Identification of new components involved in shoot gravitropism in *Arabidopsis thaliana*

by

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Abstract

Plants can reorient their growth according to environmental cues such as light, water, nutrients, gravity etc. Shoots (hypocotyls and inflorescence stems) of the plants exhibit negative gravitropism. Germinating seeds are usually not exposed to the light therefore in the dark the directional growth of stem and root is solely determined by the orientation of gravity vector. Therefore, gravitropism is essential for plant survival because if a seedling does not break the ground it will not be able to photosynthesize and it will not survive.

SCARECROW (SCR) plays key roles as transcription factor in *Arabidopsis thaliana* development. Confirmed functions of SCR include the radial patterning of axial organs, the development of endodermis and normal shoot gravitropism. The *scr* mutants exhibit abnormal internal shoot and root architecture. Analysis of shoot internal morphology indicates that both the hypocotyl and the inflorescence stem have defective radial patterning (missing one ground tissue layer) that leads to shoots to agravitropism. Functional orthologs of *Arabidopsis SCR* have already been identified in various agriculturally important crops such as maize and rice. Furthermore, at least some of the SCR functions have been found to be conserved throughout the plant kingdom.

The goal of this research is to discover novel components that function along with the *SCR* gene in the *SCR;* regulated gravitropic pathway. To achieve this goal, a detailed study of *scr1* mutant suppressors has been performed. Ten suppressors with improved hypocotyl gravitropism and three suppressors with improved root length have been identified. These suppressors show improved gravitropic response and root length respectively over *scr1* mutant but below that of the wild type

(WT) level. Most of the suppressors were isolated from different seed pools, and they show various degrees of gravitropic response as compared to WS (wild type) and *scr1*. Therefore, it is reasonable to predict that each suppressor represents a different mutation. Each mutation should point to a specific point in the gravitropic pathway in which the corresponding gene is involved. In order to confirm the number of different loci affected by *scr1* suppressors, I conducted a complementation test. The results demonstrated that six different genes are mutated in this suppressor collection.

The strongest *scr1* hypocotyl gravitropic suppressor (*shs1*) was selected for mapping analysis. The rough map positions have been determined using Simple Sequence Length Polymorphism (SSLP) markers. Pollen of *shs1* was used to cross with ovaries of *scr3* flowers (*SCR* mutant on Columbia background). F2 generation seedlings with hypocotyl gravitropic response were selected for DNA isolation and the DNAs were used for mapping. To map the position of gene two to six SSLP markers were used for each of the five chromosomes of *Arabidopsis thaliana*. Only chromosome 5 showed the linkage. The second site mutation that rescued the hypocotyl gravitropic phenotype of *shs1* maps to the lower arm of chromosome 5 about 27.9cM away from the closest SSLP marker tested.

It has been suggested that shoot agravitropism in *scr1* mutant is due to the abnormal radial pattern and/or the decreased hypocotyl cell elongation in the dark. However, my results do not support either of these hypotheses. The cell elongation in suppressors and *scr1* mutant dark grown hypocotyls is indistinguishable. Also, the hypocotyls of the all ten gravitropic suppressors have a *scr1* radial pattern that is characterized by a ground tissue deletion. These data suggest that decreased elongation and abnormal radial pattern are not responsible for *scr1* hypocotyl agravitropism. I propose SCR is more directly involved in gravitropism by regulating genes involved in early stages of gravitropism, probably the aspects of gravity sensing stage.

In order to address the function of SCR in gravitropism we have generated WS and *scr1* lines that carry *35S::SCR* and *35S::GFP::SCR* constructs. Phenotypic analysis of these transgenic plants yielded some very interesting results. Both *35S::GFP::SCR/scr1*(st1) and *35S::SCR*/*scr1*(D7) plant hypocotyls showed a gravitropic response similar to WS plants while one *35S*::*SCR*/WS (35S) line of plant hypocotyls showed complete agravitropism. The cross section analysis showed that st1 and D7 have not rescued their normal radial pattern. Both of them contain only two ground tissue layers. On the other hand hypocotyl of the 35S seedlings continued to have a normal radial pattern with three ground tissue layers. The result of radial pattern analysis is consistent with my hypothesis that the reason of agravitropism is not the absence of a cell layer but the deregulation. This finding also suggests that the *SCR* gene plays a more important role in gravity perception than previously considered.

Currently, the most favored hypothesis to describe this complex mechanism is the "Starch-statolith hypothesis" which postulates that gravity sensing involves sedimentation of amyloplasts (statoliths) in specific cells known as statocytes. I performed whole-mount amyloplast staining in order to identify the presence and location of amyloplasts in suppressors with improved gravitropic response. Staining results show that suppressors resemble *scr1* in both presence and position of amyloplasts rather than WS plants. 35S, st1 and D7 were also stained to check amyloplast sedimentation. The results demonstrated that amyloplast sedimentation in 35S is similar to WS while st1 and D7 resemble *scr1*. Our results on amyloplast sedimentation do not correlate with gravitropic responses and indicate that an alternative mechanism for sensing gravity other than amyloplast sedimentation must be operating in *Arabidopsis thaliana* hypocotyls.

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Table of Contents

List of tables

List of figures

I. Introduction and literature review

Life on earth originates and evolves in an environment where a gravitational field is omnipresent. All living organisms have accommodated to this constant force both on the structural and functional levels. Plants also orient and coordinate their growth in response to this gravitational field to maximize access to light, water and nutrients by a process called gravitropism (Blancaflor et al. 2003). Gravitropism first came to notice over two hundred years ago, when Knight showed that plants can reorient their organs in response to a centrifugal force by using a water wheel (Knight 1806). In addition to gravity, plant organs show tropic movement in response to other environmental cues like light and water. Gravitropism interacts with other tropisms such as phototropism, hydrotropism, thigmotropism to control plant growth. Plant shoots exhibit negative gravitropism by growing upward, away from the gravity vector. This growth allows plants to conduct photosynthesis while roots exhibit positive gravitropism, i.e., growing toward the gravity vector. This duality serves the crucial function of anchoring plants in soil, while orienting the plant in generally skyward direction. Thus, during seed germination, even in absence of light, young shoots keep growing upward and roots continue to grow downward.

The capability of a plant to sense gravity and bend its organ appropriately is a complex phenomenon. The response pathway of gravitropism can be divided into three organized steps: 1. perception of gravity by cells, 2. signal transduction (conversion of mechanical force into a biochemical signal), and 3. asymmetric elongation in the responding organ (Kiss 2000). Plants have specialized tissues or cells which can sense gravity and develop a biochemical signal; these

cells are known as statocytes. In eudicot shoots, cells of the endodermal layer (Fukaki et al. 1998) act as statocytes. Cells of the pulvini fulfill this role in monocot shoots (Song et al. 1988). Further, columella cells of the root cap function as statocytes in roots of the plant (Sack 1991). Eventually, the signal formed in statocyte cells is transmitted to outer layers and generates an auxin gradient through auxin transporters. According to the Cholodny-Went hypothesis, this auxin gradient triggers the differential growth rate in either side of the organ and causes asymmetric cell elongation (Went 1926; Cholodny 1927). The auxin influx carrier AUX and auxin efflux carrier PIN-formed (PIN) as well as related influx and efflux proteins all serve very important roles in auxin transport. Higher concentration of auxin (10^{-5} to 10^{-6} M) inhibits cell elongation in roots, but enhances it in shoots. Therefore, accumulation of auxin at the bottom of the shoot promotes growth of the lower region of the organ and the shoot ascends. In contrast, increased level of auxin in the bottom of the root causes upper side to elongate more than the lower side; therefore, the root descends (Haswell 2003).

Sites of gravity perception

Site of gravity sensing and response in shoots

Various aerial organs of different plant species are gravi-responsive such as *Arabidopsis thaliana* hypocotyl and inflorescence, pea hypocotyl, maize coleoptiles, barley pulvini etc (Fukaki et al. 1998; Song et al. 1988). Two common features observed in these gravi-responsive shoot organs are negative gravitopic response and the presence of sedimentable amyloplasts in their tissues. Because of the presence of the sedimentable amyloplast cells of these tissues has been considered as "statocytes", or gravity sensing cells. In eudicot stems and hypocotyls, the endodermal layer and in monocot stems the pulvini (knee-like swellings in stem) are believed to

be the specific site of gravity sensing, respectively (Fukaki et al. 1998; Song et al. 1988). *Arabidopsis thaliana* is the most well studied species and molecular genetic studies performed on this plant showed that the endodermal layer of the shoot acts as a statocyte (Fukaki et al. 1998). In *Arabidopsis thaliana*, both the root and the shoot possess a radial pattern of cell arrangement. This radial pattern organizes cell layers by the same radial plan; the central stele that includes the pericycle and vascular system, surrounded by endodermis, cortex and epidermis (Dolan et al. 1993). The SCARECROW (SCR) and SHORT ROOT (SHR) proteins are known to control the development of normal radial patterning in *Arabidopsis thaliana* (Helariutta et al. 2000). Both of the proteins belong to a well-known plant specific transcription factor family called GRAS (Pysh et al. 1999). Mutants, *scr* and *shr* both have abnormal radial pattern, they are missing a ground tissue layer. Along with this, both mutant plants show abnormal shoot gravitropism but normal root gravitropism (Fukaki et al. 1998). All *shoot gravitropism* (*sgr*) mutants of *Arabidopsis thaliana* show a defect in shoot gravitropism while they have normal root gravitropism. The *sgr3*, *sgr5* and *sgr6* have defective inflorescence gravitropism and *sgr1*, *sgr2*, *sgr4* and *sgr7* have both defective inflorescence and hypocotyl gravitropism (Fukaki et al. 1996b; Yamauchi et al. 1997). The *SGR1* and *SGR7* are allelic to *SCR* and *SHR* genes respectively (Fukaki et al. 1998). Recently orthologs of *SCR* and *SHR* have been identified in Japanese morning glory (*Pharbitis nil*) as weeping (*WE*) and weeping2 (*WE2*) genes respectively. Similar to *scr* and *shr* mutants of *Arabidopsis thaliana*, *we* and *we2* mutants also had an abnormal endodermal layer and exhibited impaired shoot but normal root gravitropic responses (Kitazawa et al. 2005; Kitazawa et al. 2008). All these results signify that the endodermal layer is involved in the gravity sensing. Gravitropism regulates the rate of cellular elongation of the stimulated organ and influences its growth in a specific direction. In shoots, both the site of gravity perception and response take place in the same

organ. In 1996, Fukaki et al. confirmed that the gravity perception site is not restricted to a specific region of the shoot and is present throughout the elongation zone. This study showed that segments of various parts of the stem were able to respond to gravity. Decapitated stems without the apical meristem, flower and leaves also exhibited similar gravitropic responses as an intact stem (Fukaki et al. 1996a).

Site of gravity sensing and response in root

The statocytes of roots differ significantly from those of shoots. In roots, the gravity sensing site is separate from the region that actually responds to gravity. The columella cells of the root cap act as statocytes while gravitropic response takes place in the elongation zone of the root (Sack 1991). The root cap covers the root tip and its meristematic region, protecting them from outer environmental hazards such as hard soil particles. In *Arabidopsis thaliana*, columella cells are present in the central region of the root cap. The root cap of *Arabidopsis thaliana* consists of four tiers of columella cells which are designated as S1, S2, S3 and S4 (with S4 nearest to the root tip) and lateral root cap cells (Fig. 1.1 C). It has been shown by performing laser ablations of specific cells in the root cap that the S2 layer contributes the most to gravity sensing. On the other hand, ablation of the lateral root cap cells did not have any significant effect on root gravitropism (Blancaflor et al. 1998). Transgenic lines of *Arabidopsis thaliana* that carry the diphtheria toxin A (DT-A) gene expressed under the control of a root-cap specific promoter, also confirmed the role columella cells in gravitropism (Tsugeki and Fedoroff 1999). Diphtheria toxin A is well known for inhibiting protein synthesis in cells. These transgenic plants showed abnormal root gravitropic response. Similar to the statocytes of shoots, columella cells also contain sendimentable amyloplasts. When roots are positioned vertically these amylopasts are located at

the end of the columella cells (Fig. 1.1 C). All of these aforementioned results lead us to believe that the root cap provides essential gravity sensing mechanism and columella cells may act as gravity sensing site in roots.

The meristem region of the root, just above the root cap, provides the specific region where cell division and initial cell differentiation occur. Distal elongation zones (DEZ) and central elongation zone (CEZ) both develop above the meristem region. The CEZ exist shootward of the DEZ and maximum elongation takes place in this zone (Ishikawa and Evans 1997). When the roots reorient, cell elongation in the central elongation zone and at the bottom side of the distal elongation zone ceases while cells of the upper side of the distal elongation zone expand massively, sloping the curvature in a downward direction at the DEZ (Ishikawa and Evans 1997).

Distal elongation zone of roots can also contribute to gravity sensing

Though columella cells are essential for gravity sensing in roots, there is a question as to whether root gravity sensing depends solely upon these columella cells. Researchers have always been curious about the possibility of dual gravity sensor sites in the root and they have been trying to dissect this phenomenon through the use of various tools. Experiments performed on maize seedlings using the specific rotation device ROTATO, which was connected to an automated camera, provided some valuable information (Wolverton et al. 2002). This device allowed the root tip to be held in a vertical position, while still gravi-stimulating the predefined region of the root. Experiments performed on *Zea* and *Arabidopsis* showed that, even when the root tip was in a vertical position, the DEZ of the root continues to curve. These results strongly suggest that DEZ functions as a secondary gravity sensing site in the root. Though twenty percent of the gravitational sensed response comes from the DEZ, columella cells are still the major sites of gravity sensing

(Wolverton et al. 2002). One more notable fact: DEZ cells do not have sedimentable amyloplasts (Baldwin et al. 2012).

Specific characteristics of statocyte cells

Large central vacuole with dynamic vacuole membrane

The statocyte cells of shoots possess some distinguishing features, most particularly a large central vacuole. Most the volume of a shoot statocyte cells is occupied by a large central vacuole (Fig. 1.1 B). The cytoplasm and cell organelles remain in the peripheral region as a thin layer between the plasma membrane (PM) and the vacuolar membrane (VM) (Morita et al. 2002). The vacuolar membrane contains various structures: trans-vacuolar strands, bulbs and sheets. These structures are inter-convertible and continuously emerge and disappear. Trans-vacuolar strands penetrate the central vacuole and develop a tunnel-like structure. Bulbs and sheets were originated by invagination and folding of vacuolar membrane into the lumen. They display a double membrane structure and surround relatively lower amount of cytoplasm. In the wild type *Arabidopsis*, amyloplasts of endodermal cells are almost entirely enclosed by the vacuolar membrane. While most of them are sedimented downward in the direction of gravity, some of them show upward saltatory movement (Fig. 1.1 B). All of the amyloplasts in shoot endodermal cells move through transvacuolar strands or narrow cytoplasmic spaces (Sack and Leopold 1985; Saito et al. 2005).

Arabidopsis shoot agravitropic mutants (sgr) indicate that flexibility of vacuolar membrane is important for shoot gravitropism

Investigation of *Arabidopsis* shoot agravitropic mutants such as *sgr2, sgr3, sgr8, sgr4* and *sgr6* provided a detailed knowledge about the gravity sensing mechanism. Both cytological and genetic studies of these mutants indicated that the flexibility of VM and membrane trafficking both contribute to normal shoot gravitropism (Morita et al. 2002; Silady et al. 2004; Yano et al. 2003; Hashiguchi et al. 2014). The position of an amyloplast was determined to be abnormal in endodermal cells of *sgr2*, *sgr3, sgr4/zig*, *sgr6* and *sgr8/grv2/kam2* mutants. The amyloplasts were not restricted to the bottom (lower side) of the cell. Instead they were distributed on the periphery of the cells. Transmission electron microscopy of *sgr3* and *zig/sgr4* also revealed that these mutants have an accumulation of abnormal vacuolar or vesicular structures in endodermal cells and that amyloplsts are no longer associated with the vacuolar membrane, and that they never demonstrate saltatory movements (Yano et al. 2003; Saito et al. 2005). This new imagery indicates that the less flexible and abnormal vacuole disturbs the normal amyloplast movement. Expression of *SGR2* and *SGR4/ZIG* genes tagged with *GFP* under control of *SCR* promoter (endodermis specific expression) rescued the shoot gravitropic response of both *sgr2* and *sgr4* mutants respectively. *SGR2* encodes a protein that has sequence similarity with bovine phosphatidic acid-preferring phospholipase A1-like protein and SGR2 protein fused with GFP was found to localize in the vacuoles and other cell organelles (Morita et al. 2002). Though these results showed a possibility of SGR2 protein to be involved in function of vacuoles, the role of *SGR2* in membrane trafficking still remains unclear.

Endoplasmic reticulum

The statocyte cells need to activate specific receptors upon gravi-stimulation in order to be able to sense the direction of the gravity vector. Not much information is available about these receptors however. Hensel and Sievers hypothesized that amyloplast sedimentation generates membrane tensions by acting upon the distal endoplasmic reticulum (ER) or plasma membranes via displacement of cytoskeleton tethers (Hensel and Sievers 1981). The tension generated in membranes could trigger the opening of mechano-sensitive ion channels. This channel opening activity would increase the concentration of Ca^{2+} in the cytoplasm, which then would initiate a calcium signal transduction pathway that has been previously linked to gravity response (Hensel and Sievers 1981). According to this theory the ER may play an important role in gravity perception. In shoots the ER is localized to the cell periphery since the central part of the cell is occupied by the vacuole and the cytoplasm is only present in the peripheral region. It is likely that amyloplasts, which are also restricted to the peripheral region, come in contact with the ER. Since the ER also functions as the intracellular reservoir of Ca^{2+}, amyloplast-ER collision may cause the release of Ca^{2+} and trigger the signal transduction upon gravi-stimulation.

Most of the volume of root columella cells is occupied by cytoplasm, with the nucleus present at the upper side of the cell. In contrast, the ER is positioned at the distal end of these cells and forms a cup shaped structure, and amyloplasts occupy the bottom of the cell (Fig. 1.1 D). The presence of a special type of nodal ER at the peripheral region of tobacco root columella cells has been previously reported (Zheng and Staehelin 2001). Recently a close contact between amyloplasts and the cortical ER was observed by using electron micrographs with high-pressure freezing and freeze-substitution methods (Leitz et al. 2009). These finding suggests a potential

involvement of the ER in gravity sensing, however no direct evidence is available still to date support this point.

Actin microfilaments arrangement

The plant cytoskeleton is primarily made up of actin filaments (AFs) and microtubules (MTs). The intricate cytoskeleton of plants plays an important role in wide range of cellular processes such as cell division, cell signaling and cell expansion. The AF network has been considered to play a critical role in gravity sensing (Perbal and Driss-Ecole 2003). In *Arabidopsis*, hypocotyls and stems have been shown to have thick bundles of actin filaments in their endodermal cells and it is hypothesized that sedimenting amyloplasts in these cells should exert mechanical tension on AFs (Yamamoto and Kiss 2002; Saito et al. 2005). Tension in the AFs has been shown to cause opening of mechano-sensing or stretch- activated ion channels in the plasma membrane (Perbal and Driss-Ecole 2003). This ion channel activation could be responsible for the sudden change in Ca^{2+}, H^{+}, and inositol 1,4,5-triphosphate ($InsP_3$) concentration in the cytoplasm observed during gravi-stimulation (Perbal and Driss-Ecole 2003). This suggests a positive role of AFs in gravity sensing and it was expected that the use of actin-destabilizing drugs would decrease the gravity sensing ability of plants. However, *Arabidopsis* hypocotyls and stems treated with Latrunculin B (Lat B), a potent actin-disrupting drug, showed enhanced gravitropic response (Yamamoto and Kiss 2002). Lat-B treatment reduced the saltatory movement of amyloplasts in shoot statocyte cells and almost every amyloplast sedimented in the direction of the gravity vector. This finding may help to explain the enhanced shoot gravitropic response in the shoot.

An *Arabidopsis* mutant, *shoot gravitropism 9 (sgr9)* with significantly reduced shoot gravitropism has been identified (Nakamura et al. 2011). SGR9 encodes RING-type ubiquitin E3

ligase, which is localized on amyloplasts and facilitates the amyloplast sedimentation in shoot endodermal cells. Since SGR9 is an ubiquitin E3 ligase, it most likely modulates amyloplasts and AFs interaction via detaching the amyloplasts from AFs. SGR9 may degrade the substances that anchor the amyloplasts with AFs. As noted above in the wild type shoot, endodermal cell amyloplasts are in equilibrium between sedimentation and saltatory movements. The *sgr9* mutant, with abnormal amyloplast and AFs interaction, showed defective amyloplast sedimentation and had increased number of amyloplasts that display saltatory movement (Nakamura et al. 2011). Interestingly Lat-B (actin-disrupting drug) treatment of *sgr9* mutants rescued its shoot gravitropic response. Kato et al, reported that a semi-dominant mutant *frizzy shoot 1 (fiz1),* with an amino acid substitution in ACT8 that causes fragmentation of AFs, showed enhanced amyloplast sedimentation and decreased saltatory movement (Kato et al. 2010). Endodermis–specific expression of $ACT8^{fiz}$ (heterozygous *fiz* mutation) in *sgr9* mutants also restored the shoot gravitropic response of this mutant (Nakamura et al. 2011). These results showed the importance of AFs in equilibrium between sedimentation and saltatory movements of amyloplast, which is essential for normal shoot gravitropic response. These results also showed that AFs do not act as a positive regulator of gravitropism. AF's may negatively control the gravitropic response by restricting the amyloplast sedimentation. The hypothesis that SGR9 detached the amyloplsts from AFs to facilitate amyloplast sedimentation in endodermal cells of shoots was also supported by these results (Nakamura et al. 2011).

In contrast to thick bundles of actin filaments in shoot endodermal cells, root columella cells have a fine network of randomly oriented actin filaments in *Arabidopsis.* In wild type plants, amyloplasts are surrounded by this fine actin network (Collings et al. 2001). To explain the role of cytoskeletal system in root gravity sensing, the restrained model has been proposed (Kumar et al.

2008). According to this model, organelles within columella cells are not free to move, they are physically linked to the cytoskeleton and their movements are controlled by the cytoskeleton. When the cell is reoriented, the organelles are proposed to exert pressure on the cytoskeleton (and the membrane receptors because they are inter-connected) thereby triggering signal formation by opening stretch activated mechano-sensitive ion channels. Consistent with this suggestion, the degradation of the cellular actin network should inhibit gravity perception and eventually the gravitropic response in roots. However, Lat B treatment, similar to shoots, displayed enhanced root gravitropic response both in maize and *Arabidopsis* (Hou et al. 2003; Hou et al. 2004). In fact, with the use of ROTATO devise, Hou et al. showed that, Lat B treated corn seedlings, when exposed to defined angle of gravistimuli, exhibited an extended root bending in comparison to untreated seedlings (Hou et al. 2003). This finding suggests that both in shoots and roots AFs play a role of negative regulator of gravitropic response by restricting displacement of amyloplast. Also AFs are required for proper vacuolar dynamics and they can modulate amyloplast sedimentation in shoot endodermal cells by controlling the arrangement of VM (Hashiguchi et al. 2013).

Proposed gravity sensing mechanisms

Although a great deal of research has been performed to explain the phenomenon of gravitropism, the precise molecular mechanism of this complex process is still unknown. Evolution of higher organisms tends not to discard what can assist fitness; it seems to supplement the pre-existing system of less complex organisms. Therefore, most likely flowering plants with higher position in the evolutionary hierarchy possess multiple systems for gravi-sensing. According to physical principles, in order to detect a gravi-stimulation, motion of two masses relative to each other is necessary. These masses should be able to perform a significant amount

of work on a receptor. Inside plant cells, starch filled amyloplasts and entire masses of protoplast have been considered as potential gravi-receptors. To explain how the direction of gravity is perceived by plant organs, two hypotheses: the starch-statolith model and the protoplast pressure model have been proposed.

Starch-statolith hypothesis

This hypothesis postulates that in higher plants, amyloplasts act as statoliths and that sedimentation in response to gravi-stimulation in statocyte cells is required to perceive the direction of gravity. This is the most favored hypothesis and it has been discussed in many reviews (Sack 1997; Caspar and Pickard 1989; Kiss et al. 1989). Amyloplasts are non-pigmented plastids and they fall under the category of leucoplasts (Weise 2007). Fundamentally, amyloplasts do not have well-developed thylakoid membrane systems and they contain many large starch granules. Electron micrographic analysis of plastids of the *Arabidopsis* shoot endodermal layer reveals that amyloplasts of light-grown seedling hypocotyls and barley pulvini statenchyma cells contain both well-developed thylakoid membrane systems and pigments along with several starch granules (Morita et al. 2002; Song et al. 1988). More precisely, these plastids commonly called amyloplasts are "starch accumulating chloroplasts". While amyloplasts of dark grow hypocotyls are similar to the fundamental definition of an amyloplast, they lack pigment and a thylakoid system (Sack 1987). However, prolamellar bodies are occasionally found in these hypocotyls. On the other hand, plastids of root columella cells have elevated counts of starch granules but they are non-pigmented and lack a thylakoid membrane system (Sack 1991). Therefore, as these plastids are almost identical to the fundamental definition of an amyloplast, surely root columella plastids are true

amyloplasts. The conclusion is that although both shoots and roots contain amyloplasts they are not identical in shoot endodermal and root columella cells.

Movement of amyloplasts in statocyte cells in shoots and roots

It is believed that amyloplasts act as "statoliths", gravity sensors. The verbal meaning of "statolith" is "stationary stone", however, behavior of amyloplasts is not very consistent with the ideal definition of a statolith, especially in the shoot statocyte cells. In a vertically positioned shoot, most of the amyloplasts are displaced toward the bottom of the statocyte cell but are unlikely to settle to the bottom (Saito et al. 2005; Sack et al 1985). Microscopic observation of both living maize coleoptiles (Sack et al. 1985) and *Arabidopsis* shoot (Saito et al. 2005) statocyte cells revealed that amyloplasts move through transvacuolar strands. The most interesting finding was that amyloplasts move both in downward and upward directions via transvacuolar strands (Fig. 1.1 B). This upward movement of amyloplasts is known as "saltatory" movement. It is a type of non-Brownian cytoplasmic streaming (Sack et al. 1985). As explained earlier, various *Arabidopsis shoot gravitropic (sgr)* mutants, exhibit abnormal shoot gravitropism. These mutants contain an accumulation of abnormal vacuolar systems along with amyloplasts distributed around the cell periphery. It was observed that in *Arabidopsis* shoot endodermal cells, amyloplasts maintain equilibrium between sedimentation and saltatory movement by interacting with F-actin filaments (Nakamura et al. 2011). Disruption of actin-filament network with the treatment of an actin depolarization drug, Latrunculin-B (Lat-B), diminishes the saltatory movement of endodermal amyloplasts in *Arabidopsis* and enhances sensitivity to gravity. Also, RING-type E3 ligase protein, encoded by SGR9, is known to promote the detachment of amyloplasts from F-actin in response to gravi-stimulation (Nakamura et al. 2011). These results provide clear evidence that amyloplasts

do not move toward the bottom of the cell by default; rather their movement is sophisticatedly controlled by their interaction with various cellular components such as F-actin and vacuolar membrane. On the other hand, in roots, the behavior of amyloplasts is closer to the fundamental definition of statolith; most of them are sedimented at the bottom of columella cells and are relatively stable. Further, saltatory movement is not a common characteristic of root columella cells. Although located in both root and shoot statocytes, the positions of amyloplasts inside the statocyte cells are not very similar in these two gravity sensitive plant organs. It seems that the mechanisms to control amyloplast movements in statocyte cells of roots and shoots are different from each other.

Evidence to support the role of amyloplast in gravity sensing

Harberlandt was first to observe sedimentation of amyloplasts in the direction of gravity within some specific cells (Haberlandt 1900). Further investigation revealed the presence of amyloplasts in almost all gravi-responsive organs of various vascular plants. It was found that in *Arabidopsis*, when the stems were moved horizontally, amyloplasts were relocated to a new basal position within three minutes (Saito et al. 2005). Various studies performed on different plants showed that in root columella cells, almost all amyloplasts are located at the bottom of the cells and, when reoriented 90^0 to the gravity vector, the amyloplasts changed their position and re-sedimented to the new base of the root within a minute of gravi-stimulation (Sack et al. 1985). These observations show that amyloplasts respond to gravity.

The amyloplast sedimentation theory got further support from experiments performed using a high-gradient magnetic field (HGMFs) (Kuznetsov and Hasenstein 1996). HGMFs laterally displace the diamagnetic amyloplasts in statocyte cells. It generates a force of 1g or greater to

displace amyloplasts by using the difference in negative magnetic susceptibility between starch granules and other cytoplasmic organelles. When shoots and roots were exposed to HGMFs, amyloplast shifted in an opposite direction from the direction of magnetic field and this shift in amyloplast location was sufficient to cause root bending in the direction of amyloplast displacement and shoot bending in the opposite direction to the amyloplast relocated side (Kuznetsov et al. 1996; Kondrachu and Hasenstein 2001). These organ bending responses were very similar to gravitropic responses and they provided direct evidence that sedimentation of amyloplasts can cause organ bending both in root and shoot.

Presence of starch in amyloplasts is crucial for optimal gravitropic responses

The most remarkable characteristic of an amyloplast that makes it a potential candidate for being a statolith is its dense starch granules. For this reason, it was interesting to analyze the gravitropic response of a mutant defective in starch synthesis. A thorough investigation of gravitroipc responses of *Arabidopsis* starchless mutant, *phosphoglucomutase (pgm)*, exhibited reduced but not completely aborted gravitropic responses, both in roots and shoots (Caspar and Pickard 1989). *PGM* encodes an enzyme phosphoglucomutase that mediates starch biosynthesis. Therefore, the *pgm* mutant amyloplast starch granules do not contain as much starch and do not sediment to the bottom of the mutant statocyte cells (Kiss et al. 1989). Impaired amyloplast sedimentation and reduced gravitropic responses of mutants positively correlate with each other. Also, when these starchless mutants were placed in HGMF, no root bending was observed unlike wild type plants (Weise and Kiss 1999). This observation clearly showed that only amyloplasts and no other cytoplasmic component can be forced to displace by HGMF and cause gravitropic response. One other noticeable result was that amyloplasts of columella cells of the *pgm* mutant

sedimented at the bottom of the statocyte cells in response to hypergravity and also exhibited root bending (Weise and Kiss 1999).

The importance of starch in gravity sensing can also be illustrated by an *Arabidopsis thaliana* mutant *sex1* (*starch excess*). This mutant is defective in an enzyme that regulates the starch degradation; therefore, *sex1* seedlings contained excess starch in some tissues such as cotyledons, hypocotyls, the hypocotyl-root transition zone and root cap cells (Vitha et al. 2007). Analysis of *sex1*, seedlings showed that the sizes of amyloplasts of these mutants are occasionally larger than in wild type, depending on organs and growth conditions. Both roots and dark grown hypocotyls of mutant seedlings contain amyloplasts similar in size to the wild type seedlings. Consistently, these seedlings showed similar gravitropic sensitivity. However, hypocotyls of *sex1* mutants grown in light have larger amyloplasts and stronger gravitropic sensitivity than the wild type. Hypocotyl of *sex1* curved much faster than wild type in response to new gravity vector. These results advocated the hypothesis that a large change in amyloplast density in comparison to wild type can also change its ability to sense gravity (Vitha et al. 2007).

Further support for the amyloplast sedimentation hypothesis comes from another *Arabidopsis thaliana* mutant, *endodermal amyloplast less1* (*eal1*), allelic to *SGR7/SHR* (Fujihira et al. 2000; Morita et al. 2007). This mutant exhibits inflorescence complete agravitropism, reduced hypocotyl gravotropism and normal root gravitropism. Interestingly, *eal1* mutants contain an endodermal layer both in roots and shoots; however, endodermal amyloplasts do not accumulate starch and expression of *SGR5* gene (mainly expressed in inflorescence endodermal layer) is markedly reduced. Only one amino acid is deleted in proteins encoded by *eal1* and the presence of an endodermal layer in *eal1* indicates that these proteins still act as a transcription factor (Fujihira et al. 2000). However, they are not able to develop gravity-responsive amyloplasts and

their shoot endodermis is also not sufficiently differentiated to respond normally to gravi-stimulation. In peanut plants, the gynophore is the gravity responsive organ while amyloplasts of the starch sheath of gynophores act as a gravi-sensor (Moctezuma and Feldman 1999). Removal of amyloplasts from gynophores with the treatment of gibberellic acid and kinetin also reduced gravi-stimulated bending by 82% in comparison to water treated gynophores. These results strongly support that starch filled amyloplasts and normal endodermal layers are required for gravity-perception.

All these data collected from the analysis of various mutants such as *pgm*, *sex1*and *eal1* strongly suggest that the strength of gravitropic response is directly proportional to the starch content of endodermal amyloplasts. Therefore, starch plays an important part in gravity perception and is required for a complete and normal gravitropic response. However, the residual gravitropic responses of both roots and shoots of the *pgm* mutants indicate that starchless amyloplasts can also trigger gravity perception and when sedimented forcefully via hypergravity, these starchless amyloplasts can trigger a full gravitropic response. Therefore, starch is an important component that provides a certain mass to the amyloplast and facilitates sedimentation in response to gravi-stimulation; however, the amyloplast itself is likely to act as a statolith and perceive the direction of gravity.

