PALACKY UNIVERSITY OLOMOUC
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MOLECULAR MECHANISM OF CELL POLARITY IN MODEL PLANT *ARABIDOPSIS THALIANA*

Diploma thesis

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I declare that I wrote this diploma thesis independently and used only information sources referred in the Literature chapter.

In Olomouc, 5. 5. 2010 ……

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SUMMARY

Auxin, as the first described phytohormone, is crucial for many developmental processes in plants and such as tropic responses. Regulation of those developmental processes is mediated also by the polar auxin transport mediated by PINFORMED (PIN) proteins. PIN function also depends on its polar subcellular localization in transport-competent cells, which is regulated by PIN constitutive cycling between endosomes and plasma membrane.

The main experimental topics of my master thesis were mechanisms of (i) regulation of the PIN endocytosis and (ii) gravitropic response in the *Arabidopsis* shoot:

(1) I used two approaches to characterise the possible pathway of PIN endocytosis. Using forward genetic approach we conducted a screen for mutants with possible defects in endocytosis. The second reverse genetic approach focused on the specific mutations in SNARE proteins. We characterise these mutations and their possible effect on PIN1 and PIN2 polar localization.

(2) In the next part of my thesis I focused on how the polar localization of PIN proteins relates to the mechanism of shoot gravitropism. Gravitropism aligns plant growth with gravity. It involves gravity perception and the asymmetric distribution of auxin. Here we provide insights into a mechanism for shoot gravitropic response. We show that PIN3 auxin transporter is key regulator of auxin distribution and gravitropism in *Arabidopsis thaliana* hypocotyls. Gravity stimulation polarizes PIN3 to the bottom sides of hypocotyl endodermis cells which correlates with increased auxin accumulation and response at the lower hypocotyl side. The PIN3 polarization and hypocotyls bending require activities of PIN polar trafficking regulators ARF-GEF GNOM and PINOID kinase. Our data showed that gravity-induced PIN3 polarization diverts auxin flow to mediate asymmetric auxin distribution and shoot gravitropic response.

SOUHRN

Auxin, jako první popsaný fytohormon, je nezbytný při vývojových procesech a zprostředkování tropické odpovědi rostlin. Regulace vývoje je zprostředkována směrovaným transportem auxinu, který je určen přítomností PINFORMED (PIN) proteinů. Ustavení PIN proteinu je regulováno konstitutivním cyklováním mezi endosomy a plasmatickou membránou.

V rámci experimentální části své diplomové práce jsem se zaměřila na (i) regulaci endocytózy PIN proteinů a (ii) gravitropní odpovědi hypokotylu rostliny *Arabidopsis thaliana*:

(1) Použila jsem dva přístupy k charakterizaci možných účastníků endocytózy. Použitím přímého přístupu jsme provedli sceen, kdy jsme hledali BFA rezistentní mutanty s možným efektem na endocytózu. Druhý reverzní přístup byl zaměřen na specifické mutace ve SNARE proteinech. Charakterizovala jsem tyto mutanty s možným efektem na PIN1 a PIN2 lokalizaci.

(2) V další části své diplomové práce jsme se zaměřila na polární relokalizaci PIN proteinů související s mechanismem gravitropismu hypokotylu. Gravitropismus umožňuje rostlinným orgánům růst ve směru nebo proti gravitaci. To vyžaduje vnímání gravitace a asymetrickou distribuci auxinu. Poskytuji zde náhled do mechanismu gravitropní odpovědi hypokotylu. Bylo prokázáno, že PIN3 protein je klíčovým regulátorem distribuce auxinu a gravitropní reakce hypokotylu *Arabidopsis thaliana*. Gravitropní stimulace polarizuje PIN3 na spodní stranu buněk endodermis v hypokotylu, která koreluje se zvýšením kumulace auxinu. Změna lokalizace PIN3 proteinu vyžaduje aktivitu regulátoru polárního targetování ARF-GEF GNOM a kinázy PINOID. Data dokazují, že polarizace PIN3 po působení gravitace přesměruje tok auxinu, zprostředkovává asymetrickou distribuci auxinu a způsobuje gravitropní odpověď hypokotylu rostliny.

CONTENT

1. INTRODUCTION

My diploma thesis particularly focuses on several, interconnected questions related to the transport of the plant phytohormone auxin in the plant model organism *Arabidopsis thaliana* and the resulted developmental regulations. Asymmetric accumulation of auxin is necessary for various developmental roles of auxin (Friml et al., 2002a; Friml et al., 2002b; Friml et al., 2003; Benková et al., 2003). The major determinant of asymmetric auxin accumulation is directional intercellular auxin transport (Friml et al., 2002a). The auxin asymmetry establishment depend on directional cell-to-cell auxin transport. The main components of the transport between cells are auxin transporters, mainly encoded by PINFORMED (PIN) genes (Petrášek et al., 2006). Various PIN proteins exhibit distinct and specific localization patterns. The localization of PINs determines the direction of auxin efflux (Wisniewska et al., 2006). The localization of PIN efflux carrier at the plasma membrane depends on the constitutive intercellular trafficking. The PIN polar localization is dynamic and consist of endo- and exocytose of PIN proteins from and back to the plasma membrane (Geldner et al., 2001). Constitutive cycling of PINs is mediated by actin cytoskeleton and by ARF-GEF GNOM activity (Geldner et al., 2003). The detailed mechanism of this PIN polar determination is still not fully characterized.

The PIN-dependent asymmetric distribution of auxin appears first in the early embryogenesis and follows during the postembryonic development and influences processes such as organ initiation (Benková et al., 2003) or tropism (Friml et al., 2003).

2. THEORETICAL BACKGROUND

2.1. Plant hormone auxin

Plant hormones are substances that influence regulation of developmental and morphological processes in plants. The plant hormone auxin (indole-3-acetic acid; IAA) has been identified as an important factor mediating tissue and organ polarity in plants, mainly on account of its strictly directional flow through plant tissues. Transport of auxin represents a prominent communication system in plants. It is involved in many developmental and morphological processes that are based on differential division and cell elongation, such as embryogenesis (Shevell et al., 1994; Friml et al., 2003), root meristem maintenance (Blilou et al., 2005; Friml et al., 2002a) differentiation of vascular tissue (Sauer et al., 2006a), apical hook formation (Zadnikova et al., 2010), apical dominance (Lehman et al., 1996), lateral organ formation (Casimiro et al., 2001) or grow responses to environmental stimuli (Woodward and Bartel, 2005).

Auxin is the first identified plant hormone; Charles Darwin described the phototropic effects, in these experiments studying the response of grass coleoptiles to unilateral light (Darwin 1880). They proposed the existence of a growth-regulating substance that is later known as auxin. The higher production of auxin occurs in young apical tissues (Ljung et al., 2005). The synthesis of auxin in *Arabidopsis* occurs mainly in leaves, cotyledons and roots (Ljung et al., 2001, 2005). Experimentally are often used synthetic auxin analogues such as 1-naphthylacetic-acid (NAA).

The important regulatory level in auxin action is its unique property, the directional intercellular transport mediating local accumulation of auxin in cells and tissues. Auxin is transported throughout the plant by two different mechanisms. For distribution of auxin serves apoplastic and symplastic transport systems of a plant. Auxin is transported for long distance with other substances in phloem, but also transported from cell to cell. Transport in phloem is faster and not directional. It proceeds in both ways, from the top to the root tip and opposite from the roots to the apex. On the other hand, transport from cell to cell, is polar, regulated and slower. The main direction of polar auxin transport is from the top to the root tip, where the flow turns back in epidermis cells to the elongation zone of the root. The flow is polarized

thanks to the asymmetric distribution of PIN auxin transporters at the plasma membrane (Friml and Palme, 2002).

The local auxin accumulation can be experimentally observed using numerous different approaches, such as by the auxin response promoter DR5 (Ulmasov et al., 1997), immunolocalization of IAA (Friml et al., 2003) or by direct auxin measurements from tissue sections (Ljung 2005).

Auxin triggers in the cell specific genomic and non-genomic responses. Regulated protein degradation is the main mechanism of auxin signalling in the genomic pathway (Paciorek and Friml, 2006). In cells, auxin induces rapidly transcription of regulators from the AUXIN/INDOL-3ACETIC ACID (AUX/IAA) family (Abel et al., 1994). In low auxin level, these repressors inhibit the transcription of genes. AUX/IAA form domains with other AUX/IAA proteins and also with AUXIN RESPONSE FACTORs (ARFs). Complex interacts with TRANSPORT INHIBITOR RESPONSE1 (TIR1) protein, which is part of the ubiquitin ligase complex SCF^{TIR1} and marks target proteins including AUX/IAA with ubiquitin and thus leads to their degradation in proteasomes. Auxin binds the TIR1 protein, the auxin receptor (Dharmasiri et al., 2005) and increase the interaction between TIR1 and AUX/IAAs (Gray et al., 2001). AUX/IAA can directly interact with the TIR1 subunit of the CSF^{TIR1} complex and after auxin application this interaction is promoted and AUX/IAA degradation is accelerated. This suggests that auxin stimulates the degradation of AUX/IAA by promoting TIR1-AUX/IAA interaction (Gray et al., 2001).

