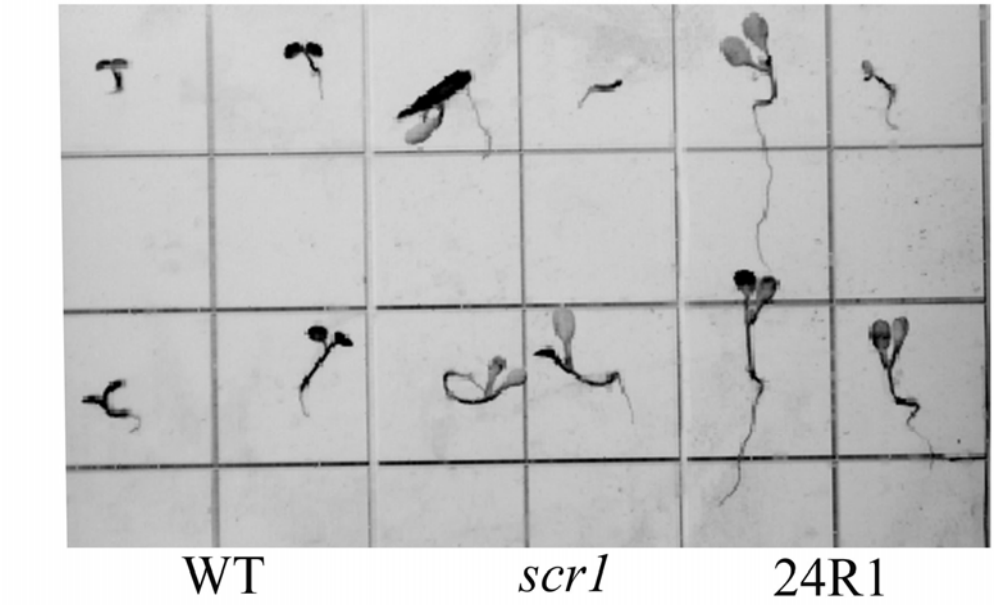

Figure 4: Schematic of *SCR* gene in *scr1* allele with the positions of T-DNA and PCR primers indicated. The orientations of the primers are indicated by the arrows. *scr1* allele amplifies a 0.9 Kb product only with SCR-R (1956) and a T-DNA primer (RBF3) whereas wildtype *SCR* allele amplifies a 1.6 kb length product only with SCR primers SCR-F (344) and SCR-R (1956) alone (Figure 5).

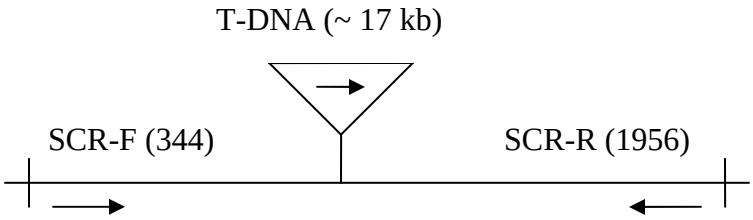

Figure 5: PCR amplification products. Blue arrow points to the 0.9 kb amplification products generated with SCR-R and T-DNA primers when using *scr1*, 24R1, 25G2, 3A2, 23C2, 11A1 and 23K1 DNAs; red arrow points to the 1.6 kb WT product generated with SCR-F and SCR-R primers that is absent in *scr1* background. The large T-DNA insertion in *scr1* allele prevents amplification with SCR primers located on either side of the T-DNA (Figure 4).