PIFs inhibit conversion of amyloplasts into other types of plastids

Phytochromes (red and far-red light photoreceptors) are well known for their involvement in regulation of various aspects of plant development. It was found that phytochromes inhibit negative gravitropism of hypocotyls in light but the mechanism of this process is poorly understood (Kim et al. 2011). Recently, a *phytochrome-interacting factor* quadruple mutant

(*pif1pif3pif4pif5or pifQ*) has been identified (Shin et al. 2009). In dark conditions, the *pifQ* mutants exhibit completely impaired negative gravitropism in hypocotyls, which indicates that by inhibiting PIFs, phytochromes inhibit the hypocotyls gravitropic response. Further analysis of *pifQ* mutants revealed that amyloplasts in the endodermal layer of dark grown hypocotyls have partially converted into etioplasts. In contrast to wild type endodermal amyloplasts that are filled with large starch granules, *pifQ* mutant amyloplasts contain small starch granules, prolamellar bodies and prothylakoids. Endodermis specific expression of *PIF1* under control of *SCR* promoter, in *pifQ* mutant, rescued gravitropic response of dark grown hypocotyls by inhibiting the conversion of amyloplasts into etioplasts (Kim et al. 2011). These results strongly suggest that *PIFs* are responsible for inhibiting the conversion of amyloplasts into other type of plastids. These results also indicate that in order to inhibit hypocotyls gravitropic response, phytochromes inhibit *PIFs* activity, resulting in conversion of amyloplasts into etioplasts or chloroplasts.

Rate of sedimentation of amyloplasts can also affect gravitropic response

Recently *Loose Plant Architecture 1 (LPA1),* a putative transcriptional factor and a functional ortholog of *SGR5* has been identified in rice (Wu et al. 2012). *LPA1* modulates the architecture of rice plants by controlling the gravitropism of leaf sheet pulvinus and lamina joint. The *lpa1* mutant showed reduced shoot gravitropism but their root exhibited a normal response upon gravi-stimulation. In WT amyloplasts sedimented to basal ends of the coleoptile parenchyma cells within 10 minutes of gravi-stimulation. On the other hand, in *lpa1* mutants within the same time period amyloplasts were located almost at the center of the cell and they only reached the bottom after 20 minutes. Therefore, *LPA1* may act in gravity perception by controlling the rate of sedimentation of amyloplasts.

Furthermore, Toyota *et al*., analyzed the correlations between the amyloplasts movements and gravitropic curvatures of wild type and various mutants of *Arabidopsis* by using custom-designed centrifuge microscope under hypergravity conditions (Toyota et al. 2013). The custom-designed microscope that contains a coupled centrifuge and microscope is specifically designed for continuous observation of specimen during centrifugal acceleration (Inoue et al. 2001). Toyota's group found that in wild type stems the sedimentary movements of amyloplasts are linearly correlated with gravitropic curvature under hypergravity conditions (Toyota et al. 2013). Also the *Arabidopsis* mutants, *sgr2*, *sgr9* and *pgm* that exhibit impaired shoot gravitropism and abnormal amyloplast sedimentation at 1g condition were gravi- stimulated under 30g conditions. Interestingly, all these mutants were able to respond to gravity and their amyloplasts were also sedimented under hypergravity conditions. Similar to wild type stems, the sedimentary movements of amyloplasts are linearly correlated with gravitropic curvature in mutants, too. These results show the link between gravisensing and amyloplast movement. However, both *scr* and *shr* mutants failed to respond to gravity under 30g condition. This result supports the hypothesis that endodermal cells are essential for gravity sensing in stems.

Impaired amyloplst sedimentation can disturb the gravistimulated lateral auxin gradient

According to the Cholodny-Went theory (1926-1927), gravity perception triggers the asymmetric distribution of auxin on opposite sides of the elongation zone. This asymmetric distribution is mediated via change in distribution of auxin efflux facilitators (PIN-FORMED or PIN proteins) in statocyte cells and causes differential growth followed by curvature of the gravi-stimulated organ. It suggests that there should be some link between amyloplast sedimentation and asymmetric auxin distribution, and to explore this possibility Leah and co-researchers analyzed

changes in auxin response reporter (DII-VENUS) on gravity stimulation in starch-less mutant *pgm-1*(Shaner et al., 2005). DII-VENUS is a quantitative Aux/IAA based reporter and it contains an auxin-binding domain (DII) of the Aux/IAA28 protein fused with fast maturing variant of YFP (yellow fluorescent protein), VENUS driven by 35S promoter. Since binding of DII-VENUS with auxin triggers reporter degradation, concentration of DII-VENUS should be co-related to endogenous auxin level. By using confocal imaging and parameterized mathematical model (Banda et al. 2012). It was found that, after 2hr of gravi-stimulation, the difference in DII-VENUS signal between upper and lower sides of root tip was at a maximum. In contrast to wild type, in root tips of *pgm-1* mutants DII-VENUS gradient formation was entirely disrupted after 2hr of gravity stimulation. These data strongly indicate a positive correlation between amyloplast sedimentation and auxin redistribution. All evidence collected from the analysis of various mutants strongly advocates the theory that the displacement of amyloplasts within specific cells (statocytes) is most probably the first step of perception of gravity and it may also be responsible for triggering the down-stream events of gravity stimulated organ curvature.

Higher plants can have multiple mechanisms of gravity perception

The amyloplast sedimentation hypothesis is a well-documented model of gravity sensing, supported with multiple lines of evidence. However, we still cannot claim that it is the one and only way to perceive gravity in higher plants. As mentioned earlier in *Arabidopsis thaliana* roots, distal elongation zone (DEZ) acts as secondary gravity sensing site but no amyloplasts are observed there (Baldwin et al. 2012). This result strongly suggest that the DEZ has an amyloplast-independent mechanism by which to sense gravity. In starch-less *pgm-1* mutants, amyloplast sedimentation is missing and they display a reduced gravitropic response. However, they are not

completely agravitropic and a residual gravitropic response is still present in these mutants. It indicates that plants can still sense the direction of gravity, even in absence of sedimented amyloplasts. In other words, amyloplast sedimentation may be essential for an optimal gravitropic response, however, it is not necessary for residual gravity sensing. By using the ROTATO device, Wolverton et al. showed that in wild type (Columbia) plants the rate of root curvature depends on the constant angle at which the root tip is held; the larger the angle of displacement, the faster the rate of curvature (Wolverton et al. 2011). In the *pgm* mutants, root tips bend with a constant rate, independent of the angle of displacement (Wolverton et al. 2011). This observation indicates the presence of an entirely different type of gravitropic pathway in *pgm* from that of the Col.

In addition, support for an amyloplast independent gravity sensing mechanism comes from analysis of gravitropic response of lateral roots of *Arabidopsis pgm-1* mutants (Bai and Wolverton 2011). Recently it was observed that in contrast to primary roots, lateral roots of *pgm-1* mutants displayed no difference in gravitropism compared to wild type lateral roots. Similar to primary roots of *pgm-1* mutants, lateral roots also have starchless amyloplasts that do not sediment in response to gravi-stimulation (Bai and Wolverton 2011). These data suggest that amyloplast sedimentation is not required for gravity sensing in lateral roots and an alternative pathway for gravity perception in these organs, independent of amyloplast sedimentation is required.

Brassinosteroids (BRs) act as negative regulators in shoot gravitropism (Vandenbussche et al. 2011). To get a better understanding of role of BR in shoot gravitropism, both *pgm* and *scr* mutants were treated with brassinazole (Brz), a compound that blocks BR synthesis. Interestingly both mutants were found to have a partially rescued gravitropic response, while their starch synthesis and amyloplast sedimentation were still defective. Therefore, deficiency of BR can partially restore gravitropic responses of both *pgm* and *scr* mutants without restoring amyloplasts

sedimentation. Though the mode of action for BR is still not clear, it is anticipated that in the absence of BR an amyloplast-independent gravity sensing pathway could be triggered in both mutants.

Further support for the presence of an alternative gravity sensing mechanism comes from analysis of expression of an auxin-responsive reporter DR5::GFP in the *pgm* mutants (Wolverton et al. 2011). Gravity stimulated root cap of the *pgm* mutants failed to generate an asymmetric expression of DR5::GFP during its residual gravi-bending. These results indicate that the plants can have two different types of gravitropic pathways, one amyloplast sedimentation and asymmetric auxin distribution dependent and the other could be amyloplast-auxin independent. All these findings indicate that the amyloplast sedimentation model for gravity perception is insufficient and requires a revision.

Protoplast pressure hypothesis

According to this hypothesis the entire cell protoplast acts as gravity receptor, and hydrostatic pressure exerted by the protoplast on the walls of statocyte cells allows them to sense the direction of the gravity vector. This hypothesis proposes that plant cells can sense their relative buoyancy to that of the external media surrounding them (Sack 1994). Wayne et al. observed that in the green alage *Chara*, the large internodal cells use gravity to control the polarity of cytoplasmic streaming and they do not contain amyloplast/statoliths (Wayne et al. 1992). It is hypothesized that internodal cells can sense the difference in upper and lower side hydrostatic pressure and this differential hydrostatic pressure can trigger the opening of mechano-sensitive ion channels. Opening of ion channels can cause the increase in cytosolic Ca^{2+} level that would eventually began to change the polarity of cytoplasmic-streaming. Based on inhibitor studies it

was also suggested that differential change in protoplasmic pressure can be sensed by integrin-like proteins, located close to and in the cell membrane (Baldwin et al. 2013). These results are exciting and researchers assumed that this protoplast pressure model may also be functional in higher plants. This assumption got some support from the gravitropic response of rice roots exposed to high density media (Staves et al. 1997). It was found that gravitropic response in roots of rice can also be changed by the external medium. However, the fact that most of the evidence for the protoplast pressure hypothesis comes from study of cytoplasmic streaming of giant *Chara* internodal cells that are much larger than the usual statocyte cells of vascular plants, should not be ignored. In conclusion, having a perspective of "all or none" about the role of amyloplast sedimentation in gravity sensing is not very fruitful. Either gravity sensing is completely amyloplast sedimentation dependent or amyloplasts have no role in gravity sensing. Neither theory looks very convincing in terms of fully explaining this phenomenon. All evidence mentioned earlier suggests that most probably plants have more than one mechanism to sense gravity, and it is also clear that starch and amyloplasts are required to have an optimal gravitropic response.

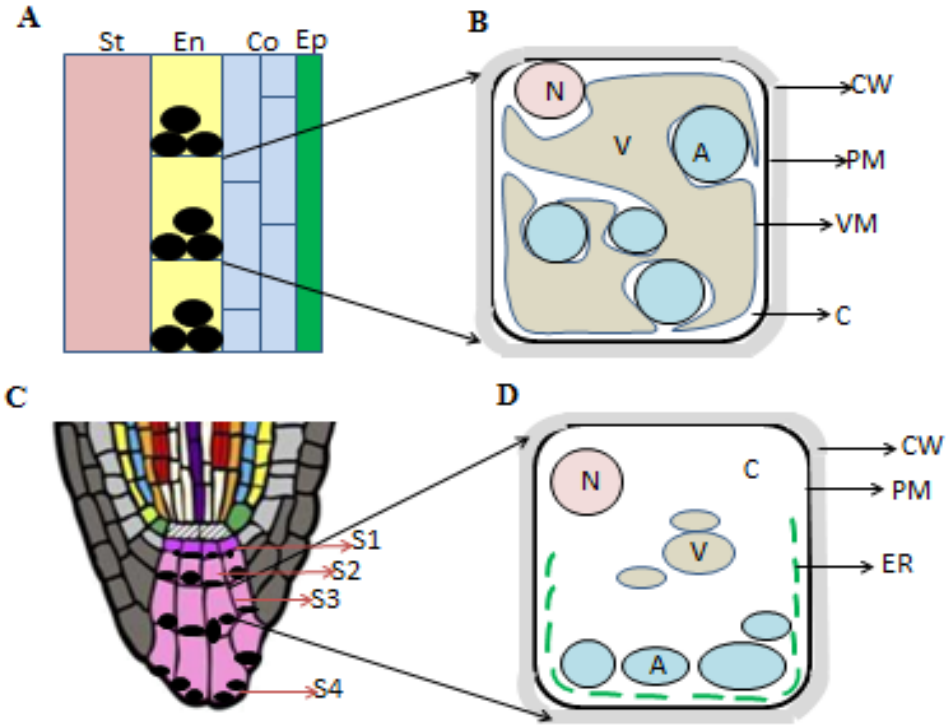

Figure 1.1 Shoot and root statocytes. (Modified version of Y. Hashiguchi et al. 2013). (A) Schematic structure of the hypocotyl of *Arabidopsis thaliana*. St, stele; En, endodermis; Co, cortex; Ep, epidermis. **(B)** Diagram of an endodermal cell (shoot statocyte cell). CW, cell wall; PM, plasma membrane; VM, vacuolar membrane; C, cytoplasm; N, nucleus; V, vacuole; A, Amyloplast. In shoot endodermal cells, amyloplasts exhibited both downward movements in the direction of gravity and upward saltatory movements. **(C)** Schematic structure of the root cap of *Arabidopsis thaliana* root. S1, the first layer; S2, the second layer; S3, the third layer; S4, the fourth layer of the columella cells. **(D)** Diagram of a columella cell (root statocyte cell). CW, cell wall; PM, plasma membrane; ER, endoplasmic reticulum; C, cytoplasm; N, nucleus; V, vacuole; A, Amyloplast. Amyloplasts [black ovals in (A) and (C)] sedimented in the direction of gravity, saltatory movement is rarely observed in root columella cells.

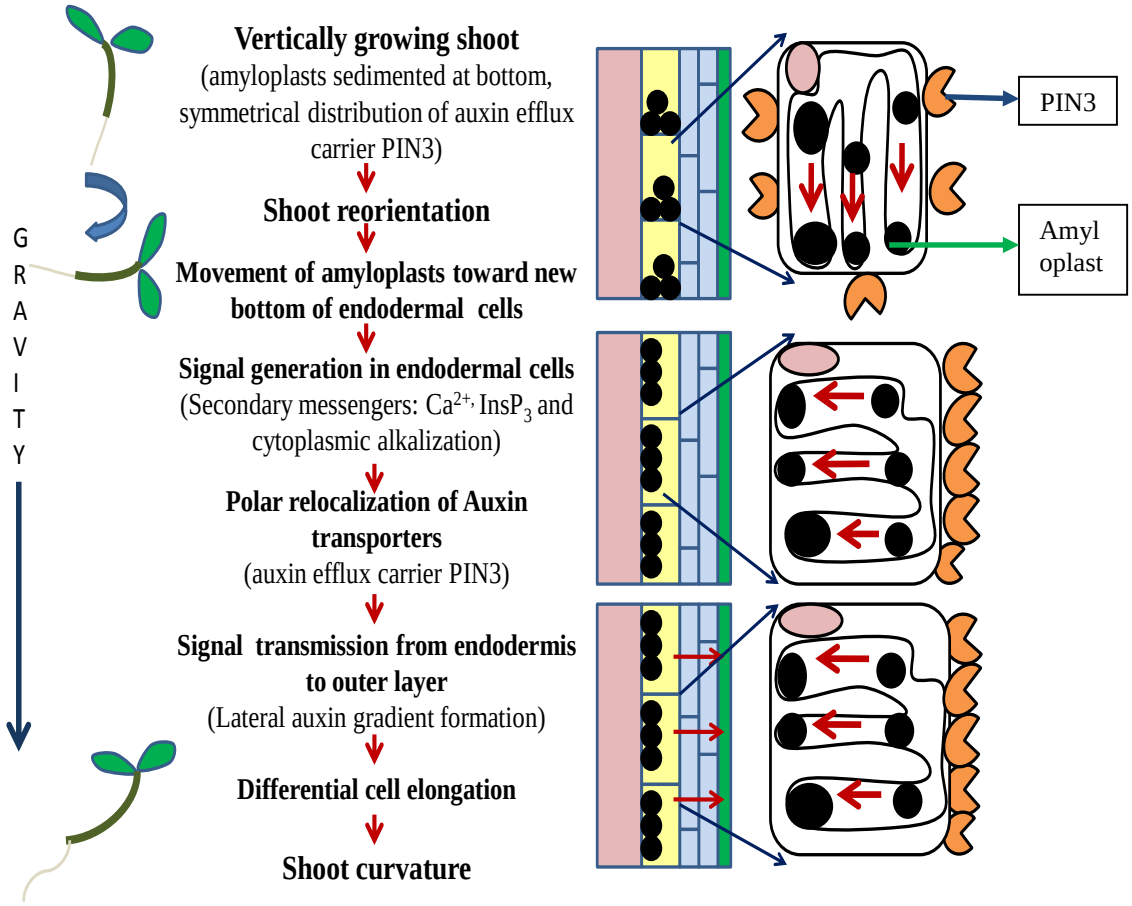

Figure1.2 Shoot gravitropism overview. In vertically growing shoot most of the amyloplasts are located at the bottom of the endodermal cells (yellow layer in figure, shoot statocyte cells) and auxin efflux carrier PIN3 is symmetrically distributed around the plasma membrane. To gravi-stimulate the shoot, plant is reoriented 90^0 relative to the gravity vector. The entire volume of endodermal cell except for a thin cytoplasmic layer is occupied by a large central vacuole. Amyloplasts travel through the transvacuolar strands and most of them move in the direction of gravity to reach the new bottom of the cell, while some of them exhibit upward saltatory movements. Flexible vacuolar membranes and SGR proteins mediate the proper movement of amyloplasts. Sedimentation of amyloplasts triggers the signal formation in endodermal cells via secondary messengers. Ca2+, InsP3 and cytoplasmic pH change possibly act as secondary messengers and somehow activate polar relocalization of auxin efflux carriers such as PIN3. PIN3 relocalization changes the direction of flow of auxin and develops a lateral auxin gradient at the lower side of shoot. This differential auxin distribution causes differential cell elongation, resulting in shoot curvature.

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II *scr1* mutagenesis and *scr* suppressor mutants screening to identify new components involved in SCR regulated pathways

Abstract

The SCR, a transcription factor, plays an important role in several developmental phenomenon in *Arabidopsis thaliana* because *scr* mutant plants have short roots, radial pattern defects in both roots and shoots, agravitropic shoots etc. To identify the new components involved in SCR regulated pathways *scr* suppressor mutants were generated. A wild type ecotype of *Arabidopsis thaliana*, Wassilewskija (WS), was used in this study. The *scr-1* mutant is on WS ecotype background. For suppressor screening we used the *scr*1 allele; it has a T-DNA insertion in the *SCR* coding region. To set up a screen of mutants that suppress the *scr*1 abnormal phenotypic characters, homozygous seeds of *scr*1 were mutagenized with Ethyl Methane Sulphonate (EMS). Mutated seeds were germinated and the resulting plants gave rise to M1 (~ 4,000 independent lines) progeny used in the *scr* suppressor screen to select the plants with improved phenotype. Thirteen suppressors, ten with improved hypocotyl gravitropism and three with improved root growth have been identified. All these 13 suppressors are homozygous for *scr*1, as was confirmed by PCR amplification of genomic DNA from each suppressor line and demonstration of the absence of WS (wild type) *SCR* allele.

Keywords: Transcription factors, *scr* suppressor mutants, EMS, Wassilewskija

Introduction

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. Suppressor mutations are the types of mutations that can hide or suppress the phenotypic effects of another mutation. Suppressor screening is a powerful tool for analyzing the characteristics of a known gene or mutation. Suppressor screening starts with mutagenesis of a known mutation followed by identification of second-site mutations (suppressor mutation occurs at a site distinct from the original mutant site) that suppress the original mutant phenotype (Fay 2006). Ethyl methane sulfonate (EMS) that preferentially induces G to A transitions, fast neutron bombardment that usually causes small deletions and insertion elements such as T-DNA or other transposons that can cause loss of function of disrupted genes are some important mutagens commonly used in *Arabidopsis* mutagenesis. EMS is one of the most powerful and frequently used chemical mutagen in plants (Brockman et al. 1984). EMS commonly induces multiple point mutations in each plant and this high rate mutagenesis makes possible the identification of plants with the phenotype of interest by screening of relatively few plants (Weigel and Glazebrook 2002). EMS mutagenesis can cause various effects on gene function such as: complete loss of function, partially reduced function, qualitatively altered function, and constitutive function. Usually positional cloning is the only technique used to isolate the genes corresponding to EMS mutations.

Role of *SCR* in Shoot Gravitropism and Developmental Processes of *Arabidopsis thaliana*

SCARECROW (*SCR*), a well characterized gene, plays the role of transcriptional regulator in *Arabidopsis thaliana* and it is essential for the radial patterning of the root, development of endodermis of the shoot, normal shoot gravitropism and indeterminate root growth (Wysocka-

Diller et al. 2000; Sabatini et al. 2003). The quiescent center (QC), a group of slowly dividing cells located at the center of root tip, is necessary for the maintenance of "stem" cells (initials in their undifferentiated state) of root tip. QC and stem cells together form the stem niche and it is required for indeterminate growth of the root (Van den Berg et al. 1997). It was reported that *scr* (*scarecrow* mutants) of *Arabidopsis thaliana* exhibited determinate mode of root growth, shoot agravitropism and abnormal internal architecture in all organs examined (Sabatini et al. 2003; Wysocka-Diller et al. 2000). Further analysis of the shoot's internal architecture also reveals that both hypocotyl and inflorescence stem have a defective radial pattern (Fukaki et al. 1998; Wysocka-Diller et al. 2000). One ground tissue layer is missing in roots because the asymmetric cell division responsible for the development of cortex and endodermis from the cortex/endodermis initials does not take place (Di Laurenzio et al. 1996). It seems that one ground tissue layer is also missing in stems but it is not as clear especially in the inflorescence because in that organ a variable number of cortical layers may be present (Wysocka-Diller et al. 2000). It is believed that in the stem the endodermis is involved in gravity sensing; therefore the missing layer could be the endodermis (Fukaki et al. 1998).

GRAS proteins are a well-defined family of plant-specific transcription factors and currently 33 GRAS genes have been described in *Arabidopsis* (Pysh et al. 1999; Lee et al. 2008). SCR is the first identified member of the GRAS family (Di Laurenzio et al. 1996). SCR along with SHORTROOT (SHR, also a member of GRAS family) plays a key role in radial patterning, root meristem maintenance and endodermal differentiation in *Arabidopsis* (Fukaki et al. 1998; Wysocka-Diller et al. 2000; Nakajima et al. 2001; Mylona et al. 2002; Sabatini et al. 2003; Heidstra et al. 2004; Gallagher et al. 2004; Paquette and Benfey 2005). *scr* mutants display defective organization of root cap and QC resulting in determinate root growth and abnormal root radial

pattern. Consistent with abnormal radial pattern and defective root growth of *scr* mutant, in WT (wild type) plant *SCR* expression was detected in the endodermal cells of root and QC (Di Laurenzio et al. 1996; Wysocka-Diller et al. 2000). Furthermore, it was demonstrated that SCR protein is essential for repression of initial differentiation in the QC (Sabatini et al. 2003). Therefore, the QC cells of *scr* mutants lose their ability to divide and differentiate early on in seedling development, leading to the "short root" phenotype. Similar to its expression in the endodermis of the root, *SCR* is expressed in the endodermal layer of the inflorescence and the hypocotyl of the wild type plant (Wysocka-Diller et al. 2000). It was observed that *SHR* that is expressed within the stele moves outward into adjacent ground tissue where it is required for QC and endodermis specification (Helariutta et al. 2000; Nakajima et al. 2001; Sabatini et al. 2003). Expression of SCR is restricted to ground tissue layer and is possibly controlled by SHR and a positive feedback loop. SHR that is transported into endodermal cells interacts directly with SCR and forms a complex in the nucleus that prevents transmission of SHR into the cortical cell layer (Cui et al. 2007). The SHR-SCR complex in the nucleus triggers production of more SCR to ensure the presence of enough SCR to bind SHR in endodermal cell layer (Cui et al. 2007). Perhaps the SHR-SCR complex formation is responsible for the formation of single layer of endodermis that surrounds the stele. Since nearly all plants contain only a single layer of endodermis and the SCR/ SHR orthologs of rice show similar expression patterns, it is likely that SHR-SCR complex formation have been conserved through evolution.

Mutations in the *SCR* gene result in shoot agravitropism, and normal endodermis starch sheaths are missing in both hypocotyls and inflorescence stems of *scr* mutant plants (Fukaki et al. 1998). Amyloplasts, starch-containing plastids, are believed to function as statoliths that sediment in response to gravity in specialized cells known as statocytes (Sack 1997). Sedimenting

amyloplasts have been observed in the endodermis of hypocotyls and inflorescence stems. In WT hypocotyls there are three ground tissue derived layers between the centrally located stele and the epidermis while *scr* mutant hypocotyls contain only two layers in that location (Fukaki et al. 1998). The appearance of the innermost layer, located in the position of WT endodermis is abnormal (Fukaki et al. 1998). Furthermore, the amyloplasts do not seem to sediment in response to gravity in *scr* hypocotyls. It was assumed that lack of amyloplast sedimentation is the cause for hypocotyl agravitropism in *scr* mutant (Fukaki et al. 1998). Inflorescence stems of WT *Arabidopsis* contain variable number of ground tissue layers but sedimenting amyloplasts have been localized only in the single innermost layer, the endodermis. No amyloplast has been localized in any cell layer of *scr* mutant inflorescence stems (Fukaki et al. 1998). It is believed that absence of amyloplasts is responsible for inflorescence stem agravitropism in *scr*. In roots, central columella cells of root cap function as gravity sensing statocyte cells. Sedimenting amyloplasts have only been localized in columella cells of the root. In *scr*, both root gravitropism and amyloplast sedimentation in columella cells appear normal (Fukaki et al. 1998).

Although much work has been done on *SCR* gene itself, very few genes that are controlled by or that interact with *SCR* are known. We hypothesized that SCR, a transcription factor, works via interacting with various target genes and gene products in different tissues involved in SCR regulated pathways. These target genes can be identified by second site mutation in *scr* mutants that restore SCR regulated phenotypes. Therefore, to identify additional components involved in *SCR* regulated pathways, we have used a forward genetic approach. We generated and screened the *scr*1 suppressors (suppressors of the *scr*1 mutant). For suppressors screening *scr*1 mutants with T-DNA insertion in *SCR* coding region has been used. This T-DNA insertion causes the loss of gene function. To set up a screen of mutants that suppress the *scr*1 abnormal phenotypic characters,

homozygous seeds of *scr*1 were mutagenized with EMS. The length of T-DNA in *scr1* is approximately 17kb while EMS usually induces point mutations so it is highly unlikely that SCR function could have been restored by EMS mutagenesis. Therefore, *scr1* suppressors with any improved phenotypic characteristic would represent second-site mutations.

Materials and Methods

Plant materials and Growth conditions

Individuals of *Arabidopsis thaliana*, [Wassilewskija (WS) and Columbia (Col)] were used as a wild type plants. The *scr* mutant on the WS ecotype background is *scr1* (Di Laurenzio et al. 1996) and on Col ecotype background is *scr3* (Fukaki et al. 1996b).To obtain the suppressors, *scr1* mutant's seeds were mutagenized with EMS. To accomplice the screening and phenotypic characterization of suppressors, seeds were sterilized and grown on Murashige and Skoog (MS) agar plates as described by Fukaki et al. (1996b).Plants were transferred to pots from MS agar plates and grown under white light at 23 ± 1^0C in long day light conditions (16 hours light). These plants were used for inflorescence stem gravitropic studies and seed collections.

Mutagenesis

The *scr1* mutant's seeds were mutagenized with EMS (ethyl methane sulfonate) and mutagenesis was performed (Levin et al. 1998). First seeds were washed with 0.1% Tween-20 for 15 minutes and then treated with 0.2% EMS (volume/volume in water) for 12 hours. Seeds treated with EMS were rinsed with water 8 times and seeds were soaked in the last rinse for 15 minutes (to allow time for the EMS to diffuse out of seeds), followed by several rinses with 0.1% Tween-20 for a total of 5.5 hours. In beginning, first three batches of mutagenized seeds were directly sown on

soil. However, the next batches of mutagenized seeds were germinated on MS agarose plates containing 4.5% sucrose. To synchronize the seed germination the plates were kept in dark at 4^0 C for 3 days. After 3 days plates were transferred to a growth chamber at 20^0 C (16 hour daylight) for germination. Two weeks after germination healthy seedlings were transferred to the soil and their seeds were harvested in pools. Each pool contained seeds collected from 40-100 plants.

Potential *scr1* suppressor mutants screening

Primary Screen

In order to perform the primary screening, mutagenized seeds were surface sterilized and plated in rows on MS agar plates containing 4.5% sucrose. These plates were stratified in the dark at 4^0 C for 3 days to synchronize their germination. After 3 days plates were transferred to a growth chamber and placed in a vertical position at 23 ± 1^0 C (16 hour daylight) for germination. Plates with germinating seedlings were covered with aluminum foil and left in a vertical position for 1day. Photographs of all plates were taken (0 hr photographs) and then they were reoriented by 90 degrees for 48 hours. Photographs at 48 hours after reorientation were also taken. These photographic images (Kodak Image Station440CF) were used to compare the gravitropic responses. Seedlings with improved hypocotyl gravitropic responses were transferred to pots. The plates without improved hypocotyl gravitropic seedlings were left in the growth chamber for approximately two more weeks. Seedlings with longer root lengths were also transferred to pots. Seedlings in pots were allowed to self-pollinate and eventually seeds were collected for secondary screening.

Secondary screen

Progeny of primary screening isolates were used to perform the secondary screening. Seeds of potential suppressors selected in primary screening were grown with *scr1* and WS on the 4.5% sucrose MS agar plates to clearly distinguish phenotypic differences. Plates were screened with similar experimental conditions as previously described in primary screening. Phenotypic responses of seedlings were compared with WS and *scr1* and seedlings with improved phenotypic responses over *scr1* mutants were selected as potential suppressors.

Suppressor's genotype confirmation

Seeds of WS, *scr1* and confirmed *scr1* suppressors were placed on MS agar plates containing 4.5% sucrose for germination. DNA was extracted from WS, *scr1* and *scr1* suppressors (Edwards et al. 1991). The DNAs were amplified by using SCRF344, SCRR1956 and T-DNA right border primer RBF3. The Polymerase Chain Reaction (PCR) contained the following reaction mixture: 0.2mM of all three primers, 1.5-3.0 mM $MgCl_2$, 0.5U Taq polymerase (Promega), 0.2mM of each dNTP (Promega) and ~100ng of DNA template. Following PCR cycle was used: denaturation at 94^0C for 1 minute followed by primer annealing at 54^0C for 1 minute, followed by extension at 72^0C for 1 minute and 30 seconds for 10 cycles. After first 10 cycles the entire program was repeated for 24 more cycles with denaturation time (30 seconds) at 94^0C, primer annealing at 54^0C for 30 seconds, extension at 72^0C for 1 minute and 30 seconds and final elongation at 72^0C for 4 minutes.

DNA Extraction

DNA extraction was performed as described by Edwards et al. (1991). For DNA extraction, plant tissues were collected in Eppendorf tubes and 300 µl lysis buffer (10mM Tris-HCl, pH 8; 25mM EDTA, pH 8 and 0.5% SDS) was added. Plant tissues with lysis buffer were ground with a pestle that fits in the eppendorf tubes followed by addition of another 300 µl lysis buffer. This mixture was incubated at 55^0 C for 15 minutes to 1 hour. After incubation, the tubes were cooled to room temperature and 3 µl RNaseA at 5mg/ml was added to digest the RNA. These tubes were incubated at 37^0 C for 15 minutes to 1 hour followed by protein precipitation by adding 200 µl of 5M ammonium acetate. Tubes were centrifuged at 14000 rpm for 5 min. The supernatants with DNA were transferred to new tubes. An equal amount of isopropanol was added in the supernatant to precipitate the DNA. Tubes were centrifuged at 14000 rpm for 5 min. Supernatants were discarded and DNA containing pellets were air dried for at least 15 minutes. Dried pellets were resuspended in 50 µl distilled water. Extracted DNA was kept at 4^0C for further studies.

Phenotypic Characterization of potential suppressors

Hypocotyl gravitropism

To test the gravitropic response of suppressors, WS, *scr*1 and each suppressor were grown on the same plate. To do this, forty seeds of each plant type were sterilized and placed on MS agar plates containing 4.5% sucrose. These plates were kept at 4^0 C for 3 days to synchronize germination. After 3 days plates were transferred to a growth chamber (16 hour daylight) for germination. Plates with germinating seedlings were covered with aluminum foil and left in vertical position for 1day. Photographs of all plates were taken (0 hour photographs) and then the plates were reoriented at a

90^0 angle. Photographs were taken after 48 hour of reorientation. These photographic images (Kodak Image Station 440CF) were used to compare gravitropic responses.

Inflorescence gravitropism

To check the inflorescence gravitropism of the suppressors, seedlings were transferred into the pots from the plate and plants with 6 to 8 cm long inflorescence stems were analyzed as described by Fukaki et al. (1996a). Pots with WS, *scr*1 and each suppressor plant (one type of plant/pot) were placed horizontally in the dark. This was referred as the beginning of the experiment (0 hour). Inflorescence gravitropic response was measured after 24 hours.

Root growth and length

To test the root growth rate and length of the suppressors and to compare them with both WS and *scr*1 plants, WS, *scr*1 and each suppressor, were placed side by side. To do this, forty seeds of each plant type were sterilized and placed on MS agar plates containing 4.5% sucrose. After 3 days in the dark at 4^0 C, plates were transferred to the growth chamber under long day conditions (16 hours) for germination and growth. Root growth was analyzed 21 day after germination.

