ABSTRACT

KAJLA, JYOTI DALAL. ROSY1, a Novel Regulator of Tropic and Stress Responses in *Arabidopsis thaliana*. (Under the direction of Dr. Heike Sederoff and Dr. Steven Clouse.)

Plants are sessile organisms, which constantly modulate their growth to their changing environment. Gravity is a constant directional force that elicits a directional growth response in plant organs, called gravitropism. Plant primary roots are positively gravitropic: they grow towards the direction of gravity. If re-oriented with respect to the vector of gravity, the roots are able to sense the change of their orientation towards the vector of gravity, and re-direct their growth towards the new direction by bending towards the direction of gravity. This bending is brought about, in part, by modulation of gene expression. In roots, gravitropic re-orientation induces specific changes in gene expression. One of the earliest transcriptional responses to gravity stimulation is the up-regulation of *ROSY1* (InteractoR Of SYnaptotagmin1) mRNA. The expression of *ROSY1* mRNA is up-regulated within one minute of gravity stimulus, but the expression is transient and recedes to basal levels within five to ten minutes of continous gravity stimulus. The *ROSY1* mRNA expression is also up-regulated in response to light stimulation in a fast and transient manner.

The hypothesis of this dissertation research is that ROSY1 plays a key role in gravitropic and phototropic responses in Arabidopsis primary roots. The research presented in this dissertation, is focused on three major objectives to answer the following questions:

1) Which signal transduction factors are required for gravitropic and phototropic up-regulation of the *ROSY1* transcript?

To characterize the signal transduction elements required for the up-regulation of the *ROSY1* mRNA in *Arabiopsis* roots apices, we used transgenic *Arabidopsis* lines with altered gravity-induced phospholipid signaling. The results of this research show that the gravity and light-induced expression of *ROSY1* mRNA is strictly dependent on Phospholipase C (PLC)-mediated signaling. This work is published in Plant Cell and Environment (2010) and summarized in Chapter 2 of this thesis.

2) Is ROSY1 required or essential for root tropic responses?

Arabidopsis insertion mutants defective in ROSY1 have a significant difference in the gravitropic and phototropic bending. Roots of ROSY1 knockout plants bend faster on gravitropic reorientation than WT roots, indicating that ROSY1 is a negative regulator of these tropic responses. This phenotype is partially brought about by changes in auxin transport rates. These results as well as the cellular and subcellular localization of the ROSY1 protein in *Arabidopsis* are described and discussed in Chapter 3 of this thesis.

3) What is the physiological function of ROSY1 in *Arabidopsis* roots?

Computational analysis identified a conserved lipid binding domain in the ROSY1 protein sequence, and a likely localization on endomembrane systems. We therefore characterized the lipid binding activity of ROSY1 in vitro and its interaction with other proteins. The results showed that ROSY1 binds specifically to stigmasterol and phosphatidylethanolamine in vitro. ROSY1 also interacts with a protein known for its function in vesicle fusion – synaptotagmin 1 (SYT1). A metabolic profile of the membrane composition of ROSY1 knockout plants showed significant changes in the sterol and phospholipid composition of the mutants. Membrane composition affects physiological characteristics of plants beyond

tropisms: it is also important for plant stress signaling, defense and survival. Taken together, these results suggest that ROSY1 is involved in the regulation of vesicle-trafficking in Arabidopsis root apices. These experiments are described and discussed in Chapter 4 of this thesis.

In summation, we have identified a novel protein ROSY1 in *Arabidopsis* that plays a key role in mediating plant gravitropic, phototropic and stress responses, possibly via specific sterol binding and interaction with the *Arabidopsis* membrane trafficking protein SYT1.

ROSY1, a Novel Regulator of Tropic and Stress Responses in *Arabidopsis thaliana*

by
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DEDICATION

This thesis is dedicated to my mother Mrs. Suresh Dalal, who believes in potency and power of education as the one treasure that is never lost, and who loves me, believes in me and stands by me like a rock in the darkest of times. Thank you mumma.

BIOGRAPHY

I was born in New Delhi, India, in the spring of 1983. I was the first child in my family. I completed my Bachelor of Science from Sri Venkateswara College, which is one of the very reputed colleges under the broad umbrella of Delhi University. Because of my interest in Biology, I was given a choice to pursue a degree in biology or an honors program in Botany and Zoology. I selected Botany in July 2000 for my B.Sc., and I am studying Botany ever since. I went on to do my M.Sc. at Maharshi Dayanand University, one of the biggest, if not the biggest university in Haryana, the original mother state for my parents and me. I tried research in molecular biology at the Department of Genetics, University of Delhi. After breaking a pipette on the first day of work (which I did not know was serious), and tearing a glove on the second day of work (which I thought was very serious), I took to molecular biology like a fish takes to water. I felt I had more patience and enthusiasm than my supervisor postdocs. I got very excited looking at the PCR machines, sequencers, the bioinformatics softwares and the whole scope of computational biology and plant molecular biology. Because I had never traveled more than 100 miles far from home, I decided to go further this time, and applied in distant reputed universities in India, and in the U.S. for a Ph.D. degree. I am so happy I chose to work with Dr. Heike Winter Sederoff. Her patience, brilliance and coolness structured me into an able scientist, inspiring me to work hard and work smart and be creative. Also, she bought us ice cream.

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working with me during our yeast two hybrid experiment, and for all her time and efforts being my mentor for all needs and purposes through the entire Ph.D. I am grateful to my other corridor buddies Mingzhu, Lissette and Cat for their friendship. I thank Dr. Sonia Herrero for the opportunity of knowing her.

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Chapter 1. Literature review

GRAVITROPISM

Overview

The direction and force of gravity is one of the very few constant physical parameters that remains unchanged throughout the life of a plant. It is the first tropic stimulus that a germinating seed perceives, and the entire initial plant structure is set with reference to gravity. In general, roots are positively gravitropic and grow towards the direction of gravity, whereas shoots are negatively gravitropic and grow away from the direction of gravity. In the root system, the primary root is positively gravitropic and always grows towards the direction of gravity (Darwin, 1868). The lateral roots are initially plagiogravitropic, which means that they grow at a fixed angle to gravity even though they do not face towards gravity, but this changes as they grow longer (for e.g. longer than 10mm in *Arabidopsis*) , after which they also grow in the direction of gravity (Kiss et al., 2002).

All plant organs grow at a specific angle with respect to the gravity vector, called Gravitropic Set point Angle, or GSA (Digby and Firn, 1995). The GSA for most roots is 0° and for shoots 180°, but it can change depending upon the type of plant and the environmental conditions. For example, the GSA for corn shoots is 180° but that for some vines, climbers and grasses can be anywhere between 0 and 180°. Primary root GSA is 0°, but that of lateral

roots can be variable. Gravitropic responses are plant movements aiming to restore a displaced GSA, whatever that GSA may be (Digby and Firn, 1995).

Gravity perception

In plant roots, the site of gravity signal perception is the root tip, specifically, the columella cells at the root tip (Darwin, 1868). The columella cells contain starch-filled plastids (amyloplasts) called statoliths (Haberlandt, 1900; Nemec, 1900), as seen in Figure 1.1 (Leitz et al., 2009).

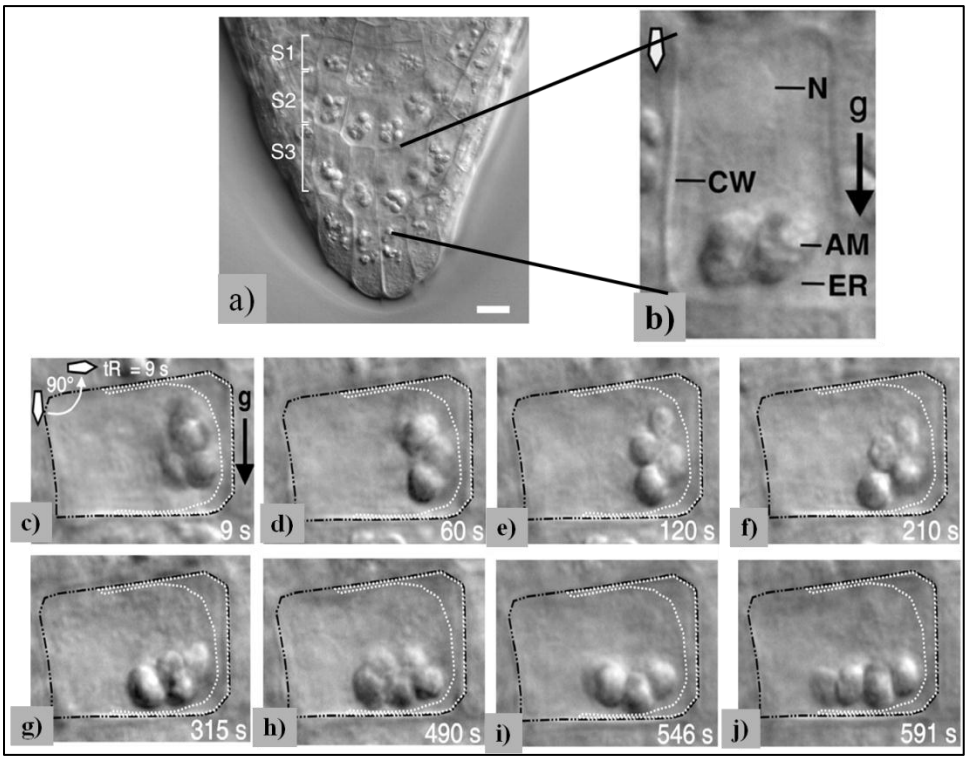

Figure 1.1 Gravity-induced sedimentation of root cap statoliths.

Root cap cells were imaged using differential interference contrast (DIC) microscopy to monitor gravity-directed statolith sedimentation. a) Three tiers of four cells each at the root cap, labeled S1, S2

and S3, are called columella cells. These cells contain amyloplasts (AM) called statoliths. b) An enlarged columella cell showing statoliths sedimented towards the direction of gravity (g), denoted by the black arrow.The cortical endoplasmic reticulum (ER), the cell wall (CW) and the nucleus (N) of the cell are also labeled. c-j) Gravitropic reorientation of the root by 90° , as shown in c) is followed by re-orientation of statoliths to the direction of gravity. Statoliths start re-orienting within 60 seconds of root re-orientation (d); within 591 seconds (~10 minutes), statoliths are complete;t reoriented to the direction of gravity (j). Image reproduced from Leitz et al. (2009) (Leitz et al., 2009).

As seen in Figure 1.1, the statoliths physically "fall down" towards the direction of gravity, and they are widely accepted as "gravity-sensors" in the root columella cells and the endodermal cells of the negatively gravitropic influorescence stem (Thimann and Pickard, 1965; Pickard and Thimann, 1966; Kiss and Hertel, 1989; Fukaki et al. 1998; Leitz et al., 2009).

If the direction of root orientation with respect to gravity changes, statoliths at the root tip reorient towards the direction of gravity within minutes, as seen in Figure 1.1 (Leitz et al., 2009) and this mechanical force is somehow converted to a biochemical signal that is transduced from the columella cells to the elongation zone of the root. The elongation zone then initiates asymmetric cell elongation. The cells on the side of the root away from gravity elongate more than the cells on the other side, and this unequal cell elongation causes a curvature towards the direction of gravity, so that, in a very short time post re-orientation, the root tip grows towards the direction of gravity again.

Lateral roots of Arabidopsis (Kiss et al., 2002), tea (Yamashita et al., 1997), sunflower (Stoker and Moore, 1984) and castor oil plant *Ricinus*(Moore and Pasieniuk, 1984) also have columella cells, though fewer than primary root tips. These cells house amyloplasts too,

which explains the gravitropic properties of lateral roots. Statoliths, are not the only factors involved in gravitropic perception, as gravitropic response is seen in systems lacking statoliths as well, such as in bean lateral roots (Ransom and Moore, 1985).

Cytoplasmic streaming in internodes of the alga Chara is gravitropically induced but is not statolith-dependent (Staves et al., 1995) and suggests that the entire protoplast may also function as gravity sensor in some organisms. It has also been shown that cellular organelles such as plastids in mutants with reduced starch (Sack, 1997) or vacuoles and liposomes in fungi (Grolig et al., 2006) can sediment in response to gravitropic stimulus and cause a gravitropic response.

The sedimentation of amyloplasts is the first root response to gravitropic reorientation, and it is considered to be important for the root bending (Blancaflor et al., 1998). Laser-mediated ablation of root cap cells causes loss of gravity perception by roots (Tsugeki and Fedoroff, 1999). The roots of starch-deficient *Arabidopsis* mutants, such as *pgm-1* have starch-less plastids in the columella cells, and display reduced gravitropic bending response (Caspar and Pickard, 1989; Kiss et al., 1989). On the other hand, the roots of excess starch-containing *Arabidopsis sex1* mutants display a hyper-gravitropic response (increased gravitropic bending) (Vitha et al., 2007).

The sedimentation of amyloplasts towards the vector of gravity is not entirely due to their higher particle density; the cellular actin cytoskeleton may have an important role to play for gravity-directed statolith sedimentation. This was demonstrated with *Arabidopsis*

*sgr9*mutants, which have reduced interaction between F-actin and amyloplasts. The amyloplasts in hypocotyl endodermis of these plants show increased jumping-like saltatory movements, but do not sediment in response to gravity (Nakamura et al., 2011), causing a reduced gravitropic response. The mutation is rescued by addition of actin de-polymerizing drug Latrunculin B (Lat B), or genetically by *fiz1* mutation, which causes fragmentation of actin filaments (Nakamura et al., 2011). Research using Lat B on Arabidopsis roots and hypocotyls indicates that actin filaments somehow impede gravitropic response, and therefore, may have some role in gravitropic signaling (Blancaflor et al., 2003; Hou et al., 2003; Hou et al., 2004).

The gravity-induced re-orientation of amyloplasts in the root tip is a mechanical response, which has to be translated into biochemical signal(s) in the columella cells that are yet uncharacterized (Peer et al., 2011). These mobile signals reach the root elongation zone, where differential cell elongation enables gravitropic bending. Over a century of research on gravitropism has identified some key components for gravitropic signal transduction, including $InsP_3$, Ca^{2+}, and the phytohormone auxin.

THE ROLE OF AUXIN IN GRAVITROPIC RESPONSES

The first and perhaps the most important gravity signaling component discussed here is the phytohormone auxin. Using various mutant studies and biochemical data, it has been demonstrated that the gravity-induced differential cell elongation in the root is caused

because of a gradient in the distribution of auxin. This gradient is formed due to polar auxin transport. Polar auxin transport and its effects of gravitropism will be discussed below.

Polar auxin transport

The asymmetric auxin distribution in plant tissues is brought about by asymmetric auxin transport, called polar auxin transport (Muday and DeLong, 2001). Generated in response to tropic stimuli and developmental cues, polar auxin transport forms an important link between environmental signals and the plant form. Asymmetric auxin distribution is responsible for asymmetrical cellular elongation and organ bending during tropic responses such as gravitropism (Swarup et al., 2005).

Auxin is transported long distances from the site of synthesis (young leaf primordial and meristematic tissues) to sink tissues such as lateral root induction sites by mass transportvia the phloem (Goldsmith et al., 1977; Marchant et al., 2002). Auxin is also transported through small distances via cell-to-cell plasma membrane-mediated active transport (Zazimalova et al., 2010). Auxin formed in leaf primordium cells is transported by short distance cell to cell transport through various cell files until it reaches phloem sieve elements (Goldsmith et al., 1977). Cell to cell transport is also utilized by transporting IAA through cambial cells from shoot to root. Phloem transports auxin by bulk flow until it reaches the central cells of the primary root, from where auxin again travels by short distance cell to cell transport to reach particular root cells (Swarup et al., 2005).

IAA molecules are freely permeable through the plasma membrane, which means that they can enter the cell through the plasma membrane in a concentration dependent manner (lipophilic diffusion) (Srivastava, 2002; Zazimalova et al., 2010). But IAA is a weak acid, which dissociates into IAA^- and H^+ in solution.

At 20°C, the pKa (acid dissociation constant) of IAA is 4.7-4.8. Apoplastic pH is about 5.5 which is close to IAA pKa. At this pH, most of the IAA will be found in dissociated form (Figure 1.2). While the undissociated IAA still can freely permeate through the plasma membrane, the negative charge on IAA^- prevents its uptake. Therefore, diffusion cannot by itself explain auxin uptake. Within the cell the pH is about 7, which shifts the equilibrium towards the dissociated form IAA^-, a form that again cannot diffuse out of the plasma membrane even if the concentration of IAA in the cell is higher than in the apoplast. This makes cells effective IAA traps (Srivastava, 2002; Zazimalova et al., 2010). The influx and efflux of IAA utilize carrier mediated active transport, as shown in Figure 1.2 reproduced from Friml, 2010 (Friml, 2010).

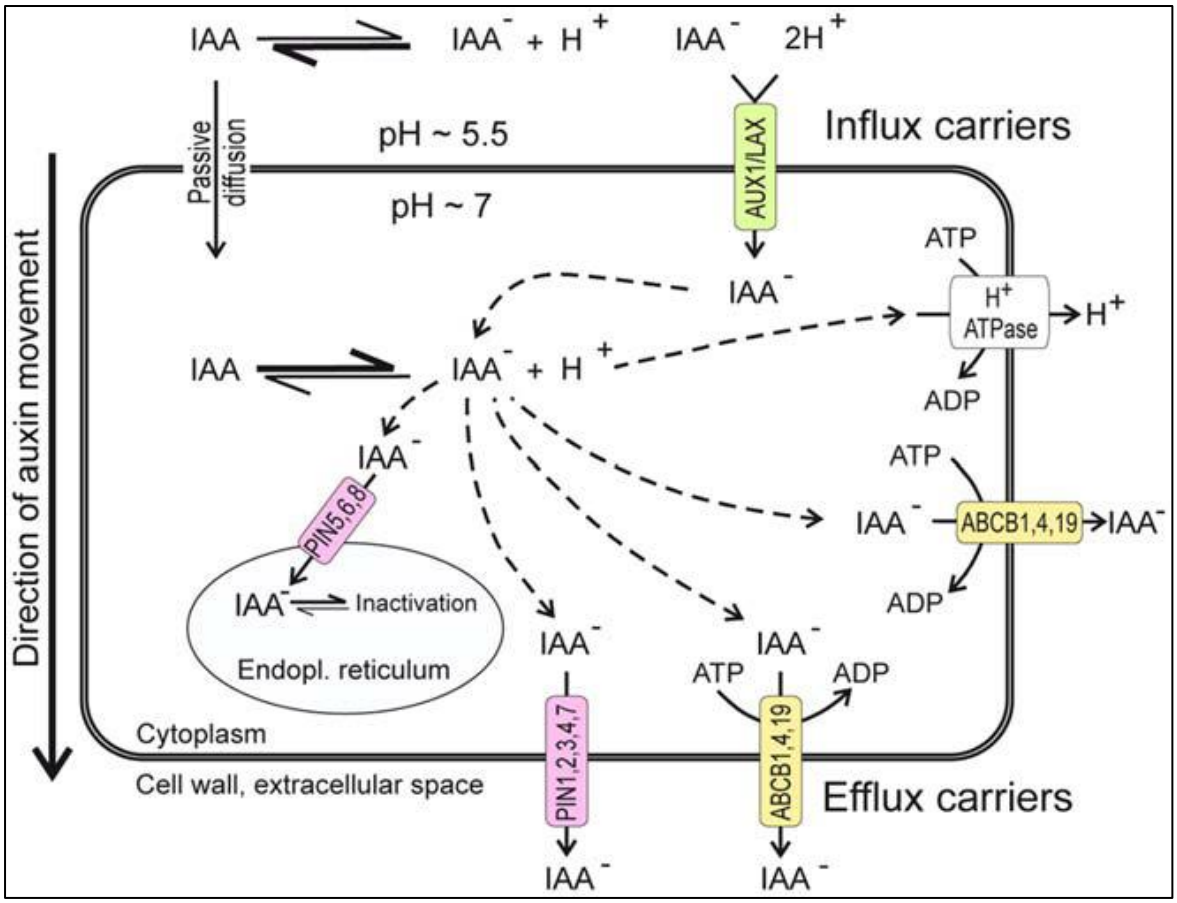

Figure 1.2 A model of intercellular auxin transport.

The image depicts that at the apoplastic pH 5.5, most of the IAA available is the undissociated form. The undissociated IAA molecules then can enter cells by passive diffusion, or by carrier-mediated transport via AUX1/LAX auxin influx carriers. Within the cells, at the pH 7.0, most of the IAA exists as the undissociated form. This form cannot readily permeate the plasma membrane, and requires auxin efflux carrier PIN proteins and the ABCB proteins for auxin efflux. PIN proteins regulate auxin efflux into the apoplast, or into intercellular organelles, as depicted in the image (reproduced from Friml, 2010).

Auxin influx carriers

Four plasma membrane proteins are known so far to function in auxin influx, namely AUXIN RESISTANT1 (AUX1) (Marchant et al., 1999), and the related LIKE AUX1

(LAX1, LAX2 and LAX3) (Young et al., 1999; Parry et al., 2001). AUX1/LAX proteins belong to the structurally conserved family of AAAP (amino acid and auxin permease) proteins (Young et al., 1999) that function as proton symporters to bring IAA^- into the cell against a concentration gradient (Ugartechea-Chirino et al., 2010). Of these, AUX1 was identified and cloned first (Bennett et al., 1996). The protein is 485 amino acids long, with eleven transmembrane spanning domains (Swarup et al., 2004). The *Arabidopsis* genome encodes four AUX1 proteins, localized in plasma membrane and Golgi apparatus, and expressed in the epidermal cells of the root apices, the lateral root caps, the columella cells and the protophloem cells (Swarup et al., 2004), and the lateral root primordia as well as in the shoot apical meristems (Marchant et al., 2002). When AUX1 is absent, cells show significantly reduced auxin uptake, in keeping with the function of AUX1 as an auxin influx carrier (Rouse et al., 1998). Consequently, *aux1* mutants are characterized by having reduced IAA levels in leaves (auxin sources) and roots (auxin sinks), and there is reduced loading of IAA into the phloem in young leaves (Marchant et al., 2002). Accordingly, *aux1* mutants have a reduced number of lateral root primordia (Marchant et al., 2002), altered phyllotactic patterning (Bainbridge et al., 2008) and an agravitropic phenotype in both roots and hypocotyls (Marchant et al., 1999). Reduced auxin uptake also has serious consequences on embryo development and organogenesis; *aux1* mutants show altered development of leaf primordia in embryonic shoots (Marchant et al., 2002) and altered cell number, size and patterning in embryonic roots (Ugartechea-Chirino et al., 2010).

AAAP proteins LAX 1, 2 and 3 are paralogs of AUX1 with auxin influx functions. LAX3 has the maximum sequence similarity with AUX1 (Parry et al., 2001). In heterologous yeast system, AUX1 has a very high auxin import activity (Yang and Murphy, 2009). LAX3 has similarly high auxin import activity while LAX1 and 2 have lower auxin import activity (Yang et al., 2006; Swarup et al., 2008; Yang and Murphy, 2009). Using mutant studies, LAX3 has been demonstrated to also be involved in lateral root emergence (Swarup et al., 2008) and leaf primordia formation (Bainbridge et al., 2008), just like AUX1. AUX1 and LAX3 together are also a link between auxin and ethylene interaction, and are involved in apical hook formation in etiolated *Arabidopsis* seedlings (Vandenbussche et al., 2010). Under nitrogen deficiency, in addition to the AUX1/LAX proteins, the nitrate transporter NRT1.1 is involved in auxin uptake and lateral root induction (Krouk et al., 2010).

Auxin efflux carriers

Auxin efflux is mainly carried out by PIN proteins, a family of carrier proteins with 9-11 transmembrane helices, named after the PINFORMED inflorescences shown by genetic mutants of the earliest discovered PIN protein, PIN1 (Okada et al., 1991). PIN proteins are conserved throughout multicellular plants, ranging from Streptophyta to mosses to modern angiosperms (De Smet et al., 2011). PIN orthologs are notably absent from unicellular algae (De Smet et al., 2011).

The *Arabidopsis* genome encodes eight PIN proteins, named PIN1-8, out of which PIN6 and PIN8 are not yet fully characterized (Krecek et al., 2009). PIN proteins have hydrophobic N and C terminal transmembrane domains separated by a central hydrophilic loop, which can be of variable lengths. Long PINs (PIN1, 2, 3, 4 and 7) contain a lengthy loop, localize to the plasma membrane and directly transport auxin from the cell to apoplast or adjoining cell. Further, these PINs enable polar auxin transport, which gives directionality to biological processes such as embryogenesis, organogenesis and tropisms. While localized to the plasma membranes, the long PINs undergo constant recycling from plasma membrane to endosomal compartments by a transcytosis-like mechanism. PIN protein containing vesicles pinch off from plasma membrane and travel to vacuole, while newly synthesized PIN protein vesicles later fuse into the plasma membrane. This brefeldin-A dependent internalization of PIN proteins is dependent on environmental cues, such as light. Long PINs are deposited very specifically to certain faces of the cell, in specific organs and carry out specialized functions (Peer et al., 2004; Krecek et al., 2009).

PIN1 is expressed both in roots and aerial parts of the plant. The protein is expressed in all cells of the young embryo, but later its expression restricts to basal plasma membranes of precambial tissues, and then to vascular tissues (Galweiler et al., 1998; Steinmann et al., 1999). The polarity of PIN1 distribution within the cell is important in controlling the direction of auxin efflux. In young *Arabidopsis* seedlings, PIN1 is localized to xylem parenchyma and surrounding cortical cells (Noh et al., 2003).

PIN2 is expressed in the root tips; at the tips PIN2 is expressed in the lateral root cap, the epidermis and the cortex. Within the cells, PIN2 is localized to the basal and anticlinal side plasma membrane (Muller et al., 1998; Boonsirichai et al., 2003). PIN3 is expressed in shoot endodermal and root columella cells. Within the cells, PIN3 is localized to lateral side plasma membranes of shoot endodermal cells, and to all sides in the columella cells (Friml et al., 2002). PIN4 is expressed in the root quiescent center and surrounding cells, and localizes on all sides of the cells (Friml et al., 2002). PIN7 at first localizes to the basal cell membranes of the embryo and then in root tip columella cells, in provascular cells in the root meristem and vascular cells in root elongation zone vascular cells (Friml et al., 2003). Within cells, PIN7 is localized to the apical membrane of the embryonic basal cell, in lateral and basal membranes in vascular cells and on all faces of columella cells, just like PIN3 (Friml et al., 2003).

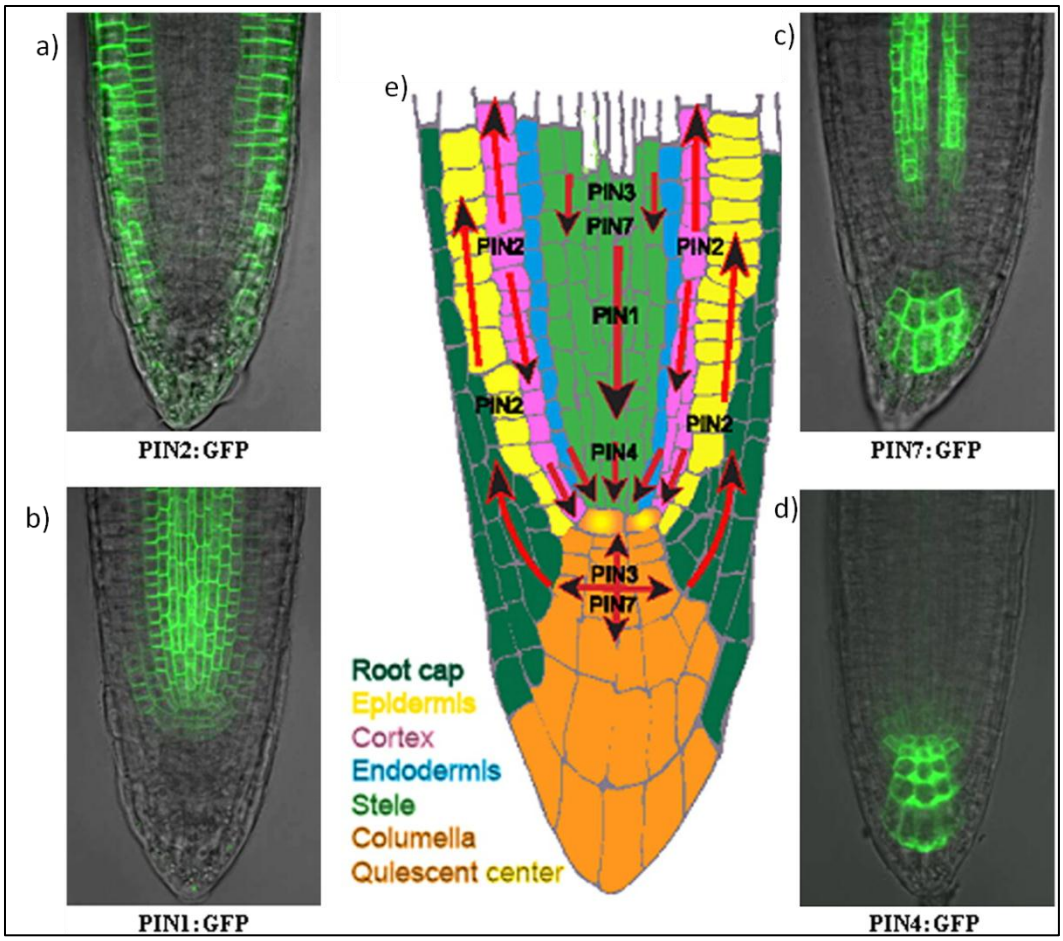

Figure 1.3 Expression localization of PIN protein in *Arabidopsis* root tip.

a)PIN2-GFP expresses in the cortical, epidermal and the lateral root cap cells, localizes to the basal plasma membrane in the cortical cells and apical plasma membrane in the epidermal and lateral root cap cells.b)PIN1-GFP expresses in the root vasculature cells, localizes to the basal plasma membrane of the cells. c) PIN7-GFP expresses in the vascular and columella cells, localizes to the basal plasma membrane in the columella cells and is apolar in the vascular cells. d) PIN4-GFP expresses in the central root meristems and the quiescent center cells, localizes to the basal plasma membrane in the central root meristems cells and has a lower polarity in the quiescent center cells. e) Polar expression of the PIN proteins results in the polar auxin transport at the root tip. The direction of net auxin transport, as affected by the PIN proteins is depicted by red arrows. Auxin is first transported rootward by the action of PIN1, PIN7, PIN3, PIN2 and PIN4. Once auxin reaches the columella cells, auxin is transported laterally and up the lateral root cap by the concerted action of PIN3, PIN2 and PIN7.Image reproduced from Feraru and Friml, 2008 (Feraru and Friml, 2008).

Short PINs include PIN5, 6 and 8. They have a much reduced central hydrophilic loop, and these proteins do not reside in plasma membranes or transport auxin out of the cell, but actually localize to cellular endomembranes, where they possibly function in homeostatic auxin compartmentalization (Peer et al., 2011). In *Arabidopsis* seedlings, PIN5 is expressed strongly in the guard cells. vasculature of cotyledons and the elongating parts of the hypocotyls, as well as weakly in the root tip and root pericycle (Mravec et al., 2009). PIN5 is ectopically expressed at a weak basal level even in mature leaves, stems and flowers. Within the cells, PIN5 is localized to the endoplasmic reticulum (ER) (Mravec et al., 2009). PIN8 expresses in root hairs (Ganguly et al., 2010) and mature pollen grains (Distefano et al., 2009). Within the cells, PIN8 is localized on both the ER and the plasma membrane (Ganguly et al., 2010). PIN8 expresses on cell plates during cytokinesis as well, which underscores its plasma membrane localization (Ganguly et al., 2010). Auxin upregulates expression of PIN proteins, with an exception of PIN5, which is down-regulated by auxin (Krecek et al., 2009).

Other than the PINs, auxin efflux is also carried out by ABCB/PGP P-glycoproteins ABCB 1, 4 and 19 (in *Arabiodpsis*) (Peer et al., 2011). These proteins help in active auxin efflux and transport through long distances in plants. ABCB1/19 regulate auxin flux out of apical tissues rootward (Bandyopadhyay et al., 2007; Titapiwatanakun et al., 2009), and ABCB4 directs shootward auxin transport at the root tip (Santelia et al., 2005; Terasaka et al., 2005), plays a

role in light/sucrose dependent root growth (Terasaka et al., 2005) and root hair elongation (Santelia et al., 2005; Cho et al., 2007).

With the help of the above mentioned carrier proteins, auxin travels directionally and increases in a polar or non-polar manner in certain tissues. How much auxin is internalized by auxin influx carriers or exported by efflux carriers depends on the regulation of these proteins at the transcriptional, translational and protein level. While auxin is known to regulate gene expression of its own carriers, protein stability of carrier proteins is also an important check point in auxin biology. PIN protein phosphorylation seems to be the key mechanism in regulating the polarity of PIN proteins on cellular membranes, and thereby, the direction of auxin transport. Discovery of broad spectrum kinase inhibitors such as K25a and staurosporine inhibit auxin efflux but not influx, gave a clue that efflux related carriers may be prone to activation by phosphorylation, which was later confirmed. It is now known that three members of AGC family of serine-threonine kinases (PID, WAG1 and WAG2) reversibly phosphorylate PIN proteins at a conserved serine residue within the central hydrophilic loop in PIN proteins (Lee and Cho, 2006; Dhonukshe et al., 2010). Lack of PID in functional mutants leads to basal delivery of PIN proteins, and overexpression leads to apical delivery of PIN proteins on plasma membrane (Kleine-Vehn et al., 2009).

The protein Phosphatase 2 A (PP2A) also has an important role in maintaining PIN polarity. The *rcn1* knockout mutants (defective in PP2A expression), or WT plants treated with cantharidin (that inhibits PP2A), both show an increased basipetal auxin transport. PP2A loss

of function mutants display an apical delivery of PIN proteins, just like PID over-expressing plants, which leads to the currently accepted model that PID and PP2A are antagonists in regulating PIN proteins phosphorylation (Michniewicz et al., 2007).

It was previously believed that plasma membrane bound PIN proteins, are static in the membrane (Paciorek and Friml, 2006), but as mentioned previously in the text, it has now been shown that the long PIN proteins constantly cycle between plasma membrane and some not very well known endocytic compartments (Kleine-Vehn et al., 2008; Kleine-Vehn and Friml, 2008). Notably, this recycling is not just limited to efflux carrier PINs. The auxin influx carrier AUX1 is also shown to recycle between plasma membrane and endocytic compartments, even though by a different internalization mechanism (Kleine-Vehn et al., 2006). This internalization of AUX1 and PIN proteins and their redelivery is an evolutionarily conserved mechanism (Kleine-Vehn and Friml, 2008), and it is an important mechanism to regulate their polarization on the plasma membrane. Further, proteins required in this cycling process add another level of regulation to PIN proteins distribution and perhaps stability in the cell.

Some aspects of the mechanism of PIN internalization and recycling are known, as summarized in Figure 1.4 (reproduced from Petrasek and Friml et al., 2009). The internalization begins with endosome formation aided by protein ARF GEF BEN1/MIN7 (Tanaka et al., 2009), internalized vesicle is coated with clathrin (Dhonukshe et al., 2007) and the process is BFA sensitive (Kleine-Vehn et al., 2008). BFA, or Brefeldin A, is a fungal

toxin that “splits” the Golgi apparatus in plant cells- the *cis* cisternae get absorbed in the ER, whereas the *trans* cisternae fuse to form a compartment called a BFA compartment (Surpin and Raikhel, 2004).

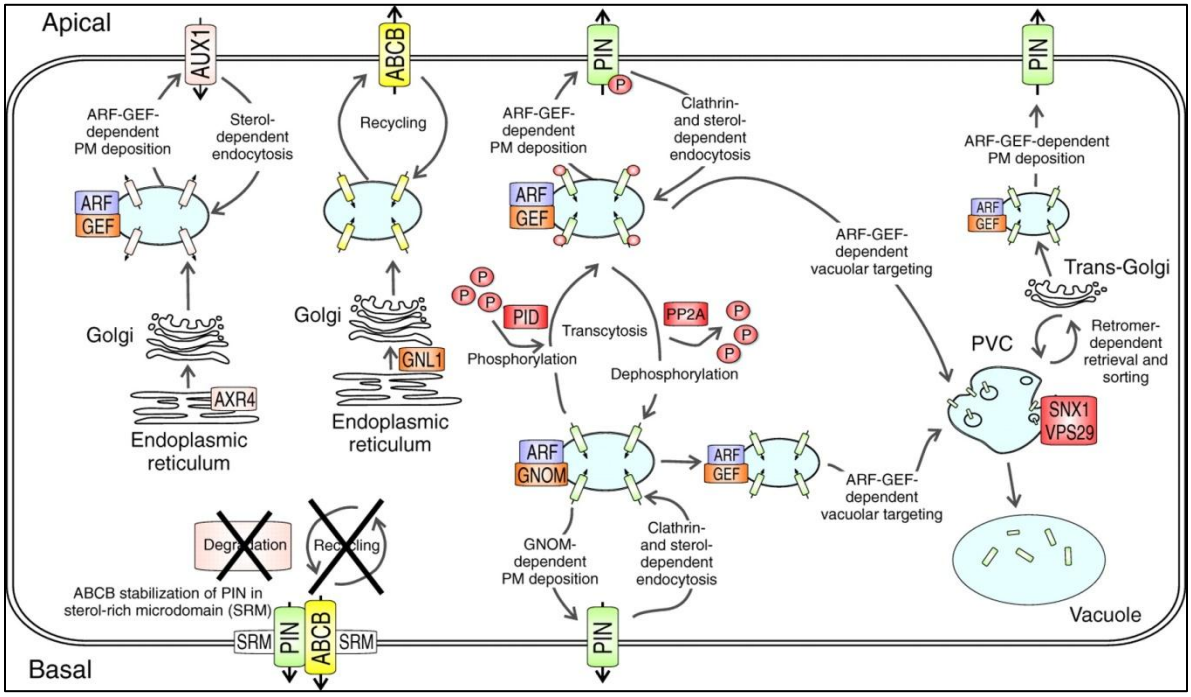

Figure 1.4Sub-cellular trafficking of auxin efflux and influx carriers.

The auxin carrier proteins are localized on the apical or basal plasma membrane (PM), and are constantly recycled between the plasma membrane and sub-cellular vesicles. The PIN proteins, as well as the AUX1 proteins are internalized by sterol-dependent endocytosis. PIN proteins get internalized in clathrin-coated vesicles. New carrier proteins are synthesized and processed through ER and *trans* Golgi network. The deposition of veciles with carrier proteins on the apical plasma membrane requires ARF-GEF activity. PIN protein delivery on basal plasma membrane requires ARF-GEF GNOM activity. PIN proteins may be targeted to the apical or basal plasma membrane depending on their phosphorylation state. The protein PID phosphorylates PIN proteins, which leads to the apical membrane delivery of PIN proteins. The protein PP2A dephopshorylates PIN proteins, which leads to basal membrane delivery of PIN proteins. The sterol composition of the membrane microdomains may be necessary to regulate PIN protein delivery on the membrane. Image is reproduced from Petrasek and Friml, 2009 (Petrasek and Friml, 2009).

The recycling, or redelivery on the basal plasma membrane requires ARF GEF GNOM (Geldner et al., 2003), as seen in Figure 1.4, and its possible antagonist ARF GAPs SCF/VAN3 (Sieburth et al., 2006), and is less BFA sensitive (Kleine-Vehn et al., 2008). The redelivery on apical membrane depends on PID1, WAG1 and WAG2 kinases. The currently accepted model postulates that the competitive recycling of PINs basally by GNOM and apically by PID, WAG1 and WAG2 is what actually causes polarization of PIN proteins (Dhonukshe et al., 2010). Auxin itself also regulates recycling of PIN proteins. Putative auxin receptor AUXIN BINDING PROTEIN1 (ABP1) has been shown to be important to mediate auxin-dependent inhibition of endocytosis of clathrin-coated vesicles (Robert et al., 2010).

Auxin and root gravitropism

Gravitropism is defined as directed growth of plant organs with respect to the direction of gravity, manifested as curvature of the organ concerned if its GSA is disturbed. Formation of curvature involves asymmetric cell elongation on the two sides of the organ. In the case of roots, cells in the elongation zone of the side away from gravity elongate faster than the side facing towards the vector of gravity, thereby orienting the root tip at 0° with respect to the vector of gravity. The role of auxin in this differential elongation may be summarized into two phases – first the formation of an auxin gradient at the gravistimulated root tip followed by auxin induced inhibition of cellular elongation and consequent curvature. These phases are discussed in more depth below:

Gravity-induced formation of auxin gradient at the root tip

Gravity-induced asymmetrical cell elongation is caused by asymmetric accumulation of auxin. Using radio-labeled auxin (Young et al., 1990) or the auxin inducible promoter DR5 linked with reporters GFP and GUS (Rashotte et al., 2001; Boonsirichai et al., 2003; Ottenschlager et al., 2003; Wisniewska et al., 2006) showed that upon gravistimulation of a horizontally oriented root, auxin accumulates at the side facing gravity in the root tip up to the elongation zone.

In *Arabidopsis* seedlings, auxin travels from the shoots rootward via phloem in an acropetal transport. At the root tip, as the auxin reaches columella cells, it is redirected shootwards in a basipetal transport mode. This changes in basipetal transport causes an auxin distribution gradient. The auxin distribution gradient is caused by concerted action of auxin transporters AUX1, PIN3, PIN2 and ABCB4 also known as MDR4 or PGP4 (Friml, 2010; Peer et al., 2011). AUX1 is an important auxin influx carrier, for which null mutants and conditional mutants have reduced auxin transport and exhibit an agravitropic phenotype (Marchant et al., 1999; Swarup et al., 2004). By action of AUX1 and PIN1, auxin moves through the vasculature to the columella cells. Then PIN3, which is expressed in columella cells, is responsible for lateral flux of auxin into the epidermis and the lateral root cap. As a result of gravitropic reorientation, PIN3 localizes to the lower side of the root within less than 2 minutes and its polarization changes so that it transports more auxin towards the lower side

of the root (Friml et al., 2002), as seen in Figure 1.5. PIN3 loss of function mutants, as expected, have agravitropic roots.

Two transmembrane J-domain proteins ALTERED GRAVITY1 (ARG1) and ARG LIKE1 (ARL1), have been shown to be important for PIN3 accumulation at the lower side of columella cells and lateral auxin redistribution, possibly by interaction with actin (Harrison and Masson, 2008 (a); Harrison and Masson, 2008 (b)). Like PIN3 mutants, ARG1 and ARL1 mutants also show agravitropic root tips. Hypocotyls of PIN3, ARG1 and ARL1 loss of function mutants are also less response to gravity, but the hypocotyl gravitropic mechanisms are not yet fully understood. Once in the lateral root cap, AUX1 and PIN2 transport the auxin through cortical cells at the root tips to the epidermis and lateral root cap in the root elongation zone.

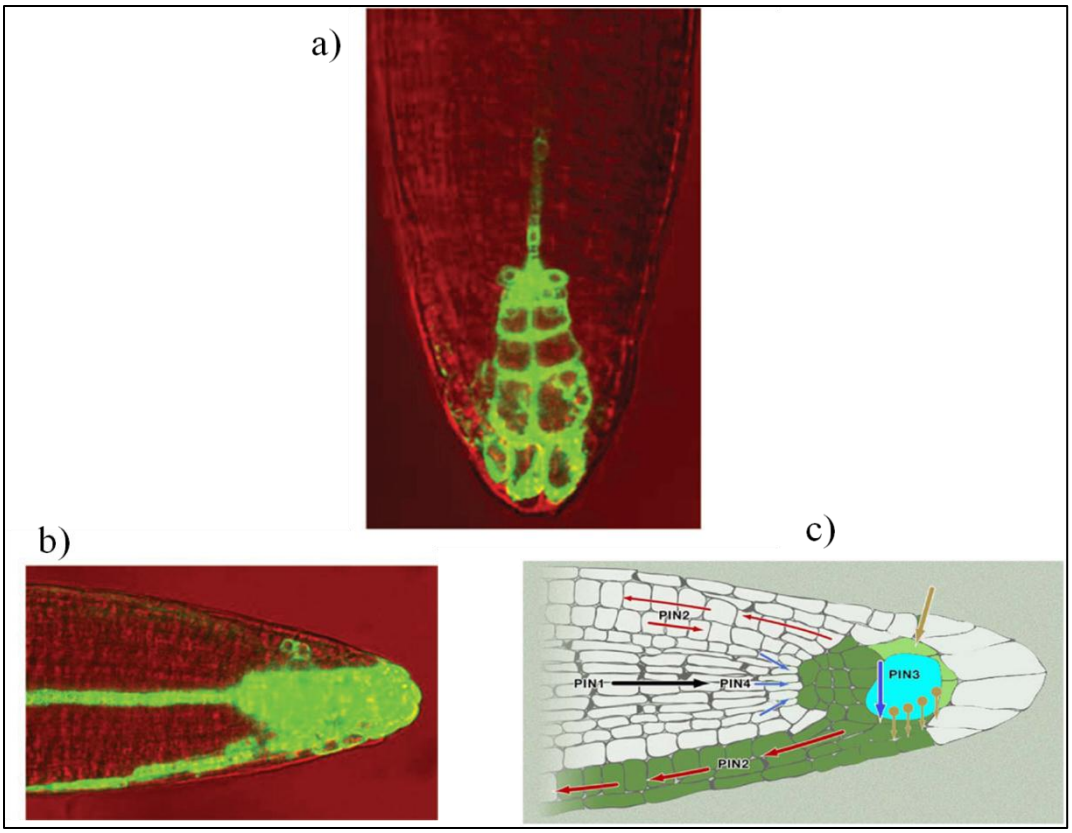

Figure 1.5Gravity-induced asymmetric auxin distribution.

a) *Arabidopsis* root expressing the *DR5:GFP* construct. The *DR5* promoter is activated by auxin, and the green color of the in the root indicates regions of local auxin concentration in the vasculature and the columella cells. b) Upon gravitropic reorientation, auxin accumulates on the lower side of the root within an hour of gravity stimulus. c) PIN proteins are responsible for the gravity-induces auxin redistribution. Auxin is transported to the columella cells by PIN1 (black arrow), PIN2 (red arrows) and PIN4 (blue arrows). There is a high auxin concentration at the columella cells (light blue pool).When the root is re-orientated to the vector of gravity (indicated by solid blue arrow), PIN3 in the columella cells (indicated by brown arrow) transports more auxin to the lower part of the root. Lesser auxin in the upper part of the root leads to PIN2 degradation in the upper part of the root, causing a steeper gradient in auxin distribution. Images are reproduced from Friml, 2010 and Kleine-Vehn and
Friml, 2008(Kleine-Vehn and Friml, 2008; Friml, 2010)

The role of PIN2 during gravitropism is key, as it is responsible for asymmetrical transport of auxin between the upper and the lower root flanks. Auxin has been shown to stabilize PIN2

and prevent its degradation in the lower side of the root, whereas PIN2 undergoes degradation in the upper side of the root, thereby polarizing auxin flow through the lower side of the reoriented root (Abas et al., 2006). Cell files seem to be really important in auxin transport during gravity response: lateral root cap, epidermis and cortical localization of PIN2 seems to be critical for correct gravitropic responses (Swarup et al., 2005; Rahman et al., 2010).

MDR4/PGP4/ABCB4 is a multidrug resistance P-glycoprotein, a member of ABC (for ATP binding cassette) superfamily of integral membrane transporters. Strongly expressed in root cap and epidermal cells, PGP4 also enables auxin efflux in basipetal auxin transport (Terasaka et al., 2005). Null mutants for PGP4 show reduced basipetal auxin transport, and exhibit an enhanced gravitropic phenotype (Lewis et al., 2007).

Recently, it has been shown that flavonoids are responsible for enabling polar PIN shifts during gravitropic response (Santelia et al., 2008). PIN protein localization is dependent on kinases PID1, WAG1 and WAG2, perhaps acting redundantly (Dhonukshe et al., 2010). Gravitropic curvature is mildly reduced in mutant *pid1* roots (Sukumar et al., 2009), but triple mutants *pid1wag1wag2* show highly agravitropic phenotypes (Dhonukshe et al., 2010).

Generation of auxin induced curvature

The exact mechanism of how auxin inhibits cellular elongation in roots is not yet completely known. As discussed earlier in this chapter, it is well known that auxin regulates gene expression. A score of genes, including some transcription factors, are regulated by auxin. Examples of auxin induced transcription factors are *ARF*s, homeodomain transcription factor *WOX5*, AP2 transcription factors *PLETHORA*genes *PLT1* and *PLT2* that maintain root tip stem cell niche (Ding and Friml, 2010), some*Class III HD-ZIP* gene *ATHB8 (Donner et al., 2010)* and *KANADI* transcription factors (Ilegems et al., 2010). Some examples of auxin induced genes are the *PIN*s, cell wall modifying enzymes xyloglucan endotransglycosylase and endo-1, 4-beta-glucanase (Catala et al., 1997, 2000; Catala et al., 2001), *REVOLUTA*, a gene responsible for interfascicular fibers and secondary xylem (Zhong and Ye, 2001)and *PHAVOLUTA*, necessary for adaxial-abaxial axis organization (Ilegems et al., 2010).

Auxin differentially upregulates the expression of inwardly rectifying K^+-channels in *Zea mays* coleoptiles (Philippar et al., 1999) which can affect turgor, phospholipase PLA2 which directs phospholipid signaling and cell growth (Lee et al., 2003), and the enzyme invertase that cleaves sucrose (Wu et al., 1993). Because of pieces of evidence such as those described above, it is understood that auxin aids long term cellular elongation by regulating gene expression, even though the exact genes involved in this elongation process are not yet documented.

Another piece of this puzzle is the hormone ethylene. Mutants in auxin biosynthesis enzyme TAA1 and TAR1 are ethylene insensitive (*wei2*, *wei7* mutants) (Stepanova et al., 2005; Stepanova et al., 2008). At the same time, ethylene up-regulates auxin biosynthesis in roots, and thereby inhibits root cell elongation. It remains to be determined if auxin accumulation by transport and not localized biosynthesis in turn up-regulates ethylene as well to inhibit cellular elongation.

Shoot gravitropism

Shoot are negatively gravitropic, but the mechanism of hypocotyl gravitropism is not clear. In shoots, the endodermis is the site of perception for gravity-stimulation because starch filled amyloplasts are located in the endodermis (Fukaki et al., 1998). Mutants lacking SCARECROW and SHORTROOT transcription factors lack endodermis, and are agravitropic. In starch deficient mutants, such as phosphoglucomutase mutant *pgm*, gravitropism was reduced (Caspar and Pickard, 1989; Kiss and Sack, 1990; Saether and Iversen, 1991; Kiss et al., 1997; Weise and Kiss, 1999; Tanimoto et al., 2008) and in mutants with higher amount of starch like the *sex1* mutant, hypocotyl negative gravitropism was increased (Vitha et al., 2007). It is known that auxin accumulates laterally in gravistimulated hypocotyls in the side of the hypocotyl away from gravity (Friml et al., 2002). But interestingly, high auxin promotes cell elongation in hypocotyl tissue rather than inhibiting it. Therefore, shoots do bend, just in the direction opposite to gravity. PIN3 does not show lateral re-distribution as a response to shoot gravity-stimulation.

Role of membrane composition in gravitropic responses.

The cellular membrane composition as well as vesicle trafficking have recently been shown to be important to maintain auxin transport, and have a role in plant abiotic and biotic stress responses. The distribution of membrane lipids and sterols, and their role in gravitropism will be reviewed in this section.

Membrane lipid composition

Plant membranes can be divided into seven categories (Leshem, 1992) - plasma membrane, tonoplast or the vacuolar membrane, inner and outer plastid membranes, thylakoid membranes, mitochondrial membranes, highly folded ER membrane system, stomatal guard cell membranes and miscellaneous membranes consisting of membranes surrounding nuclei, lysosomes and peroxisomes.

While most membranes including plasma membrane, ER membrane, tonoplast and thylakoid membrane are single bilayers, chloroplast and mitochondrial organelles have two membranes. Oleosomes in seeds with high triglyceride-rich fatty acids are surrounded by half unit membranes, not bilayers at all. Membranes are composed of lipids, sterols and proteins. Lipids are made of a glycerol backbone, where the first two carbons bind to fatty acids and the third carbon (sn-3) forms bonds with usually hydrophilic groups (Ohlrogge and Browse, 1995). When sn-3 is esterified with a phosphate group it forms phosphatidic acid (PA), parent compound to all phospholipids. Phospholipids are named according to the moiety

binding PO_4^-, such as phosphatidylethanolamine (PE) which is formed when phosphate is followed by ethanol, which is then followed by an amino group, as seen in Figure 1.6.

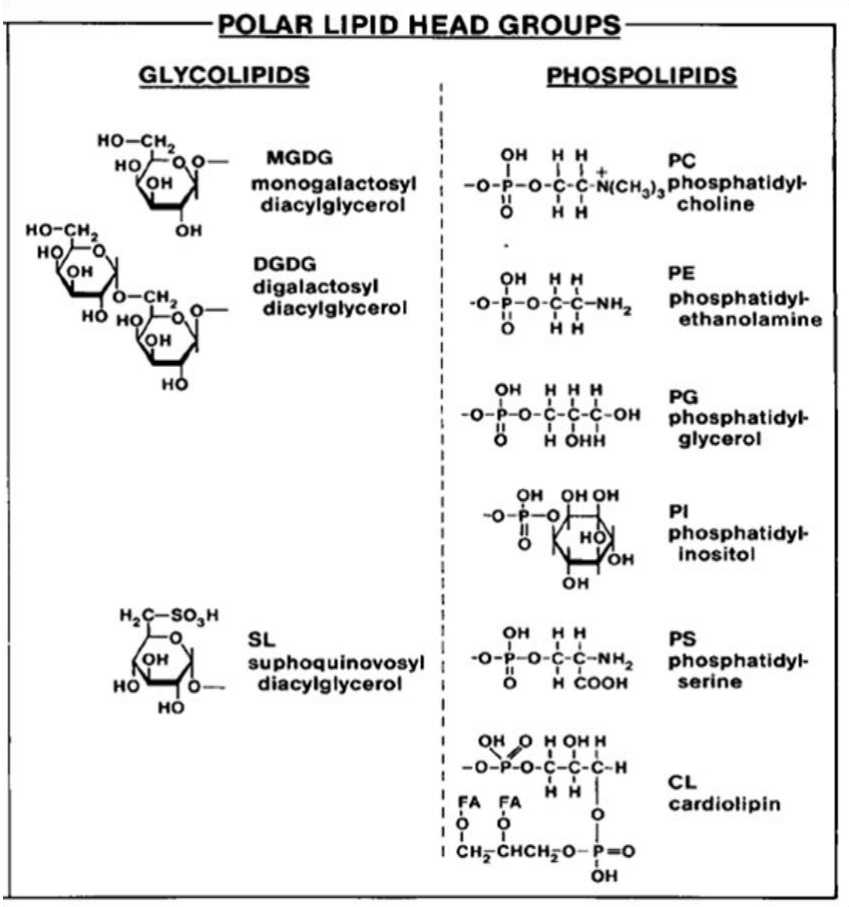

Figure 1.6 Structure of major lipids in plant membranes.