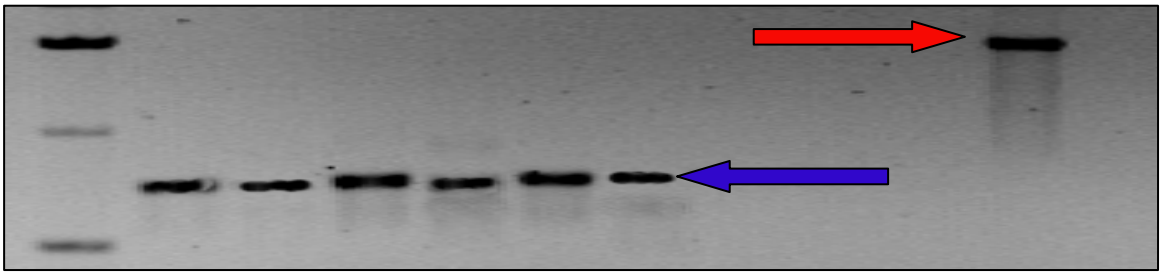

Figure 6: Hypocotyl gravitropic responses of WT, *scr1* and confirmed *scr1* suppressors at several time points to new gravity vector, graphical representation of Table 3. The X-axis represents the time interval in hours and the Y- axis represents the hypocotyl curvature in degrees.

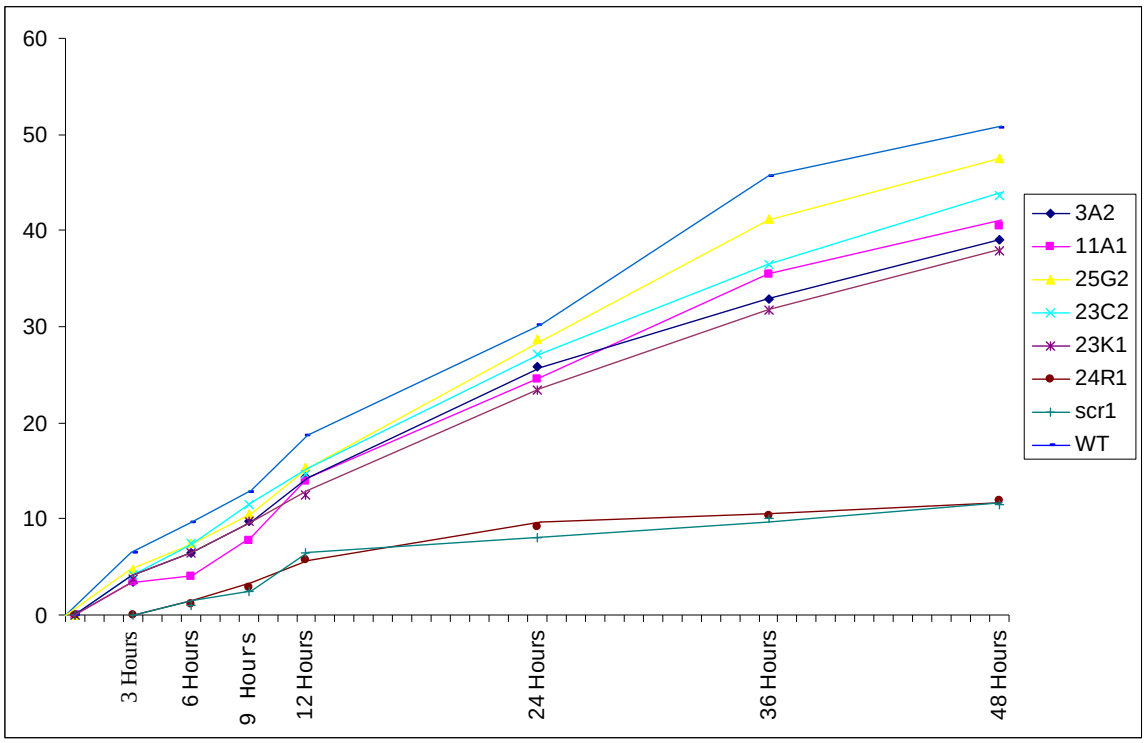

Figure 7: Hypocotyl gravitropic responses of the strongest gravitropic *scr1* suppressor 25G2, 48 hours after plate reorientation in the dark. Arrows indicate the orientation of gravity vector. The same *scr1*, 25G2 and WT seedlings are shown before reorientation (top) and 48 hours later (bottom).