Results

Mutagenesis and seed collection

For the identification of genes involved in SCR regulated pathways, the *scr1* mutants carrying T-DNA insertion mutation were mutagenized with EMS. Mutagenesis was performed in batches of 2000 seeds per batch every other week. EMS-treated seeds were directly sown in soil at a density of approximately 50 seeds per pot for first three batches. Pots were transferred to the growth

chamber under long day conditions (16 hours) for germination and growth. Seeds were collected from the mature plants in pools. Very few EMS- treated seeds were able to germinate in soil and many plants died after germination before being able to produce seeds. Furthermore the surviving plants were able to produce very few seeds. Therefore, only 21 seed pools representing 40-50 plants per pool, were collected from the first three batches of seeds (approximately 6000 seeds) that were sown directly into soil. Maximum of 1000 independent lines were represented by these 21 pools.Since the first procedure was not efficient, to enhance seedling survival from the next batch onwards seeds were plated on MS agar plates and allowed to grow on plates for 2 weeks and only robust seedlings were transferred to soil. The plants were transferred to soil at a density of 15 seedlings/pot. Eventually 30 additional pools were generated representing ~3000 independent lines, each containing thousands of seeds. Seedlings with improved phenotype such as long root or larger leaves were selected as potential suppressors and transferred to soil for seed collection.

Primary and Secondary Screening

In the primary screening 438 seedlings with improved phenotypic characters were selected and transferred to the soil for seed production. Seeds were collected from only 235 of these primary screened suppressors. A three part name was given to each primary suppressor that indicated its origin. The first, second and third part of the name represents pool number, plate number (indicated by a letter) and isolate number from the plate respectively. These 235 seedlings, selected as potential suppressors, which represent 30 pools out of 44 pools screened (Table 2.1). In order to confirm the real suppressors, seeds collected from all of the selected 235 primary suppressors were subjected to secondary screening. In secondary screening to compare suppressors with both WS and *scr*1 plants, WS, *scr*1 and each suppressor, were grown on the same MS agar plate containing

4.5% sucrose. All primary isolates were selected for improved hypocotyl gravitropic response, root length and leaf size. After secondary screening 38 potential suppressors with improved hypocotyl and/or improved root length over *scr1* mutants were identified. Seedlings of these 38 lines were transferred in soil and next generation seeds were collected. The seeds collected from 38 potential suppressor lines were re-screened as described earlier to confirm the stability of the potential suppressor phenotypes and only the suppressors that were able to retain their improved phenotypes for two generations were selected as confirmed suppressors. Eventually, only 13 lines were able retain their suppressor phenotypes for next two generation. These 13 lines come from 11 different pools and probably represent at least 11 different loci.

Suppressor's genotype confirmation

The genetic background of all 13 confirmed suppressors was evaluated. The presence and/or absence of WS (wild type) and mutant *scr1* alleles of *SCR* in the genomes of suppressor lines was determined by PCR test. 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 *scr1* allele and do not carry a WS copy of *SCR* gene different primers were designed (Table 2.2). The primers are designed in such a way that *SCR* forward and reverse are positioned on either side of T-DNA insertion of *scr1* allele (Fig. 2.1). Genomic DNA was extracted from all thirteen potential suppressors, WS and *scr1* and PCR amplification analysis was performed. A single band of ~ 1.6 kb is produced by the WS DNA with two *SCR* specific primers (Fig. 2.2 A). A single, ~ 0.7 kb band with T-DNA right border primer and *SCR* reverse primer was yielded by the *scr1* DNA (Fig. 2.2 A). Similar to WS and *scr1*, genomic DNA of all thirteen potential suppressors was amplified with *SCR* forward, reverse and T-DNA right border primers. A single band of ~ 0.7 kb was

amplified by all thirteen potential suppressors DNA, with only *SCR* reverse and T-DNA right border primers (Fig. 2.2 A and Fig. 2.2 B) confirming the absence of wild type *SCR* allele and the presence of *scr1* genetic background. Therefore, the PCR analysis confirmed that all these thirteen isolated lines are one on *scr1* background and represent second site mutations.

Hypocotyl gravitropism

It has been reported earlier that *scr* mutants show hypocotyl agravitropism (Fukaki et al. 1996a; Fukaki et al. 1996b and Fukaki et al. 1998). Hypocotyl gravitropic response of all the confirmed suppressors was test and compare with WS and *scr1* seedlings. The results showed that 10 suppressors 23C2, 25G2, 3A2, 11A1, 23K1, 30I1, 30X1, 31F1, 39Aa20 and 48B16 (renamed as *shs1, shs2, shs3, shs4, shs5, shs6, shs7, shs8, shs9* and *shs10* respectively) have improved gravitropic response over *scr1* mutant but below the level of wild type plants (Fig. 2.3 A and Fig. 2.3 B). One of the suppressors, *shs1*, has a very similar response to WS hypocotyls. The most surprising result was that contrary to the previous reports *scr1* hypocotyl showed some residual hypocotyl gravitropic response (Fig. 2.3 A) (Fukaki et al. 1996a; Fukaki et al. 1996b and Fukaki et al. 1998). However, the responses showed by *scr1* hypocotyls were much weaker than WS response. The remaining three suppressors 24R1, 39A9 and 41C30 (*srs1, srs2* and *srs3)* exhibited the same level of hypocotyl gravitropic response as *scr1*, no improvement in their hypocotyl gravitropic response was noticed (Fig. 2.3 C).

Inflorescence gravitropism

In order to check inflorescence gravitropic response, plants turned by 90^0 by placing pots on their sides in dark, the inflorescence stems with negative gravitropism will reorient soon in response to

new gravity vector. Inflorescence stems of WS plants can turn 90^0 upward within two hours after reorientation. It has been reported that *scr* mutants exhibit complete inflorescence agravitropism (Fukaki et al. 1996a and Fukaki et al. 1998). To check that suppressors have been able to rescue their inflorescence gravitropic response or not and to compare the response of WS, *scr1* and thirteen confirmed suppressors, plants with 6-8 cm long inflorescence stem were selected and placed in dark on their sides (Fig. 2.4 A, Fig .2.4 B and Fig. 2.4 C). Within first three hour of reorientation WS inflorescence stem bend 90 degrees upward. As reported earlier inflorescence stems of *scr1* mutants showed complete inflorescence agravitropism (Fig. 2.4 A). None of the confirmed *scr1* mutant suppressors showed any improvement in their inflorescence gravitropic response within 24 hours of reorientation. Their inflorescences were completely agravitropic.

Root length

Roots of *Arabidopsis thaliana* grow continuously throughout their life and this type of root growth is known as indeterminate root growth. However, *scr1* shows determinate type of root growth, thus their roots stop growing in length at early stage of life and exhibit short root phenotype (Sabatini et al. 2003). In order to test if suppressors were able to rescue indeterminate root growth phenotype, all of the confirmed suppressors were tested for the type of root growth they show. As expected WS shows indeterminate (long roots) and *scr1* exhibits determinate root length (short roots). Only three suppressors 24R1, 39A9 and 41C30 (*srs1, srs2* and *srs3*) exhibit significantly longer roots than *scr1* (Fig. 2.5 A). The other ten suppressors did not show any significant improvement in root lengths, they have roots of similar length to *scr1* (Fig. 2.5 B).

Rename Suppressors

Ten suppressors with improved hypocotyl gravitropism have been renamed as *scr* hypocotyl gravitropic suppressors *(shs): shs1 to shs10.* The hypocotyl suppressors 23C2, 25G2, 3A2, 11A1, 23K1, 30I1, 30X1, 31F1, 39Aa20 and 48B16 were renamed as *shs1, shs2, shs3, shs4, shs5, shs6, shs7, shs8, shs9* and *shs10* respectively. The other three suppressors with improved root length have been renamed as *scr* root length suppressors *(srs): srs1 to srs3*. The root length suppressors 24R1, 39A9 and 41C30 were renamed as *srs1, srs2* and *srs3* respectively.

Discussion

SCR, a transcription factor, works via interacting with various target genes and gene products in different tissues involved in several developmental processes such as shoot gravitropism, indeterminate root growth and normal radial patterning of *Arabidopsis thaliana*. Therefore, we used the *SCR* gene as an entry point to identify other components involved in SCR regulated developmental pathways. We hypothesized that these interacting genes can be identified by generating second site mutations in *scr* mutants that will restore phenotypic defects of *scr* mutants. These *scr* suppressor mutants would represent SCR target or interacting genes. The *SCR* gene is expressed in different plant organs and at different times and its activity is required in different developmental pathways. Therefore, it is reasonable to conclude that different SCR-regulated pathways are controlled by different sets of genes.

Thousands of *scr1* mutant seeds were mutagenized with EMS and over 4000 independent lines were screened to identify potential *scr* suppressors with phenotypic improvement. Two hundred thirty five potential suppressors were selected in a primary screen. However, secondary screening yielded only 38 suppressors from the whole collection of primary isolates. Out of 38

potential suppressors only 13 suppressors were able to retain their phenotypes for more than two generation. Therefore, these 13 suppressors were selected as confirmed suppressors. Out of all 13 suppressors, 10 suppressors named as 23C2, 25G2, 3A2, 11A1, 23K1, 30I1, 30X1, 31F1, 39Aa20 and 48B16 rescued only hypocotyl gravitropic phenotype and renamed as *scr* hypocotyl suppressor *(shs): shs1, shs2, shs3, shs4, shs5, shs6, shs7, shs8, shs9* and *shs10,* respectively. The other 3 suppressors named as 24R1, 39A9 and 41C30 exhibited only long root phenotype were renamed as *scr* root length suppressors *(srs): srs1, srs2* and *srs3* respectively. Plants from different pools mostly likely would represent different mutations while plants from same pools might carry the same mutation. The suppressors *shs1, shs5* (23C2, 23K1) came from the same pool and rescued the similar phenotype; similarly, suppressors *shs6, shs7* (30I1, 30X1) were selected from the same pool and exhibit similar phenotype. It is possible that *shs1, shs5* represent the same mutation and *shs6, shs7* also carry the same mutation. To determine the number of loci affected by suppressor mutations, complementation tests will be performed. Also, to locate the position of mutated gene in selected suppressors, the genes will have to be mapped. Therefore, thirteen suppressors that probably represent eleven different loci involved in at least two different SCR regulated pathways (hypocotyl gravitropism and indeterminate root growth) have been identified after suppressor screening.

The *scr* hypocotyl gravitropic suppressors (*shs)* showed improved gravitropic response over *scr1* but below WS level (Fig. 2.3 A and Fig. 2.3 B). Detailed analysis of all ten *shs*s gravitropic responses on different time intervals will be performed. The other three *scr* root length suppressors (*srs*) showed gravitropic response similar to *scr1* mutants. No improvement in their hypocotyl gravitropic responses were observed (Fig. 2.3 C). One important observation was the residual gravitropic response showed by hypocotyl of *scr1* mutant seedlings (Fig. 2.3 A). In contrary to the

previous report (Fukaki et al. 1998), we observed that *scr1* mutant hypocotyls are not completely agravitropic and still retain some residual gravitropism. Since amyloplast sedimentation is completely absent in *scr* mutants (Fukaki et al. 1998), it is possible that hypocotyl gravitropism is partially independent of amyloplast sedimentation. The results also indicate that abnormal amyloplast sedimentation is the primary defect of *scr1* mutant hypocotyl while the other aspects of hypocotyl gravitropic responses are still functional. Another possibility is that the contribution of amyloplast sedimentation in gravity stimulated signal formation is not very significant. In order to assess these possibilities the morphological analysis and comparison of gravity sensing cells of both *scr1* mutants and *shs*s is required. Since *shs*s rescued hypocotyl gravitropic response it is possible that some of the mutated genes are related with sedimentation of amyloplasts in endodermal cells of the hypocotyl. Therefore, analysis of radial pattern and localization of amyloplasts in suppressors is essential.

Several genes that play key roles in gravitropism of one, two or all three organs have already been identified with the help of mutational analysis (Fukaki et al. 1997). Characterizations of these genes suggest that root, hypocotyl and inflorescence stem do not have identical molecular mechanisms for their gravitropic responses. At least some factors involved in gravitropic pathways of these three organisms are genetically different (Tasaka 1999). Mutation in the *SCR* gene is only responsible for shoot (hypocotyl and inflorescence stem) agravitropism because *scr* roots have normal (wild type) root gravitropism in response to a gravity vector (Fukaki et al. 1996a, Fukaki et al. 1998). All the thirteen suppressors exhibited complete inflorescence agravitropism similar to *scr1* inflorescence (Fig. 2.4 A, B and C). As expected, roots of all thirteen suppressors have wild type positive gravitropic responses. These observations support the theory that root, hypocotyl and inflorescence stem do not have identical molecular mechanisms for their gravitropic

responses. At least some factors involved in gravitropic pathway of these three organs are genetically different. 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. Root length of all three root suppressors is greater than *scr* mutants but below the level of wild type plants (Fig. 2.5 A). The other ten hypocotyl suppressors did not show any significant improvement in their root growth. They still showed determinate type of root growth similar to *scr* mutants. The only phenotype rescued by the *srs*s is long root length; therefore, it is very possibly that root suppressor genes are involved in root development. In order to have the indeterminate type of root growth *SCR* gene needs to be expressed in the quiescent center (QC). It has also been reported that when the *SCR* gene is only expressed in QC of *scr* mutant roots it only rescued indeterminate root growth; however, plants still expressed an abnormal radial pattern (Sabatini et al. 2003). Therefore, it is important to analyze the radial pattern of *srs*s roots. If *srs*s has only rescued the root length but not the normal radial pattern it will suggest that root suppressor genes are functional in QC and possibly involved in meristem maintenance.

In conclusion, thirteen confirmed suppressors have been identified by *scr* suppressor screening of *Arabidopsis thaliana*. Ten suppressors have rescued hypocotyl gravitropic response while other three suppressors exhibited improved root length phenotype. None of the suppressors has exhibited both hypocotyl gravitropic response and long root phenotype. No suppressor with improved inflorescence gravitropism was identified; therefore, characterization of these suppressor genes will lead to identification of the components involved in at least two SCR regulated pathways.

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	24
30	18
31	58
34	13
36	5
38	9

39	2
40	14
41	1
42	14
44	15
45	11
48	1
49	6

Table 2.1. Number of primary screened potential suppressors isolated from different pools.

Primer	Tm	Length	Aim	Sequence (5'-3')
SCRF344	65	24	Upstream to T-DNA insertion in *SCR*	ACCGTGGTGGTCGGAATGTTATGA
SCRR1956	65	24	Downstream to T-DNA insertion in *SCR*	AGTCGCTTGTGTAGCTGCATTTCC
RBF3	59	21	T-DNA right border	CCAAACGTAAAACGGCTTGTC

Table 2.2. Primers used for the confirmation of genotype of suppressors.

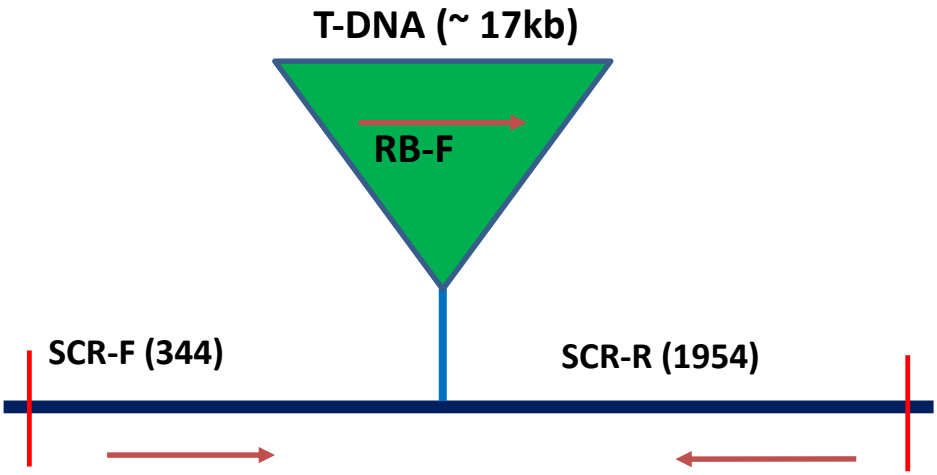

Figure 2.1. Schematic representation of *SCR* gene in *scr1* allele with the T-DNA insert and location of PCR primers. Arrows indicated the orientation of the primers. Wild type *SCR* allele amplifies a 1.6 kb length product with SCR-F (344) and SCR-R (1954) alone while *scr1* allele amplifies a 0.7 kb product with T-DNA primer (RBF) and SCR-R (1954) only.

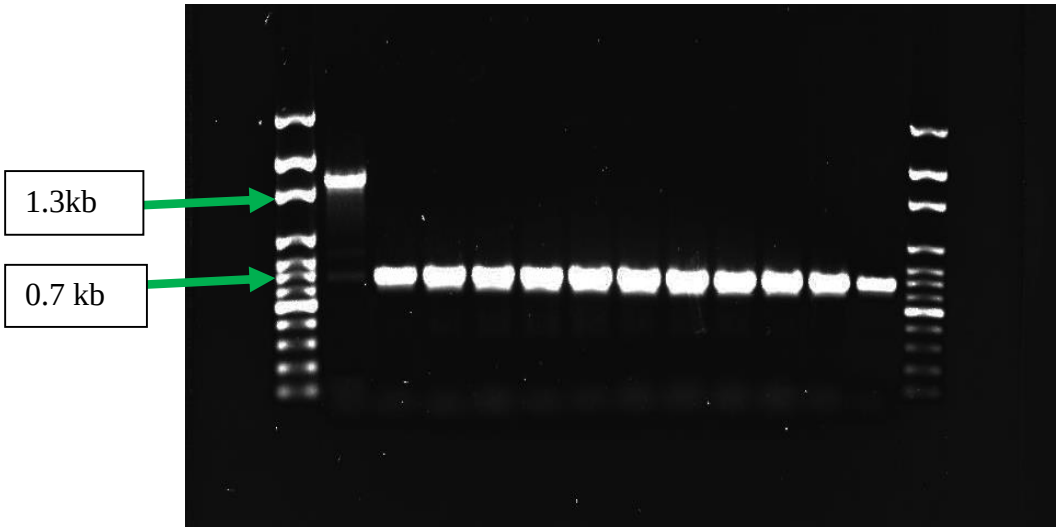

Figure 2.2 A. PCR amplification. Lane 1. 100bp ladder Lane 2. WT (1.6 kb amplification products generated with SCR-F and SCR-R primers) Lane 3-12. *scr* hypocotyl gravitropic suppressors, 1 to 10 (0.7 kb amplification products generated with T-DNA F and SCR-R primers) Lane 13. *scr1* (0.7 kb amplification products generated with T-DNA F and SCR-R primers) Lane 14. 100bp ladder. SCR-F and SCR-R primers cannot amplify *scr1* allele because of the presence of large T-DNA insertion in genomic region. Green arrows points to 1.3kb and 0.7kb marker bands.

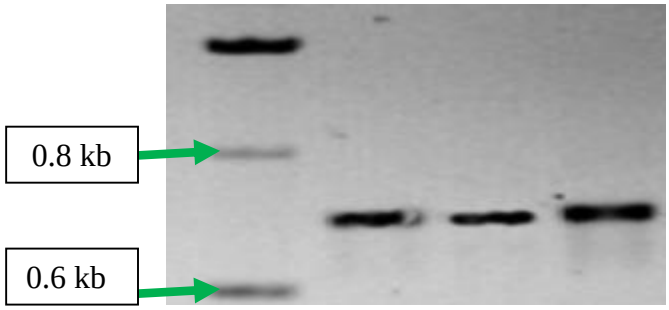

Figure 2.2 B. PCR amplification. Lane 1. 1kb ladder Lane 2-4. *scr* root growth suppressors, 1 to 3 (0.7 kb amplification products generated with T-DNA F and SCR-R primers). Green arrows points to 0.8kb and 0.6kb marker bands.

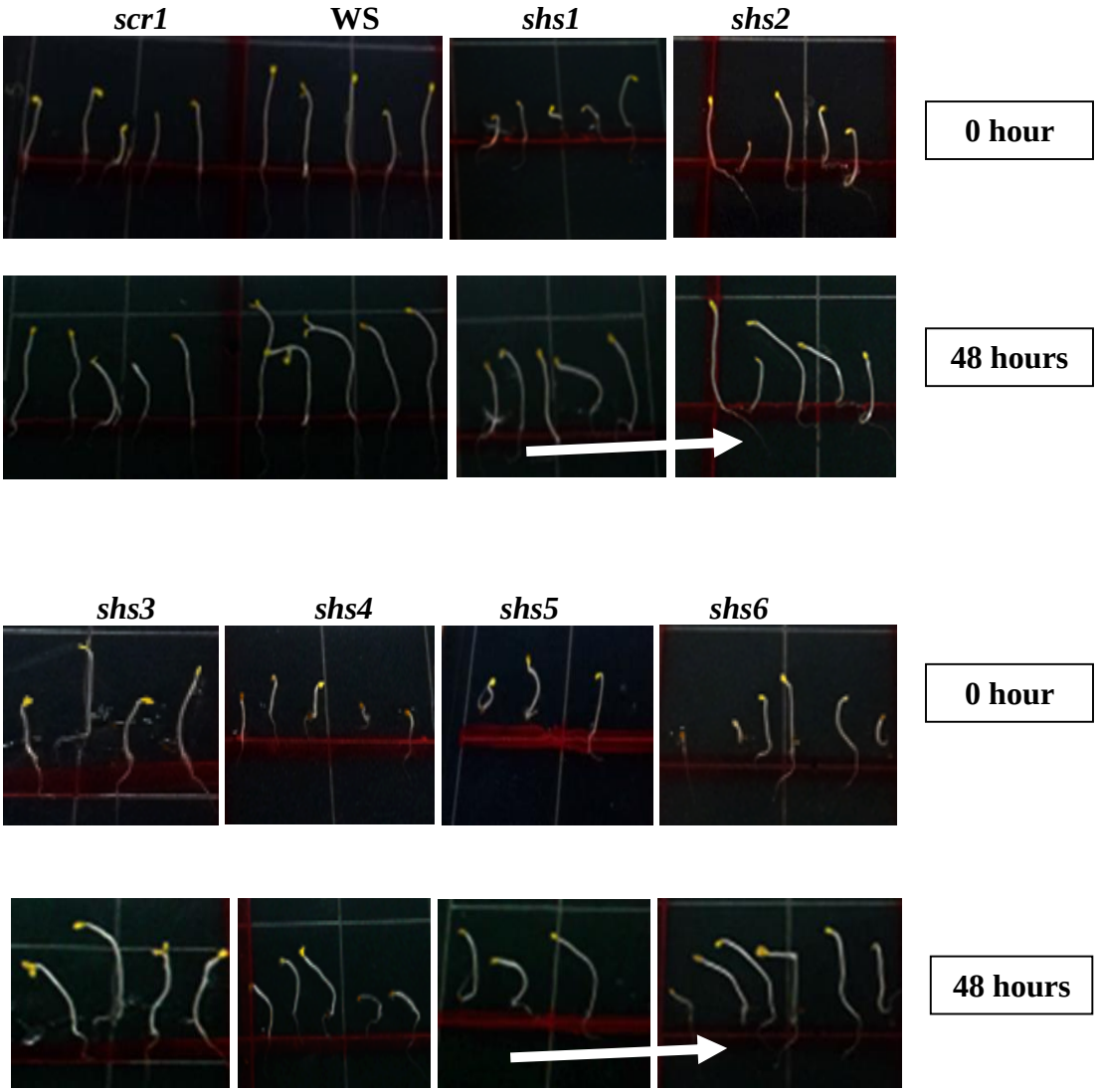

Figure 2.3 A. Hypocotyl gravitropic response of seedlings after 48 hours of reorientation. Hypocotyl gravitropic response of the *scr1*, WS and *scr* hypocotyl gravitropic suppressors' *shs1*, *shs2*, *shs3*, *shs4*, *shs5* and *shs6*, 48 hours after plate reorientation in the dark. Arrows indicate the direction of the gravity vector. The seedlings of *scr1*, WS and *shs*s are shown before reorientation (0 hour) and 48 hours after reorientation.

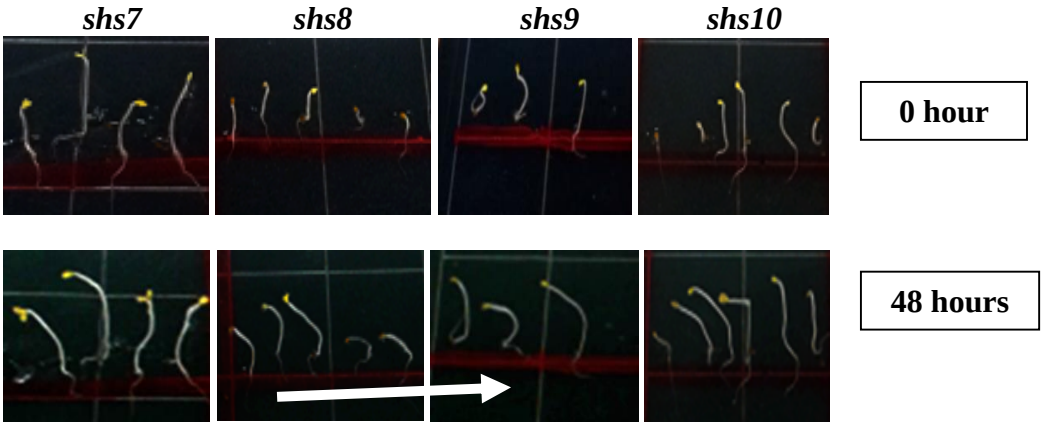

Figure 2.3.B. Hypocotyl gravitropic response of seedlings after 48 hours of reorientation. Hypocotyl gravitropic response of the *scr* hypocotyl gravitropic suppressors' *shs7*, *shs8*, *shs9* and *shs10*, 48 hours after plate reorientation in the dark. Arrows indicate the direction of the gravity vector. The seedlings of *shs*s are shown before reorientation (0 hour) and 48 hours after reorientation.

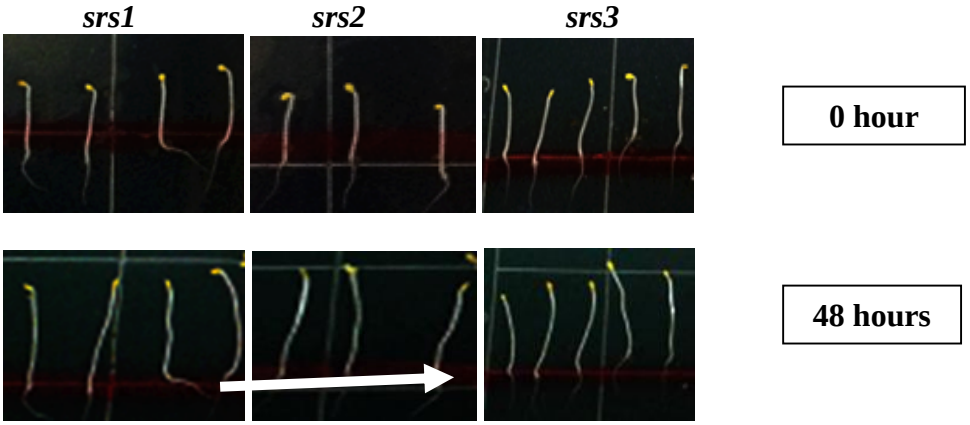

Figure 2.3.C. Hypocotyl gravitropic response of seedlings after 48 hours of reorientation. Hypocotyl gravitropic response of the *scr* root growth suppressors' *srs1*, *srs2*, and *srs3*, 48 hours after plate reorientation in the dark. Arrows indicate the direction of the gravity vector. The seedlings of *srs*s are shown before reorientation (0 hour) and 48 hours after reorientation.

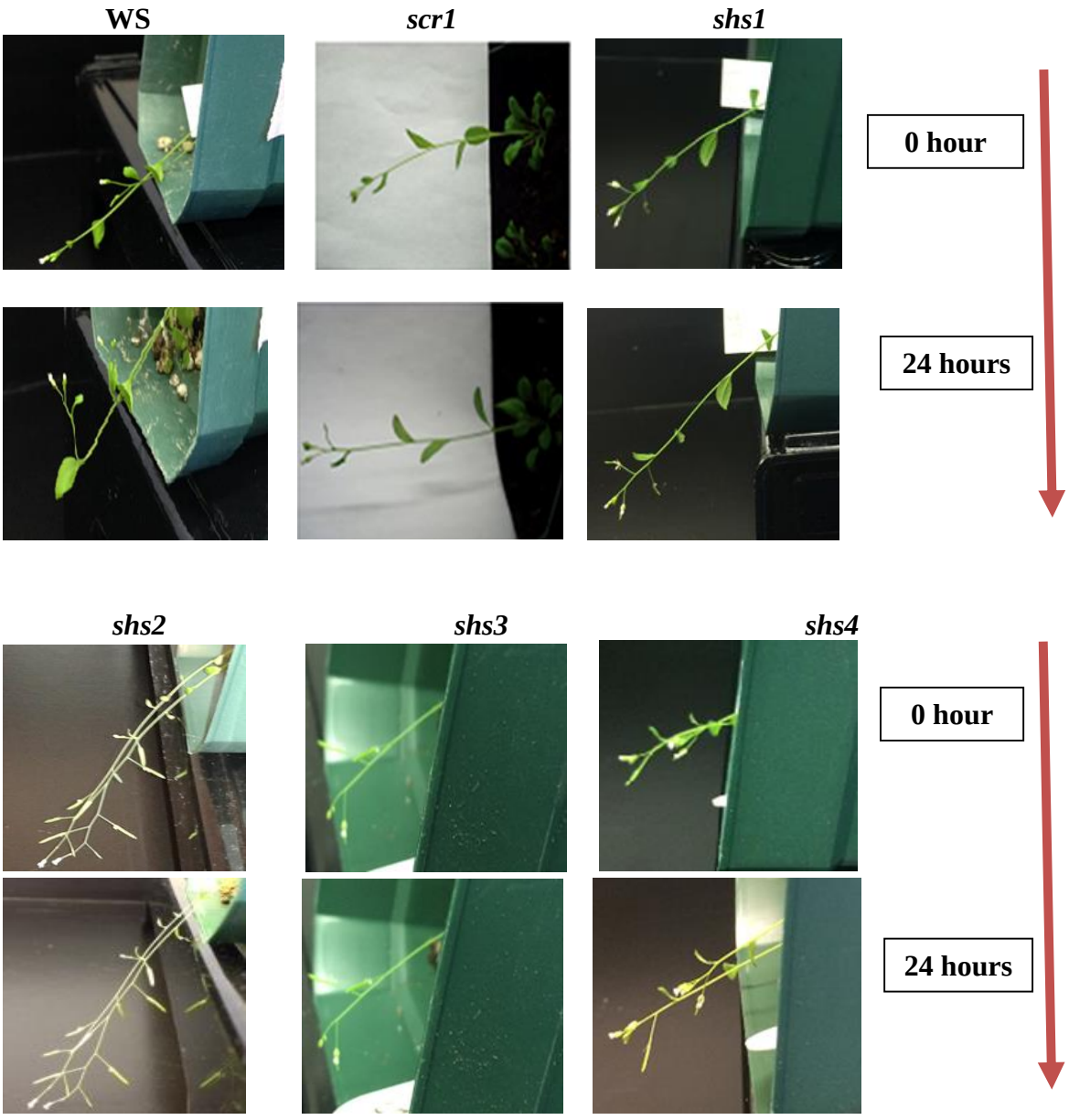

Figure 2.4.A. Inflorescence gravitropic response of the suppressors to the new gravity vector. Inflorescence gravitropic response of the WS, *scr1* and *scr* hypocotyl gravitropic suppressors' *shs1*, *shs2*, *shs3* and *shs4*, 24 hours after plants reorientation in the dark. Arrows indicate the direction of the gravity vector. The inflorescence stems of *scr1*, WS and *shs*s are shown before reorientation (0 hour) and 24 hours after reorientation.