Auxin regulates expression of genes containing regulation sequence TGTCTC in promoter part called Auxin-Response Element (AuxRE). ARF proteins bind this sequence, form the complex with other AFRs or with AUX/IAA protein and inhibit transcription. ARF proteins can be activators and for other genes inhibitors of their transcription, it depends on the central domain (Woodward and Bartel, 2005). By contrast, the accumulation of AUX/IAA proteins in the cells turns off the auxin signalling. AUX/IAA proteins have very short half-time which means that they are very rapidly degraded. This complicated system enables short-term and temporary auxin signalisation (Gray et al., 2001). AUX/IAA binds directly DNA (Morgan et al., 1999) and thus might also directly regulate gene transcription.

There is another possible way of auxin-mediated regulation. The protein named auxin-binding protein1 (ABP1) show the strong ability to bind the auxin (Palme et al., 1992). Loss of function *abp1* mutant is lethal (Chen et al., 2001) suggesting important role in plants. We know, that ABP1 bind the auxin and initiate cell elongation and regulate embryogenesis, but the detailed role of ABP1 remains unclear.

2.1. Compounds regulating auxin flow

Formulation of a chemiosmotic hypothesis for polar auxin transport (Rubery and Sheldrake, 1974, Raven 1975) explained the mechanism of cell-to-cell polar auxin transport (Fig. 1). Model proposed the existence of plasma membrane localized auxin carrier proteins that facilitate auxin uptake and auxin efflux out of the cells. This model also proposed that the asymmetric localization of the efflux carriers at one side of the cells determines the direction of the intracellular auxin movement. Three decades later, this hypothesis has been verified and molecularly supported with the identification of several proteins that have the capacity to transport auxin and are polar localized. The chemical property of IAA suggests, that auxin efflux is the limiting step, the auxin efflux carriers became the main objective, therefore I will focus to efflux carriers in my theoretical background.

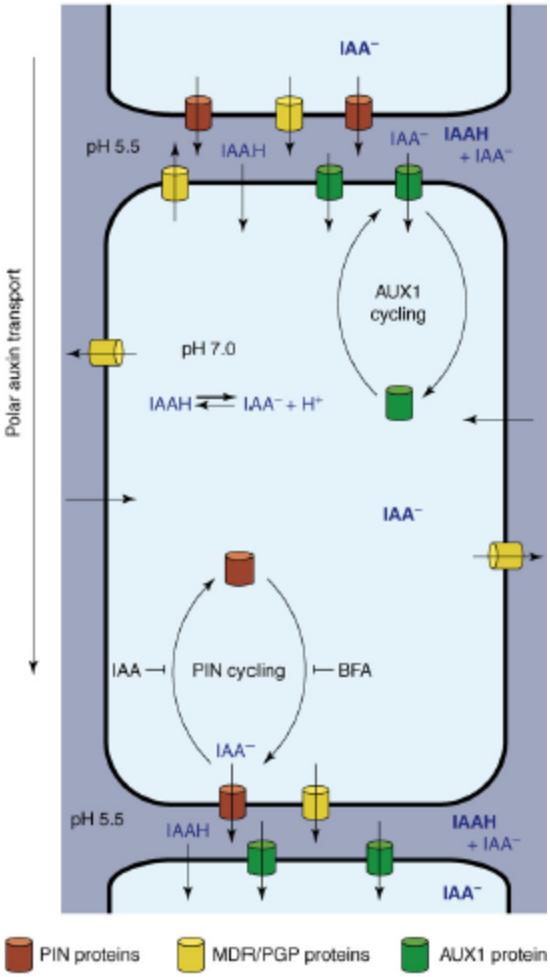

Fig. 1 Model of chemiosmotic hypothesis.

Plasma membrane proton pumps create at the plasma membrane proton gradient and cause low pH in the apoplast. Auxin as a weak acid in the apoplast dissociates only partially. Non dissociated molecules IAAH are hydrophobic and can go freely and passively through the plasma membrane. Whereas, dissociated forms IAA- are polar and to the cells can be transported only actively with transporter proteins at the plasma membrane. In the cytosol, the auxin is also dissociated and that's way it can't go passively through membrane and need transport proteins to transport it out of the cell. This implies that the rate-limiting step of the transport is amount and localization of auxin efflux carriers. An asymmetric, polar distribution of auxin efflux transporters leads to unidirectional auxin transport.

Edited according to Vieten et al., 2007.

Molecular genetics and physiological studies led to the discovery of genes coding for auxin influx and efflux carriers. At the level of the individual cell the polar auxin transport is mediated by transporters of the AUX/LAX (Bennett et al, 1996), PGP (Geisler and Murphy, 2006) and PIN (Petrášek et al, 2006) families.

2.1.1. Efflux carriers

Discovery and characterisation of the auxin efflux carriers was an important step in understanding regulation and mechanism of auxin transport. The auxin efflux carriers are phosphoglycoproteins (PGP), plant orthologues of mammalian ABC-transporters (ATP-binding cassette transporters) (Terasaca et al., 2005). Members of the multi-drug-resistant/P-glycoprotein (MDR/PGP) subfamily of ABC proteins have been demonstrated to transport auxin from the cells (Geisler et al. 2005). PGP proteins localize symmetrically in almost all cells, this suggests their role in a non polar transport at a long distance. In addition, in several cells, such as endodermis of the elongation zone of the root, PGP proteins, specifically PGP1 and PGP4, localize asymmetrically suggesting their role in the polar auxin transport (Geisler et al., 2005).

The PIN FORMED (PIN) proteins have been identified and characterized as key regulators of auxin-mediated developmental processes. The first identified was PIN1; the *pin-formed1* (*pin1*) mutant forms needle-like stems that lack flowers. The same phenotype was found in the wild type treated by chemical inhibitors of auxin transport (Okada et al., 1999). PIN proteins belong to the protein family with eight members in Arabidopsis; they show tissue-specific expression and are localized polarly at different sides of the various cell types (Vieten et al., 2007). PIN proteins are plasma membrane proteins that act as auxin efflux carriers (Petrášek et al., 2006) and they are typically polarly localized at the plasma membrane, although some of them can be found in specific cell types without pronounced polarity. All PIN proteins are auxin transporters, but they participate in different development processes. In most cases, developmental processes, like establishment of new organs (flowers, leaves, lateral roots etc.) are accompanied by local auxin accumulations, or at least auxin activity maxima (Luschnig et al., 1998; Muller et al., 1998; Friml et al., 2002a; 2002b; Benková et al. 2003, Friml et al. 2003, Scarpella et al. 2006).

PIN proteins exhibit cell type and PIN amino-acid sequence specific polar localization which determines the direction of auxin flow (Wisniewska et al., 2006). PIN proteins

transport auxin also in non-plant systems lacking auxin transport machinery such as yeast or human cells, which provides evidence that PIN proteins do not require any cofactors for their efflux activity (Petrášek et al., 2006).

Because PIN proteins are homologous and functionally redundant, in some cases they can substitute each other and therefore single *pin* mutants typically don't have so strong phenotypes. The multiple mutations, however, show strong, at some cases lethal phenotypes (Friml et al., 2003; Blilou et al., 2005; Vieten et al., 2005). For example, during embryogenesis, PIN1, PIN4, and PIN7 show polar localizations and specify the apical-basal axis of the embryo (Friml et al., 2003). Postembryonically, PIN proteins have different plasma membrane localizations. The basally localized (root apex-facing) side of the vasculature and stele cells are PIN1, PIN3, PIN4, and PIN7, whereas apically localized (shoot apex-facing side) are PIN1 in the shoot apex epidermis or PIN2 in the lateral root cap and epidermis cells. In the shoot endodermis and root pericycle cells, PIN3 localizes at the inner lateral side, whereas it has a symmetric localization in columella cells (Fig. 2). PIN5, PIN6 and PIN8 have not been fully functionally characterized but they presumably regulate auxin homeostasis at the endoplasmic reticulum (Mravec et al., 2009). Another PIN functions were described already. For example, PIN1 mediates organogenesis and vasculature formation, PIN2 and PIN3 are responsible for the root gravitropic growth, PIN3 also phototropic and gravitropic response of the hypocotyl. PIN4 regulates the activity of the root meristem and PIN7 plays a role mainly during embryogenesis (Petrášek and Friml, 2009).

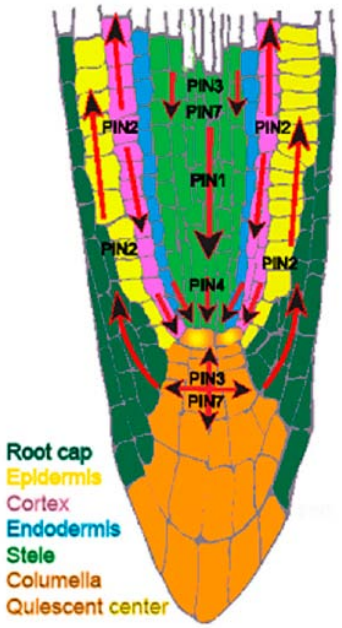

Figure 2. Polar localization of PIN proteins in the *Arabidopsis* root tip.