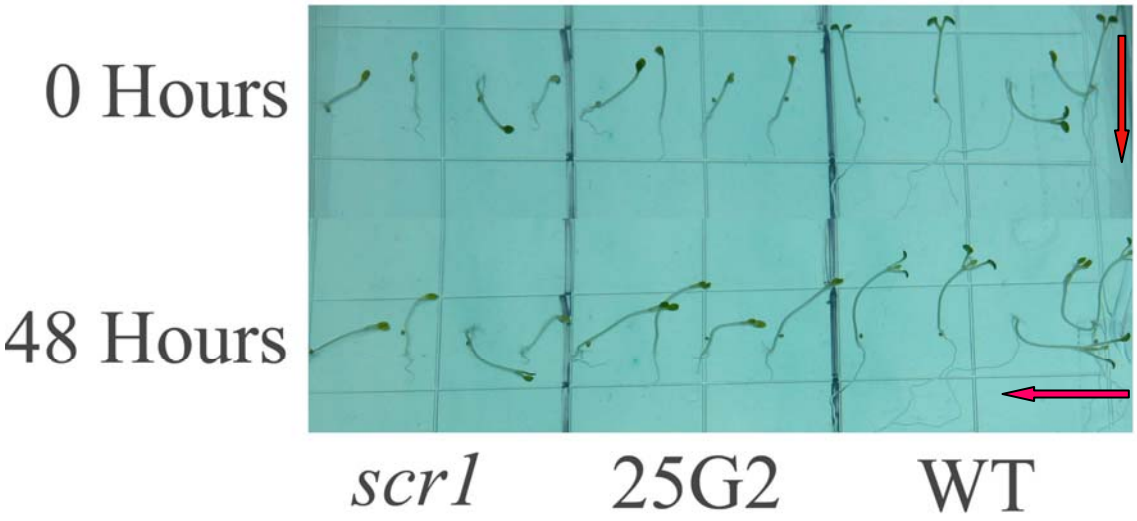

Figure 8: Inflorescence gravitropic response of WT, *scr1* and 24R1 to the new gravity vector. Arrows indicate the gravity vector. Top photos were taken at start of the experiment. Bottom photos were taken at 3 hours after reorientation for WT and 15 hours after reorientation for *scr1* and 24R1.