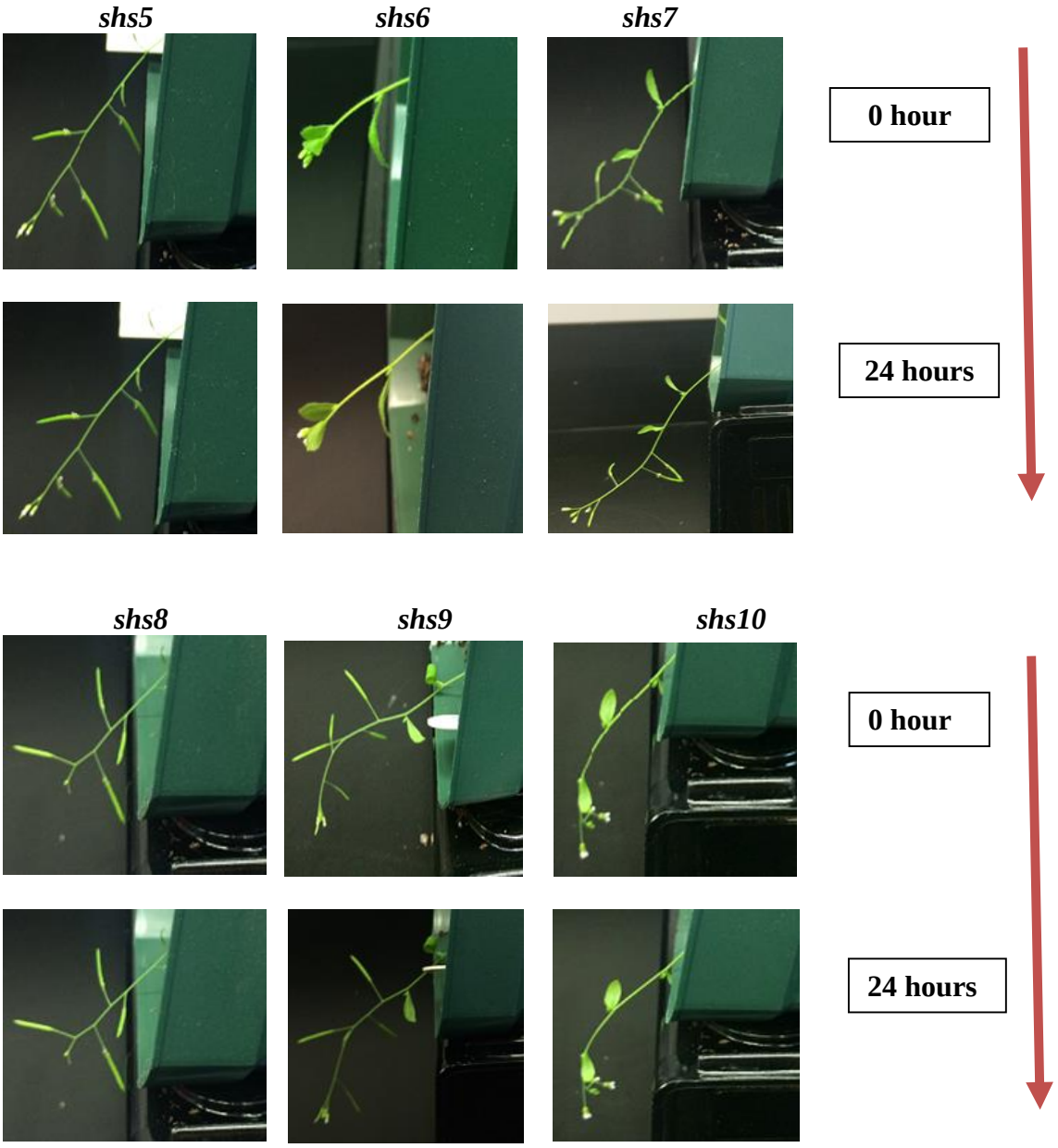

Figure 2.4 B. Inflorescence gravitropic response of the suppressors to the new gravity vector. Inflorescence gravitropic response of the *scr* hypocotyl gravitropic suppressors' *shs5*, *shs6*, *shs7*, *shs8*, *shs9* and *shs10*, 24 hours after plants reorientation in the dark. Arrows indicate the direction of the gravity vector. The inflorescence stem of *shs*s are shown before reorientation (0 hour) and 24 hours after reorientation.

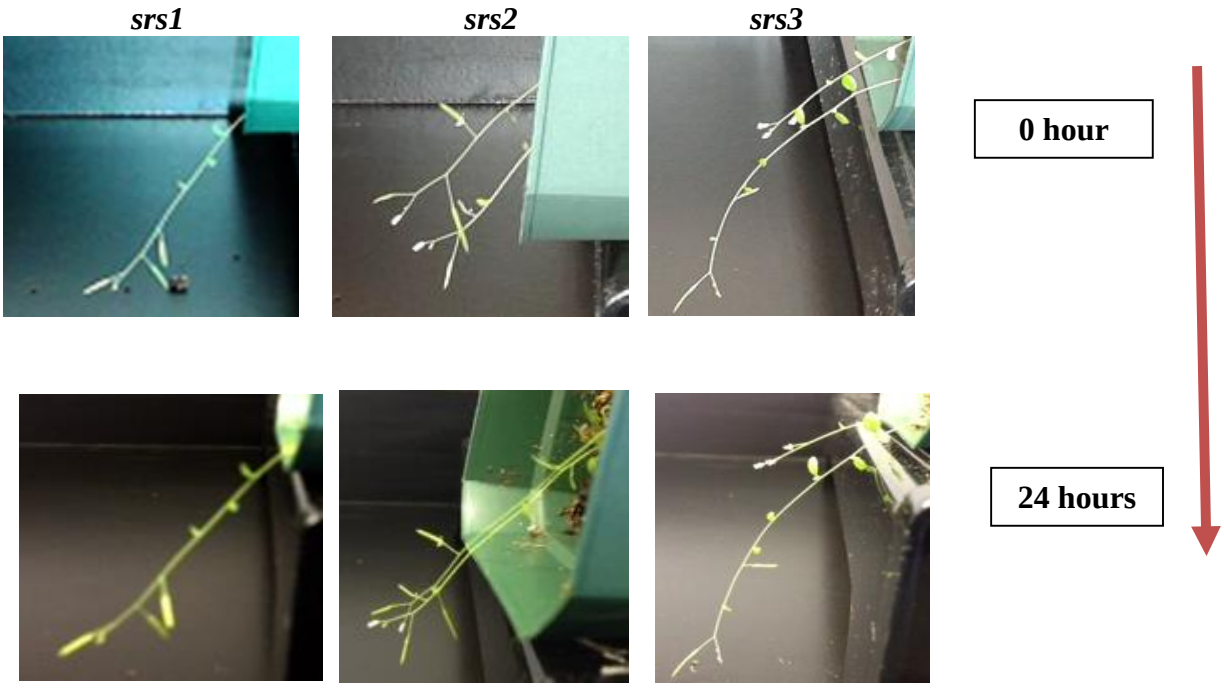

Figure 2.4 C. Inflorescence gravitropic response of the suppressors to the new gravity vector. Inflorescence gravitropic response of the *scr* root growth suppressors' *shs1*, *shs3* and *shs3*, 24 hours after plants reorientation in the dark. Arrows indicate the direction of the gravity vector. The inflorescence stem of *srs*s are shown before reorientation (0 hour) and 24 hours after reorientation.

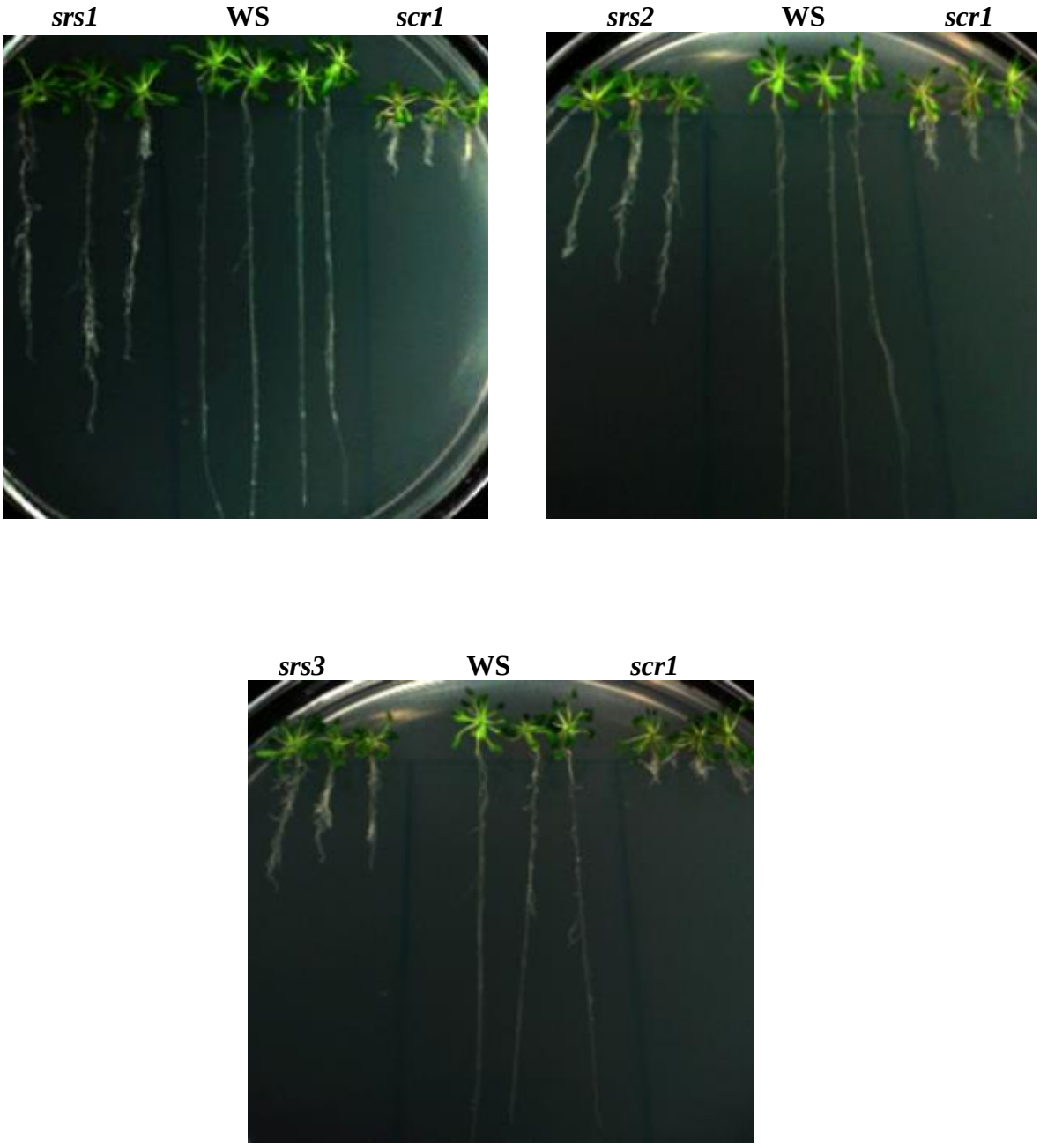

Figure 2.5 A. Root growth of suppressors. Root growth of WS, *scr1* and *scr* root growth suppressors (*srs*s), *srs1*, *srs2* and *srs3*, 21 day after germination (DAG).

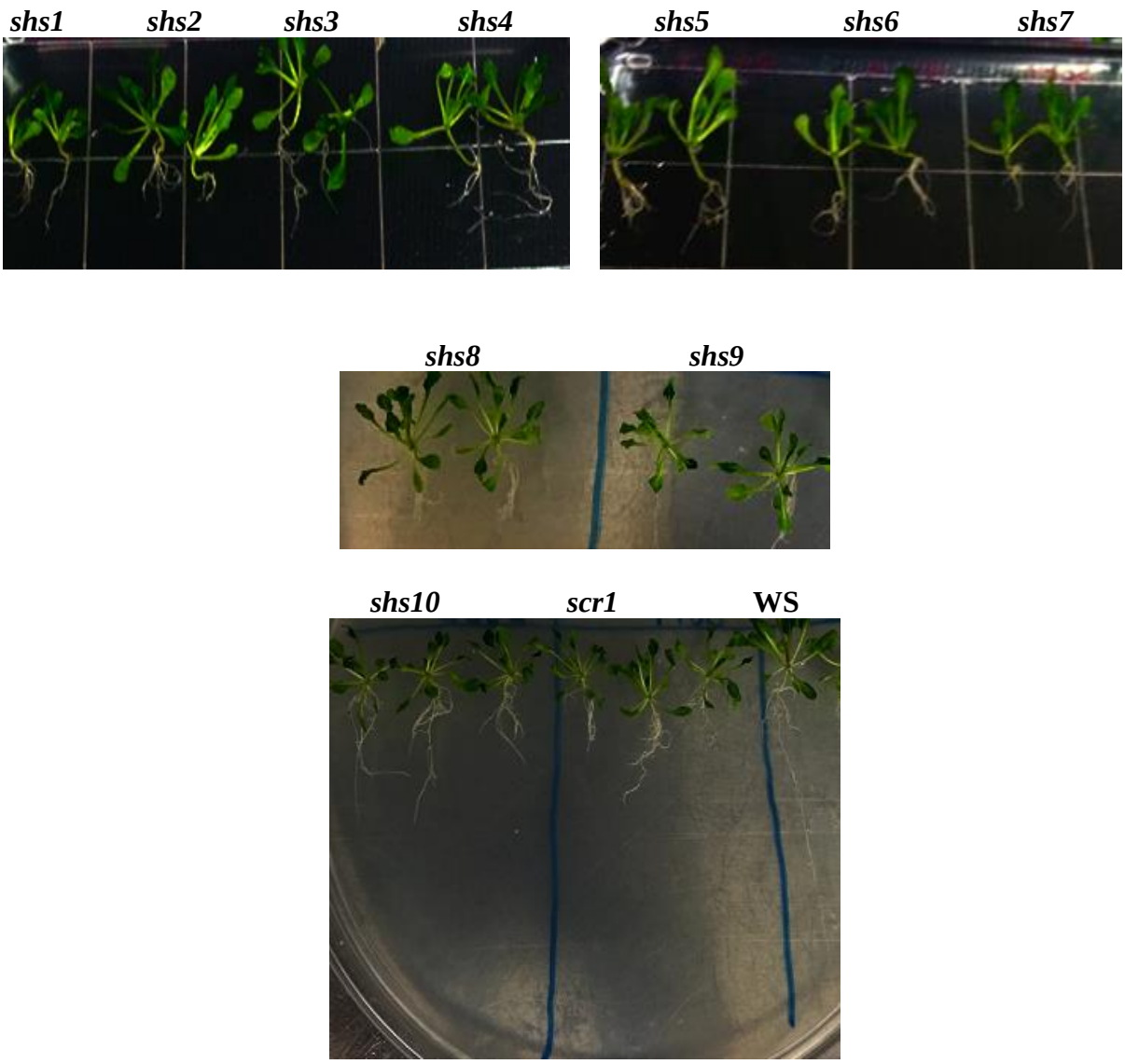

Figure 2.5 B. Root growth of suppressors. Root growth of *scr* hypocotyl gravitropic suppressors (*shs*s) *shs1, shs2, shs3, shs4, shs5, shs6, shs7, shs8, shs9, shs10, scr1* and WS 21 day after germination (DAG).

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III Phenotypic and molecular characterization of *scr1* hypocotyl gravitropic suppressors (*shs*)

Abstract

All higher plants are capable of gravitropism that is reorienting their growth in response to gravity. The shoots of the plants show negative gravitropism by growing upward. The *SCARECROW* (*SCR)* gene is essential for normal radial patterning and shoot gravitropism in *Arabidopsis thaliana*. The *scr* mutant exhibits shoot agravitropism and defective radial pattern (missing one ground tissue layer). SCR works via interacting with various target genes and gene products in different tissues involved in shoot gravitropism. These target genes can be identified by second site mutations in *scr* mutant background that restore shoot gravitropism. We have identified 10 independent *scr* hypocotyl gravitropic suppressor (*shs*) lines that may represent novel genes involved in this pathway. These ten suppressors fall into six complementation groups representing six different gene loci. The hypocotyls of confirmed suppressors exhibit improved gravitropic response over the *scr1* mutant indicating that the corresponding genes play a role in hypocotyl gravitropism. Positional mapping of one of the strongest hypocotyl gravitropic suppressor, *shs1*, suggested that mutated suppressor allele might be located on the lower arm of chromosome 5. Hypocotyl cross sections of all ten suppressors revealed that they still have the radial pattern defect. The precise molecular mechanism of gravitropism is still unknown but the most favored hypothesis is the "Starch-statolith hypothesis". Starch staining of the hypocotyls with I-KI solution shows an absence of amyloplast granules in suppressors grown on medium with no sucrose.

However, suppressors grown on 1% sucrose medium show the presence of some amyloplasts but they are not sedimented. These results indicate that amyloplast sedimentation in the endodermal layer is not essential for hypocotyl gravitropism and there could be an alternative pathway other than amyloplast sedimentation for at least hypocotyl gravitropism.

Keyword: hypocotyl, *scr* hypocotyl gravitropic suppressors, amyloplast sedimentation.

Introduction

All higher plants can reorient their growth according to environmental cues such as light, water, nutrient, gravity etc. In order to maximize the access to light, water and nutrients plants use gravity to orient and coordinate their growth (Blancaflor and Masson 2003). Plant shoots exhibit negative gravitropism (grow upward), while roots show positive gravitropism (grow downward). The process of gravitropism can be divided into three steps: perception of the gravity by the cell, signal transduction and asymmetric elongation in the responding organ (Kiss 2000).

Although many hypotheses have been proposed to explain the phenomena of gravitropism, the precise molecular mechanism of this complex process is still unknown. One of the most favored hypotheses of graviperception is "Starch-statolith hypothesis" which postulates that gravity sensing involves sedimentation of amyloplasts (statoliths) in specific cells (statocytes) (Caspar and Pickard 1998; Kiss et al. 1989; Sack. 1997). Columella root cap cells in roots and endodermis of shoots (both hypocotyl and inflorescence stem) act as statocyte cells (Vitha et al. 1997; Fukaki et al. 1998). Statocyte cells are specific cells that sense gravity. Numerous studies have shown the importance of amyloplast sedimentation in graviperception however, the starch-statolith hypothesis still requires revision. It has been found that roots of starchless mutants of *Arabidopsis*

are gravitropic (Casper and Pickard 1989), and roots of a starch-deficient mutant of *Nicotiana* also show gravitropism (Kiss et al. 1989). These starchless gravitropic roots indicate that starch and amyloplast sedimentation are not necessary for gravitropic sensing. An alternative hypothesis for gravisensing is the "Gravitational pressure model". According to this hypothesis, reorientation of the organ can cause a subtle change in pressure of cytoplasm and it is sufficient to generate a perceivable signal (Staves 1997). One more hypothesis is that, in addition to the amyloplast, a second mass is also involved in gravity sensing. This mass could be nucleus or the cell itself (Sack 1994). The possibility that the cell or protoplast plays a role in gravity sensing is supported by data on gravity-dependent polarity of cytoplasmic streaming in *Nitellopsis* (Wayne et al. 1992). All these studies suggest that there may be more than one mechanism of gravity sensing in plants.

The second step of gravitropism is the dissipation of the potential energy of gravity perception into the generation of a chemical signal that leads to signal transduction eventually resulting in a growth response (Kumar et al. 2011). The precise mechanism of signal transduction is still unknown; however, it has been proposed that cytosolic ions and change in pH mediate gravity sensing (Morita et al. 2004).

Differential growth of the organ that leads to the gravitropic curvature is defined as the third stage of gravitropism (Perrin et al. 2005). It has been accepted that a lateral auxin gradient that enhances differential cell elongation is responsible for the gravitropic curvature in root and shoot, known as Cholodny-Went model (Trewavas 1992). The plant growth hormone auxin inhibits cell elongation in roots, but enhances it in shoots. Therefore, accumulation of auxin at the bottom of the shoot promotes growth of the lower region of the organ and leads to upward bending, while increased level of auxin in the bottom of root causes downward bending (Haswell 2003).

It has been shown that along with the auxin, brassinosteroid (BR) phytohormones, also play an important role in gravitropism. BRs inhibit the gravitropic response of etiolated *Arabidopsis* hypocotyls via modulating auxin homeostasis (Nakamoto et al. 2006). It was demonstrated that BR–treated etiolated seedlings, grown on glucose-free medium, failed to show hypocotyl gravitropism, but in the presence of exogenous glucose (3%-5%) their gravitropic response was restored. This result suggests that sugars antagonize the negative effect of BR on hypocotyl gravitropic responses (Gupta 2012).

To explore the molecular mechanism of gravitropism, scientists have been analyzing the mutants with defective gravitropic responses in *Arabidopsis thaliana* (Okada and Shimura 1994; Fukaki et al. 1996b, c; Yamauchi et al. 1997). Three organs of *Arabidopsis thaliana* root, hypocotyl and inflorescence stem show gravitropism (Okada and Shimura 1992; Fukaki et al. 1996a, b). Several genes that play key roles in gravitropism of one, two or all three organs have already been identified with the help of mutational analysis (Fukaki et al. 1996c). Characterizations of these genes suggest that root, hypocotyl and inflorescence stem do not have identical molecular mechanisms for their gravitropic responses. At least some factors involved in the gravitropic pathway of these three organs are genetically different (Tasaka 1999).

In *Arabidopsis thaliana* both root and shoot possess a radial pattern of cell arrangement. All cell layers are organized on same radius, the stele that includes vascular system, surrounded by endodermis, cortex and epidermis (Dolan et al. 1993). Still, hypocotyl cell arrangement is not identical to root arrangement. In roots single layers of epidermis, cortex and endodermis are present while in hypocotyls, there is an extra layer of cortex. In addition, the hypocotyl stele has a larger number of cells the hypocotyl's epidermis consists of almost twice the number of cells as compared to the root epidermis (Dolan et al. 1993).

SCR plays a key role in plant growth and *scr* mutant plants show many abnormal phenotypic characters such as short roots, radial pattern defects, smaller leaves, shoot agravitropism etc. (Di Laurenzio et al. 1996; Fukaki et al. 1998; Wysocka-Diller et al. 2000).To identify the other components involved in SCR regulated pathways, screening of *scr* mutant suppressors has already been performed. To screen the mutants that suppress the *scr*1 abnormal phenotypic characters, homozygous seeds of *scr*1 were mutagenized with Ethyl Methane Sulphonate (EMS). Ten suppressors with improved hypocotyl gravitropic responses than *scr1* but below the level of WT have been identified. These suppressors are named as *scr* hypocotyl gravitropic suppressors (*shs*). All these ten suppressors are homozygous for *scr*1 and do not carry a functional copy of *SCR* gene. The detailed phenotypic analysis of these suppressors revealed that *shs*s have only rescued hypocotyl gravitropic response while they still have abnormal radial patterns. Along with this they also do not show amyloplast sedimentation and they resemble *scr1* in the presence and the positioning of amyloplasts rather than WT plants. These results supported the hypothesis that starch and the presence of normal statocyte cells and/or amyloplast sedimentation are not necessary for gravitropic sensing.

Materials and Methods

Plant materials and Growth conditions:

Individuals of *Arabidopsis thaliana*, [Wassilewskija (WS) and Columbia (Col)] were used as a wild type plants. The *scr* mutant on the WS ecotype background is *scr1* (Di Laurenzio et al. 1996) and on Col ecotype background is *scr3* (Fukaki et al. 1996b). For phenotypic characterization of suppressors, seeds were sterilized and grown on Murashige and Skoog (MS) agar plates as described by Fukaki et al. (1996b).

Quantitative analysis of hypocotyl gravitropic responses of *shss*

To do the quantitative analysis of gravitropic response of suppressors and to compare them with both WS and *scr*1 plants, WS, *scr*1 and each suppressor, were grown on the same plate. To do this, forty seeds of each plant type were sterilized and placed on MS agar plates containing 4.5% sucrose. Photographs of all plates were taken (0 hour photographs) and then the plates were reoriented at 90^0 angle. Photographs were taken at three different time intervals (12hr, 24hr, and 48hr) within 48 hour of reorientation. These photographic images (Kodak Image Station 440CF) were used to compare the gravitropic responses. "Imagej 1.48v" software was used to calculate curvature as the increment over the initial angle of each individual hypocotyl. Curvature's mean and SD values were calculated and used to categorize the suppressors.

Reverse-transcriptase PCR to confirm the absence of SCR transcript in suppressors

To confirm the absence of SCR transcript in suppressors, RNA was extracted from the hypocotyl of WS, *scr1* and *scr* hypocotyl gravitropic suppressors according to the RNeasy Plant Mini Kit (Qiagen, USA). cDNAs were synthesized with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen^{TM}). The first strands of cDNAs were diluted to get a final concentation of 100ng/µl before they were used in the reverse transcriptase PCR (RT-PCR) reaction. *SCR* gene specific primers were used to perform RT-PCR (Table 3.2). The RT-PCR Reaction contained the following reaction mixture: 0.2 mM of each primer, 1.5-3.0 mM $MgCl_2$, 0.5U Taq polymerase (Promega), 0.2mM each dNTP (Promega) and ~100ng of DNA template. Following PCR cycle was used: denaturation at 94^0C for 1 minute followed by primer annealing at 54^0C for 1 minute, followed by extension at 72^0C for 1 minute and 30 seconds for 10 cycles. After first 10 cycles the entire program was repeated for 24 more cycles with denaturation time (30 seconds) at 94^0C, primer

annealing at 54^0C for 30 seconds, extension at 72^0C for 1 minute and 30 seconds and final elongation at 72^0C for 4 minutes.

Inheritance pattern analysis of suppressors

In order to confirm if suppressors are homozygous recessive or dominant and if they represent a single gene mutation, backcrosses with *scr1* mutant were performed. Pollen of *scr1* mutant (male) was used to cross the ovary of each suppressor. If the suppressor is single nuclear homozygous recessive:

suppressor X *scr1* (Backcross)

F1 **All *scr1*** phenotype (heterozygous agravitropic)

F2 **3 agravitropic *scr1*: 1 gravitropic suppressor** (3:1) - recessive

Complementation Test

Each mutation should point to the specific point in the gravitropic pathway in which corresponding gene is involved. To confirm the number of different loci affected by mutations identified in the screen, a complementation test was performed. In order to accomplish the complementation test, pair wise crosses were performed. Each suppressor was crossed with the other nine. On the basis of the results of these crosses, suppressors were placed in their complementation group.

Suppressor 1 x Suppressor 2	**F1**	sup1/sup2 (allelic and fail to complement)
		scr1 (alleles of different genes and complement)

Hypocotyl length of suppressors

To analyze the hypocotyl length of suppressors in dark and to compare it with WS and *scr1*, seeds of each suppressor, WS and *scr1* were plated on MS agar plates containing 4.5% sucrose. These plates were kept on 4^0 C for 3 days to synchronize germination. After 3 days plates were transferred to a growth chamber (16 hour day light) for germination. Plates with germinating seedlings were covered with aluminum foil and left in a vertical position for 7 days. Hypocotyl length of seedlings grown in dark conditions for 7 days was measured using "Imagej 1.48v" software.

Presence and position of amyloplasts in suppressor hypocotyls

To identify the presence and location of amyloplasts in suppressors with negative gravitropic response, whole-mount amyloplast staining was performed as described by Fukaki et al. 1998. In order to do this, seeds were sterilized and placed on both MS agar plate without sucrose and MS agar plate supplemented with 1% sucrose. To induce germination plates were kept in a growth room (16 hr. daylight). Plates with germinated seedlings were kept in the dark for 24 hr. to enhance elongation of the hypocotyl. Only those seedlings which were growing in an up-right direction were fixed in FAA (formalin, acetic acid, alcohol) solution for 24 hr. at 4^0C. After fixation, seedlings were rinsed with 50% [v/v] ethanol and stained with I-KI 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 gram chloral hydrate, 2 ml water, and 1 ml glycerol). Slides with mounted seedlings were examined and pictures were taken with the use of Nikon Biophot microscope with a Nikon D-70 digital camera attachment.

Radial pattern analysis of suppressors

For cross sections, hypocotyl fragments were embedded in plastic media as follows. Seedlings grown in the dark for 3 days after germination were cut and hypocotyl fragments were selected for further procedure. Samples were fixed at 4^0C overnight in fixative containing 3% glutaraldehyde in 0.05 M potassium phosphate buffer. Thereafter, samples were washed with phosphate buffer and dehydrated in a graded ethanol series. Samples were treated 15-30 minutes in each step with the following alcohol concentrations: 10%, 30%, 50%, 75%, 95% and 95%. JB-4 embedding kit (Sigma-Aldrich) was used for infiltration and embedding. Dehydrated samples were infiltrated and embedded in plastic embedding medium. Finally, the embedded tissues were mounted and sectioned by using microtome. The cross sections of 2-3 µm were heat fixed to slides, stained with toluidine blue O (TBO). A Nikon Biophot microscope with a Nikon D-70 digital camera attached was used for examination of cross sections.

Mapping

To map the position of mutated gene in suppressor *shs1*, *shs1* was crossed with *scr*3 (in ecotype Col). The *shs1* was used as a male parent and *scr*3 as a female parent. Therefore, pollen of *shs1* was used to pollinate the carpels of *scr*3. The second generation progeny with the suppressor's phenotype was used for mapping. Suppressors are resistant to kanamycin (T-DNA, inserted in *SCR* gene of *scr1* mutants have a kanamycin resistance gene) but *scr3* mutants are not. Therefore, F1 generation seeds (heterozygous for kan resistance gene) should be resistant to kanamycin. F1 seeds of crosses were collected and plated on 4.5 % sucrose MS agar plates containing kanamycin. Kanamycin resistant seedlings were transferred to soil. Seeds were collected from mature F1 (self-pollinated) plants. Seeds were plated on 4.5% sucrose MS agar plates and tested for their hypocotyl

gravitropic responses. Seedlings with hypocotyl gravitropic response were selected for DNA extraction. DNA of 20-34 plants were used in mapping with the help of SSLP markers (Table 3.7).

Results

Quantitative analysis of hypocotyl gravitropic responses of *shs*

In the absence of light, orientation of the germinating seedling is solely based on the gravity vector. The preliminary hypocotyl gravitropic response analysis suggested that all ten *scr* hypocotyl gravitropic suppressors have gravitropic responses above the level of *scr1* but below WS. Furthermore, results also indicated that *scr1* mutant seedlings may have some residual hypocotyl gravitropic responses. Therefore, the degrees and the time courses of gravitropic responses of all ten *shs*s, *scr1* and WS were determined and compared (Table 3.1 and Fig. 3.1). For quantification of hypocotyl gravitropic response 35 seedlings of all ten *shs*s, *scr1* and WS were used. The average values with standard deviations of hypocotyl gravitropic responses were calculated in degrees and presented both in table form (Table 3.1) and graphically (Fig. 3.1). WS hypocotyls showed negative gravitropic responses by reorientation of growth by 39.8 degrees after 12 hours of reorientation. All the ten suppressors show significant but lower than WS gravitropic responses in range of 20-28 degrees after 12 hours reorientation. One suppressor, *shs1* exhibit stronger response than other suppressors and closest to WS response at all three time points used in this analysis (Table 3.1 and Fig. 3.1). WS and *shs1* hypocotyls exhibited the final angel of deflection in the range of 53-59 degrees after 48 hours reorientation to a new gravity vector (Table 3.1 and Fig. 3.1). The other nine suppressors showed hypocotyl gravitropic responses significantly above the level of *scr1* with the range of 36-47 degrees after 48 hours of reorientation (Table 3.1 and Fig. 3.1). The most exciting observation was that *scr1* hypocotyls' still possess some residual

gravitropic responses contrary to the previous reports of *scr1* hypocotyls agravitropism (Fukaki et al. 1996a; Fukaki et al. 1996b and Fukaki et al. 1998). However, the response is much weaker, slower and exhibit only 9 degrees of angles of deflection after 12 hours of reorientation. After 48 hours of reorientation to a new gravity vector *scr1* hypocotyls showed only 16 degrees of deflection that represents only approximately 25% of the WS response. These results suggest that *scr1* retains a low level of hypocotyl gravitropism. They also indicate that all the hypocotyl gravitropic suppressors have significantly improved hypocotyl gravitropism over *scr1* but below the level of WS. One of the suppressors, *shs1* is stronger than the other suppressors and shows a very similar response to the WS hypocotyl gravitropic response.

Reverse-transcriptase PCR to confirm the absence of SCR transcript in suppressors

To confirm that improved hypocotyl gravitropic responses of *scr* hypocotyl gravitropic suppressors are solely because of second-site mutation and no SCR transcript is present in confirmed suppressors, RT-PCR was performed. cDNAs of all the samples were used for PCR amplification performed with SCRF836 (before intron) and SCRR1870 (after intron) primers (Table 3.2). A single band of ~ 0.9 kb was produced by the WT cDNA (no intron) with two *SCR* primers (Fig. 3.2). However, cDNAs of *scr1* and all confirmed hypocotyl suppressors failed to produce any amplification product (Fig. 3.2). The RT PCR results confirmed the absence of any SCR transcript and showed that all ten suppressors are on *scr1* background.

Inheritance pattern analysis of suppressors

Genetic inheritance pattern of an offspring can be determined by doing a backcross. Therefore, to confirm if suppressors are homozygous recessive or dominant and if they represent a single gene

mutation all *shs*s were backcrossed with *scr1* mutants. If the suppressor is a single nuclear homozygous recessive, all the F1 progeny should be hypocotyl agravitropic (heterozygous). While the F2 generation should show the phenotypic segregation in a 3:1 ratio (3 agravitropic: 1 gravitropic). All the progeny of backcrosses of suppressors with *scr1* mutant in F1 generation were agravitropic and in the F2 generation they fit the ratio of 3 (agravitropic): 1 (gravitropic) (Table 3.3). The results confirmed that each suppressor represents a single locus and it is a recessive allele.

Complementation test

The complementation test is a way 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 relevant for recessive mutants and as shown earlier all the suppressors are single gene recessive alleles. In order to perform the complementation test, the suppressors are crossed with each other. If the mutations of both suppressors are in the same gene all F1 progeny should have suppressor phenotype. However, if suppressors have mutation in two different genes all F1 progeny should have the parent (*scr1*) phenotype and thus the two genes complement each other. Failure to complement suggests that two mutations lie in the same gene, therefore, are part of the same complementation group.

The pair wise crosses between all suppressors were performed. F1 generation progeny of *shs1*X*shs2*, *shs1*X*shs5*, *shs6*X*shs7* and *shs6*X*shs4* showed hypocotyl gravitropic response (Fig. 3.3). Thus, *shs1*, *shs2* and *shs5* have the mutation in the same gene and belong to the same complement group. Similarly, *shs4*, *shs6* and *shs7* are all in the same complementation group (Table 3.5). The F1 progeny of all other pair wise crosses between *shs1, shs3, shs4, shs8, shs9* and *shs10* showed hypocotyl agravitropism. Their F2 generation progeny segregation fit 9:7 ratios for

agravitropic and gravitropic seedlings respectively (Table 3.4). These results suggest that *shs1, shs3, shs4, shs8, shs9* and *shs10* are alleles of different genes. Eventually all ten *scr* hypocotyl gravitropic suppressors were grouped into six complementation groups (Table 3.5) that represent six different loci (genes) involved in hypocotyl gravitropic pathway.