The directionality of auxin transport (arrows) is determined by the polar localization of PIN proteins. PIN1 is localized at the basal side of the root vasculature, PIN2 predominantly localizes at the basal side of the cortical cells and at the apical side of the epidermal cells and root cap cells; PIN3 in an apolar manner in the columella cells of the root; PIN4 at the basal side of cells in the central root meristem and with less pronounced polarity in the cells of the quiescent center; and PIN7 localize at the basal side of the stele cells and apolar in the columella cells.

Edited according to Kleine-Vehn and Friml, 2008.

Polar targeting of PIN proteins has a clear developmental output, because the polarity of the PIN localization at the single-cell level determines the direction of intercellular auxin transport and the directional signalling to neighbouring cells (Wisniewska et al., 2006).

Several PGP proteins also interact with PIN proteins and probably stabilize them at the plasma membrane (Bandyopadhyay et al., 2007). Taken together, PGP contribute to non-directive auxin transport at the long distances, whereas PIN protein rather polar transport for short distance. In several cells they also interact and cooperate. In addition, it seems that PGP and PIN proteins define distinct transport systems, because both of them have different sensitivity to inhibitors (Petrášek et al., 2006).

2.1.2. Influx carriers

Auxin at the low pH in the apoplast is only partially dissociated and thus can pass the plasma membrane and enter the cell; however, in addition auxin is also transported by specific auxin transporters. One of already described auxin influx carriers is the AUXIN RESISTANT1 (AUX1) permease (Bennet et al., 1996; Swarup et al., 2001) and it's homologous (AUX/LAX) that transports auxin into the cells.

The *auxin resistant1* (*aux1*) mutant was identified in a screen for auxin resistant and agravitropic plants (Maher and Martindale, 1980). Plants mutant for *aux1* are agravitropic and less sensitive to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (Bennett et al. 1996). The agravitropic phenotype can be rescued by exogenous application of membrane permeable auxin analogous NAA (Marchant et al., 1999). The role of AUX1 as auxin influx carrier has been established by several experiments (Marchant et al., 1999; Parry et al., 2001). AUX1 has several homologues, the LIKE AUX (LAX) genes, which might act in a redundant way, but their function has not been fully determined yet.

The AUX1/LAX proteins localize at the plasma membrane (Bennett et al, 1996; Yang et al, 2006). Amount of AUX1 on the plasma membrane is regulated by dynamic cycling between plasma membrane and endosomal compartments. In addition, this protein has opposite polar localization at the plasma membrane than PIN proteins in the same cells. However, the mechanism of its trafficking appears to be distinct from PIN1, as it does not require the ARF-GEF GNOM (Kleine-Vehn et al. 2006). In protophloem cells in AUX localized asymmetrically on the upper side of the cells whereas on the lower side are proteins

transported auxin out of the cells. This system support the direction auxin flow from phloem to the root tip (Swarup et al., 2001). AUX1 play a crucial role in unloading auxin in protophloem cells from non-polar transport system (Swarup et al., 2001).

2.2. Regulation of PINs

Regulation the activity of auxin transporters namely PIN proteins occurs at different levels. PIN activity is affected by auxin via transcriptional regulation, which also accounts for their wide functional redundancy (Vieten et al., 2005; Blilou et al., 2005), and also by posttranslational degradation (Abas et al., 2006), vesicle trafficking (Steinmann et al., 1999; Geldner et al., 2001; Paciorek et al., 2005) and phosphorylation (Friml et al., 2004).

2.2.1. Regulation of PIN proteins abundance

The transcription of various PIN proteins has been shown to be regulated by auxin. Regulation depends on the auxin stimulated repression of transcription factors ARF (Woodward, 2005). Auxin can increase its own efflux inducing expression of PIN transporters (Paponov et al., 2008). Gene expression regulation is mediated AUX/IAA pathway. Auxin increase degradation of AUX/IAA repressors, release ARFs transcription factors to activate transcription of PIN proteins (Blilou et al., 2005).

Regulation of PIN abundance occurs also with their specific degradation. The regulation of PIN activity with the degradation was described for PIN2 efflux carrier. PIN2 is responsible for basipetal auxin transport from the root tip to the elongation zone of the root and thus cause gravitropic response. After establishment of the auxin accumulation at the lower side of the gravity stimulated root, auxin inhibits root growth and cause bending of the root. PIN2 abundance decreases with the decrease of auxin concentration at the upper side of the root. This degradation of PIN2 protein is regulated by AXR1 protein playing role in ubiquitin-dependent proteolysis in proteasomes; thus the degradation of PIN2 occurs in a proteasome-dependent manner (Sieberer et al., 2000; Abas et al., 2006). Other PIN proteins, for example PIN1 and PIN4, are regulated by degradation in the response to increase of auxin concentration (Vieten et al., 2005).

2.2.2. Subcellular trafficking and targeting of PIN proteins

Subcellular targeting of PIN proteins is crucial for the establishment of asymmetric auxin accumulation. The polar localization on the plasma membrane directs the auxin flow. The main processes to send the PIN proteins to the correct cell side are constitutive cycling and transcytosis (Kleine-Vehn et al., 2008). It seems that also these pathways are connected and regulated. The polar localization of PIN proteins is highly dynamic and seems to rely on the constitutive endo- and subsequent exocytose of PIN proteins from and back to the plasma membrane (Geldner et al., 2001). The decision between apical or basal targeting is based on the phosphorylation stage of PIN proteins (Wishniewska et al., 2006).

There are at least two different polar targeting machineries given that AUX1 and PIN trafficking shows different sensitivities to various inhibitors and that AUX1 and PIN proteins are controlled by different subsets of vesicle trafficking pathways (Kleine-Vehn et al., 2006).

Plant and animal cells might differ fundamentally in the manner by which the polar-component cargoes are kept at their polar domain. Animal epithelial cells are a preferred model system, because their plasma membrane is divided into two distinct domains, the apical domain facing the lumen and the basolateral domain (Mostov et al., 2003). Tight junctions form a physical barrier and limit lateral diffusion of proteins between adjacent polar plasma membrane domains (Brown and Stow, 1996), to which new proteins are directly send or delivered by secretion or transcytosis (Fig. 3).

2.2.2.1 Phosphorylation

Reversible phosphorylation is one of the most often regulation of the protein activity. It is ensured by kinases and phosphatases. Phosphorylated stage influences the localization of auxin transporter in the cells and thus regulates the auxin transport.

The experiments showed that PIN1 ectopically expresses under PIN2 promoter localize at the lower side of the epidermis cells suggesting that the sequence-specific signal contribute to the control of polar PIN localization. The GFP insertion to this specific place of the PIN1 protein cause the switch to the upper side in the epidermis cells (Wisniewska et al., 2006). It was observed, that different polar cargos such as PIN1 or PIN2, localize to different polar domain of the same cells. These findings imply a PIN sequence-based signal for

decision on subcellular localization. The PIN polarity signals are related to the phosphorylation sides found in the PIN sequence (Zhang et al., 2009).

One of the regulators on the PIN polarity, the Ser/Thr protein kinase PINOID (PID; Christensen et al., 2000; Benjamins et al., 2001) regulates PIN targeting. Over-expression of PID leads to a preferential apical PIN localization, proteins are shifted to the apical side of the cell. On the other hand, low activity of PID kinase in the *pid* mutant leads to the preferential basal PIN targeting; PIN proteins that normally localize to the apical side shifted to basal side (Friml et al., 2004). Over-expressed PID causes change of the polar localization of PIN1, PIN2 and PIN4 during embryogenesis and in the primary root. In the *Arabidopsis* seedlings, PID over-expression leads to transport PIN2 and PIN4 to the apical side of cells and auxin flow is not routed to the root tip, auxin is depleted and the collapse of the root meristem occurs (Friml et al., 2004). Furthermore, the shift of PIN polarity is observed within a few hours following the increase of PID expression, indicating the direct regulation of polar PIN targeting by PID kinase. PID kinase is the only factor that has been already identified to mediate decisions about the polarity of PIN targeting. It was shown that the level of PID activity is controlled by auxin (Benjamins et al., 2001). Increased expression of PID gene after auxin incidence suggests that this gene belongs to the auxin induced genes (Benjamins et al., 2001). In addition to these findings, the *AuxRe* (auxin responsive elements) in the promoter sequence of protein kinase PID was found (Michniewicz et al., 2007).

PP2A phosphatase has been shown to antagonize this action (Michniewicz et al., 2007). Thus, dephosphorylated PIN is preferentially recruited by the basal targeting machinery and phosphorylated PIN is trafficked by the apical pathway. The phenotype of *rcn1* (mutant in PP2A gene) is similar to the over-expressed PID kinase. The seedlings are agravitropic and the collapse of the root tip occurs. Moreover, in *rcn1* mutants, basal localized PINs are shifted to apical side in cortex cells of the root. Both, PID and PP2A, are localized at the plasma membrane and both partially colocalize with PIN protein (Michniewicz et al., 2007).

Moreover, the fact that the manipulation of PIN polarity by PID over-expression leads directly to changes in auxin flow, reversal of auxin gradients and corresponding patterning effects occurs (Friml et al, 2004).