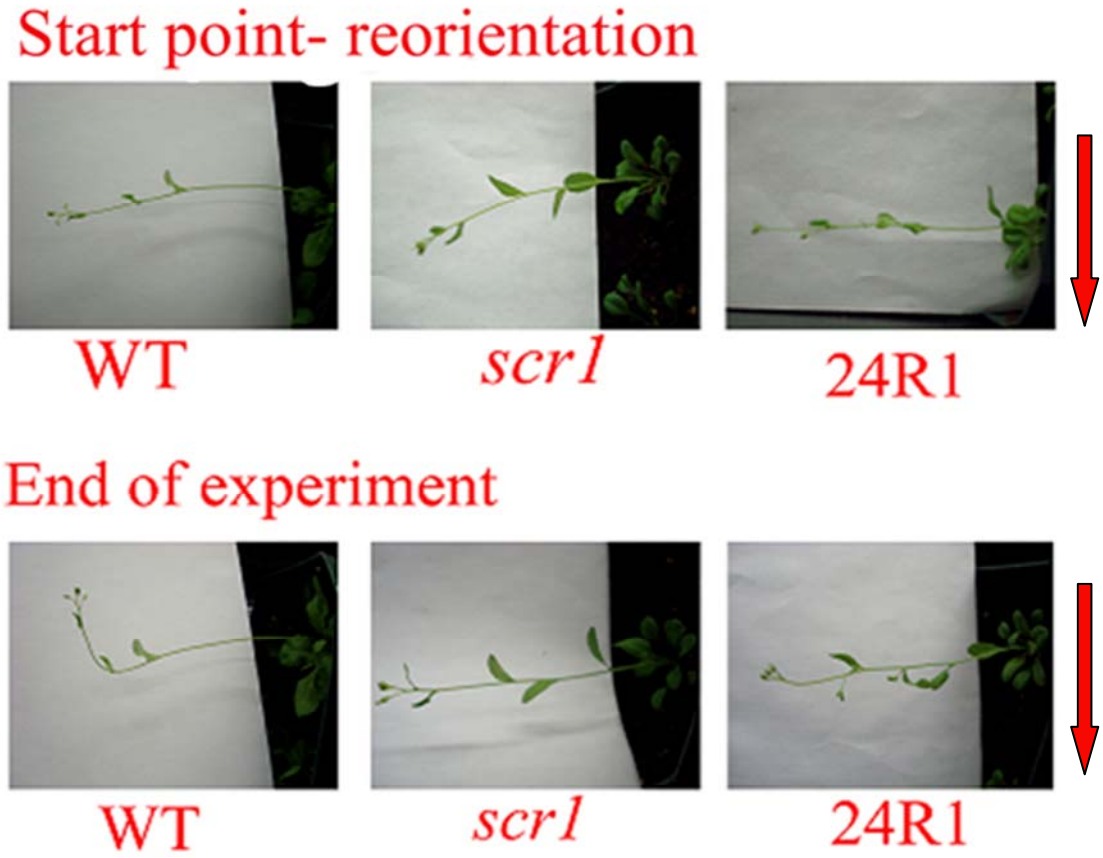

Figure 9: Primary root growth of WT, *scr1* and confirmed *scr1* suppressors, graphical representation of Table 4. The X- axis represents time in days (DAG) and Y- axis represents the root length in mm.