The structures of major glycolipids and phospholipids present in plant membranes are presented here. Structures of the lipids are arranged in approximately in decreasing order of their abundance in plant leaf samples. Image is reproduced from Ohlrogge and Browse (1995) (Ohlrogge and Browse, 1995)

Phospholipids may be electroneutral, such as phosphatidylcholine (PC) and PE or may be anionic such as PA, phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylinositides (PI). When sn-3 binds carbohydrate, generally glucose or galactose, it

forms galactolipids (Ohlrogge and Browse, 1995). When sn-3 binds sulfur, sulpholipids are generated. When the backbone is not glycerol but sphingosine, with its first two carbons binding groups, sphingolipids are formed (Ohlrogge and Browse, 1995). The enzyme phospholipase D (PLD) hydrolyzes phospholipids back to PA (Munnik et al. 1995). PA is an important signaling molecule as well, as will be discussed later in this section. Other than PA, PI is perhaps the most functionally important phospholipid, as the inositol group can be phosphorylated to phosphoinositide phosphate (PtInsP) at different positions by different phosphoinositide kinases (PIK). Phosphorylation at position D-3 by PI3K generates PtIns3P, at position D-4 by PI4K generates PtIns4P, at position D-5 by PI5K generates PtIns5P, and sequential phosphorylation by PI4P5K or PI5P4K generates PtIns(4,5)P_2 (PIP_2) (Gonzales et al., 2005) $PIP_2$is hydrolyzed by phospholipase C (PLC) to yield Ins(1,4,5)P_3 ($InsP_3$) and diacyl glycerol (DAG) (Pfaffmann et al., 1987). $InsP_3$ induces Ca^{2+} release in the cells, and Ca^{2+} further serves as an important secondary messenger.

Different membranes vary in specific lipids and protein composition (Ohlrogge and Browse, 1995). For example, the plasma membrane is rich in phospholipids. Chloroplast membranes on the other hand, have low phospholipids, no PE, and a majority of the membrane is made of the glycerolipids (galactolipids and sulpholipids) (Schleiff et al. 2001). Furthermore, the fatty acyl species vary in the chloroplast membranes of the same plant as an adaption to cold exposure (Leshem, 1992). Double bonds at carbon 6 are replaced by double bonds at carbon 5 in frost acclimated conifers. The mitochondrial membrane has phospholipids comparable to

plasma membrane, but completely lacks galactolipids and sulpholipids (Leshem, 1992). In the bilayers itself, the two leaflets differ drastically in the protein composition and share partial to absolute asymmetry in their phospholipids (Leshem, 1992). For example, 63 to 75% of PC is present in the outer monolayer of most membranes. In guard cells, Ca^{2+} when in contact with the inner bilayers leads to stomatal closure. In plasma membranes of leaves and fruits, Ca^{2+} when present in the interstitial space between the two leaflets retards senescence, but when it enters or crosses the inner leaflet, it promotes senescence (Leshem, 1992; Schleiff et al. 2001).

Phospholipids in membranes serve many critical biological roles as secondary messengers in signal transduction and regulatory molecules for plant stress signaling and adaptation. *Arabidopsis* mutants deficient in PC biosynthesis induce cell death and reduction of cell growth phenotypically manifested by small roots and short epidermal cells (Testerink and Munnik, 2005).

Deficiency of $InsP_3$ leads to smaller roots, and overexpression or external application of $InsP_3$ leads to enhanced root growth. $InsP_3$ levels can be reduced in plants to levels lower than 5% of wild type by expressing human type I Inositol1,4,5 -triphosphate 5-ptase (InsP-5ptase) gene in plants, or pharmacologically by application of PLC inhibitor U73122 (Perera et al., 1999; Perera et al., 2001; Perera et al., 2006; Salinas-Mondragon et al., 2010). Reducing $InsP_3$ levels in the plant leads to significant stress tolerance. In transgenic *A. thaliana*, reduced $InsP_3$ levels resulted in drought tolerance and changes in ABA metabolism

(Perera et al., 2008). Transgenic tomato plants, with $InsP_3$ levels lower than 30% of wild type plants, were more drought tolerant, had higher vegetative biomass, and had increased lycopene and hexose concentrations in fruits (Khodakovskaya et al., 2010).

In fact, on transcript profiling of root, leaf and fruit tissues in tomato (Khodakovskaya et al., 2010) and roots apices in *A. thaliana*(Salinas-Mondragon et al., 2010) [Chapter 2], it was seen that the mRNA profiles were changed in the absence of $InsP_3$. Some of these genes have roles in cell wall growth, tropic signaling and photomorphogenesis. It has been proposed that $InsP_3$ itself, or the Ca^{2+} released by it downstream, or even any other protein(s) activated or repressed downstream is/are capable of regulating transcriptional activation or repression of certain genes (Salinas-Mondragon et al., 2010) [Chapter 2], and by this mechanism $InsP_3$ regulates plant growth and development.

PA plays a role in root growth, lateral root formation and root hair formation (Testerink and Munnik, 2005). Long range PA responses include actin polymerization (Lee et al., 2003), auxin distribution (Lanteri et al., 2008), abscisic acid-induced inhibition of growth as well as vesicle trafficking (Testerink and Munnik, 2005; Li and Xue, 2007). Because PA regulates actin microfilament polymerization as well as vesicle transport, PA deficiency is very pronounced in pollen tube growth (Monteiro et al., 2005). Seedlings deficient in PLD do not produce sufficient amounts of PA and show reduced primary root elongation and absence of lateral root formation, whereas PLD overexpressing seedlings display branched and swollen root hairs (Ohashi et al., 2003).

Other than developmental roles, phospholipids have important roles during stress tolerance. On exposing *A. thaliana* seedlings to NaCl and KCl (salt stress) and sorbitol (osmotic stress) and cold stress, both PIP_2 and IP_3 levels were found to increase. Salt stress has been reported to induce PIP_2 internalization by clathrin coated vesicles (Lee et al., 2009). All PLD isoforms have been shown to be required for salt and drought stress tolerance. Both PLC and PLD pathways are activated on freezing stress but different PLD isoforms have different roles in freezing tolerance. While *A. thaliana* seedlings deficient in PLDα1 have improved tolerance for freezing, mutants in PLDδ have reduced freezing tolerance which may be because they induce distinct gene expression (Wang, 2005). Loss of PLDα1 induces expression of genes that code for osmolytes that help plants recover from freeze-induced injury (Rajashekar et al., 2006). PLDδ mutants also have strongly reduced H_2O_2 tolerance (Zhang et al., 2003), suggesting that PA may be important for plants to counter oxidative stress induced cell death. Wounding also upregulates several PLD isoforms (Wang et al., 2000).

It has been reported that PLζ2 and its product PA are necessary for cycling PIN2 from vesicular compartments back into the plasma membrane, which is very important considering the central role of auxin on plant life (Li and Xue, 2007). Lipids also confer functionality to proteins by association. As will be reviewed later in this Chapter, membrane trafficking protein SYT1 cannot be activated in absence of PIP_2 in the membrane, because the lipid changes affect the conformation of SYT1.

Membrane sterol composition

Sterols are important membrane components in both plant and animal membranes. Sterol basic structure is composed of a polar hydroxyl head group, a steroid skeleton and a variable aliphatic chain. Different sterols are formed owing to the differences in the structure of the aliphatic side chain and the number and position of double bonds in the tetracyclic steroid skeleton. In *A. thaliana* seedlings, sitosterol is the most prevalent sterol (64%), followed by campesterol (11%), stigmasterol (6%), brassicasterol (2%) and 24-methylcholesterol (2%) (Dyas L., 1993).

In most higher plants, the polar head group does not form conjugates, and sterols are thus called free sterols, but this group can be esterified or acylated, forming steryl esters, steryl glucosides and when the sugar in steryl glucosides is further esterified, it forms acylated steryl glucosides (Hartmann, 1998). To start sterol biosynthesis, acetyl-coA condenses to 3-hydroxymethyl-3-glutaryl coenzyme A (HMG-CoA), which is converted to mevalonic acid (MVA) by HMG-coA reductase. MVA is converted into squalene in a multistep process which is the precursor of all the different plant sterols (Hartmann, 1998). Sterols are present in the highest amount in plasma membrane, and in low amounts in other membranes like the ER, tonoplast, mitochondrial and chloroplast membranes, whereas they are completely absent from thylakoid membranes (Schleiff et al., 2001).

Sterols have important structural roles in biological membranes, primarily as condensing agents. It has been reported that the area occupied by an artificial membrane made up of a

phospholipid and a sterol is less than the sum of both the components (Hofsass et al., 2003). For membrane function, typical sterols need to satisfy certain structural requirements (Grunwald, 1971). The hydroxyl group needs to be free and not conjugated, the tetracyclic structure needs to be planar, and the aliphatic side chain should be 8-10 carbon atoms long (Hartmann, 1998).

The sterol hydroxyl group faces water interface between membranes or towards the cytoplasm or cell wall. The tetracyclic core and the side chain extend into the hydrophobic membrane. Needless to say, different sterols with their variable fatty acid chains localize differently in membranes. Integrating into the membrane and interacting with the lipids and proteins present there, chemically and spatially restricts the movement of the side chains and the surrounding lipids, in a phenomenon called membrane ordering, which governs the liquid to crystalline phase transition temperature for the membrane (Schuler et al., 1991). Sitosterol and 24-methylcholesterol side chains are planar and these sterols are the most efficient in membrane ordering, reducing movement of surrounding lipids and in reducing water permeability through the membrane (Dufourc, 2008). While the rest of the sterol structure is the same between sitosterol and stigmasterol, the presence of one trans oriented double bond in the stigmasterol side chain makes the chain staggered, reduces the ordering ability of stigmasterol in a membrane and also makes the membrane poorly impermeable to water (Schuler et al., 1991).

Plant cells absolutely require sterols for growth and development. Plants mutated in HMG-CoA Reductase grow much smaller and weaker compared with wild type plants (Jang et al., 2000). Experiments on celery cells prove that particular sterols may have some importance in cell growth and development, because selectively blocking stigmasterol biosynthesis arrested growth, even though other sterols were being synthesized (Griebel and Zeier, 2010). Sterols have also been reported to be important for regulating function of certain proteins (Goad, 1990). In maize roots, low concentrations of stigmasterol and cholesterol stimulate proton pumping from plasma membrane bound H^+-ATPases, whereas all other sterols including sitosterol inhibit H^+-ATPases at all concentrations (Grandmougin-Ferjani et al., 1997). This is very interesting, because it means that the exact membrane lipid and sterol surrounding dictate if a protein will be active or not. At the same time proteins can change membrane composition, as new membrane lipids and sterols are incorporated in the membrane by vesicle fusion, while sterols are removed from the membrane by clathrin coated pits.

In a typical membrane, lipids and proteins are constantly in motion and not statically bound in one place. Sometimes, a particular sterol lipid protein complex is more energetically stable bound to each other, so these float in the membrane together, as structures named lipid rafts, or membrane microdomains, focal points of a myriad of membrane related biological processes. It is said that most membrane proteins and lipids are constantly recycled, and a typical membrane renews itself in an hour or so. Plasma membrane from kidney cells in baby hamster renews itself every 30 minutes (Griffiths et al., 1989). Whether this is also true for

plants or not, the versatile nature of the membrane and its great biological importance make membrane dynamics an important field of study.

Membrane composition and gravitropism

Membrane composition has often been implicated in gravity response. It was shown in azuki bean (*Vigna angularis*) that in hypergravity conditions of 300g, membrane composition changes, and to a great extent sterols and to a much lesser extent phospholipid amounts increased in the membrane (Koizumia et al., 2007). Sterols were seen to provide membranes of azuki epicotyls resistance against gravity, and slowed the gravitropic growth. Gravitropic growth was much faster after treating seedlings with lovostatin, a sterol biosynthesis inhibitor (T. Koizumia, 2007). Other than their roles in structural membrane support during gravity, sterols have also been shown to be necessary for correct positioning of PIN proteins.

Arabidopsis seedlings mutated in the sterol-biosynthesis enzyme CYCLOPROPYLSTEROL ISOMERASE1-1 (CPI1-1) have increased portion of cyclopropylsterols (sterol precursors), and almost no mature sterols, including stigmasterol, sitosterol and campesterol (Men et al., 2008). This gross change in the membrane sterol composition affected the growth and development of the plant, and the mutant plants were very small and sterile. The cells in these mutant plants failed to localize PIN2 properly post cell division. PIN2 localization is key to directional auxin transport into the lateral root cap and epidermis during gravitropism. Consequently, *cpi1-1* mutant roots were defective in gravitropism (Men et al., 2008).

Similarly, plants mutated in STEROL METHYLTRANSFERASE1 (SMT1) lack most major membrane sterols, show a dwarfed phenotype, and mis-localization of PIN1 and PIN3 (Willemsen et al., 2003). The tropic responses of *smt1*-1 roots are random as compared with the straight bending response of wild type roots.

The effect of phospholipids on gravitropic responses has also been investigated in some detail. It is known that within five minutes of gravitropic stimulation, $InsP_3$ levels rise about 3 times in the lower side of pulvini of maize and oats, where auxin accumulates post gravity stimulus (Perera et al., 2001). As mentioned before, $InsP_3$ levels have been reported to be reduced to less than 5% of wild type by expressing human type I InsP 5-ptase in *Arabidopsis*, and by application of PLC inhibitor U73122 (Perera et al., 1999; Perera et al., 2001; Perera et al., 2006; Salinas-Mondragon et al., 2010). When $InsP_3$ levels are reduced in the plant, $InsP_3$ gradient cannot form post gravity stimulus, and these plant show a reduced bending to gravity stimulus compared with wild type plants (Kimbrough et al., 2004; Kimbrough et al., 2005; Salinas-Mondragon et al., 2005). This data was confirmed by an alternate approach, where *Arabidopsis* seedlings mutated in Ins5Ptase (which we assume leads to higher $InsP_3$ levels) causes increased bending to gravity, as compared with wild type seedlings (Wang et al., 2009).

Gravity-stimulation induces gene expression (McClure and Guilfoyle, 1989; Kimbrough et al., 2004; Massa and Gilroy, 2003; Kimbrough et al., 2005; Salinas-Mondragon et al., 2005). It has been reported that gravistimulation of *Arabidopsis* root apices up-regulates a wide

variety of genes, including some transcription factors, membrane transporters, cell wall modifying enzymes, cell cycle related proteins and stress related proteins (Kimbrough et al., 2004; Kimbrough et al., 2005). This analysis uncovered several unknown proteins, such as SAMT-like and ROSY1 which show fast and transient up-regulation in response to gravity; these proteins may also be important for plant tropic signal transduction, and general plant growth and development. This is indeed the case, as *Arabidopsis* seedlings mutated for SAMT-like (unpublished data) and ROSY1 (discussed in Chapter 2) display faster bending kinetics to gravity stimulus. Interestingly, some of the genes (including *ROSY1*) showing differential transcriptional response to gravity, showed similar differential expression in response to light stimulus (Salinas-Mondragon et al., 2005).

But even though it was known that some gravity and light regulated genes are also regulated by different hormones such as auxins, cytokinins and brassinosteroids (Kimbrough et al., 2005), the molecules downstream of gravity that influenced gene expression were not described. In the recent years, $InsP_3$ has been identified as a key metabolite that can modulate-gravity induced gene expression (Salinas-Mondragon et al., 2010) (Chapter 2). We do not know why PLC generates more $InsP_3$ post gravity stimulus, but it must be a very fast response, because $InsP_3$ levels are seen raised within 30 seconds of gravity stimulation.

Gravity-induced gene expression was compared between wild type plants and plants expressing human Type I InsP-5ptase causing very low $InsP_3$ levels (Salinas-Mondragon et al., 2010). It was seen that expression of some gravity inducible genes was indeed

$InsP_3$dependent; these genes were not up-regulated in response to light or gravity in transgenic plants (Salinas-Mondragon et al., 2010)(Chapter 2). One of these $InsP_3$ dependent genes is *ROSY1*, as discussed in Chapter 2.

Membrane trafficking and gravitropism

Recently, membrane trafficking has received a lot of attention in the field of auxin biology, and consequently, in the field of gravitropism. The discovery that the long PINs (PIN1,2,3,4,7) recycle between the plasma membrane and endosomal compartments and that short PINs (PIN5,6,8) are actually localized to ER has brought protein machinery involved in vesicle fusion, endocytosis and exocytosis into focus in auxin biology. Membrane steroid binding protein MSBP1 was reported to increase cycling of PIN2-containing vesicles, thereby increasing gravitropism in both roots and shoots (Yang et al., 2008). In plants defective in the expression of PLDζ2, PIN2 cycling by vesicles is reduced and consequently reduces gravitropism, whereasPLDζ2 overexpression increases PIN2 vesicular cycling and enhances gravitropism (Li and Xue, 2007).

Interestingly, neither MSBP nor PLDζ2 change PIN2 localization, they only facilitate better cycling of these proteins. But what does cycling really mean? Cycling has two basic components. The first is removal from part of the plasma membrane, aided by special proteins into a clathrin coated pit, which is then incorporated into other compartments. This holds true for *Arabidopsis* PINs, as already documented earlier in this chapter. The second is

fusion of vesicles with PINs or any other proteins to fuse into the plasma membrane. This fusion process requires SNARE (**s**oluble ***N***-ethylmaleimide-sensitive fusion protein **a**ttachment protein **re**ceptor) complex. This complex consists of a set of proteins present on two separate membrane leaflets, whose proteins interact and in the process, bring the leaflets together and finally fuse the membranes, often in a Ca^{2+} dependent manner (Bassham and Blatt, 2008).

This process of docking and fusion is very specific and occurs selectively, but is quite potent, as SNARE complexes can fuse two cells together if the complex proteins are on the outer membranes of two neighboring cells, and are able to physically come in contact with each other to interact (Rothman, 1994). In *A. thaliana* SNARE proteins SYP22, SYP5 and VTI11 are present in gravity-perceiving endodermal cells of the shoot. SYP22 is involved in anterograde trafficking between plasma membrane and vacuole. Loss of SYP22, SYP5 or VTI11 leads to defects in vesicle trafficking, and pronounced reduction in shoot gravitropism (Kato et al., 2002; Stoelzle et al., 2003). In fact, even statolith sedimentation is said to be disrupted when SNARE functionality is compromised in some mutants (Saito et al., 2005). But with such a critical role for SNARE complexes in plant survival, protein isoforms have been observed to take on roles of mutated proteins in some cases (Surpin et al., 2003). The protein SYT1 in Arabidopsis interacts with the SNARE complex and mediates vesicle fusion and recycling. SYT1 is reviewed in detail later in this chapter.

PHOTOTROPISM

Phototropism is defined as directed growth in response to directional light stimulus. Aerial plant parts such as the stem and leaves exhibit both tropic and nastic movements to take advantage of available light while optimizing water loss by evapo-transpiration. Interestingly, roots also exhibit phototropic movements, possibly helping the root system gauge its depth in soil by how much light passes through. Unlike statoliths in root gravity responses, no organelles are yet known to have a role in phototropic perception even though hypocotyl chloroplasts have been implicated to be important for phototropic bending (Jin et al., 2001).

Sunlight, as it reaches the earth is a mixture of light rays of various wavelengths, ranging from high energy violet, indigo and blue wavelengths to the lower energy red light (Gilroy et al., 2008). Red light (R) to Far red Light (FR) ratio in sunlight is 1:2. As light rays filter through material substances, like tree canopies, collisions with other molecules causes high energy photons to lose energy and the rays that stream through have lower energy, and thereby longer wavelength. Under the canopies, the ratio of R:FR is 0.13. It is safe to assume that by the time photons represent FR, they have lost most of their energy and capacity to power photosynthesis. Under the soil surface, very little light passes through and the FR component still increases; under 5 mm the soil R:FR is 0.88. Plants perceive R:FR ratio and direct their growth accordingly (Gilroy et al., 2008).

Some seeds, especially small seeds such as lettuce require a certain R:FR ratio for germination. These seeds do not germinate if the FR component is too high. Pine trees in coniferous forests also have a minimum light requirement for germination (Atzet and Waring, 1970). This ensures that the seeds don't germinate too deep in the soil or in places extremely shaded by overhead foliage (Batschauer, 1998). Other life processes that involve red light perception are floral induction and maintenance of circadian rhythms.

Red light (R) and far red (FR) light are received by receptor pigment molecules called phytochromes. *Arabidopsis* encodes five phytochromes from phyA-E (Batschauer, 1998). Phytochromes exist in two spectral forms Pr and Pfr (Butler et al., 1959; Quail, 1997). Pr absorbs red light at 666 nm peak, upon which its structure changes into Pfr form. Pfr absorbs Far Red light at 730 nm peak, upon which, it changes to Pr. Pfr also reverts to Pr form in dark (Gilroy et al., 2008).

High energy blue and UV light is perceived by cryptochromes 1 and 2 (CRY1 and CRY2), and phototropins 1 and 2 (PHOT1 and PHOT2), earlier called NON-PHOTOTROPIC HYPOCOTYL1 and 2 (NPH1 and 2) respectively (Briggs and Huala, 1999). The proteins PHOT1 and PHOT2 are autophosphorylating serine-threonine kinases with a single kinase domain, and two flavin mononucleotide (FMN) binding LOV (light, oxygen, voltage) domains (Cho et al., 2006). Both FMN and LOV domains are somehow activated by blue light, so that the kinase is activated which then phosphorylates PHOT proteins and other unknown proteins (Cho et al., 2006). Potential PHOT phosphorylation targets are ABCB and

PIN proteins, particularly ABCB19 and PIN3, both of which have been implicated in phototropism (Titapiwatanakun and Murphy, 2008). Light sensitivities of PHOT1 and PHOT2 vary, while PHOT1 is very sensitive to low light, PHOT2 is activated only by light intensities higher than 10 μmol $m^{-2}s^{-1}$(Harada et al., 2003). Originally plasma membrane localized, PHOT proteins can dissociate on blue light application (Briggs and Huala, 1999; Harada et al., 2003).

Because different proteins sense different wavelengths, plants are able to sense their light environment more precisely and take advantage of it. Exceptions are a few polypodaceous ferns and the green alga *Mougeotia*, which have one protein NEOCHROME that functions as both red light sensing phytochrome and blue light sensing phototropin, and takes advantage of any available light in the very shaded microhabitats of these plants (Kawai et al., 2003; Suetsugu et al., 2005). But in most land plants, blue and red light have different perception and signaling pathways as discussed above and different roles to play.

Blue light, which has much higher energy than red light, is used to power photosynthesis and it has been observed that small amounts of blue light can remarkably increase photosynthesis (Hogewoning et al., 2010). Saturating blue light also does not inhibit photosynthesis (Hogewoning et al., 2010). Blue light is used to maintain transpiration rates as blue light mediates stomatal opening (Talbott et al., 2002). When white light is split into an action spectrum of various wavelength lights, blue light elicits the strongest phototropic responses known in plants (Wiesner, 1878). Red light on the other hand, powers photosynthesis to a

much lesser extent, and indeed inhibits photosynthesis under saturating conditions (Hogewoning et al., 2010). Red light-induced phototropism is also quite mild, and its role seems to be primarily in seed germination and floral induction. Known aspects of plant phototropism are discussed below.

Red light phototropism

Red light traditionally has not been known to induce a phototropic response, because the response is very weak (Millar et al., 2009). In etiolated *A. thaliana* seedlings, red light induces positive phototropism in roots (Kiss et al., 2003), a response lacking in mutants for PHYA and PHYB, suggesting that red light root phototropism is phytochrome-mediated. But red light-induced hypocotyl phototropism in the model plant *Arabidopsis* is hard to see in normal conditions, which may be because this response is weak, and is masked by the overriding gravitropic response. With this in mind, experiments were conducted on *Arabidopsis* under microgravity conditions in space flight experiments and it was seen that red light induces positive phototropism in hypocotyls as well (Millar et al., 2009). Phytochrome mediated red light phototropism has also been reported for maize mesocotyls (Iino and Schafer, 1984). Phytochrome-mediated FR phototropism in shoots is not yet fully characterized: while cucumber shoots exhibit positive phototropism to FR light (Ballare et al., 1992), *Cuscuta planiflora* shoots are negatively phototropic to FR.

Blue light phototropism

Blue light induces strong positive phototropic response in hypocotyls even in a normal 1g gravity environment (Tsuchida-Mayama et al., 2010). Roots are negatively phototropic to white light and blue light, though the negative phototropism to blue light is a weak response, not easily discernible in wild type plants, but more pronounced in starchless mutants (Ruppel et al., 2001). Blue light negative phototropism in roots also increased in microgravity in plants grown in The International Space Station (Millar et al., 2009).

Regulation of phototropism

Phytochromes and cryptochromes are important for phototropic sensing, and therefore, phototropic curvature. Mutants *phyAcry1cry2* and *phyAphyBcry1cry2* hypocotyls show reduced curvature in directional blue light (Tsuchida-Mayama et al., 2010). PHY and CRY have been shown to aid phototropic bending by suppressing ABCB19, an auxin efflux carrier, and a negative regulator of phototropic bending (Nagashima et al., 2008). PHYA increases expression of PHYTROCHROME KINASE SUBSTRATE1 (PSK1) in blue light, which contributes towards a negative curvature in roots as a response to directional blue light stimulation. PKS1 mutants do not show phototropic bending, while the bending is exaggerated in PKS1 over-expressing plants (Boccalandro et al., 2008). In root phototropism, another protein of importance is ROOT PHOTOTROPISM 2 (RPT2), which modulates the

activity of blue light receptor PHOT1 (Inada et al., 2004). RPT2 is also regulated by PHY and CRY proteins (Tsuchida-Mayama et al., 2010).

Asymmetrical cell elongation that causes phototropism involves asymmetrical auxin accumulation. As seen with an auxin inducible DR5:GUS construct, auxin accumulates on the shaded side of a hypocotyl exposed to directional white light (Friml et al., 2002). In hypocotyls, auxin accumulation increases cell elongation, and causes curvature in the direction of white light. Auxin induced transcription factors NPH3 and NPH4/ARF7 are reported to be involved in phototropism (Molas and Kiss, 2008). Some proteins that have been shown to aid asymmetric auxin distribution following directional illumination are PIN3 (Friml et al., 2002), ABCB19 (Noh et al., 2001), PIN1 (Noh et al., 2003) and AUX1 (Stone et al., 2008). At the shoot apex, unilateral light already forms an auxin gradient, where PIN1 and ABCB19 play an important role in transporting auxin rootwards; ABCB19 mutants mis-localize PIN1 in the shoot apex and hinder fast rootward auxin transport, This causes a steep auxin gradient near the apex and results in hyper-phototropic hypocotyls (Noh et al., 2001). Lower in the hypocotyl, lateral relocation of auxin to the shaded side is also important for phototropic bending, as seedlings mutated in PIN3 exhibit reduced phototropism (Ding et al., 2011).

Another aspect of phototropism, as has been recently revealed, is the role of phospholipid signaling (Salinas-Mondragon et al., 2010) [Chapter 2]. In transgenic plants with reduced $InsP_3$, there was a marked reduction in tropic responses, including phototropism to blue light.

The blue light perception gene *CRY2* and the red light perception genes *PHYB* and *PHYE* were differentially expressed in $InsP_3$ deficient plants compared with wild type plants. Many genes involved in photomorphogenesis were found differentially expressed. Therefore, $InsP_3$ mediated transcriptional regulation (directly or through downstream proteins/metabolites) also in part accounts for phototropic responses (Salinas-Mondragon et al., 2010) [Chapter 2].

In spite of some proteins recognized for phototropic signal transduction, the picture is yet far from clear. While PHY, CRY and phot holoprotein are accepted to be the light receptors, it is not known how these proteins affect auxin redistribution. It is hypothesized that PHOT proteins phosphorylate auxin efflux carriers, but this has not been experimentally investigated. Furthermore it is not known how the light gradient across the tissue is recognized by the plant at a molecular level. This is important because when plants face directional light, the darker side of the plant is also not completely dark compared with the illuminated side, it is just comparatively dark. It is interesting to note that the two PHOT proteins are activated by different fluence rates oflight. Whether all light receptor proteins have isoforms like PHOT proteins that turn on at different light fluence rates, or if the same proteins change spectral forms depending on the light intensity is not known. It is not known why auxin concentration, while inhibiting elongation in roots, promotes cellular elongation in shoots.

Discovery of novel proteins involved in phototropic signal transduction by approaches like mutant analysis is rendered difficult by the fact that phototropism is a weak response

dominated by gravitropism, and there may not be an easily discernible phenotype even if the proteins are involved in the pathway. One way to solve this problem is to conduct mutant screening experiments in microgravity conditions at the International Space Station.

Interaction between gravitropism and phototropism

Mature plants have a primary shoot apex and a primary root apex which are vertically oriented in the direction of gravity. In even light, the shoot apex grows straight upwards, but in a light gradient, it exhibits phototropism by bypassing gravitropism and growing towards the light. Root tips grow straight down. But most of the plant biomass is actually created by the lateral organs. Branches with leaves are responsible for almost all of the photosynthesis and the elaborate lateral root system is responsible for almost all water and mineral uptake. These lateral organs do not have a 0° GSA, but a variable and versatile angle of growth which is in a dynamic equilibrium with many directional responses, such as gravity, light, touch, water, oxygen etc. Some components in these pathways interact. All of them involve auxin redistribution, though recently other hormones such as GA (Wolbang et al., 2007; Ross and Wolbang, 2008), brassinosteroids (Li et al., 2005; Vandenbussche et al., 2011), cytokinin and ethylene (Golan et al., 1996) are also being implicated in tropic responses. There is also evidence that tropic responses compete with each other. Red light, through phytochromes, has been reported by several authors to negate gravitropism in certain species (Liscum and Hangarter, 1993; Parks et al., 1996; Poppe et al., 1996; Hangarter, 1997).

While gravitropic perception may not be affected by red light, as statoliths sedimentation was seen to be unaffected by red light stimulation in moss protonemata (Kern and Sack, 1999), red light exposure promotes randomization of shoot growth direction. Previous red light exposure actually sensitizes hypocotyls to gravitropism (Britz and Galston, 1982). Further, red light exposure increases blue light-induced phototropism, perhaps by countering competing gravitropism (Liscum and Hangarter, 1993; Parks et al., 1996; Poppe et al., 1996; Hangarter, 1997). While the mechanisms of tropic interactions at molecular level are yet far from known, their knowledge is crucial for developing plants which can resume normal growth in extreme situations, like in shaded environments on earth, and in life support systems in space.

SYNAPTOTAGMIN

Arabidopsis Synaptotagmin 1, AtSYT1, belongs to the large Synaptotagmin family of proteins. The SYT family is evolutionarily very conserved, and 98 SYT genes have been identified from ten sequenced genomes of *C. elegans*, *Anopheles*, fruit fly, *Danio*, *Ciona*, *Fugu*, mouse, humans, rice and *Arabidopsis*(Craxton, 2004). The SYT proteins are transmembrane proteins first discovered because of their importance in causing synaptic vesicles to fuse with presynaptic membranes (Littleton and Bellen, 1995).

All SYT proteins contain a short intervesicular sequence at the N-terminal region followed by transmembrane helices, a linker sequence, two cytoplasmic calcium binding C2 domains

(C2A and C2B) and a WHXL motif (Sutton et al., 1995) and finally a C-terminal region. The transmembrane helices are important for transmembrane anchoring of SYT family proteins while the C2 domains bind Ca^{2+}. The WHXL motif is needed for plasma membrane association in vesicle docking process (Fukuda et al., 2000; O'Connor and Lee, 2002).

SYT proteins are involved in both, vesicle exocytosis and endocytosis (Schwarz, 2004; Tang et al., 2006).With other key proteins, namely vesicle-associated soluble *n*-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE), target membrane-localized (t-SNARE), SNAP-25, syntaxin and synaptobrevin, SYT proteins carry out neuronal membrane fusion in animal systems (Broadie et al., 1994; Weber et al., 1998; Jahn and Scheller, 2006; Fox and Sanes, 2007; Brunger et al., 2009). The mechanism of vesicle fusion and the protein machinery involved is conserved through *Drosophila*(Littleton et al., 1993), *C. elegans*(Nonet et al., 1993), mouse (Geppert et al., 1994) and human (Gao et al., 2000). In the presence of Ca^{2+}(Baram et al., 1999; Kuo et al., 2009), the C2 domains are configured to bind two membrane bilayers and bridge them together for fusion (Herrick et al., 2009). Neuronal firing happens in response to elevated cytosolic Ca^{2+}, and Ca^{2+} sensitivity is actually provided by SYT proteins alone in the SNARE complex (Lynch et al., 2007), and they are therefore called Ca^{2+} sensors. It is important to remember, though, that not all predicted SYT proteins have Ca^{2+} sensing and vesicle trafficking functions.

Mammals have 16 members in the SYT family, out of which eight are capable of binding Ca^{2+}. In mammals, SYT 1, II, III, V, VI, VII, IX and X function as Ca^{2+} sensors and carry out

exocytosis or endocytosis or both (Xu et al., 2007). SYT1 binds with and 'bends' membrane leaflets and interacts with other members of the SNARE complex in a Ca^{2+} dependent manner to bring down the energetic cost of membrane fusion (Lynch et al., 2008) for membrane exocytosis and endocytosis. Recently, single vesicles have been analyzed to quantify the exact proportion and quantities of seven integral proteins of brain synaptic vesicles SYT1, SV2, the proton ATPase, Vglut1, synaptobrevin2, synaptophysin and synaptogyrin (Mutch et al., 2010).

SYT1 is sorted into vesicles with high precision; the number of SYT1 molecules found per vesicle showed little variation. The Ca^{2+} dependency and modes of vesicle docking for SNARE-Synaptotagmin driven fusion has been studied at a single vesicle level (Christensen et al., 2010), and Ca^{2+} accelerated the vesicle docking more than two orders of magnitude, though not all docked vesicles ended up in membrane fusion. Vesicle docking and fusion is dependent not only on the presence of synaptic vesicle proteins, but also on the lipid composition of the plasma membrane (Kuo et al., 2011). SYT proteins bind phosphatidylinositol, 3,4,5-trisphosphate (PIP_3) in the absence of Ca^{2+} and in presence of Ca^{2+} SYT binds phosphatidylinositol, 4,5-bisphosphate (PIP_2). In presynaptic membranes, this PIP3 binding by Syt1 is actually necessary for Ca2+ binding and coupling of Ca2+ influx with vesicular fusion (Paddock et al., 2008). Decreased PIP_2 levels at nerve endings causes impairment in vesicle trafficking and lethality in mice (Di Paolo et al., 2004). PIP_2 increased SYT1 affinity to Ca2+ in *Rattus norvegicus*(Radhakrishnan et al., 2009). When bilayers

made of phosphatidylcholine (PC) and phosphatidylserine (PS) were supplemented with PIP_2, the C2A domain of Syt1 penetrated deeper in the membrane, altering the C2B domain orientation and pushing it towards the bilayer interface where it bridges bilayers (Kuo et al., 2011).

SYT protein isoforms are present in all plants tested to date, including the evolutionary ancient bryophyte *Physconitrella,* which actually has 19 predicted SYT coding genes in its haploid genome (Craxton, 2007). *Arabidopsis thaliana* encodes five AtSYT proteins named AtSYTA-E. AtSYT B-E proteins are predicted endomembrane proteins with unknown functions, but information is available about roles of protein AtSYTA, now called AtSYT1. Just like its mammalian isoforms, AtSYT1 has been shown to be an important vesicular recycling protein (Yamazaki et al., 2010). While all mammalian SYT proteins localize to endomembranes such as ER and vesicles but not plasma membrane, AtSYT1 localizes to the plasma membrane (Yamazaki et al., 2010). The protein is processed in the ER and Golgi, but specifically localizes to the plasma membrane (Yamazaki et al., 2010). Tandem C2 domains in AtSYT1 are required for localization of the protein to plasma membrane (Yamazaki et al., 2010). The protein regulates plasma membrane-derived vesicle endocytosis in plants. In a study aiming to understand the role of AtSYT1 in virus endocytosis and trafficking within the plant, it was seen that AtSYT1 bound directly with viral movement proteins for *Cabbage leaf curl virus* (CaLCuV) and *Tobacco mosaic virus* (TMV). In plants lacking AtSYT1, cell to cell trafficking of viral movement proteins was inhibited (Lewis and Lazarowitz, 2010).

Therefore, AtSYT1 has a role in trafficking proteins within the plant which has important implications for plant growth, development and stress responses.

Vesicular fusion also has an important role in maintaining and regaining plasma membrane integrity after biotic and abiotic stresses. Being sessile, plants need to develop mechanisms to tolerate stresses such as soil salinity, osmotic shock, freezing and thawing with fluctuating temperatures between day and night. Stresses such as these cause breakage in the plasma membrane, and just like in animal systems, the resealing process needs a specialized assembly of proteins accompanied with Ca^{2+} influx at the wound site (Schapire et al., 2009). In mammalian systems, SYT VII has a role in plasma membrane repair in the presence of Ca^{2+}, post-mechanical tearing of the membrane (Reddy et al., 2001; McNeil and Kirchhausen, 2005). In plants AtSYT1 was the first identified component in the plasma membrane repair apparatus (Schapire et al., 2009). AtSYT1 helps in fusing vesicles back to the membrane to help reseal it after salt stress (Schapire et al., 2008) and freezing stress (Yamazaki et al., 2008). Insertional mutants and RNAi lines with knock-out or silenced AtSYT1 were more susceptible to stress damage compared with wild type plants.

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Chapter 2. Copy of the published paper "Role of inositol 1,4,5-triphosphate signaling in gravitropic and phototropic gene expression" Plant Cell and Environment (2010) 33, 2041-2055.

MY CONTRIBUTION

Generating and analyzing data for Figures 4 and 9.

SIGNIFICANCE TO THE DISSERTATION

Gravity and light stimulate $InsP_3$ generation

The earliest known biochemical response to gravity signal is the Phospholipase C (PLC)-mediated spike in the levels of inositol 1,4,5-trisphosphate ($InsP_3$) in gravity-sensing tissues (Record et al., 1999; Perera et al., 2001). The enzyme PLC hydrolyses the plasma membrane phospholipid phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and $InsP_3$ (Meijer and Munnik, 2003). Within 10 seconds of gravistimulation, there is a five-fold spike in $InsP_3$ levels in the lower halves of both oat and maize pulvini (Perera et al., 1999; Perera et al., 2001). $InsP_3$ elicits the release of Ca^{2+} from cellular stores such as the ER and the vacuole (Alexandre and Lassalles, 1990), and Ca^{2+} in turn may serve as a secondary messenger for gravitropic signal transduction and amplification (Poovaiah and Reddy, 1993). PLC mediates generation of $InsP_3$ in both phototropic (Harada et al., 2003) and gravitropic responses (Perera et al., 1999; Perera et al., 2001).

Chemical inhibition of PLC by aminosteroid U73122 blocks $InsP_3$ accumulation and leads to reduced gravitropism in both roots and stems of *Arabidopsis*. Reduction in $InsP_3$ levels has also been achieved by expressing human type I InsP 5-ptase in *Arabidopsis*, which specifically hydrolyses soluble inositol phosphates $InsP_3$ and $InsP_4$ (Majerus et al., 1999), reducing $InsP_3$ levels in transgenic plants to less than 5% of that of wild type plants (Perera et al., 2006). Gravitropic bending is reduced in both roots and hypocotyls of transgenic *Arabidopsis* plants with low $InsP_3$ levels (Perera et al., 2006). This demonstrates that gravitropic signal transduction has a PLC mediated $InsP_3$ dependent component.

Gravity and light up-regulated gene expression

To identify novel genes playing a role in gravitropic signal transduction, microarray experiments were conducted by several groups, to identify genes specifically regulated by gravity stimulation (Moseyko et al., 2002; Kimbrough et al., 2004). Gravity induces increases in transcript abundances of 65 genes in *Arabidopsis* root apices (Kimbrough et al., 2004). These genes fall into many functional categories such as cell wall elongation, cell cycle maintenance, stress and defense signaling, even photomorphogenesis as well as novel genes for which functions aren't yet established, such as *ROSY1* (mentioned as At2g16005 MD2-Related in the paper) (Kimbrough et al., 2005). Some of these genes, including *ROSY1*, are also transcriptionally up-regulated by phototropic stimulus (Salinas-Mondragon et al., 2005).

Gravity and light-induced regulation of *ROSY1* mRNA abundance is $InsP_3$-dependent

Gravity induces transcriptional up-regulation of *ROSY1* mRNA within less than 2 minutes of gravity stimulus (Kimbrough et al., 2004). Light also induces transcriptional up-regulation of *ROSY1* mRNA within less than two minutes (Salinas-Mondragon et al., 2005).

In our publication "Role of inositol 1,4,5-triphosphate signaling in gravitropic and phototropic gene expression" included in this chapter, Salinas-Mondragon et al. have shown that the gravity and/or light-induced up-regulation of some genes is dependent on the levels of $InsP_3$ in the plant. They compared expression of light and gravity-regulated genes between WT and transgenic plants having very low levels of $InsP_3$. We found that some genes tested, including *ROSY1*, had different expression levels in transgenic plants compared with WT plants. This data puts $InsP_3$ and Ca^{2+} upstream of the transcriptional regulation of some light and/or gravity-induced genes, including *ROSY1*.

Contributions of this publication specifically in context with *ROSY1* and this dissertation are highlighted below:

1. In transgenic plants with lower $InsP_3$ levels, *ROSY1* mRNA is not transcriptionally up-regulated in response to gravity (Figure 3) or light (Figure 6) stimulation. Therefore, $InsP_3$ and Ca^{2+} are upstream of light and gravity-induced *ROSY1* transcriptional up-regulation.
2. The levels of *ROSY1*mRNA in the roots of seedlings grown in red and far red light are different between wild type plants and transgenic plants with reduced $InsP_3$ levels.

Therefore, $InsP_3$ is upstream of *ROSY1* transcriptional regulation in different light conditions in soil.

COPY OF THE PUBLICATION

Publication “Role of inositol 1,4,5-triphosphate signalling in gravitropic and phototropic gene expression” (Salinas-Mondragon et al., 2010).

Plant, Cell & Environment

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Role of inositol 1,4,5-triphosphate signalling in gravitropic and phototropic gene expression

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ABSTRACT

Plants sense light and gravity to orient their direction of growth. One common component in the early events of both phototropic and gravitropic signal transduction is activation of phospholipase C (PLC), which leads to an increase in inositol 1,4,5-triphosphate ($InsP_3$) levels. The $InsP_3$ signal is terminated by hydrolysis of $InsP_3$ through inositolpolyphosphate-5-phosphatases (InsP 5-ptases). *Arabidopsis* plants expressing a heterologous InsP 5-ptase have low basal $InsP_3$ levels and exhibit reduced gravitropic and phototropic bending. Downstream effects of $InsP_3$-mediated signalling are not understood. We used comparative transcript profiling to characterize gene expression changes in gravity- or light-stimulated *Arabidopsis* root apices that were manipulated in their $InsP_3$ metabolism either through inhibition of PLC activity or expression of InsP 5-ptase. We identified $InsP_3$-dependent and $InsP_3$-independent co-regulated gene sets in response to gravity or light stimulation. Inhibition of PLC activity in wild-type plants caused similar changes in transcript abundance in response to gravitropic and phototropic stimulation as in the transgenic lines. Therefore, we conclude that changes in gene expression in response to gravitropic and phototropic stimulation are mediated by two signal transduction pathways that vary in their dependence on changes in $InsP_3$.

Key-words: gravitropism; inositol phosphate; phospholipase C; photomorphogenesis; phototropism; transcription.

INTRODUCTION

The direction of plant growth is mainly determined by light and gravity. Primary roots grow towards the vector of gravity (positive gravitropism) and red light (positive phototropism), but away from white and blue light (negative phototropism). The known sensing and signal transduction pathways of light and gravity responses are very different. The vector of gravity is sensed via sedimentation of starch-containing amyloplasts (statoliths) in the columella cells of the root tip (Juniper *et al.* 1966; Sack & Kiss 1989; Blancaflor, Fasano & Gilroy 1998, 1999), while light is sensed by chromophores in the cytosol (reviewed in Chen, Chory & Fankhauser 2004).

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How the sedimentation of statoliths initiates the gravitropic signal transduction is unclear. Mutant screens identified components of the translocon of outer membrane of chloroplast (TOC) to be involved in gravity sensing (Stanga *et al.* 2009), which can interact with the actin cytoskeleton (Jouhet & Gray 2009). The actin cytoskeleton and other actin-binding proteins are required for gravitropic bending (Hou, Mohamalawari & Blancaflor 2003; Hou *et al.* 2004; Wang *et al.* 2004). It was postulated that either disruption of the actin cytoskeleton or interaction of the statolith with the endoplasmic reticulum could activate mechano-sensitive ion channels (Evans, Moore & Hasenstein 1986; Pickard & Ding 1993; Yoder *et al.* 2001; Blancaflor 2002; Leitz *et al.* 2009).

The fastest responses after gravity stimulation were measured for phospholipase C (PLC)-mediated changes of inositol 1,4,5-triphosphate ($InsP_3$) levels in oat and maize pulvini. A transient fivefold increase in $InsP_3$ within 10 s of gravity stimulation in the lower half of the pulvinus was detected (Perera, Heilmann & Boss 1999; Perera *et al.* 2001). Increases of $InsP_3$ concentrations are generated by activation of PLC which hydrolyses phosphatidylinositol-4,5 bisphosphate [PtdIns(4,5)P_2] into diacylglycerol and $InsP_3$ (Fig. 1). Hydrolysis of $InsP_3$ through inositolpolyphosphate-5-phosphatases (InsP 5-ptases) or by phosphorylation by inositol phosphate kinases reduces $InsP_3$ levels (Berdy *et al.* 2001; Stevenson-Paulik, Odom & York 2002).

Inhibition of PLC by the aminosteroid U73122 blocked $InsP_3$ accumulation and reduced gravitropic bending (Perera *et al.* 1999, 2001). Changes in cytosolic $InsP_3$ levels have been shown to regulate changes in cytosolic Ca^{2+} concentrations (Gilroy, Read & Trewavas 1990; Shacklock, Read & Trewavas 1992; Berridge 1993; Tang *et al.* 2007). Distinct cytosolic Ca^{2+} signals occur within 90 s after gravity stimulation in *Arabidopsis* seedlings and hypocotyls, with biphasic kinetics very different from cytosolic Ca^{2+} transients evoked by other mechanical stimuli (Plieth & Trewavas 2002; Toyota *et al.* 2008). Two components downstream of $InsP_3$ and Ca^{2+}-mediated signalling known to be involved in plant gravitropism or phototropism are the calcium/calmodulin-dependent protein kinase (MCK1) that is required for light-dependent gravitropic responses in maize roots and the auxin receptor transport inhibitor response 1 (TIR1) that requires binding of $InsP_6$ for its activity (Lu, Hidaka & Feldman 1996; Tan *et al.* 2007).

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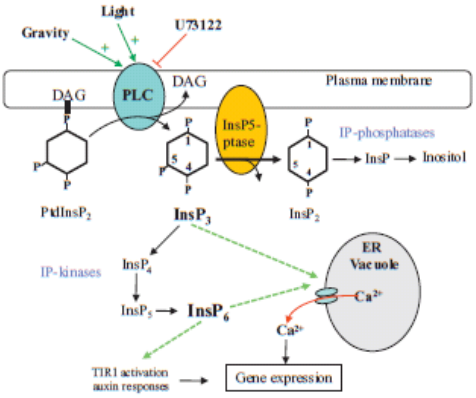

Figure 1. Gravity or light activates phospholipase C (PLC), which hydrolyses phosphatidylinositol-4,5 bisphosphate [PtdIns(4,5)P_2] into diacylglycerol DAG and inositol 1,4,5-triphosphate ($InsP_3$). The $InsP_3$ signal is terminated either by hydrolysis of $InsP_3$ through inositolpolyphosphate-5-phosphatases (InsP 5-ptases) or by phosphorylation through inositol phosphate kinases (IP-kinases). Inhibition of PLC by the aminosteroid U73122 inhibits PLC activity and blocks $InsP_3$ accumulation. Changes in cytosolic $InsP_3$ or $InsP_6$ levels have been shown to regulate changes in cytosolic Ca^{2+} concentrations, thereby activating downstream responses including changes in gene expression.

PLC activation, $InsP_3$ increases and changes in cytosolic Ca^{2+} concentrations also have been described in response to light stimulation by white, blue, red and far-red light (Morse *et al.* 1989; Shacklock *et al.* 1992; Volotovski *et al.* 1993; Harada, Sakai & Okada 2003). Light is sensed through chromophores in the cytosol. In *Arabidopsis*, a small family of phytochromes (PHYA-E) absorb red, far-red and low-fluency blue light (Clack, Mathews & Sharrock 1994; Quail 2002). Blue light/UV-A responses are primarily mediated by cryptochromes (CRY1,2), phototropins (PHOT1,2) and ZLP/ADO (Ahmad & Cashmore 1996; Huala *et al.* 1997; Ahmad *et al.* 1998; Briggs & Christie 2002; Imaizumi *et al.* 2003; Ohgishi *et al.* 2004; Kanegae *et al.* 2006). Light-induced chromophore activation has been shown to regulate changes in gene expression through either phosphorylation of transcriptional regulators or protein degradation (Chen *et al.* 2004). Blue light increases cytosolic calcium concentrations through PHOT1 and PHOT2 activities through a mechanism that requires PLC activity and the endogenous InsP 5-ptase 13 (Baum *et al.* 1999; Folta *et al.* 2003; Harada *et al.* 2003; Chen *et al.* 2008).

The downstream consequences of changes in PLC activity and the associated increases in $InsP_3$ and cytosolic Ca^{2+} concentrations on gene expression in gravitropism and phototropism are unknown. We analysed gravity- or light-induced gene expression changes in transgenic plants constitutively expressing the human type I InsP 5-ptase (InsP 5-ptase). We chose this heterologous enzyme because it specifically hydrolyses the soluble inositol phosphates $InsP_3$ and $InsP_4$, and not the inositol phospholipids (Majerus, Kisseleva & Norris 1999). The known plant InsP 5-ptases can hydrolyse both lipid substrates and soluble inositol phosphates, and are not specific for $InsP_3$ hydrolysis (Gillaspy 2010). Expression of the heterologous InsP 5-ptase in *Arabidopsis* reduced $InsP_3$ in roots and hypocotyls to less than 5% of wild-type (WT) levels. In these transgenic plants, increases in $InsP_3$ levels upon gravity stimulation of the inflorescence stem were abolished, and gravitropic bending in roots, hypocotyls and inflorescence stems was delayed. Basipetal auxin transport in the root tips of the transgenic lines was inhibited in a naphthylphthalamic acid (NPA)-independent mechanism, and the development of a gravity-induced asymmetric auxin gradient was delayed, showing that $InsP_3$ signalling is upstream of auxin and regulates its transport capacity (Perera *et al.* 2006), possibly by regulation of vesicle trafficking that is required for auxin efflux carrier activation (Wang *et al.* 2009). Rapid transient Ca^{2+} responses to a cold or salt stimulus are reduced by ~30% in the transgenic plants (Perera *et al.* 2008).

We analysed gravity- and light-induced changes in transcript abundances in root apices of WT and transgenic *Arabidopsis* lines expressing InsP 5ptase. In the transgenic roots, known light-regulated genes were significantly up-regulated compared to WT. After gravity stimulation, changes in transcript abundances clustered into two distinct groups of co-expressed genes: those that exhibited the same response in the transgenic and WT roots, and those that showed significant differences between the two lines. Gravity-regulated changes in transcript abundances in *Arabidopsis* root apices are mediated by two signal transduction pathways that differ in their dependence on changes in $InsP_3$ metabolism. Genes that were gravity-regulated by an $InsP_3$-dependent pathway were also light-regulated in an $InsP_3$-dependent manner. This suggests that $InsP_3$-dependent signal transduction mediates regulation of gene expression to integrate growth responses to light and gravity stimulation in *Arabidopsis* root apices.

MATERIALS AND METHODS

Plant growth and experimental design

Seeds [Col-0 for WT, vector control C2 and two independent *Arabidopsis* lines expressing Ins P5-ptase (2–8, 2–6; both T5 generation)] were sterilized and then stratified for 2 d at 4 °C in the dark on vertical agar plates (8 g L^{-1}) containing 4.3 g L^{-1} Murashige and Skoog salts (Sigma, St Louis, MO, USA), 0.5 g L^{-1} MES and 1 g L^{-1} sucrose (Sigma). For the gravitropism experiments, seeds were germinated in the dark for 7 d at 22 °C. Eighteen hours prior to the experiment, the plates were transferred and placed vertically in front of a dim green light (bandpass, 525 ± 15 nm; <0.1 μmol m^{-2} s^{-1}) and randomized. The transverse orientation of the light relative to the vertical plates ensured that the direction of the light relative to the seedling did not change after gravity stimulation. Reorientation, harvesting

and microarray analysis was carried out as described by Kimbrough *et al.* (2004). In short, gravity stimulation was induced by clockwise 135° reorientation of the plates. Root apices were harvested either before (0 min = vertical control) or 2, 5, 15, 30 and 60 min after reorientation of the plates. Seedlings were harvested by placing the plates in a horizontal position and immediately pouring RNAlater (Ambion, Austin, TX, USA) onto the plates. While submerged in RNA later, the seedlings were transferred onto a chilled glass slide, and ca. 0.7 mm of the root apices was separated from the seedlings and stored for a day at −20 °C. For each time-point and replicate, root tips from ca. 150 seedlings were pooled. Microarray analysis was carried out on two independent experiments.