2.2.2.2 Role of a secretion and recycling in polar targeting

Genetic suppression of the ARF-GEF function in *gnom* mutant or using chemical inhibitors revealed that PIN proteins are constantly moving between plasma membrane and endosomes, termed constitutive cycling (Geldner et al., 2001). The delivery of PIN proteins to the plasma membrane is sensitive to fungi toxin brefeldin A (BFA), a known inhibitor of secretion and subcellular trafficking (Steinmann et al., 1999; Geldner et al., 2001). Trafficking from the plasma membrane to the endosomal compartments seems to be, on the other hand, insensitive to BFA. These differential effects of BFA on exocytosis and endocytosis lead to the internalization and subsequent accumulation of constitutive cycling protein in the BFA compartments (Fig. 3) (Geldner et al., 2001; 2003). A molecular target of the BFA action is GNOM. GNOM functions as the GDP/ GTP exchange factor for the small G proteins of the ARF class which mediates formation of exocytic vesicles at endosomes responsible for carrying PIN proteins and possibly other cargos to the plasma membrane (Geldner et al., 2001; 2003).

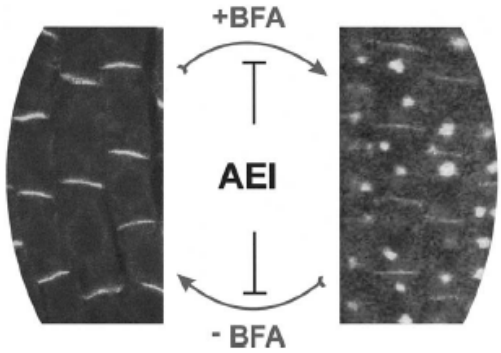

Figure 3. Re-cycling of PIN1 proteins.
The reversible internalization of PIN1 upon Brefeldin A (BFA) treatment suggesting that PIN1 cycles between the plasma membrane and an endosomal compartment. Polar auxin transport inhibitors (AEI) such as TIBA block PIN1 cycling.
Edited according to Friml and Palme, 2002c.

The BFA induced internalization occurs also when protein synthesis or protein degradation are inhibited (Geldner et al., 2001; Paciorek et al., 2005), confirming, that internalized protein in BFA compartments were not *de novo* synthesized but originated from the plasma membrane. An auxin-dependent increase in the amount of plasma membrane localized PIN proteins would provide a mechanism, by which auxin can control its own transport. GNOM seems to be more crucial for basal polar targeting (Kleine-Vehn et al., 2008).

Direct delivery of secreted proteins and other cargos to distinct polar domains at the plasma membrane is one of the basic possibilities for generating an asymmetric distribution at the cell surface. PIN proteins seem to be delivered originally in a non-polar fashion after the

synthesis, and their apical or basal polarity is then established in the next step involving internalization from the plasma membrane and polar cycling. This recycling is important in the generation of the PIN polar localization (Kleine-Vehn and Friml, 2008).

It seems that PIN cycling is a crucial part of auxin transport and is necessary for the rapid changes in PIN polar localization observed during embryogenesis, organogenesis and tropic growth (Friml et al., 2003; Benková et al., 2003; Friml et al., 2002b). Not fully clear mechanism is how the different polar-competent proteins are recognized and delivered to the correct side of the cell.

Endocytosis is an essential process in eukaryotic cells and it is involved in the internalization of molecules from the plasma membrane extracellular environment, plasma membrane recycling, including uptake and the degradation of signal molecules (Mellman, 1996). The plant endomembrane system is highly dynamic, with extensive interactions between diverse endocytic compartments (Fig. 4; Šamaj et al., 2005). The protein cargos usually first enters the endomembrane system at the endoplasmic reticulum before moving on to the Golgi apparatus. After sequential passage through the Golgi, proteins arrive at the tran-Golgi network (TGN). At the TGN, vesicles are distributed to go to the prevacuolar compartments, or to the vacuoles; or they are targeted back to the plasma membrane. It is known, that endosomes interact with the TGN during protein sorting. Moreover, endosomes and TGN share endocytic molecules such as dynamin, clatrin, endocytic SNAREs, Rabs, ARFs, ARF-GEF GNOM (Uemura et al., 2004; Šamaj et al., 2005). SNAREs localize more or less specifically on the plasma membrane, recycling endosomes, early or late endosomes, trans-Golgi network or vacuoles (Šamaj et al., 2005) and mediate vesicle fusion in several levels of endocytic network. Actually, proteins called soluble N-ethylmaleimide-sensitive factor adaptor proteins (SNAP) receptors (SNARE) are binding partners. The role of SNARE appears in the assembling the complex of “t-SNARE” (target-SNARE) and “v-SNARE” (vesicle-SNARE) which drives the fusion of vesicle with the target membrane (Weber et al., 1998).

In *Arabidopsis*, localization of N-ethyl-maleimide-sensitive factor attachment protein receptor (Sanderfoot et al., 2000; Uemura et al., 2004), concretely VAMP727 is sensitive to ARF-GEF GNOM (Ueda et al., 2004). The endomembrane system is difficult and still not fully clear, but the cooperation in the endocytic pathway is possible.

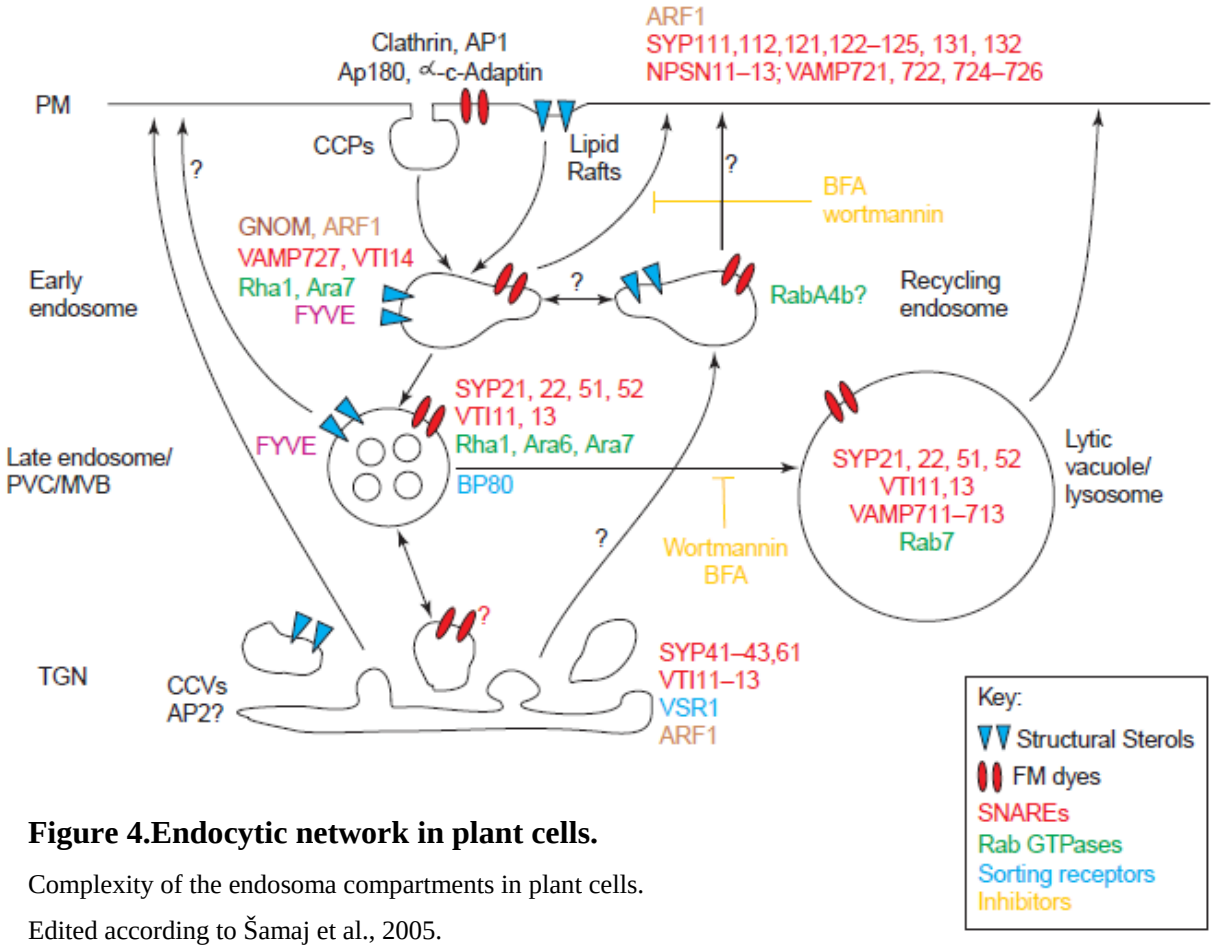

Figure 4.Endocytic network in plant cells.

Complexity of the endosoma compartments in plant cells.

Edited according to Šamaj et al., 2005.

Constitutive PIN cycling probably facilitates rapid changes in PIN polarity (for example needed during tropic responses) and also provides a means for controlling the occurrence of PIN proteins at the plasma membrane and thus regulates the rate of auxin efflux from the cells (Paciorek et al., 2005; Zažímalová et al., 2007). Constitutive cycling includes two repeating processes – endocytosis, vesicle transport from plasma membrane to the vesicle compartments; and exocytose, transport back to the plasma membrane. The endocytic step of the cycling is clathrin-dependent (Dhonukshe et al., 2007). Another important structure that participates in transport of cargoes containing PIN proteins is actin cytoskelet (Geldner et al., 2001).