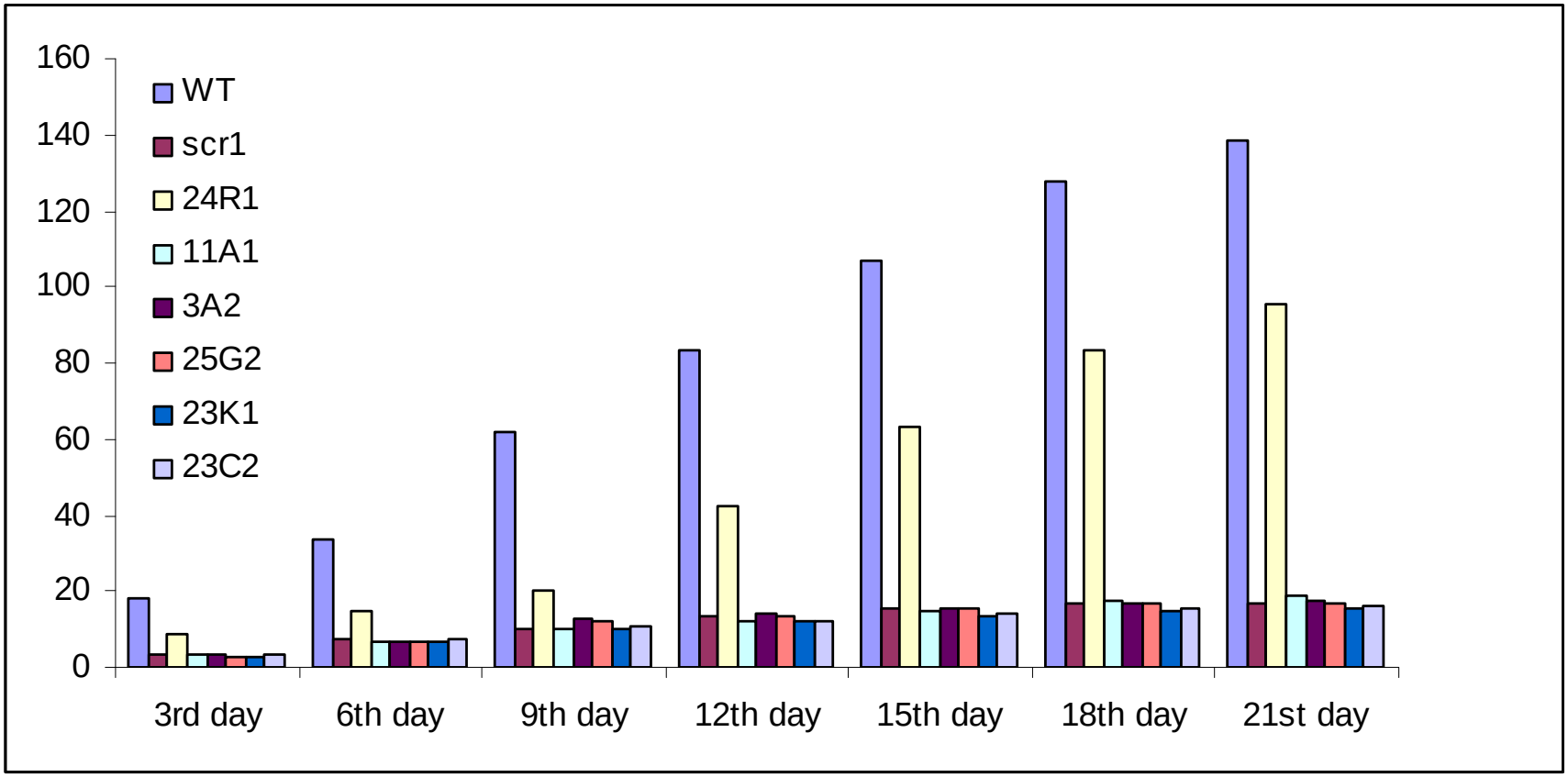

Figure 10: Root growth of *scr1*, 24R1 and WT seedlings at 15 days of germination (DAG) that were grown on the same MS plate containing 4.5% sucrose. The tips of primary roots are indicated by horizontal bars.