Hypocotyl lengths of suppressors (*shs*)

The hypocotyl is the embryonic stem that bears the cotyledons (embryonic leaves) and plumule and in *Arabidopsis,* from apex to base, hypocotyl have approximately 20 cells and after germination no significant cortical and epidermal divisions occur (Gendreaau et al. 1997). However, the hypocotyl may increase more than 10 fold in length and cell elongation is entirely responsible for this postembryonic hypocotyl growth. Various factors such as light, temperature, touch and plant hormones etc. have very strong influence on hypocotyl elongation (Collett et al. 2000). This organ is significantly elongated in dark growth conditions. When grown in dark condition WT hypocotyls are at least 1.5 fold longer than *scr1* (Table 3.6 and Fig. 3.5). However, all of the suppressors have similar hypocotyl length as *scr1* mutant (Table 3.6 and Fig. 3.4.). No significant difference between the hypocotyl lengths of *scr1* and any of the suppressors was observed on 7 day after germination in dark.

Presence and position of amyloplast in *shs*'s hypocotyls

Currently the most favored hypothesis of gravity sensing mechanism is the "Starch-statolith hypothesis" which postulates that gravity sensing involves sedimentation of amyloplasts (statoliths) in specific cells (statocytes). To gain a better understanding of the gravity sensing mechanism in plant shoots and to test the "Starch-statolith hypothesis" whole-mount amyloplast

staining was performed. The results show that suppressors resemble *scr1* for the presence and the position of amyloplast rather than the WT plants. As shown in Fig. 3.6 when seedlings were grown on MS agar plate without sucrose, were stained with I-KI solution, the presence of amyloplasts was detected only in WS. These were found adjacent to the vascular tissue in WS while in *scr1* and all other *shs* no amyloplasts could be detected (Fig. 3.6). When seedlings grown on MS agar plate containing 1% sucrose were stained with I-KI solution, amyloplasts were found in all WS, *scr*1 and *shs* hypocotyls. However, in WS the amyloplasts were sedimented and present only in cells near the vascular system while in *scr1* and all other *shs* amyloplasts were scattered into cell layers (Fig. 3.7). Importantly the presence and position of amyloplasts in *scr1* and *shs* hypocotyls were indistinguishable.

Radial Pattern analysis of suppressors

In *Arabidopsis thaliana* both root and shoot possess a radial pattern of cell arrangement. All cell layers are organized on the same radius, the stele that includes vascular system, surrounded by endodermis, cortex and epidermis (Dolan et al. 1993). It was reported *scr* (*scarecrow* mutants) of *Arabidopsis thaliana* exhibited abnormal internal architecture (one missing ground tissue layer) in all organs examined (Wysocka-Diller et al. 2000). To determine the radial pattern of *scr* hypocotyl gravitropic suppressors, cross sections of WT, *scr1* and all ten *shs* were generated and observed under a microscope (Fig. 3.8). The radial pattern of all the 10 suppressors is similar to the *scr1* mutant. They have not rescued the missing ground tissue layer.

Mapping analysis of *scr* hypocotyl suppressor1 (*shs1*)

scr hypocotyl gravitropic suppressor1 (*shs1)* is the strongest hypocotyl gravitropic suppressor identified in suppressor screening with a strong and reproducible phenotype. Since *shs1* has only rescued the hypocotyl gravitropic phenotype and still exhibits inflorescence agravitropism and short roots, it is probable that the gene mutated in *shs1* is involved in hypocotyl gravitropism and not in the other SCR regulated pathways. Therefore, *shs1* was selected for mapping analysis.

Positional cloning is based upon sequentially excluding all the other regions in the genome and systematically narrowing down the genetic interval containing a specific mutation (Lukowitz et al. 2000). This approach relies on the availability of genetic markers that are polymorphic between the accessions used for developing the mapping population. *Arabidopsis thaliana* is an ideal model system for genetic mapping because of the availability of a comprehensive set of resources such as annotated reference genome, sequenced alternative accessions, and a multitude of molecular markers. The chromosomal position of a mutant gene can be determined by recombination frequencies of molecular markers. The *shs1* mutation is a suppressor of *scr1* (WS background), and to map the mutated gene *shs1* was crossed with *scr*3 (Col background). The T-DNA insertion in *SCR* coding region of *scr1* mutant contains Kan resistance gene, therefore *scr1* is kanamycin resistant but *scr3* is sensitive. The difference in kanamycin sensitivity was used for selection of "real" crosses. The *shs1* was used as a male parent and *scr*3 as a female parent. Therefore, pollen of *shs1* was used to pollinate the carpels of *scr*3. F1 seeds were collected from the successful crosses and plated on kanamycin containing MS agar plates with 4.5% sucrose. Seedlings able to grow in presence of kanamycin were transferred to soil and F2 generation seeds were collected for mapping.

F2 seeds were plated on MS agar plate containing 4.5% sucrose and tested for their hypocotyl gravitropic responses. Approximately a quarter of F2 seedlings had hypocotyl gravitropic response while the rest of progeny were hypocotyl agravitropic. These seedlings with hypocotyl gravitropic responses were selected and transferred to soil. Their DNA was extracted and used for mapping.

SSLP (Simple Sequence Length Polymorphism), PCR based markers that show polymorphism between Col and WS, were used for *shs1* mapping (TAIR, www.arabidopsis.org). The SSLP are easy to use, PCR based, co-dominant and relatively abundant markers, also easily accessible at The Arabidopsis Information Resource (TAIR, www.arabidopsis.org). The markers were selected based on their location along the chromosome. A total of 30 markers were selected for initial mapping and tested on *shs1*, *scr3* and heterozygous (*shs1/scr3*) seedlings DNAs. Only 21 of the markers gave reproducible results with all three *shs1*, *scr1* and het DNAs. The 21 sets of primers were used on the DNAs from 20-34 F2 seedlings with hypocotyl gravitropic response (Table 3.7). The recombination frequencies data suggest that the most likely position of the *shs1* locus is on the lower arm of chromosome V approximately 27.9cM away from NGA129 (Table 3.8 and Fig. 3.9). The markers, their chromosomal positions, primer sequences and product sizes are provided in Table 3.7, Table 3.8 and Fig. 3.9.

Discussion

All higher plants are capable of orienting their growth in response to gravity, a process termed gravitropism. Germinating seedlings in the soil are not exposed to the light therefore, in the absence of light directional growth of the hypocotyl (embryonic stem) and roots can be determined

only by orientation of the gravity vector. Three organs of the *Arabidopsis thaliana;* root, hypocotyl and inflorescence stem show gravitropism (Okada and Shimura 1992; Fukaki et al. 1996a, b).

In this research the *SCR* gene was used as an entry point to identify other components involved in SCR regulated hypocotyl gravitropic pathways. To do so *scr* mutant suppressors were generated. Ten confirmed *scr* hypocotyl gravitropic suppressors (*sh*ss) were identified after primary and secondary screening. All ten suppressors showed improved gravitropic responses above the level of *scr1* but below the level of WS at all three different time points: 12 hours, 24 hour and 48 hours (Table 3.1). The suppressor line *shs1* has a stronger gravitropic response than other suppressors and is similar to WS in that respect. In addition to identification of *scr* hypocotyl suppressors, it was also observed that contrary to the previous report (Fukaki et al. 1998), *scr1* itself is not completely agravitropic and retains some residual hypocotyl gravitropic response. These results also suggest that although SHR is essential and SCR is very important for hypocotyl gravitropic response, you can still mutate many different genes involved in the hypocotyl gravitropic pathway and get the response on *scr* mutant background.

Before starting any further genetic and phenotypic characterization of these suppressors, it was important to make sure that the improved hypocotyl response of these suppressors was not due to the reversion of the *scr* mutation. Therefore, RNAs were extracted from all the suppressors and used for cDNAs synthesis. These cDNAs were used for PCR amplification with *SCR* specific primers. Only WS cDNA gave a product of ~0.9 kb, *scr1* and all ten *sh*ss cDNAs failed to amplify using *SCR* specific primers. Results of RT-PCR showed that no SCR transcript is present in the hypocotyl of suppressors and the improved hypocotyl gravitropism is not due to the reversion of *scr* mutation but because of the second-site-mutation.

scr hypocotyl gravitropic suppressors were backcrossed with *scr1* mutants to confirm if suppressors are homozygous recessive or dominant and if they represent single gene mutations. The F1 progeny of backcrosses between suppressors and *scr1* mutants were all agravitropic (similar to *scr1*) and the F2 generation progeny segregations fit the 3:1 (agravitropic: gravitropic) ratio (Table 3.3). It showed that the allele of each *scr* hypocotyl gravitropic suppressor represents a recessive single locus.

A complementation test was performed to establish how many genes were affected by ten mutations associated with the hypocotyl gravitropism phenotype. Each mutation should point to the specific point in the gravitropic pathway in which a corresponding gene is involved. Results of complementation test analysis showed that the ten *scr* hypocotyl gravitropic suppressors fall into six complementation groups (Table 3.5). It suggested our suppressors correspond to six different genes that are involved in hypocotyl gravitropic pathway. The orientation of germinating seedlings in the absence of light is mostly dictated by hypocotyl gravitropism therefore, hypocotyl gravitropism is an essential phenomenon for survival of the plant. Exploration of six different genes involved in the hypocotyl gravitropic pathway during suppressors screening supports the hypothesis that hypocotyl gravitropism is very important for germinating seedlings therefore, there may be more than one alternative pathway to ensure survival.

scr1 plants have a much shorter hypocotyl than WS (Fig. 3.4). Since the number of hypocotyl cells are fixed from the embryonic stage, the hypocotyl length only depends upon cell elongation. It suggests that *scr1* hypocotyl may be defective in cell elongation. Because the asymmetric cell elongation is essential for gravitropic response, it could be speculated that one of the reasons for the very weak gravitropic response of *scr1* mutant hypocotyls is impaired cell elongation as compared to wild type plants. To address this hypothesis, hypocotyl lengths of all these

suppressors grown in the dark were examined. Hypocotyl lengths of all suppressors were very similar to *scr1* but significantly shorter than WS (Table 3.6 and Fig. 3.4). These results indicate that hypocotyl length and thus normal cell elongation is not an essential factor for gravitropic response. However, it also suggests that *SCR* is essential for normal hypocotyl cell elongation, and that none of the suppressor genes are involved in hypocotyl cell elongation.

Amyloplast sedimentation is entirely absent in *scr1* mutants however it was observed that, *scr1* itself retains some residual gravitropic response (Fig. 2.3 and Table 3.1). These results indicate that hypocotyl gravitropism is partially independent of amyloplast sedimentation. Because amyloplast-sedimentation is believe to play important role in gravity signaling, it can be argued that improved hypocotyl gravitropic responses of suppressors occur because they have restored the amyloplast sedimentation. Therefore, to check the presence and location of amyloplasts, all the suppressors were stained with I-KI solution. Results showed that the presence and the position of amyloplasts in all the suppressors is similar to *scr1* rather than WS (Fig. 3.6 and Fig. 3.7). Because the amyloplasts sedimentation takes place in the endodermal cell layer, absence of amyloplast sedimentation in suppressors suggests that they have rescued only gravitropic responses and not the hypocotyl architecture such as the radial pattern. To confirm this hypothesis cross sectioning of suppressors was performed and it was found that one ground tissue layer is still missing in all the suppressors. These findings support the hypothesis that starch and 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. It has been postulated that the gravireceptors lie in between the plasma membrane and the cell wall and would be mechanically stimulated for initial sensing of gravity vector in any mechanism (Guo et al. 2008). According to the "Gravitational pressure model", reorientation of the organ can cause a subtle

change in cytoplasmic pressure sufficient to generate a perceivable signal (Staves 1997). The gravireceptors could perceive subtle changes in compression pressure resulting from reorientation of the protoplast in the displaced organs (Telewski 2006). In the absence of amyloplast sedimentation, as is the case of *scr1* mutants and hypocotyl gravitropic suppressors, perhaps change in cytoplasmic pressure is responsible for gravity perception. The stronger hypocotyl gravitropic responses of suppressors, even though they have not rescued the amyloplast-sedimentation and don't have the right architecture, could be because of a mutation where the gravireceptors have enhanced sensitivity to detect any subtle change in cytoplasmic pressure and gravity vector. It is also possible that involvement of amyloplast sedimentation in gravity perception is less than expected. Furthermore, it can be speculated that since SCR is a transcription factor and it is required for expression of downstream genes in several developmental pathways, 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 SCR. The mutation may eliminate the need for SCR to turn it "on" or "off". Another possibility is that the gene products needed to interact with SCR protein are directly activated and due to mutation no interaction is required. Until the identities of mutated genes are revealed we can only speculate what type of product they encode and their involvement in gravity sensing. Finally, these data clearly indicate that at least hypocotyls can perceive gravity even in the absence of sedimenting amyloplasts. It also suggests that *scr1* mutant are primarily defective in gravity sensing mechanism and no other aspects of the gravitropic responses.

One suppressor, *shs1* was selected for positional cloning and a rough map position for *shs1* has been determined. The mapping results indicate that the gene corresponding to *shs1* mutation is located on the lower arm of chromosome V (Fig. 3.9). Most probably it is located 27.9cM away

from the Nga129 SSLP marker. However, this distance is too large to come to any conclusion about a most probable candidate gene. Mapping by next generation sequencing can be an alternative way to identify the mutated genes (Lister et al. 2009).

In summary, ten hypocotyl gravitropic suppressors that represent six different genes involved in hypocotyl gravitropic pathway have been identified. Each of these suppressors represents a recessive allele of a single locus. These suppressors have rescued only hypocotyl gravitropic phenotypes but they still have abnormal radial pattern and they 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 amyloplast sedimentation.

Samples	Time interval		
	12 hour	**24 hours**	**48 hours**
WS	39.8 ± 11.7	48.7 ± 10.6	59.6 ± 12.3
scr1	9.0 ± 8.0	14.5 ± 10.4	16.7 ± 11.1
shs1	28.4 ± 7.1	39.8 ± 9.4	53.6 ± 12.8
shs2	26.4 ± 13.8	37.2 ± 13.0	47.0 ± 12.5
shs3	27.6 ± 14.2	36.9 ± 12.9	42.8 ± 15.6
shs4	25.5 ± 14.6	38.4 ± 13.7	47.3 ± 16.3
shs5	24.6 ± 14.3	35.0 ± 9.5	45.2 ± 11.9
shs6	27.3 ± 13.0	37.8 ± 14.7	45.7 ± 15.0
shs7	25.8 ± 13.0	37.9 ± 14.4	47.7 ± 14.4
shs8	23.4 ± 13.8	31.5 ± 14.2	41.6 ± 18.1
shs9	20.6 ± 13.6	31.6 ± 12.8	37.0 ± 14.8
shs10	25.6 ± 11.9	34.5 ± 14.9	36.7 ± 13.0

Table 3.1. Quantitative analysis of hypocotyl gravitropic response of WS, *scr1* and ten confirmed *scr* hypocotyl gravitropic suppressors. Angle of deflection were measured at three different time intervals 12 hours, 24 hours and 48 hours after reorientation.

Primer	Sequence (5'-3')	Product length	Tm
SCRF836	AGGCAGAAGCAAGACGAAG	920bp	57.5C
SCRR1870	CTTCACCGCTTCTCGATGGT	920bp	60.5

Table 3.2 SCR specific primers used in RT- PCR of suppressor hypocotyls.

F2 generation of cross	Sample size	Hypocotyl agravitropic	Hypocotyl gravitropic	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	104	46	2.8:1.2	$\chi^2 = 2.4$	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 3.3 Backcross of *scr* hypocotyl gravitropic suppressors with *scr1*. F2 generation progeny of *scr1* X *shss* were analyzed to test hypocotyl agravitropic/gravitropic ratio.

F2 generation of cross	**Sample size**	**Hypocotyl agravitropic**	**Hypocotyl gravitropic**	**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*femalX *shs10* male	165	100	65	9.7:6.3	$\chi^2 = 1.272$	P> 0.05
shs8 male X *shs9* female	258	158	100	9.7:6.02	$\chi^2 = 2.4$	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

Table 3.4 Complementation test analysis of hypocotyl gravitropic suppressers. F2 generation progeny of *crossed suppressors* were analyze to test hypocotyl agravitropic/gravitropic ratio.

Complementation Group	***scr* hypocotyl gravitropic suppressors (*shs*)**
1	*shs1, shs2,shs5*
2	*shs6, shs7, shs4*
3	*shs3*
4	*shs8*
5	*shs9*
6	*shs10*

Table 3.5. Six different complementation groups and suppressors that belong to each group.

Sample	Hypocotyl length of seedlings (in mm)
WS	16.7 ± 1.8
scr1	10.6 ± 1.7
shs1	11.3 ± 1.2
shs2	10.1 ± 2.6
shs3	9.4 ± 2.2
shs4	11.8 ± 1.5
shs5	10.7 ± 2.7
shs6	10.1 ± 2.7
shs7	11.1 ± 1.1
shs8	8.5 ± 3.0
shs9	10.2 ± 1.8
shs10	10.5 ± 2.1

Table 3.6. Hypocotyl length of WS, *scr1* and *scr* hypocotyl gravitropic suppressors 7 days in dark after germination. After germination seedlings were transferred into dark for 7 days. Hypocotyl lengths were measured in millimeter (mm).

Marker Name	Position (bp or kb or cM)	Forward Primer/Reverse Primer	Annealing Tempe. of primers (F/R)	Product size
JV 26/27	4015243 bp	CAAGAGATTGCAACATCCAC A/AAGCTCCTTGGATCCGATTT	57.5^{0}C/56. 4^{0}C	Col= 171bp WS= smaller than Col
CIW12	9621357 bp	AGGTTTTATTGCTTTTCACA/C TTTCAAAAGCACATCACA	50.2^{0}C/50. 9^{0}C	Col= 128bp WS= 115bp
CIW1	1836388 1bp	ACATTTTCTCAATCCTTACTC/ GAGAGCTTCTTTATTTGTGAT	53.4^{0}C/53. 4^{0}C	Col= 159bp WS= 130bp
Nga280	83.8cM 20456.0k b 2087369 bp	GGCTCCATAAAAAGTGCACC/ CTGATCTCACGGACAATAGTG C	58.4^{0}C/62. 1^{0}C	Col= 105bp WS= 85bp
Nga111	115.55c M 26693.0k b 2735321 2bp	TGTTTTTTAGGACAAATGGCG/ CTCCAGTTGGAAGCTAAAGG G	55.4^{0}C/61. 2^{0}C	Col= 128bp WS= 146bp
ATPase	117.86c M 2853383 7bp	GTTCACAGAGAGACTCATAA ACC/CTGGGAACGGTTCGATT CGAGC	60.9^{0}C/65. 8^{0}C	Col= 85bp WS= 69bp
UPSC_ 2-1401	1401050 bp	GTTTGGATCAGTCCCAGCTC/T GAAAAAGTGGTGGAACCAA	60.5^{0}C/54. 3^{0}C	Col= 211bp WS= 300bp
Nga168	73.77cM 1629184 1bp	GAGGACATGTATAGGAGCCT CG/TCGTCTACTGCACTGCCG	64.2^{0}C/54. 3^{0}C	Col= 151bp WS= 135bp
Nga172	6.91 cM 786296 bp	CATCCGAATGCCATTGTTC/AG CTGCTTCCTTATAGCGTCC	55^{0}C/61.2^0 C	Col= 162bp WS= 138bp
UPSC_ 3-20956	2095695 4bp	TCTGTTGGTGCGTCATGAAT/T GCAAACGAGATTGATTTGG	56.4^{0}C/54. 3^{0}C	Col= 794bp WS= 2000bp
UPSC_ 4-764	764195b p	TTTTTAATTAAGGGAACAAAA TGGA/TTGTGTCATATGTCAAG TCTGTCG	55.9^{0}C/62^0 C	Col= 224bp WS= 151bp
UPSC_ 4-6222	6222296 bp	CAGAACCAAGCTGCAATGAA/ CCTTCGATGTCTTCGCTGAT	56.4^{0}C/58. 4^{0}C	Col= 236bp WS= 140bp

UPSC_4-17110	17110453bp	ATCGCTAACCCTCTCACGAA/TGGCTGTGAGTGAGTGAAGA	58.4^{0}C/58.4^{0}C	Col= 280bp WS= 225bp
DHS1	18539227 bp	GAGCTTTGTAAATCAACAACC/GATATTTTTCAGGCGACGTGGAAGC	55.4^{0}C/65.8^{0}C	Col= 194bp WS= 165bp
Nga158	18.12cM 1698613 bp	ACCTGAACCATCCTCCGTC/TCATTTTGGCCGACTTAGC	59.5^{0}C/55^{0}C	Col= 108bp WS= 120bp
CA72	29.6cM 4254759 bp	CCCAGTCTAACCACGACCAC/AATCCCAGTAACCAAACACACA	62.5^{0}C/58.4^{0}C	Col= 124bp WS= 110bp
Nga139	50.48cM 8863.0 kb 8428133 bp	GGTTTCGTTTCACTATCCAGG/AGAGCTACCAGATCCGATGG	59.5^{0}C/60.5^{0}C	Col= 174bp WS= 132bp
Nga76	68.4 cM 11162.0 kb 10418610 bp	AGGCATGGGAGACATTTACG/GGAGAAAATGTCACTCTCCACC	58.4^{0}C/62.1^{0}C	Col= 231bp WS= 199bp
SO191	13754.0 kb 15004685bp	CTCCACCAATCATGCAAATG/TGATGTTGATGGAGATGGTCA	56.4^{0}C/57.5^{0}C	Col= 148bp WS= 162bp
Nga129	105.41 cM 19007.0 kb	CACACTGAAGATGGTCTTGAGG/TCAGGAGGAACTAAAGTGAGGG	62.1^{0}C/62.1^{0}C	Col= 177bp WS= 165bp

Table 3.7.Markers used in positional mapping. Location of markers on chromosomes, their sequences, annealing temperature and PCR product size of Columbia (Col.) and Wassilewskija (WS).

Marker	Colombia chromosome/Total chromosome	Recombination Frequency (%)
Chromosome I		
JV 26/27	21/46	45.6
CIW12	30/56	53.5
CIW1	16/40	40
Nga280	20/40	50
Nga111	21/57	36.8
ATPase	23/58	39.4
Chromosome II		
UPSC_2-1401	23/44	52.2
Nga168	37/70	53.1
Chromosome III		
Nga172	22/34	64.7
UPSC_3-20956	20/40	50

Chromosome IV

UPSC_4-764	34/56	64.7
UPSC_4-6222	26/64	40.62
UPSC_4-17110	24/72	44.44
DHS1	16/40	40

Chromosome V

Nga158	17/46	36.95
CA72	22/62	35.48
Nga139	18/40	37.5
Nga76	21/54	38.8
SO191	22/68	32.3
Nga129	19/68	27.9

Table 3.8. SSLP markers on chromosomes I to V. Recombination frequencies with SSLP markers on different chromosomes used in *shs1* mapping analysis.

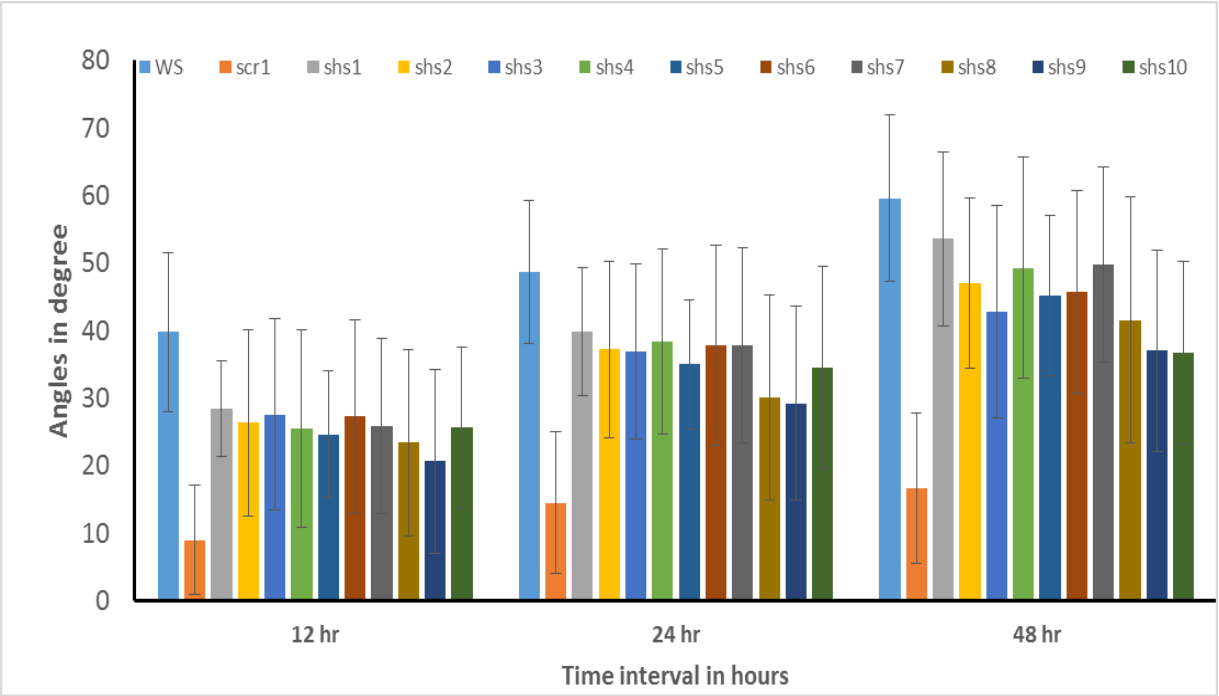

Figure 3.1. Hypocotyl gravitropic responses of WT, *scr1* and ten confirmed *scr* hypocotyl gravitropic suppressors (*shs1* to *shs10*). Gravitropic responses of all the samples were measured at several time points to new gravity vectors in degrees, graphical representation of Table 3.1. The X-axis represents the time interval in hours and the Y-axis represents the angle of deflection of hypocotyls in degrees.

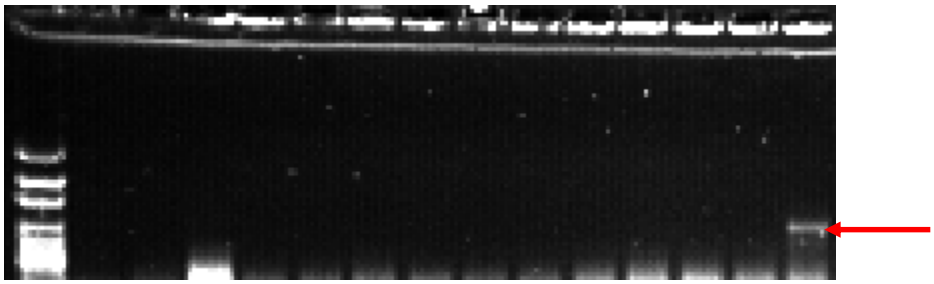

Figure 3.2. RT-PCR analysis of suppressors. Lane 1. 100bp ladder Lane 3. *scr1* (failed to produce any band with SCR specific primers). Lane 4-14. *scr* hypocotyl gravitropic suppressors, 1 to 10 (no amplification with *SCR* specific primers). Lane 15. WS (~ 0.9kb product with *SCR* specific primers, red arrow indicates the product).

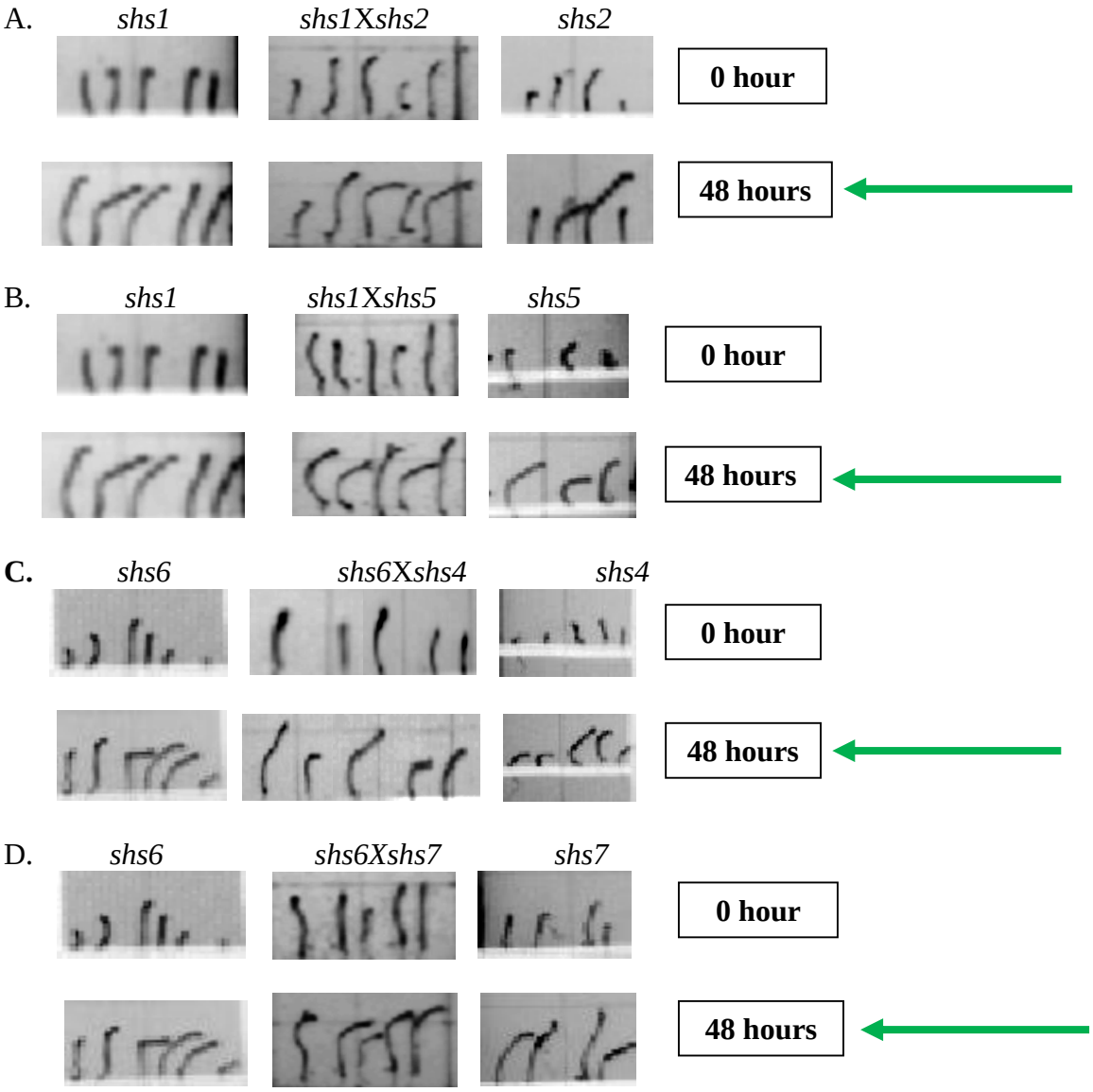

Figure 3.3 Complementation test analysis. Hypocotyl gravitropic responses of the parental *scr* hypocotyl gravitropic suppressors (*shs*s) and F1 generation progeny of their crosses, 48 hours after plate reorientation in the dark. Arrows indicate the direction of the gravity vector. The seedlings of *shs*s are shown before reorientation (0 hour) and 48 hours after reorientation. **A.** Hypocotyl gravitropic response of *shs1*, *shs2* and F1 generation of *shs1*X*shs2*. **B.** Hypocotyl gravitropic response of *shs1*, *shs5* and F1 generation of *shs1*X*shs5*. **C.** Hypocotyl gravitropic response of *shs6*, *shs4* and F1 generation of *shs6*X*shs7*. **D.** Hypocotyl gravitropic response of *shs6*, *shs7* and F1 generation of *shs6*X*shs7*.

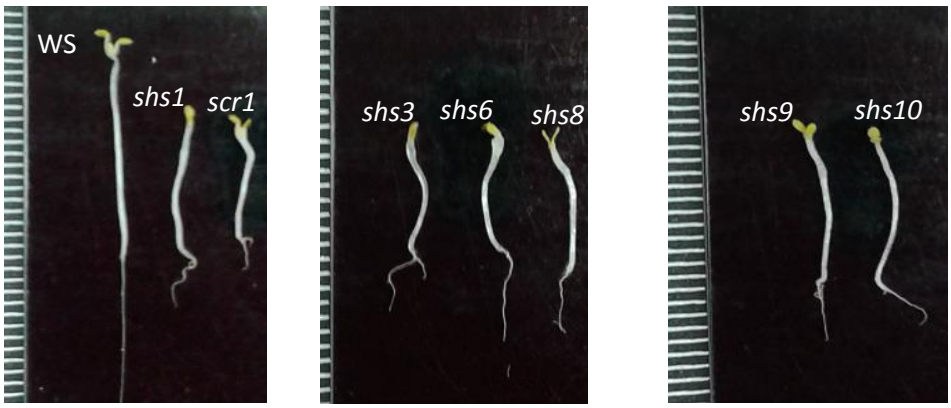

Figure 3.4. Hypocotyl length of WS, *scr1* and *scr* hypocotyl gravitropic suppressors 7 days in dark after germination. After germination seedlings were transferred into dark for 7 days. Hypocotyl lengths of one suppressor from each complementation group is shown in picture.