2.2.3. Auxin regulates its own transport

The canalization hypothesis proposed feedback regulation between auxin levels and throughput and directionality of auxin flow as more universal mechanism regenerative and patterning processes in plant (Sachs, 1991). During the vascular formation or regeneration,

local auxin accumulation leads to the rearrangement of PIN polarity and subsequently redirection of auxin flow. Interestingly, the application of auxin seems to be enough to elicit changes in PIN polar localization. This demonstrates, that auxin is a sufficient polarizing factor in the mechanism involving PIN polar targeting (Sauer et al., 2006a).

By inhibiting the internalization of PIN constitutive cycling, auxin cause increase levels of PIN proteins at the plasma membrane. Auxin promotes its own efflux from cell by a vesicle trafficking-dependent mechanism. By modulating PIN protein trafficking, auxin regulates PIN abundance at the cell surface, providing a mechanism for the feedback regulation of auxin flow. Auxin mediated stabilization of PIN proteins on the particular cell side can be mechanism by which auxin influences the polar localization of its transporters. Thus auxin can influence the rate and also the directionality of its own flow (Paciorek et al., 2005).

This effect is also show during gravitropism, where it correlates with decreased PIN2 internalization. Asymmetric auxin accumulation correlates spatially with the inhibition of endocytosis during the root gravitropic response at the lower side of the root. The direct effect of how auxin regulates its own efflux has not been shown yet.

Auxin itself plays important role also in the regulation of its own transport mediated by activation of expression genes for auxin transporters such as AUX1/LAX, PGP, PIN and genes coding protein kinase PID. Auxin also inhibits endocytosis of cycling PIN proteins (Paciorek et al., 2006).

2.2.4. Transcytosis mechanism for PIN polarity switches

Transcytosis involves cargo movement between different sides of the cells. In animal cells transcytosis involves trafficking of polar cargos from one side of the cell to the other. In the animal epithelium, this process is crucial for the polar delivery of multiple cargos (Leibfried and Bellaiche, 2007). In plants, the directional translocation of PIN1 protein from the basal to the apical side of plasma membrane can be induced by BFA treatment. After BFA removal, the basal localization of PIN proteins is restored by translocation in the opposite direction from the apical to the basal cell side (Kleine-Vehn et al., 2008). These results demonstrate that apical and basal targeting pathways in plants are interconnected and can be used by PIN proteins to move between the apical and basal sides of cells (Fig. 5). This alternative PIN recruitment to distinct ARF-GEF dependent targeting pathways may enable

synchronous apical-basal targeting of PIN proteins, redefining PIN polarity or causing rapid PIN polarity alterations. The rapid PIN polarity switches, which can be observed during different developmental processes, are presumably realized by the transcytosis mechanism (Petrášek and Friml, 2009).

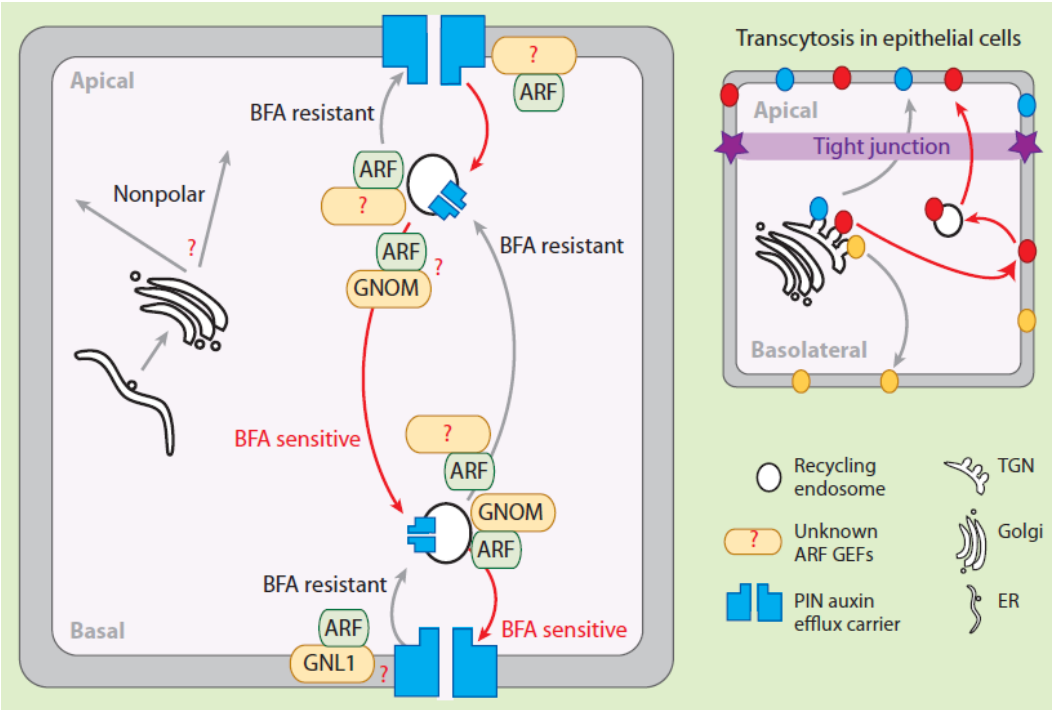

Figure 5. Apical and basal targeting and transcytosis of PIN-FORMED (PIN) proteins.

Polar PIN distribution is regulated by distinct ARF GEF dependent apical and basal targeting pathways. Alternative utilization of both apical and basal targeting pathways enables dynamic translocation of PIN cargos between different cell sides. Inhibition of the GNOM component of the basal targeting pathway genetically or by BFA leads to the preferential recruitment of cargos by the apical pathway and to a reversible PIN polarity shift.

A similar process occurs in animal epithelial cells, in which several polar-competent proteins (depicted in *red*) are initially targeted to the basolateral cell side and subsequently transcytosed to their final destination (the apical cell side). However, other polar cargos (depicted in *yellow* and *blue*) do not require transcytosis for polar localization. The difference between plant and animal is tight junction presence, protein complex forming an impermeable barrier to fluid.

Abbreviations used: ARF GEF, GDP/GTP exchange factor for adenosyl ribosylation factors; BFA, brefeldin A; ER, endoplasmic reticulum; GNL1, GNOM-LIKE1; TGN, trans-Golgi network.

Edited according to Kleine-Vehn et al., 2008.

2.3. Developmental and environmental modulation of PIN polarity

In plants, cell polarity provides means by which they maintain developmental continuity and adapt their development to optimally conform to environmental conditions. Directional flow of auxin mediates developmental processes such as tropic growth (Chen et al., 1998; Friml et al., 2002b), axis formation in embryogenesis (Friml et al., 2003), postembryonic organogenesis (Benková et al., 2003), root meristem maintenance (Friml et al., 2002a; Blilou et al., 2005), and vascular tissue differentiation and regeneration (Sauer et al., 2006; Scarpella et al., 2006).

2.3.1. Auxin transport during embryogenesis

Embryogenesis is the fundamental process where the basic body plan is set up and bilateral and radial symmetries, as well as apical-basal axis are established. The earliest known switches in PIN polarity occur during embryogenesis (Fig. 6).

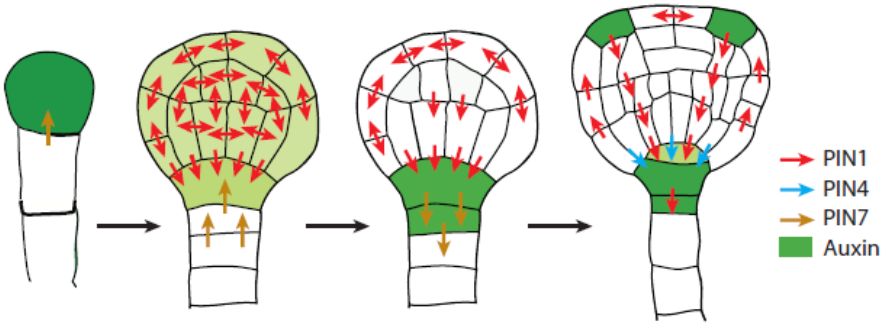

Figure 6. Alterations of apical-basal polarity during *Arabidopsis* embryogenesis.
Schematic model representing PIN distribution and polar orientation during embryo development. At early stages, PIN7 localizes to the apical sides of suspensor cells mediating auxin flow into the proembryo, where PIN1 is localized first in an unpolar manner. Afterwards, PIN1 and PIN7 undergo a GNOM-dependent polarity switch at the globular stage. The shift of PIN7 polarity together with the basal accumulation of PIN1 in proembryo cells, correlates with a rearrangement of auxin maxima from the apical part of proembryo to basal accumulation at the root pole. These PIN polarity rearrangements result a dramatic change in the apical-basal auxin gradient. A new auxin maximum is established at the position of the future root, contributing to the initiation of the root specification. At later stages, PIN4 expression in the central root meristem aids the establishment of local auxin accumulation in the centre of developing root meristems. Later, PIN1 relocates at the apical surface of the proembryo to establish two symmetrically auxin accumulation locating the formation of cotyledons.
Edited according to Feraru and Friml, 2008.