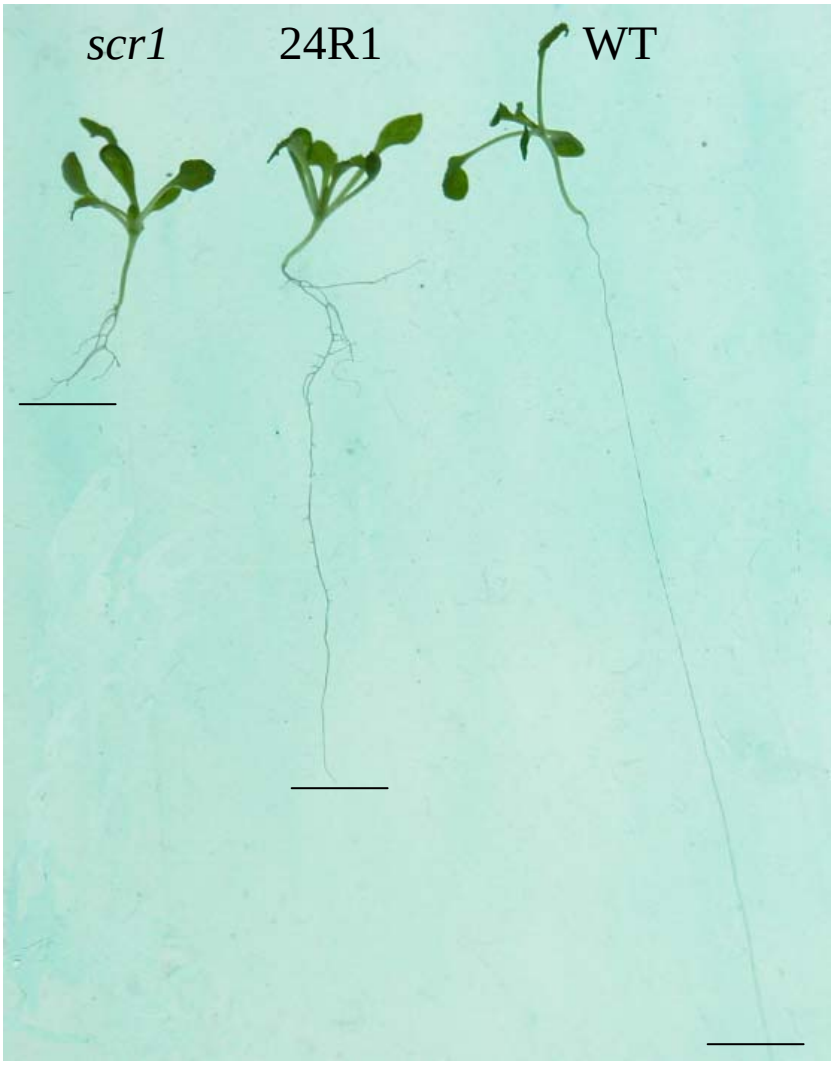

Figure 11: Hypocotyl lengths of WT, *scr1* and confirmed *scr1*suppressors, graphical representation of Table 5. The X- axis represents the genotype and Y-axis represents the length in mm.