RNA profile preservation and isolation

Homogenization of tissue was performed using FastRNA (Qbiogen, Carlsbad, CA, USA) lysing matrix tube with 450 μL of extraction buffer. The homogenate was poured directly into a Qiashredder spin column (Qiagen, Valencia, CA, USA). After DNA digestion, purified RNA was eluted from the column with 20 μL of water.

Sample processing and analysis

Amplified complementary RNA was synthesized according to Affymetrix GeneChip eukaryotic small sample target labelling assay II (Affymetrix, Santa Clara, CA, USA). Ten micrograms of fragmented biotin-labelled cRNA was hybridized to an Affymetrix ATH1 whole-genome array for 16 h at 45 °C. The arrays were washed and stained with a streptavidin R phycoerythrin conjugate (Molecular Probes, Eugene, OR, USA) on a GeneChip Fluidics Station 400 (Affymetrix) and scanned with a GeneArray scanner (Agilent, Palo Alto, CA, USA).

Analysis of microarray data

Microarray data were analysed using MAS5 (Affymetrix). Scanned arrays were normalized to baseline array with median overall expression using dCHIP (Li & Wong 2001). Transcripts with a mean fluorescence intensity value of <50 for each line were considered not expressed above background. TIGR TM4 Microarray software suite (3.1) was used to analyse significant changes between experiments [analysis of variance (ANOVA) or *t*-test] and for hierarchical cluster analysis (Saeed *et al.* 2003). Only genes with transcript abundance changes in at least two consecutive time-points and with the same tendency in both biological replicates were considered significant in this study.

Real-time RT-PCR

Harvesting, RNA extraction and cDNA synthesis were carried out as described above. PCR primers were designed using OligoAnalyzer version 3.0 (http://www.idtdna.com/scitools/scitools.aspx) to create amplicons 100–150 bp in length from regions within 500 bp from the 3′ end of cDNA. Quantitative PCR was performed using an ABI7900HT sequence detection with SYBR Green (ABI, Foster, CA, USA). Data were analysed using ABI SDS software (ABI), and gene expression data were calculated using the $2^{\wedge}\Delta C_T$ method (Livak & Schmittgen 2001). Actin-8 was quantified as an internal control (F: 5′ CTTTCCGGTTACAGC GTTTG 3′; R: 5′ GAAACGCGGATTAGTGCCT 3′). Changes in transcript abundances of the specific genes were normalized to their abundance prior to gravity stimulation or to their respective dim green light controls.

PLC experiments

U73122 and U73343 (Biomol Research Laboratory, Plymouth, PA, USA) were dissolved in chloroform, dispensed in aliquots and the solvent was evaporated by a stream of nitrogen according to the manufacturer's protocol. Immediately before use, the compounds were dissolved in 100% dimethylsulphoxide (DMSO) for a final concentration of 10 mM. This stock solution was diluted 1:1000 (v/v) in water to a final concentration of 10 μM. Maintaining the vertical orientation of the plates, the seedlings were sprayed with the 10 μM solutions of U73122 or U73343 or 0.1% (v/v) DMSO in water as a control, and incubated at room temperature for 30 min prior to gravity or light stimulation.

Light exposure

In all the experiments with light sources, the plates were placed 20 cm away from the source of light. For exposure to directional light, the plates were placed vertically into wooden boxes (31 × 31 × 31 cm) with one side open for unfiltered incandescent white light (25.77 μmol m^{-2} s^{-1}) or with filters for specific wavelengths. The light intensity was quantified with a Traceable Enviro-Meter (Fisher Scientific, Suwanee, GA, USA). For dark conditions, the open side of the box was covered with aluminium foil. Dark-grown *Arabidopsis* seedlings on vertical plates were exposed to white light (25.8 μmol m^{-2}s^{-1}), red light (650 nm, 1.2 μmol m^{-2} s^{-1}), far-red (750 nm, 0.4 μmol m^{-2} s^{-1}), blue light (450 nm; 0.32 μmol m^{-2}s^{-1}) or dim green light (545 nm, <0.1 μmol m^{-2}s^{-1}) for 5 min from above or as indicated in Fig. 9. For gravitropism experiments, the dark-grown seedlings on the vertical plates were placed vertically, transverse to the dim green light source for 18 h prior to reorientation. This way, the relative orientation of the seedling towards the light source remained the same after reorientation of the plates.

Phototropic response

Seeds of WT, and transgenic lines 2–6 and 2–8 were germinated and grown for 4 d on vertical plates (12 seedlings per line) under an incandescent white light from above, and then transferred for 24 h into a box with unidirectional blue

light (450 nm; 0.32 μmol m^{-2} s^{-1}) transverse to the longitudinal axis of the seedlings as indicated in Fig. 9. The plates were photographed with a digital camera (Konica Minolta Dimage A2, 8 megapixel, Marunouchi, Tokyo, Japan) before and after blue light exposure, and the differences in hypocotyl orientation were quantified from four independent biological replicates. The photographic images were analysed using the software ImageJ 1.42 (http://rsbweb.nih.gov/ij/).

RESULTS

Expression of InsP 5-ptase affected abundance of specific transcripts in *Arabidopsis* roots

To identify changes in gene expression that were caused by the transgene in the absence of tropistic stimulation, we compared whole-genome transcript abundances in vertical root apices from two independent InsP 5-ptase expressing transgenic lines (2–8, 2–6), the vector control line (C2) and WT prior to reorientation (0 min time-point). Transcripts showing significant ($P < 0.0001$; *t*-test) differences between the control lines (WT, C2) and the transgenic lines (2–8, 2–6) were subjected to hierarchical clustering.

We found 95 genes with steady-state transcript abundance levels significantly higher in the root apices of both transgene-expressing lines (2–8, 2–6) compared to the controls (WT, C2), while transcripts of only 44 genes were significantly lower in the transgenic plants (Supporting Information Fig. S1 for annotated genes; full data set in Supporting Information Table S1). Of those 95 genes up-regulated in the transgenic plants, 14 were plastid encoded and four were encoded on the mitochondrial chromosomes, while all 44 down-regulated transcripts were nuclear encoded. Several transcripts that were strongly up-regulated in the transgenic lines are known light-induced genes (Fig. 2a).

Because we used green light to handle the experiments, we compared our array results to the green light-regulated genes (Dhingra *et al.* 2006). Seventeen of the 139 differentially expressed genes from our set were also regulated by green light. Plastid-encoded transcripts (eight genes) that were down-regulated by green light in Dhingra's experiment showed increased abundance in the transgenic lines compared to the WT under green light. None of the genes further investigated in this study were shown to be affected by green light.

We compared transcript abundances from root apices of WT and transgenic lines of known light receptor genes and genes with key roles in light-regulated photomorphogenesis. Expression of InsP 5-ptase affected

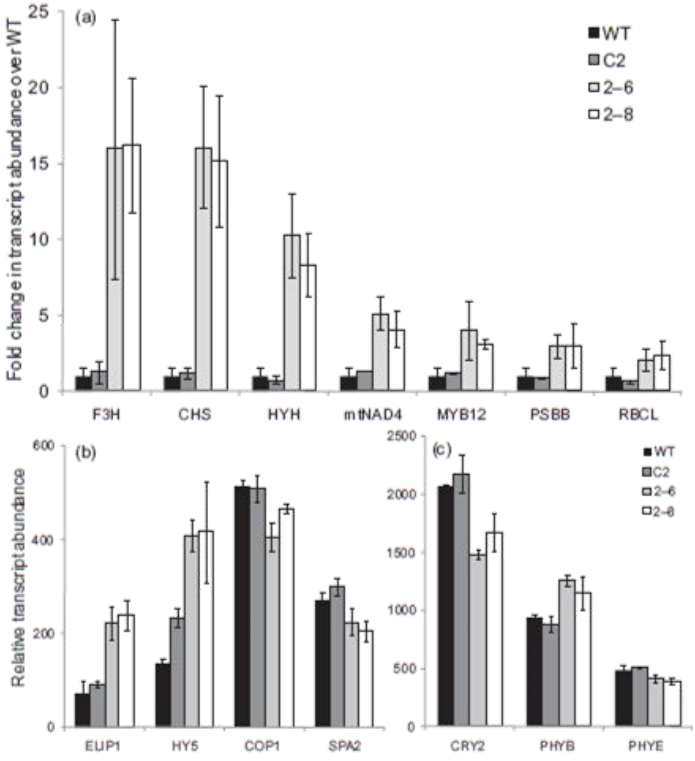

Figure 2. Expression of inositolpolyphosphate-5-phosphatase (InsP-5ptase) affects transcript abundances of several genes involved in photomorphogenesis and light perception. Transcripts from known light-induced genes were several-fold higher in the root apices of the transgenic lines (a). Transcripts from genes with known function in the activation (*HY5*, *ELIP*) or repression (*COP1*, *SPA2*) of photomorphogenesis (b) and the receptors for red/far-red (*PHYB*, *PHYE*), as well as the blue light (*CRY2*), were also differentially expressed between the control and transgene-expressing lines (c). Error bars represent SD; $P < 0.005$ between control [wild-type (WT), C2] and transgenic lines (2–8, 2–6).

transcript abundances of several genes with known function in activation (*HY5*) or repression (*COP1,SPA2*) of photomorphogenesis in dark-grown *Arabidopsis* root apices (Fig. 2b). The red/far-red light receptor genes *PHYB* and *PHYE*, as well as the blue light receptor *CRY2*, were also differentially expressed between the control and transgene-expressing lines (Fig. 2c), while transcript levels of *PHYA*, *PHYC*, *PHYD* and *CRY1* or phototropins were not affected by expression of the transgene.

Changes in transcript abundances in response to gravity

Because previous work had shown that rapid changes in transcript abundance occur with gravity stimulation in WT *Arabidopsis* root apices (Kimbrough *et al.* 2004), we carried out whole-genome microarray analysis of root apices from the transgenic line (2–8) in a time-course before and 2, 5, 15, 30 and 60 min after gravity stimulation by reorientation under the same experimental conditions. Gravity-regulated transcript abundances in line 2–8 were identified by ANOVA as the same (up or down) changes in at least two consecutive time-points in both biological replicates (time/line $P < 0.05$). Out of the 22 766 transcripts analysed, 958 responded to gravity stimulation in the transgenic line during the first hour after reorientation (Supporting Information Table S2). We analysed the differences between gravity-induced transcript abundance changes in root apices from the InsP 5-ptase expressing 2–8 and the equivalent data set from WT root apices (Kimbrough *et al.* 2004). Raw fluorescence intensity data were normalized together and analysed by two-factor ANOVA (WT versus 2–8/time) and identified line-specific, time-specific and interaction-specific transcriptional changes (Supporting Information Table S3b–d).

This analysis yielded two major groups of regulated transcripts. The first group comprises 153 transcripts that responded in the same way to gravity stimulation in WT and transgenic lines (Fig. 3a). Gravity-induced transcript abundances of these genes were apparently not affected by expression of the InsP 5-ptase, and therefore the lowered basal InsP$_3$ levels in the transgenic lines. The second group shows significant changes between WT and transgenic lines (Fig. 3b). Gravity-induced transcript abundances of genes in this group appear to be affected by expression of the InsP 5-ptase and changes in basal InsP$_3$ levels.

Hierarchical clustering distinguished major groups of gravity-regulated InsP$_3$-dependent transcripts (Fig. 3b). In cluster A, the transcript abundances were higher in the transgenic lines than in WT. Transcripts encoded by mitochondrial (23) and chloroplast (30) genes were over-represented in cluster A. Transcript abundances of genes in cluster B showed the opposite trend with up-regulated transcript abundances in the WT plants, while in the transgenic lines these transcripts were mostly down-regulated (Fig. 3b). Interestingly, transcript abundance of *PHYA*-activated transcription factor *HFR1* showed significant increases in response to gravity stimulation in WT, but not the transgenic line 2–8, indicating that *HFR1* expression requires InsP$_3$-mediated signalling (Supporting Information Fig. S1). To investigate further the role of InsP$_3$-mediated signal transduction in transcriptional regulation of tropistic signal transduction in roots, we focused on four representative genes from cluster B (*At2g16005-MD2-related lipid recognition domain protein*, *At4g11310-cysteine protease*, *At4g23670-major latex protein*, *At5g38020-SamT-like*) and two representative genes from cluster A (*ATMG00630-orf110b*, *AtMg00690-orf240a*) (Fig. 3c). These genes were selected because they represent two different clusters, show fast and transient changes in transcript abundances and represent different genomes (nuclear and mitochondrial).

PLC activity is required for transcriptional regulation of the gravitropic response

An alternative approach to reduce InsP$_3$ levels is to inhibit PLC activity. PLC hydrolyses PtdInsP$_2$ to produce InsP$_3$ and diacylglycerol (Berridge 1993). The aminosteroid U73122, but not its analog U73343, has been shown to effectively inhibit plant PLCs *in vitro* (Staxen *et al.* 1999), and reduce InsP$_3$ levels *in vivo* in *Arabidopsis* and other plants (Takahashi *et al.* 2001; Vergnolle *et al.* 2005; Parre *et al.* 2007). Inhibition of PLC by U73122 abolished the differential increase in InsP$_3$ levels in gravity-stimulated oat pulvini, and attenuated its gravitropic growth response without affecting growth (Perera *et al.* 2001). We analysed the effect of PLC inhibition on growth, gravitropic bending and differential expression of specific transcripts after gravity stimulation in the root apices of WT plants. Application of 10 μM U73122, 10 μM U73343 or 0.1% (v/v) DMSO did not significantly affect vertical root growth (elongation) within 24 h (Fig. 4a), while bending of those root apices was significantly and specifically reduced by the inhibitor U73122 after 6 h of gravity stimulation. The inhibitory effect was no longer observed after 24 h of gravity stimulation (Fig. 4b).

We analysed the changes in transcript levels of the representative genes (Fig. 3c) in root apices of the control [0.1% (v/v) DMSO; inhibitor analog U73343] and treated (inhibitor U73122) samples using real-time PCR (Fig. 5a,b; Supporting Information Fig. S3c,d). Overall, inhibition of PLC had the same effect as expression of InsP 5-ptase in the transgenic lines on gravity-induced transcript abundance changes. Transcripts that were up-regulated by gravity stimulation in WT roots showed reduced transcript abundances in the transgenic line (2–8), as well as after PLC inhibitor treatment (Fig. 5a; Supporting Information Fig. S3c), while those transcripts that were up-regulated in the InsP$_3$-dampened transgenic lines showed the same response in inhibitor-treated WT lines (Fig. 5b; Supporting Information Fig. S3d). This is shown not only for nuclear genes, but also transcripts encoded on the mitochondrial genome. The inactive inhibitor analog or DMSO alone did not significantly affect the gravity-induced transcript changes in the WT plants.

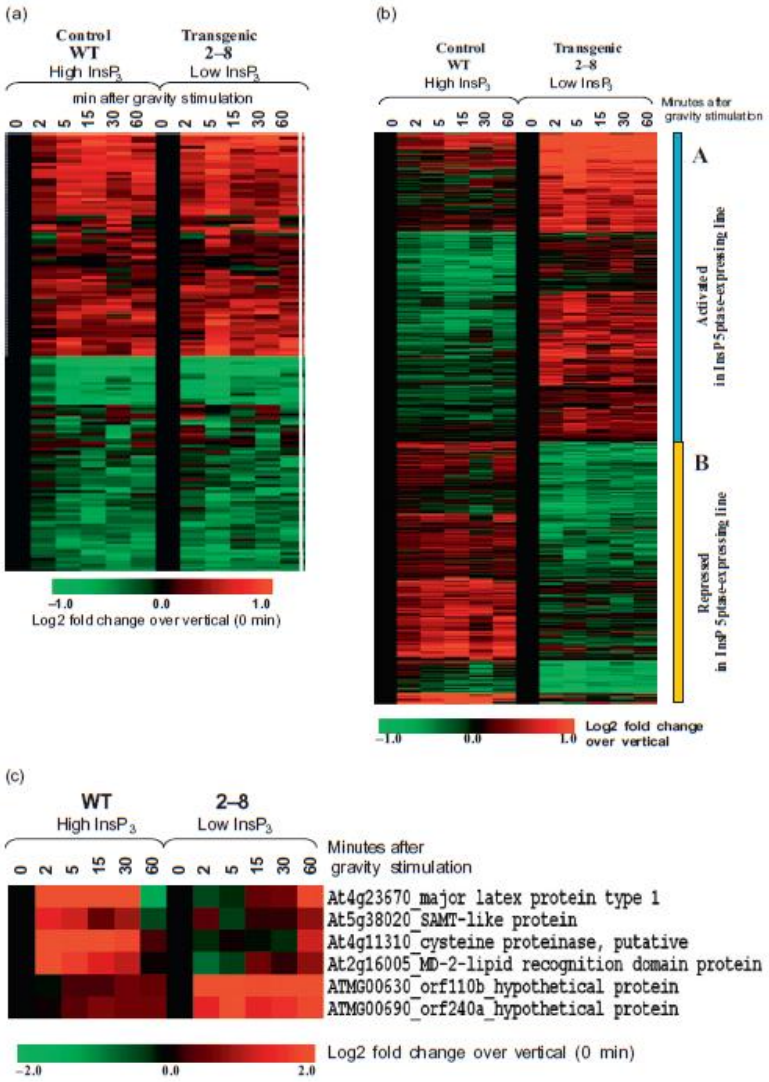

Figure 3. (a) Gravity-induced transcript abundance changes independent of transgene expression. Transcript abundances of 153 genes showed the same quantitative and temporal changes in a time-course after gravity stimulation in the root apices from wild type (WT) and inositolpolyphosphate-5-phosphatase (InsP 5-ptase)-expressing lines. (b) InsP 5-ptase mediates changes in transcript abundances in response to gravity stimulation. Hierachical cluster analysis identified two major clusters that differ in their overall gravity-induced gene expression between WT and 2–8 root apices. (c) Selected transcripts from clusters B (top four) and A (bottom two) from Fig. 2b showing differential Ins 5-ptase-mediated activation or repression of transcription in response to gravity stimulation. These genes were further analysed for their response to inhibition of phospholipase C (PLC) and light stimulation.

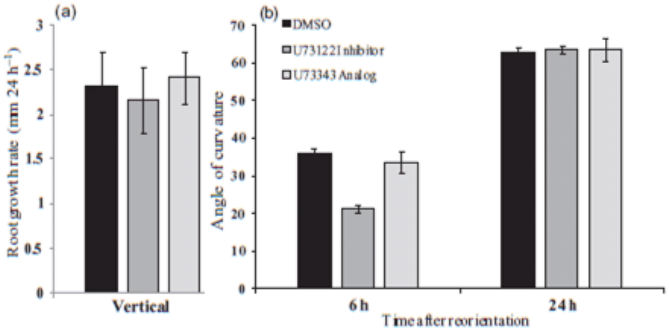

Figure 4. U73122 inhibition of phospholipase C (PLC) transiently reduced the gravity response in wild-type (WT) roots in the dark. Inhibition of PLC by U73122 did not reduce root elongation rates (a), but transiently and significantly reduced the bending response in root apices after 90° reorientation compared to dimethylsulphoxide (DMSO) and inactive analog U73343 (b). Error bars represent SD.

Light regulation of specific gravity-induced genes

When dark-grown WT and transgenic *Arabidopsis* seedlings were stimulated by continuous white light from above, the same transcripts that responded to reorientation in the dark in the WT root apices were also fast and transiently up-regulated in the WT root apices in response to the light exposure (Fig. 6). These transient changes were not detected in the transgenic 2–8 line in response to gravity and were also not seen in response to white light (Figs 3c & 6, respectively). We further delineated the light-induced changes of transcript abundance for specific genes with respect to light quality. Dark-grown *Arabidopsis* seedlings (WT, 2–8) were exposed to white light, red light, far-red light, blue light or dim green light from above for 5 min. Transcript abundance changes in the root apices for the selected genes (*At2g16005*, *At5g38020*, *At4g11310*) were analysed using real-time PCR and normalized to the respective WT or transgenic controls under dim green light. In the wild-type plants, transcript abundances of these genes were significantly up-regulated in response to white (Fig. 6) or far-red light, but either not significantly regulated or down-regulated in response to blue or red light. These

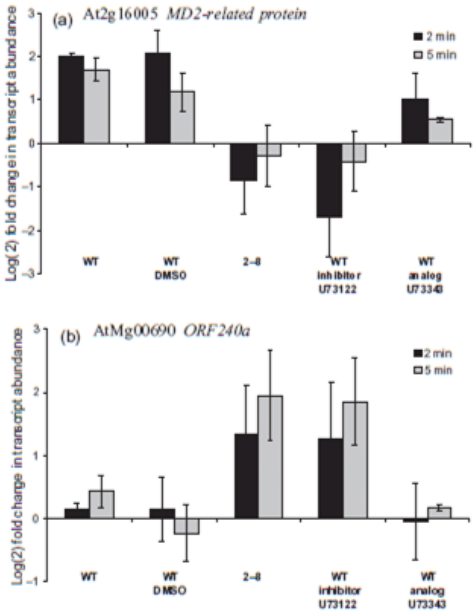

Figure 5. Inhibition of phospholipase C (PLC) by U73122 in gravity-stimulated wild-type (WT) roots mimicked the effect of transcript abundance changes in gravity-stimulated root apices in the transgenic line 2–8 (a,b). Root apices from WT or 2–8 plants were grown on vertical plates and treated 30 min before gravity stimulation. Root apices were harvested either before (reference), or 2 or 5 min after 135° reorientation in dim green light. Error bars represent SD. Additional examples are shown in Supporting Information Fig. S3c,d.

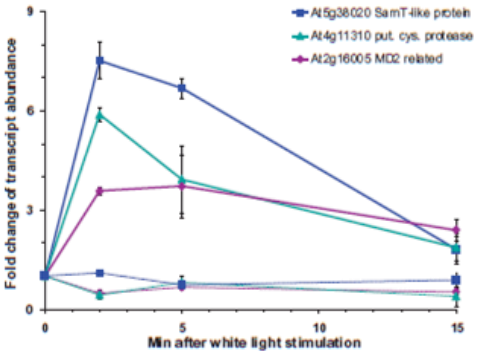

Figure 6. Light-induced changes in gene expression. Transcripts that responded to gravity stimulation in wild-type (WT) apices were also up-regulated in WT root apices when etiolated seedlings were exposed to white light (solid lines), but not in the transgenic line 2–8 (dashed lines). Changes in transcript abundances were calculated relative to their abundances prior to stimulation of the respective line. Error bars represent SD.

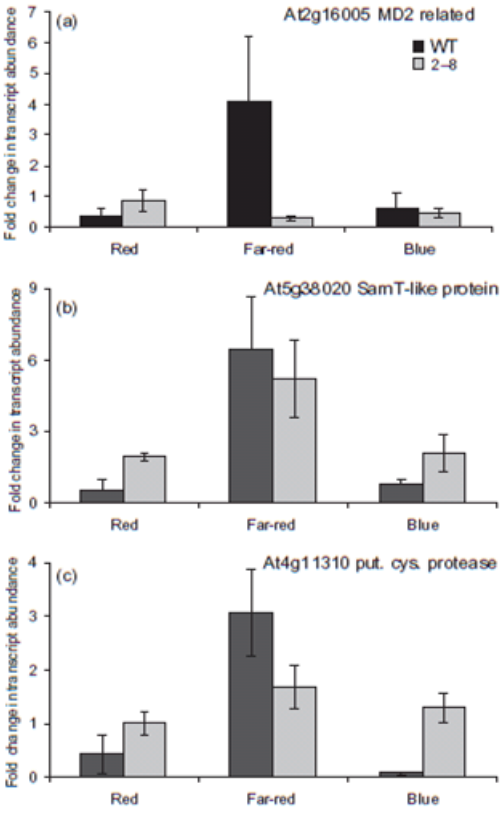

Figure 7. Changes in transcript abundances relative to dim green light in root apices from wild type (WT) and line 2-8 after 5 min of exposure to red, far-red or blue light. (a–c). Error bars represent SD.

transcript abundance changes in response to light quality were significantly different in the root apices of the transgenic lines (Fig. 7a–c).

To test whether these differences in response to different wavelengths were caused by changes in $InsP_3$ metabolism, we pretreated root tips of WT and transgenic 2–8 with the PLC inhibitor (U73122) prior to exposure to white or far-red light. Similar experiments with blue or red light were not carried out because of the relatively small transcript abundance changes. Using white and far-red light treatments, we compared the transcripts levels in the WT and the transgenic line (2–8) to those in the WT with and without prior treatment with the inhibitor U73122. Transcript abundances of At2g16005 (MD2-related protein) and At4g11310 (cysteine proteinase) in WT root apices after treatment with U73122 showed a similar relative response to white and far-red light as in the root apices of the transgenic line (2–8) without the inhibitor (Fig. 8a,b).

Photomorphogenic and phototropic growth is modified in transgenic hypocotyls

To test if the transgenic plants with reduced $InsP_3$ levels show any phenotypic differences in their growth and phototropic responses, we analysed their skotomorphogenic and photomorphogenic growth, as well as their reponses to unidirectional blue light. Seedlings were grown for 4 d on vertical plates in low-fluency white light from above or in the dark. The seedlings were then exposed to unidirectional low fluency (0.32 μmol m^{-2} s^{-1}) blue light (450 nm) from a 90° transverse angle as indicated in Fig. 9. Hypocotyl length, root length and angles of hypocotyl bending were quantified before or after 24 h of blue light exposure.

Light-grown transgenic seedlings had significantly longer hypocotyls and roots compared to WT seedlings (Fig. 9a,c), while dark-grown transgenic seedlings did not show any significant growth phenotype compared to WT (Fig. 9e,g).

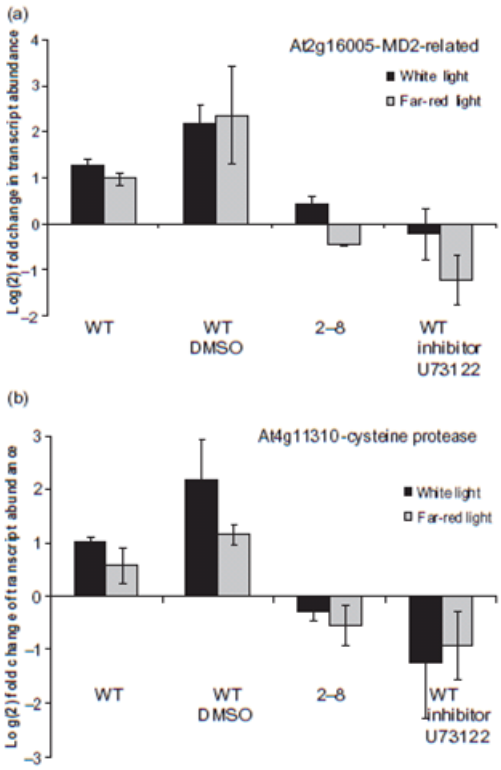

Figure 8. Inhibition of phospholipase C (PLC) by U73122 in wild-type (WT) root apices prior to exposure to white or far-red light reduced transcript abundance of these genes with the same trend as found the transgenic line 2–8 (a,b). Changes are plotted as log (2)-fold changes over transcript levels in dim green light for each line. Error bars represent SD.

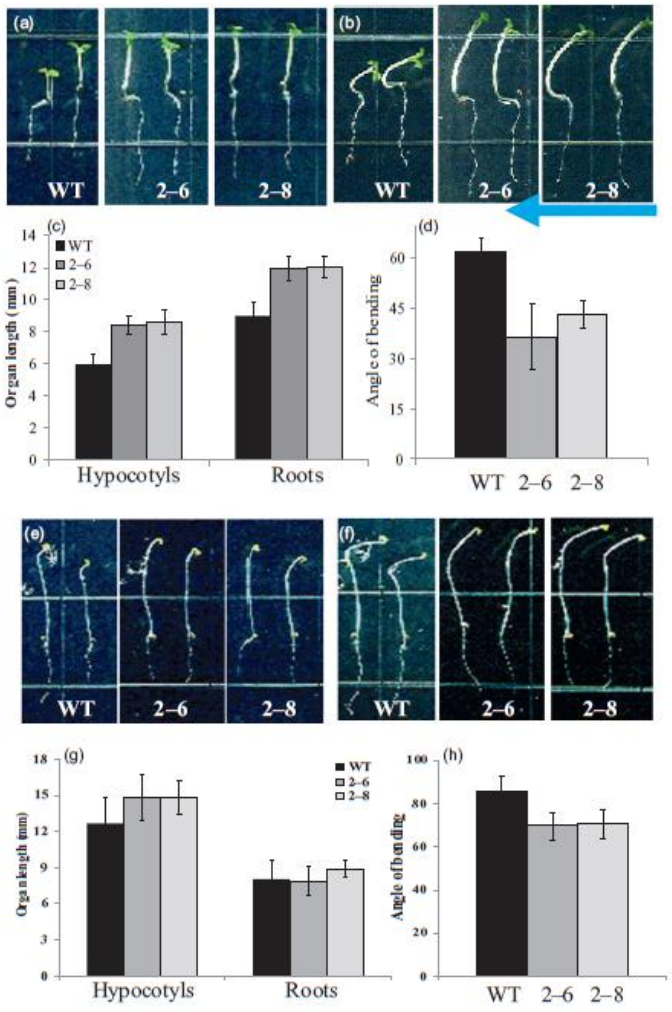

Figure 9. Inositolpolyphosphate-5-phosphatase (InsP 5-ptase)-expressing lines 2–6 and 2–8 grow significantly faster than wild-type (WT) seedlings in white light (a,c), but not in the dark (e,g). The phototropic response of the hypocotyls from the transgenic seedlings to blue light was significantly reduced in light-grown and dark-grown seedlings (b,d,f,h); blue light direction indicated by arrow. Reduced hypocotyl bending was significantly different for both transgenic lines and WT, but not between the transgenic lines (four independent bioreps, 12 seedlings per line each, $P < 0.001$, *t*-test). Error bars represent SD.

Hypocotyls from the transgenic seedlings grown under light or dark conditions showed a reduced angle of phototropic bending after exposure to directional blue light for 24 h (Fig. 9b,d,f,h). We did not quantify phototropic responses in the root apices because they are much weaker than in the hypocotyl and more variable at this low-fluence rate blue light. Red and blue light responses in roots are weak compared to responses to gravity, while in the hypocotyl the phototropic responses are stronger than the gravitropic response (Kiss, Ruppel & Hangarter 2001; Ruppel, Hangarter & Kiss 2001). At fluence rates of about 0.1 μmol m^{-2} s^{-1}, WT roots were shown to bend less than (−20°) in 48 h, while hypocotyls under the same condition bend more than 70° in only 12 h of exposure to blue light (Sakai *et al.* 2000).

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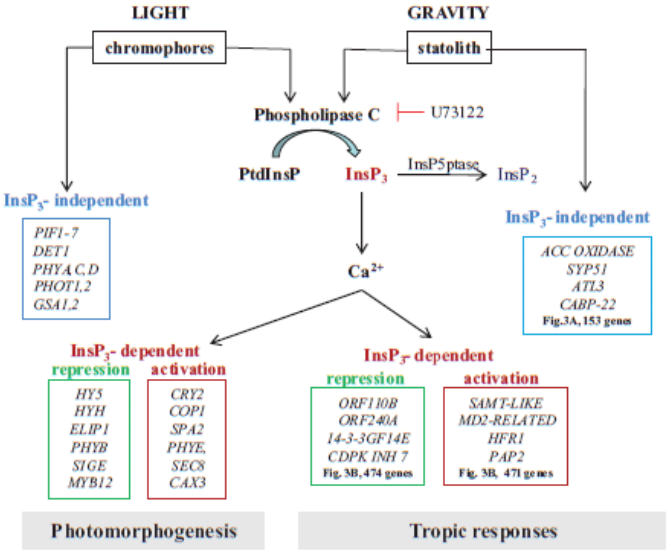

Figure 10. Model of inositol 1,4,5-triphosphate ($InsP_3$)-mediated regulation of transcription. Light- and gravity-induced gene expression is mediated through two signal transduction pathways. The integration of both pathways is regulated through an $InsP_3$-dependent pathway in roots, while $InsP_3$-independent signal transduction regulates stress-specific gene expression. Basal levels of $InsP_3$ mediate changes in gene expression that reduce cell elongation during photomorphogenesis.

DISCUSSION

Transcriptional regulation of gravitropism: dual pathways or dual receptors?

Comparison of transcript abundance changes in root apices of the WT and InsP 5-ptase expressing lines revealed 153 transcripts with no significant differences (Fig. 3a) after gravity stimulation. Gravity-induced expression of these genes apparently was not affected by changes in $InsP_3$ metabolism. On the other hand, 1031 transcripts showed line-specific differences after gravity stimulation (Fig. 3b). This indicates that expression of these genes includes a component that involves $InsP_3$-mediated signal transduction after gravity stimulation.

Therefore, gravity-induced transcriptional regulation apparently has an $InsP_3$-mediated component and a component that is gravity induced, but does not require increases in $InsP_3$ levels for its regulation of gene expression (Fig. 10). Reduction of the $InsP_3$ concentration through expression of InsP 5-ptase or inhibition of PLC never completely abolished the gravitropic or phototropic bending, but reduced the kinetics or degree of its response (Perera *et al.* 2001, 2006; Repp *et al.* 2004).

Our results show that two pathways ($InsP_3$ mediated and $InsP_3$ independent) are involved in the regulation of gravity-induced gene expression. It is not clear if both pathways are activated by the same or two different sensing mechanisms. The molecular mechanism of gravity sensing is not known, although the sedimentation of statoliths is clearly involved in the perception of gravity. The concept of dual receptors for sequential gravity sensing has been suggested before (LaMotte & Pickard 2004a,b), but cannot be distinguished in our approach from the possibility of a single receptor activating two separate signal transduction pathways. Most of those transcripts that are regulated independent of $InsP_3$ metabolism (i.e. are regulated similarly in WT and 2–8, Fig. 3a) are annotated as transcription factors or protein kinases. Examples include several transcripts of Ca^{2+}-regulated and Ca^{2+}-regulating protein families, and the ethylene-forming enzyme 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase At1g05010) which were up-regulated in response to gravity stimulation.

$InsP_3$ mediates repression and activation/de-repression of transcription

Temporal changes of transcript accumulation between WT and 2–8 in response to gravity stimulation distinguished the presence of at least two mechanisms by which $InsP_3$-mediated signalling affects gravity- and light-induced changes in transcript abundance. One group of genes showed increased transcript levels in the transgenic lines compared to the WT (Fig. 3b, cluster A), while the other group of genes showed increased transcript abundances after gravity stimulation in the WT compared to the transgenic lines (Fig. 3b, cluster B). For select transcripts from both clusters, we showed that the same responses can be brought about either by increasing the hydrolysis of $InsP_3$ (using the InsP 5-ptase transgenics) or by inhibition of $InsP_3$ synthesis through PLC activity (using the inhibitor U73122). For these transcripts, the difference in gravity-induced transcript levels seems to be caused by lower $InsP_3$ levels. High levels of $InsP_3$ in the WT plants mediated the repression of genes from cluster A, while genes from cluster B required high $InsP_3$ levels for their transcriptional activation.

Compared to all other clusters and to the whole genome, transcripts encoded in plastidal or mitochondrial genome were over-represented in cluster A. Thirty out of the 128 plastid encoded genes were up-regulated in response to gravity stimulation in the transgenic line only, and 23 out of the 57 mitochondrial encoded transcripts in *Arabidopsis* were up-regulated. Application of the PLC inhibitor, but not the inactive analog, prior to gravity stimulation in WT seedlings, mimicked the effect on mitochondrial encoded transcript abundances (Fig. 5b; Supporting Information Fig. S3d). This result shows that high concentrations of InsP$_3$ in WT mediate repression of the transcription of genes encoded in the plastid and mitochondrial genomes, possibly through changes in Ca^{2+} and/or expression of nuclear-encoded transcriptional regulators of plastid encoded genes like sigma factors.

Inositol phosphate signalling in light responses

Light induces the transition from skotomorphogenic to photomorphogenic growth and development in dark-grown seedlings. This transition is characterized by inhibition of hypocotyl growth, expansion of the cotyledons and differentiation of etioplasts into photosynthetically active chloroplasts. Differences in growth rates between the InsP 5-ptase-expressing lines and WT were light dependent. When grown in low white light for 4 d, hypocotyl and root length of the transgenic seedlings were significantly increased compared to those of WT seedlings (Fig. 9c). These differences were not observed in dark-grown seedlings (Fig. 9g). This shows that InsP$_3$ signalling was involved in light-regulated gene expression in roots. Several genes known to be key regulators of photomorphogenesis were differentially expressed in the dark between WT and InsP 5-ptase transgenic seedlings (Figs 2 & 10). Although we did not observe any differences in the cotyledon morphology or chlorophyll content during light-induced photomorphogenesis between the control and transgenic lines, many known light-induced genes were significantly up-regulated in the vertical dark-grown transgenic lines 2–6 and 2–8 (Fig. 2a; Supporting Information Fig. S1) (Ma *et al.* 2001; Tepperman, Hwang & Quail 2006; Sellaro *et al.* 2009). Increased transcript abundances of plastid-encoded transcripts like ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (*RBCL*); photosystem I and II reaction centre proteins (*PSAA*, *PSAB*, *PSAC*); and light-induced nuclear encoded genes for chalcone synthesis (*CHS*, *F3H*), sigma factor E (*SIGE*), the bZIP transcription factor Hy5-like (*HYH*) and myb transcription factor (*MYB12*) in the InsP$_3$-dampened transgenic lines indicate that InsP$_3$-mediated signalling is involved in a wide coordination of transcription in response to light. The nuclear encoded sigma factor SIGE is up-regulated by red light (Monte *et al.* 2004), and regulates the activity of the plastid RNA polymerase and transcription of specific plastid encoded genes (Favory *et al.* 2005). The bZIP transcription factors HY5 and HYH are predominantly involved in bluelight regulation of nuclear transcription for photomorphogenesis (Sellaro *et al.* 2009), and themselves light-regulated as a target of dark-specific protein degradation by COP1 (Holm *et al.* 2002). Transcript abundances of *COP1* and its co-regulator *SPA2* (Fittinghoff *et al.* 2006) were both down-regulated in the transgenic lines, which is consistent with up-regulation of light-induced genes in the dark. The light induction of the R2R3-Myb family transcription factor *MYB12* (At2g46470) is dependent on *HY5* expression (Stracke *et al.* 2010). *MYB12* was strongly up-regulated in the transgenic lines as were its known target genes *chalcone synthase* (*CHS*) and *flavanone-3 hydroxylase* (*F3H*) (Mehrtens *et al.* 2005). However, up-regulation of chalcone synthase mRNA itself was highly variable between experiments. The expression of transcription factor LONG HYPOCOTYL IN FAR-RED1 (*HFR1*) is also regulated in response to light and gravity in an InsP$_3$-dependent mechanism (Supporting Information Fig. S2). HFR1 has been shown to play a role in both gravity and light responses. *Arabidopsis* seedlings over-expressing an N-terminal-truncated version of HFR1 showed photomorphogenic development in the dark and randomization of gravitropic growth in hypocotyls and roots in the dark (Yang *et al.* 2003).

Several gravity-induced genes that were up-regulated in response to white light in WT roots did not respond to light stimulation in the InsP 5-ptase transgenic lines or in WT root tips treated with the PLC inhibitor (Figs 6 & 8). Up-regulation of these transcripts in response to gravity or white light requires InsP$_3$-mediated signalling. This poses the question of the function of these genes in phototropic or gravitropic responses. Light-grown seedlings of knockout mutants for either the MD2-related protein (At2g16005) or the SamT-like protein (At5g38020) had longer roots compared to WT seedlings, and showed significantly faster bending responses to gravitropic stimulation compared to roots from WT seedlings (Kajla *et al.*, unpublished data). This indicates that those proteins act as repressors of gravitropic bending and root growth.

Our results are in agreement with the analysis of *Arabidopsis* knockout mutants defective in endogenous InsP 5-ptase genes. These knockout lines have increased concentrations of InsP$_3$ and exhibit increased hypocotyl elongation rates when grown in the dark, but not in light-grown seedlings, while over-expressing lines show a slight growth inhibition compared to WT seedlings in blue light (Gunesekera *et al.* 2007; Chen *et al.* 2008).

Taken together, these results suggest a correlation between growth rates, light and InsP$_3$ levels: low InsP$_3$ levels are involved in skotomorphogenic hypocotyl elongation in the dark, while light-induced increases in InsP$_3$ levels inhibit cell elongation in roots and hypocotyls during photomorphogenesis. Basipetal auxin transport capacity was reduced and delayed in InsP 5-ptase expressing transgenic line 2–8 compared to WT plants, placing InsP$_3$ signalling upstream of auxin-mediated changes in cell elongation (Perera *et al.* 2006). Auxin transport capacity is determined by auxin

efflux carriers. It has recently been shown that the auxin efflux carrier PIN2 is regulated by InsP 5-ptase 13 and HY5-dependent endocytotic trafficking between the vacuolar compartment and the plasma membrane, regulating light-dependent auxin transport capacity (Laxmi *et al.* 2008; Wang *et al.* 2009).

On the other hand, many well documented light-regulated genes and proteins were not affected by changes in $InsP_3$ levels. Chlorophyll biosynthesis genes like *glutamate-1-semialdehyde aminotransferase* (*GSA12*), the photoreceptors (*PHYA*, *PHYC* AND *PHYD*), the blue light receptors (*PHOT1*, *PHOT2* AND *CRY1*), as well as the phytochrome-interacting transcription factors (*PIF1–7*) and de-etiolated1 (*DET1*), are key regulators of light perception and light-induced transcriptional regulation, but their transcript abundances were independent of $InsP_3$-mediated gene expression (Fig. 10, Supporting Information Table S2). This shows that light-responsive gene expression is regulated by two separate pathways that differ in their dependence on $InsP_3$ metabolism.

Blue light

Of the five identified blue light receptors in *Arabidopsis*, only phototropin 1 (PHOT1 formerly NPH1) is required for the phototropic response to low intensity blue light (Lasceve *et al.* 1999; Sakai *et al.* 2001). Hypocotyls of PHOT1-deficient seedlings are agravitropic under low-fluence-rate blue light (Lariguet & Fankhauser 2004). Blue light-induced changes in cytoplasmic Ca^{2+}concentrations require PHOT1 (Baum *et al.* 1999; Babourina, Newman & Shabala 2002; Folta *et al.* 2003). Increases in cytosolic Ca^{2+} concentrations probably occur via two (possibly consecutive) mechanisms. In *Arabidopsis* leaves and mesophyll protoplasts, blue light stimulates a phototropin-activated voltage-dependent Ca^{2+} channel in the plasma membrane (Harada *et al.* 2003; Stoelzle *et al.* 2003). Blue light-induced changes in cytosolic Ca^{2+} concentrations were strongly reduced by pharmacological inhibition of PLC in wild-type and the phot1 mutants. Blue light-induced *PHOT1* activation increases Ca^{2+}concentrations in the cytosol via PLC. The endogenous InsP 5-ptase13 in *Arabidopsis* has been shown to modulate phototropin1-mediated blue light responses by altering cytosolic Ca^{2+} levels. The knockout mutant of InsP 5-ptase13 has slightly increased concentrations of $InsP_3$ and shows reduced hypocotyl elongation in response to blue light (Chen *et al.* 2008). However, the endogenous InsP 5-ptase enzymes are not membrane associated and are not specific for Ins 1,4,5-P_3 as a substrate as the human type I enzyme used in this study. We show here that blue light-induced phototropic bending in *Arabidopsis* hypocotyls was dramatically reduced (but not completely inhibited) in the InsP 5-ptase-expressing lines compared to the WT (Fig. 9), although overall cell elongation was not affected. Again, this suggests that $InsP_3$-mediated signalling is involved in the inhibition of cell elongation.

Red and far-red light

In etiolated *Arabidopsis* seedlings, the hypocotyls are negatively gravitropic, but upon exposure to red or far-red light, hypocotyl growth orientation becomes random with respect to the vector of gravity (Liscum & Hangarter 1993). The randomization of hypocotyl growth direction in response to far-red light is mediated only by phytochrome A (PHYA), while randomization under red light is mediated by PHYA or PHYB (Poppe *et al.* 1996; Robson & Smith 1996). Red light sensed by PHYA and PHYB induces positive phototropism in *Arabidopsis* roots, but there is little or no phototropic response in roots to unidirectional far-red light (Ruppel *et al.* 2001; Kiss *et al.* 2003). A possible role for $InsP_3$ metabolism in phytochrome signalling was also shown in oat leaf protoplasts, where red or far-red light induced cytoplasmic Ca^{2+} oscillations. Red light increased the synthesis of phosphatidylinositol-4,5 bisphosphate ($PtdInsP_2$), while far-red light significantly reduced the synthesis of $PtdInsP_2$ (Volotovski *et al.* 1993). Red light induced a transient increase (<1 min) in cytosolic free Ca^{2+} in etiolated wheat leaf protoplasts, causing a 20% swelling. The increase of cytosolic free calcium and subsequent swelling was mimicked by release of caged $InsP_3$. Far-red light prevented the red light-induced increases in cytosolic Ca^{2+} (Shacklock *et al.* 1992).

We show here that transcript abundances of selected genes were up-regulated in response to far-red light in WT root apices (Fig. 7a–c), but less or not at all in the transgenic lines. The far-red light-induced increase in transcript abundance required PLC activity (Figs 7 & 8). Far-red light regulates expression of hundreds of genes, but a mechanism for this regulation is not known (Wang *et al.* 2002; Devlin, Yanovsky & Kay 2003; Sato-Nara *et al.* 2004). Our data show that PLC activity and $InsP_3$-mediated signalling are involved in far-red light-induced gene expression.

CONCLUSION

When $InsP_3$ levels were reduced in *Arabidopsis* either through a transgenic approach (hydrolyzing $InsP_3$) or by pharmacological inhibition of its synthesis through PLC, gravitropic and phototropic responses are reduced or delayed. To delineate the role of $InsP_3$ in the regulation of gene expression of tropistic responses, we compared changes in transcript abundances of after light and gravity stimulation in *Arabidopsis* root apices. Our data show that gravity-induced gene expression and light-induced gene expression were regulated by an $InsP_3$-dependent and an $InsP_3$-independent signal transduction pathway (Fig. 10). Genes that responded to gravity in an $InsP_3$-dependent manner also responded to light in an $InsP_3$-dependent way. This suggests that $InsP_3$ signalling might be involved in the integration of both gravitropic and phototropic growth responses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparison of steady-state transcript abundances in root apices of control wild-type (WT, C2) and transgenic (2–6,2–8) lines grown vertically under dim green light. Transcripts of 139 genes exhibited significant differences in their abundance between the two groups ($P < 0.0001$). Known light-regulated genes (yellow circle), as well as genes from the mitochondrial and plastid genome, are increased in their transcript abundance. Only data from transcripts with functional annotation are presented here (Supporting Information Table S1 for full data set).

Figure S2. Gravity-induced changes in *HFR1* transcript abundances require InsP$_3$-mediated signalling. Transcript abundance of the light-regulated transcription factor *HFR1* is up-regulated in wild-type (WT) root apices in response to gravity stimulation, but not in the transgenic line 2–8. Average of two bioreps with similar results.

Figure S3. (c,d) Inhibition of gravity-induced transcript abundance changes in 2–8 can be mimicked by phospholipase C (PLC) inhibitor (additional examples). Error bars represent SD.

Table S1. Transcript abundance changes in vertically grown *Arabidopsis* root apices from transgenic and wild-type (WT) lines.

Table S2. Microarray data of gravity-stimulated root apices from transgenic line 2–8.

Table S3. Comparison of transcript abundance changes in response to reorientation between root apices of wild-type (WT) and inositolpolyphosphate-5-phosphatase (InsP 5-ptase) expressing line 2–8. (a) Same response; (b) line-specific different; (c) time-specific different; (d) interaction-specific different.

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Chapter 3. ROSY1-mediated regulation of tropic bending

INTRODUCTION

Plants are sessile organisms with their roots anchored in the soil. This lack of movement is in part compensated for by their plastic development. Plants are able to redirect their growth in response to changes in the environmental conditions. Gravity is a directional environmental stimulus, which induces a directional growth response in plant organs, called gravitropism (Charles Darwin, 1881). Roots are positively gravitropic, which means that at any given time, a given primary root grows towards the direction of gravity. Being positively gravitropic also means that if the root is reoriented with respect to the direction of gravity, it is able to sense the direction of gravity, and reorient its growth towards the direction of gravity. The site of gravity perception in roots is the columella cells at the root tip (Haberlandt, 1900; Nemec, 1900; Sack, 1991; Kiss et al., 1996).

While columella cells in the root tip are the sites of gravity perception, the site of gravity response is the root elongation zone. The cells in the root elongation zone elongate differentially on opposite sides to enable bending towards the direction of gravity (Ishikawa and Evans, 1993). Because the site of gravity perception and gravity response are spatially separated, upon gravitropic re-orientation, biochemical signal(s) are required to be transported from the columella cells to the elongation zone (Masson, 1995). Some of these biochemical signals are known, but some are yet unknown. Among the known signals that

originate in root tips on gravity stimulus are asymmetric basipetal auxin transport, oscillations in the levels of cytoplasmic Inositol trisphosphate ($InsP_3$) and Ca^{2+}, as well as changes in apoplastic pH at the root tip and in the root elongation zone.

As discussed in chapter 1, upon the gravitropic re-orientation of an *Arabidopsis* root, within two minutes, the auxin efflux carrier PIN3 re-localizes in columella cells (Friml et al., 2002) thereby shuttling more auxin through the lower side of the root compared with the upper side of the root (Ottenschlager et al., 2003). Furthermore, lower auxin concentrations on the upper side of the root leads to increased proteasome-mediated degradation of PIN2 in these cells, causing a steeper gradient in auxin distribution between the upper and the lower root flanks (Abas et al., 2006). In roots, higher auxin concentrations inhibit cell elongation (Rayle et al., 1970). Therefore, the elongation zone cells on the upper side of the root elongate faster than the lower side, enabling the root to bend towards the direction of gravity. But redirection of auxin transport is not the earliest response to gravity stimulus.

The earliest known biochemical response to gravity signal is a spike in the levels of $InsP_3$ in gravity-sensing tissues, generated by the action of light and gravity-regulated enzyme PLC (Record et al., 1999; Perera et al., 2001). Within 10 seconds of gravistimulation, there is a five-fold spike in $InsP_3$ levels in the lower halves of both oat and maize pulvini (Perera et al., 1999; Perera et al., 2001). In transgenic *Arabidopsis* plants with low $InsP_3$ levels, both roots and hypocotyls exhibit reduced gravitropic bending upon re-orientation (Perera et al., 2006). This demonstrates that gravitropic signal transduction has a PLC mediated InsP3 dependent

component. As documented in Chapter 2, the metabolite $InsP_3$ regulates expression of certain light and/or gravity-induced genes in *Arabidopsis*(Salinas-Mondragon et al., 2010).

In gravistimulated tissues, asymmetrical increase in $InsP_3$ levels can explain the gravity-induced asymmetric increase in apoplastic Ca^{2+} levels. In *Arabidopsis* roots, gravity-induced changes in cytosolic Ca^{2+} levels were monitored in plants expressing a fluorescence resonance energy transfer (FRET) based Ca^{2+} sensor yellow cameleon YC3.6 (Monshausen et al., 2011). In these plants, the enhanced-cyan fluorescent protein (ECFP) is expressed in fusion with the enhanced-yellow fluorescent protein (EYFP) with a calcium binding protein Calmodulin in between the two fluorescent proteins. In presence of free cytosolic Ca^{2+}, Calmodulin changes confirmation to bring the two fluorescent proteins together and ECFP excitation results in EYFP fluorescence emission. In *Arabidopsis* seedlings subjected to 90° gravitropic stimulation, within 2-4 minutes the cytosolic Ca^{2+} levels decrease in the epidermal cells of the upper root flank, and within 3-6 minutes, cytosolic Ca^{2+} levels rise in the epidermal cells of the lower root flank. The changes in Ca^{2+} levels are seen to originate at the root cap and spread to the elongation zone within 2 minutes of gravity-stimulation, thereby, directly connecting gravistimulation with Ca^{2+} increase in root tips. The wave of Ca^{2+} reaching the elongation zone is hypothesized to somehow regulate proton fluxes which changes apoplastic pH, and is related to cell expansion in the elongation zone (Monshausen et al., 2011). Presence of free Ca^{2+} in cells is critical for bending, as has been demonstrated

by abolished bending response in tissues treated with calcium chelators such as EDTA(Lee et al., 1983; Poovaiah et al., 1987; Poovaiah and Reddy, 1987; Reddy et al., 1987).

Recent studies have uncovered the importance of $InsP_3$ as a central molecule regulating gravitropic responses in plants. Levels of Myo-inositol , $InsP_3$ and Ca^{2+} have all been shown to be necessary for maintaining polarity of auxin transport carrier PIN proteins, thereby for maintaining auxin transport and gravitropic responses (Luo et al., 2011; Zhang et al., 2011). Excess levels of $InsP_3$ and Ca^{2+}, as seen in *supo1* mutants, do not affect the localization of PIN2 in the cell. But pharmacological inhibition of $InsP_3$ (by U731220) or Ca^{2+} (using chelator EGTA or by blocking Ca^{2+} channels by $LiCl_3$), causes a largely non-polar localization of PIN2 in the lateral root cap cells (Zhang et al., 2011). Generally, the apical or basal localization of PIN proteins is dependent on the reversible phosphorylation of a conserved Cysteine residue in the hydrophilic loop of the PIN proteins (Lee and Cho, 2006; Dhonukshe et al., 2010). Phosphorylation of PIN proteins is brought about by PINOID (PID) Ser/Thr kinase, which leads to apical delivery of PIN proteins (Kleine-Vehn et al., 2009). The action of phosphatase PP2A causes basal delivery of PIN proteins (Michniewicz et al., 2007). It is interesting to note that the PID kinase is itself regulated by a phosphoinositide binding protein PDK1 (Zegzouti et al., 2006), further implicating the involvement of phospholipid signaling in gravitropic signal transduction.