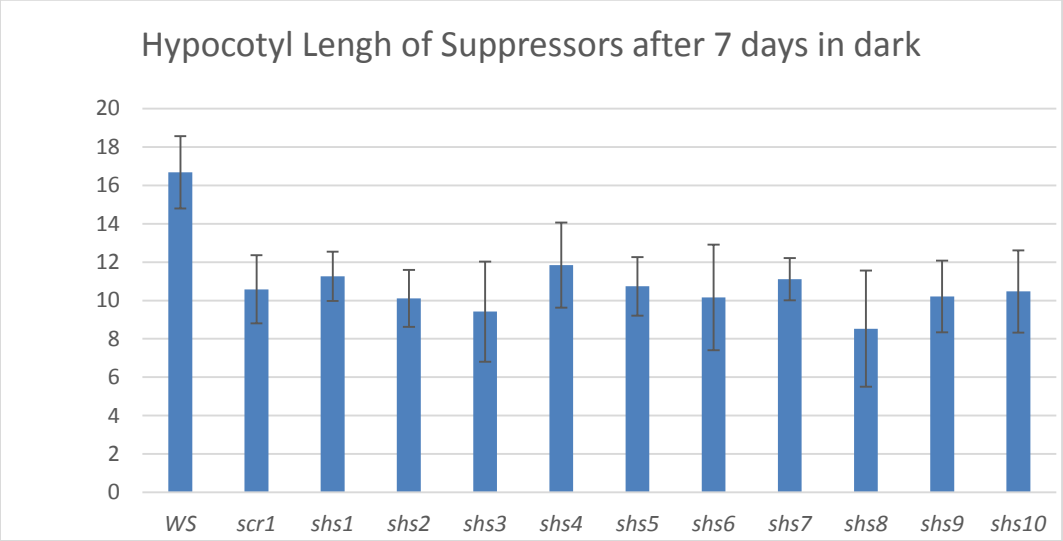

Figure 3.5. Graphical representation hypocotyl lengths of WS, *scr1* and *shs*s. Seedlings were grown in dark for 7 days after germination. Length of hypocotyls were measured in mm (Table. 3.5). The X-axis represent the type of seedling (genotype) and Y-axis represent the length in mm.

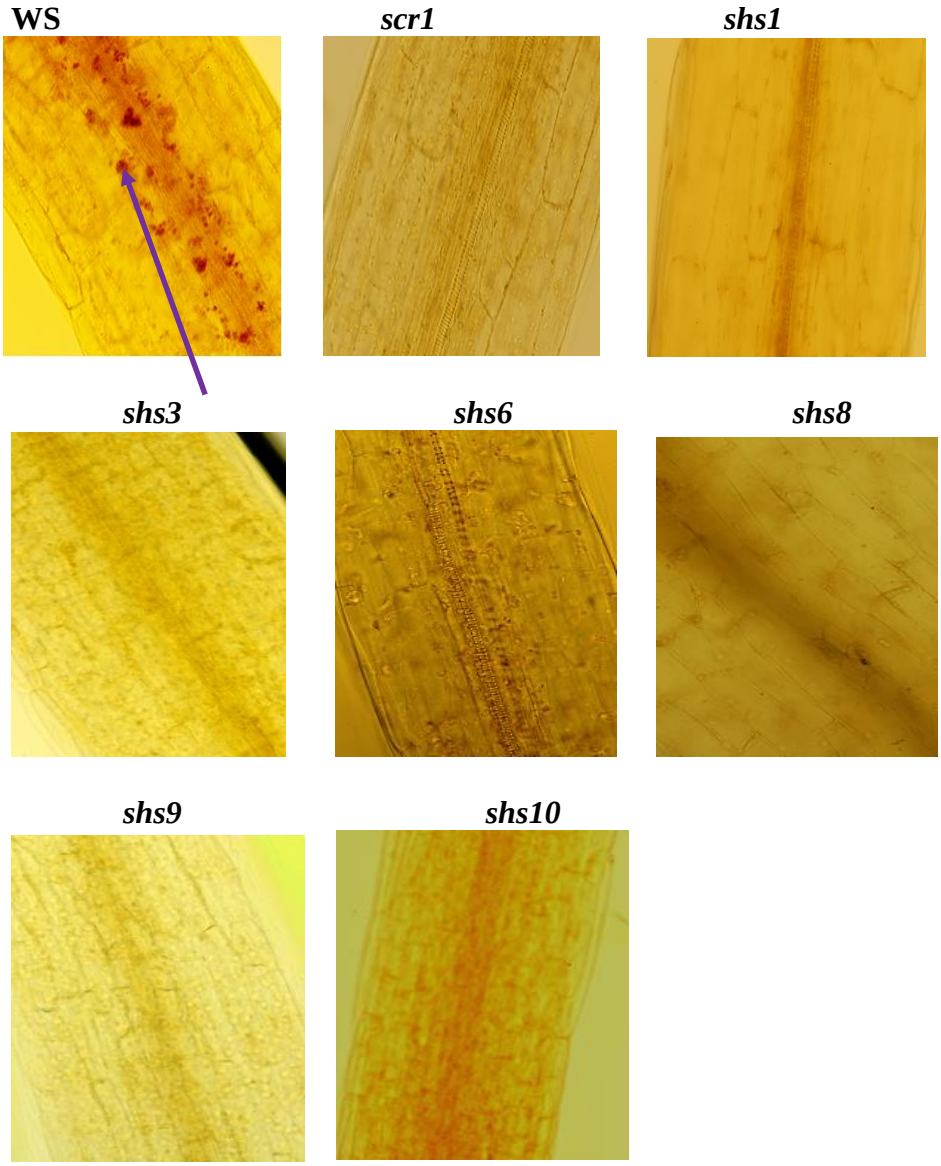

Figure 3.6. Presence and position of amyloplast in WS, *scr1* and *shs*s. Seedlings grown on MS agar plates containing no sugar were stained with IKI solution and analyzed under light microscope. Purple arrow indicates the sedimented amyloplast in endodermal layer of WS. One suppressor from each complementation group is shown in figure.

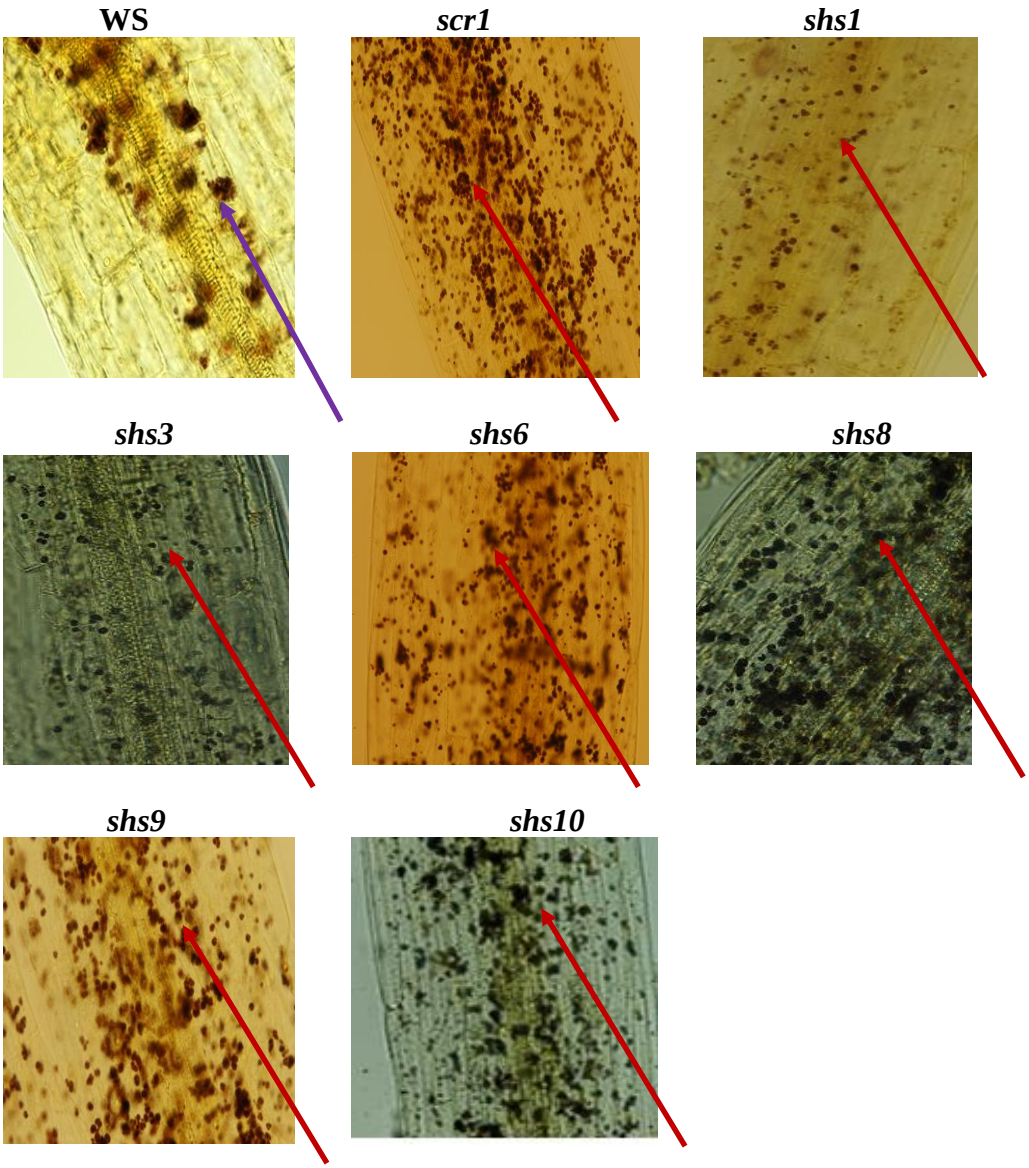

Figure 3.7. Presence and position of amyloplast in WS, *scr1* and *shs*s. Seedlings grown on MS agar plates containing 1% sugar were stained with IKI solution and analyzed under light microscope. Purple arrow indicates the sedimented amyloplast in endodermal layer of WS and red arrows indicate the distribution of amyloplasts. (One suppressor from each complementation group is shown in Fig.).

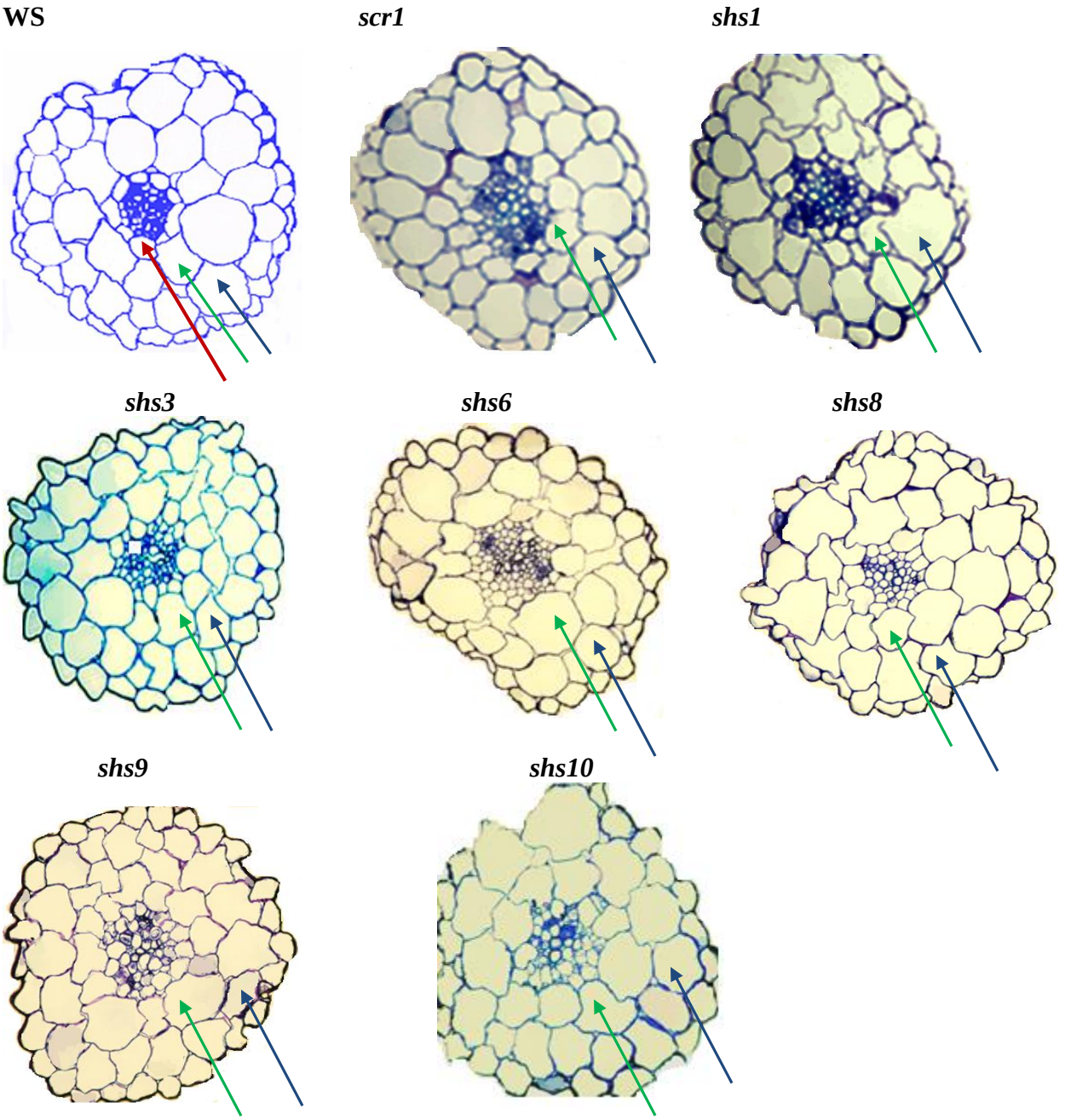

Figure 3.8. Cross section of WS, *scr1*, *shs1*, *shs3*, *shs6*, *shs8*, *shs9* and *shs10* hypocotyls. Seedlings were grown in dark for 3 days after germination. Red arrow points to the endodermal layer in WS. Green and blue arrows point to ground tissue layer two and three respectively in WS, *scr1* and all *scr* hypocotyl suppressors (one suppressor from each complementation group is shown).

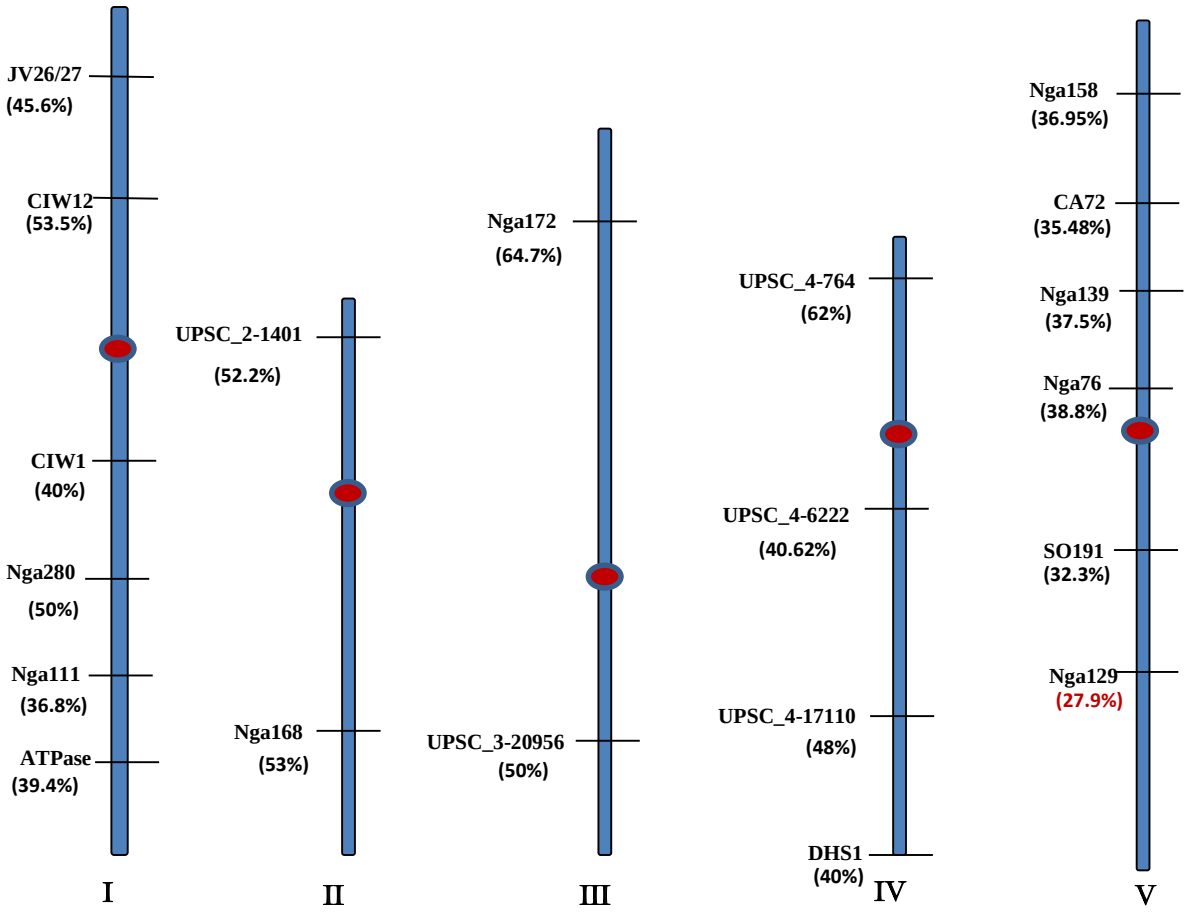

Figure 3.9. Relative position of the markers on the chromosomes.

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IV Accessing function of SCR through the analysis of *Arabidopsis thaliana* transgenic lines with altered *SCR* expression

Abstract

The *SCR* gene is expressed in the endodermis of shoot, endodermis and quiescent center of root and bundle sheath of leaves. *scr* mutant plants exhibit shoot agravitropism, abnormal radial pattern in both shoot and root and short root phenotype. In order to expand our knowledge about *SCR* gene function and to identify the location of *SCR* expression pattern/level driven by 35S promoter in *Arabidopsis thaliana*, a transgenic approach was used. To do that *scr* mutant and WS plants were transformed with *35S::SCR* and *35S::GFP::SCR* inserts. Hypocotyls of two lines *35S::GFP::SCR/scr1*(st1) and *35S::SCR*/*scr1*(D7) showed gravitropic response similar to WS plants while one *35S*::*SCR*/WS (35S) line showed having complete agravitropism. The cross section analysis revealed that st1 and D7 have not rescued normal radial pattern. Both of them contain only two ground tissue layers. However, 35S hypocotyls have a normal radial pattern with all three ground tissue layers present. The amyloplast sedimentation analysis of 35S, st1 and D7 demonstrated that presence and location of amyloplasts in 35S is similar to WS while st1 and D7 resemble *scr1*. These results support our hypothesis that normal radial pattern is not essential for normal gravitropic responses and that in the hypocotyl there is at least one amyloplast independent pathway for gravity sensing.

Keyword: Transgenic lines, 35S promoter, overexpression, hypocotyl gravitropism.

Introduction

SCARECROW (*SCR*), a putative transcriptional factor, is essential for the radial patterning of the root, development of endodermis of the shoot, normal shoot gravitropism and indeterminate root growth in *Arabidopsis thaliana* (Wysocka-Diller et al. 2000; Sabatini et al. 2003). It was reported that *scr* (*scarecrow* mutants) of *Arabidopsis thaliana* exhibited a determinate mode of root growth, shoot agravitropism and abnormal internal architecture in all organs examined (Sabatini et al. 2003; Fukaki et al. 1998; Wysocka-Diller et al. 2000).The *scr* mutants display defective organization of root cap and quiescent center (QC) resulting in determinate root growth. Mutant roots fail to undergo a formative cell division that is responsible for normal endodermis and cortex development from the cortex/endodermis (C/E) initials, resulting in abnormal radial pattern (Di Laurenzio et al. 1996). Both hypocotyls and inflorescence stems also have a defective radial pattern in *scr* mutant plants (Fukaki et al. 1998; Wysocka-Diller et al. 2000). It seems that one ground tissue layer is also missing in stems but it is not as clear (especially in the inflorescence stem) because in that organ a variable number of cortical layers may be present (Wysocka-Diller et al. 2000). Consistent with abnormal radial pattern and defective root growth of *scr* mutants in WS plants, *SCR* expression was detected in the endodermal cells, E/C initials and QC of roots (Di Laurenzio et al. 1996; Wysocka-Diller et al. 2000). Similarly to its expression in the endodermis of the root, *SCR* is expressed in the endodermal layer of the inflorescence stem and the hypocotyl of wild type plants (Wysocka-Diller et al. 2000). Furthermore, it was demonstrated that SCR protein is essential for repression of initial differentiation in the QC (Sabatini et al. 2003). It was reported that when *SCR* is expressed only in the QC the roots grow better than *scr*, but that QC expression was not able to restore normal radial patterning of the root (Sabatini et al. 2003). However, expression of *SCR* in C/E initial cells alone rescued normal radial pattern. The root

growth of these plants is faster than *scr* mutant but below the level of wild type. Expression of *SCR* in both the domains, QC and C/E initials, was able to restore both indeterminate root growth and normal radial pattern phenotype (Sabatini et al. 2003). These results suggest that change in the domain of *SCR* expression can alter the phenotype of plants. Therefore, to identify the location of the *SCR* expression pattern driven by 35S promoter in *Arabidopsis thaliana* and to examine the broader effects of the *SCR* gene missexpression in the *scr* mutant and WS plants, a transgenic approach was used. Hypocotyls of two lines *35S::GFP::SCR/scr1*(st1) and *35S::SCR*/*scr1*(D7) have regained the negative gravitropic response. However they still have an abnormal radial pattern and do not exhibit sedimentation of amyloplasts. While in one *35S*::*SCR*/WS (35S) line plant hypocotyls showed complete agravitropism, they still have a normal radial pattern and exhibit amyloplast sedimentation in the endodermal layer. Therefore, hypocotyl gravitropic responses of these transgenic lines do not correlate with a normal radial pattern and amyloplast sedimentation.

Material and Methods

Plant materials and growth conditions

Individual of *Arabidopsis thaliana*, Wassilewskija (WS) was used as wild type plant. The *scr1* is on WS ecotype background (Di Laurenzio et al. 1996). Already available transgenic *Arabidopsis thaliana* lines *35S::SCR/WS* (35S) and *35S::SCR/scr1* (D7) were used as overexpression *SCR* lines on WS and *scr1* backgrounds respectively. For phenotypic characterization transgenic lines, seeds were sterilized and grown on Murashige and Skoog (MS) agar plates as described by Fukaki et al. (1996b).

Overexpression plasmid construction and transformation to develop new transgenic lines

An entry vector G10761 containing cDNA of the *SCR* gene was purchased from Arabidopsis web site (TAIR, www.arabidopsis.org). That entry vector was used to transfer the *SCR* gene into pMDC44 via the LR reaction (Gateway^{R} Entry vector, Life Technologies) using the manufacturer's protocol. Recombinant pMDC44 plasmids containing *35S::GFP::SCR* insert were used for transformation into *Agrobacterium tumefaciens* LBA4404. *Arabidopsis thaliana* plants both WS (wild type) and *scr1* mutants were transformed via the floral dip method (Clough and Bent 1998). Seeds were collected from these plants and plated for selection on hygromycin-containing MS agar medium. Hygromycin selection marker was used to develop the homozygous transgenic lines. After four generations homozygous lines of *35S::GFP::SCR/WS* (Wt lines) and *35S::GFP::SCR/scr1* (st lines) were developed. PCR analysis of the transgenic lines and segregation analyses based on the presence or absence of the hygromycin selection marker were used to determine the homozygosity of transgenic plants.

Transgenic lines' genotypic background confirmation

WS, *scr1*, 35S, D7, Wt and st line seeds were placed on MS agar plates as described by Fukaki et al. (1996b). DNA was extracted from WS, *scr1*, 35S, D7, Wt and st lines as described by Edwards et al. (1991). The respective DNAs were amplified by using SCRF836 and SCRR1870 primers. The Polymerase Chain Reaction (PCR) contained the following reaction mixture: 0.2mM of each primer, 1.5-3.0 mM $MgCl_2$, 0.5U Taq polymerase (Promega), 0.2mM each dNTP (Promega) and ~100 ng of DNA template. Following PCR cycle was used: denaturation at 94^0C for 1 minute followed by primer annealing of 54^0C for 1 minute, followed by extension at 72^0C for 1 minute and 30 seconds for 10 cycles. After first 10 cycles the entire program was repeated for 24 cycles

with denaturation time (30 seconds) at 94^0C, primer annealing at 54^0C for 30 seconds, extension at 72^0C for 1 minute and 30 seconds and final elongation at 72^0C for 4 minutes.

Root growth analysis of newly developed transgenic lines

To test the root growth rate and length of both type of newly developed transgenic lines (Wt and st lines) and to compare them with both WS and *scr*1 plants respectively, Wt and st lines were plated with WS and *scr1* side by side. To do this thirty five seeds of each plant type were sterilized and placed on MS agar plates containing 4.5% sucrose. After 3 days in the dark at 4^0 C plates were transferred to the growth chamber under long day conditions (16 hours) for germination and growth. Root growth was monitored by marking the positions of the growing root tips at 5 day intervals starting 5 days after germination (DAG) until 20 DAG. Root lengths were measured in mm and their average values and standard deviation values were calculated.

Root growth analysis of selected transgenic lines

As described earlier the similar experimental conditions were used and root growth of Wt2, st1 (selected lines of Wt and st respectively), D7, and 35S with both WS and *scr1* was analyzed and compared.

Quantitative analysis of hypocotyl gravitropic responses of all different transgenic lines

Quantitative analysis of gravitropic response of already available transgenic lines (35S and D7) and selected lines of Wt and st (Wt2 and st1) was performed. To do this thirty five seeds of each plant type were sterilized and placed on MS agar plates containing 4.5% sucrose. Photographs of all plates were taken (0 hour photographs) and then they were reoriented at 90^0 angle. Photographs

were taken at three different time intervals (12hr, 24hr, and 48hr) within 48 hours of reorientation. These photographic images (Kodak Image Station 440CF) were used to compare the gravitropic responses. "Imagej 1.48v" software was used to calculate curvature as the increment over the initial angle of each individual hypocotyl. Curvatures' average and SD values were calculated.

Inflorescence gravitropism

Inflorescence stem gravitropic responses of transgenic plants were analyzed. To do that the 35S, Wt2, D7 and st1 seedlings were transferred into pots from the plate and an experiment was performed on plants with inflorescence stems that were 6 to 8 cm in length as described by Fukaki et al. (1996a). Pots containing WS, *scr*1 and each transgenic plant were placed in the dark on horizontally. This was marked as the beginning of the experiment and was referred to as time 0 hour. Inflorescence gravitropic responses was measured after 48 hours.

Reverse-transcriptase PCR to confirm the over-expression of SCR in transgenic lines

To confirm the overexpression of SCR transcripts in transgenic lines RNA was extracted from the hypocotyls (3 days in dark after germination), roots (20 days after germination), and inflorescences (35 days after germination) of WS, *scr1*, 35S, Wt2, D7 and st1 plants according to the RNeasy Plant Mini Kit (Qiagen, USA). cDNAs were synthesized with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen^{TM}). The first strand of cDNAs was diluted finally to a concentration 100ng/µl before it was used in the reverse transcriptase PCR (RT-PCR) reaction. *SCR* gene specific primers were used to perform RT-PCR (Table 4.1). The RT-PCR Reaction had 0.2mM of each primer, 1.5-3.0 mM $MgCl_2$, 0.5U Taq polymerase (Promega), 0.2mM each dNTP (Promega) and ~100ng of DNA template. Following PCR cycle was used for amplification: denaturation at 94^0C

for 1 minute followed by primer annealing temperature of 54^0C for 1 minute, followed by extension at 72^0C for 1 minute and 30 seconds for 10 cycles. After first 10 cycles the entire program was repeated for 24 cycles with denaturation time (30 seconds) at 94^0C, primer annealing at 54^0C for 30 seconds, extension at 72^0C for 1 minute and 30 seconds and final elongation at 72^0C for 4 minutes.

Hypocotyl length of transgenic lines

The hypocotyl length of transgenic seedlings grown in the dark was analyzed and compare with WS and *scr1*. To do so, seeds of 35S, Wt2, D7, st1, WS and *scr1* were plated on MS agar plates containing 4.5% sucrose. These plates were kept on 4^0 C for 3 days to synchronize seed germination. After 3 days plates were transferred to growth chamber (16 hour daylight) for germination. Plates with germinating seedlings were covered with aluminum foil and left in a vertical position for 7 days. Hypocotyl lengths of seedlings grown in the dark for 7 days were measured using "Imagej 1.48v" software.

Presence and position of amyloplasts in hypocotyls of transgenic plants

To identify the presence and position of amyloplasts in transgenic lines, whole-mount amyloplast staining was performed. In order to do this, seeds of 35S, Wt2, D7 and st1 were sterilized and placed on MS agar plate with 1% sucrose. To induce the germination, plates were kept in growth room (16 hour daylight). Plates with germinated seedlings were kept in the dark for 24 hrs. to enhance hypocotyl elongation. Only those seedlings which were growing in an upright direction were fixed in FAA solution for 24 hrs. at 4^0C. 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 gr. chloral hydrate, 2 ml water, and 1 ml glycerol) (Fukaki et al. 1998). Slides with mounted seedlings were examined and pictures were taken using of Nikon Biophot microscope with a Nikon D-70 digital camera attached.

Radial pattern analysis of transgenic hypocotyls

For cross sections, hypocotyl fragments of 35S, Wt2, D7 and st1 were embedded in plastic media as follows. Seedlings grown in dark for 3 days after germination were cut and hypocotyl fragments were selected for further procedure. Samples were fixed at 4^0C 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 minutes in each step with following alcohol concentrations: 10%, 30%, 50%, 75%, 95% and 95%. A JB-4 embedding kit (Sigma-Aldrich) was used for infiltration and embedding. Dehydrated samples were infiltrated and embedded in plastic embedding medium. Finally, the embedded tissues were mounted and sectioned using a microtome. Cross sections of 2-3 µm were heat fixed to slides, stained with toluidine blue O (TBO). Cross sections were examined and pictures were taken using Nikon Biophot microscope with a Nikon D-70 digital camera attached.

SCR::GFP expression analysis in Wt2 and st1 lines

Wt2 (*35S::GFP::SCR/WS*) and st1 (*35S::GFP::SCR/scr1*) lines have inserts fused with GFP markers at the N-terminal of the *SCR* gene. Over-expression of SCR was analyzed in hypocotyl and root of Wt2 and st1 lines. For hypocotyl analysis seedlings grown in the dark for 3 days after germination and roots of 20 day old seedlings were used and examined under a Nikon Eclipse 801

epifluorescence microscope. A GFP filter was used to block chlorophyll autofluorescence and a UV filter was used to detect fluorescence under UV light. All photographs were taken with a Qimaging Fast 1394 digital camera (Imaging).

Results

Selection of transgenic lines transformed with *35S::GFP::SCR* construct

Arabidopsis thaliana plants both WS (wild type) and *scr1* mutants were transformed with *35S::GFP::SCR* construct. Seeds were collected from these plants and for selection plated on hygromycin-containing MS agar medium. Hygromycin selection marker was used to develop the homozygous transgenic lines. After four generations eight homozygous lines of *35S::GFP::SCR/*WS (Wild type transgenic or Wt lines) and five *35S::GFP::SCR/scr1* (*scr1* transgenic or st lines) were selected.

Transgenic lines genotypic background confirmation

The genetic background of eight Wt, five st lines, 35S and D7 was evaluated. The presence and/or absence of an uninterrupted *SCR* gene and cDNA of *SCR* in the genomes of transgenic lines was determined by a PCR test. The *SCR* gene has an 114bp long intron in the coding region. To confirm that 35S and Wt lines carry both the gene and the cDNA of the *SCR* while D7 and st lines carry only the transgene cDNA of the *SCR*, *SCR* gene specific primers were designed (Table 4.1). The primers are designed in such a way that *SCR* forward and reverse are positioned on either side of the intron (Fig. 4.1 A). Genomic DNA was extracted from all of the transgenic lines, WS and *scr1* and PCR amplification analysis was performed. WS DNA gives a single band of 1035bp but *scr1* DNA failed to produce any band because of the T-DNA insert in *SCR* coding region (Fig. 4.1 B

and Fig. 4.2 B). Double bands of 1035bp and 921bp were produced in the Wt lines and 35S DNA with two *SCR* primers (Fig. 4.2 A). The st lines and D7 DNA gives a single 921bp band (Fig. 4.2 B). These results confirm the presence of both endogenous and transgene (cDNA) *SCR* in 35S and Wt lines and the presence of only functional transgene (cDNA) *SCR* in D7 and st lines.