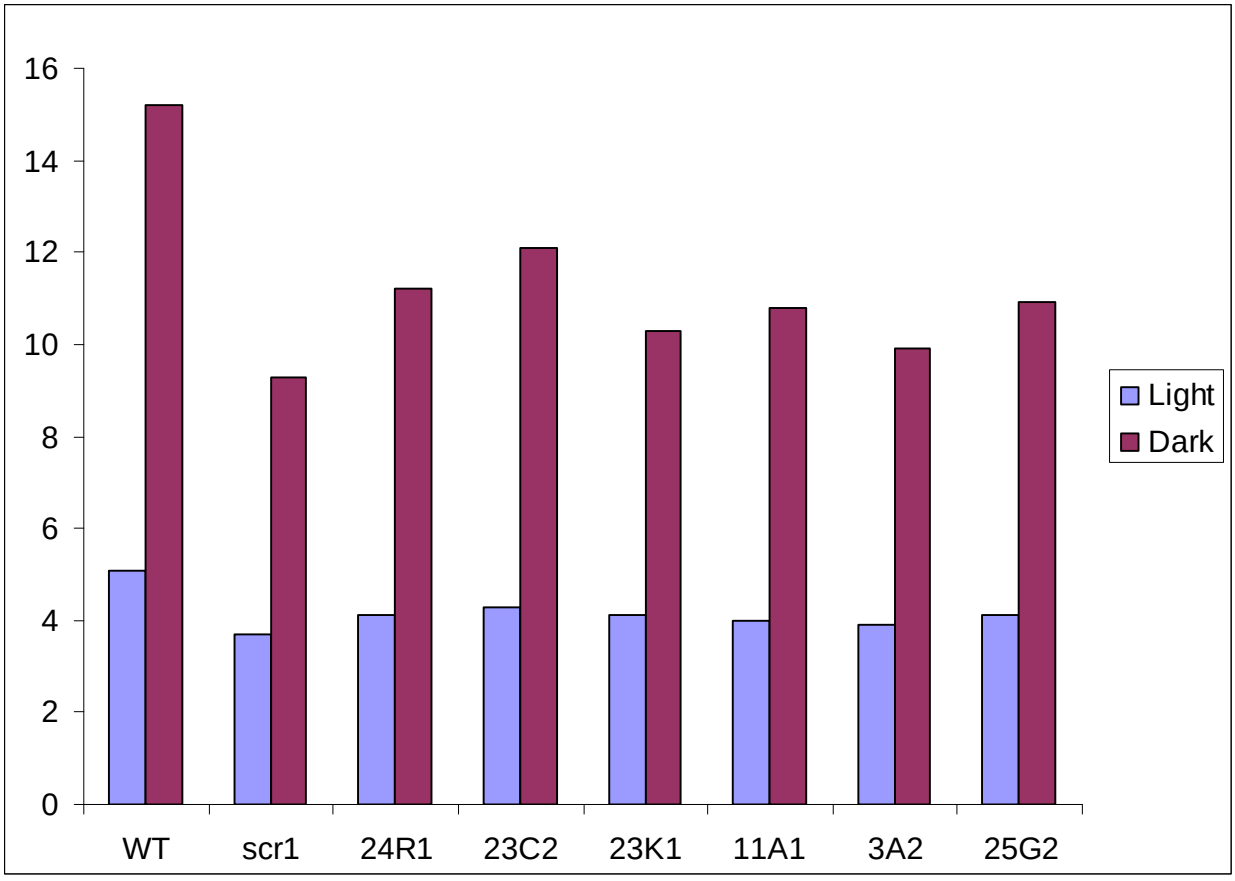

Figure 12: Cross sections of WT, *scr1* and 24R1 primary roots at 4 DAG. Blue arrow points to endodermis in WT and the yellow arrow points to mutant layer in *scr1* and in 24R1.

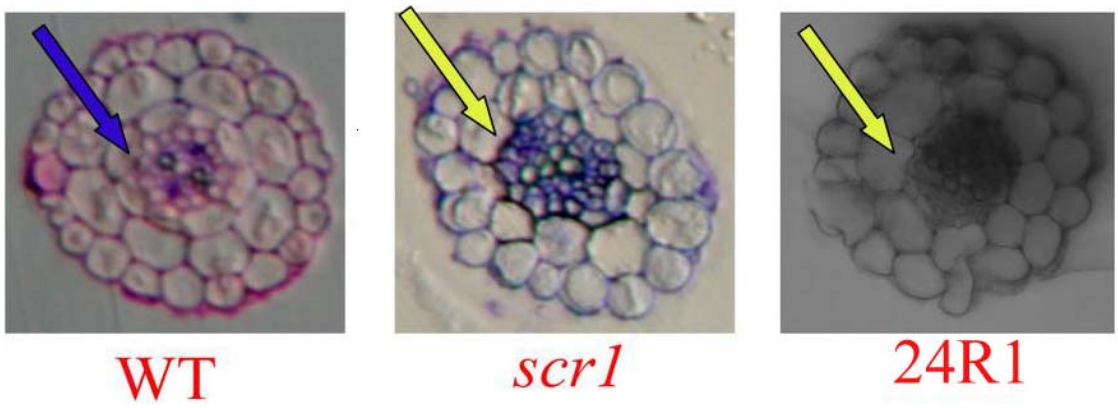

Figure 13: Longitudinal optical sections of WT, *scr1* and 24R1 primary roots at 4 DAG. Blue arrow points to endodermis in WT and the yellow arrow points to mutant layer in *scr1* and in 24R1.