But even as it is understood that gravitropic signal transduction involves auxin-mediated differential growth and $InsP_3$-dependent mechanisms, additional components need to be

identified to understand how the gravity signal is transduced from columella cells to the elongation zone. In this regard, it is interesting to note that gravity induces specific gene expression in plants (Moseyko et al., 2002; Kimbrough et al., 2004). Because in root tips, gravity is perceived at the root cap, studying early gene expression in response to gravity at the root cap can help us identify the proteins immediately required by the plant specifically in response to gravity, and help us understand gravitropic signal transduction better. It has been reported by Kimbrough et al. that in *Arabidopsis* root tips, sixty five transcripts are up-regulated specifically in response to gravity, whereas no transcripts are down-regulated specifically in response to gravity, exclusive of the mechanical stress of movement caused during gravitropic re-orientation (Kimbrough et al., 2004). The transcripts up-regulated by gravity encode a variety of proteins, such as transcription factors, membrane transporters, cell wall modifying enzymes, cell cycle-related proteins and stress related proteins (Kimbrough et al., 2005). Out of these sixty-five transcripts, five genes show a very fast and transient up-regulation in response to gravity stimulus (Kimbrough et al., 2004).

One of these fast and transient expressing genes, At2g16005 (later named *ROSY1*), is especially interesting because of the very fast up-regulation of this transcript in response to both light and gravity stimuli. Upon gravitropic re-orientation of dark-grown *Arabidopsis* seedlings, *ROSY1* mRNA is up-regulated by about eight-fold within two minutes after gravity stimulus, as seen in Figure 3.1 (a) (Kimbrough et al., 2004). Upon finer time-course analysis, it can be seen that *ROSY1* mRNA is up-regulated within the first 45 seconds of gravitropic

stimulus, as seen in Figure 3.1 (b) (Kimbrough et al. unpublished data), which makes *ROSY1* mRNA up-regulation an extremely fast response. As seen in Figure 3.1(a), the gravity-induced *ROSY1* mRNA up-regulation is not only fast, but also very transient – *ROSY1* mRNA expression levels come back down to basal levels within only 5 minutes of the application of gravity stimulus. It may be noted here that in light-grown *Arabidopsis* roots which are re-oriented to gravity, the bending response can be seen after 2-3 hours of gravitropic re-orientation. Therefore, if ROSY1 has a role in mediating plant tropic responses, it may be during very early phases of the signal transduction.

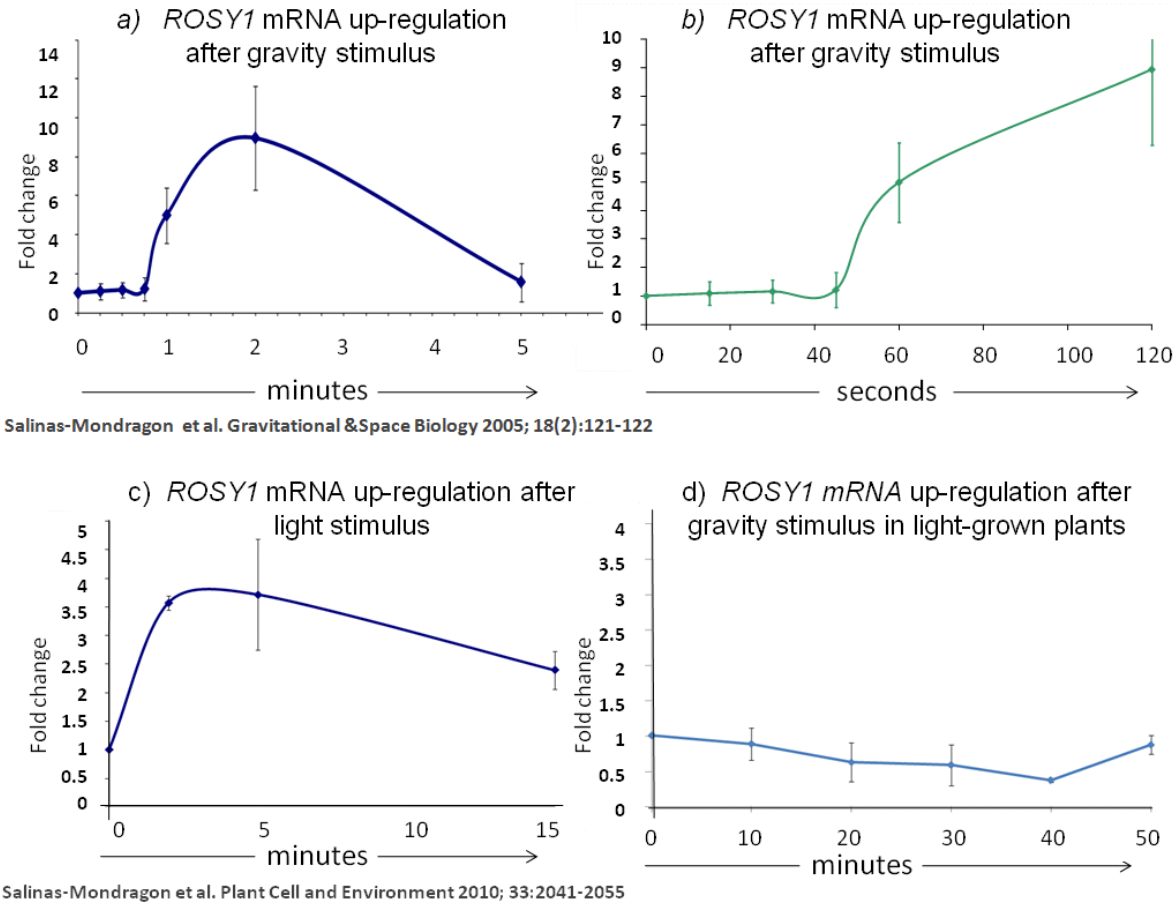

Figure 3.1Gravity and light-induced*ROSY1* expression.

Seven-day-olddark-grown *Arabidopsis thaliana* seedlings were either gravity-simulated by re-orientation (135°) or stimulated by unidirectional white light. At different time points after stimulus, root tips were harvested and RNA was extracted. Using qRT-PCR, transcript abundance of *ROSY1* mRNA was measured. a) *ROSY1* mRNA is up-regulated 8-fold over basal levels within two minutes of gravity stimulus, and the expression level falls back to basal level within 5 minutes of gravity stimulus. b) *ROSY1* mRNA is up-regulated within the first 45 seconds of gravity stimulus. c) *ROSY1* mRNA is up-regulated 3.5-fold over basal levels within the first minute of unidirectional white light stimulus, and the expression level approaches basal level after 15 minutes of light stimulus. d) *ROSY1* mRNA is not up-regulated in response to gravity in roots of seven-day-old light-grown seedlings.

The *ROSY1* mRNA shows fast and transient up-regulation not only in response to gravity stimulus, but also in response to light stimulus in dark-grown plants, as seen in Figure 1(c).

These data imply that ROSY1 is not a gravity-specific protein, but involved in general tropic responses (Salinas-Mondragon et al., 2005). Further, as seen in Figure 3.1(d), *ROSY1* mRNA is not up-regulated by gravity stimulus in light-grown *Arabidopsis* seedlings. This indicates that the protein may somehow integrate plants' gravitropic and phototropic responses, though whether it does that and how it may do so is yet unknown.

The fact that *ROSY1* mRNA is up-regulated within the first 45 seconds in response to gravity stimulus poses interesting questions about how this gene is regulated. Temporally, *ROSY1* mRNA up-regulation is downstream only of gravity induced spike in $InsP_3$ levels. Therefore, it is interesting to note that transcriptional up-regulation of *ROSY1* mRNA by tropic stimuli is actually dependent on $InsP_3$ levels in the plant. In transgenic *Arabidopsis* plants expressing human Type I Insp 5-ptase having <5% $InsP_3$ levels compared with wild type plants, *ROSY1* mRNA did not exhibit the gravity or light-induced transcriptional up-regulation, as seen in Figure 3.2 (Salinas-Mondragon et al., 2005). These data indicate that gravity or light-induced *ROSY1* mRNA up-regulation is dependent on the levels of $InsP_3$ in the plant.

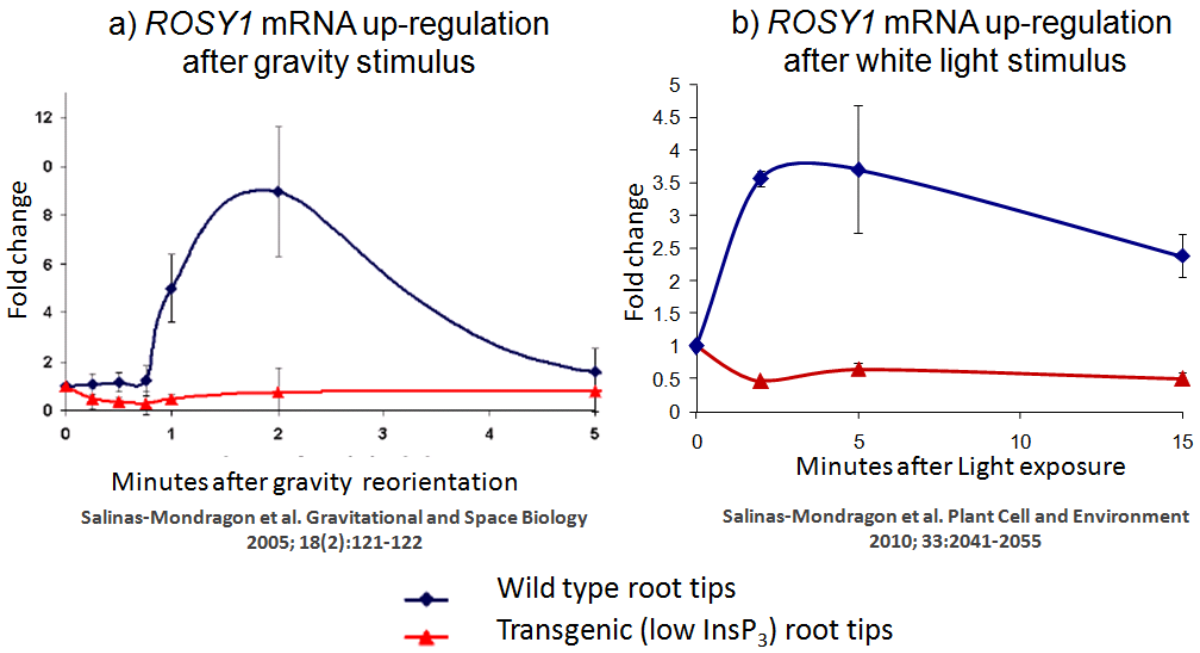

Figure 3.2 Gravity and light-induced up-regulation of *ROSY1* mRNA is $InsP_3$-dependent.

Seven-day-old dark-grown *Arabidopsis thaliana* seedlings (WT or transgenic seedlings with low-$InsP_3$) were given gravity (re-orientation by 135°) or unidirectional white light stimulus. At different time points after stimulus, root tips were harvested and RNA was extracted. Using qRT-PCR, transcript abundance of *ROSY1* mRNA was measured and gravity or light-induced *ROSY1* mRNA expression was compared between WT and transgenic low-$InsP_3$ expressing roots. *ROSY1* mRNA does not show fast and transient up-regulation in response in low $InsP_3$ plants in response to a) gravity, or b) light stimulus.

Considering the $InsP_3$ dependent fast and transient expression kinetics of *ROSY1* mRNA in response to both light and gravity, we sought to investigate the gene and its role in gravitropic signal transduction further. Computational analysis was done to gather phylogenetic and structural information available for ROSY1.

RESULTS

Computational Analysis of*ROSY1*

The gene *ROSY1* is a novel *Arabidopsis* protein coding gene, which is transcribed into a 483bp long mRNA which in turn encodes a 17 KDa protein. To learn more about the protein and its functions, in-silico analysis was done on the ROSY1 amino acid sequence using bioinformatics software. Based on the hydrophobicity analysis of the ROSY1 amino acid sequence, the PSIPRED program (Jones, 1999) predicts that the first 20 amino acids in ROSY1 form a transmembrane helix, as seen in Figure 3.3.

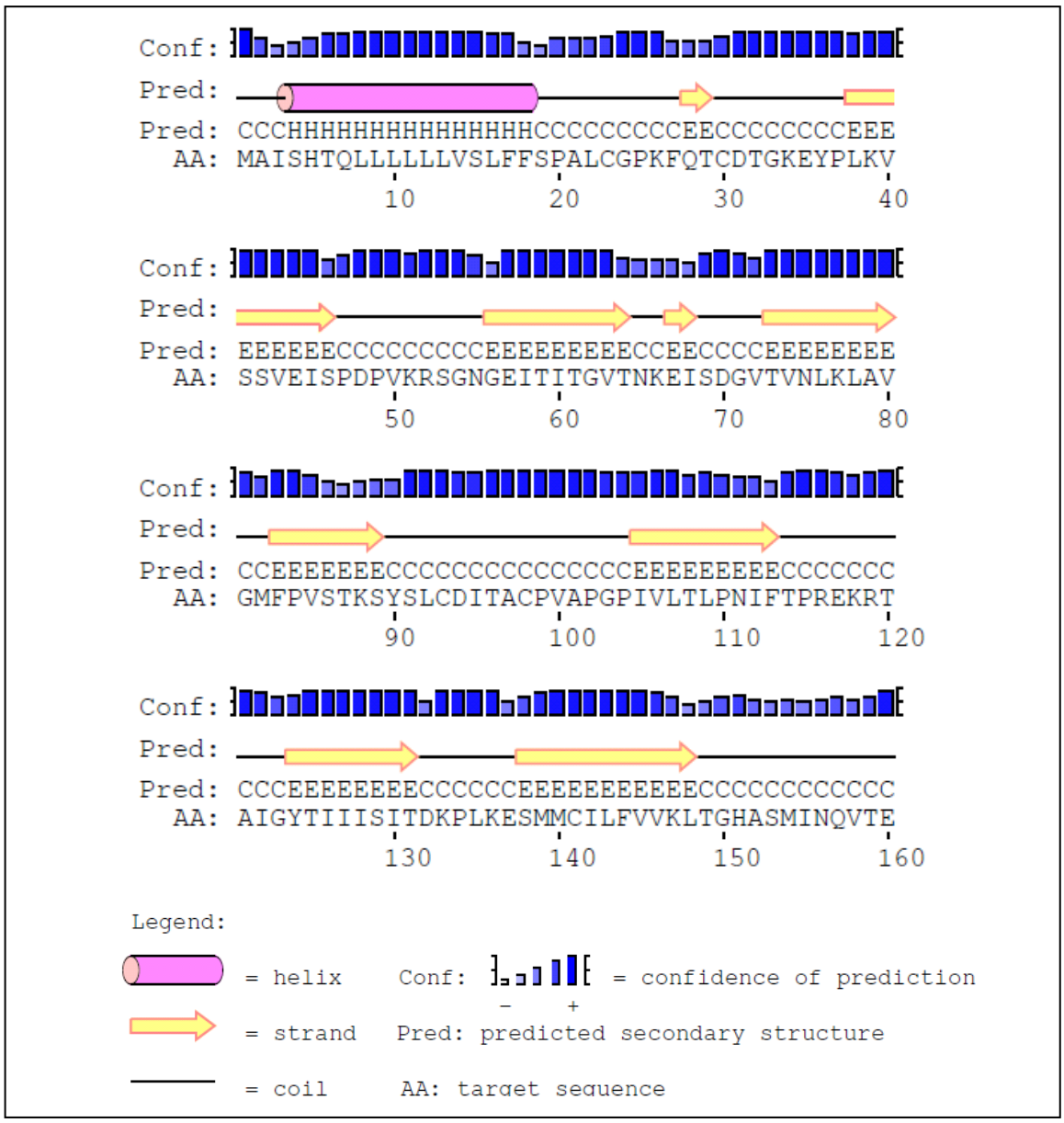

Figure 3.3Predicted ROSY1 secondary structure.

Using the ROSY1 amino acid sequence, the presence and position of transmembrane helices and strands was detected using position-specific scoring matrices on the PSIPRED program (Jones, 1999).

Within the protein sequence, a signal peptide for protein cleavage was detected using the SignalP 3.0 program(Nielsen et al., 1997; Bendtsen et al., 2004). Using both neural networks and Hidden Mark Models (HMMs) trained for eukaryotic protein sequences, the program estimated that ROSY1 has a cleavage site between amino acids 23 (Cysteine) and 24 (Glycine) (Nielsen et al., 1997; Bendtsen et al., 2004). The probability of protein cleavage at this site is 0.97 using neural networks and 1.0 using HMMs. Using PREDOTAR, a program entrained on thousands of *Arabidopsis* protein sequences to identify proteins targeted to cellular organelles such as the ER, mitochondria or plastids, the ROSY1 protein was shown to have a high probability (0.99) for being targeted to ER (Small et al., 2004).

Using the ELM (Eukaryotic Linear Motif) program, which identifies short functional sites within proteins, the positions 124-127 in ROSY1 were identified to be a tyrosine-based sorting signal responsible for endocytosis (Puntervoll et al., 2003).

Finally, an NCBI-BLAST search (using default parameters) to identify domains of interest in ROSY1 based on sequence similarity, reveals that the amino acids 26-148 constitute an ML domain (MD2 lipid binding domain), as seen in Figure 3.4. The ML domain is a highly conserved domain throughout phyla (Inohara and Nunez, 2002). Characteristics of the ROSY1 ML domain will be discussed at greater length in Chapter 4, where molecular aspects of ROSY1 action will be discussed.

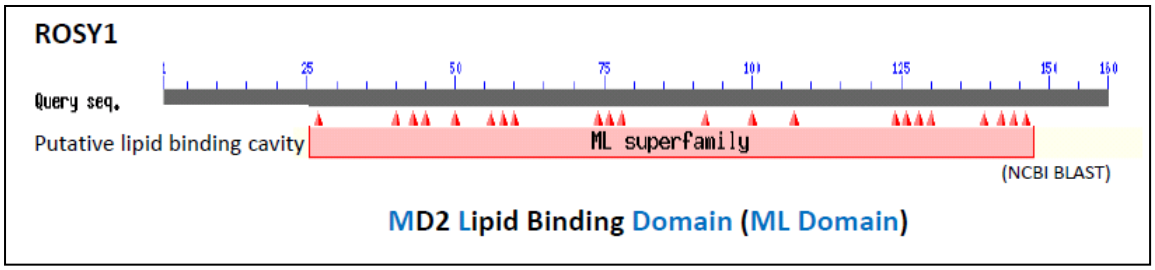

Figure 3.4Position of the ROSY1 ML domain.

Using NCBI BLAST, the ROSY1 protein sequence (160 amino acids long) was found to contain a central conserved ML domain, belonging to the ML superfamily.

Phylogenetic analysis of*ROSY1*

Owing to the highly conserved ML domain, potential *ROSY1* functional homologs exist in all eukaryotic systems tested. Potential *ROSY1* homologs exist in all plant systems sequenced, including dicots such as *Populus*, monocots such as rice and maize, and even in lower eukaryotes, such as green alga *Chlamydomonas* and fungi such as the yeast *Saccharomyces*. Potential *ROSY1* isoforms exist in animal systems as well, such as the human proteins NPC2 and GM2A. Interestingly, the protein seems to have evolved with the eukaryotic evolution, as there are no *ROSY1* homologs present in prokaryotic bacteria. Taxonomic relationships of ROSY1 with some other ML domain proteins is presented in Figure 3.5

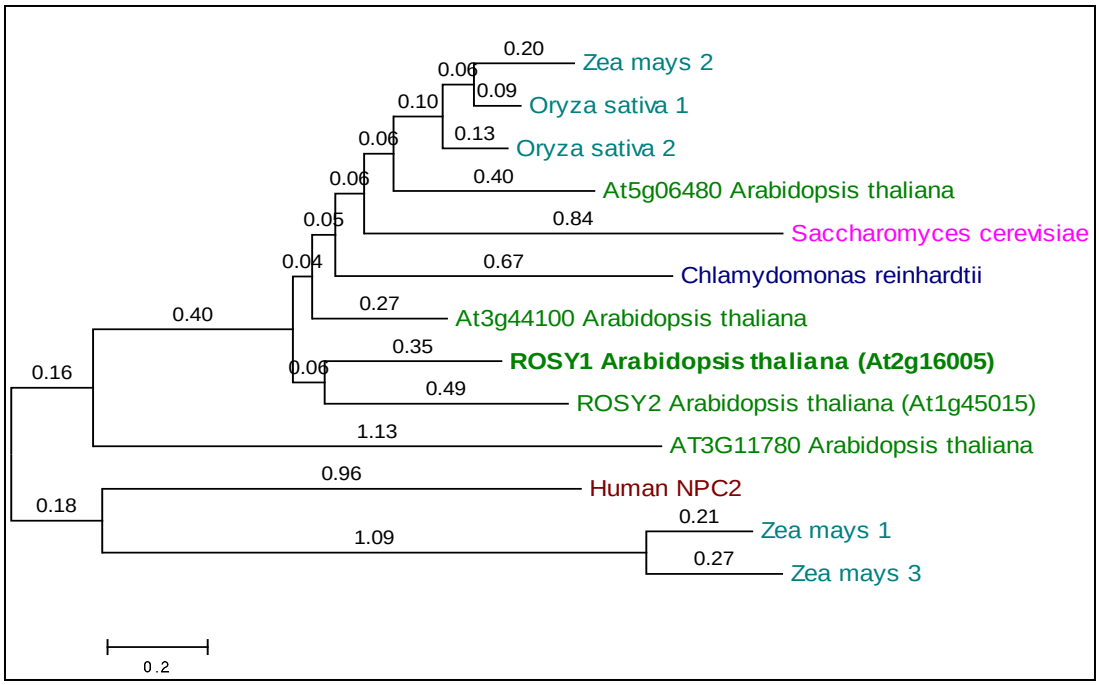

Figure 3.5 Phylogenetic analysis of *ROSY1*.

Using ROSY1protein sequence, BLAST analysis was performed using SMART program (http://smart.embl-heidelberg.de/) to identify other genes that share local regions of high sequence similarity with ROSY1. Default BLAST algorithm parameters were used to identify conserved domains in the ROSY1 sequence, and then to identify other proteins with these domains. Representative genes from different phyla were selected, and coding sequences were used for multiple sequence alignment using MEGA5 program (Tamura et al., 2011). The evolutionary distances were computed using the coding sequences of all selected genes by Maximum Likelihood Method and the taxonomic tree was constructed using Neighbor-Joining method using MEGA5 software. The tree is to scale and branch lengths are representative of taxonomic relationship of the coding sequences.

Within *Arabidopsis*, two proteins- At1g45015 and At3g44100 share a high sequence similarity with *ROSY1*mRNA coding sequence (Figure 3.6 (a)). These proteins may be functional paralogs of ROSY1 in *Arabidopsis*. Using the TAIR database (http://www.arabidopsis.org/), it was seen that the three genes- *ROSY1*, At1g45015 and At3g44100 have distinct tissue-specific expression. Using semi-quantitative RT-PCR, the

mRNA expression for the three genes was investigated in different *Arabidopsis* tissues (Figure 3.6 (b)).

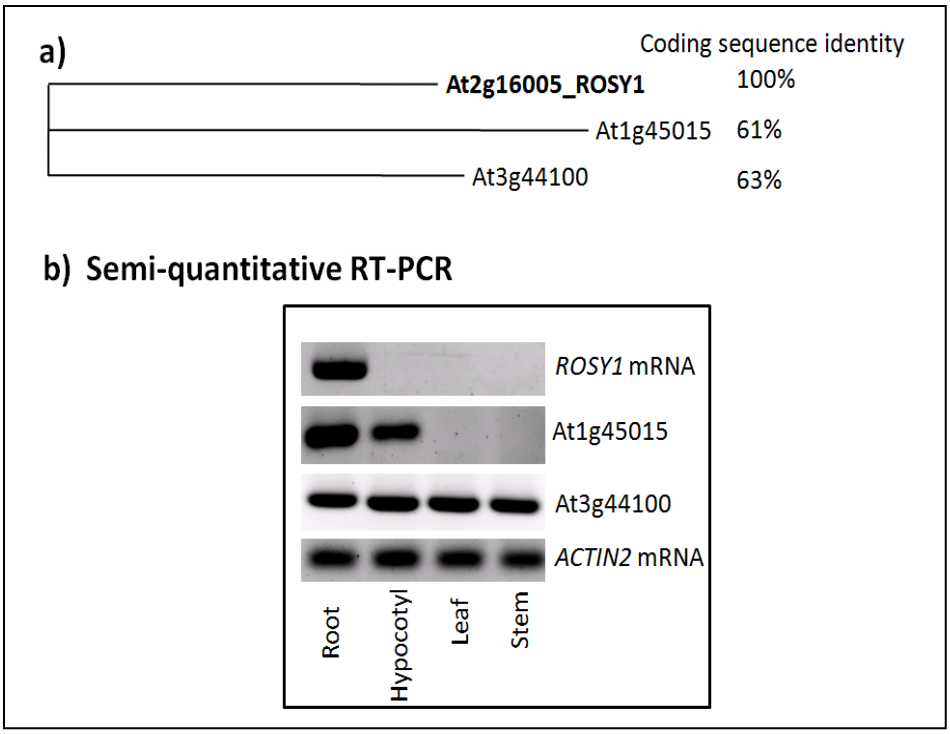

Figure 3.6*ROSY1* potential isoforms and their expression localization.

a) Protein sequences of ROSY1, At1g45015 and At3g44100 were aligned using CLUSTAL W and a cladogram was generated as depicted (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The coding sequences were also aligned using CLUSTAL W generating the percentage identity values depicted. b) RNA was extracted from roots and hypocotyls of seven-day-old*Arabidopsis thaliana* seedlings and from leaf and stem of 3 weeks old *Arabidopsis* plants. Semi-quantitative PCR was conducted with sequence specific to investigate tissue-specific expression localization. Primer design and establishment of their sequence-specificity is documented in the materials and methods section.

ROSY1 mRNA is expressed specifically in root tissue (Figure 3.6 (b)). The isoform At1g45015 mRNA is expressed more in root and less in hypocotyl of *Arabidopsis* seedlings, but the expression was not detected in any mature tissues tested. The other potential isoform At3g44100 is seen to express in all the tissues tested. These data indicate that if these proteins are indeed functional homologs of each other, they can carry their function in

different plant organs. Also, there can be functional redundancy between the proteins in some tissues, for example, in the root where all three transcripts are expressed. These hypotheses can be investigated once the function of ROSY1 in *Arabidopsis* is better understood.

To investigate if ROSY1 has a function in mediating plant gravitropic and phototropic responses, we identified insertional mutant knockouts for *ROSY1* in *Arabidopsis*.

Identification of *rosy1-1* knockout mutant

To identify the role of ROSY1 in gravitropic and/or phototropic signaling, we identified an insertional mutant line ET11532 *rosy1-1* in *Arabidopsis thaliana* (Ler-0) backgroundfrom Cold Spring Harbor Labs (http://genetrap.cshl.edu/) (Springer and Martienssen, 1998). The transgenic plants have a 6kb insertion in the fourth exon, as seen in Figure 3.7 (a). The homozygous *rosy1-1* plants were identified using PCR on genomic DNA extracted from the insertional mutants (Figure 3.7 (b)). To investigate if *ROSY1* mRNA is transcribed in the mutant seedlings, RNA was extracted from seven days old *rosy1-1* and WT seedlings. RNA was extracted from whole seedlings, or seedling roots or hypocotyls. All seedlings were gravity-stimulated for 2 minutes before tissue extraction to isolate maximum yield of *ROSY1* mRNA. Using semi-quantitative RT-PCR, *ROSY1* mRNA was found to be completely absent in the insertional mutant as seen in Figure 3.7 (c), therefore *rosy1-1* was identified as a true knockout.

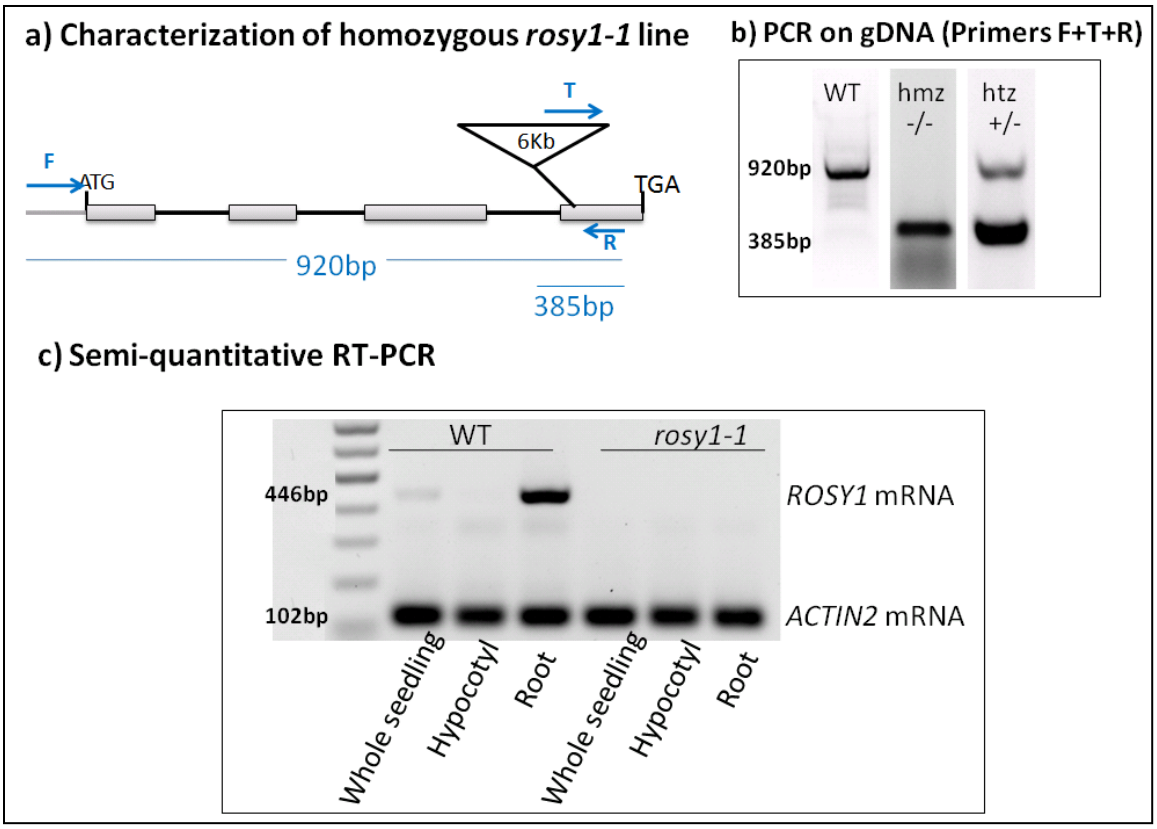

Figure 3.7 Identification of *rosy1-1* mutant.

a) In *rosy1-1* plants, the *ROSY* gene has a 6kb insertion in the 4th exon. For identification of homozygous mutants, forward (F) and reverse (R) primers were designed in the *ROSY1* gene, and one forward primer was designed within the insertional element. b) All three primers were used together in each PCR to distinguish WT plants, and homozygous (hmz) or heterozygous (htz) insertional mutants. c) *ROSY1* coding region-specific primers were used to investigate presence of *ROSY1* mRNA in *rosy1-1* knockout mutants. *ROSY1* mRNA was found to be not transcribed in the knockout mutants.

Genetic rescue of *rosy1-1* knockout mutant

Transformants were generated to genetically rescue the*rosy1-1* mutation for phenotypic studies. Previous attempts to generate 35S:ROSY1 by Kimbrough et al.were unsuccessful (data not published), possibly because over-expression of ROSY1 by the constitutive promoter may be lethal in the embryo development. Therefore, the native *ROSY1* promoter needed to be identified to drive the *ROSY1* gene in mutants. 5kb of DNA sequence upstream of the *ROSY1* start codon was analyzed to search for promoter motifs using bioinformatics software Softberry TSSP (Solovyev and Shahmuradov, 2003). The TATA box for *ROSY1* gene is predicted to be 64 bp upstream of the start codon. Using neural networks entrained on animal promoters at the BDGP server (Reese, 2001), the entire promoter region is predicted to be within 1500 bp upstream of the start codon. The DNA sequence 1.6 kb upstream of the start codon was amplified as the *ROSY1* promoter. *ROSY1* gene was amplified from the genomic DNA including the 1.6 kb promoter region, exons, introns and a 200bp 3'UTR region. The construct *ProROSY1:ROSY1* was generated, cloned into binary vector pEarleyGate 302 and transformed into homozygous *rosy1-1* knockout plants. T3 generation homozygous plants with the insertion were isolated and used as a genetic rescue control group for phenotypic experiments using *rosy1-1* mutants.

For localization of *ROSY1* promoter activity within the plant, WT plants were transformed with *ProROSY1:GFP* construct. For localization of *ROSY1* expression, homozygous *rosy1-1*

knockout plants were transformed with the *ProROSY1:GFP-ROSY1* construct for N-terminal GFP fusion and *ProROSY1:ROSY1-EGFP* construct for C-terminal EGFP fusion.

The knockout *rosy1-1* plants were used to study the role of ROSY1 in plant gravitropic and phototropic responses, with WT plants and *ProROSY1:ROSY1* rescued mutant plants as control groups.

Morphology of *rosy1-1* knockout mutant

Before using the knockout mutant, *rosy1-1*to study gravitropic and phototropic bending in absence of ROSY1, we needed to study the growth of *rosy1-1* mutants compared with WT plants in "normal" growth conditions. Phenotypically, *rosy1-1* mutants were indistinguishable from wild type plants atthe seedling stage (Figure 3.8).

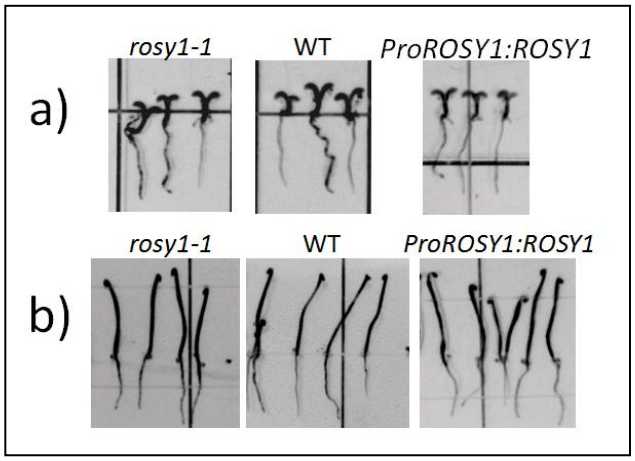

Figure 3.8Seedling growth phenotype of *rosy1-1* vs. WT seedlings

Four-day-old light-grown (a) and dark-grown (b) *rosy1-1*, WT and *ProROSY1:ROSY1* seedlings were observed for differences in growth phenotype. There was no significant difference in growth phenotypes of *rosy1-1* plants compared with WT and control plants.

No significant morphological difference was observed between *rosy1-1*,WT and *ProROSY1:ROSY1* plants with respect to plant stature, development, flowering, silique formation etc. (data not shown).

ROSY1 is involved ingravitropic and phototropic response

Transcriptional up-regulation of*ROSY1* mRNAis one of the first and fastest transcriptional responses to both gravity and light stimuli. The *ROSY1* mRNAis expressed in roots, and roots' response to gravitropic signal transduction is bending towards the direction of gravity. Therefore, it follows that if ROSY1has a central role in mediating gravitropic signal transduction, the absence of ROSY1 in *rosy1-1* mutants may compromise the mutant roots' ability to bend in response to gravitropic re-orientation. Similarly, if ROSY1 has a central role in mediating roots' phototropic responses, the absence of ROSY1 in *rosy1-1* mutant may also affect mutant roots' ability to bend in response to unidirectional light of specific wavelengths.

But it may be remembered at this point that ROSY1 does have potential isoforms in *Arabidopsis*, namely At1g45015 and At3g44100, which are not restricted to the root in their expression. If the absence of ROSY1 in knockout mutants affects gene expression or regulation of ROSY1 isoforms in *Arabidopsis*, the phenotypes in *rosy1-1* mutants may not only be attributed to the absence of ROSY1. As a result, it is possible to see phenotypic

differences in hypocotyl responses to phototropic or gravitropic bending in *rosy1-1* mutants, even though ROSY1 is itself expressed in root tissue.

Gravitropic and phototropic bending experiments were conducted to investigate how knockout *rosy1-1* seedlings (both roots and hypocotyls) respond to gravitropic re-orientation and phototropic stimulation, as compared with wild type and *ProROSY1:ROSY1* plants.

Gravitropic bending phenotype

To analyze the gravitropic bending of WT, *rosy1-1* and *ProROSY1:ROSY1*plants, homozygous seeds were sterilized and seedlings were grown either in complete dark or light (16h day/8h night cycles). When the seedlings were four-day-old, they were reoriented by turning the plates 90° clockwise, and the angle of bending was observed every hour for light-grown seedlings and every four hours for dark-grown seedlings. It was seen that in both light-grown and dark-grown seedlings, *rosy1-1*roots bend towards gravity faster than WT and*ProROSY1:ROSY1*plants. In dark-grown seedlings, *rosy1-1* hypocotyls respond faster to gravity-stimulation than hypocotyls of WT plants. As seen in Figure 3.9, in light-grown seedlings, *rosy1-1* roots bend to gravity faster than WT - the difference being clearest at 4 hours post reorientation. After 4 hours of gravity stimulus, there is a small but significant greater degree of bending (15°) in *rosy1-1* roots compared with WT and *ProROSY1:ROSY1* roots ($n=143$, $p<0.0001$) (Figure 3.9 (b)).

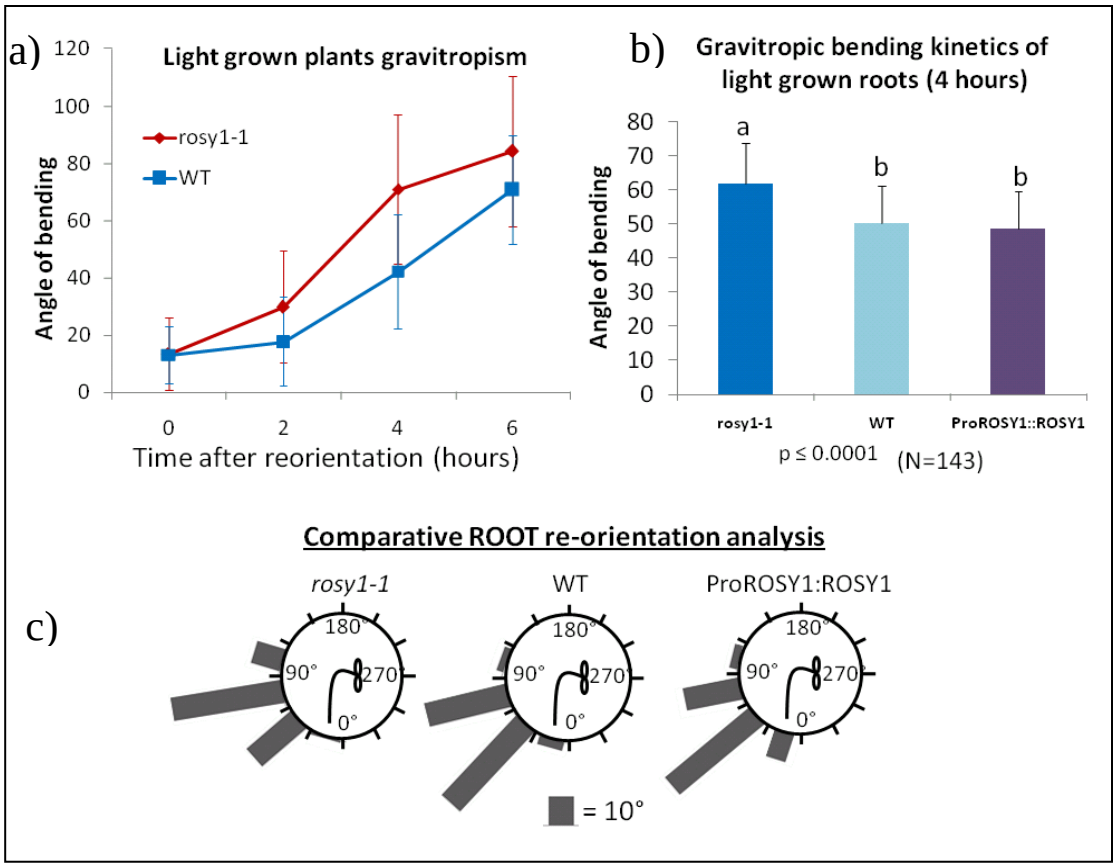

Figure 3.9 Root gravitropic bending analysis of light-grown seedlings.

Four-day-old seedlings were re-oriented with respect to gravity and the angles of root bending were measured at (a) different time points, and there was an increase in bending of *rosy1-1* roots compared with WT roots after 4 hours of re-orientation. b) The 4 h time point was analyzed in detail and *rosy1-1* roots bend significantly faster than WT or *ProROSY1:ROSY1* roots. c) In a comparative distribution of angles of bending, after 4 h of gravity stimulus, *rosy1-1* roots show higher angles of bending than WT and *ProROSY1:ROSY1* roots.

Because phototropism and gravitropism are inter-dependent tropic responses (Hangarter, 1997; Kiss et al., 2003), the presence of incandescent light from the top may interfere with gravitropic bending. Furthermore, *ROSY1* mRNA is up-regulated in response to gravity in only in dark-grown roots. Therefore, the bending phenotype of dark-grown *rosy1-1*

seedlingswas also tested. In this analysis, *rosy1-1*, WT and *ProROSY1:ROSY1* seeds were germinated in dark, and seedlings were grown in dark until they were four-day-old. The seedlings were reoriented by 90° for 24 hours in the dark, and the angles of gravitropic bending were measured and compared. As seen in Figure 3.10, the*rosy1-1* seedlings show higher bending both in hypocotyls and in roots compared to wild type and *ProROSY1:ROSY1* seedlings, as seen in Figure 3.10.

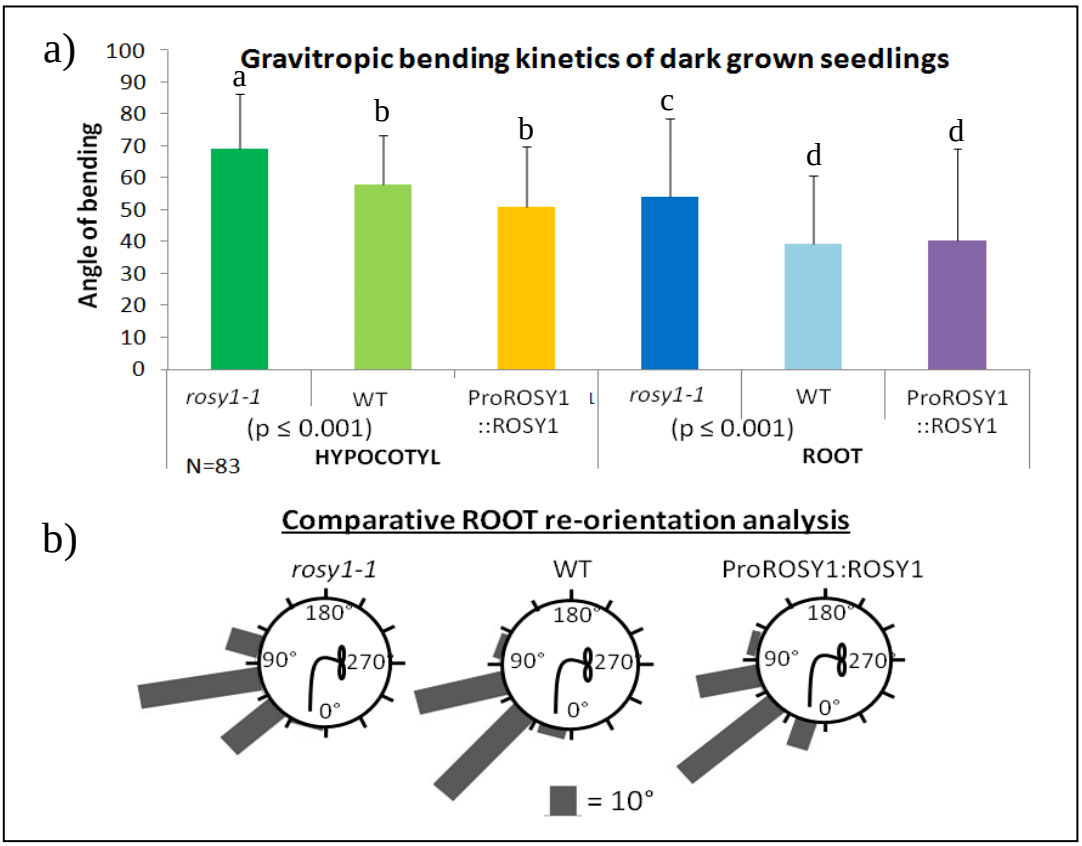

Figure 3.10 Root and hypocotyl gravitropic bending analysis of dark-grown seedlings.

Four-day-olddark-grown seedlings were re-oriented with respect to gravity and the angles of root and hypocotyl bending were measured. (a) After 24 h of gravitropic re-orientation, *rosy1-1* roots and hypocotyls show increased bending compared with WT and *ProROSY1:ROSY1* roots and hypocotyls

after 4 hours of re-orientation. b) In a comparative distribution of angles of bending, after 24 h of gravity stimulus, *rosy1-1* roots and hypocotyls show higher angles of bending than WT and *ProROSY1:ROSY1* roots and hypocotyls.

As seen in Figure 3.10, there is a small but significant 10° increase in bending of *rosy1-1* hypocotyls compared with WT hypocotyls, and a 20° increase in bending of *rosy1-1* roots compared with WT roots ($p<0.001$, $n=83$).

Increased gravitropic bending in *rosy1-1* knockout plants, where ROSY1 is essentially not present, infers that when present, ROSY1 acts as an inhibitor of gravitropic bending. In the *ProROSY1:ROSY1* seedlings, which are *rosy1-1* knockout seedlings transformed with native *ROSY1* gene, the gravitropic bending phenotype was restored to the WT bending phenotype. This strengthens the hypothesis that, when ROSY1 is present, it inhibits roots and hypocotyls bending to gravity. It is interesting to observe that ROSY1, an apparent inhibitor of tropic bending, should be one of the first genes to be transcribed in response to gravity and light stimuli. This implies that the plant invests resources into not bending to gravity stimulus. The only other known protein that inhibits the roots' bending to gravity too fast is the actin protein in root columella cells, particularly, the actin microfilaments in the columella cells. Upon application of Lat-B, which disrupts actin microfilaments, the gravitropic bending in roots is increased (Hou et al., 2003).

Phototropic bending phenotype

ROSY1 mRNA has fast and transient up-regulation in response to white light stimulus (Kimbrough et al., 2004). *ROSY1* mRNAalso displays $InsP_3$-dependent changes in transcript abundance in response to unilateral exposure to specific light wavelengths, namely- dim far-red, red and blue light (Salinas-Mondragon et al., 2010). Roots have a phototropic response to different light wavelengths (Wiesner, 1878; Kiss et al., 2003; Millar et al., 2009; Tsuchida-Mayama et al., 2010).We investigated if ROSY1 had any role in mediating root or hypocotyl phototropic response to different light wavelengths. Wild type, *rosy1*-1 and *ProROSY1:ROSY1* seeds were sterilized and plates were covered with aluminum foil. The seeds were grown in complete darkness for three days, after which the plateswere taken out of the foil and in the dark, placed in wooden boxes with different wavelength light filters on one side. The three day old seedlings were exposed to dim unilateral illumination using red (650nm, 0.2 µmol m^{-2} s^{-1}), far-red (750nm, 0.01 µmol m^{-2} s^{-1}) and blue light (450nm, 0.04 µmol m^{-2} s^{-1}) filters for 15 hours. The unidirectional light was incandescent and decreased in intensity as it went deeper in the growth chamber away from the light source, for example, blue light intensity ranged from 0.04-0.01 µmol m^{-2} s^{-1}, so seedlings of each line tested were spread evenly on the plates to receive the full gradient of unilateral illumination. The phototropic responses of the seedlings were found to not be different from WT based on how far the seedlings were from the light source in the growth chamber, so it appears that the light intensity used was saturating for the phototropic responses tested. In red and far-red light, the

tropic responses of *rosy1-1* were not found to be significantly different from WT plants (data not shown). But in blue light, *rosy1-1* seedlings showed a significantly faster bending response in hypocotyls compared to WT plants (n=60, p<0.0001) and a higher percentage of *rosy1-1* hypocotyls showed a nearly complete reorientation in response to blue light at the end of 15 hours, as seen in Figure 3.11.

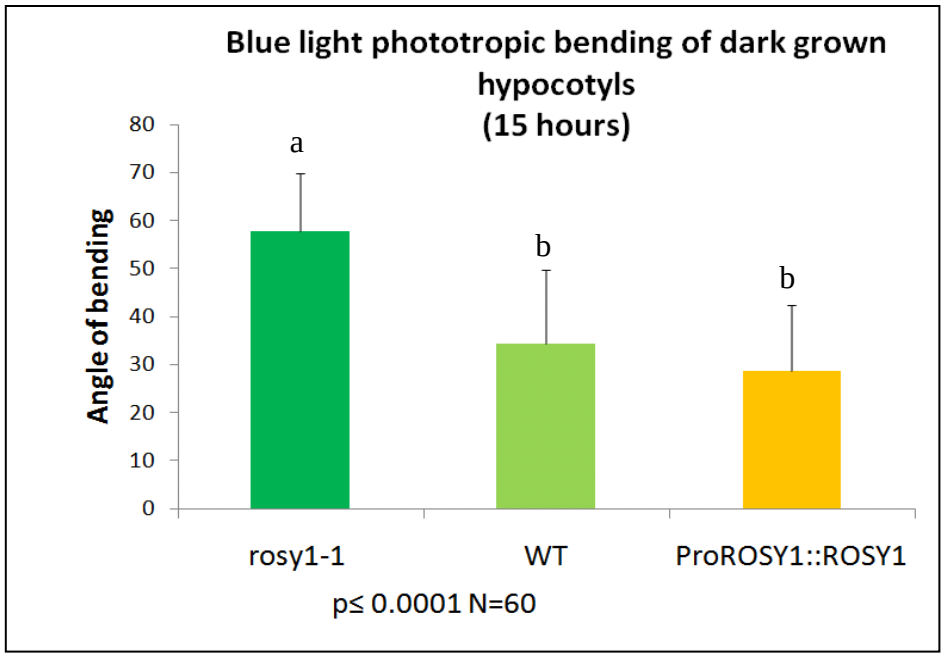

Figure 3.11 Hypocotyl gravitropic bending analysis of dark-grown seedlings.

Three day old seedlings were exposed to unilateral blue light and the angles of hypocotyl bending were measured. After 15 h of light stimulus, *rosy1-1* hypocotyls show increased bending compared with WT and *ProROSY1:ROSY1* hypocotyls.

The faster rate of *rosy1-1* hypocotyl bending, was even further increased after blue light stimulation, compared to gravitropic stimuli on dark-grown plants, as seen in photographs of 15 h blue light stimulated dark-grown hypocotyls vs. 24 h gravity stimulated dark-grown hypocotyls as seen in Figure 3.12.

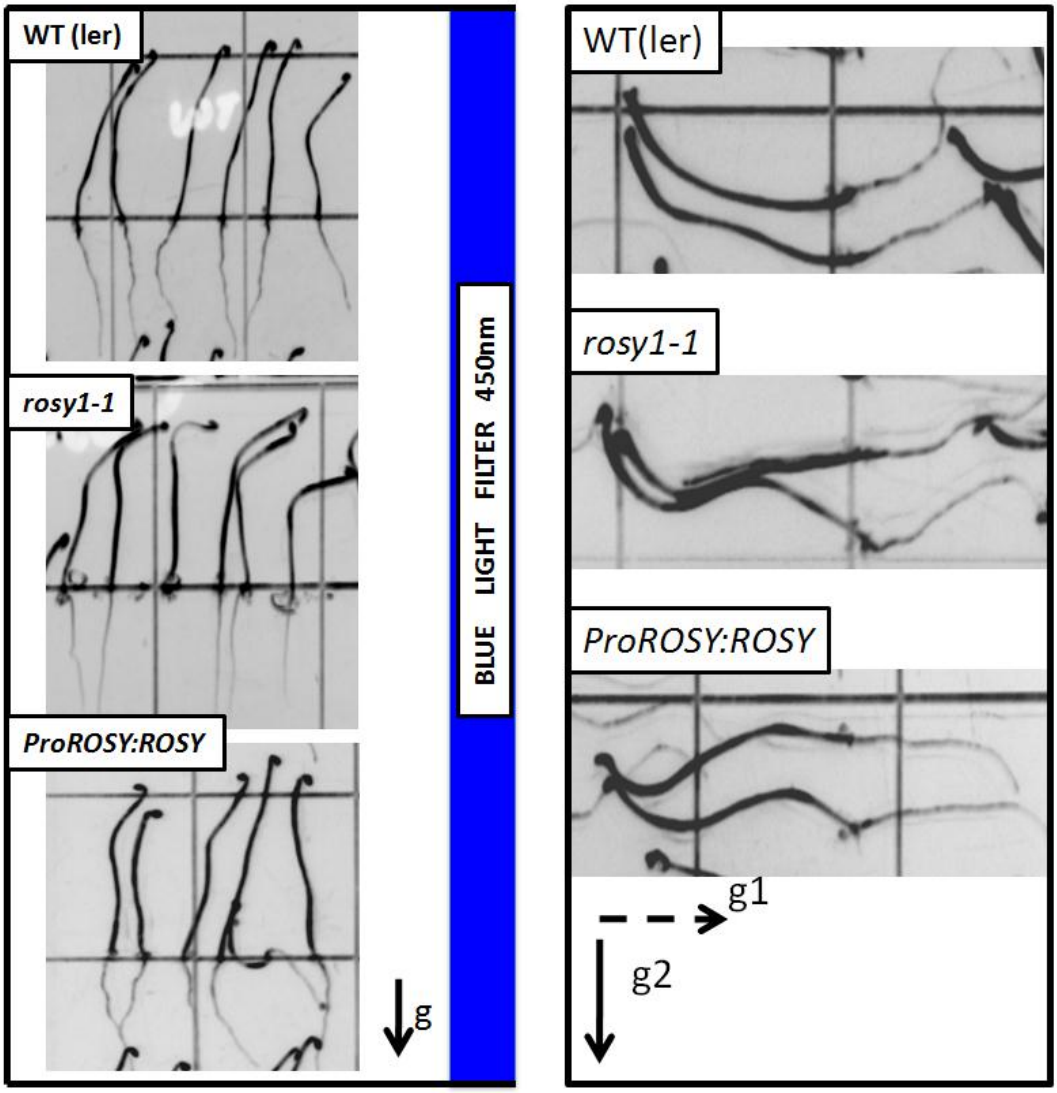

Figure 3.12 Gravitropic and phototropic bending of *rosy1-1* hypocotyls.