At the early embryogenesis stages, PIN7 is localized at the apical side of the suspensor cells, where it mediates the auxin flow toward the small apical cell to specify it as a proembryo. PIN1 is first expressed in a non-polar manner in the proembryo. At the defined moment of embryo development, PIN1 polarizes toward the basal side of provascular cells and PIN7 changes its localization to the basal side of the suspensor cells. These PIN polarity rearrangements redirect the auxin flow toward the area of the future root meristem, where auxin accumulates and contributes to the specification of the root meristem (Friml et al., 2003).

2.3.2. Auxin transport during organ formation and development

During postembryonic organogenesis, for example by the formation of lateral roots or leaves and flowers at the shoot apical meristem, PIN polarity undergoes rearrangements that determine the position of the future organ relative to the preexisting ones and the new growth axis of the organ primordium (Benkova et al., 2003; Reinhardt et al., 2003). The changes of PIN polarity consequently redirect the auxin flow, which plays a role in root and shoot organs development (Petrášek and Friml et al., 2009). Auxin with the forming accumulation marks the position of organ initiation and development (Benková et al., 2003). In addition, the local application of auxin initiates the formation of flowers and leaves (Reinhadrt et al., 2000) and of lateral roots (Dobrovsky et al., 2008). The auxin flow is change and coordinated by auxin influx and efflux carriers. The establishment of the auxin transporters is relevant to initiate the new organs (Petrášek and Friml, 2009).

Additionally, formation of vasculature is mediated also by PIN polarity changes, which is related to the canalization hypothesis (Sachs, 1981), which assumes that auxin can induce its own transport and form the auxin channels. The polar auxin transport through a cell increases this cell’s transport capacity and also enhances the polarity of this cell. This rearrangement of PINs has been identified during leaf vasculature formation (Scapela et al., 2006).

2.3.3. Auxin routes in tropisms

Tropisms are typical example of auxin mediated post-embryonal development which is regulated by environmental cues. Both shoots and roots direct their growth in response to external signals such as light and gravity (Estelle, 1996; Fellner et al., 2003). These responses enable adaptive adjustments of growth according to the changes in the direction of gravity or light to optimize organs positioning for better light perception of the shoot and root growth orientation for water or nutrient acquisition and provides plants with extraordinary flexibility in terms and survival. Therefore, shoot show negative gravitropism and grow upwards, whereas roots show positive gravitropism manifested by the downward growth.

Accepted models for tropism of plants are based on the classical Cholodny-Went theory that proposes differential auxin distribution underlying the unequal growth at the two sides of a bending organ (Went F., 1974). Lateral asymmetry in auxin distribution is formed during various tropism responses (Rosen 1999; Friml, 2002). Accordingly, the asymmetry in the auxin distribution and auxin response has been detected in gravity-stimulated bending organs in different plant species as a tomato (Harrison and Pickard, 1989) or maize (Young L.M et al, 1990). Multiple experiments independently demonstrated (Winslow R. Briggs 1963*;* Epel BL et al, 1992) the lateral, intercellular auxin transport is essential for this asymmetric auxin distribution during tropisms.

2.3.3.1. Phototropism

Phototropism enables plants to growth towards light. This was the first studied tropic response (Darwin 1880). Charles Darwin and his son Francis discovered (in 1880) that the phototropic stimulus is detected at the tip of the plant. Studies on tropism have been also instrumental to formulate the concept of plant hormones and led to the identification of the first of them, the auxin. Phototropic response involves light perception and the asymmetric distribution of the plant hormone auxin. Light polarizes the PIN3 auxin transporter in shoot endodermis cells and subsequent auxin redistribution mediates asymmetric growth during phototropism (Ding et al., 2010). In response to a directional light cue, differential trafficking at the shaded and illuminated sides redirect PIN3 according to the light direction and diverts auxin flow towards the shaded side where auxin promotes growth causing shoots to bend towards the light.

2.3.3.2. Gravitropism

Gravitropism is one of well visually obvious adaptation responses of plants to the changing environment. It allows plant shoots to grow upon gravity and roots to avoid it. Gravitropism involves gravity perception and mediate accumulation of auxin at one side and form lateral gradient across stimulated organs. It leads to differential cell elongation in that side. Evidences have been showed previously. First, mutation that affects auxin synthesis, transport, or response has been shown to affect gravitropism (Muday and Rahman, 2008). A number of current studies in the laboratory focus on how auxin transport is regulated during gravitropic bending. Second, lateral transport toward the lower side of gravistimulated shoot (Parker and Briggs, 1990) or root (Young et al., 1990) leading to auxin redistribution has been shown to precede the gravity response.

The asymmetric auxin accumulation at the lower side implies a directional auxin flow across the organ aligned with the gravity direction. The elevated auxin on the lower root would then inhibit growth on the lower side and allow bending to occur. In the other hand in hypocotyl, the gradient forms on the lower side upon gravistimulation and initiate the cell division and prolongation resulting shoot bending upwards.

Gravity in plants is perceived by sedimentation of specialized starch-containing organelles statoliths in specialized cells of the root cap and shoot endodermis. The previous work showed that in the gravity-sensing root cap cells, the statoliths sedimentation is followed by the preferential localization of PIN3 to the lower side of cells (Friml et al., 2002a) that might be a mechanism for the subsequent auxin accumulation at the lower side of the roots. Nonetheless, what is the mechanism of the PIN3 relocation and, whether it is required for asymmetric auxin distributions, remain unclear.

Supporting role in gravitropic response of the *Arabidopsis* root has PIN2 protein. PIN2 protein is localized apically in epidermis cells and guides the auxin flow from the root tip to the elongation zone of the root. The *pin2* mutant shows defects in gravitropism of the root (Muller et al., 1998).

3. MATERIALS AND METHODS

3.1. Plant materials and growth conditions

For mapping and sequencing of mutant *Arabidopsis thaliana* found during EMS mutated plant screening, the wild type Columbia (Col-0) was used as a control. For comparison the SALK 076199 line of AGO1 was used. To establish the mapping population *Landsberg* ecotype (Ler) was used.

Seeds of wild type *Arabidopsis thaliana* (ecotype *Columbia*), *PIN1::PIN1:GFP* lines or mutants were used as a control for BFA analysis. Seeds were mutated with EMS-mutagenesis to get the Brefeldin A resistant mutant lines.

Snare mutant SALK lines were ordered (Tab.1). Combination with PIN2:GFP was generated through crossing with the *PIN2::PIN2:GFP* (Wisniewska et al., 2006).

Tab. 1 Mutant lines of SNAREs.

GENE	number	SALK line
SYP31	At5g05760	N654206
SGR3/SYP22	At5g46860	N662665
ZIP1/VTI12	At1g26670	N663764
NPSN12	At1g48240	N664298, N663340
NPSN13	At3g17440	N662392
SFT11	At4g14600	N662461, N654232
OSM1/SYP61	At1g28490	*osm1*
VAMP714	At5g22360	N661737
TYN12	At4g35560	N662326

Published transgenic and mutant lines: *DR5rev::GFP* (Friml et al., 2003); *PIN3::PIN3-GFP* (Friml et al., 2003); *PIN7::PIN7-GFP* (Blilou et al., 2005); $GNOM^{M696L}$ (Geldner et al., 2003); $gnom^{R5}$ (Geldner et al., 2004); *pin3*, *pin7* (Zadnikova et al., 2010); *35S::PID* (Benjamins et al., 2001) were used for gravitropic experiments. All pin mutants were in Columbia (Col-0) background. *Pin3*/*DR5rev::GFP*, *pin7*/*DR5rev::GFP*, *35S::PID*/*PIN3::PIN3-GFP*; $GNOM^{M696L}$ /*PIN3::PIN3-GFP* and $gnom^{R5}$ /*PIN3::PIN3-GFP* were generated by introducing *DR5rev::GFP* or *PIN3::PIN3-GFP* into mutants through crossing. The double mutants *pin4/pin7* and *pin3/pin4* have been described previously (Friml et al., 2003).

Seeds were surface-sterilized sterilized with chloral gas. To the bottle with 100 ml of 13% sodim hypochlorite (NaClO) was add 5 ml concentrated HCl. Seeds were incubated with developed chloral gas under underpressure for at least 3 hours. Or seeds were sterilized with 70% ethanol in the paper seed bag. Paper bag with the seeds was spray with ethanol and let dry.

Seeds were placed on the 1/2 strength MS culture medium (Murashige and Skoog, 1962) without vitamins and (non)containing 1% sucrose (AM+/AM-), pH 5.8 that was solidified by 0.8 % phytagel. Plates with seeds were stored at 4°C for 2-4 days to break dormancy and then vertically mounted under light (7-23 h light) or in darkness at 19 °C. For growing in dark use initialisation of germination and growth, before transfer to dart let 5-6 hours on light.

For gravitropic stimulations, 4-day old seedlings were turned 90°. Seedlings were either imaged for the measurement or imaged with a confocal microscopy. Each experiment was conducted at least in triplicates.

All *Arabidopsis thaliana* plants were than grown in the greenhouse, on soil.

3.2. Phenotype analysis

For phenotype analysis were used 7 or 10 days old seedling growth on AM+/AM- under light or in darkness at 19 °C. Seedlings were either scanned with a scanner for the measurement or imaged with a confocal microscopy.