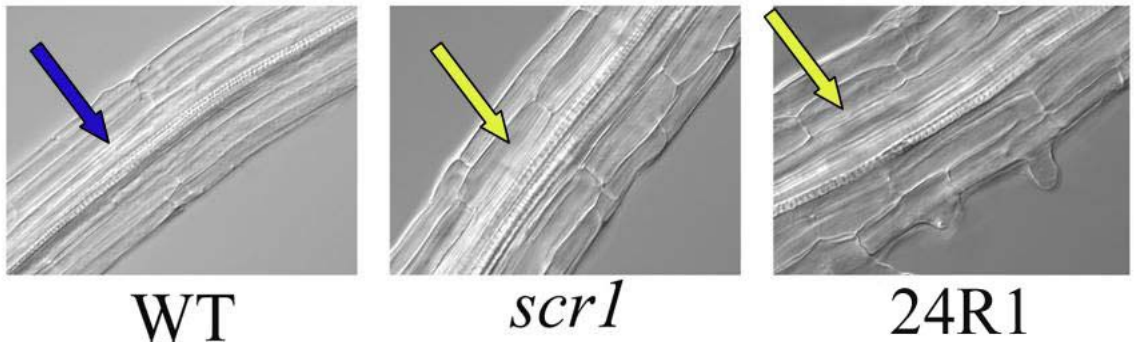

Figure 14: Relative position of the markers on the chromosomes

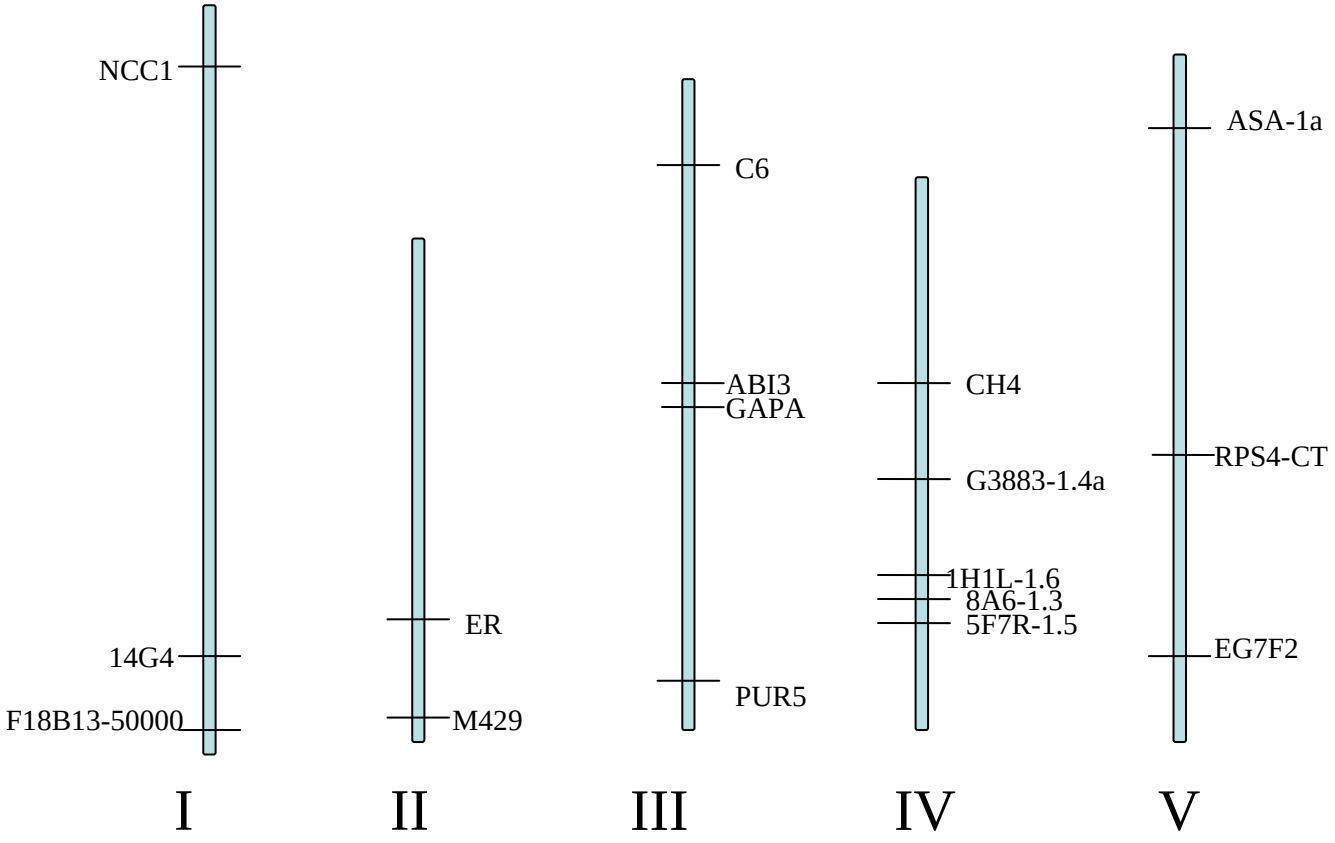

Figure 15: Possible locus position of 24R1 on Chromosome IV relative to all the markers used in our study.