Differential bending in *rosy1-1* hypocotyls was found to be much more pronounced in response to unilateral blue light than in response to gravitropic re-orientation.

With the blue light intensity we used in our experiment, we could not detect substantial root phototropism in majority of seedlings tested.

Root Cell Layer and Starch phenotype

We asked if the differential tropic response of mutant seedlings was because of developmental or architectural defects in cell layers of the mutant roots, or because of abnormal levels of starch grains in those roots. We found that the mutant seedlings have the same basic root architecture as WT plants, as seen in Figure 3.13.

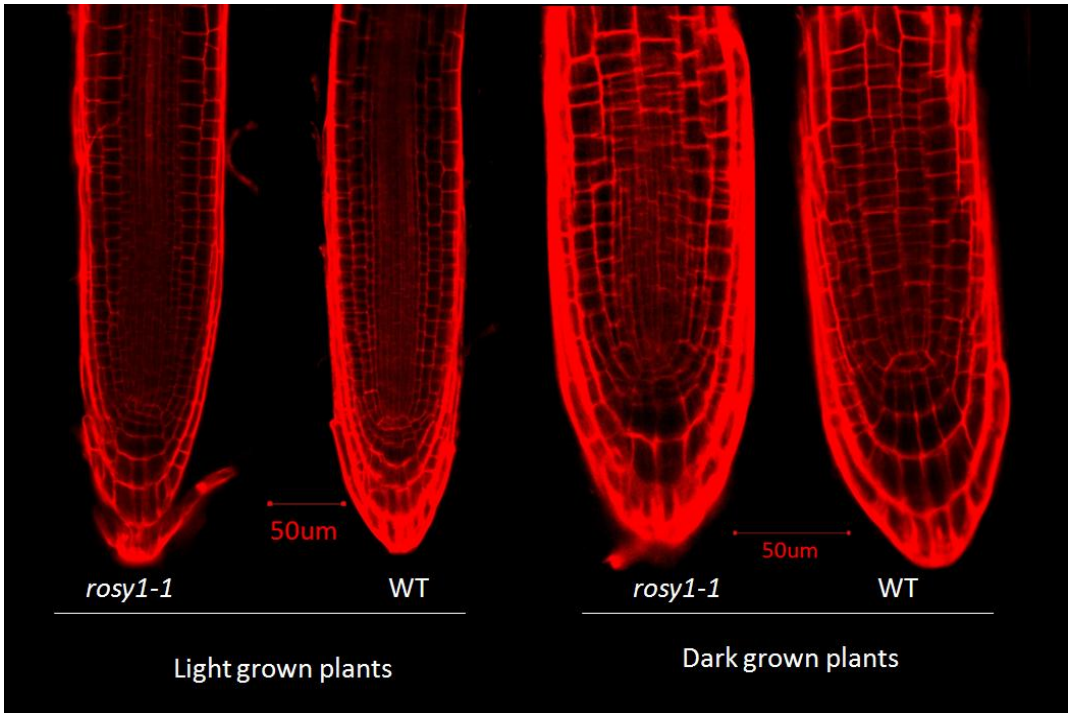

Figure 3.13 Root cell layer comparison between *rosy1-1* and WT roots.

The basic root structure was visualized by staining 3 day old *rosy1-1* and WT roots with propidium iodide. The roots of *rosy1-1* mutants have same basic architecture and cell layers as WT roots.

Mutant *rosy1-1* seedling roots also appear to have comparable number and distribution of starch grains in root tips aswild type seedlings (Figure 3.14).

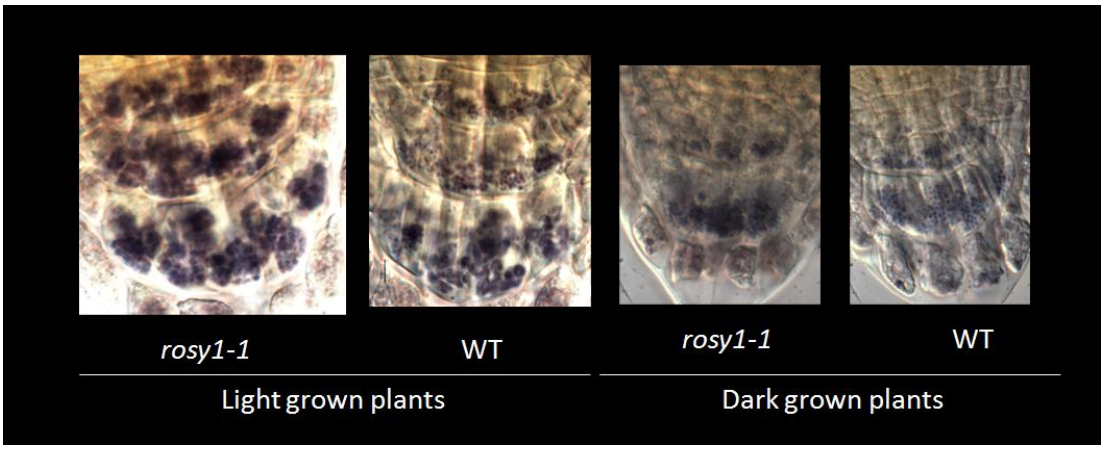

Figure 3.14Root tip amyloplasts comparison between *rosy1-1* and WT roots.

Amyloplasts in the root tips of 3 day old *rosy1-1* and WT roots were stained with KI/I_2. The number and distribution of amyloplasts is similar between *rosy1-1* and WT root tips.

ROSY1 Expression Localization

Localization of *ROSY1* mRNA expression, based on microarray studies has been predicted to be in *Arabidopsis* seedling roots. Using semi-quantitative RT-PCR, the *ROSY1* mRNA was indeed found to be expressed exclusively in *Arabidopsis* seedling roots, as seen in Figure 3.15.

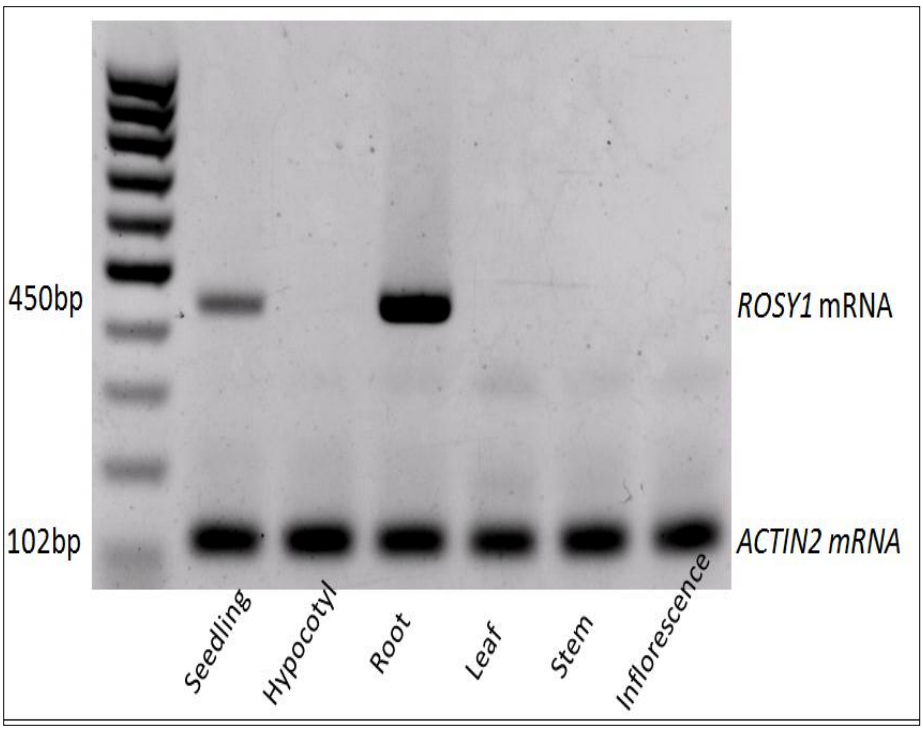

Figure 3.15 Tissue-specific *ROSY1* mRNA localization using semi-quantitative RT-PCR.

RNA was extracted from whole seedlings, roots and hypocotyls of seven-day-old*Arabidopsis thaliana* seedlings and from leaves, stems and inflorescences of flowering *Arabidopsis* plants. Semi-quantitative PCR was conducted with sequence specific to investigate tissue-specific *ROSY1* mRNA expression localization.

To investigate in which root cells the *ROSY1* promoter was activated, transgenic plants expressing the Green Fluorescent Protein (GFP) driven by the *ROSY1* promoter(*ProROSY1:GFP*) construct were generated and analyzed.

ROSY1 Protein localization

Using GFP fluorescence as an indicator of cellular activity, the *ROSY1* promoter activity was observed in the cortical and epidermal cells in the mature zone of *Arabidopsis* root and in the lateral root caps at the root tips, as seen in the confocal micrographs in Figure 3.16.

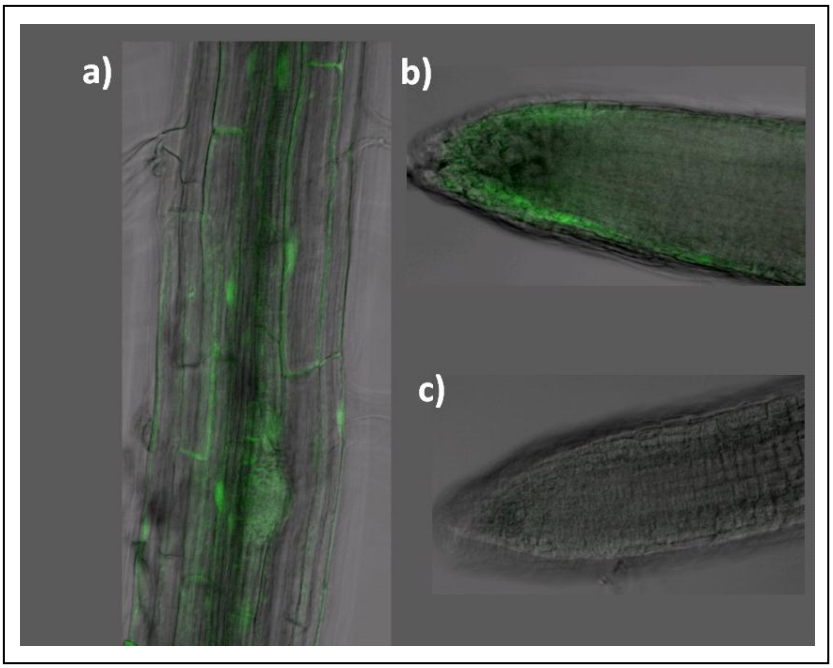

Figure 3.16*ProROSY1:GFP* expression.

ROSY1 promoter is activity in the a) cortical and epidermal cells of the mature root zone and in the b) lateral root cap at the root tip. c) WT root tip as a control for GFP fluorescence.

To visualize the intracellular localization of ROSY1 protein, transgenic plants were generated, in which the native *ROSY1* promoter was used to drive the *ROSY1* gene fused with an N-terminal GFP (*ProROSY1:GFP-ROSY1*) or C-terminal EGFP(*ProROSY1:ROSY1-EGFP*). The *ProROSY1:GFP-ROSY1* plants expressed the correct size fusion GFP-ROSY1

protein. The protein expressed in the epidermal and cortical cells in the elongation zone (Figure 3.17 (a)), in addition to sometimes being present in the lateral root cap cells at the root tip (Figure 3.17(b)). The expression of GFP:ROSY1 in the lateral root cap had lower intensity, and the protein seems to be considerably more labile in the lateral root cap than in the epidermal and cortical cells of the mature regions of the root. ROSY1 expression is notably absent in the hypocotyl, and the zone of protein expression ends exactly at the root-hypocotyl junction, only a few hypocotyl epidermal cells at the rootward end show the presence of ROSY1 (Figure 3.17(c)). At the intracellular level, the protein was found to be localized in the cytoplasm, around the nucleus and in small vesicular structures (Figure 3.17(d,e,f)).

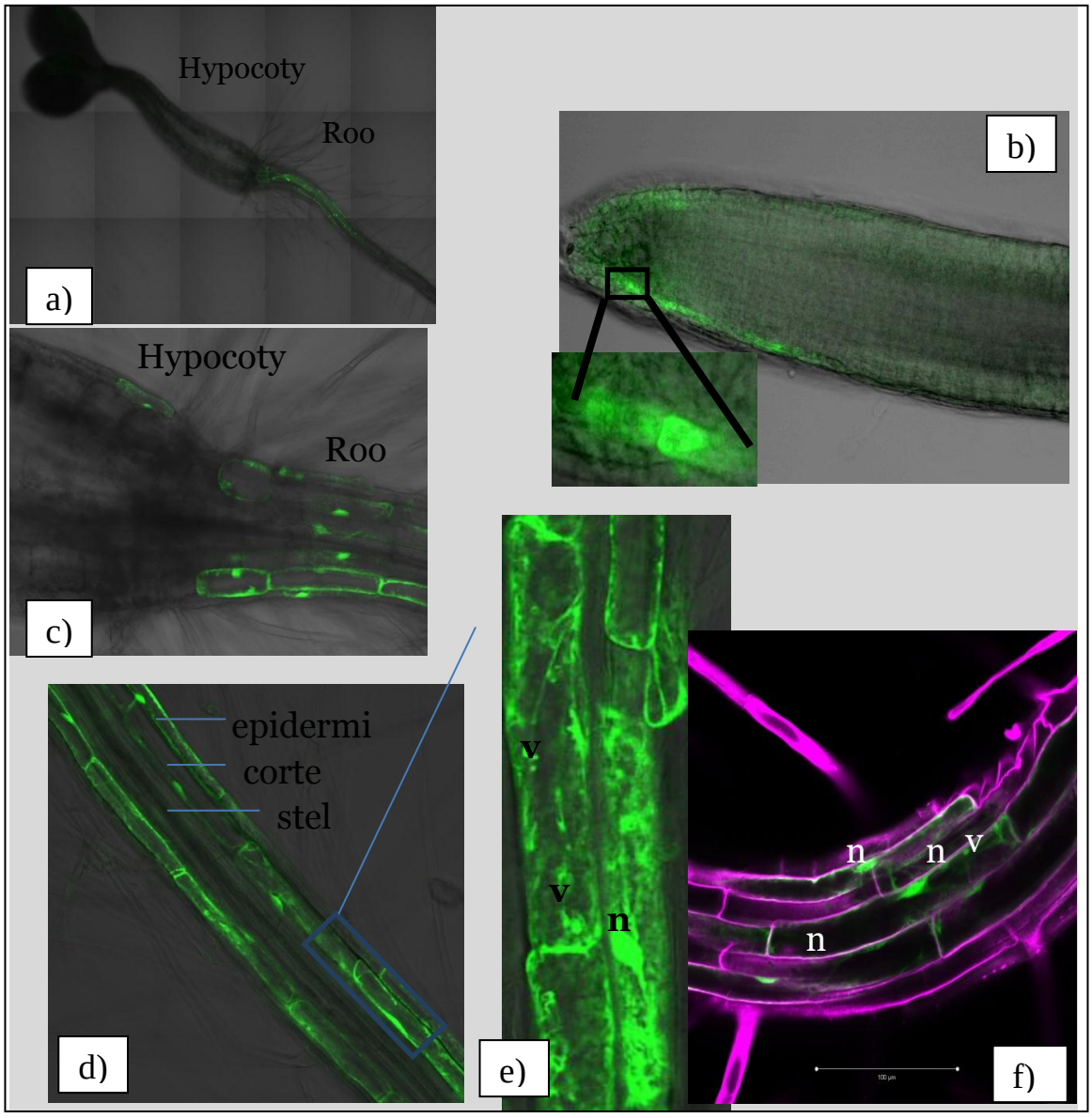

Figure 3.17 ProROSY1:GFP-ROSY1 expression.

ROSY1 was seen to express in epidermal and cortex cells of root (a,c-f), a few epidermal cells at the rootward end of the hypocotyl (c) and in the lateral root cap (b). Protein is seen in the ER around nucleus(n) in several vesicles(v) and in cytoplasm of the expressing cells (e,f). Green color represents GFP, Magenta represents PI staining for plasma membrane.

In plants which were transformed with C terminal ROSY1:GFP fusion construct driven by the *ROSY1* promoter(*ProROSY1:ROSY1-EGFP*), the localization of ROSY1 expression was seen to be completely different. The EGFP localization was in the vascular zone and in the lateral root cap, as seen in Figure 3.18. In the lateral root cap, ROSY1:EGFP expression was much brighter and consistent in between samples indicating a stable expression of the fusion protein. In comparison, the GFP:ROSY1 expression in the lateral root cap had a much lesser intensity and was not very consistent in between samples, which indicates an unstable or labile expression of the fusion protein.

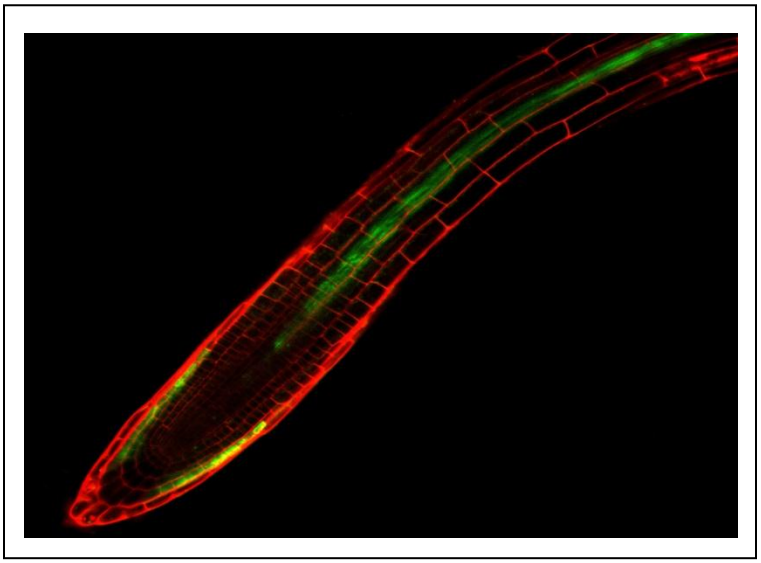

Figure 3.18*ProROSY1:ROSY1*-EGFP localization.

In three day old *Arabidopsis* seedlings, the possible break-down product of ROSY1-EGFP fusion, the 27KDa EGFP (green) can be visualized in the stele and in the lateral root cap. The root tips are stained with propidium iodide to mark the plasma membranes (red).

On probing the size of the fusion protein using anti-GFP antibody, there was found to be a single band at 27KDa corresponding to EGFP alone (Figure 3.19(a)). The fusion mRNA was

found to be of correct size (Figure 3.19(b)), which meant that there was *in-vivo* protein degradation.

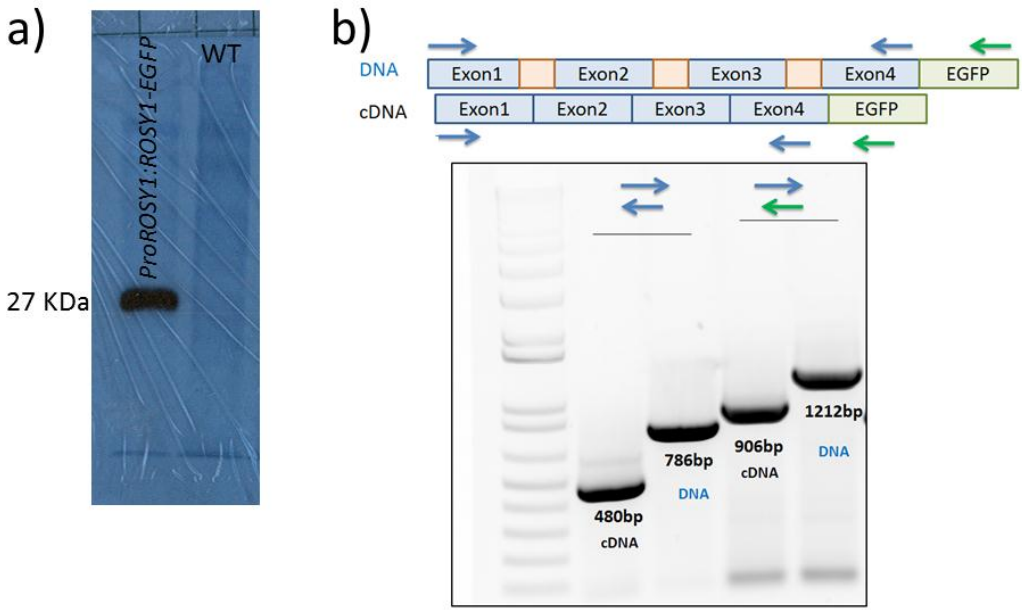

Figure 3.19ROSY1-EGFP fusionprotein, DNA and mRNA in *ProROSY1:ROSY1-EGFP* plants

Seven-day-old *ProROSY1:ROSY1-EGFP* expressing seedlings were used to investigate the fusion protein, DNA and mRNA sizes. **(a)** Total proteins were extracted from the transgenic and WT seedlings. The proteins were separated on an SDS-PAGE gel, transferred to a PVDF membrane and probed with an anti-GFP antibody. The size of ROSY1 is 17 KDa, whereas the size of EGFP is 27 KDa. Therefore, the size of ROSY1-EGFP is expected to be 44 KDa. However, the transgenic seedlings showed a 27 KDa band, corresponding only to EGFP. **(b)** Total DNA and RNA were extracted from the transgenic seedlings. The RNA was used to generate cDNA. Using *ROSY1* coding sequence (blue) and EGFP sequence specific primers (green), PCR was conducted on the DNA and the cDNA. The *ROSY1* coding sequence on the DNA is 789 bp, encoding a 483 bp mRNA. PCR was also conducted using forward ROSY1 sequence-specific primers, and reverse EGFP sequence-specific primers. The coding sequence-specific primers detected ROSY1-EGFP fusion at both DNA and mRNA level.

It is unclear why the cleaved EGFP from degraded protein was not degraded as well, but in fact gave a very bright fluorescent signal. It is also not understood why the localization of ROSY1 expression was completely different upon using this construct. Both WT and *rosy1-*

*1*plants were transformed with *ProROSY1:ROSY1-EGFP* construct, and 10-15 independent transformants from each line consistently showed the same vascular and lateral root cap localization of ROSY1 expression, and the same 27KDa size on western blot. Dark-grown plants from which protein was extracted in green light also showed 27KDa band in the western blot, which means that the protein degradation is not a light-induced mechanism. Because the ROSY1 expression localization seen in this construct does not confirm with the *ProROSY1:GFP* expression, we considered the *ProROSY1:GFP-ROSY1* construct expression as the correct ROSY1 expression.

In the case of both N-terminal and C-terminal GFP fusions, ROSY1 is expressed in the lateral root cap. This is interesting because lateral root cap is the site of basipetal auxin transport, and basipetal auxin transport is critical for gravitropic bending (Swarup et al., 2005). Because ROSY1 clearly has a role in gravitropic signal transduction, and the protein is expressed in the lateral root cap where basipetal auxin transport takes place, we investigated if auxin transport rates were altered in *rosy1-1* mutants.

ROSY1 and basipetal auxin transport

In collaboration with Dr. Daniel Lewis at Wake Forest University, wecompared the rates of auxin transport between WT and *rosy1-1* mutants using radioactive IAA. It was seen that in light-grown 5 day old seedlings, mutant *rosy1-1* roots have slower auxin transport compared with WT plants (Figure 3.20), which means that ROSY1 has a role in auxin transport. The slower auxin transport persisted even after NPA application, which means that ROSY1

probably does not have a role in auxin efflux, and it may have a role in auxin influx (Figure 3.20).

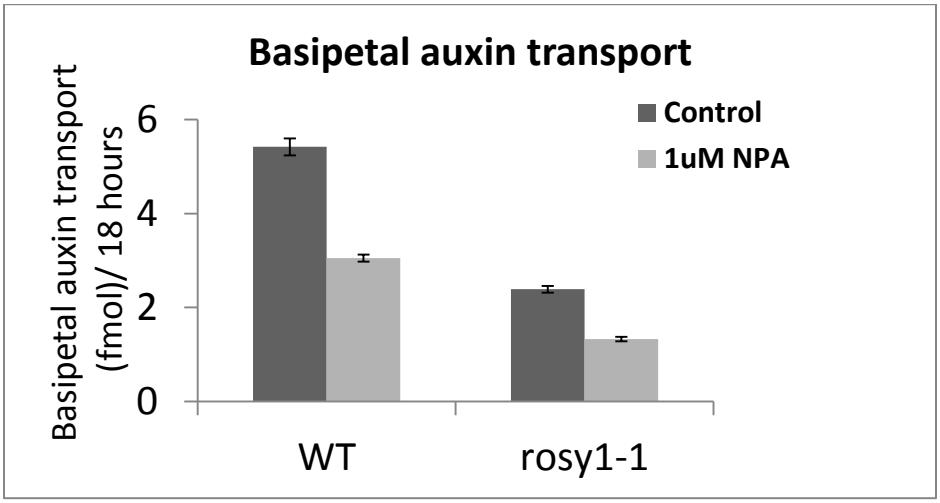

Figure 3.20 Measurement of basipetal auxin transport in *rosy1-1* roots.

Upon measuring the rate of basipetal auxin transport in *rosy1-1* and WT root tips, the basipetal auxin transport rate in *rosy1-1* roots was found to be lesser than WT roots. The reduced rate of auxin transport was found to be NPA-independent.

Slower basipetal transport in the root tip is known to cause a faster bending root phenotype, as shown in a variety of auxin transport mutants, such as the *mdr4* mutants (Lewis et al., 2007). These authors attribute this fast bending response to formation of steeper local auxin gradient at the root tip because of slowed transport up the lateral root cap (Lewis et al., 2007). The data above show that ROSY1 has a role in auxin transport, does not have a role in NPA-dependent auxin efflux, but may well have a role in auxin influx. Auxin efflux is carried out by the auxin efflux carrier PIN proteins. To investigate if the*rosy1-1* mutants have

altered auxin efflux in roots caused by a PIN-protein dependent mechanism, the cellular localization of several root-localized PIN proteins were investigated in *rosy1-1* mutants.

ROSY1 and localization of PIN proteins

The protein ROSY1 is localized in the cortical cells in the mature zone of the root, which coincides with the zone of PIN1 expression. ROSY1 is also localized in lateral root cap, its zone of expression coincides with that of PIN2. PIN4 is expressed in quiescent center and cell around it, which may also coincide with ROSY1 expression at the root tip. In collaboration with Dr. Olaf Teitz and Dr. Klaus Palme at University of Freiburg, Germany, we compared the localization of proteins PIN1, PIN2 and PIN4 between *rosy1-1* and wild type plant roots. As seen in immunolocalization images in Figure 3.21, the localization of PIN proteins does not differ between *rosy1-1* and wild type roots.

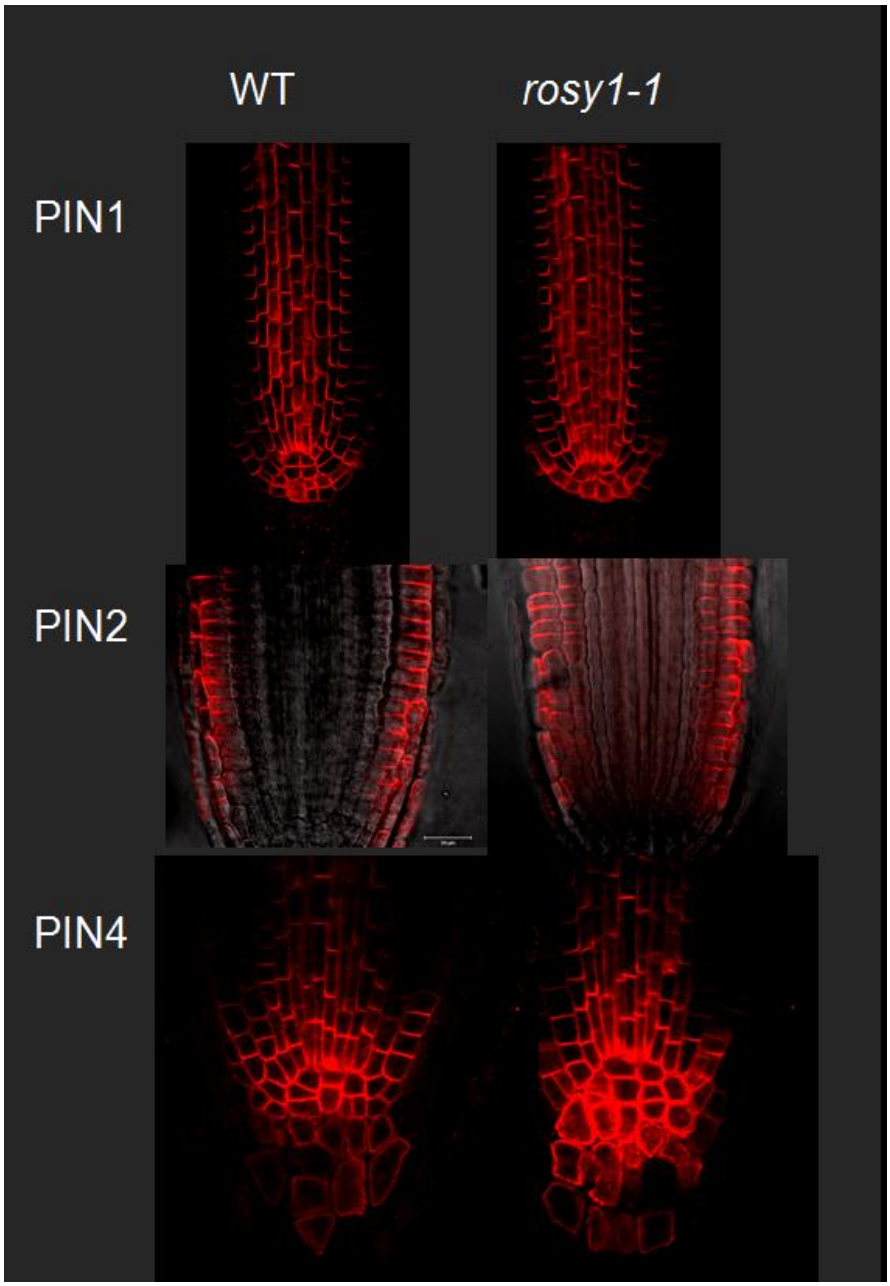

Figure 3.21 PIN1, PIN2 and PIN4 localization in *rosy1-1*vs. WT roots.

The localization of proteins PIN1, PIN2 and PIN4 were investigated by immuno-localization in 4-5 day old *rosy1-1* and WT seedlings. No difference in PIN protein localization was observed between *rosy1-1* and WT roots.

As seen earlier in this chapter, basipetal auxin defect in *rosy1-1* seedlings was NPA independent, and therefore, possibly related to auxin influx. Correct localization of the auxin efflux carrier PIN proteins in *rosy1-1* roots adds weight to the theory that ROSY1 may function solely in auxin influx.

DISCUSSION

One of the ways that plants respond to changing environmental conditions is by changing their gene expression. Various regulators such as transcription factors, microRNAs etc regulate gene expression in response to environmental conditions. Plant gene promoter sequences are targeted by a variety of regulating proteins that determine when and where a particular gene will be expressed. Light and gravity regulate gene expression for tropic and photomorphogenic responses, but the exact mechanism is unclear.

The expression of ROSY1 is very tightly controlled; the gene is expressed in very specific cells, at low basal levels, but the expression increases up to nine fold on providing tropic stimuli (Kimbrough et al., 2004). This gravity and light-induced up-regulation of *ROSY1* mRNA is dependent on $InsP_3$levels in plants, as demonstrated by Salinas-Mondragon et al. by using transgenic *Arabidopsis* plants expressing low $InsP_3$ (Salinas-Mondragon et al., 2010). Therefore, the transcriptional regulation of *ROSY1* mRNA is possibly dependent on $InsP_3$ and Ca^{2+}/calmodulin based transcription factors, but that cannot be ascertained without a yeast one-hybrid analysis or other appropriate analyses. Even though such an analysis has

not been performed for *ROSY1*, light and gravity-induced transcription factors have been identified by yeast-one-hybrid analysis of another gravity and light sensing gene, *AtTHAS1* (de Silva et al., 2010).*AtTHAS1* (At5g48010) encodes the enzyme thalianol synthase, which is needed for synthesis of the triterpenoid thalianol in *Arabidopsis* (Field and Osbourn, 2008). In response to gravity stimulus, *AtTHAS1* is one of the five fastest up-regulated transcripts along with the *ROSY1*transcript, up-regulating within 2 minutes of gravity stimulus (Kimbrough et al., 2004). Also like *ROSY1*, the *AtTHAS1* mRNA is up-regulated by light stimulus (Salinas-Mondragon et al., 2005). Furthermore, the light and gravity-induced *AtTHAS1* mRNA up-regulation is also dependent on $InsP_3$ levels in the plant (Salinas-Mondragon et al., 2010). Using the yeast-one-hybrid approach, 240 bp promoter region of *AtTHAS1* gene was used to identify transcription factors interacting with this promoter area (de Silva et al., 2010). The screen identified a calcium-dependent lipid binding protein (AtCLB) as a transcription factor for the gene *AtTHAS1*. The AtCLB protein has a conserved C2 Ca^{2+} binding domain. Tertiary structure prediction for the protein AtCLB by homology modeling using the PHYRE server indicates that the protein is structurally close to the Synaptotagmin proteins, which are well known Ca^{2+} sensing membrane fusion proteins. This data directly links Ca^{2+} with up-regulation of gravity and light-induced genes, and $InsP_3$ mediated regulation of gravitropic gene expression (Salinas-Mondragon et al., 2010) may in part be because of Ca^{2+}-regulated transcription factors.

Similar analyses with *ROSY1* promoter sequence will be very useful, and will help in identifying common transcription factors that regulate gravity and light signaling. Because AtCLB has already been identified as a transcription factor regulating light and gravity-induced gene expression, investigating the interactions of *ROSY1* promoter sequence or the protein itself with AtCLB will be interesting experiments for the future.

One of the most interesting features of the gravity-induced *ROSY1* mRNA up-regulation is that while the transcript is up-regulated within 45 seconds of gravity stimulus, it also reverts back to basal levels within only 5-15 minutes after the stimulus. Therefore, high amounts of this protein are needed, but only for very short time. As seen by transformation of *Arabidopsis* plants with the 35S:ROSY1 construct, the constitutive expression of ROSY1 is toxic to plants. The tropic bending analysis of *rosy1-1* seedlings shows that the high amounts of the protein are probably used by the plant to inhibit either cell elongation itself, or some other aspect of gravitropic signaling. This aspect could well be auxin transport, as ROSY1 has a possible role in regulating auxin influx.

But the role of ROSY1 in auxin transport cannot be readily predicted as the protein does not share homology with any auxin efflux or influx carrier proteins. Being only 17KDa, the protein is too small to serve as a transmembrane carrier for auxin transport. Auxin transport is mediated by auxin efflux carriers (the PIN proteins) and auxin influx carrier (the AUX1 protein). But these proteins are not stationary on the membrane; PIN proteins have been shown to constantly recycle between the plasma membrane and intracellular vesicular

compartments (Kleine-Vehn et al., 2008). Therefore proteins with key roles in vesicular fusion or recycling may also have a role in mediating auxin transport.

Because the ROSY1 protein has the conserved MD2 lipid binding (ML) domain, we investigated how the ROSY1 ML domain interacts with lipids, sterols and other proteins. Such studies help us understand how ROSY1 may have a role in inhibiting tropic bending in *Arabidopsis*roots and hypocotyls, regulating auxin transport and maintaining general plant growth and stress responses.

These results are documented in the next chapter of this dissertation.

MATERIALS AND METHODS

Computational Analysis of ROSY1

Protein sequences of ML proteins in Arabidopsis were aligned by homology extended alignment using PRALINE server (http://www.ibi.vu.nl/programs/pralinewww/) (Pirovano et al., 2006, 2008; Pirovano et al., 2009). Transmembrane secondary structure prediction was carried out using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) (Bryson et al., 2005), signal peptide and cleavage site prediction was carried out using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004), (Nielsen et al., 1997). PREDOTAR (http://urgi.versailles.inra.fr/predotar/predotar.html) and ELM server (http://elm.eu.org/). Protein threading to find homologous structures was carried out

usingprotein fold recognition with PHYRE (http://www.sbg.bio.ic.ac.uk/~phyre/) (Kelley and Sternberg, 2009).

Taxonomic Conservation of ROSY1

A list of ML domain proteins from various databases was obtained from SMART (http://smart.embl-heidelberg.de/) (Schultz et al., 2000). Coding sequences of ML domain proteins were aligned using MEGA5 software (Tamura et al., 2011). The taxonomic tree was created with coding sequences of selected proteins using Neighbor-joining method in MEGA5. The taxonomic tree obtained was confirmed in PAUP making taxonomic tree with both Neighbor-joining and parsimony method (Wilgenbusch and Swofford, 2003).

Semi-quantitative RT-PCR of *ROSY1*, At1g45015 and At3g44100 mRNA.

Arabidopsis WT (Ler-0) seeds were surface sterilized with 50% bleach as described before (Valvekens et al., 1988) and plated on square petriplates containing 1X MS medium with 0.8% agar. The plates with seeds were incubated for 3 days at 4°C and then transferred to the growth chamber (Percival Scientific, IA) at 22°C set at long day (16 hours day/8 hours night) conditions. When the seedlings were seven-day-old,, they were used for tissue extraction. Plates with seven-day-oldseedlings were kept at a stationary vertical position for one hour prior to tissue extraction. The seedlings were sprayed with RNAlater (Qiagen), and seedlings were used directly for RNA extraction or used to cut off roots and hypocotyls while immersed in RNAlater solution.Leaf and stem tissues were harvested from 3 weeks old

Arabidopsis plants. RNA was isolated using RNeasy plant mini kit (Qiagen), and 3µg of total RNA was used for 20µl cDNA synthesis reaction using Superscript II Reverse Transcriptase (Invitrogen, CA). 1µl cDNA was amplified. Primer sequences are as follows: *ROSY1* coding sequence primers ROSY1 FP (forward primer) 5'- CAC CCA GCT TTT GCT TCT TC -3' and ROSY1 RP (reverse primer) 5'- TAG ACG CAT GAC CAG TGA GC-3'; At1g45015 coding sequence primers At1g45015 FP 5'- GTG GAG ATT TCT CCC GAT GA -3' and At1g45015 RP 5'- ATT CCA CTG GGC CAC GAC TAC -3'; At3G44100 coding sequence primer At3g44100 FP 5'- CAA GCT CAG CCT CTG CTT CT -3' AND At3G44100 RP 5'- GAT CTT CAC ACC CGT TGA CCT -3'; *ACTIN2* coding sequence primers ActinF 5'- GCC ATC CAA GCT GTT CTC TC -3', ActinR 5'- ACC CTC GTA GAT TGG CAC AG -3'

Because the *ROSY1* coding sequence primers had been used by me before to amplify the coding sequence for cloning in entry vectors for recombinant protein synthesis, the specificity of these primers had been proven. They amplify the *ROSY1* gene to yield a single band on agarose gel, which upon sequencing is the *ROSY1* coding sequence. The specificity of the primers amplifying At1g45015 and At3g44100 was verified by sequence alignment.

Using CLUSTAL W, the coding sequences of the three genes, *ROSY1,* At1g45015 and At3g44100 were aligned and the position of the primers selected of At1g45015 and At3g44100 amplifications were analyzed for specificity, as presented below. Color coding- At3g44100 FP, At3g44100 RP, At1g45015 FP, At1g45015 RP.

Alignment between At2g16005 (*ROSY1)* and At3g44100 coding sequence:

```
At2g16005       ATGGCGATATCTCACACCCAGCTTTTGCTTCTTCTCCTTGTGTCACTCTTTTTCTCACCT 60
At3g44100       ATGGCGATTTCTCAAGCTCAGCCTCTGCTTCTTCTCCTCTTATCAGTCTTCTTCTTACCT 60
                ******** *****  * **** * *************  * *** **** **** ****

At2g16005       GCTTTATGCGGCCCCAAATTCCAAACTTGCGATACTGGTAAAGAGTATCCCCTAAAGGTC 120
At3g44100       GCTTTGCACG---CCACTTCTTTCACCTACTGCGATAAGAGGCTCGATCCCGTTAAGGTC 117
                *****   **   ***  *     ** * *     *   *      ***** * ******

At2g16005       AGTTCTGTGGAGATATCTCCAGACCCGGTTAAGCGTAGCGGTAATGGAGAGATTACGATA 180
At3g44100       ACGGGTGTGAAGATCTCCCCTGACCCTGTTGTGAGTGGTGCAGCCGCAACATTTAAGATT 177
                *    **** **** ** ** ***** ***  * ** * *     * *    *** ***

At2g16005       ACCGGTGTTACAAACAAAGAAATCTCTGATGGAGTAACCGTAAATCTCAAGCTCGCGGTA 240
At3g44100       TTTGGTTCTACTGGCGAAGACATCTCTGGAGGAAAAGTAGTGA---TCAGGGTTTTATAC 234
                   ***  ***   * **** *******  ***  *   ** *   *** * *


At2g16005       GGCATGTTTCCGGTCTCTACAAAAAGTTACTCCCTCTGTGATATAACAGCATGCCCTGTT 300
At3g44100       GTTGGGATTCCTGTCCATACCGAAACTCATGACCTCTGCGATGAGACGGCATGTCCGGTT 294
                *    * **** ***  ***  *** * *   ****** ***   ** ***** ** ***

At2g16005       GCACCTGGCCCGATTGTGCTTACTCTCCCTAACATATTCACTCCGCGTGAAAAAAGAACA 360
At3g44100       GCACCTGGCAGCTTTGTGCTTTCTCATTCTCAAACACTCCCTTC------AATTACACCA 348
                *********    ******** ***   ** * * * ** ** *      **  * * **

At2g16005       GCAATTGGATATACTATCATAATAAGCATCACCGATAAGCCTCTGAAAGAGTCAATGATG 420
At3g44100       CCTGGTACTTACACGCTAAAGATGACAATCAACGACAAGAAT---GGCGGAAGACTAACG 405
                 *   *   ** **  * *  ** *  **** *** ***  *      *    * * * *

At2g16005       TGCATCCTTTTTGTTGTTAAGCTCACTG---GTCATGCGTCTATGATTAATCAAGTTACC 477
At3g44100       TGTATCAGCTTCAAATTCAAGATCACAGTAGGTTCTGCGGTCTTTGCTAGTTAA------ 459
                ** ***   **     * *** **** *   **  ****    *   ** * **

At2g16005       GAGTGA 483
At3g44100       ------
```

Alignment between At2g16005 (*ROSY1)* and At1g45015 coding sequence:

```
At2g16005       ATGGCGATATCTCACACCCAGCTTTTGCTTCTTCTCCTTGTGTCACTCTTTTTCTCACCT 60
At1g45015       ATGGCGAAATCTCACTACCAGCCTCTGTTTCTTCTCCTTGCATCACTCTTGTTCTTGCCT 60
                ******* *******  ***** * ** ************  ******** ****  ***

At2g16005       GCTTTATGCGGCCCCAAATTCCAAACTTGCGATACTGGTAAAGAGTATCCCCTAAAGGTC 120
At1g45015       GCTTTGCATGCCGTCGATTTTAAATACTGCAAC---GGTATTGGATACGTCGATAAAATC 117
                *****    * *  * * **  **   *** *    ****  *  **   *   **  **

At2g16005       AGTTCTGTGGAGATATCTCCAGACCCGGTTAAGCGTAGCGGTAATGGAGAGATTACGATA 180
At1g45015ACTAAAGTGGAGATTTCTCCCGAT--GATCC--CTCAACTGTTAT--------TACAATC 165
                * *   ******** ***** **   * *    *  * * ** **        *** **

At2g16005       ACCGGTGTTACAAACAAAGAAATCTCTGATGGAGTAACCGTAAATCTCAAGCTCGCGGTA 240
At1g45015       TCCGGTCTTACAAGAAGAGGACTCGTCTATGCTGGAACTGTAGTCGTGGCCAGTGGAATT 225
                 ***** ******  * ** * **    ***  * *** ***    *       *   *

At2g16005       GGCATGTTTCCGGTCTCTACAAAAAGTTACTCCCTCTGTGATATAACAGCATGCCCTGTT 300
At1g45015       GGCGAATTTAATATCCCTTTTAAATACTACGACTTTTGTCAGTTGTGTAAATGCCCTATG 285
                ***   ***    ** **   ***   ***  * * *** *  *      ******* *

At2g16005       GCACCTGGC-CCGATTGTGCTTACTCTCCCTAACATATTCACTCCGCGTGAAAAAAGAAC 359
At1g45015       CTAAGTGGCACCAACTTTGTTTTCACTCTTTCTCAAATTCTCATTCCAAAAGCTTTTGCG 345
```

```
                 *  **** ** * * ** ** * ***  *  ** **** *    *   *

At2g16005        AGCAATTGGATATACTATCATAATAAGCATCACCGATAAGCCTCTGAAAGAGTCAATGAT 419
At1g45015        AGTGACAAGCT-TGCTGTTACGTTGAGTTTAATATCTAGAGATCAAACAGA---AGGAGT 401
                 **  *   * * * ** * *   * **  * *    **    **  * ***   *    *

At2g16005        GTGCATCCTTTTTGTTGTTAAGCTCACTGGTCATGCGTCTATGATTAATCAAGTTACCGA 479
At1g45015        GTGCGTCTACTTCGATTTC---CCTACTTCAGCTAACTCTATGCTTAATCAAGCTAGCGA 458
                 **** **   ** * * *    *  ***     *   ****** ********* ** ***

At2g16005        GTGA 483
At1g45015        ATGA 462
                  ***
```

Alignment between At1g45015 and At3g44100 coding sequence:

```
At3g44100        ATGGCGATTTCTCAAGCTCAGCCTCTGCTTCTTCTCCTCTTATCAGTCTTCTTCTTACCT 60
At1g45015        ATGGCGAAATCTCACTACCAGCCTCTGTTTCTTCTCCTTGCATCACTCTTGTTCTTGCCT 60
                 *******  *****    ********* **********   **** **** ***** ***

At3g44100        GCTTTGCACGCCACTTCTTTCACCTACTGCGATAAGAGGCTCGATCCCGTTAAGGTCACG 120
At1g45015        GCTTTGCATGCCGTCGATTTTAAATACTGCAACGGTATTGGATACGTCGATAAAATCACT 120
                 ******** ***     *** *  ****** *    *       *   ** ***  ****



At3g44100        GGTGTGAAGATCTCCCCTGACCCTGTTGTGAGTGGTGCAGCCGCAACATTTAAGATTTTT 180
At1g45015        AAAGTGGAGATTTCTCCCGATGATCCCTCAACTGTTA------------TTACAATCTCC 168
                    *** **** ** ** **   *      * ** *             ***  ** *

At3g44100        GGTTCTACTGGCGAAGA---CATCTCTGGAGGAAAAGTAGTGATCAGGGTTTTATACGTT 237
At1g45015        GGTCTTACAAGAAGAGGACTCGTCTATGCTGGAACTGTAGTCGTGGCCAGTGGAATTGGC 228
                 ***  ***  *   **    * *** **  ****  *****  *      *  *   *

At3g44100        GGGATTCCTGTCCATACCGAAACTCATGACCTCTGCGATGAGACGGCATGTCCGGTTGCA 297
At1g45015        GAATTTAATATCCCTTTTAAATACTACGACTTTTGTCAGTTGTGTAAATGCCCTATGCTA 288
                 *   **  * *** *    **    * *** * **  *   *     *** **  *   *

At3g44100        CCTGGCA---GCTTTGTGCTTTCTCATTCTCAAACACTCCCTTCAATTACACCACCTGGT 354
At1g45015        AGTGGCACCAACTTTGTTTTCACTCTTTCTCAAATTCTCATTCCAAAAGCTTTTGCGAGT 348
                   *****    ******  *  *** ********  ***  * ***   *     *  **

At3g44100        -ACTTACACGCTAA-AGATGACAATCAACGACAAGA-ATGGCGGAAGACTAACGTGTATC 411
At1g45015        GACAAGCTTGCTGTTACGTTGAGTTTAATATCTAGAGATCAAACAGAAGGAGTGTGCGTC 408
                  **   *  ***   *  *     * **   * *** **     *  *  *  ***  **

At3g44100        AGCTTCAAATTCAAGATCACAGTAGGTTCTGCGGTCTTT---GCTAGTTAA--- 459
At1g45015        TACTTCGATTTCCCTACTTCAGCTAACTCTATGCTTAATCAAGCTAGCGAATGA 462
                   **** * ***   *   ***     ***  * *   *   *****  **
```

The primers were found to be specific enough to use for the semi-quantitative PCR analysis, which was confirmed when they yielded single size-specific bands on cDNA amplification.

Identification of *rosy1-1*knockout

The *Arabidopsis thaliana* (Ler-0) enhancer trap line ET11532 was obtained from Arabidopsis Genetrap Website Cold Spring Harbor Lab, NY (Springer and Martienssen, 1998). Homozygous knockout mutants were screened using primers DNA FP 5'- CCG CTT AAG GGT GTC TAT GAA -3', DNA RP 5'- CAC ACC CAG CTT TTG CTT CTT C -3', insertion primer Ds5-2 (5′-CCG TTT TGT ATA TCC CGT TTC CGT-3′). For checking the presence of *ROSY1* mRNA, seven-day-old *Arabidopsis* seedlings were used and roots and hypocotyls were cut off in RNAlater (Qiagen) as explained above. RNA was isolated using RNeasy plant mini kit (Qiagen), and 3µg of total RNA was used for 20µl cDNA synthesis reaction using Superscript II Reverse Transcriptase (Invitrogen, CA). 1µl cDNA was amplified with actin primers ActinFP 5'- GCC ATC CAA GCT GTT CTC TC -3', ActinRP 5'- ACC CTC GTA GAT TGG CAC AG -3' and *ROSY1* cds primers ROSY1 FP 5'- CAC CCA GCT TTT GCT TCT TC -3' and ROSY1 RP 5'- TAG ACG CAT GAC CAG TGA GC-3'.

Cloning of Constructs

***rosy1-1* Complementation Construct:**

For genetic complementation, the*ROSY1*gene was amplified complete with the native promoter and 3' UTR sequences. DNA was isolated from *Arabidopsis thaliana* (Ler-0) seedlings using the DNeasy Plant DNA isolation kit (Qiagen, CA). The *ROSY1* gene was

amplified from the DNA starting from 1584 bp upstream of start codon (to include the promoter) up to 628 bp downstream of the stop codon. Primers used are: using ROSY1DNA FP 5'- CACC TTC CGA TCA TAG TAA AAA A -3'; ROSY1DNA RP 5'- CTA TAG TAT AAA TAC ATG TAC ATT ACA -3'. The PCR product was cloned into pENTR/D-Topo entry vector (Invitrogen, CA) to create the*ProROSY1:ROSY1*entry vector. LR Clonase II (Invitrogen, CA) was used to clone the*ROSY1*gene in the pEarleyGate302 (Earley et al., 2006) to create binary vector.

Cloning of *ProROSY1:GFP* Construct:

The *ROSY1*promoter was amplified from the*ProROSY1:ROSY1* entry vector using the following primers: ProROSY1 FP 5'- CACC TTC CGA TCA TAG TAA AAA A -3' and ProROSY1 RP 5'- TCC TTT ACT AGT CAG ATC TAC CAT GGA TAT CAC TAG AGA AAA ATA CT -3'. The GFP sequence was amplified from pEarleyGate103 vector (Earley et al., 2006) using the following primers: GFPFP 5'- AGT ATT TTT CTC TAG TGA TAT CCA TGG TAG ATC TGA CTA GTA AAG GA -3', GFPRP 5'- TCA CAC GTG GTG GTG GTG GT -3'. Using the overhangs of the ProROSY1 RP and GFP FP, fusion PCR was conducted and *ProROSY1:GFP* was cloned in pENTR/D-Topo entry vector (Invitrogen, CA). LR Cloning was performed to clone *ProROSY1:GFP* in pEarleyGate103 (Earley et al., 2006) using LR Clonase II (Invitrogen, CA).

Cloning of ProROSY1:GFP-ROSY1 Construct:

ProROSY1:GFP was amplified from the *ProROSY1:GFP* construct using primers: ProROSY1 FP 5'- CACC TTC CGA TCA TAG TAA AAA A -3' and GFPRP2 5'- TGG GTG TGA GAT ATC GCC AT CAC GTG GTG GTG GTG GT -3'. The stop codon of GFP was excluded from amplification. The*ROSY1*gene was amplified from the*ProROSY1:ROSY1*entry vector using primers ROSY1-2 FP 5'- AC CAC CAC CAC CAC GTG ATG GCG ATA TCT ACA CCC A -3', ROSY1-2 RP 5'- CTA TAG TAT AAA TAC ATG TAC ATT ACA -3'. Using the overhangs of GFPRP2 and ROSY1-2 RP, Fusion PCR was conducted. The PCR product was cloned in the pENTR/D-Topo vector (Invitrogen, CA) to create the*ProROSY1:GFP-ROSY1* entry vector. The construct was cloned into pEarleyGate302 (Earley et al., 2006).