Root growth analysis of newly developed transgenic lines

Roots of *Arabidopsis thaliana* grow continuously throughout their life thus exhibiting indeterminate growth. However, *scr1* plants shows determinate root growth, thus their roots stop growing at early stage of seedling development and exhibit a short root phenotype (Sabatini et al. 2003). In order to test if st lines able to rescue indeterminate root growth phenotype or had any effect on root growth of Wt lines, all the lines were tested for the root growth phenotype. As expected, Wt lines showed indeterminate root growth but the rate of root growth was faster than WS. Three strongest Wt lines (Wt1, Wt2 and Wt3) are shown in Table 4.2 A and Fig. 4.3 A. On the other hand all five st lines showed root length above the level of *scr1* but below WS (Table 4.2 B and Fig. 4.3 B). For further phenotypic characterization one Wt line: Wt2 and one st line: st1 were selected.

Root growth analysis of selected transgenic lines

Root length of 35S, D7, St1 and Wt2 with WS and *scr1*, was compared and results showed that Wt2 exhibited the fastest root growth, *scr1* showed the slowest root growth while the root growth of other three (D7, st1 and 35S) was intermediate (Fig 4.5). Five days after germination WS, Wt2, 35S, D7, st1 and *scr1* showed 11.4mm, 13.2mm, 4.1mm, 3.57mm, 6.8mm and 2.6mm average root length respectively (Table 4.3). Final average root lengths of WS, Wt2, 35S, D7, st1 and *scr1*

after 20 days of germination were 62.9mm, 100mm, 19.5mm, 24.8, 36 and 6.7mm respectively (Table 4.3).

Quantitative analysis of hypocotyl gravitropic responses of selected transgenic lines

The degrees and the time courses of gravitropic responses of Wt2, 35S, st1, D7, *scr1* and WS were determined and compared (Table 4.4 and Fig. 4.6). For quantification of hypocotyl gravitropic responses 35 seedlings of Wt2, 35S, st1, D7, *scr1* and WS were used. The average values with standard deviations of hypocotyl gravitropic responses were calculated in degrees (Table 4.4 and Fig. 4.7). WS, Wt2, st1, D7 and *scr1* hypocotyls showed negative gravitropic responses by reorientation of growth by 39.8, 37.8, 32.6, 36 and 9 degrees respectively after 12 hours of reorientation (Table 4.4 and Fig. 4.7). WS, Wt2, st1 and D7 hypocotyls exhibited the final angle of deflection in the range of 53-59 degrees after 48 hours of reorientation to a new gravity vector (Table 4.4 and Fig. 4.7). However, *scr1* hypocotyls showed only 16 degrees of deflection after 48 hours. These results indicated that no significant difference between hypocotyl responses of WS and Wt2 was observed at all three time points. However, st1 and D7 have rescued *scr1* hypocotyl gravitropic responses and their responses were very similar to WS at all three time points (Table 4.4 and Fig. 4.7). Surprisingly, hypocotyls of 35S exhibited no gravitropic response even after 48 hours of reorientation to new gravity vector (Fig 4.6).

Inflorescence gravitropic responses of transgenic lines

Inflorescences of *Arabidopsis thaliana* exhibit negative gravitropism. To compare the inflorescence gravitropic responses of WS, *scr1* Wt2, st1, D7 and 35S, plants with 6-8 cm long inflorescence stems were selected and placed in the dark in a horizontal orientation (Fig. 4.8).

Within the first three hours of reorientation WS inflorescence stems bend 90 degrees upward. As reported earlier, inflorescence stems of *scr1* mutants showed complete inflorescence agravitropism (Fig. 4.8). Wt2, D7 and st1, all three of them showed negative hypocotyl gravitropic responses and their inflorescence stem bend 90 degrees upward. Interestingly, inflorescences of 35S plant were completely agravitropic.

Reverse-transcriptase PCR to confirm the over-expression of *SCR* in transgenic lines

RT-PCR was performed to confirm and compare the expression of *SCR* in WS, *scr1*, D7, 35S, Wt2 and st1 lines. No SCR transcripts were detected in hypocotyls, roots, and inflorescences of *scr1* mutant plants (Fig. 4.9 A, B and C). *SCR* expression was detected in all three organs of WS, D7, 35S, Wt2 and st1 plants (Fig. 4.9 A, B and C). *SCR* expression was strongest in Wt2 and st1 in all three organs. However, D7 and 35S showed the *SCR* expression stronger than WS but below the level of Wt2 and st1 (Fig. 4.9 A, B and C).

Hypocotyl length of transgenic seedlings

Hypocotyl lengths of Wt2, st1, D7, 35S, WS and *scr1* were analyzed. Hypocotyl lengths were measured on the 7^{th} day after germination in dark (Table 4.5 and Fig. 4.10 C). Both st1 and D7 showed similar hypocotyl length as *scr1* mutant. No significant difference between hypocotyl lengths of *scr1*, st1 and D7 was observed on after germination 7 day in the dark (Table 4.5 and Fig. 4.10 B). Surprisingly hypocotyls of Wt2 and 35S were also similar to *scr1* and significantly shorter than WS hypocotyls (Table 4.5 and Fig. 4.10 A).

Presence and position of amyloplasts in transgenic hypocotyls

Whole-mount amyloplast staining was performed to analyze the presence and position of amyloplasts in hypocotyls of Wt2, st1, D7 and 35S (Fukaki et al. 1998). As expected Wt2 showed the presence and position of amyloplasts similar to WS, the amyloplasts were clumped at the bottom of cells near the vascular system (Fig. 4.11). However, both D7 and st1 resemble *scr1* for the presence and the position of amyloplasts rather than the WS plants (Fig. 4.11). Similarly *scr1* amyloplasts were scattered between cell layers and within cells in both D7 and st1 hypocotyls (Fig. 4.11). The presence and position of amyloplasts in D7, st1 and *scr1* were indistinguishable (Fig. 4.11). Most interestingly 35S exhibited presence and position of amyloplasts very similar to WS. Clumps of amyloplasts were located at the bottom of the cells present adjacent to the vascular system only (Fig. 4.11).

Radial Pattern analysis of transgenic lines

To determine the radial pattern of Wt2, st1, D7 and 35S cross sections of their hypocotyls were generated and observed under the light microscope (Fig. 4.12). The radial pattern of both Wt2 and 35S was similar to WS. Both 35S and Wt2 possess the normal radial pattern with three ground tissue layers (Fig. 4.12). On the other hand both D7 and st1 exhibited an abnormal radial pattern similar to *scr1*. One ground tissue layer is still missing in both D7 and st1 (Fig. 4.12).

***GFP::SCR* expression analysis in Wt2 and st1 lines**

In WS plants *SCR* expression was detected in the endodermal cells, C/E initials and the QC of roots and endodermal layer of hypocotyls (Di Laurenzio et al. 1996; Wysocka-Diller et al. 2000). The transgene expression (*35S::GFP::SCR)* was detected predominantly in vascular tissues of

both Wt2 and st1 hypocotyls (Fig. 4.13 A and B). In Wt2 roots *35S::GFP::SCR* expression was mostly detected in vascular tissues and root tip (Fig. 4.13.A). However, in st2 roots *35S::GFP::SCR* expression was detected in almost the entire root (Fig. 4.13 B).

Discussion

To supplement the suppressor work in SCR-regulated function in gravitropism, I have also analyzed transgenic lines with different domains of *SCR* expression that show different levels of gravitropism. One transgenic *Arabidopsis thaliana* line *35S::SCR/*WS (overexpression of *SCR* gene on WS background) named as 35S and other line *35S::SCR/scr1*(overexpression of *SCR* gene on *scr1* mutant background) named as D7 were already available in my lab. First, 35S lines were developed by transforming WS plants with *SCR* cDNA driven by a 35S promoter. 35S plants were crossed with *scr1* mutants and in F3 generation homozygous lines were selected and named as D7. Therefore, both transgenic lines, 35S and D7 have the same insert. However, the insert 35S::SCR does not contain any visible marker, thus location of overexpression of *SCR* cannot be determined in 35S and D7 lines. Therefore, to determine the location of the overexpression/missexpression of the *SCR* gene in transgenic plants, new transgenic lines of WS plant and *scr1* mutant carrying a *35S::SCR* construct tagged with GFP reporter were generated by using a Gateway cloning kit and *Agrobacterium tumefaciens* mediated gene transfer method. Eight independent homozygous *35S::GFP::SCR/*WS Wt lines and five independent homozygous *35S::GFP::SCR/scr1* st lines were developed. Root growth of both Wt lines and st lines was analyzed. All eight Wt lines showed indeterminate root growth and their root growth was faster than WS plants. Fastest root growth was observed in the Wt2 line, and it was selected for further genetic and phenotypic analysis (Table 4.2 A and Fig. 4.4 A). All eight Wt lines exhibited hypocotyl and inflorescence gravitropic

responses (data not shown). All five st lines showed improved root length compared to *scr1* mutants but below the level of WS plants (Table 4.2 B and Fig. 4.4 B). Three lines st1, st2 and st3 showed significant improvement in root length (Fig. 4.3 A). All five st lines showed negative hypocotyl gravitropic response (data not shown). However, only st1 line showed inflorescence gravitropism, other four lines (st2, st3, st4 and st5) failed to show inflorescence gravitropic responses (Fig. 4.8 A and 4.8 B). Therefore, st1 with improved root growth, hypocotyl and inflorescence gravitropism was selected for further studies.

Root growth of already available lines 35S and D7 and newly developed lines Wt2 and st1 was compared with WS and *scr1*. Results showed that both D7 and st1 have similar root lengths at all four different time point used, starting 5 days after germination (DAG) (Table 4.3). Both lines show improved root length over *scr1* but fail to restore the WT root phenotype. However, Wt2 and 35S exhibited very different root phenotypes from each other. Wt2 showed faster root growth than WS at all four time points but 35S showed retarded root growth at all four time points (Table 4.3). Root growth rate of 35S plants was in the ranges of D7 and st1, faster than *scr1* but below the level of WS (Table 4.3). These data indicate that change in the level and/or domain of *SCR* expression can affect several aspects of root growth. The indeterminate root growth is not rescued in either D7 or st1 lines, which suggests that probably the 35S promoter does not drive the expression of *SCR* to QC. Although the exact reason for the retarded root phenotype of 35S plants is not known, it is clear that the amount and location of *SCR* expression plays an important role in root growth. The SHORTROOT (SHR), also a GRAS family transcription factor, along with SCR, plays a key role in radial patterning, root meristem maintenance and endodermal differentiation in *Arabidopsis* (Fukaki et al. 1998; Wysocka-Diller et al. 2000; Nakajima et al. 2001; Mylona et al. 2002; Sabatini et al. 2003; Heidstra et al. 2004; Gallagher et al. 2004; Paquette and Benfey 2005).

It was reported that *SHR* that is expressed within the stele moves outward into adjacent ground tissue where it is required for QC and endodermis specification (Helariutta et al. 2000; Nakajima et al. 2001; Sabatini et al. 2003). Expression of *SCR* is restricted to the ground tissue layer and is possibly controlled by SHR that occurs directly upstream of *SCR* in the root development pathway (Nakajima et al. 2001). SHR that moves into endodermal cells interacts directly with SCR and forms a complex in the nucleus that prevents transmission of SHR into the cortical cell layer (Cui et al. 2007). We hypothesized that the reason behind the retarded root growth of 35S plants can be the overexpression of *SCR* in the stele. SCR proteins may form a complex with SHR and prevent the movement of SHR from the stele into adjacent ground tissue where it is required for normal root development. However, it is not possible to localize the overexpression of *SCR* in 35S plants (not tagged with visible marker). Therefore Wt2 plants, tagged with GFP were analyzed to locate the *SCR* overexpression in roots. The results showed that *GFP::SCR* were overexpressed in stele and root tip of Wt2 roots (Fig. 4.13 A). But Wt2 plants exhibit faster root growth than WS (Table 4.3), which suggests that overexpression of *SCR* in the stele is not responsible for retarded root growth.

Quantitative analysis of hypocotyl gravitropic responses of WS, *scr1*, 35S, Wt2, st1 and D7 was performed. Two independent *SCR* overexpression lines driven by 35S promoter on *scr1* mutant background, st1 and D7 showed gravitropic responses similar to the level of WS at all three different time points: 12 hours, 24 hour and 48 hours (Table 4.4 and Fig. 4.7). Both D7 and st1 lines have clearly rescued their hypocotyl gravitropic responses. One of the new *SCR* overexpression WS background lines, Wt2, showed no significant difference in hypocotyl gravitropic response than WS (Table 4.4 and Fig. 4.7). However, interestingly hypocotyls of 35S, the other *SCR* overexpression line driven by 35S promoter on WS background, did not respond to

gravistimulation even after 48 hours of reorientation (Fig. 4.6 A). Along with hypocotyl gravitropic responses, inflorescence gravitropic responses of WS, *scr1*, Wt2, 35S, D7 and st1 were also assessed. It was observed that both D7 and st1 have rescued their inflorescence gravitropic responses (Fig. 4.8). Inflorescence stems of both D7 and st1 turned 90^0 upward after reorientation to a new gravity vector. Similarly to their hypocotyl gravitropic response Wt2 showed wild type inflorescence gravitropic response too. However, inflorescence stems of 35S plants (similar to their hypocotyls) were completely agravitropic (Fig. 4.8 A). These results suggested that, similar to root growth phenotype change in SCR, transcript amount and location can alter the shoot gravitropic response, too.

Reverse transcriptase (RT) PCR was carried out to access and compare the expression level of *SCR* in WS, *scr1*, Wt2, 35S, D7 and st1. RNAs were extracted from hypocotyl, root and inflorescence stem of WS, *scr1*, Wt2, 35S, D7 and st1. The cDNAs were synthesized and used for PCR with *SCR* specific forward and reverse primers. The cDNA of *scr1* mutants failed to amplify any product with *SCR* specific primers in all three organs (Fig. 4.9 A, B and C). These results confirm the absence of SCR transcript in hypocotyl, root and inflorescence stem of *scr1* mutant plants. Wt2 and 35S showed stronger *SCR* expression than WS in all three organs. However, *SCR* expression was stronger in Wt2 than 35S in all three organs (Fig. 4.9 A, B and C). Similarly D7 and st1 also showed *SCR* expression in all three organs and expression in st1 was stronger than D7. It is noticeable that there is no endogenous *SCR* expression in 35S and D7 (*scr1* mutant background) and only exogenous *SCR* expression is represented in RT-PCR (Fig. 4.9 A, B and C).

Results of hypocotyl length analysis of *scr* hypocotyl gravitropic suppressors (*shs*s) indicate that the hypocotyl length and thus the normal cell elongation is not an essential factor for gravitropic response. To analyze the effect of overexpression of *SCR* on length of hypocotyls,

hypocotyl length analysis was performed. The hypocotyl lengths of both D7 and st1 were very similar to *scr1* but significantly shorter than WS (Table 4.5 and Fig. 4.10 B). Therefore, D7 and st1 have normal hypocotyl gravitropic response but still possess shorter hypocotyl lengths.

More interestingly Wt2 with normal hypocotyl gravitropic response showed significantly shorter hypocotyl length than WS (Table 4.5), while the hypocotyl length of 35S (hypocotyl agravitropic) was shorter than *scr1* mutants (Table 4.5 and Fig. 4.10 A). These results clearly suggest and support our hypothesis that the hypocotyl length and thus normal cell elongation is not an essential factor for hypocotyl gravitropic responses. Furthermore, these data also suggest that hypocotyl length can also be altered by changing the level and location of *SCR* expression. Also, for wild type hypocotyl length phenotype, both optimum level and correct location of *SCR* expression is required.

Results of both presence and position of amyloplasts and radial pattern analysis of suppressors hypocotyls helped us to hypothesize that starch and the presence of normal statocyte cells and/or amyloplast sedimentation are not necessary for gravitropic sensing, and that hypocotyl gravitropic perception relies on at least two separate mechanisms. For the confirmation of this hypothesis, transgenic seedlings grown on 1% sucrose MS agar plates were stained with IKI solution. Results showed that the presence and position of amyloplasts in both D7 and st1 are similar to *scr1* rather than the WS (Fig. 4.11). However, the presence and position of amyloplasts in both Wt2 and 35S are similar to WS (Fig. 4.11). Therefore, these results revealed that amyloplast sedimentation in hypocotyls does not correlate with gravitropic response. Radial pattern analysis of hypocotyl of transgenic lines showed that both D7 and st1 have not been able to recover the normal radial pattern with three ground tissue layers (Fig. 4.12). D7 and st1 hypocotyls still contain only two ground tissue layers similar to *scr1* mutants. On the other hand, both Wt2 and 35S showed

the normal radial pattern similar to WS (Fig. 4.12). These results clearly support our hypothesis that normal radial pattern is neither essential nor sufficient for hypocotyl gravitropic response. Furthermore, sedimentation of amyloplasts cannot be the only mechanism of gravity sensing and hypocotyl gravitropic perception relies on at least two separate mechanisms.

In summary two independent transgenic lines carrying *35S::SCR* and *35S::GFP::SCR* on *scr1* background named as D7 and st1 respectively, and two independent transgenic lines with *35S::SCR* and *35S::GFP::SCR* on WS background named as 35S and Wt2 respectively were analyzed. D7 and st1 show normal gravitropic responses but no amyloplast sedimentation and still have abnormal radial pattern and short length hypocotyls. On the other hand, Wt2 is hypocotyl gravitropic, exhibits normal amyloplasts sedimentation and normal radial pattern. However, Wt2 also has a short length hypocotyl phenotype. Most interestingly, 35S is hypocotyl agravitropic but still exhibits normal amyloplast sedimentation and a normal radial pattern but severely retarded hypocotyl length. These results strongly suggest the presence of an amyloplast-independent gravity sensing pathway in the hypocotyl and also suggest that the level and/or domain of *SCR* expression can affect several aspects of root and shoot phenotype. As mentioned earlier D7 lines were developed by crossing 35S plants with *scr1* mutant plants therefore, 35S and D7 lines have the same insert. However, Wt2 and st1 are two independent lines. In future Wt2 plants will be crossed with *scr1* mutant plants and st1 plants will be crossed with WS plants. Characterization of *SCR* overexpression lines on WS and *scr1* backgrounds, developed by crossing st1 with WS and Wt2 with *scr1* respectively, will be the future lines of research.

Primer	Sequence (5'-3')	Tm
SCRF836	AGGCAGAAGCAAGACGAAG	57.5C
SCRR1870	CTTCACCGCTTCTCGATGGT	60.5

Table 4.1. *SCR* specific primers used in genotypic background confirmation and RT- PCR analysis of transgenic lines.

Sample	Time interval			
	5 DAG	**10 DAG**	**15 DAG**	**20 DAG**
WS	10.8 ± 3.4	27.3 ± 7.2	49.5 ± 7.4	61.4 ± 9.5
Wt1	12.4 ± 2.4	32.7 ± 5.2	62.7 ± 6.4	79.9 ± 11.0
Wt2	13 ± 2.3	36.9 ± 7.1	69.6 ± 9.8	96.5 ± 8.9
Wt3	14 ± 1.7	37.5 ± 3.9	65.1 ± 7.4	87.1 ± 7.2

Table 4.2 A. Root length analysis of Wt transgenic lines. Average root lengths of WS, Wt1, Wt2 and Wt3 measured at 5 day intervals starting 5 days after germination. Root lengths were measured in mm.

Sample	Time interval			
	5 DAG	**10 DAG**	**15 DAG**	**20 DAG**
scr1	1.7 ± 0.6	1.9 ± 0.5	5.0 ± 1.6	7.1 ± 0.5
st1	6.7 ± 1.6	17.8 ± 3.5	22.3 ± 3.3	36.1 ± 8.0
st2	6.7 ± 2.9	18.3 ± 6.1	23.6 ± 7.3	38.2 ± 2.7
st3	6.5 ± 3.2	14 ± 2.1	17.8 ± 3.5	33.3 ± 5.1
st4	4.3 ± 1.3	8.0 ± 2.1	10.1 ± 1.9	12.0 ± 3.0
st5	4.6 ± 0.5	8.2 ± 2.9	11.8 ± 3.5	14.5 ± 3.2

Table 4.2 B. Root length analysis of st transgenic lines. Average root lengths of *scr1*, st1, st2, st3, st4 and st5 measured at 5 day intervals starting 5 days after germination. Root lengths were measured in mm.

Sample	Time interval			
	5 DAG	**10 DAG**	**15 DAG**	**20 DAG**
WS	11.4 ± 3.3	28.8 ± 7.7	49.9 ± 6.8	62.9 ± 9.2
scr1	2.6 ± 1.4	2.8 ± 1.2	4.9 ± 1.3	6.7 ± 1.2
Wt2	13.2 ± 2.3	35.7 ± 6.9	71.4 ± 7.3	100.6 ± 7.1
35S	4.1 ± 2.2	10.2 ± 2.1	13.4 ± 3.3	19.5 ± 4.0
D7	3.5 ± 1.1	9.3 ± 2.1	14.4 ± 2.3	24.8 ± 5.7
st1	6.8 ± 1.6	16.5 ± 3.1	21.7 ± 4.8	36.0 ± 7.1

Table 4.3. Root length of selected transgenic lines. Average root lengths of WS, *scr1*, Wt2, 35S, D7 and st1 measured at 5 day intervals starting 5 days after germination. Root lengths were measured in mm.

Samples	Time interval		
	12 hour	**24 hours**	**48 hours**
WS	39.8 ± 11.7	48.7 ± 10.6	59.6 ± 12.3
scr1	9.0 ± 8.0	14.5 ± 10.4	16.7 ± 11.1
Wt2	37.8 ± 11.9	47.6 ± 8.7	59.6 ± 11.4
St1	32.6 ± 12.3	43.5 ± 13.9	53.6 ± 14.5
D7	36 ± 16.1	45.9 ± 16.9	54.8 ± 16.8
35S	0	0	0

Table 4.4. Quantitative analysis of hypocotyl gravitropic responses of WS, *scr1*, Wt2, st1, D7 and 35S. Angles of deflection were measured at three different time intervals 12 hours, 24 hours and 48 hours after reorientation.

Sample	Hypocotyl length of seedlings (in mm)
WS	16.7 ± 1.9
scr1	10.5 ± 1.7
Wt2	11.9 ± 1.6
st1	11.1 ± 2.9
D7	8.3 ± 2.0
35S	6.1 ± 1.5

Table 4.5. Hypocotyl length analysis of selected transgenic lines. Hypocotyl lengths of WS, *scr1*, Wt2, st1, D7 and 35S seedlings grown in dark for 7 days after germination. Hypocotyl lengths were measured in mm.

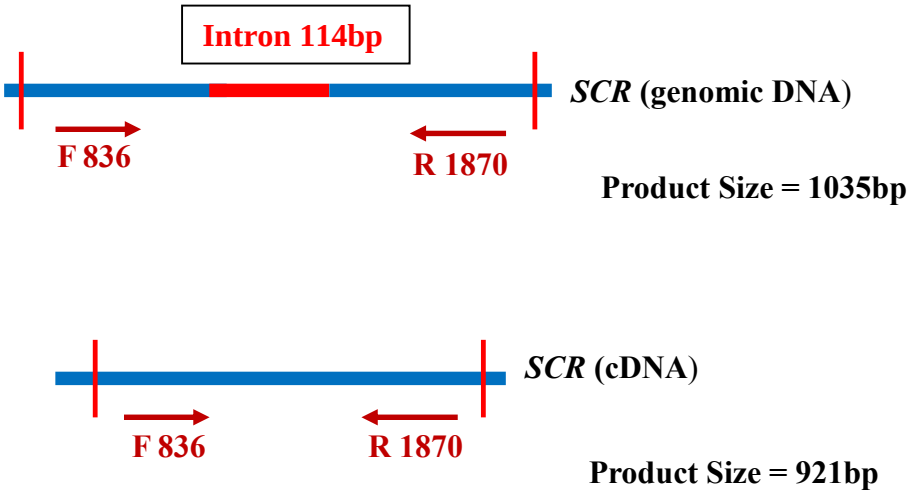


Figure 4.1 A. Schematic representation of *SCR* gene with 114 bp intron and cDNA of *SCR* gene. *SCR* specific forward and reverse primers are positioned before and after intron respectively. The orientations of primers are indicated by the arrows. Genomic DNA (with intron) amplifies a 1035bp product with SCR F836 and SCR R1870 primers. cDNA (without intron) amplifies a 921dp product with SCR F836 and SCR R1870 primers. Wt lines (*35S::GFP::SCR*/WS) and 35S (*35S::SCR*/WS) amplify two bands of 1035bp and 921bp corresponding to endogenous gene and cDNA transgene respectively. WS amplifies only one band of 1045bp corresponding to endogenous gene.

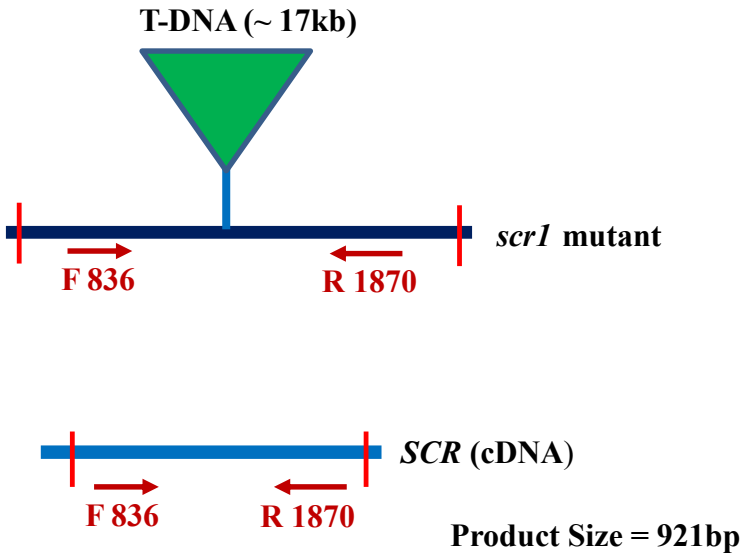


Figure 4.1 B. Schematic representation of *SCR* gene in *scr1* allele with T-DNA insertion and cDNA of *SCR* gene. *SCR* specific forward and reverse primers are positioned before and after T-DNA respectively. The orientations of primers are indicated by the arrows. *scr1* allele DNA (with T-DNA) fails to amplify any product with SCR F836 and SCR R1870 primers. cDNA (without intron) amplifies a 921dp product with SCR F836 and SCR R1870 primers. All st lines (*35S::GFP::SCR/scr1*) and D7 (*35S::SCR/scr1*) amplify a single band of 921bp corresponding to a cDNA transgene.

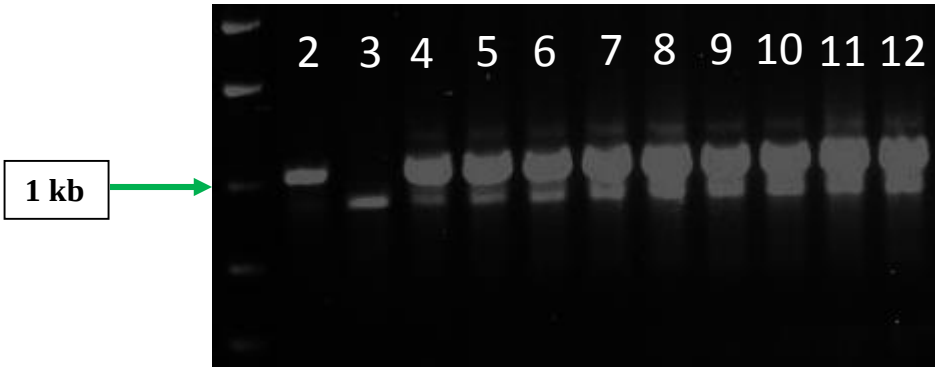


Figure 4.2 A. Genotype background confirmation of Wt lines.WS (only endogenous WT *SCR*), D7 (only transgenic cDNA of *SCR*), and Wt1 toWt8 (both endogenous WT and transgenic cDNA of *SCR*) were amplified with *SCR* gene specific primers (F' primer before the intron and R' primer after the intron). Lane 1. 1kb ladder, Lane 2. WS: 1 band of 1035 bp, Lane 3. D7: 1 band of 921 bp, Lane 4 to 12. Wt1 to Wt8: 2 bands of 1035 bp and 921 bp. Green arrow point at 1kb marker.

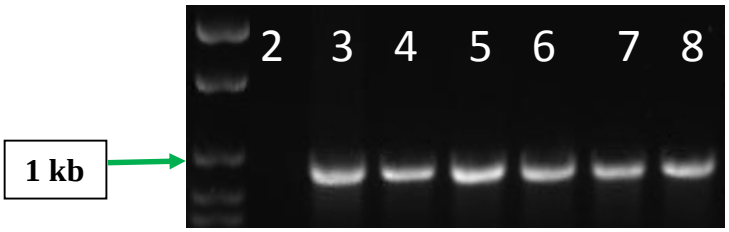
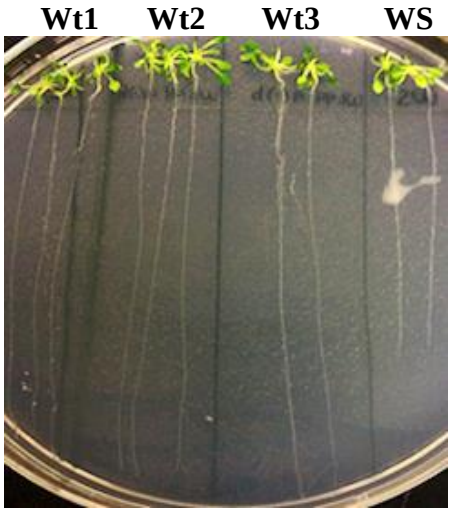


Figure 4.2 B. Genotypic background confirmation of st lines. *scr1* (T-DNA insertion), D7 (only transgenic cDNA of *SCR*), and st1-st5 (only transgenic cDNA of *SCR*) were amplified with *SCR* gene specific primers (F' primer before the intron and R' primer after the intron) Lane 1. 100 bp ladder Lane 2. *scr1* (no product) Lane 3. D7: 1 band of 921 bp Lane 4 to 8. st1 to st5 : 1 band of 921 bp. Green arrow point at 1kb marker band.

A.



B.

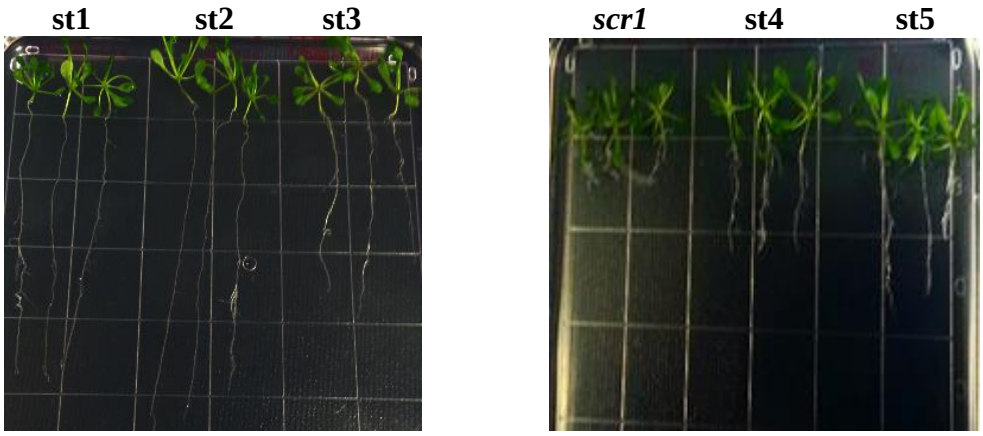
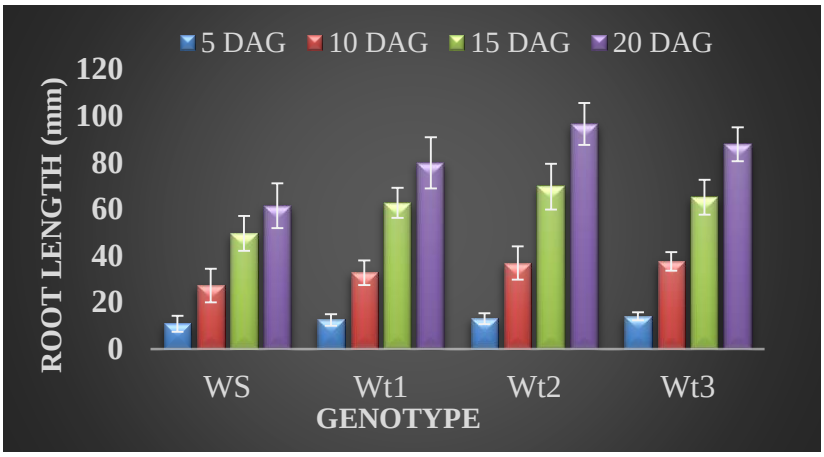


Figure 4.3. Root length analysis of Wt and st lines. A. Root length of Wt1, Wt2, Wt3 and WS 20 days after germination. **B.** Root length of st1, st2, st3, st4, st5 and *scr1* 20 days after germination.

A.



B.