For gravitropism experiments, plates with 3-day old seedlings were turn 90°.

All treatments were performed at least in duplicate, with a minimum of 100 hypocotyles/roots evaluated in total in each treatment.

Real-time analysis and statistics of hypocotyl angle kinetics during gravity stimulation: Development of seedlings was recorded at 1-hour intervals for 1 day at 19°C with an infrared light source (880 nm LED; Velleman, Belgium) by a spectrum-enhanced camera (EOS035 Canon Rebel Xti, 400DH) with builtin clear wideband-multicoated filter and standard accessories (Canon) and operated by the EOS utility software. Angles of hypocotyls, formed after gravity stimulation, were measured by ImageJ. Fifteen seedlings with synchronized germination start were processed.

3.3. Clearing of cotyledons vasculature

Ten days old seedlings were used. Chlorophyll was removed with 100 % ethanol, over night. Ethanol removed and seedlings washed with water. Slides were prepared with clearing solution. Clearing solution is prepared: 250 g chloral hydrate; 93,75 g H_2O; 31,25 g glycerol.

3.4. Pharmacological treatments

Genetic screen: A mutant screen to isolate plants for resistance to brefeldin A (BFA) effect on PIN protein internalization was performed on the ethylmethane sulphonate-mutagenized PIN1:GFP population. Seedlings were grown on plates with AM+ medium and supplemented with 20µM BFA and subsequently 10 day old plants with visible resistance (bigger cotyledons, longer roots) in comparison to PIN1:GFP control were used for next analysis.

Resistance BFA – to check the resistance to brefeldin A was used different concentration of BFA in liquid medium or in solid medium under light conditions in RT. Control treatments contained an equal amount of solvent (dimethylsulphoxide). Seedlings, 4 days for immunolocalization and 5 days old for life imaging, were treated with 25 µM or 50 µM BFA for different time conditions, and the BFA-induced internalization of PIN1:GFP or PIN1 and PIN2 immunolocalization was analysed with confocal microscope.

PIN polarization and internalization of *snare* mutants were observed following BFA treatment similar to previous, or with 5 µM NAA for 30 min, followed by concomitant treatment with 25 µM or 50 µM BFA for 90 min, and the BFA-induced internalization of PIN2:GFP or PIN1 and PIN2 immunolocalization were analysed with confocal microscope. Following chemicals were used: Brefeldin A (Invitrogen) stock solution 50mM, final concentration 25µM or 50µM. All treatments were performed at least in triplicate, with a minimum of 20 roots evaluated in total in each treatment.

BFA treatment and gravity stimulation - BFA treatment in dark was performed by incubation of 3-day old etiolated seedlings on solid AM medium supplemented with brefeldin A (50 µM). Pre-treated seedlings were subsequently gravity stimulated for bending angle measurement or to check relocation of PIN3 protein. Control treatments contained an equivalent amount of solvent (dimethyl sulphoxide). For all comparisons, at least three independent experiments were performed giving the same significant results.

Visual evaluation of phenotype: For growth and curvature measurements seedlings were observed directly on the Petri dishes - were placed on a standard PC scanner. Analysis and measurements were made with the open source software Image-J (http://rsb.info.nih.gov/ij/). For processing the movie, Windows Movie Maker was used. Pictures were made every hour.

3.5. Immunolocalization of PIN1 and PIN2 proteins

Immunolocalization of PIN proteins in *Arabidopsis* roots were performed as described (Sauer et al., 2006b). The following antibodies and dilutions were used: the anti-PIN1 rabbit and anti-PIN2 rabbit antibody were used at a dilution of 1:1000 and CY3-conjugated anti-rabbit secondary antibodies (Dianova) were used at a dilution of 1:600. The Insitu Pro robot (Intavis) was used.

Fixation was done with 4% Paraformaldehyde in PBS for 1h in vacuum.

PBS 10 x (per 1 l), pH 7.4

KCl..... 2g
Na Cl.....80 g
Na_2HPO_4 $2H_2O$.....17,8 g
KH_2 PO_4.....2.4 g

For digestion/permeabilization were used 1,5% driselase in PBS, incubated at 37°C for 30-40 min and than washed 4 times for 7 min with PBS. 1% NP40, 10% DMSO in PBS, incubated at RT for 40 min -1 h,than washed 6 times for 5 min with PBS. Blocking was done with 2% BSA in PBS for 1-2 h at 37°C.

1st Antibody: in 2% BSA in PBS (blocking solution), incubated at 37°C for 4h and washed 6-8 times for 5 min PBS.

2nd Antibody: in 2% BSA in PBS, incubated at 37°C for 3h, washed 4 timer for 7 min PBS and washed twice for 7 min H2O.

Slides were prepared with embedding solution for Immunofluorescence samples (Michifluor). Solution prepared mixing 90 % Glycerol and 10 % PBS, add DAPCO (from SIGMA): 25 mg/ml, pH 8,5 or 9.

3.6. Staining by the endocytosis tracer FM4-64

The FM4-64 (Molecular Probes) uptake experiments were performed with 5 days old *Arabidopsis* seedling roots of wild type and *snare* mutant lines. Roots were incubated for 5 min with 4μM FM4-64. Worked with FM-dye at 4°C to slow down endocytosis and then washed before observation in liquid AM+ medium using different time of incubation. To monitor the red fluorescent dye, 488 nm excitation and 620 nm or 710 nm emission filters were used.

3.7. Light and confocal microscopy

For microscopy 4-10 day-old seedlings were transferred to microscopic slides with 50 % glycerol and cover with cover-slips. The Leica-DF (DMLB) light microscope was used to detect lateral root primordial, and Leica MZ16 binocular with Nikon camera to image the cotyledons vasculation.

For confocal microscopy images, Zeiss LSM 510 or Zeiss Exciter (Carl Zeiss, Germany) confocal scanning microscopes were used. The red fluorescent dye FM4-64 was excited by the 488 nm laser line and emission was filtered between 620 and 710 nm. GFP fluorescence was imaged using excitation with the 488 nm line of an argon laser and a 505-530 nm emission filter. Samples were examined using 40x or 63x water-immersion objectives. Confocal microscopy evaluation of the PIN1 and PIN2 signal or fluorescent of FM4-64 dye were performed with *Leica LCS* software or *ZEN 2008 Light Edition* software.

The scans were performed with identical microscope and laser settings for all experiments. Analysed cells on scans were selected interactively of the same root region. Images were processed in Adobe Photoshop.

3.8. RNA/DNA isolation

CTAB- DNA extraction: For the DNA isolation we used the method yields relatively clean, high molecular weight DNA. A single leaf or the whole seedlings provided starting material (if necessary, the tissue was stored at 20 °C before processing). For grinding the Qiagen milling machine with adaptors was used. Immediately before homogenizing, the plant

material was frozen quickly in liquid nitrogen. 400 µl of CTAB extraction buffer is added and the sample is incubated at 37 °C for 45 min. After cooling down, 400 µl of chloroform is added and mixing. The two phases are separated by centrifuging 15 min in the centrifuge equipped with adaptors for plates at 4000 rpm. The upper, aqueous phase (300 µl) is transferred to a fresh tube and mixed well with 400 µl 2- propanol. The pellet DNA is obtained by centrifuging 45 min, 4000 rpm at 4 °C. The supernatant is discarded and the white pellet is washed with 200 µl of 70% ethanol, then spinned down again. Ethanol is removed and the pellet is allowed to air-dry, being dissolved at the end in 50-200 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 1-2 µl of DNA used for a PCR reaction.

CTAB buffer for DNA isolation from plant material:
2% (w/v) Cetyltrimethylammoniumbromid (CTAB)
100 mM TrisHCl pH 8,0
20 mM EDTA pH 8,0
1,4 M NaCl
1% (w/v) Polyvinylpyrolidone (PVP 40) Mr 40000.

Total RNA from *Arabidopsis thaliana* plants was isolated with RNeasy Plant Mini kit (Qiagen) using the protocol: Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi. RNA was isolated from the whole 10 days old seedlings (for AGO1 mutant 14 days old) frozen with the liquid nitrogen and mechanically homogenized.

Measurement of RNA/DNA concentration purity was done with NanoDrop.

3.9. Genotyping

We used selection markers such as phenotype characteristics or PCR primers detecting the mutations to characterise the mutants. Amplification of DNA fragments was performed via polymerase chain reaction (PCR). The DNA markers were amplified by PCR using following conditions:

A standard PCR mixture (30 µl volume) comprises:
10X PCR reaction buffer.....3 µl
MgCl2 (50 mM stock solution).....1,4 µl
dNTP mixture (stock of 10 mM).....0,4 µl
Primers mixture (forward and reverse, 10 µM each).....2 µl
DNA template.....1 µl
Taq polymerase (5U/ µl stock).....0,2 µl
Sterile H2O.....up to 30 µl

A standard programme for a PCR reaction:
1) Initial denaturation…….94 °C for 3 min
35 cycles from step 2 to 4:
2) Denaturation……...94 °C for 30 seconds
3) Annealing……...55 °C for 1min.
4) Primer extension……72 °C for 90 seconds
5) Final extension……...72 °C for 10 min.