Agrobacterium mediated Arabidopsis transformation

All binary vectors were transformed in electrocompetent *Agrobacterium tumefaciens* (strain UIA143/pMP90 Rec-). Arabidopsis plants were transformed by floral dip method as described before (Clough and Bent, 1998). The T-0 seed were spread on 1X MS medium supplemented with BASTA Phosphinothricin (Gold Biotechnologies, MO) to 20µg/ml final concentration. The transformed seedlings were transferred to soil and the transgene insertion was confirmed by PCR. For phenotypic rescue experiments, T3 homozygous rescue seeds were collected.

Gravitropic and Phototropic bending

Arabidopsis WT (Ler-0) seeds were surface sterilized with 50% bleach as described before (Valvekens et al., 1988) and plated on square petriplates containing 1X MS medium with 0.8% agar. The plates with seeds were incubated for 3 days at 4°C (stratification) in darkness, and then transferred to the growth chamber to be grown in either "light-grown" or "dark-grown" conditions.

Plates for the light-grown seedlings were placed in the growth chamber (Percival Scientific, IA) at 22°C set at long day (16 hours day/8 hours night) conditions. When the seedlings were four-day-old, they were used for gravitropic bending experiments.

Plates for the dark-grown seedlings were covered with aluminum foil to keep out light. The plates were placed in the growth chamber (Percival Scientific, IA) for four days after which they were used for gravitropic bending experiments, or for three days after which they were used for the phototropic bending experiments.

For the gravitropic bending experiments, the plates containing the four-day-old light-grown or dark-grown seedlings were taken out of the growth chamber and placed in a laminar hood with light from top. All plates were re-oriented by 90° clockwise rotation. For the light-grown seedlings, the plates were picked up every one hour until 8 hours post re-orientation, and scanned using the Gel Doc imager (Bio-Rad, CA). The plates containingthe dark grown

seedlings had been wrapped in aluminum foil, so to image gravitropic bending, separate plates were opened at 4 hours, 8 hours and 24 hours post re-orientation to scan the plates.

For phototropic bending experiments, the plates with the three-day-old dark-grown seedlings were used. In very dim light, the aluminum foil was removed from the plates and the plates were paced in wooden boxes closed from three sides and with specific color filters (Red/FarRed/Blue) on one side. The specific color filter provided unilateral illumination to the plates. The plates were placed in the filter-boxes within 1 minute of uncovering the aluminum foil. The fluorescent lights placed outside the box were turned on for 15 hours after which the plates were taken out of the boxes and scanned using the Gel Doc imager (Bio-Rad, CA).

All the images taken by the scanner were exported to TIFF images, and the angles of gravitropic or phototropic bending were measured using the ImageJ program (Abramoff et al., 2004).

Microscopy

Cell Layers Visualization

For cell layer visualization, three-day-old light-grown or dark-grown seedlings were placed in 0.5% propidium iodide (PI) stain (Sigma-Aldrich, MO) (excitation/emission maxima: 514/617 nm). Each seedling was kept in the solution for 1 minute and gently washed in water. The seedling was then mounted on a glass slide (Fisher-Scientific, MO) in a drop of

water, and hypocotyls were cut off and removed. The roots were imaged using the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Germany). PI staining on plasma membranes was visualized and imaged at 20X resolution.

Starch Grains Visualization

Starch grains were stained in 3 day old light or dark grown seedlings with 1X I_2/KI solution for 10 seconds, washed with water for 2-5 minutes and mounted on slides. Hypocotyls were cut off and the roots were imaged using Zeiss Axiovert 100 TV (Carl Zeiss, Germany) inverted microscope at 100X with oil.

GFP imaging with Confocal microscopy

Three-day-old transgenic *Arabidopsis* seedlings expressing GFP were imaged using Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Germany), using the 488nm excitation laser. The plasma membranes of the GFP-expressing seedlings were stained with 0.5% propidium iodide (PI) stain (Sigma-Aldrich, MO) (excitation/emission maxima: 514/617 nm). Both the GFP and PI were imaged using sequential line scan using 488nm and 514 nm excitation lasers for GFP and PI respectively.

Auxin measurement

Basipetal transport of auxin was measured by applying agar droplets containing ^{3}H-IAA to the root apex of seedlings grown under 100 µmol/m^{2}sec constant cool white light after five

days of growth. Measurement of IAA movement was carried out as described by (Lewis and Muday, 2009). Plants were individually transferred to plates containing 1 µM NPA or 0.01% DMSO as a solvent control eight hours before the start of the assay. ^{3}H-IAA concentration in the application agar was 100nM, and the assay duration was 18 hours from source application to sectioning. Roots were cut 2mm and 7mm from the root apex and the resulting 5mm section was immersed in scintillation fluid, where it was incubated overnight in the dark before detection of radioactivity in a Beckman LS6500 scintillation counter (Beckman-Coulter, Brea, CA). Each measurement represented the average of three independent assays, each of which was performed with 8-12 seedlings per genotype/treatment condition.

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Chapter 4. Molecular aspects of ROSY1 function

INTRODUCTION

Plant gravitropism has captured in the interest of plant biologists for more than a century. Even though we are still far from understanding how plants perceive directional stimuli, some aspects of gravitropism signal transduction and responses have been identified, such as the gravity-induced rise in cytosolic Ca^{2+} (Goswami and Audus, 1976; Lee et al., 1983; Poovaiah et al., 1987; Poovaiah and Reddy, 1987; Reddy et al., 1987). Recent research has shown that levels of cytosolic Ca^{2+} rise in specific cells in the gravity-stimulated roots (Monshausen et al., 2011), and that the Ca^{2+} spike is caused, at least in part, by Phospholipase C (PLC) mediated inositolphosphate signaling (Perera et al., 2006).

The levels of Inositol-1,4,5-triphosphate ($InsP_3$) are not only necessary for releasing Ca^{2+} from intracellular stores (Alexandre and Lassalles, 1990), but also for regulating the positioning of the auxin efflux carrier PIN proteins on the plasma membrane and thereby regulating the direction of auxin flux (Luo et al., 2011; Zhang et al., 2011). It has been shown that the positioning of PIN proteins on the plasma membrane is also dependent on the sterol composition of the plasma membrane (Willemsen et al., 2003; Men et al., 2008). Using *Arabidopsis* mutants defective in sterol biosynthesis, it was demonstrated that when the membrane sterol composition is altered, it affects the positioning of PIN proteins on the

plasma membrane, which affects auxin efflux, and in turn affects gravitropism (Willemsen et al., 2003; Men et al., 2008).

Because of the importance of membrane sterol composition and lipid signaling in gravitropic responses, it is interesting to note that ROSY1, which we characterized as a repressor of gravitropic bending (chapter 3), has a conserved lipid/sterol binding ML (MD2 Lipid-binding) domain. In animal systems, proteins with the ML domain have been shown to be important for recycling cellular lipids and sterols, thereby maintaining the sterol and lipid composition in the cellular membranes (Inohara and Nunez, 2002).

Proteins containing the ML domain are relatively small (~20KDa). The ML domain consists of multiple β-strands that form two β-sheets and a central cavity region -(Figure 4.1). This cavity binds specific lipids or sterols.In the case of the ML domain human protein NPC2 depicted in Figure 4.1, the cavity binds sterols, specifically, cholesterol (Okamura et al., 1999).

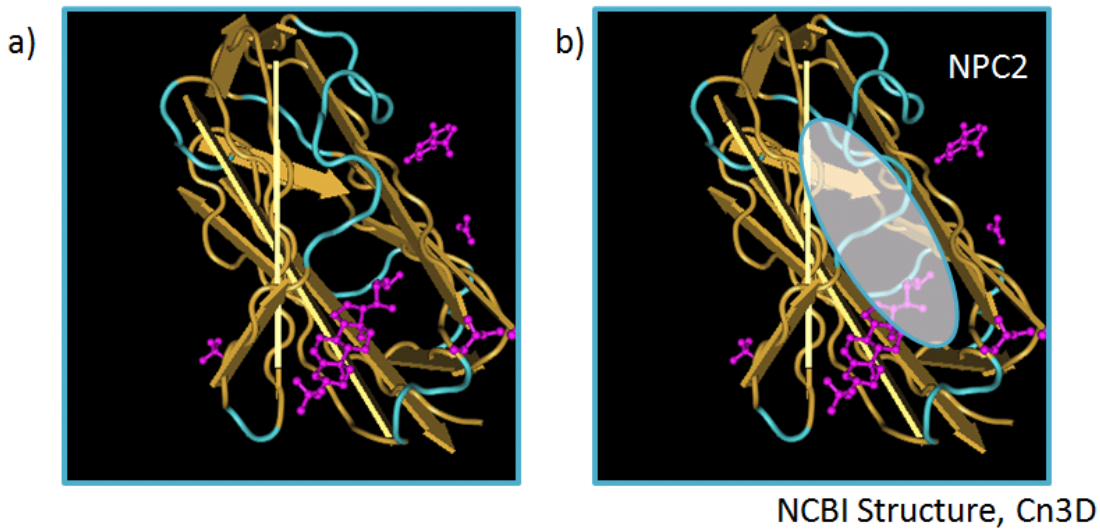

Figure 4.1 Model based on the crystal structure of NPC2 depicting the lipid binding cavity.

The lipid-binding cavity in the structure of the protein NPC2 was studied by Friedland et al. (2003). (a) The β-sheets (denoted by the golden arrows) were reported to form a central region where the lipid binding cavity exists, denoted by the oval in (b). The cavity in this region binds cholesterol (denoted by the pink ball and stick steroid structure interacting with the cavity). The blue tube worm structure denotes coils in the protein structure. Image is obtained from the NCBI Cn3D database.

The domains have been shown to have very specific lipid or sterol binding affinities. TheML-domains bind lipopolysaccharides in the human MD1 and MD2 proteins (Viriyakosol et al., 2001), phosphatidylinositol and phosphatidylglycerol in*Aspergillus* PG/PI-TP protein(Record et al., 1999) and GM2 ganglioside in human GM2A protein (Conzelmann and Sandhoff, 1979).

This specificity of binding is determined by the conformation of specific amino acids in the lipid binding cavity. All ML domain proteins, including ROSY1, have four conserved cysteine residues (Inohara and Nunez, 2002). These cysteine residues form disulfide bonds

inthe homologous human proteins MD1, MD2 (Schromm et al., 2001), Der f2 (Ichikawa et al., 1998) and GM2A (Wright et al., 2000). Mutations in the conserved cysteine residues lead to a loss in lipopolysaccharide binding activity. Other amino acids in the binding cavity also promote or inhibit lipid binding. Within the lipid binding cavity of the protein NPC2, the cholesterol molecules can bind at three different regions(Figure 4.2) and specific amino acids within the cavity promote cholesterol binding (Ko et al., 2003).

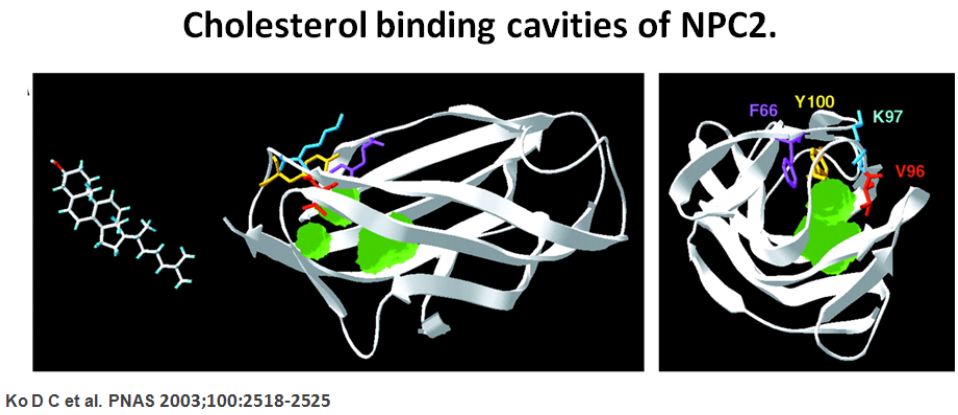

Figure 4.2Lipid binding cavities in NPC2.

Three possible cholesterol binding cavities (denoted by green) were detected (Ko et al., 2003) in the structure of the crystallized NPC2 (Friedland et al., 2003). Ko et al. identified four key residues key residues- phenylalanine at position 66, lysine at position 97, valine at position 96 and tyrosine at position 100 were shown to be necessary for cholesterol binding. The NPC2 protein is shown as ribbons, cholesterol and the required amino acids are denoted by ball and stick figures. Image reproduced from (Ko et al., 2003).

The specific lipid binding characteristics of these proteins are important for their biological function. The biological functions of some ML domain proteins such as the human GM2A and NPC2 protein are well characterized. In lysosomes (organelles for fat-storing, breakdown

and recycling) of the brain and spinal cord cells, these proteins bind specific lipids or sterols and transfer them to other proteins for processing (Naureckiene et al., 2000).

In case of GM2A, the protein binds the sphingolipid GM2 ganglioside, and presents it to another enzyme, the β-hexosaminidase for degradation, thereby acting as a substrate specific cofactor for degradation of GM2 ganglioside (Sandhoff and Kolter, 1998; Mahuran, 1999). Mutation on the GM2A protein results in build-up of GM2 ganglioside to toxic levels in the cells, resulting in cellular degradation and causing the Tays-Sachs disease in humans (Mahuran, 1999). Tays-Sachs disease is an infant-lethal disease. The neuronal degradation due to ganglioside build-up begins at six months of age, and lead to a severe loss of mental and physical capacities and death within four years of affliction (Cutz et al., 1974). The protein NPC2 shares a high structural similarity with ROSY1, and the function of this protein related to its sterol binding characteristics is documented in the next section.

Because ROSY1 contains an ML domain, elucidating the lipid binding specificities of the protein are necessary to understand the function of this protein.

RESULTS

ROSY1 lipid/sterol binding

Because the protein ROSY1 has the conserved ML domain, we hypothesized that it may have lipid binding activity. To understand ROSY1 function, structural homologs of ROSY1 were identified by sequence-based homology modeling. ROSY1 protein sequence was used toidentify structural homologs based on homology modeling using the PHYRE server (http://www.sbg.bio.ic.ac.uk/~phyre/) (Kelley and Sternberg, 2009). We found 15% amino acid identity, and 100% predicted tertiary structure homology (E-value $1.1e^{-10}$; estimated precision 100 %) to the human protein NPC2. NPC2 was named after the Neimann-Pick disease Type C (NP-C), a fatal inherited human neurovisceral cholesterol storage disorder (Naureckiene et al., 2000). NPC is characterized by LDL-cholesterol accumulation in late endosomal compartments and defective movement of cholesterol to other cellular locations particularly plasma membrane. The glycoproteins NPC2 and NPC1 play a role in correct cholesterol trafficking from lysosomes (Pentchev et al., 1984; Pentchev et al., 1985; Pentchev et al., 1986; Pentchev et al., 1986; Pentchev et al., 1987; Sokol et al., 1988; Liscum et al., 1989; Pentchev et al., 1994; Vanier and Millat, 2003; Ioannou, 2005). NPC2 is a small 16 KDa protein that binds free lysosomal cholesterol and makes it available to NPC1, a membrane trafficking protein.

To investigate if ROSY1 also binds sterols like its human homolog, recombinant ROSY1 protein was generated. ROSY1 expression in bacterial cells was lethal. Because ROSY1 is only 17 KDa in size, at first, a 6X His tag was fused to the protein using vector pDEST17, (Invitrogen, CA), for aiding purification from *E. coli*. The ROSY1-His expression was toxic to a variety of bacterial strains investigated (Table 1), including bacterial strains better-suited for toxic protein purification. ROSY1 fusion with larger tags, such as GST and MBP was tried for protein expression, but the protein was either not generated, or was always severely truncated. Use of chaperone proteins to stabilize ROSY1 expression in the pCold expression system (Roche) also did not yield ROSY1 recombinant protein.

Table 1.A summary of ROSY1 recombinant protein expression efforts.

ROSY1 RECOMBINANT PROTEIN EXPRESSION EFFORTS

Intracellular Accumulation in E.coli - FAILED

E. coli	Vector	Tag	Construct	Result
BL21	pDEST17	His	Complete protein	-
BL21 AI	pDEST17	His	Complete protein	-
C41DE3	pDEST17	His	Complete protein	-
C43DE3	pDEST17	His	Complete protein	-
C41DE3plys	pDEST17	His	Complete protein	-
C43DE3plys	pDEST17	His	Complete protein	-
BL21codon-plus	(pCold vector)5 + pDEST15	GST/MBP	Complete protein	-
C43DE3	(pCold vector)5 + pDEST15	GST	Complete protein	-
C43DE3	(pCold vector)5 + pDEST15	GST	Soluble protein	-

Extracellular Secretion in Kluveromyces lactis - WORKED

Yeast	Vector	Tag	Construct	Result
K. lactis	pKLAC2	HA	Soluble protein	+

ROSY1 complete coding sequence was expressed in all the *E.coli* expression strains listed. Only the soluble part of the sequence (amino acids 26-148; the ML domain) was also cloned with GST expression tag in *E. coli* strain C43DE3 (Lucigen Corporation, WI). No recombinant protein was recovered from *E. coli*. The protein was recovered when the soluble part of ROSY1 was cloned in vector pKlac2 and transformed in the yeast,*K. lactis*.

Failure to generate recombinant ROSY1 in bacteria is interesting, considering that ROSY1 is a eukaryotic protein, and homologs to ML domain proteins do not exist in bacteria. Because of the failure to generate recombinant protein in bacteria, a eukaryotic model system, the yeast *Kluveromyces lactis* was used to generate recombinant ROSY1. Toxicity in yeast following intercellular accumulation of ROSY1 on induction of protein synthesis, was

avoided by cotranslational secretion out of the yeast cells. To enable secretion, the ROSY1 protein coding sequence was cloned following theα-MF secretory domain driven by the LAC4 promoter (NEB).To successfully secrete the ROSY1 recombinant protein from yeast, only the soluble part of the protein – the ML domain was used for protein synthesis. To enable protein purification, the ROSY1 coding sequence was fused with a C-terminal HA tag(Figure 4.3a). Chemically competent*Kluyveromyces lactis* cells were transformed with the pKlac2 plasmid containing the AtROSY1ML-HA construct. Using this strategy, the recombinant protein AtROSY1ML-HA was successfully secreted by yeast cells.*K. lactis*cells (untransformed controls and expressing AtROSY1ML-HA) were induced with galactose for 24 hours, and the total cellular proteins were separated using SDS-PAGE. The tagged recombinant ROSY1 was detected on a western blot with anti-HA-HRP antibody (Genscript, NJ), (Figure 4.3c).

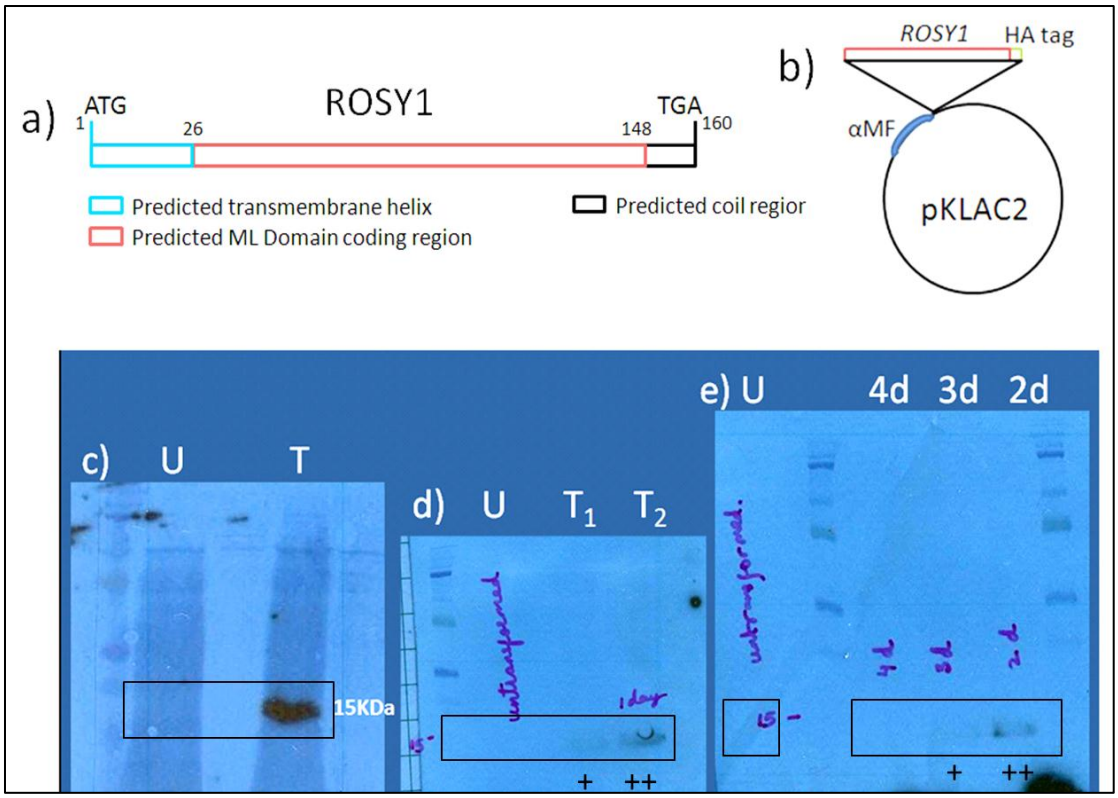

Figure 4.3ROSY1 protein expression from *K. lactis.*

a) Model of *ROSY1* coding sequence. Numbers indicate positions of amino acids. The ML domain (red) was used to clone in the pKlac2 vector, as shown in **b).**The HA tag is denoted in green. The model is not to scale. **c)** 3ml medium with *K. lactis* cells, untransformed (U) as well as transformed with AtROSY1ML-HA construct (T) were induced with galactose for 1 day. The culture was shaken and an aliquot was taken, mixed with 2X SDS buffer and boiled at 95°C for 3 minutes. The total proteins were separated on SDS PAGE gel and incubated with 1:1000 anti-HA-HRP (Genscript, NJ) for 1 hour. Signal was developed using SuperSignal West Femto chemiluminiscent substrate (Fisher Scientific, MO) and probed on Kodak autoradiography films (Sigma-Aldrich, MO), which were then developed with Kodak GBX developer and fixer solutions (Sigma-Aldrich, MO). The un-transformed yeast cells do not show any signal with the antibody on the film. After the western blot analysis, the PVDF membranes were stained with amido-black to stain all the proteins on them. The film was overlapped with the membrane and scanned. The image shows that the 15KDa recombinant ROSY1 protein is synthesized by induced transformed *K. lactis* cells, and that the antibody is specific. **d)** Time course analysis was done to see protein expression levels using multiple transformed *K. lactis* cells. Two representative transformed strains are named T_1 and T_2 here. Clearly, T_2 generates more protein (++) than T_1 (+). There is no band in the untransformed control sample lane. The membranes used for the western analysis were stained with amido-black, but as can be seen in the image, very few proteins are secreted out of yeast, and very few proteins can be seen on the membrane. ROSY1 may be the major protein in the yeast secretion medium. **e)** Time course analysis with another yeast

strain showing protein expression levels 2 days (2d), 3 days (3d) and 4 days (4d) after induction. After 2 days, the maximum amount of recombinant protein was generated (++). The protein production decreased 3 days after induction (+) and no protein was detected in the medium 4 days after induction. Using multiple colonies, the time point of 2 days after induction was selected for recovering maximum ROSY1 recombinant protein.

The anti-HA-HRP antibody recognized a single 15 KDa band of the expected size of the AtROSY1ML-HA protein and does not detect other *K. lactis* proteins. A time course analysis was done to identify how fast after induction, maximum protein is secreted out into the medium by the *K. lactis* cells. Two days after induction was the optimal time point to obtain AtROSY1ML-HA protein (Figure 4.3 (d) and (e)).

Because ROSY1 is a close structural homolog of the cholesterol-binding NPC2, we investigated if ROSY1also binds sterols. The protein AtROSY1ML-HA was used for sterol binding experiments using overlay blots. Various sterols found in plant membranes were used for dilution series from 0.5 pmol to 100 pmol. The sterols were spotted onto a PVDF membrane. The only major plant sterol not used for the analysis was campesterol, because of the low availability and prohibitive cost of pure campesterol needed for the analysis. Because there were not many other proteins secreted by yeast into the medium, and the antibody was specific to AtROSY1ML-HA protein (Figure 4.3 (d) and (e)), the protein was not purified from the growth medium of induced yeastbut directly used for the sterol-binding experiment. 10X TBST was added to the medium to make the final concentration of the medium 1X TBST, and the solution was used for incubation with the sterol-displaying PVDF membranes.

Upon probing the membrane with anti-HA-HRP antibody, it was seen that ROSY1 specifically binds stigmasterol(Figure 4.4.).

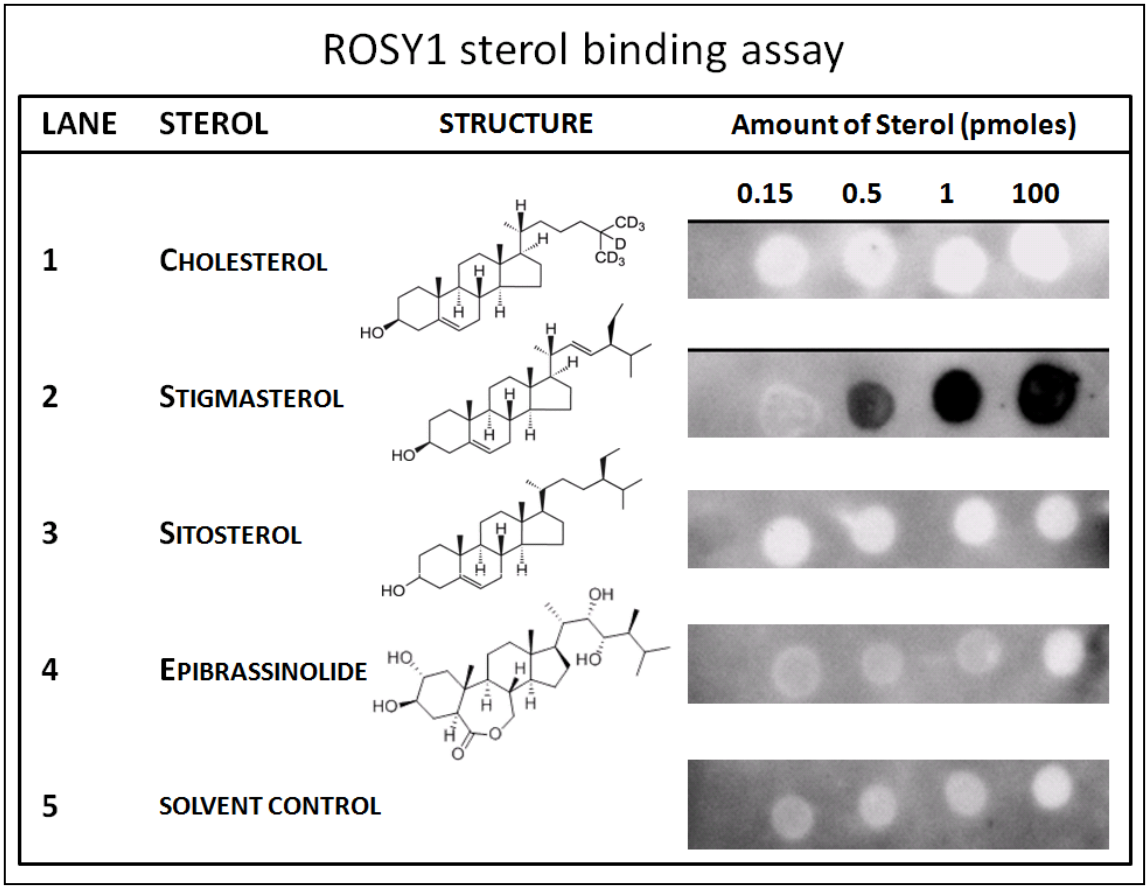

Figure 4.4ROSY1 Sterol Binding Assay.

Chemically competent *K. lactis* cells were transformed with pKlac2 vector expressing AtROSY1ML-HA construct. The yeast were grown on selective medium with 3mM acetamide, and positive colonies were streaked on fresh plates and screened for presence of AtROSY1ML-HA construct by PCR. Six colonies were selected and protein expression was induced by growing colonies in YPD/Galactose medium for two days. 10ml culture was taken from transformed yeast expressing AtROSY1ML-HA construct and control untransformed *K. lactis* cells. The culture medium was centrifuged to pellet down yeast cells, and the supernatant medium was mixed with 1ml 10X TBST to make 1X TBST medium. The medium was then used to bind on PVDF membranes, spotted with different quantities of sterols. The membranes were washed with 1X TBST 3X for 90 minutes, incubated with 1:1000 anti-HA-HRP (Genscript) for 1 hour, and washed 3X for 90 minutes. Signal was developed using SuperSignal West Femto chemiluminiscent substrate (Thermo Scientific) and probed on Kodak autoradiography films (Sigma), which were then developed with Kodak GBX

developer and fixer solutions (Sigma).The untransformed yeast culture does not give any signal on the film.ROSY1 specifically binds stigmasterol. The structures of different sterols can be seen. Stigmasterol has a staggered side-chain owing to double bond between C22 and C23.

Unlike NPC2, ROSY1 does not bind cholesterol. ROSY1 also does not bind epibrassinolide, which is interesting because brassinosteroids are important plant-specific growth regulators, which affect plant gravitropic response (Kim et al., 2000). However, ROSY1 does not bind sitosterol which is structurally close to stigmasterol (Figure 4.4), except that the side chain in sitosterol is more planar, whereas the side chain of sitosterol has a more staggered conformation owing to the double bond. These results indicate that sterol-binding to ROSY1 ML domain does not depend on the structure of the tetracyclophenanethrene ring system, but on the structure and conformation of the sterol side chain. It has been reported that within the lipid binding cavity of the protein NPC2, several amino acids play a key role in binding cholesterol (Ko et al., 2003). Therefore the tertiary structure of ROSY1, and specific amino acids within the protein's lipid binding domain will probably determine which lipids/sterols ROSY1 binds.

The ML domain is equally capable of binding lipids and sterols, as shown by binding preferences of other ML domain proteins such as NPC2 and GM2A. In the NCBI database (http://www.ncbi.nlm.nih.gov/), the protein ROSY1 has been annotated as phosphatidyl glycerol/phosphatidyl inositol (PG/PI) binding protein, because some homologs have been shown to bind PG and PI (Record et al., 1999), and carry out their exchange on the membrane. To investigate if ROSY1 binds to PG, PI or other phospholipids in vitro,

AtROSY1ML-HA was used to bind lipids on commercially available membrane strips with membrane phospholipids (Echelon Biosciences Inc., UT, USA). AtROSY1ML-HA bindsspecifically with phosphatidyl ethanolamine (PE), (Figure 4.5.).

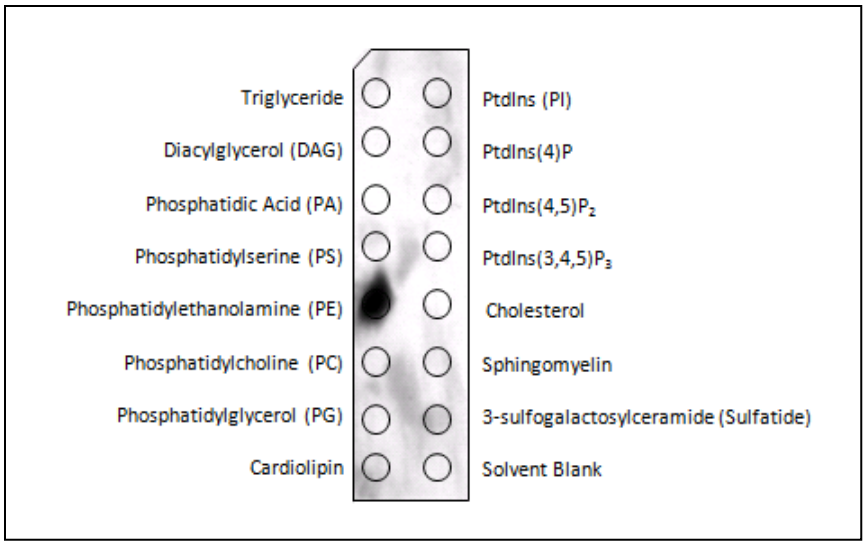

Figure 4.5 AtROSY1ML-HA Lipid Binding Assay.

Interaction of ROSY1 was tested with common membrane phospholipids and sterols using a commercial membrane strip (Echelon Biosciences, UT). 200 pmol of each lipid or sterol was spotted on a PVDF membrane. ROSY1 specifically binds to phosphatidylethanolamine (PE).

The specific PE spotted on the membrane lipid strip is 1,3-Dipalmitoyl-sn-glycero-2-phosphoethanolamine (DPPE). To test if the biding of ROSY1 with DPPE was also dependent on the structural conformation of DPPE, another PE variant, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was acquired. DOPE has a double bond in its side chain, whereas the structure of DPPE has a comparatively planar conformation(Figure 4.6 (b) and 4.6 (c)). It was shown that ROSY1 specifically binds DPPE, but does not bind DOPE and binds with a much higher affinity with stigmasterol (Figure 4.6).

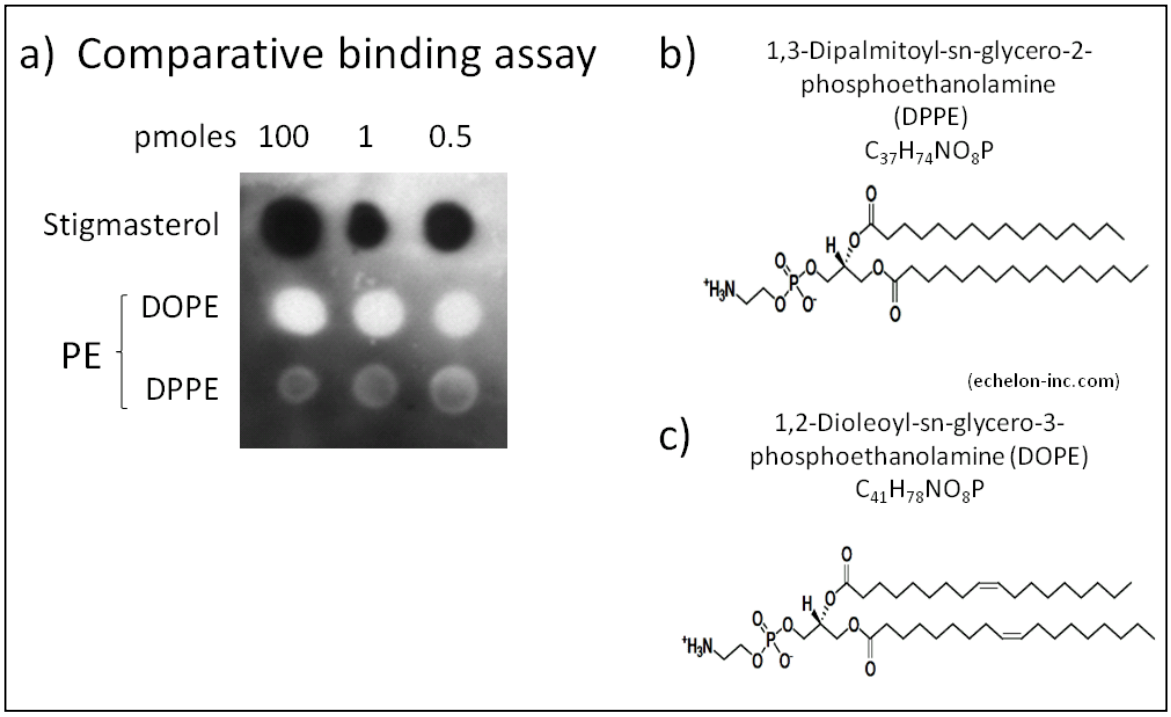

Figure 4.6 Comparison of ROSY1 binding with Stigmasterol, DPPE and DOPE.

a) Stigmasterol, DPPE and DOPE were spotted on to a PVDF membrane, and binding affinity of ROSY1 was investigated with the three. ROSY1 binds with high affinity with stigmasterol, binds with lesser affinity with DPPE and does not bind with DOPE. b) and c) indicate the structures of DPPE and DOPE. The side chains of DOPE contain double bonds that make the structure of the lipid more staggered, and comparatively less planar.

ROSY1 and membrane sterol and lipid composition

ROSY1 binds stigmasterol, which constitutes about 6% of total sterols in *Arabidopsis*, and DPPE, a plasma membrane phospholipid. Because it is know that ROSY1 homologs in mammalian systems are involved in vesicle transport and membrane recycling, we investigated if theabsence of ROSY1 affects lipid/sterol composition in the membranes of

rosy1-1Arabidopsis knockout lines (chapter 3). To extract lipids, WT and *rosy1-1*seedlings were grown in the dark until they were seven days old. The roots and hypocotyls were separated, weighed, and total sterols and lipids of the roots or hypocotyls were extracted by a chloroform-methanol based extraction method. The dried lipids were sent to Kansas State Lipidomics Facility (Manhattan, KS) for comparative sterol and lipid profiling. It was seen that the proportion of specific lipids and sterols was indeed different between *rosy1-1* and WT samples. Comparative sterol profiling showed that quantitatively (sterols nmol/mg dry weight) all sterols were lower in *rosy1-1* roots compared with WT roots (Figure 4.7). A two-tailed T-test was used to calculate significance of differences between the quantities of different sterols between *rosy1-1* and WT root samples. Setting p-value < 0.05 as a cut-off for significance, there was no significant difference in the nmol/mg dry weight of cholesterol ($p=0.5$), brassicasterol ($p=0.09$) and campesterol ($p=0.15$) between *rosy1-1* and WT roots. There was a significant difference in the nmol/mg dry weight of stigmasterol ($p=0.04$) and sitosterol ($p=0.05$) between *rosy1-1* and WT roots. There was no significant difference in sterol levels between in WT and *rosy1-1* hypocotyls (Figure 4.7).

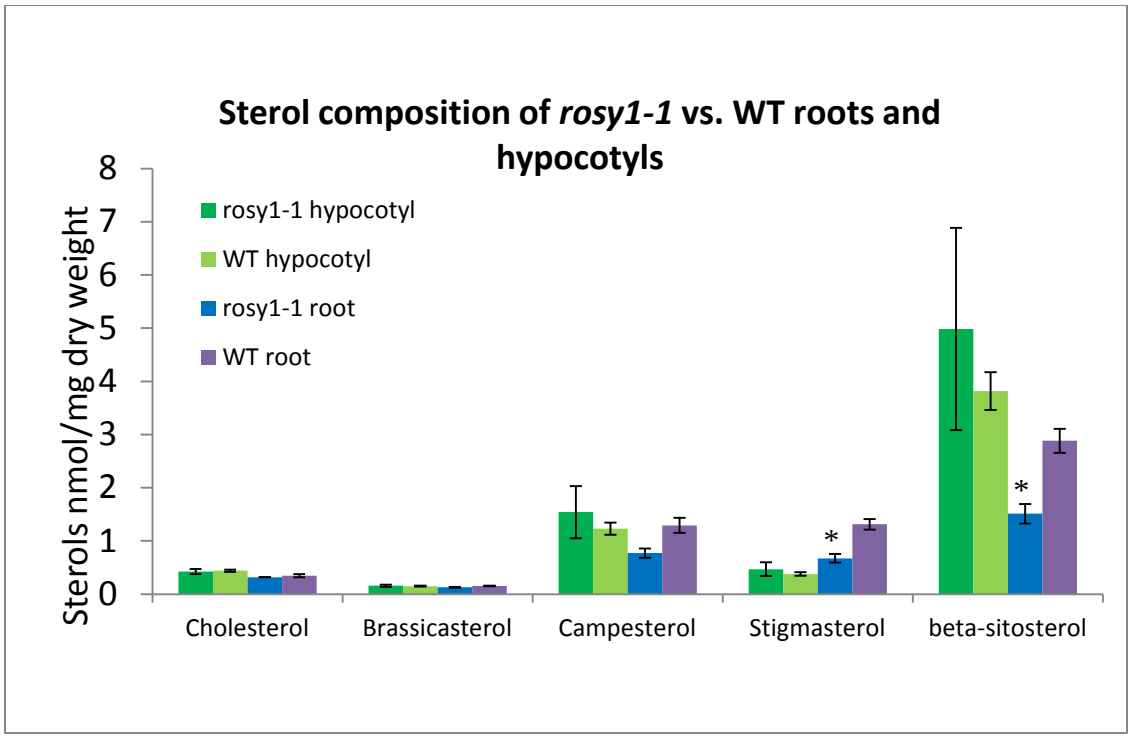

Figure 4.7Sterol composition of *rosy1-1* vs. WT roots and hypocotyls.

Seven-day-old*rosy1-1* and WT dark-grown seedlings were used to harvest roots and hypocotyls in hot isopropanol to prevent PLD activation and lipid degradation. Using chloroform- methanol extraction, the total lipids were harvested and sent to Kansas State Lipidomics Facility (Manhattan, KS) for ESI MS based comparative quantification using API 4000 LC /MS/MS system (AB Sciex, Foster City, CA). Each sample had three biological replicates and three technical replicates. Levels of total membrane sterols (nmol/mg dry weight) were compared from root and hypocotyl samples. In hypocotyl samples, levels of sterols between *rosy1-1* and WT were not significantly different. In roots, there was a significant difference in the levels of different sterols between *rosy1-1* and WT samples. Asterisks indicate significance of $p < 0.05$ (two-tailed paired T-test).

When the relative amounts of different sterols are plotted with respect to total membrane sterols, the proportion of stigmasterol and sitosterol in *rosy1-1* seedling roots was lower than in wild type roots (Figure 4.8). Consequently, the proportion of all other sterols was higher in *rosy1*-1 roots than in wild type roots. A two-tailed T-test was used to calculate significance of difference between the ratios of different sterols to the total sterols in the sample between

rosy1-1 and WT root samples. Setting p-value < 0.05 as a cut-off for significance, there was no significant difference in the ratios of cholesterol (p=0.1), brassicosterol (p=0.1) and campesterol (p=0.4) between *rosy1-1* and WT roots. There was a significant difference in the nmol/mg dry weight of stigmasterol (p=0.004) and sitosterol (p=0.04) between *rosy1-1* and WT roots.

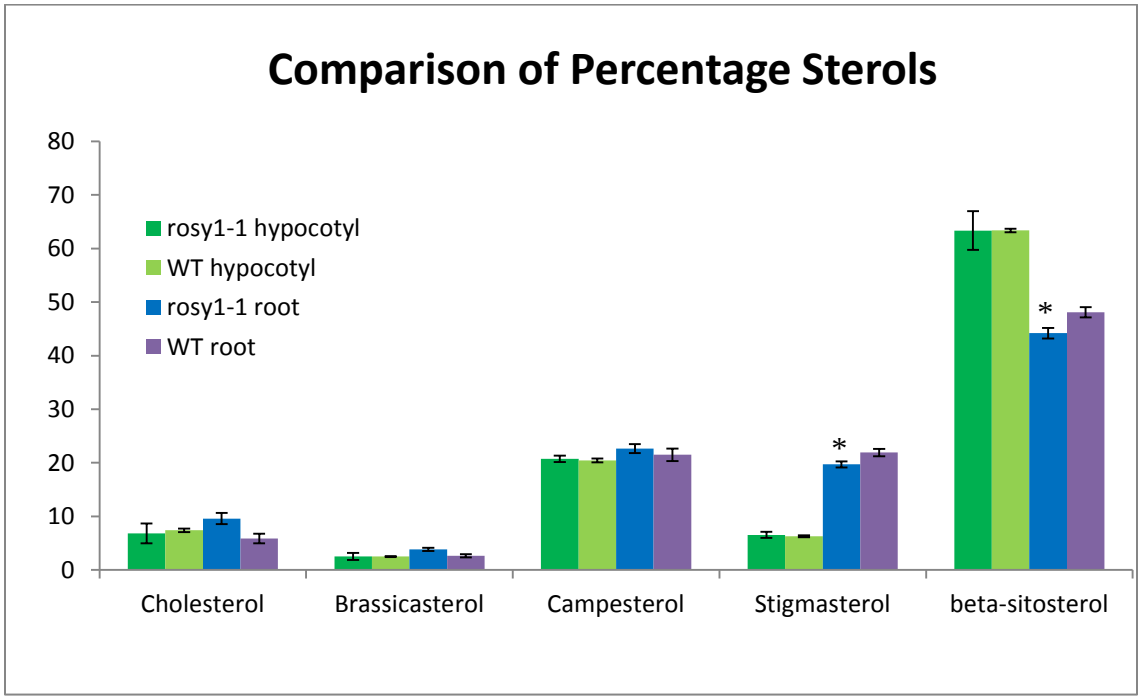

Figure 4.8 Molar percentages of sterols of *rosy1-1* vs. WT roots and hypocotyls.

The ratio of stigmasterol and sitosterol to total membrane sterols is lower in *rosy1-1* roots compared with WT roots. Levels of sitosterol are the highest in both hypocotyls and roots compared with all other sterols. In WT roots, 48.1% of total membrane sterols are sitosterol, but in *rosy1-1* roots only 44.2% of total membrane sterols are sitosterol. In WT roots, 5.9% of total sterols are cholesterol, whereas in *rosy1-1* roots, 9.6% of total root sterols are cholesterol. Therefore the sterol composition has changed in the *rosy1-1* mutant. Asterisks indicate significance of p< 0.05 (two-tailed paired T-test).

In addition to the changed membrane sterol composition in *rosy1-1* seedlings, there was also change in the lipid composition of the membranes in *rosy1-1* seedlings compared with WT. In fact,there were differences in the ratio of lipids in both*rosy1-1* roots and hypocotyls compared with WT roots and hypocotyls.

Although ROSY1 binds DPPE, there was no significant difference between PE levels in *rosy1-1* roots and hypocotyls compared with WT seedlings (Figure 4.9).

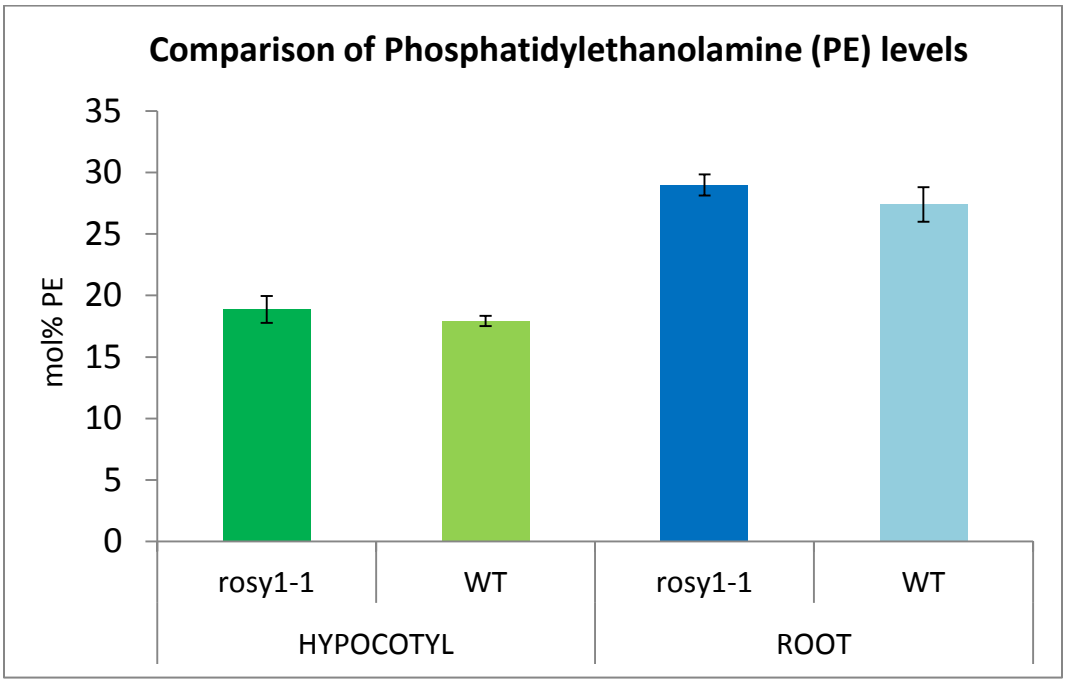

Figure 4.9 PE composition of *rosy1-1* vs. WT roots and hypocotyls.

Lipid samples were prepared from *Arabidopsis* seedlings as explained in Figure 4.7. Using ESI-MS/MS based lipid profiling, the levels of various PE species were not found to be significantly different between WT and *rosy1-1* root and hypocotyl samples. The results from all PE species were added to obtain the graph above, showing no significant difference in PE levels between *rosy1-1* and WT samples.

The most remarkable difference between *rosy1-1* and WT samples was the difference in phosphatidic acid (PA) levels, even though ROSY1 does not bind PA *in vitro*. While*rosy1-1* hypocotyls had significantly higher PA than WT hypocotyls (two-tailed T-test p=0.02), *rosy1-1* roots had significantly lower PA levels (two-tailed T-test p=0.009) (Figure 4.10).

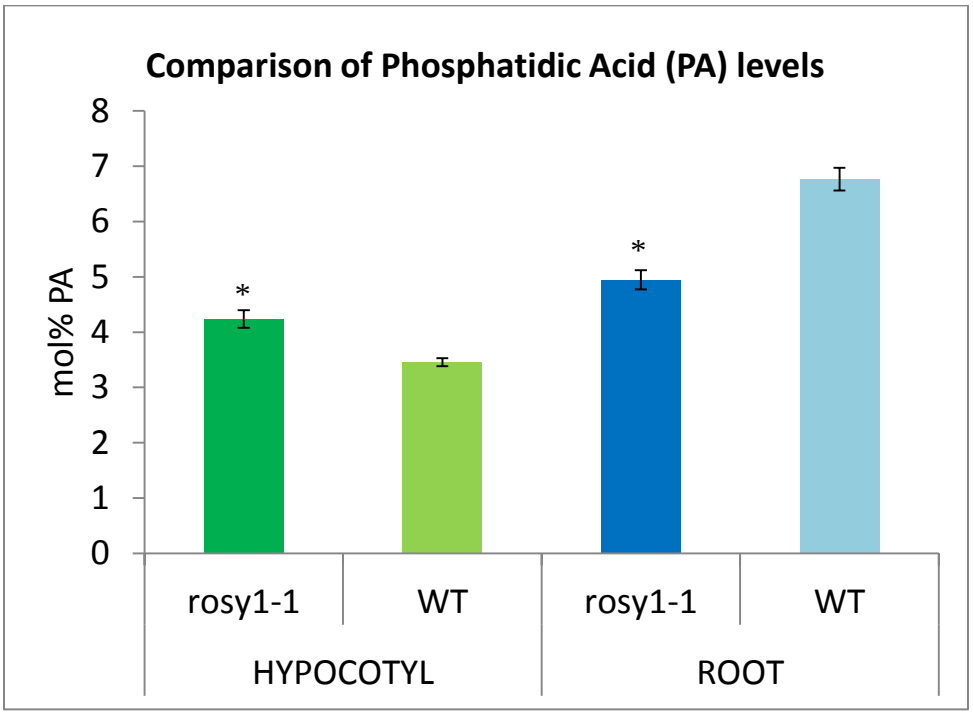

Figure 4.10 PA composition of *rosy1-1* vs. WT roots and hypocotyls.

The levels of PA were found to be significantly different between *rosy1-1* and WT samples. While the mol% of PA in WT hypocotyls is 3.4% of membrane lipids, the mol% of PA in *rosy1-1* hypocotyls is increased to 4.2% of total membrane lipids. Conversely, in WT roots the mol% of PA is 6.8% of membrane lipids, the mol% of PA is reduced to only 4.9% of membrane lipids in *rosy1-1* roots. Asterisks indicate significance of $p<0.05$ (two-tailed paired T-test).

Interestingly, all species of PA showed the same trend of being higher in *rosy1-1* hypocotyls and lower in *rosy1-1* roots compared with WT samples (Figure 4.11).

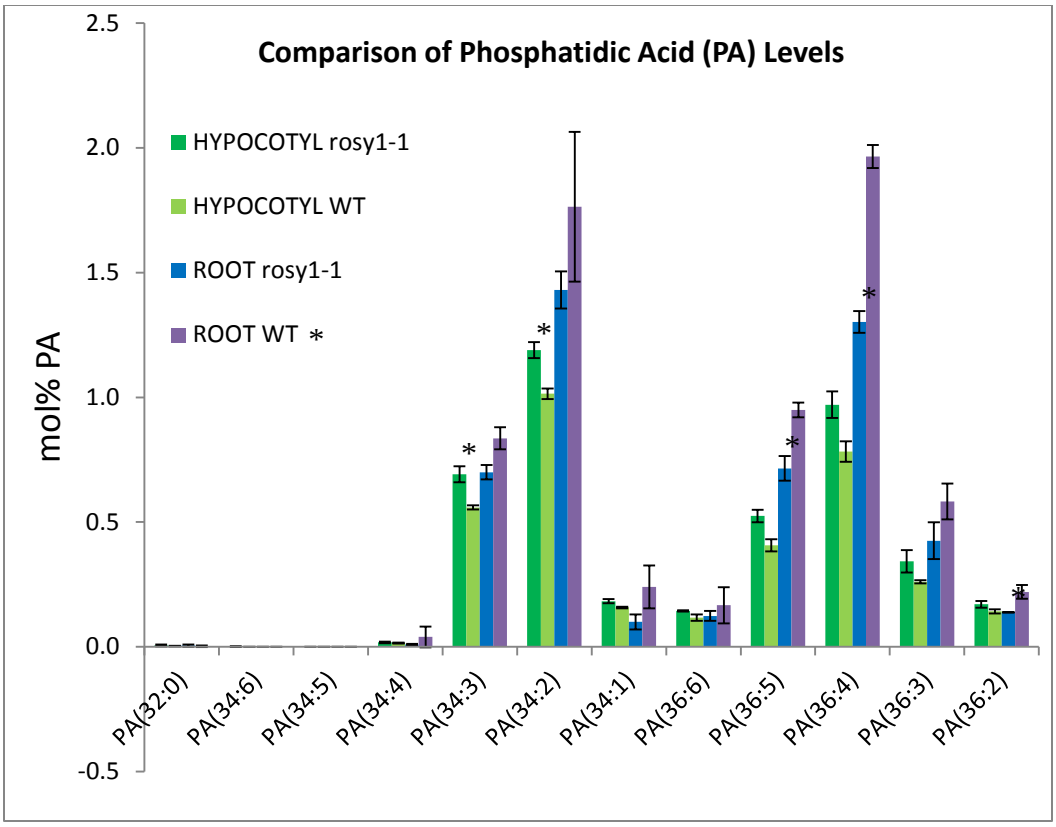

Figure 4.11 Different PA composition of *rosy1-1* vs. WT roots and hypocotyls.