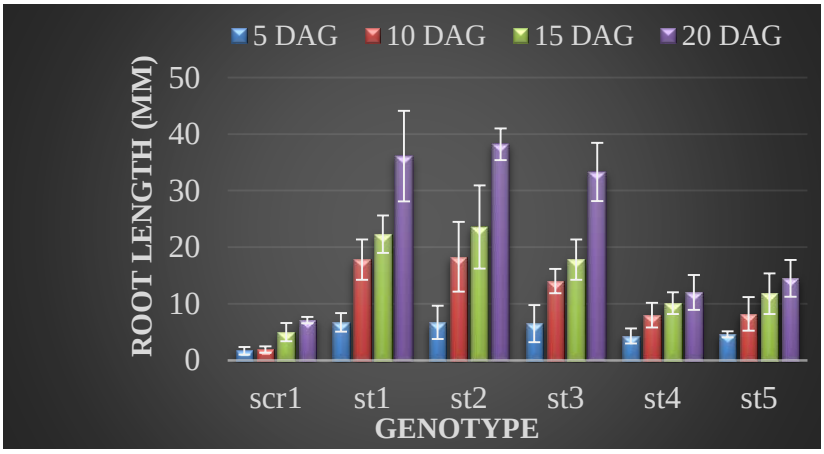


Figure 4.4. Root length analysis of Wt and st lines. A. Root growth of WS, Wt1, Wt2 and Wt3 20 days after germination, graphical representation of Table 4.2.A. **B.** Root growth of *scr1*, st1, st2, st3, st4 and st5 20 days after germination, graphical representation of Table 4.2.B. For each genotype 4 time points are indicated: 5, 10, 15 and 20 DAG.

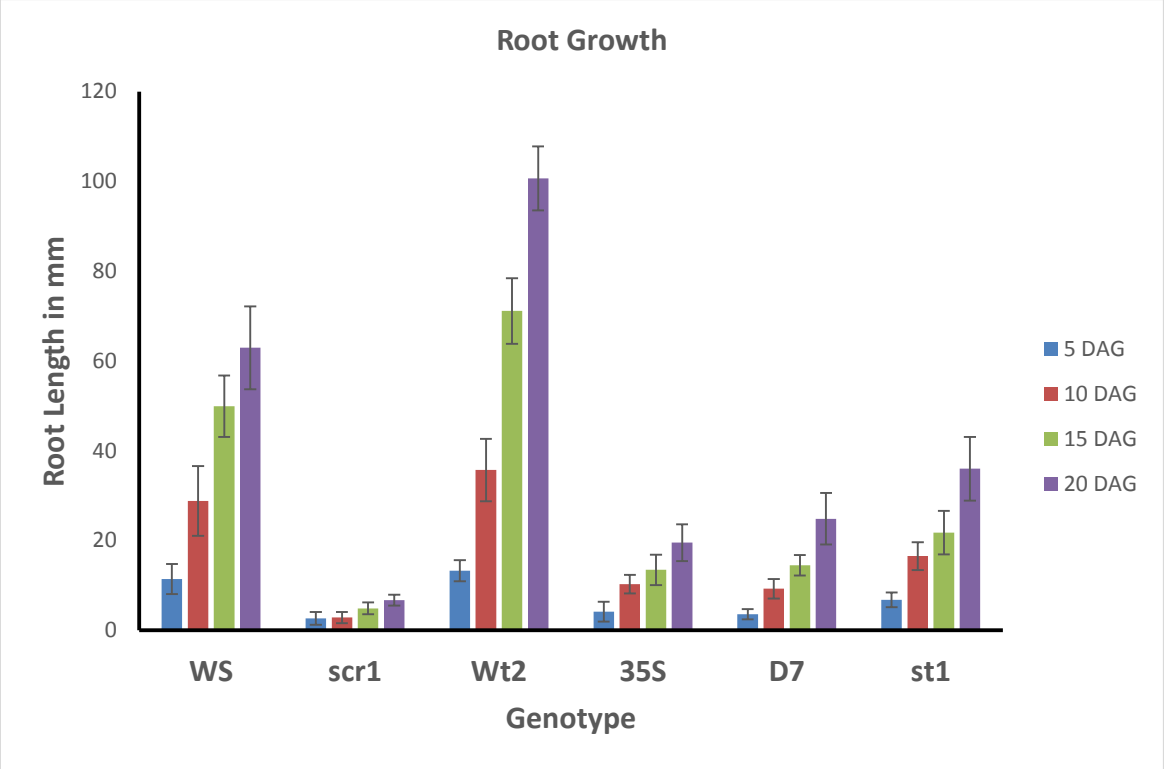


Figure 4.5. Root growth analysis of selected transgenic lines. Root growth of WS, *scr1*, Wt2, 35S, D7 and st1 20 days after germination, graphical representation of Table 4.3. For each genotype 4 time points are indicated: 5, 10, 15 and 20 DAG.

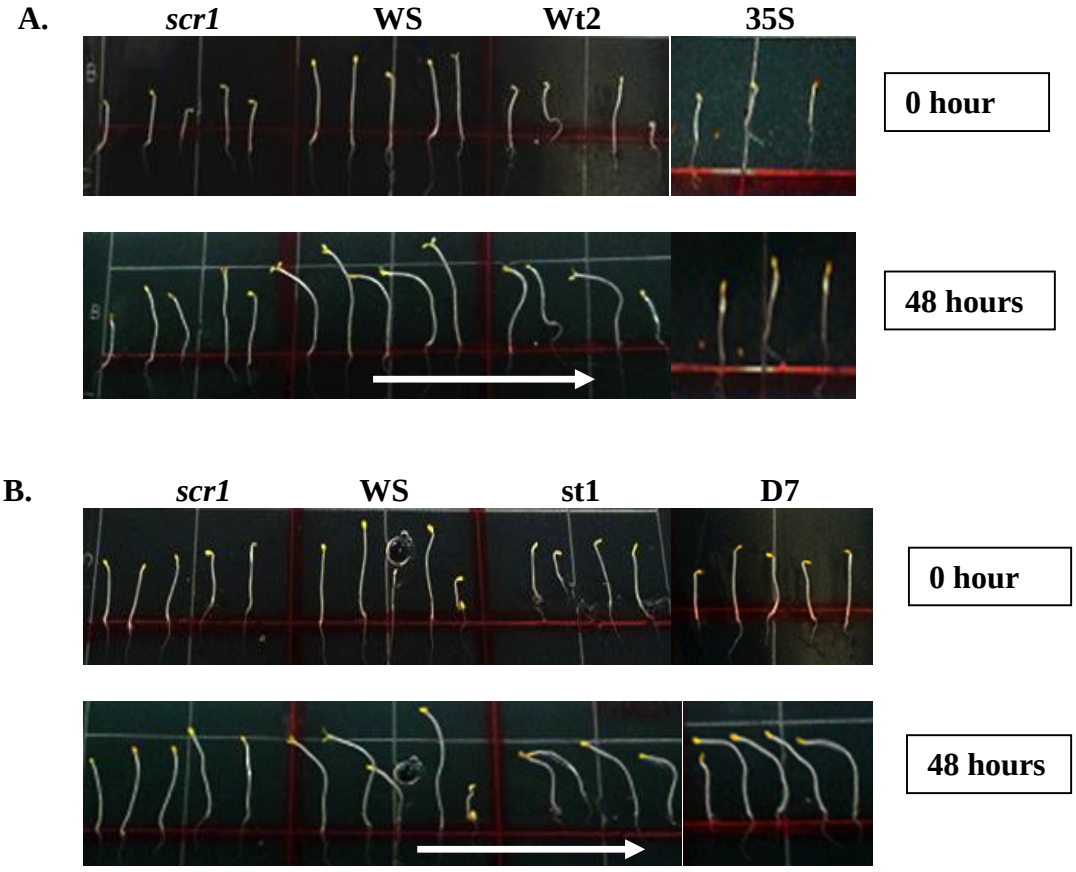


Figure 4.6. Hypocotyl gravitropic responses of seedlings after 48 hours of reorientation.

A. Hypocotyl gravitropic responses of the *scr1*, WS, Wt2 and 35S, 48 hours after plate reorientation in the dark**. B.** Hypocotyl gravitropic responses of the *scr1*, WS, st1 and D7, 48 hours after plate reorientation in the dark**.** Arrows indicate the direction of the new gravity vector. The same seedlings are shown before reorientation (0 hour) and 48 hours after reorientation.

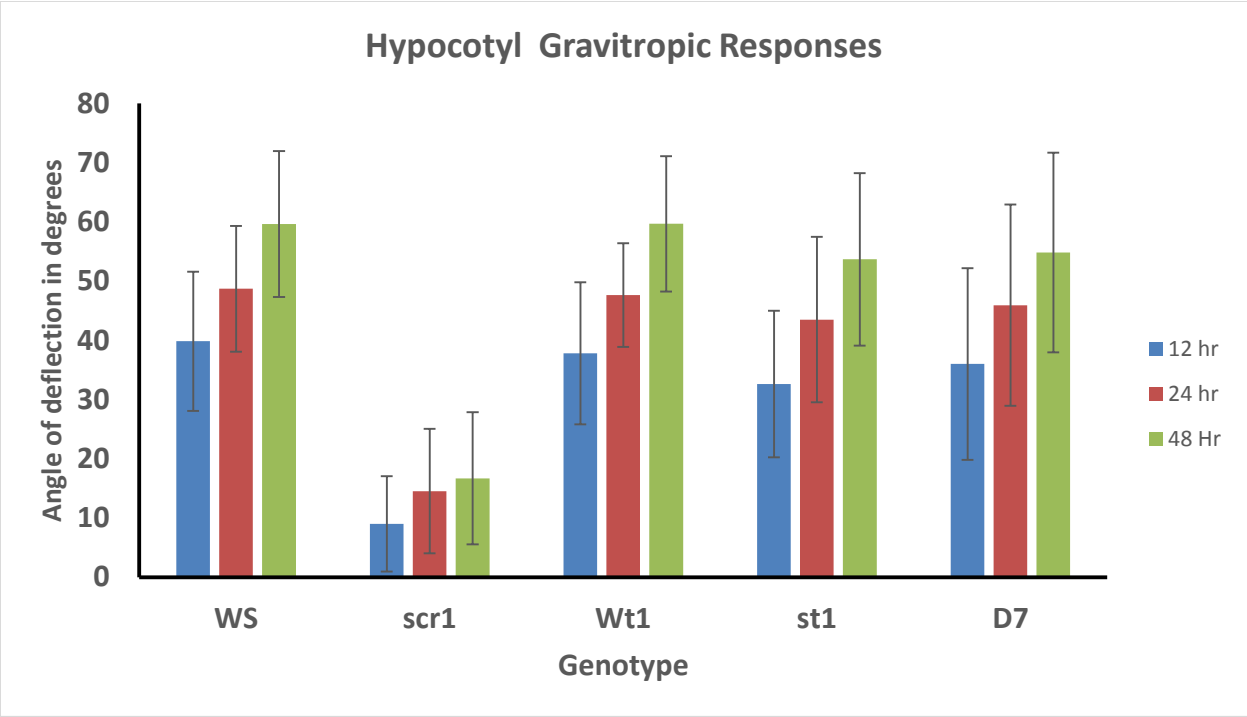


Figure 4.7. Graphical representation of hypocotyl gravitropic responses of transgenic lines. Gravitropic responses to new gravity vector of WS, *scr1*, Wt1, st1 and D7 were measured in degrees at three different time points, graphical representation of Table 4.4. For each genotype 3 time points are indicated: 12, 24 and 48 hours.

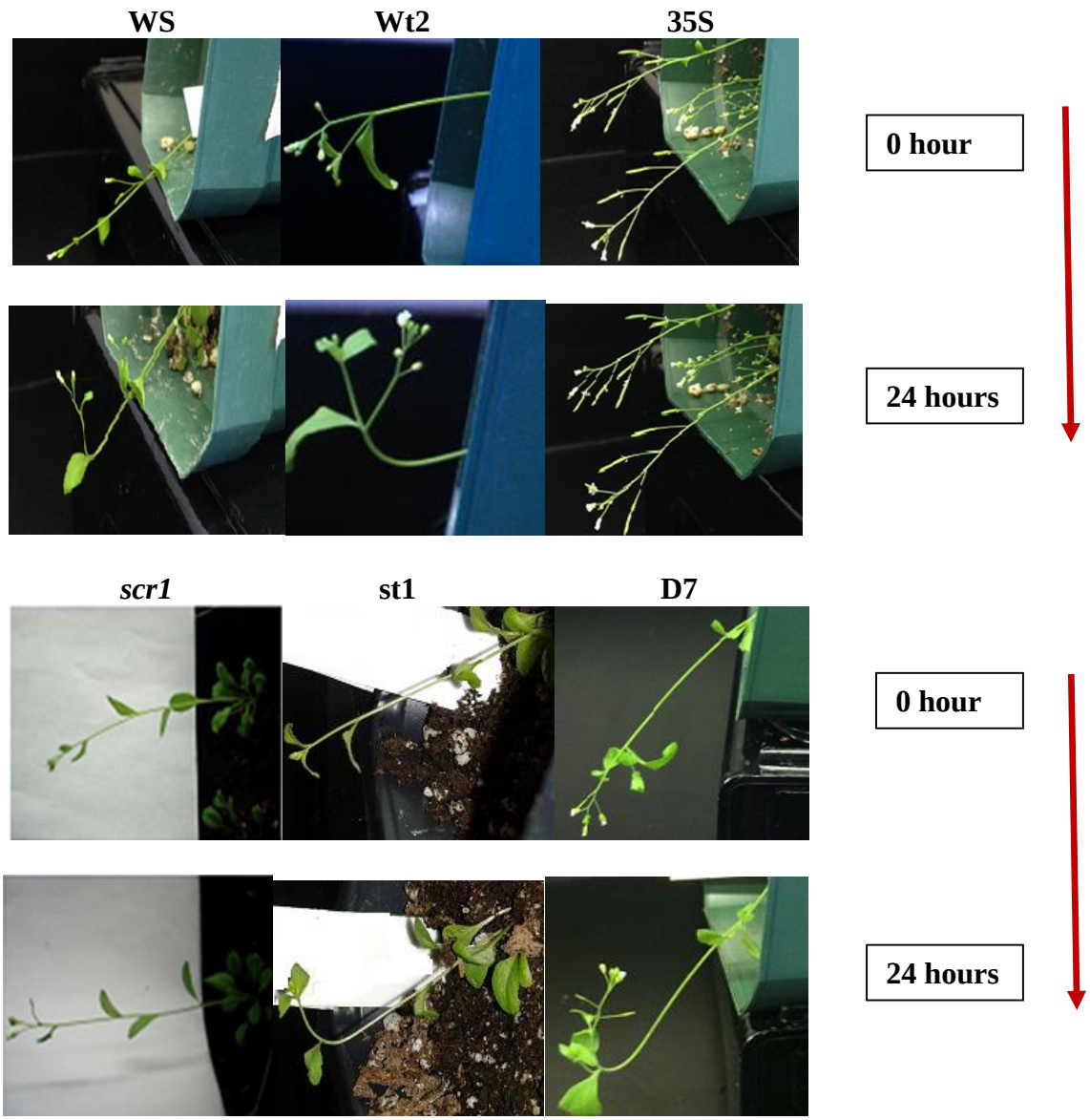


Figure 4.8 A. Inflorescence gravitropic responses of the transgenic lines to the new gravity vector. Inflorescence gravitropic response of the WS, Wt2, 35S, *scr1*, st1 and D7 24 hours after plants reorientation in the dark. Arrows indicate the direction of the gravity vector. The inflorescence stem of plants are shown before reorientation (0 hour) and 24 hours after reorientation.

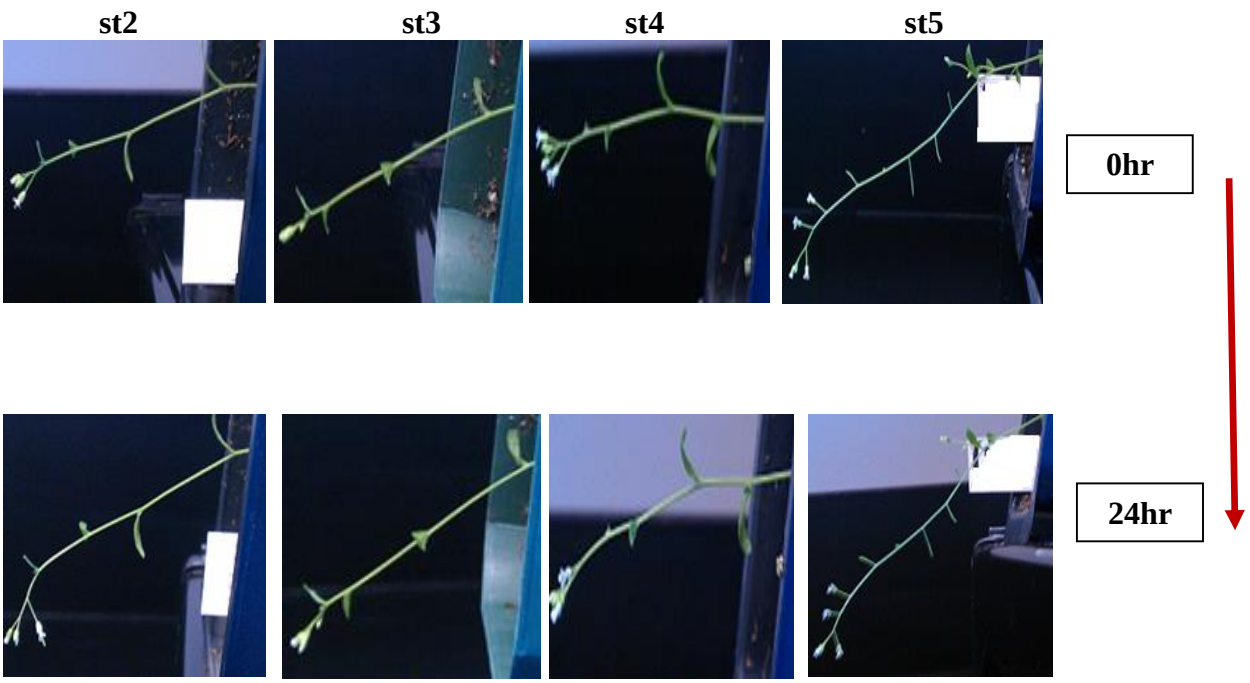
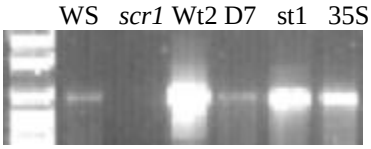
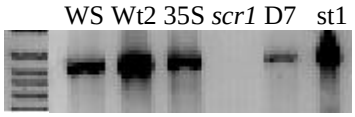


Figure 4.8 B. Inflorescence gravitropic response of the st transgenic lines to the new gravity vector. Inflorescence gravitropic response of the st2, st3, st4 and st5, 24 hours after plants reorientation in the dark. Arrows indicate the direction of the gravity vector. The inflorescence stem of plants are shown before reorientation (0 hour) and 24 hours after reorientation.

A.



B.



C.

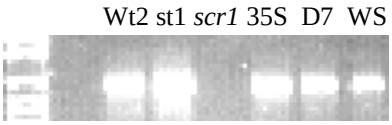
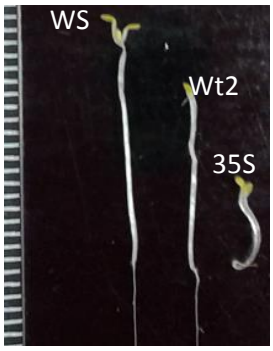
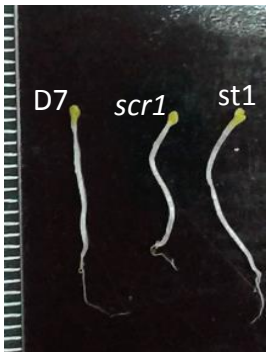


Figure 4. 9. RT-PCR analysis for *SCR* expression in WS, Wt2, *scr1*, D7, 35S and st1. A. RNAs were extracted from hypocotyls (3 days in dark after germination) of WS, *scr1*, Wt2, D7, st1 and 35S. cDNAs were synthesized from RNA and used for amplification with *SCR* specific F and R primers. **B.** RNAs were extracted from roots (20 days after germination) of WS, *scr1*, Wt2, D7, st1 and 35S. cDNAs were synthesized from RNA and used for amplification with *SCR* specific F and R primers. **C.** RNAs were extracted from inflorescence stems (35 day old plants) of WS, *scr1*, Wt2, D7, st1 and 35S. cDNAs were synthesized from RNA and used for amplification with *SCR* specific F and R primers.

A.



B.



C.

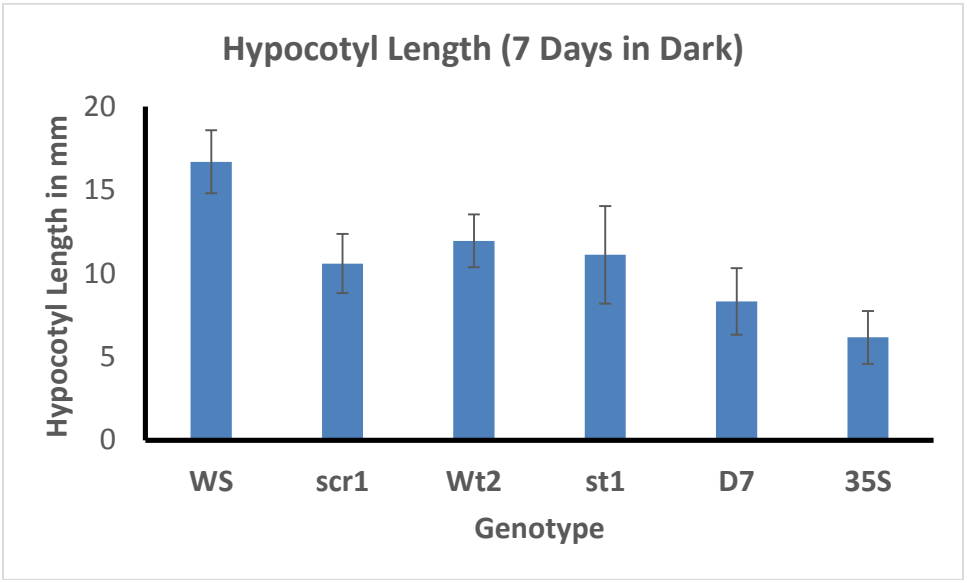


Figure 4.10. Hypocotyl lengths of transgenic seedlings. A. WS, Wt2 and 35S **B. D7,** *scr1* and st1 **C.** Graphical representation of Table 4.5.

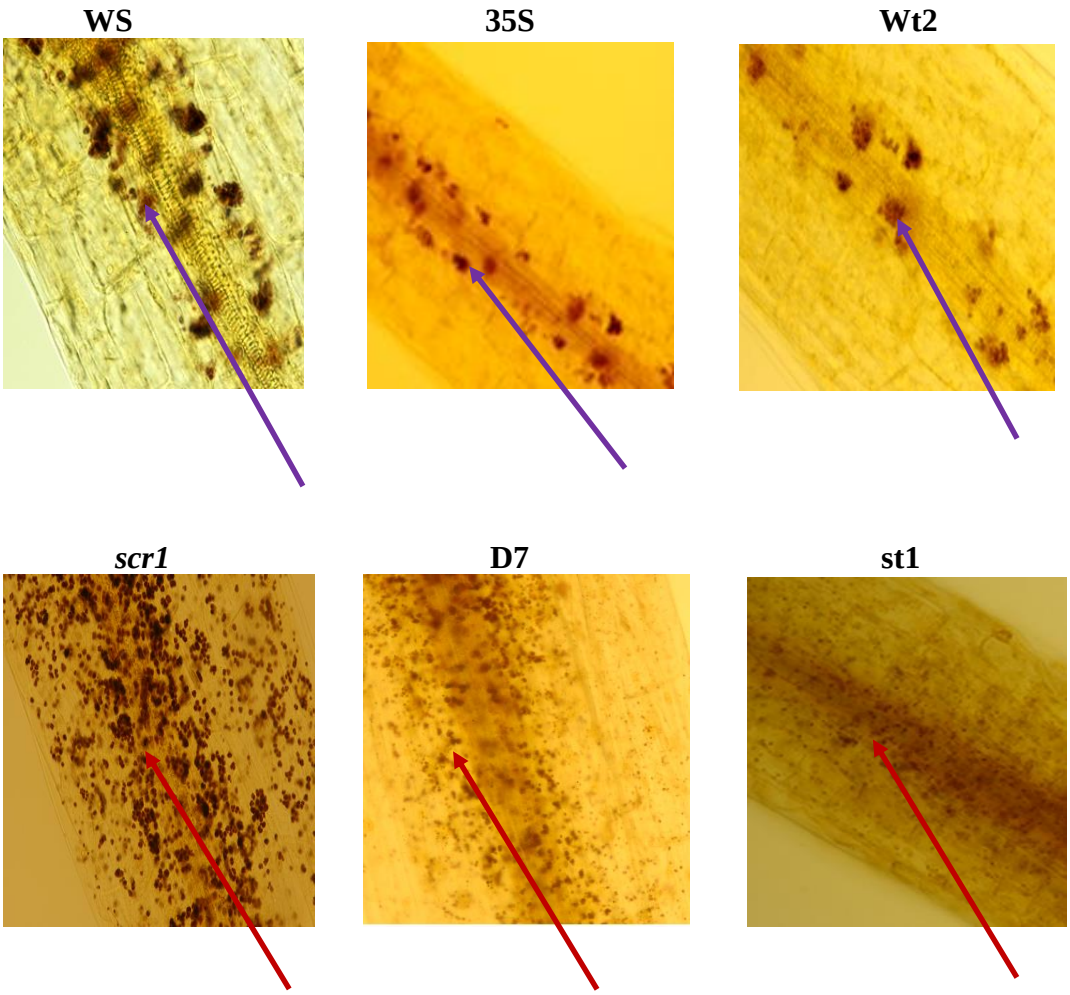


Figure 4.11. Presence and position of amyloplasts in WS, 35S, Wt2, *scr1*, D7and 35S. Seedlings grown on MS agar plates containing 1% sugar were stained with IKI solution and analyzed under light microscope. Purple arrow indicates the sedimented amyloplasts in endodermal layer of WS, 35S and Wt2 and red arrows indicate the distributed amyloplasts in *scr1*, D7 and st1.

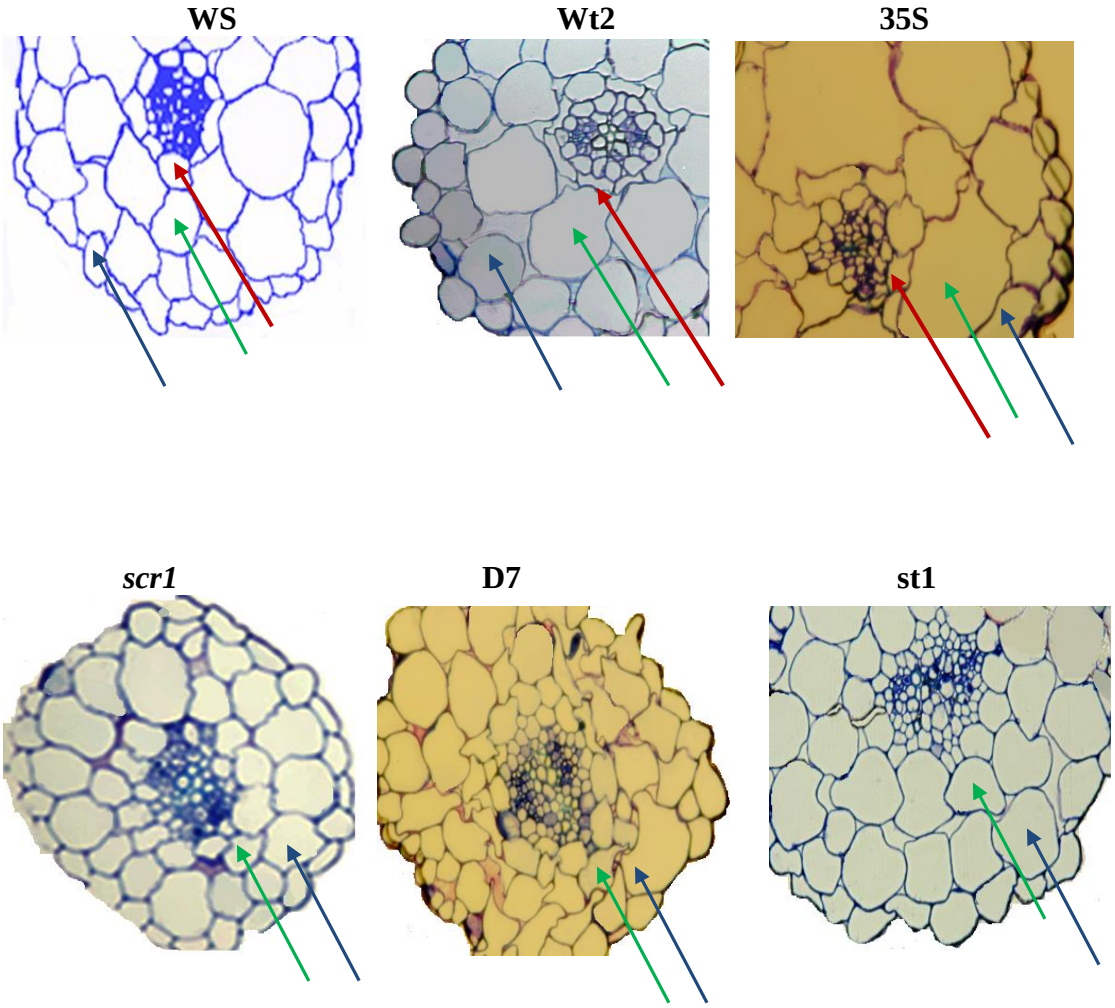


Figure 4.12. Cross sections of WS, Wt2, 35S, *scr1*, D7and st1 hypocotyls. Seedlings were grown in dark for 3 days after germination. Red arrows point to the endodermal layer in WS, Wt2 and 35S. Green and blue arrows point to ground tissue layer one and two respectively in WS, Wt2, 35S, *scr1*, D7 and st1.

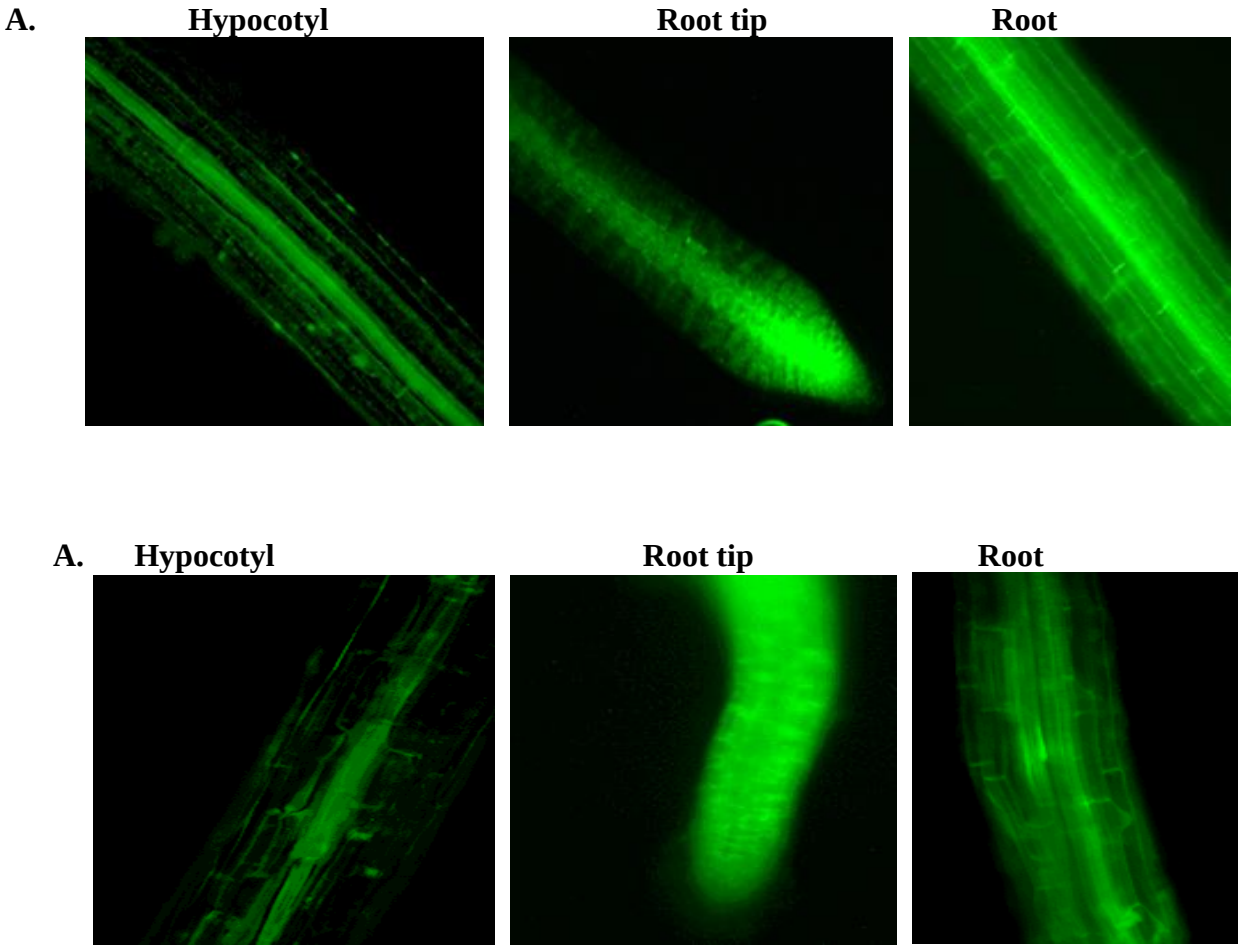


Figure 4.13. *35S::GFP::SCR* expression in transgenic lines. A. *GFP::SCR* expression driven by 35S promoter in hypocotyl and root of Wt2 seedlings. B. *GFP::SCR* expression driven by 35S promoter in hypocotyl and root of st1 seedlings.

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