The membrane proportion of all species of PA has reduced in *rosy1-1* roots compared with WT roots, and has increased in *rosy1-1* hypocotyls compared with WT hypocotyls.Asterisks indicate significance of $p<0.05$ (two-tailed paired T-test).

Other quantified membrane lipids in *rosy1-1* roots and hypocotyls, as compared with their wild type counterparts are presented in Appendix 1. The overall comparison of levels of lipids between *rosy1-1* and WT samples is tabulated below in Table 2.

Table 2 Comparative quantitities of various lipids and sterols in rosy1-1 and WT.

Lipid/Sterol	Hypocotyl		Root		P<0.05
	rosy1-1	WT	rosy1-1	WT	
DGDG	-	+	+	-	
MGDG	-	+	-	+	
PG	-	+	+	-	*
LysoPG	+	-	+	-	*
PE	+	-	+	-	
PI	+	-	-	+	
PS	-	+	+	-	
PA	+	-	-	+	*
Cholesterol	=	=	+	-	
Brassicasterol	equal	equal	+	-	
Campesterol	equal	equal	+	-	
Stigmasterol	equal	equal	-	+	
β-sitosterol	equal	equal	-	+	

The results from comparative quantitative analysis of all species of same lipid were added together, and the overall increase or decresase of the mol% of the particular lipid was compared with the total membrane lipids. The net increase or decrease of a class of lipids between *rosy1-1* and WT samples is tabulated here. The overall increase or decrease of mol% of different sterols compared with the total sterols in the sample have been tabulated here. In the table, (+) sign means that here is comparatively more mol% of a particular sterol or lipid in a given sample, (-) sign means it is lesser, and the (equal) means the level of that lipid or sterol is comparable between samples. Asterisks indicate significance of $p<0.05$ (two-tailed paired T-test).

These results indicate that ROSY1 not only binds specific lipids and sterols, but the absence of the protein in *rosy1-1* seedlings changes the sterol and lipid composition of these seedlings compared with WT. To understand how ROSY1 may affect membrane composition, we sought to identify proteins that interact with ROSY1.

ROSY1 protein-protein interactions

To identify ROSY1 protein-protein interactions, a yeast-two hybrid experiment was performed. Two cDNA libraries were generated using mRNA from roots of seven-day-old light-grown or dark-grown *Arabidopsis*seedlings. Prior to mRNA extraction, all seedlings were gravity-stimulated for 2 minutes. This was done to identify any transiently expressed mRNAs that encode proteins that may interact with ROSY1. To generate the bait vector, the complete ROSY1 mRNA sequence was used, but using this construct, no functional protein-protein interactions were identified. This could be because ROSY1 has a transmembrane helix on the N-terminus. This could also be because the full-length ROSY1 protein may be toxic in yeast. Therefore, only soluble ROSY1 sequence which is the central ML domain (AtROSY1-ML)was used to generate the bait vector. During two independent mating events (Y8 and Y16, Figure 4.12(b)) using the cDNA library generated from dark-grown *Arabidopsis* seedlings, one functional interaction was obtained from the screen (Figure 4.12).

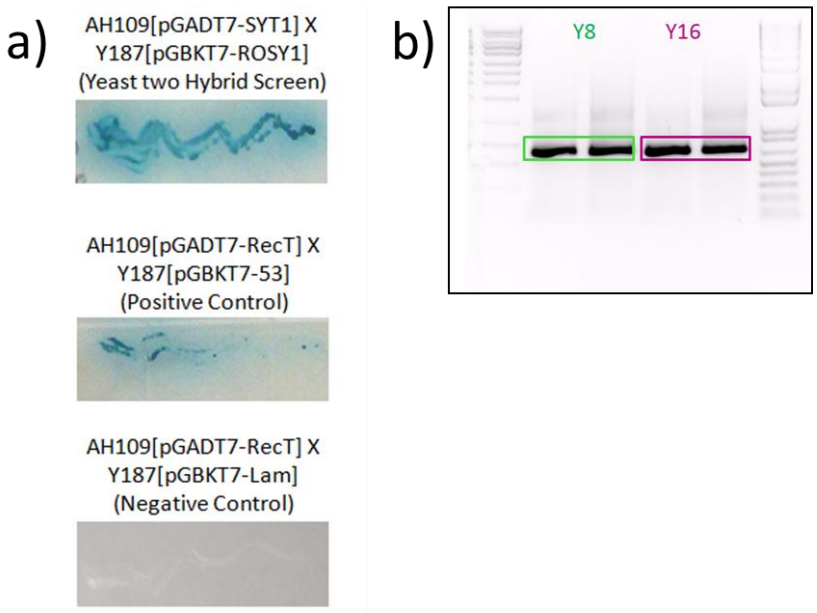

Figure 4.12Interaction between ROSY1 and SYT1 in yeast.

Soluble ROSY1 (AtROSY1-ML) was cloned in vector pGBKT7 (Clontech, CA) and transformed into *S. cerevisiae* strain Y187. The ROSY1 protein is expressed in fusion with the DNA binding domain, that interacts with the *GAL4* recognition sequence up-stream of reporter genes *MEL1, HIS2 and ADE2*. The transformed Y187 colonies are able to grow on yeast growth medium without Tryptophan (SD/-Trp). Gravi-stimulated *Arabidopsis* root cDNA library was cloned into Clontech vector pGADT7-Rec, and transformed into *S. cerevisiae* strain AH109. The vector pGADT7-Rec has a DNA activation domain and transformants can grow on selective medium lacking Leucine (SD/-Leu). If fusion proteins with the DNA activation domain interact with bait (ROSY1) bound on the *GAL4* promoter region by the DNA binding domain, it results in transcriptional activation of the *GAL4* gene and the reporter genes downstream. Positive interactions were identified by growth on quadruple drop-out medium lacking Histidine, Adenine, Leucine and Tryptophan (SD/-Ade/-His/-Leu/-Trp). Streaking positive colonies on medium with X- α-Gal activates the reporter gene *MEL1*. MEL1 encodes α-galactosidase, which hydrolyzes X-α-Gal into galactose and 5-bromo-4-chloro-3-hydroxyindole which dimerizes into a blue end product.**a)** The blue colony seen above is result of a positive interaction between ROSY1 and an unknown protein. **b)**The positive colonies were used for colony PCR using AD vector-specific primers. The PCR products were eluted from the gel and sequenced with AD vector-specific primers. Sequencing of the 850bp PCR product from both colonies Y8 andY16 revealed partial coding sequence of*Arabidopsis* Synaptotagmin1. The positive and negative controls are known proteins supplied by Clontech, which interact or do not interact with each other, used to check the validity of the experimental system.

The positive colonieswere used for colony-PCR with AD vector-specific primers, and the PCR product was sequenced using the same primers. The sequenced region from both Y8 and Y16 was found to encode the 66 amino acid long C-terminal region of *Arabidopsis* Synptotagmin1 (SYT1), a membrane trafficking protein. This interaction gave the name ROSY1 (InteractoR Of SYtnaptotagmin1) to the gene At2g16005.

The sequence cloned within the interacting SYT1 construct included only the last 66 amino acids of the 541 amino acids long SYT1 protein. Three other colonies also activated the *MEL1* reporter gene, indicating a positive interaction between ROSY1 and the protein expressed in fusion with the activation domain in these colonies. Upon sequencing with AD vector-specific primers, the DNA from these three colonies was found to encode translation elongation factor EF 1-α. Because any expressed protein is expected to interact with translation-related proteins in the cell, we focused on the interaction of ROSY1 with SYT1 to understand ROSY1 functions.

SYT1 is a member of the large SYT family of plasma membrane localized calcium sensing proteins that have been shown to regulate calcium dependent endocytosis and exocytosis of vesicles in mammalian system (Reddy et al., 2001; Schwarz, 2004; McNeil and Kirchhausen, 2005) as well as in *Arabidopsis* (Lewis and Lazarowitz, 2010; Yamazaki et al., 2010). All SYT proteins contain transmembrane helices and two calcium binding C2 domains, C2A and C2B. In the presence of Ca^{2+}(Kuo et al., 2009), and facilitating membrane environment such

as presence of PIP_2(Kuo et al., 2011), the C2 domains are configured to bind two bilayers and bridge them together for fusion (Herrick et al., 2009).

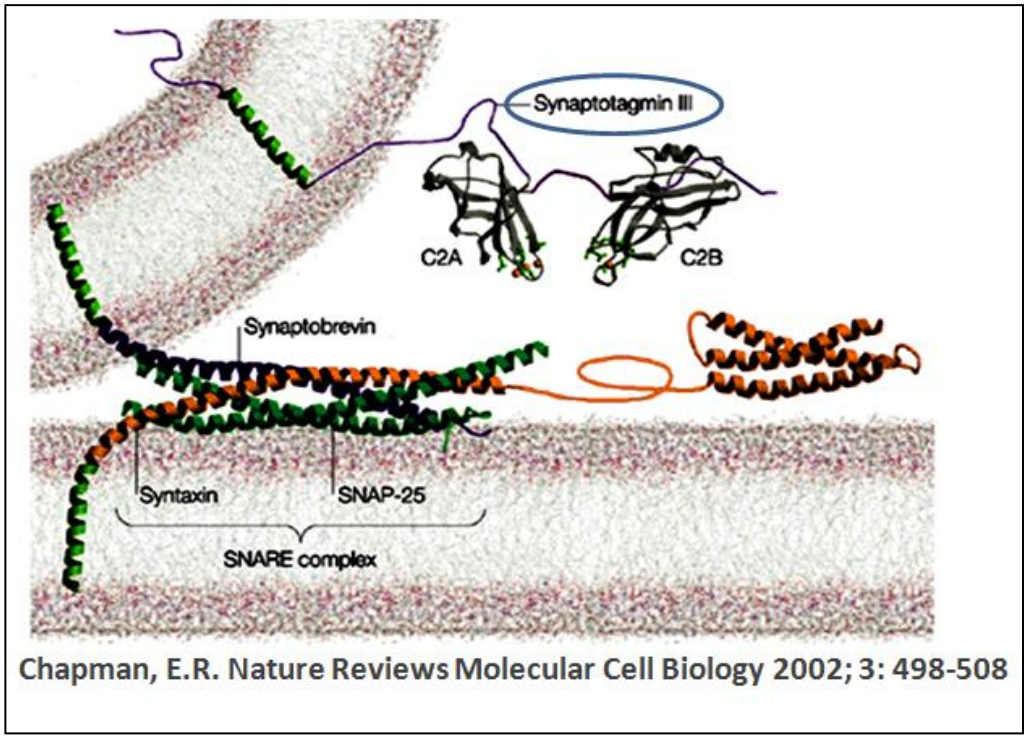

Figure 4.13 Synaptotagmin and the SNARE complex.

SYT proteins, such as SYTIII shown above, have one membrane spanning transmembrane domain as shown above, and two Ca^{2+} binding domains.The protein is activated for membrane fusion in presence of cytosolic Ca^{2+}, which enables a conformational change that helps the protein bring two bilayers together and reduce the energy for membrane fusion along with other proteins in the SNARE complex. Presence of PIP_2 in the bilayers where SYT proteins are docked is also necessary for interaction with Ca^{2+} and membrane fusion.

ROSY1 interacted with partial (65 amino acids long) sequence of SYT1 protein (amino acids 477-541) to give a positive interaction in the yeast-two-hybrid screen. The ROSY1

interacting domain in protein SYT1 is spatially distinct of the C2 domain, as seen in the *SYT1* gene cartoon in Figure 4.13.

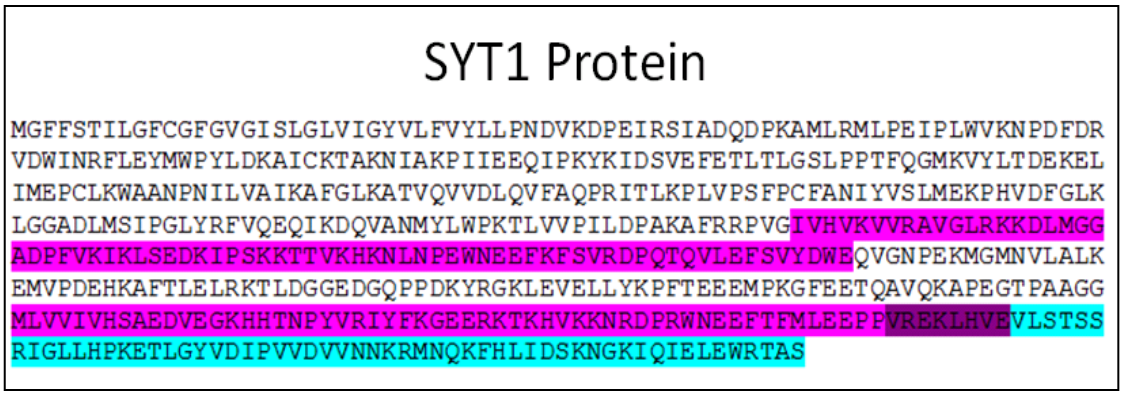

Figure 4.14*Arabidopsis* SYT1 protein sequence showing ROSY1 interacting domain.

The pink regions represent the C2 Ca^{2+} binding domain in the SYT1 protein. The blue region represents the region on SYT1 that interacts with ROSY1. The purple region in between represents the overlap between the C2 domain and the sequence pulled out of the ROSY1 interacting bait vector. It is not known which amino acids in the ROSY1 interacting region are responsible for the interaction.

The activation of *MEL1* reporter was checked by addition of X-α-Gal in the plating medium. The interaction was confirmed in yeast by co-transformation of reporter yeast strain with ΔROSY1 and SYT1 plasmids, as seen in Figure 4.15. While preparing constructs for ROSY1 co-transformation with SYT1 in yeast cells, constructs were also made to investigate interaction of the other two isoforms, At1g45015 and At3g44100 with SYT1. At1g45015 was also found to interact with ROSY1 whereas At3g44100 does not(Figure 4.15,). This demonstrates that At1g45015 may be a true functional isoform of ROSY1, and the protein At1g45015 was named ROSY2 (InteratoR Of SYnaptotagmin 2). It may be remembered at this point that while *ROSY1* mRNA was specifically root-expressed, *ROSY2* mRNA is expressed both in root and in hypocotyls.

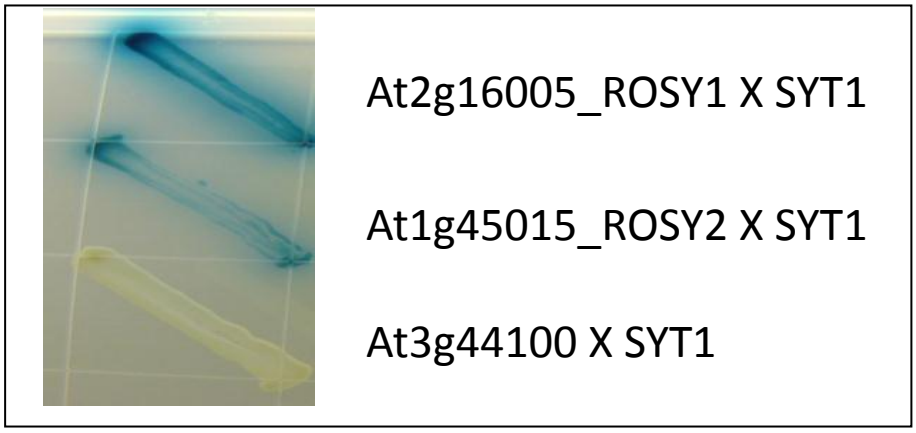

Figure 4.15 Co-transformation of SYT1 and ROSY1 in yeast.

Co-transformation of yeast strain AH109 with SYT1-pGADT7 vector was performed, along with the ROSY1 or At1g45015 or At3g44100 coding sequence cloned in the pGBKT7 vector. The positive interactions were identified by activation of *MEL1* reporter gene by picking colonies surviving on quadruple drop-out medium SD/-Ade/-His/-Leu/-Trp and streaking them on SD/-Ade/-His/-Leu/-Trp supplemented with X-α-Gal. True interactions show a blue color because of the X-α-Gal hydrolyzing activity of *MEL1* product α-galactosidase.

Function of ROSY1 in salt stress

Changes in membrane composition has distinct effects on salt stress signaling in plants (Wang et al., 2006). Phosphatidic acid is a central plant signaling lipid, implicated in signaling abiotic and biotic stresses, including salt stress (Testerink and Munnik, 2005; Wang et al., 2006; Bargmann et al., 2009; Xue et al., 2009). In plants, Phospholipase D is responsible for hydrolyzing membrane phospholipids, such as phosphatidylcholine to phosphatidic acid. Virtually all isoforms of PLD have been implicated in stress responses, and it is clear from studies on mutants of PLD isoforms that changes in PA levels can cause changes in the plant's abiotic stress responses (Sang et al., 2001; Zhang et al., 2005; Wang et al., 2006; Bargmann et al., 2009). Differences in phosphatidic acid levels in *rosy1-1* roots

and hypocotyls compared with WT samples can have an effect on abiotic stress signaling in *rosy1-1* plants. Furthermore, ROSY1 interacts with SYT1. SYT proteins along with the rest of the SNARE complex, conductvesicular fusion and recycling. *Arabidopsis* SYT1 has been shown to maintain plant plasma membrane integrity (Schapire et al., 2009). After abiotic stresses such as salinity and freezing that damage the plasma membrane, *Arabidopsis* SYT1 is required for membrane repair and resealing (Schapire et al., 2008; Yamazaki et al., 2008). Loss of function mutants *syt1-1* and *SYT1*-RNAi lines (Schapire et al., 2008; Yamazaki et al., 2008) showed an increased susceptibility to abiotic stresses. Because of the change in the lipid and sterol composition of *rosy1*-1 membranes, and the interaction of ROSY1 and SYT1, the role of ROSY1in plant abiotic stress signaling was investigated. Experiments with supplementing MS medium with a range of NaCl (0mM to 200mM) with or without $CaCl_2$, *rosy1-1* seedlings were found to be more tolerant to salt stress.

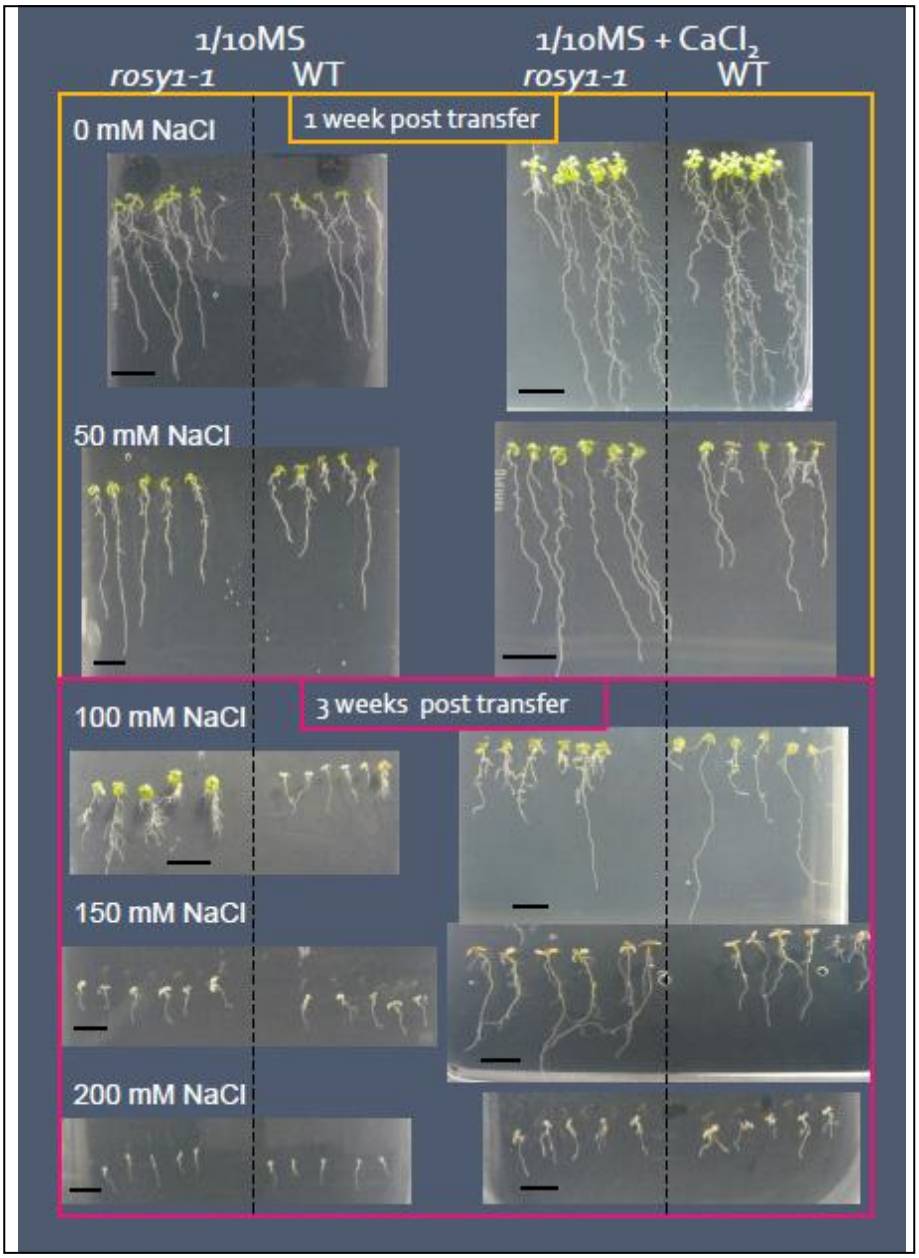

Figure 4.16NaCl salt stress phenotype of *rosy1-1* vs. WT seedlings.

WT and *rosy1-1* seedlings were grown on 0.5X MS medium until they were three day old. Three day old seedlings were transferred to plates with 0.1XMS supplemented with different quantities of NaCl, ranging in concentrations from 1 mM to 200 mM with or without 3 mM calcium chloride, as shown above. The growth of seedlings in different salt concentration was monitored. In medium not supplemented with NaCl (0mM NaCl above), the growth of WT and *rosy1-1* seedlings was

comparable. WT seedlings show lesser salt stress survival than *rosy1-1* seedlings in media with 50 mM NaCl (with or without $CaCl_2$), 100 mM NaCl (with or without $CaCl_2$) and 150 mM NaCl (with $CaCl_2$). None of the seedlings survived a high salt stress of 150 mM NaCl (without $CaCl_2$) and 200 mM NaCl (with or without $CaCl_2$). Results here are representative of four biological replicates, with 40-60 seedlings tested for each WT and *rosy1-1* per salt concentration per experiment. Scale bars represent 1 cm.

Stressed *rosy1-1* seedlings produced more root hair compared to WT seedlings and showed better overall stress tolerance (Figure 4.16). Therefore, in *rosy1-1* seedlings, some mechanism of NaCl stress sensing or response is altered. Interestingly, the improved stress tolerance phenotype of *rosy1-1* seedlings was limited to NaCl stress. Improved stress tolerance was not observed for *rosy1-1* seedlings in plates supplemented with KCl or mannitol (Figure 4.17).

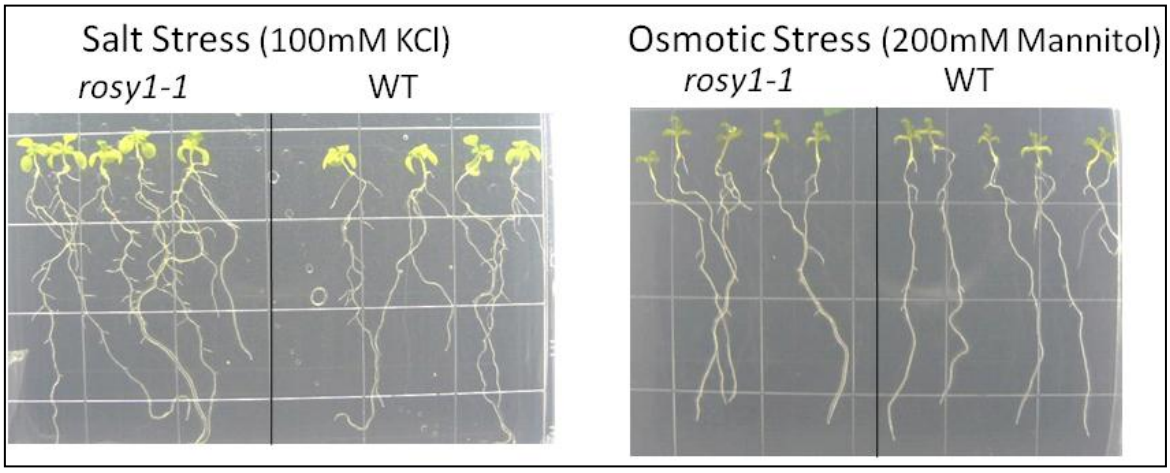

Figure 4.17KCl and osmotic stress phenotype of *rosy1-1* and WT seedlings.

WT and *rosy1-1* seedlings were grown on 0.5 X MS medium until they were three day old. Three day old seedlings were transferred to plates with 0.1XMS supplemented with different quantities of KCl or mannitol, ranging in concentrations from 1 mM to 200 mM. The growth of seedlings in different salt concentration was monitored. There were no detectable differences in growth of *rosy1-1* seedlings compared with WT seedlings. Experiments were done with 40-60 seedlings each of *rosy1-1* and WT for each KCl and mannitol concentration tested. Representative data is shown above.

The data presented above indicates that ROSY1 responds specifically to Na^+, and the Na^+ tolerant phenotype is not resultant of general salt stress (such as presented by KCl). The phenotype is also not a resultant of a general osmotic imbalance in the medium because of reduced water availability in presence of the salts, as shown by lack of phenotype in high concentrations of the osmolyte mannitol.

Membrane sterols are important signaling molecules, that can regulate gene expression. Because ROSY1 has such a pronounced effect on the membrane composition, it was investigated if there were changes in gene expression in *rosy1-1* mutants compared with WT. In particular, it was tested if the expression levels of the isoform *ROSY2* mRNAor At3g44100 mRNA differend in *rosy1-1* seedlings compared with WT.

Feedback control in ROSY1 isoform expression

To compare the expression levels of *ROSY2* mRNA and At3g44100 mRNA in wild type plants and *rosy1-1* knockouts, a qRT-PCR experiment was conducted. Seven-day-old light-grown seedlings were used for RNA extraction from whole seedlings and from roots alone. Using qRT-PCR analysis (Figure 4.18), it was seen that in the root tissue, there were differences in *ROSY2* mRNA expression.

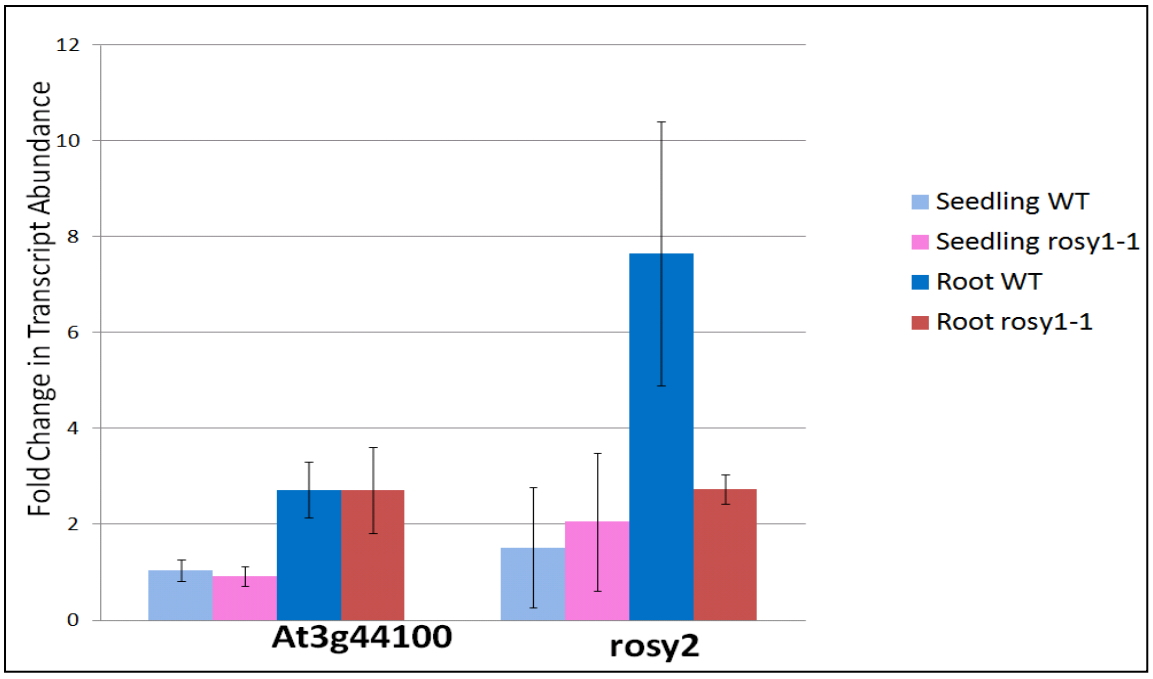

Figure 4.18 Characterization of isoform transcriptional levels in *rosy1-1* mutants.

Seven-day-old light grown seedlings were used to isolate RNA. The plates were kept stationary before the procedure, RNAlater was sprayed on the seedlings prior to picking them out and putting them on slides with RNAlater for root extraction, or putting in liquid nitrogen for seedling sample collection. qRT-PCR analysis was conducted on the samples using gene-specific primers to identify *ROSY2* mRNA expression as well as At3g44100 mRNA expression in both *rosy1-1* and WT plants. The data was normalized using *Actin2*.

While the expression level of At3g44100 mRNA in rosy1-1 mutants and WT was the same, the levels of *ROSY2* mRNA abundance decreased about threefold in *rosy1-1* seedlings(Figure 4.18). Therefore, the *ROSY1* mutation in *rosy1-1* seedlings affects gene expression of at least one ROSY1 isoform in *Arabidopsis*. The changing expression levels of isoforms in *rosy1-1* mutants may serve to explain why *rosy1-1* seedlings show phenotypic differences in hypocotyls even though ROSY1 is a root-localized protein (Figure 3.17, chapter 3).*ROSY2* is expressed in the hypocotyl (Figure 3.6. chapter 3).

DISCUSSION

ROSY1 has been identified in this thesis as a novel inhibitor of plant tropic and stress responses. The *ROSY1* mRNA expression is up-regulated by light and gravity in an $InsP_3$-dependent manner (Kimbrough et al., 2004; Salinas-Mondragon et al., 2010). We have shown that the protein is root specific, and very important for regulating gravity or light-induced tropic growth. ROSY1 acts as an inhibitor of tropic bending not only in *Arabidopsis* roots, but also in hypocotyls where *ROSY1* is not expressed. The protein has a conserved lipid binding ML domain, and binds stigmasterol with a high affinity and the phospholipid PE with a lower affinity. The protein also interacts with *Arabidopsis* SYT1, which is a protein mediating vesicular fusion and recycling. Because of the specific sterol/lipid binding characteristics of ROSY1, and its interaction with SYT1, ROSY1 is expected to play a role in regulating SYT1-mediated vesicular fusion and recycling. Because SYT1 deficiency causes salt stress susceptibility (Schapire et al., 2008), whereas ROSY1 deficiency causes salt stress tolerance, ROSY1 may be an inhibitor of SYT1-mediated vesicular fusion for plasma membrane repair.

The Na^+ stress tolerant phenotype of *rosy1-1* seedlings can be explained by two theories. In the first one, the phenotype can be attributed to the interaction of ROSY1 and SYT, and ROSY1 is predicted to be an inhibitor of SYT1 function. Because SYT1 is a membrane trafficking protein, and by virtue of the N-terminal transmembrane helix and the sterol binding ML domain, ROSY1 likely localizes on vesicles or on the plasma membrane. SYT1

carries out vesicular fusion in response to salt stress, which is necessary for salt stress tolerance (Schapire et al., 2008). If ROSY1 interaction with SYT1 inhibits the vesicular fusion carried out by SYT1, it would explain why WT plants which have functional ROSY1 will respond to salt stress more, compared with *rosy1-1* knockout plants, where owing to the absence of inhibitor ROSY1, the function of SYT1 is enhanced.

The second theory postulates that ROSY1 and SYT1 together regulate exocytosis and endocytosis of specific proteins in specific lipid environments on *Arabidopsis* membranes. In absence of ROSY1, the dynamics of exocytosis and endocytosis somehow change, which not only affect the membrane sterol and lipid composition, but also the distribution of specific plasma membrane proteins, such as the Na^+- ATPase. If there is less Na^+- channel protein in the plasma membrane of *rosy1-1* seedlings, that may result in reduced internalization of Na^+ in *rosy1-1* seedlings, and may explain the salt stress phenotype.

The *Arabidopsis* genome encodes nine ML domain proteins. Out of these, at least one, ROSY2, also interacts with SYT1. The *rosy1-1* mutants are defective in the sterol and lipid composition of their cellular membranes. Particularly, comparative levels of stigmasterol, sitosterol and PA are significantly different between *rosy1-1* and WT samples. The changed membrane composition in *rosy1-1* seedlings does not cause a change in localization of PIN1, PIN2 and PIN4 proteins on the plasma membrane. The *rosy1-1* mutants also show important NaCl stress signaling defects, and exhibit better growth in high NaCl conditions, but not in high KCl or mannitol conditions. Finally, the *rosy1-1* mutants demonstrate a dramatic

decrease in basipetal auxin transport rate. The rate of auxin transport is about half of that in WT seedlings, and the difference persists on NPA application, which blocks auxin efflux. NPA does not block all auxin efflux completely, and some auxin efflux is NPA-independent, but the decrease in the auxin transport rates of *rosy1-1* mutants is too large to be caused by the residual auxin efflux; consequently, ROSY1 very likely has a role in auxin influx. $InsP_3$ has been reported to be involved in regulating auxin efflux (Luo et al., 2011; Zhang et al., 2011). Because the light and gravity-induced up-regulation of *ROSY1* mRNA is $InsP_3$-dependent, and ROSY1 likely affects auxin influx, our data presents an $InsP_3$-mediated auxin influx branch. Several aspects and implications of the results summarized above will be discussed in this section.

ROSY1 affects membrane sterol composition.

In *rosy1-1* seedlings, the relative composition of different sterols was changed, which can have important biological implications for the plant. Complete absence of mature sterols in the *cpi* (Men et al., 2008)and *smt* (Willemsen et al., 2003) mutants causes mis-localization of auxin efflux carrier PIN proteins on the plasma membrane, and agravitropic roots. It is interesting to note that in the *cpi* mutants, only PIN1 and PIN2 were found to be mis-localized, but not the plasma membrane localized auxin influx carrier, the AUX1 protein (Men et al., 2008). Therefore, even as the localization of PIN proteins is not changed in *rosy1-1* roots, it is possible that some other plasma membrane proteins related to auxin transport are mis-localized. But while in the *cpi* mutants, all mature sterols are absent (Men

et al., 2008), in the *rosy1-1* mutants, only the relative proportion of stigmasterol and sitosterol is changed. Therefore, the mis-localization phenotypes may be more subtle in *rosy1-1* mutants, even though the basipetal auxin transport phenotype is quite remarkable in *rosy1-1* roots.

It should also be remembered that in *rosy1-1* hypocotyls, the relative levels of sterols are not different compared with WT, yet these hypocotyls have a differential tropic response phenotype. For some plant genes, such as the sterol biosynthesis gene *HYDRA*, the genes may have non-overlapping cell type-specific expression, but the gene product may be non-cell-autonomous, and transported to the other cells or organs in the plant (Souter et al., 2002). Therefore, it is possible that in *rosy1-1* roots, the expression of some signaling element downstream of or independent of the sterols spreads throughout the plant, and it is that signaling element that causes the tropic bending phenotype of *rosy1-1* hypocotyls. This signaling element could well be lipid based, as the relative lipid levels are different between *rosy1-1* and WT hypocotyls or other small molecules.

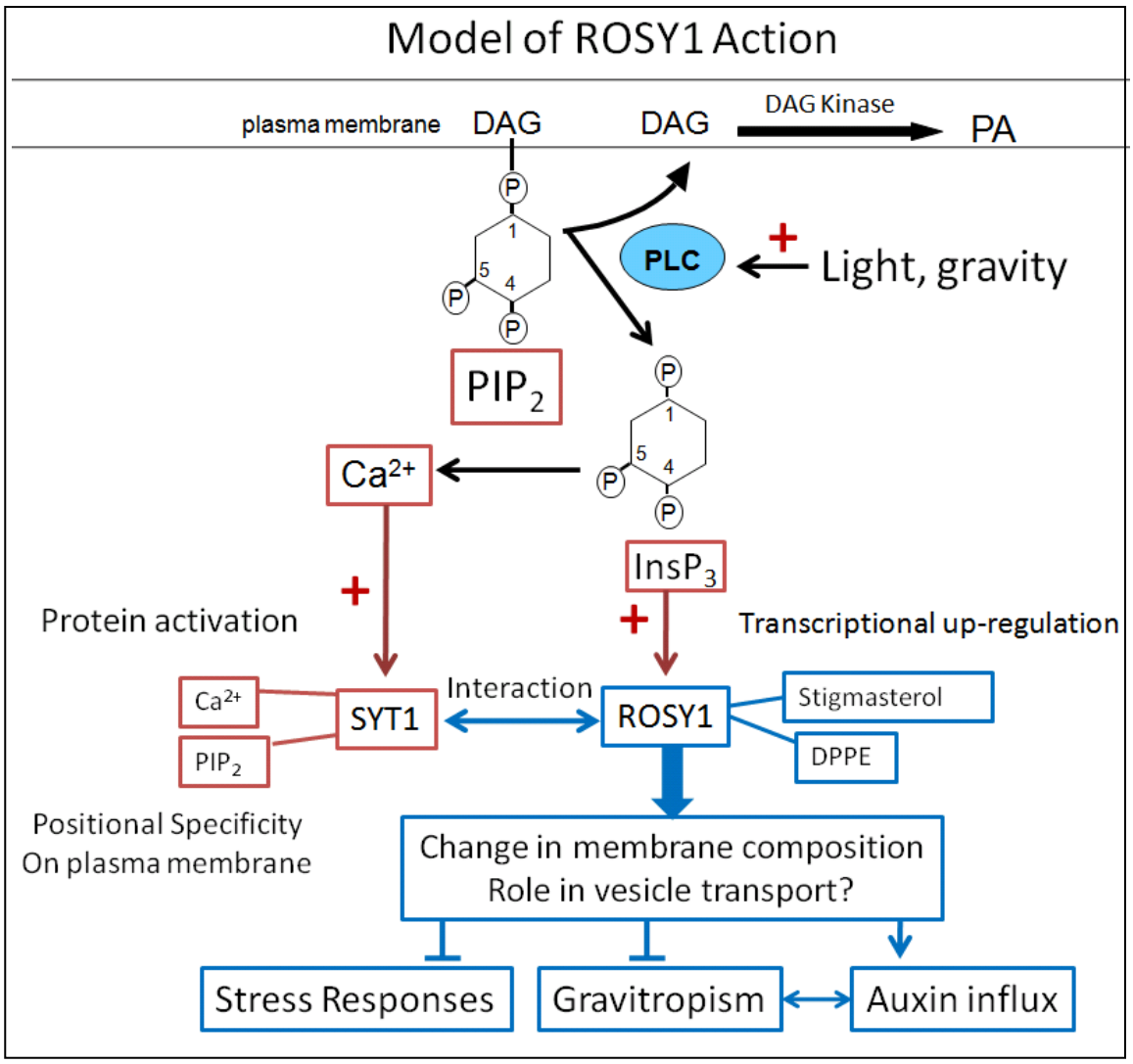

Figure 4.19 Model of ROSY1 action.

Transcriptional up-regulation of *ROSY1* mRNA is dependent on $InsP_3$ levels in the plant. The protein ROSY1 binds stigmasterol and DPPE, and interacts with SYT1. ROSY1 may have a role in vesicle fusion and recycling, by which it may regulate plasma membrane composition. A changed sterol and lipid membrane composition seen in *rosy1-1* mutants may affect localization/activation of other proteins on the membranes. Through its postulated vesicle trafficking function, ROSY1 assists basipetal auxin transport (most likely influx), and inhibits both NaCl stress signaling and gravitropic bending. The information in the red boxes or no boxes has been published in literature before. The information in the blue boxes has been contributed by this research.

It is interesting to note that in the roots of *rosy1-1* mutants, there is a relative reduction in amounts of both stigmasterol and sitosterol, even as ROSY1 binds stigmasterol *in vitro*. Therefore, it must be remembered that the binding analysis presented here is performed not using the complete ROSY1 protein sequence, but partial protein sequence, which will affect how the protein folds and the tertiary structure it forms. The recombinant AtROSY1ML-HA protein was secreted in yeast growth medium, which has the same pH as the cystosol. But, in the cell, the protein ROSY1 will possibly be docked onto a vesicle or on to the plasma membrane, which will further affect the tertiary structure of the protein as well as accessibility of certain residues in the ROSY1 ML domain for lipid binding. These factors will affect which sterols ROSY1 binds *in vivo*. It is even possible that ROSY1 may bind sitosterol *in vivo*, in addition to stigmasterol, but that cannot be ascertained using our current data. While it is possible to visualize *in vivo* sterol-proteins interaction by incubating transgenic plants expressing GFP-ROSY1 with fluorescent BODIPY tagged sterols, the structural change induced by ROSY1, a small 17 KDa protein, when it is fused with GFP, a 27 KDa protein can be substantial. The protein folding could be compromised because of the attached GFP.

Change in sterol composition is also important considering that sterols affect gene expression as well as protein function. Sterols have been known to regulate gene expression, either directly, such as in fungal elicitin gene expression (Yousef et al., 2009), or via the SREBP

(Sterol Regulatory Binding Protein) transcription factors (Field et al., 2001). Sterols regulate expression of genes related to cell expansion in *Arabidopsis* (He et al., 2003), which has implications in gravity-induced asymmetrical cell elongation. In *rosy1-1* roots, there is a change in sterol composition. The transcriptional down-regulation of *ROSY2* mRNA in *rosy1-1* roots may be because of the activity of sterol-regulated transcription factors, but again, that cannot be ascertained without further experiments.

Sterols also affect activation of different proteins. For example, the animal protein HEDGEHOG is activated upon binding cholesterol (Rohatgi and Scott, 2007). Sterols are also important for regulating the activity of plasma membrane bound H^+/ATPases (Serrano, 1993; Grandmougin-Ferjani et al., 1997). In maize roots, low concentrations of stigmasterol and cholesterol stimulate H^+ pumping from plasma membrane bound H^+-ATPases, whereas at high concentrations, they stimulate H^+ secretion. All other sterols including sitosterol inhibit H^+-ATPases at all concentrations (Grandmougin-Ferjani et al., 1997). This is very interesting, because it shows how changed proportions of different sterols in the membranes can affect the activity of certain plasma membrane bound proteins. Sterol-mediated regulation of H^+-ATPases is especially interesting: proton pumps can change membrane pH, and affect activity of other proteins. Gravity stimulation causes H^+-ATPase-mediated pH changes in columella cells and in the lateral root cap and root epidermal cells. Hyper-gravity also induces increase in sterol composition in membranes of azuki bean (*Vigna angularis*) (Koizumia, 2007). But it is still unlikely that gravity-induced pH changes, which take place

within seconds of gravity stimulus, are downstream of changes in membrane composition post-gravity, which will probably take longer time to establish.

ROSY1 interacts with SYT1

The interaction of SYT1 and ROSY1 is crucial to understanding the function of ROSY1. The interaction, though enabled constitutively in the yeast-two-hybrid system, will probably be more selective in the plant system, partly because ROSY1 is a labile and short-lived protein, especially in the lateral root cap cells, where *ROSY1* expression is seen occasionally and with difficulty. The protein appears to be stable in the mature zone of the root.

Within the cells, the SYT1 protein is activated for membrane fusion by binding Ca^{2+}, and it can bind Ca^{2+} only when the bilayers in which SYT1 is docked contains the lipid PIP_2 (Radhakrishnan et al., 2009; Kuo et al., 2011). Upon gravity stimulation, PIP_2 is hydrolyzed by PLC to generate $InsP_3$ (Record et al., 1999; Perera et al., 2001; Meijer and Munnik, 2003), it can be assumed that membrane microdomains rich in PIP_2 will harbor activated SYT1, as well as be surrounded by higher $InsP_3$ and cytosolic Ca^{2+}. It may therefore be hypothesized that gravity induces SYT1 protein activation. Gravity also up-regulates *ROSY1* mRNA abundance (Kimbrough et al., 2004). Therefore, it is possible that in the lateral root cap cells, sufficient ROSY1 expression and SYT1 activation may happen only upon gravity stimulation.

Furthermore, gravity-induced PLC hydrolysis and $InsP_3$ up-regulation is asymmetric across the root (Perera et al., 2006), therefore, *ROSY1* expression as well as the ROSY1 and SYT1 interaction will likely take place only on one side of the root. Transgenic plants expressing ROSY1-EGFP fusion protein had a very strong ROSY1 expression in the lateral root cap, not seen in plants expressing GFP-ROSY1 fusion protein. These plants had been used to visualize ROSY1 expression after gravity stimulus, and the data indicated that ROSY1 was expressed more on the upper side of the root (that elongates faster) than the lower side of the root. The ROSY1-EGFP protein from these constructs did not express in the root cortical and epidermal cells, and also the protein was found to be cleaved on western blot analysis, so these data could not be used. The same experiment was attempted using transgenic plants GFP-ROSY1 fusion protein, but in these plants, the protein expression in lateral root cap was much lower, sometimes absent, and not consistent enough to attribute expression changes to gravity stimulation.

The interaction between SYT1 and ROSY1 is likely to effect the exocytosis or endocytosis dynamics at the plasma membrane. Because of the lipid/sterol binding specificities of ROSY1 and SYT1, they may interact at very specific lipid/sterol rich membrane microdomains, and affect exocytosis or endocytosis there. In absence of ROSY1 in *rosy1-1* mutants, the specificity of vesicles released from the membranes for recycling may be compromised, which may lead to changes in membrane composition. A changed membrane composition may lead to mis-localization of proteins such as the AUX1 protein for auxin

influx and the sodium ATPase protein for internalizing salt from a high salt medium, which will explain reduced basipetal transport and enhanced NaCl tolerance of *rosy1-1* seedlings.

Whether the interaction of ROSY1 and SYT1 is indeed responsible for ROSY1 function cannot be ascertained by our current data. To investigate if *rosy1-1* phenotypes are caused by loss of ROSY1 and SYT1 interaction, it will first need to be established which amino acids in each protein are responsible for the interaction using an array of point mutants in yeast. For ROSY1, the amino acids responsible for sterol binding will also need to be identified, using another array of ROSY1 site-specific mutants for recombinant protein synthesis. Then, *rosy1-1* knockout plants will need to be genetically rescued with a mutant ROSY1 construct having a point mutation to ensure the protein does not interact with a) SYT1, but still binds stigmasterol, or b) stigmasterol but still interacts with SYT1, or c) does not interact with SYT1 and does not bind stigmasterol. The tropic bending and NaCl tolerance related phenotypes of these plants will elucidate the functional importance of the interaction between ROSY1 and SYT1.

The *Arabidopsis* genome encodes nine ML domain proteins. Other than ROSY1, we tried interaction of two other proteins, ROSY2 and At3g44100 with SYT1. It will be necessary to check the interaction of the other six proteins with SYT1 as well. Further, because SYT1 in *Arabidopsis* is quite redundant because of presence of four other isoforms, the interaction of *Arabidopsis* ML domain proteins will need to be tested with the various SYT isoforms. The lipid and sterol binding specificities of the other *Arabidopsis* ML domain proteins will need

to be ascertained, along with their expression profiles. When all of these data are available double or triple mutants can be made with ROSY1 isoforms of interest, and the functional importance of these genes in the plant system can be better understood.

Future Experiments

Based on the overall data presented in this dissertation, we can summarize that we have identified a novel protein, ROSY1 in *Arabidopsis*, which via unknown vesicle fusion and recycling mechanisms maintains the membrane composition in *Arabidopsis* roots and hypocotyls. The changes in membrane composition in *rosy1-1* mutants may affect localization or activity of membrane based proteins, which in turn affects the transport of auxin and gravitropic and phototropic bending. It also results in defects of NaCl internalization or signaling. The following future experiments to investigate these functions of ROSY1 can be suggested.

The role of ROSY1 in membrane trafficking can be examined by testing if ROSY1 affects endocytosis or exocytosis (secretion). To check for the role of ROSY1 in endocytosis and a vesicle internalization defect in *rosy1-1* roots, the mutant and WT roots can be treated with the endocytic marker FM4-64 (Invitrogen) (Vida and Emr, 1995). FM4-64 stain labels internalized membrane vesicles; in WT cells stained with FM4-64, vesicular compartments and provacuoles can be seen forming in the cells within 1-3 hours of staining. Within 4 hours and 30 minutes of staining, the vacuole tonoplast is labeled in the WT cells with normal

endocytosis (Bolte et al., 2004). These rates of the internalization of FM4-64 labeled vesicles can be compared between *rosy1-1*, WT and *ProROSY1:ROSY1* samples. Furthermore, if point mutants are available for ROSY1 that do not interact with SYT1 or stigmasterol, they can be used as samples as well. Changes in the overall FM4-64 labeled vesicle internalization between *rosy1-1* and WT samples would mean that *rosy1-1* mutants have a defect in endocytosis, which means that ROSY1 has a role in endocytosis. The use of point mutants for ROSY1 in this case would indicate the interaction of ROSY1 with SYT1 and/or stigmasterol affect the role of ROSY1 in mediating endocytosis.

To check for the role of ROSY1 in exocytosis and a secretory defect in *rosy1-1* roots, secretion to several organelles can be tested and compared between WT and *rosy1-1* roots. For checking a secretory defect to the cell wall, *rosy1-1* and WT plants can be transformed with secretory GFP (secGFP), and the GFP fluorescence can be monitored and compared between *rosy1-1* and WT samples (Zheng et al., 2005). In WT samples, secGFP is targeted to be secreted to the cell wall, where the GFP fluorescence is quenched due to the acidic apoplastic pH. In cells with a cell wall-secretory defect, GFP would accumulate in the cell, and partly colocalize with the ER marker BiP (Chen et al., 2002).

For checking secretory defect to the vacuole, the vacuolar transportation of Aleurain-GFP can be compared between *rosy1-1* and WT samples, as has been shown for *Echidna* mutants (Gendre et al., 2010, 2011).

Finally, an exocytosis defect to the plasma membrane can be checked by comparing the localization of plasma membrane proteins such as AUX1 and sodium ATPase between WT and *rosy1-1* samples. The mis-localization of these proteins in *rosy1-1* plasma membrane would means that ROSY1 has a role in exocytosis and plasma membrane targeted secretion, in addition to explaining some of *rosy1-1* phenotypes. The mis-localization of AUX1 will explain the auxin transport defect in *rosy1-1* knockouts. The mis-localization of sodium ATPase would mean that the NaCl resistant phenotype in *rosy1-1* seedlings is owing to the reduced NaCl internalization in *rosy1-1* cells. This data could then be further supported by measuring the levels of sodium accumulation in *rosy1-1* and WT tissues after significant but non-lethal salt stress, say 50mM NaCl. Levels of sodium or potassium can be measured by using radioactive sodium or potassium in the plant growth medium and later comparing the levels of radioactivity internalized by the plant tissues (Ding and Zhu, 1997).

These experiments will help shed light on how ROSY1 functions in *Arabidopsis*. On the basis of the research presented in this dissertation, we conclude that we have identified a novel protein in *Arabidopsis* that plays a key role in plant gravitropic, phototropic and stress responses, possibly via sterol binding and interaction with *Arabidopsis* membrane trafficking protein SYT1.

MATERIALS AND METHODS

Protein-Lipid Overlay Assay

Protein Production

The recombinant protein was generated using yeast *K. lactis* protein expression system (New England Biolabs, Ipswich, MA) The ROSY1 ML domain (amino acids 27-151) was amplified with forward primer introducing an *Xho1* restriction site at the 5' end (ML FP 5'-CAT CAG CTC GAG AAA AGA TTC CAA ACT TGC GAT ACT GGT-3'), and using two overlapping reverse primers designing an HA–tag, followed by a stop codon, in turn followed by *Not1* restriction site at the 3' end (ML RP1 5'- TGC ATA ATC TGG AAC ATC ATA TGG ATA ATG ACC AGT GAG CTT AAC AAC AAA -3'; ML RP2 5'-CAT CAG GCG GCC GCT TAT GCA TAA TCT GGA ACA TCA TAT GGA TA-3'). The PCR product and pKLAC2 vector were restriction digested with *Xho1* and *Not1* (NEB, MA) and ligated with T4 DNA Ligase (NEB, MA). The recombinant protein AtROSY1ML-HA was cloned downstream of αMF secretory domain in vector pKLAC2 and the sequence verified construct was linearized with *SacII*, and used to transform *K. lactis* GG799 competent cells (NEB, MA) using the manufacturer's protocol. Protein production was induced on transformed colonies, and non-transformed GG799 cells by growing the yeast for 2 days in YPD medium supplemented with 40% galactose (Sigma-Aldrich, St. Louis, MA). After two days, the cells were spun down by centrifugation, and the medium was pipetted out. Western blotting analysis was done to confirm presence and correct size of protein in the secreted

medium using anti-HA-HRP antibody (Genscript, Piscataway, NJ). While transformed colonies showed correct size protein secreted in the medium, no bands detected with the control GG799 cells. The medium from transformed cells and control GG799 cells was used for lipid/sterol binding assays.

Sterol and Lipid Binding Assay

Sterol binding protocol was adapted from Dowler et al., 2002. For sterol blots in Figures 4.4 and 4.6, 100mM solutions were prepared with cholesterol (Sigma Aldrich, St. Louis, MO) in chloroform/methanol (Sigma) 1:1 and in DMSO (Sigma), stigmasterol (Sigma Aldrich, MO) in chloroform and DMSO, Sitosterol (Sigma Aldrich, MO) in chloroform and 24-epibrassinolide (PhytoTechnology Laboratories, Overland Park, KS) in chloroform and DMSO. While cholesterol dissolved best in chloroform: methanol, stigmasterol and sitosterol dissolved best in chloroform alone, and 24-epibrassinolide dissolved in DMSO best. All the solutions were used to make dilutions of 100mM, 1mM, 500µM and 150µM in the respective solvents. For Figure 4.5, membrane lipid strips (Echelon Inc., Salt Lake City, UT) were used. For Figure 4.6 and stigmasterol (Sigma-Aldrich, MA), DPPE and DOPE (Echelon Inc., UT) were dissolved in chloroform and diluted to 100mM, 1mM, 500µM concentrations. PVDF membranes (Bio-Rad Laboratories, Hercules, CA) were cut out and dipped in methanol, then washed 2X with 1X TBST (tris-buffered saline tween-20). The membranes were placed in trays on stacks of kimwipes soaked with 1X TBST to prevent drying of the membrane. Sterol solutions (1µl) were spotted on the membrane and the spots were allowed to dry for 30

minutes. One spotted membrane, or lipid strip was made for each culture to be used for the binding assay.

Cultures of untransformed GG799 cells (NEB, MA), and strains transformed with AtROSYML-HA grown in YPD/Gal for 2 days (OD_{600}~1.0) were spun down. Supernatant medium (15ml) was extracted out of each culture and 1.5ml 10X TBST was added. Separate lipid strips were incubated with separate medium solutions for 40 minutes at 4°C and 20 minutes at room temperature. The solution was decanted and the membranes were vigorously washed 3X 30 minutes each with 1X TBST. The membranes were then incubated for 1 hour with shaking at room temperature with 1:1000 anti-HA: HRP (Genscript, NJ) in 1X TBST. After 1 hour, the antibody solution was decanted and the membranes were washed vigorously 3X 30 minutes each. Signal was developed using SuperSignal West Femto chemiluminiscent substrate (Fisher Scientific, MO) and probed on Kodak autoradiography films (Sigma Aldrich, MO), which were then developed with Kodak GBX developer and fixer solutions (Sigma Aldrich, MO).

The experiment was performed with six cultures expressing AtROSYML-HA and one untransformed GG799 sample, and was repeated 3 times with separate transformed and non-transformed colonies each time. The binding showed same sterol/lipid affinity for all transformed colonies tested, and none of the non-transformed GG799 strips showed any signal on the film.

Lipid and Sterol measurement

The lipid extraction was performed as suggested by Kansas State Lipidomics Facility (Manhattan, KS).

WT and *rosy1-1* seeds were surface sterilized as described above and plated on 1X MS medium with 1% sucrose and 0.8% agar. The plates were incubated at 4°C for four days and then placed in growth chamber with long day conditions, as described above. After 4 hours, the plates were covered with foil, and the seedlings were allowed to grow for one week. The plates were opened in green light; seedlings were placed on a glass slide on ice, and covered with isopropanol. A bunch of seedlings were placed together and the roots and hypocotyls were cut out very fast. Immediately, triplicates of WT and *rosy1-1* roots and hypocotyls were placed in 3ml 75°C pre-heated isopropanol with 0.01% butylated hydroxytoluene (BHT). The samples were then incubated with 4ml 2:1 chloroform-methanol with shaking for lipid extraction. Four extractions were performed with each sample, three for 1 hour each and the last one overnight. All extractions were pooled together, mixed with1M KCl followed by 2ml water. The lipids were dried out with stream of nitrogen gas, dry weights were taken, and samples were shipped to Kansas State Lipidomics Facility (Manhattan, KS) for analysis.

ESI-MS/MS lipid profiling (Kansas State Lipidomics Center)

An automated electrospray ionization-tandem mass spectrometry approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (Devaiah et al., 2006) with the following modifications. The samples were dissolved in 1 ml chloroform. An aliquot of 5 to 300 µl of extract in chloroform was used.

Internal standards, obtained and quantified as previously described (Welti et al., 2002), were added in the following quantities (with some small variation in amounts in different batches of internal standards): 0.6 nmol di12:0-PC, 0.6 nmol di24:1-PC, 0.6 nmol 13:0-lysoPC, 0.6 nmol 19:0-lysoPC, 0.3 nmol di12:0-PE, 0.3 nmol di23:0-PE, 0.3 nmol 14:0-lysoPE, 0.3 nmol 18:0-lysoPE, 0.3 nmol di14:0-PG, 0.3 nmol di20:0(phytanoyl)-PG, 0.3 nmol 14:0-lysoPG, 0.3 nmol 18:0-lysoPG, 0.23 nmol 16:0-18:0-PI, 0.16 nmol di18:0-PI, 0.2 nmol di14:0-PS, 0.2 nmol di20:0(phytanoyl)-PS, 0.3 nmol di14:0-PA, 0.3 nmol di20:0(phytanoyl)-PA, 0.49 nmol 16:0-18:0-DGDG, 0.71 nmol di18:0-DGDG, 2.01 nmol 16:0-18:0-MGDG, and 0.39 nmol di18:0-MGDG. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.4 ml.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 μl/min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: PC and lysoPC, [M + H]+ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); PE and lysoPE, [M + H]+ ions in

positive ion mode with Neutral Loss of 141.0 (NL 141.0); PG, [M + NH4]+ in positive ion mode with NL 189.0 for PG; lysoPG, [M – H]- in negative mode with Pre 152.9; PI, [M + NH4]+ in positive ion mode with NL 277.0; PS, [M + H]+ in positive ion mode with NL 185.0; PA, [M + NH4]+ in positive ion mode with NL 115.0; DGDG, [M + NH4]+ in positive ion mode with NL 341.1; and MGDG, [M + NH4]+ in positive ion mode with NL 179.1. The scan speed was 50 or 100 u per sec. The collision gas pressure was set at 2 (arbitrary units). The collision energies, with nitrogen in the collision cell, were +40 V for PC, +28 V for PE, +20 V and PG, +25 V for PI, PS and PA, +24 V for DGDG, and +21 V for MGDG. Declustering potentials were +100 V for PE, PC, PA, PG, PI, and PS, and +90 V for DGDG and MGDG. Entrance potentials were +15 V for PE, +14 V for PC, PG, PI, PS, and PA, and +10 V for DGDG and MGDG. Exit potentials were +11 V for PE, +14 V for PC, PG, PI, PS, and PA, and +23 V for DGDG and MGDG. The mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode. The source temperature (heated nebulizer) was 100°C, the interface heater was on, +5.5 kV or -4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software. After isotopic deconvolution, the lipids in each class were quantified in comparison to the two internal

standards of that class (Brügger et al., 1997; Welti et al., 2002). The first and typically every 11th set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the "internal standards only" spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each "internal standards only" set of spectra was used to correct the data from the following 10 samples. Finally, the data were corrected for the fraction of the sample analyzed and normalized to the sample "dry weights" to produce data in the units nmol/mg.

Sterol Analysis at Kansas State Lipidomics (Dr. Richard Jeannotte, Kansas State Lipidomics) The methodology used to analyze plant sterols in Arabidopsis tissues was adapted from Ryu et al. (2006). Lipid extracts were stored in -70°C freezer until analysis. An equivalent of 0.2-0.3 mg of lipid was used per sample. Cholesterol-d6 (5.102 nmoles) (C/D/N Isotopes Inc, Pointe-Claire, Quebec, Canada) was added to each sample prior to hydrolysis. Each lipid extract was dried under nitrogen. 125 µl of 3N HCL in methanol and 625 µl of chloroform were added to each sample, then heated at 50°C for 5 hours. Hydrolysates were dried under nitrogen, re-dissolved in pyridine (70 µL) and derivatized by addition of a silylation reagent (30 µL) such as N-trimethylsilyl-N-methyltrifluoroacetamide with trimethylchlorosilane MSTFA + 1%TMCS and incubated at 50°C for 1 h. GC–MS analysis

of the silylated extracts was performed on an Agilent model 6890N GC coupled to an Agilent model 5975N quadrupole mass selective detector (MSD). Separation was achieved on a HP-5MS fused silica capillary column (5% phenyl methyl siloxane; column length, 60 m; internal diameter, 0.25 mm; film thickness, 0.25 μm). The GC operating conditions were as follows: initial temperature of 150°C, increasing to 300°C a rate of 25°C/min, then from 300 to 325°C at a rate of 3°C/min with a final isothermal hold at 325°C for 5 min (total run duration is 19.33 minutes). Helium was used as carrier gas at an initial flow of 1.0 mL/min. The samples (1 μL) were injected in a splitless mode with an Agilent 7683 autosampler. The temperature of the injector and the mass spectrometry transfer line was set at 280°C. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV ionization energy, scanning for selective ions for each metabolite: cholesterol (m/z 329, 368, 458), cholesterol-d6 (m/z 333, 374, 464), brassicasterol (m/z 255, 380, 470), β-sitosterol (m/z 357, 396, 486), stigmasterol (m/z 255, 394, 484), campesterol (m/z 343, 382, 472). The sums of the three ions per compound were used in the quantification. Data were acquired and processed with Agilent Chemstation software, AMDIS (www.amdis.net), and MET-IDEA (Broeckling et al., 2006; http://www.noble.org/plantbio/ms/MET-IDEA/index.html).

Yeast-two-hybrid

The experiment was performed using Matchmaker Library Construction & Screening kit (Clontech, CA). For gravity-stimulated root library construction, seven-day-old *Arabidopsis* seedlings were gravity-stimulated by 90° reorientation for 5 minutes, the seedlings were

placed on a glass slide on ice and covered with RNAlater (Qiagen, CA), and roots were harvested and placed in RNAlater solution. RNA extraction was done using RNeasy plant mini kit (Qiagen, CA) and used for cDNA construction using SMART cDNA construction kit (Clontech, CA). The SMART double-stranded cDNA was cotransformed in yeast strain AH109 with the SmaI linearized vector pGADT7-Rec and the cDNA was cloned at the 3'end of the GAL4 activation domain in the vector. For the bait vector, ROSY1 coding was amplified without the transmembrane domains, and creating a 5' Nde1 restriction site using Forward primer 5'- TAT ATA CAT ATG GCT TTA TGC GGC CCC AAA TTC CAA ACT TGC -3'and EcoRI restriction site using Reverse Primer 5'- CAT GTG AAT TCA TGA CCA GTG AGC TTA ACA ACA AA -3'. The construct was cloned in yeast DNA BD vector pGBKT7 via restriction-digestion and ligation (NEB, MA). The library mating and screening was performed using the manufacturer's protocol from Matchmaker II yeast two hybrid system (Clontech, CA).

Salt stress experiment

Salt stress experiment was carried out as described for the *syt1-1* salt stress seedlings (Schapire et al., 2008). WT and *rosy1-1* seeds were surface sterilized as described above and plated on 0.5X MS medium with 1.5% sucrose and 1% agar, and stratified for four days at 4°C. Three-day-old seedlings were transferred to plates with 0.1XMS medium supplemented with different quantities of KCl or mannitol, for final concentrations from 1 mM to 200 mM. The growth of seedlings in different salt concentrations was monitored by taking pictures.

There were no detectable differences in growth of *rosy1-1* seedlings compared with WT seedlings. For NaCl stress, four biological replicates, with 40-60 seedlings tested for each WT and *rosy1-1* per salt concentration per experiment. Scale bars represent 1 cm.For KCl and mannitol stress, two separate sets of experiments were performed with 40-60 seedlings each of *rosy1-1* and WT for each KCl and mannitol concentration tested. Plates were photographed at different time points through a course of three weeks.

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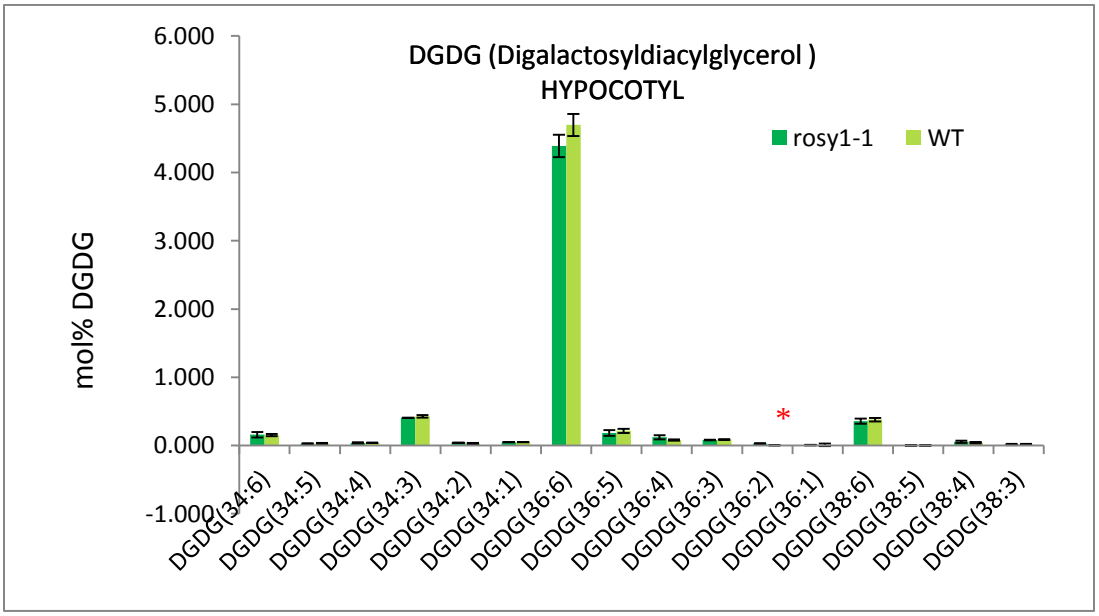
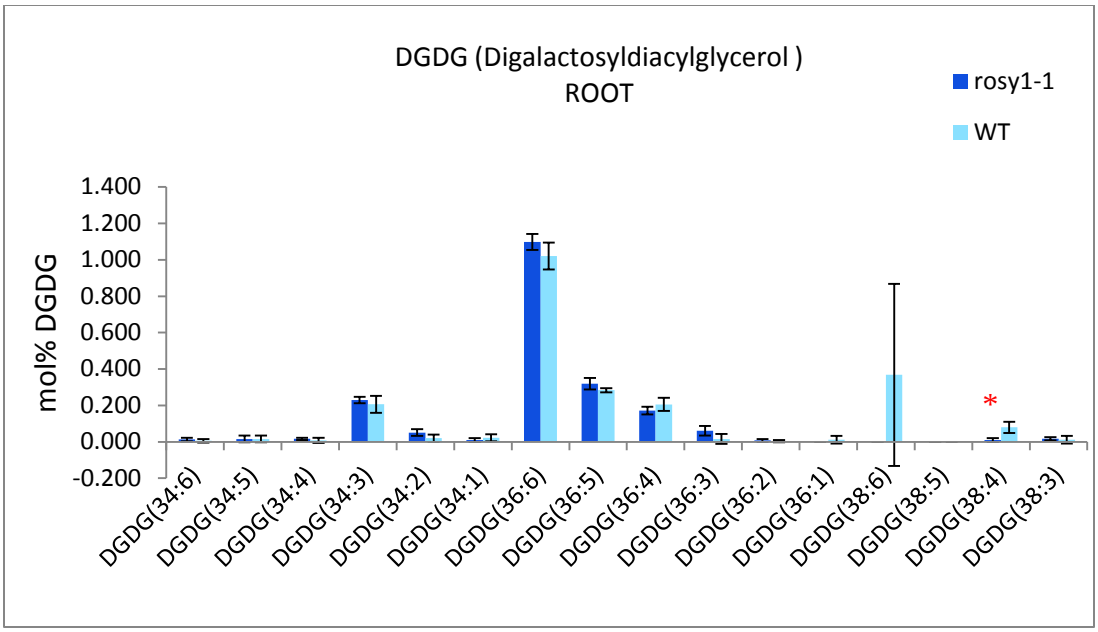
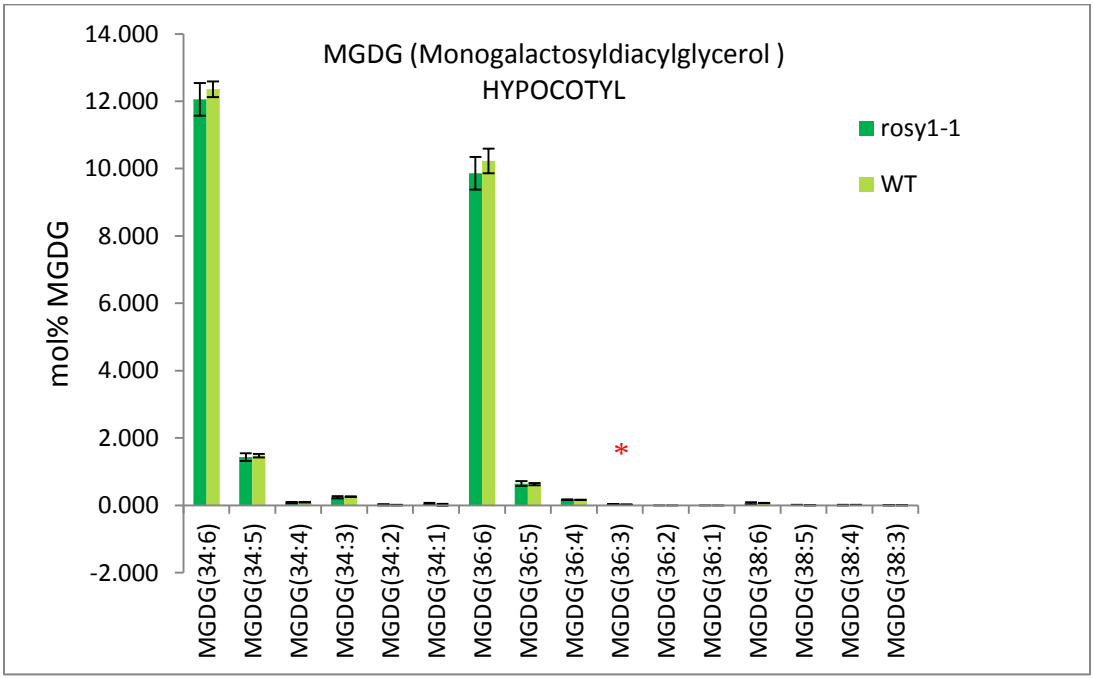
APPENDIX

APPENDIX 1 Comparison of lipid profiles between WT and *rosy1-1*

The complete lipid composition of *rosy1-1* hypocotyls and roots was studied and compared with WT hypocotyls and roots. The distribution of various lipids between the samples is presented in the graphs below. Asterisks indicate significance of $p<0.05$ (two-tailed paired T-test).




DGDG (Digalactosyldiacylglycerol)
ROOT
rosy1-1
WT
mol% DGDG
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-0.200
*
DGDG(34:6)
DGDG(34:5)
DGDG(34:4)
DGDG(34:3)
DGDG(34:2)
DGDG(34:1)
DGDG(36:6)
DGDG(36:5)
DGDG(36:4)
DGDG(36:3)
DGDG(36:2)
DGDG(36:1)
DGDG(38:6)
DGDG(38:5)
DGDG(38:4)
DGDG(38:3)


MGDG (Monogalactosyldiacylglycerol)
HYPOCOTYL
rosy1-1
WT
mol% MGDG
14.000
12.000
10.000
8.000
6.000
4.000
2.000
0.000
-2.000
*
MGDG(34:6)
MGDG(34:5)
MGDG(34:4)
MGDG(34:3)
MGDG(34:2)
MGDG(34:1)
MGDG(36:6)
MGDG(36:5)
MGDG(36:4)
MGDG(36:3)
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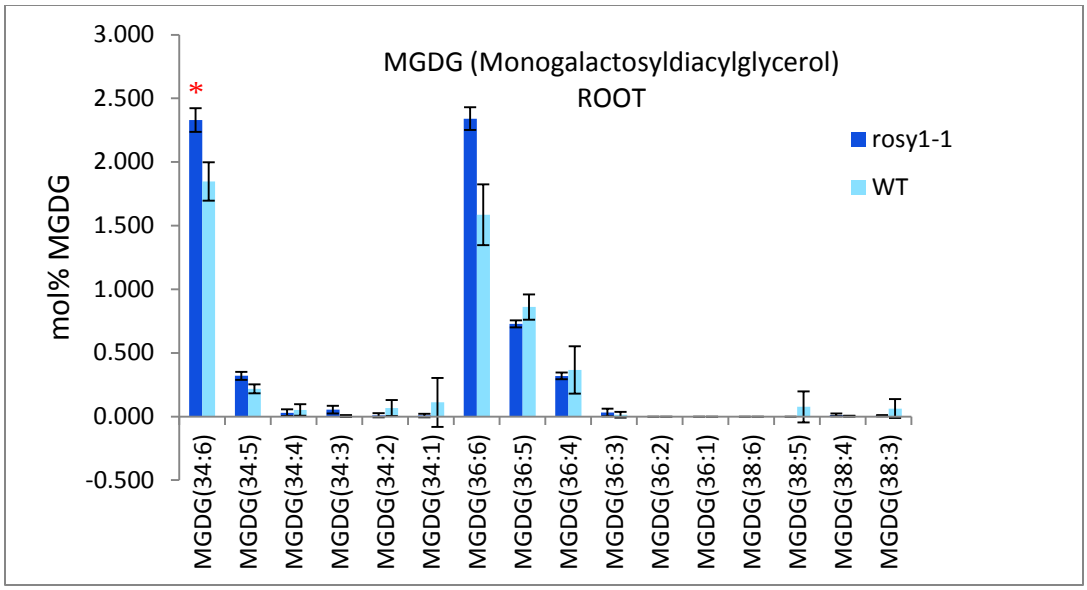
MGDG(38:3)


MGDG (Monogalactosyldiacylglycerol)
ROOT
mol% MGDG
3.000
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
*
rosy1-1
WT
MGDG(34:6)
MGDG(34:5)
MGDG(34:4)
MGDG(34:3)
MGDG(34:2)
MGDG(34:1)
MGDG(36:6)
MGDG(36:5)
MGDG(36:4)
MGDG(36:3)
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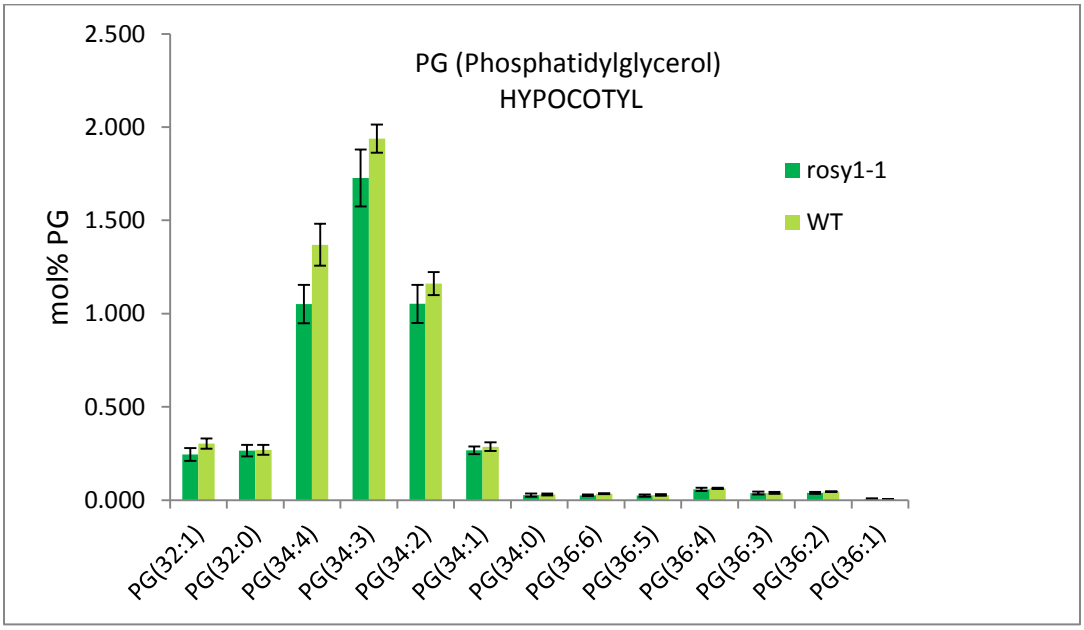
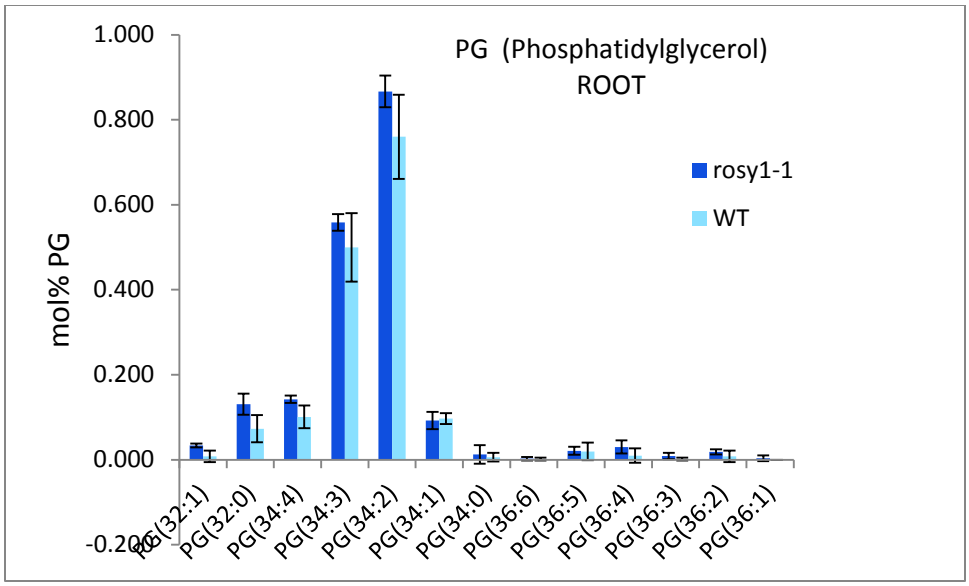
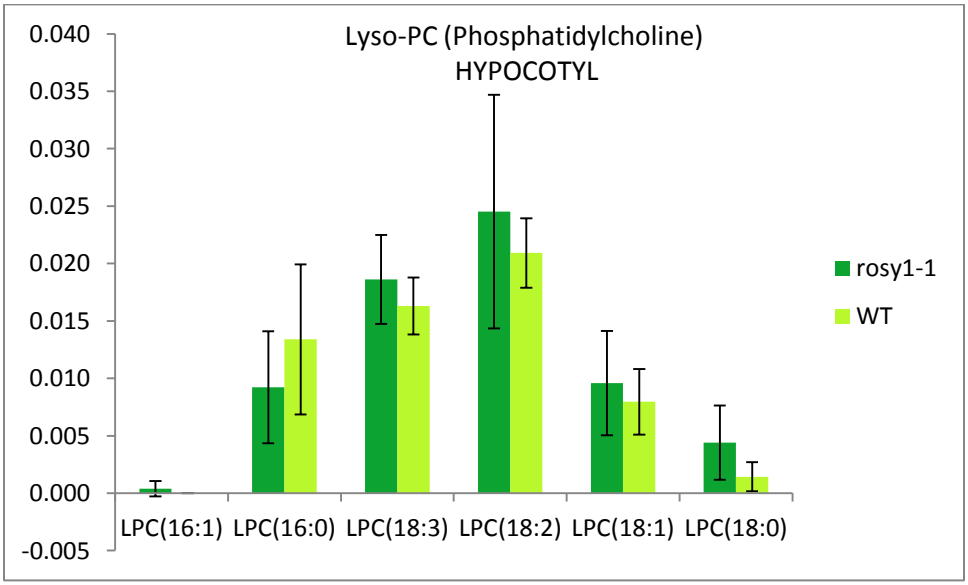
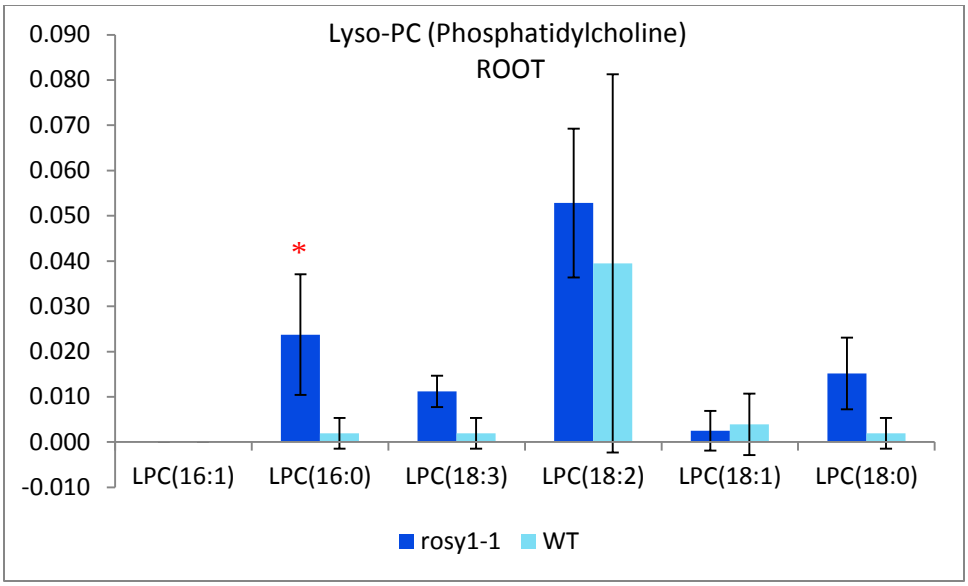
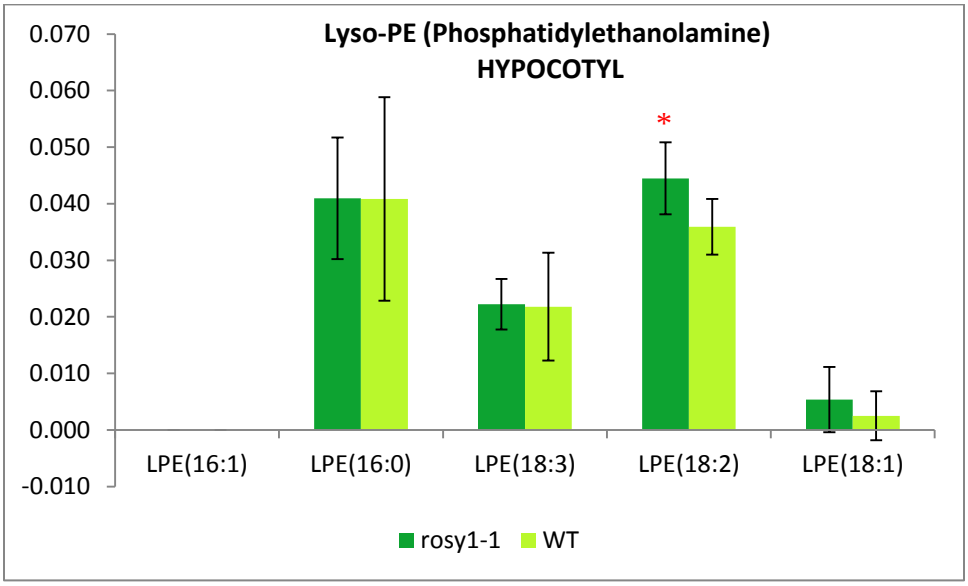
MGDG(38:3)

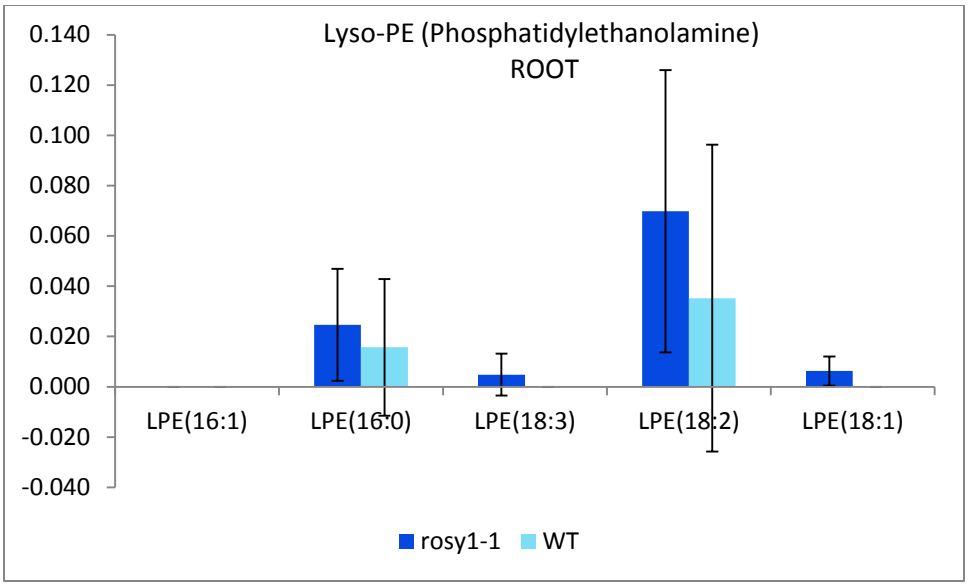

PG (Phosphatidylglycerol)
HYPOCOTYL
mol% PG
2.500
2.000
1.500
1.000
0.500
0.000
rosy1-1
WT
PG(32:1)
PG(32:0)
PG(34:4)
PG(34:3)
PG(34:2)
PG(34:1)
PG(34:0)
PG(36:6)
PG(36:5)
PG(36:4)
PG(36:3)
PG(36:2)
PG(36:1)

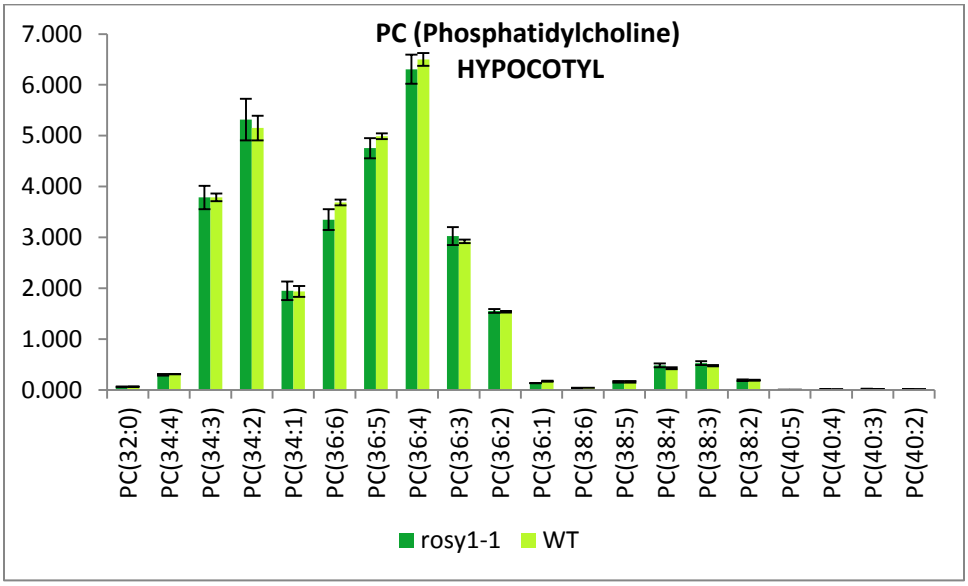

PG (Phosphatidylglycerol)
ROOT
mol% PG
1.000
0.800
0.600
0.400
0.200
0.000
-0.200
rosy1-1
WT
PG(32:1)
PG(32:0)
PG(34:4)
PG(34:3)
PG(34:2)
PG(34:1)
PG(34:0)
PG(36:6)
PG(36:5)
PG(36:4)
PG(36:3)
PG(36:2)
PG(36:1)

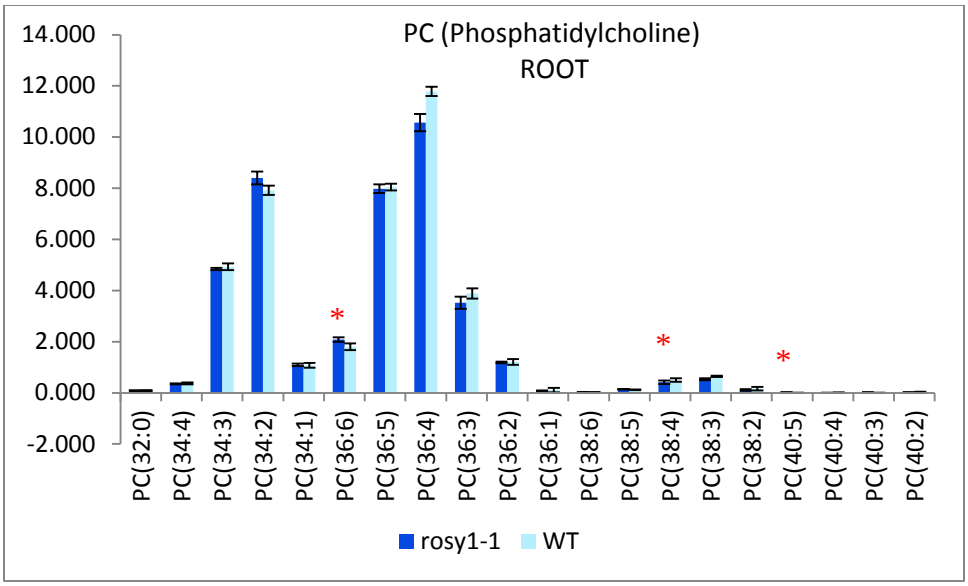

Lyso-PC (Phosphatidylcholine)
HYPOCOTYL
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
rosy1-1
WT
LPC(16:1)
LPC(16:0)
LPC(18:3)
LPC(18:2)
LPC(18:1)
LPC(18:0)

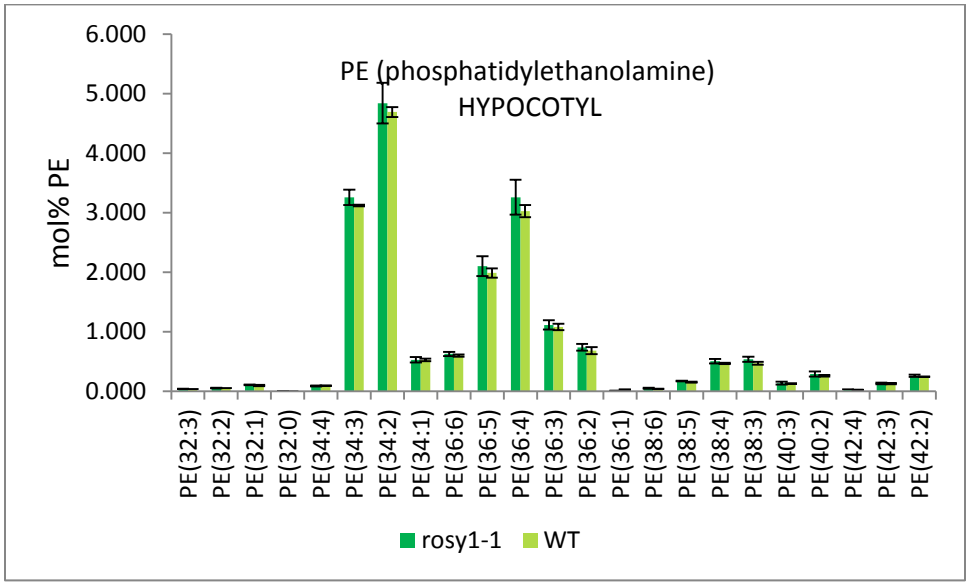

Lyso-PC (Phosphatidylcholine)
ROOT
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
-0.010
*
LPC(16:1)
LPC(16:0)
LPC(18:3)
LPC(18:2)
LPC(18:1)
LPC(18:0)
rosy1-1
WT

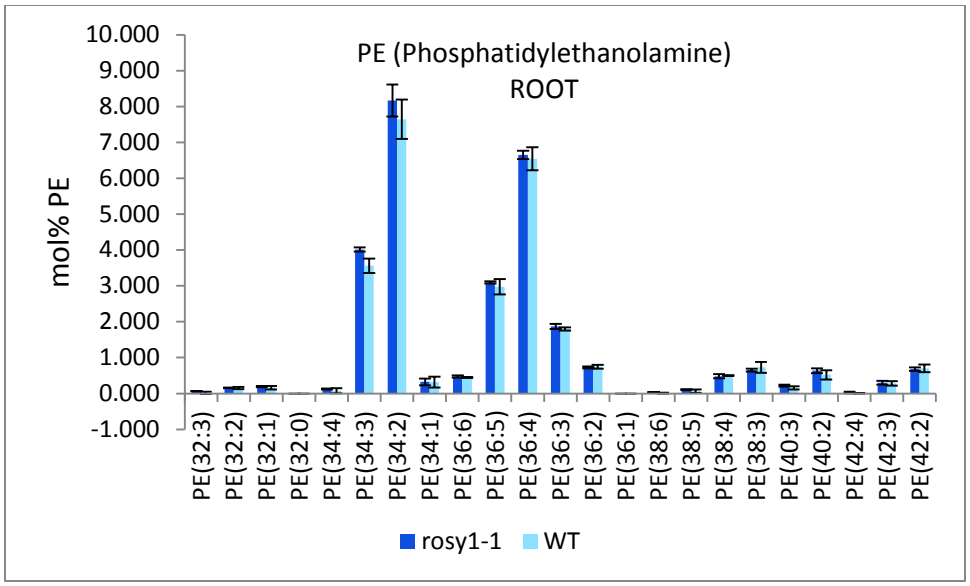

Lyso-PE (Phosphatidylethanolamine)
HYPOCOTYL
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
-0.010
*
LPE(16:1)
LPE(16:0)
LPE(18:3)
LPE(18:2)
LPE(18:1)
rosy1-1
WT

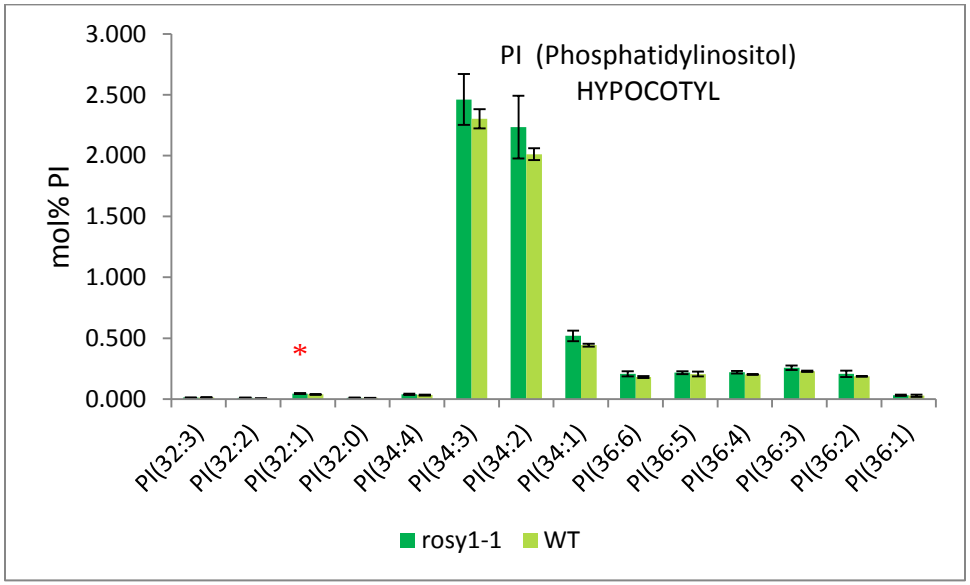

Lyso-PE (Phosphatidylethanolamine)
ROOT
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
-0.020
-0.040
LPE(16:1)
LPE(16:0)
LPE(18:3)
LPE(18:2)
LPE(18:1)
rosy1-1
WT

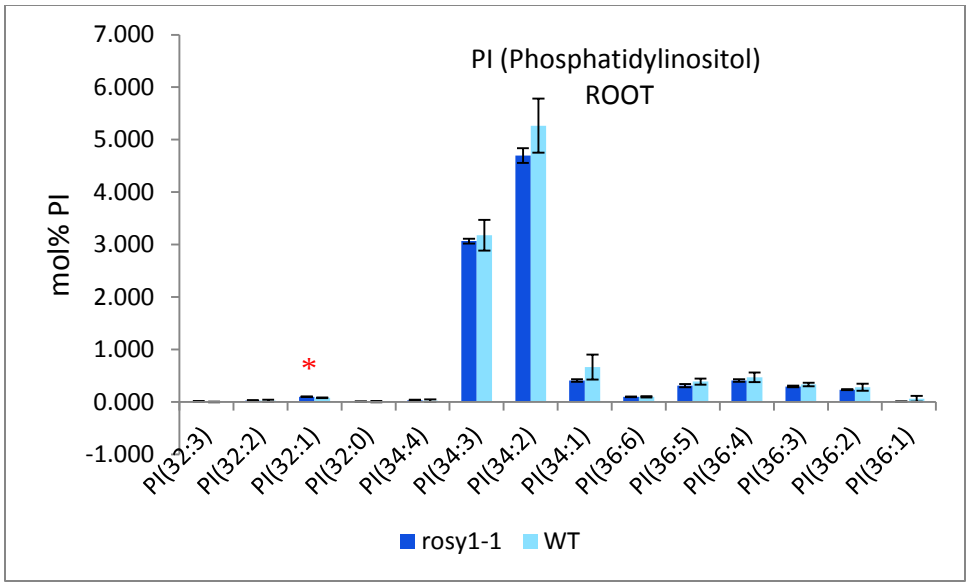

PC (Phosphatidylcholine)
HYPOCOTYL
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
PC(32:0)
PC(34:4)
PC(34:3)
PC(34:2)
PC(34:1)
PC(36:6)
PC(36:5)
PC(36:4)
PC(36:3)
PC(36:2)
PC(36:1)
PC(38:6)
PC(38:5)
PC(38:4)
PC(38:3)
PC(38:2)
PC(40:5)
PC(40:4)
PC(40:3)
PC(40:2)
rosy1-1
WT

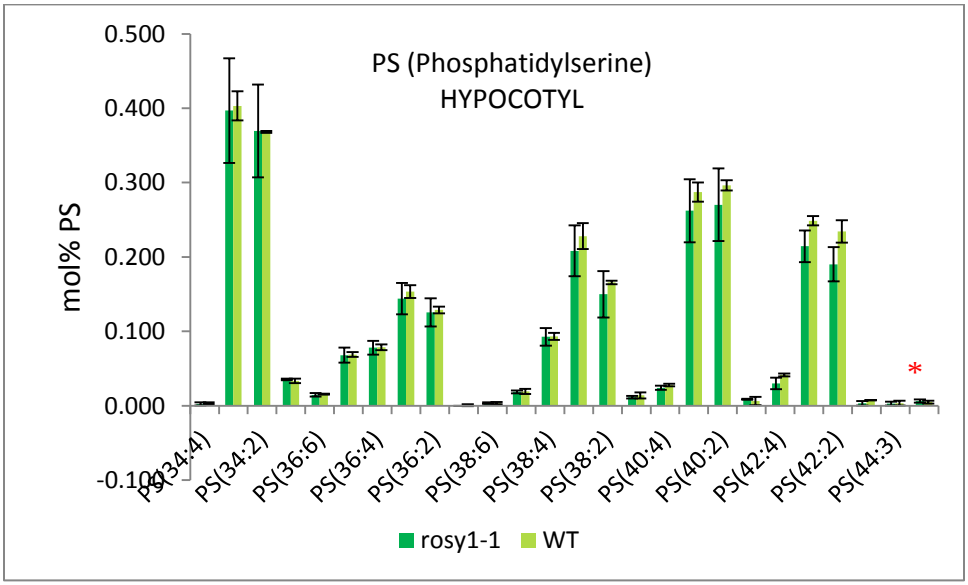

PC (Phosphatidylcholine)
ROOT
14.000
12.000
10.000
8.000
6.000
4.000
2.000
0.000
-2.000
PC(32:0)
PC(34:4)
PC(34:3)
PC(34:2)
PC(34:1)
PC(36:6)
PC(36:5)
PC(36:4)
PC(36:3)
PC(36:2)
PC(36:1)
PC(38:6)
PC(38:5)
PC(38:4)
PC(38:3)
PC(38:2)
PC(40:5)
PC(40:4)
PC(40:3)
PC(40:2)
rosy1-1
WT

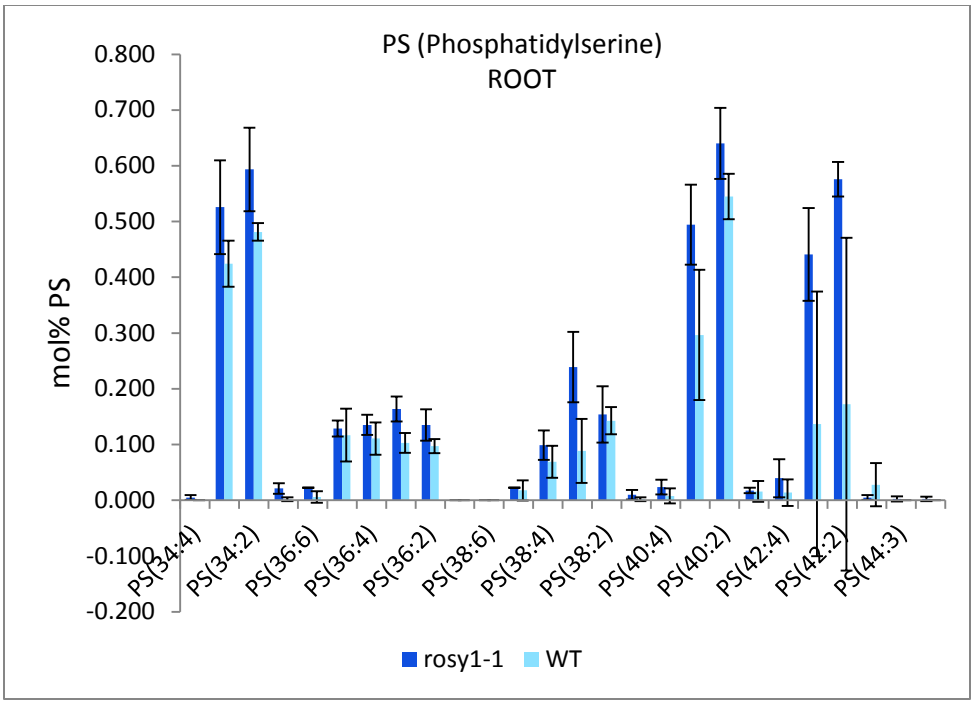

PE (phosphatidylethanolamine)
HYPOCOTYL
mol% PE
6.000
5.000
4.000
3.000
2.000
1.000
0.000
PE(32:3)
PE(32:2)
PE(32:1)
PE(32:0)
PE(34:4)
PE(34:3)
PE(34:2)
PE(34:1)
PE(36:6)
PE(36:5)
PE(36:4)
PE(36:3)
PE(36:2)
PE(36:1)
PE(38:6)
PE(38:5)
PE(38:4)
PE(38:3)
PE(40:3)
PE(40:2)
PE(42:4)
PE(42:3)
PE(42:2)
rosy1-1
WT


PE (Phosphatidylethanolamine)
ROOT
mol% PE
10.000
9.000
8.000
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
-1.000
PE(32:3)
PE(32:2)
PE(32:1)
PE(32:0)
PE(34:4)
PE(34:3)
PE(34:2)
PE(34:1)
PE(36:6)
PE(36:5)
PE(36:4)
PE(36:3)
PE(36:2)
PE(36:1)
PE(38:6)
PE(38:5)
PE(38:4)
PE(38:3)
PE(40:3)
PE(40:2)
PE(42:4)
PE(42:3)
PE(42:2)
rosy1-1
WT


PI (Phosphatidylinositol)
HYPOCOTYL
mol% PI
3.000
2.500
2.000
1.500
1.000
0.500
0.000
*
PI(32:3)
PI(32:2)
PI(32:1)
PI(32:0)
PI(34:4)
PI(34:3)
PI(34:2)
PI(34:1)
PI(36:6)
PI(36:5)
PI(36:4)
PI(36:3)
PI(36:2)
PI(36:1)
rosy1-1
WT


PI (Phosphatidylinositol)
ROOT
mol% PI
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
-1.000
*
PI(32:3)
PI(32:2)
PI(32:1)
PI(32:0)
PI(34:4)
PI(34:3)
PI(34:2)
PI(34:1)
PI(36:6)
PI(36:5)
PI(36:4)
PI(36:3)
PI(36:2)
PI(36:1)
rosy1-1
WT


PS (Phosphatidylserine)
HYPOCOTYL
mol% PS
0.500
0.400
0.300
0.200
0.100
0.000
-0.100
*
PS(34:4)
PS(34:2)
PS(36:6)
PS(36:4)
PS(36:2)
PS(38:6)
PS(38:4)
PS(38:2)
PS(40:4)
PS(40:2)
PS(42:4)
PS(42:2)
PS(44:3)
rosy1-1
WT


PS (Phosphatidylserine)
ROOT
mol% PS
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
-0.100
-0.200
PS(34:4)
PS(34:2)
PS(36:6)
PS(36:4)
PS(36:2)
PS(38:6)
PS(38:4)
PS(38:2)
PS(40:4)
PS(40:2)
PS(42:4)
PS(42:2)
PS(44:3)
rosy1-1
WT