Primers used for amplification DNA fragments and thus selection of mutant lines of *snare* mutants we designed forward primers of the SNARE gene and reverse primer complement to T-DNA.

Resulted PCR products, were run on a agarose gels (1-2%), in TAE buffer.

10x Loading dye for gel electrophoresis
1x TAE
50% Glycerol -> dilute with the TAE
0,25% Orange G

50x TAE
volume: 5 liters

EDTA	73 g
Acetic acid:	285.5 ml
Tris base:	1210 g

3.10. First strand cDNA synthesis

For cDNA synthesis of *ago1* mutant and wild type, SuperScript III First-Strand Synthesis System (Invitrogen) kit for RT-PCR was used. We changed subsequently the description:

To 2 µg of RNA in 15 µl of water we add 1 µl of oligo dT and 1 µl of dNTP (10mM) and incubated first 5 min in 65 °C and than 5 min on ice. After we add 33 µl of "Master mix" and incubated 2 hours in 50 ° and 15 min in 70 °.

A "Master mix" mixture (33 µl volume) comprises:
10 RT buffer……...5 µl
MgCl2 (25 mM stock solution)…… .10 µl
DTT (stock of 0,1 M)…….5 µl
Rnase OUT…..2,5 µl
Suprerserift (SS III)……...1 µl
Sterile H2O…….....up to 33 µl

We add 1 µl of RNAseH, incubated 20 min in 37 °C and stored cDNA at 20 °C.

For cDNA synthesis of *snare* mutants and wild type lines, iScript™ cDNA Synthesis Kit (BIO-RAD) was used. The synthesis was performed according to description.

The resulted cDNA was used as a template for PCR amplification.

3.11. Reverse transcription-PCR

Total RNA was isolated from the whole seedlings and corresponding cDNAs were synthesized and amplified by the PCR using primers for the indicated genes as follows:

AGO1 primers:

AT1G48410F10	TCGGCATAGTCTCTCAATGTT
AT1G48410F11	TGATTGGGTTTTAGCTTTAGC
AT1G48410R11	AGATTTTAGAGTCCACAACAG

Tub primers:

FP	ACTCGTTGGGAGGAGGAACT
RP	ACACCAGACATAGTAGCAGAAATCAAG

The amplified products (~600 bp) were analyzed by 2% agarose gel electrophoresis.

The experiments were repeated two times and identical results were obtained. The expression level of tubulin (Tub) gene was used as internal control to confirm that equal amount of RNA was used for RT-PCR.

3.12. Genetic mapping

Mapping population performed from F2 population of mutant crossed with *Landsberg* ecotype (Ler). For rough mapping bulk DNA was used prepared from DNA samples of 50 mutant seedlings and used 3 markers per each chromosome (Appendix 2).

The DNA of individual plants were isolated and used as template for PCR screening with flanking genetic markers.

PCR was set-up in a 30 µl final volume:
10X PCR reaction buffer…….....3 µl
$MgCl_2$ (50 mM/Invitrogen)…….....1,4 µl
dNTP mixture (stock of 10 mMeach)…….…....0,4 µl
Primers mixture (forward and reverse, 10 µM each).....2 µl
Taq polymerase (Invitrogen)…….....0,2 µl
DNA template……...1 µl
Sterile H2O……...up to 30 µl

Programme used for amplification:
1) Initial denaturation……94 °C for 3 min.
39 cycles from step 2 to 4:
2) Denaturation……..94 °C for 20 sec
3) Annealing……...53 °C for 20 sec
4) Primer extension……72 °C for 1min
5) Final extension……...72 °C for 7 min.

Resulted PCR products, short in length were run on a high resolution agarose gel (4%), in TAE buffer.

3.13. Sequencing

All DNA sequencing of PCR products, were performed by Lab Services in VIB (Antwerpen, Belgium).

DNA sequencing analysis was performed by the dideoxy chain termination method of Sanger et al. (1977). Prior to sequencing, the PCR products were treated using the ExoSAP-IT kit from USB (Product No. 78202), after which the treated PCR products were sequenced by using fluorescent dye terminators from the cycle sequencing kit from Applied Biosystems (part No. 4337456) on an ABI3730*xl* DNA Analyzer. For sequencing were used 10 µl of 5 µM primers (Appendix 1).

The raw output DNA sequence data from these PCR products were analyzed using the Phred/Phrap software.

4. RESULTS

4.1. Identification of genes involved auxin-mediated development

4.2. Identification of players in endocytic pathway – forward genetic approach

4.3. Identification of players in endocytic pathway – reverse genetic approach

4.4. Polarization of PIN-dependent auxin transport for shoot gravitropism

5. DISCUSSION

5.1. Identification of genes involved in auxin-mediated development

5.2. Identification of players in the endocytic pathway

5.3. Polarization of PIN-dependent auxin transport for shoot gravitropism

6. CONCLUSION

Research efforts in the last decade contributed significantly to elucidation of the mechanisms underlying regulation of the auxin flow in plants. Important role of PIN proteins was described, but still several points of the PIN targeting and polarization remains unclear.

During my work we characterize possible candidate genes that participate in PIN polar targeting and constitutive cycling in *Arabidopsis thaliana* plants. We identified mutant lines resistant to the trafficking inhibitor BFA. Three mutants from the screen were further analyzed and the mapping population was established for consequent mapping.

In a different screen, a mutant that forms apical hook in light and shows lethal phenotype was found, characterized, mapped and sequenced. Possible role of the identified AGO1 protein in PIN targeting or function is still being investigated.

Another possible way to get the candidate players of endocytic pathway of PIN cycling is the reverse genetic approach. Using this method, we focused on isolating and characterising mutant lines in the SNAREs, proteins mediating vesicle fusion. We focused on SNARES that are expressed in roots and identified a possible effect on PIN2 polarization in *osm1* and *tyn12* and *npsn12* SNARE mutants.

Furthermore, we studied effects of external cues on PIN polarity; specifically the mechanism of gravitropic response of the hypocotyl of *Arabidopsis* seedlings. The PIN3 auxin transporter is required for asymmetric auxin distribution and gravitropism in *Arabidopsis thaliana* hypocotyls. We found that gravity stimulation polarizes PIN3 to the lower sides of hypocotyl endodermis cells which correlates with increased auxin accumulation and response at the lower hypocotyl side. The PIN3 polarization and hypocotyls bending require activities of PIN polar trafficking regulators GNOM ARF GEF and PINOID kinase. Our data suggest that gravity-induced PIN3 polarization mediates asymmetric auxin distribution leading to hypocotyl bending.

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LIST OF ABBREVIATION

ABC	mammalian ATP-binding cassette transporters
ABP1	auxin binding protein
AGO1	argonaut1
AUX1	auxin resistant1 permease
ARF	auxin response factor
BFA	brefeldin A
BSA	Bovine serum albumin
Col-0	*Arabidopsis* ecotype *Columbia* 0
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DR5	promoter consisting of 7 tandem repeats of an auxin-responsive TGTCTC element and a minimal 35S CaMV promoter
EDTA	ethylenediamine tetraacetic acid
Fig	figure
FM4-64	N-3-triethylammoniumpropyl-4-(6-(4-diethylaminophenyl)-hexa-trienyl)pyridinium dibromide
GEF	guanine-nucleotide exchange factor
GFP	green fluorescent protein
GNOM	the GDP/ GTP exchange factor for the small G proteins of the ARF class
GUS	s-Glucoronidase
h/hrs	hour/hours
HCl	hydrogen chloride
IAA	indole-3-acetic acid
Ler	*Arabidopsis* ecotype *Landsberg*
MDR/PGP	the multi-drug-resistant/P-glycoprotein
NAA	1-naphthalene acetic acid
NaClO	sodim hypochlorite
PGP	phosphoglycoproteins
PID	pinoid
PIN	pin-formed
RNA	ribonucleic acid
RT-PCR	reverse transcriptase- PCR
SNARE	soluble N-ethylmaleimide-sensitive factor adaptor proteins (SNAP) receptors
SSLPs	simple sequence length polymorphisms molecular markers
TGN	trans-Golgi network
TIR1	transport inhibitor response1
µl	microliter
µm	micrometer
µM	micromol
µg	microgram
WT	wild type

APPENDIX

Appendix 1: Primers used for sequencing of AGO1 gene

AT1G48410F1	GTTATCTCGTTTGTTCGGAGT
AT1G48410R1	CAAACATTGAAAACAAGCACA
AT1G48410F2	TGGGCTGTTGAATTTGGTTTT
AT1G48410R2	GCTGAGAAGACACCGCTTGAT
AT1G48410F3	GTACCAAGGAAGAGGAAGAGG
AT1G48410R3	ACCTCCAGCCCCTACTTCTTC
AT1G48410F4	CGTCTTCCAGCGTATGATGGT
AT1G48410R4	TGTAATACAGCAAGCCATCAC
AT1G48410F5	CTTGGAGAGCTGGCGTGGATT
AT1G48410R5	AAGCATGGTAGTTGAGTGTGC
AT1G48410F6	AGATTTACTACTCTTATTTCG
AT1G48410R6	CCTTTCAGCCACAAGTTTATA
AT1G48410F7	TGGTGATAAAGCCTCATTGTG
AT1G48410R7	CCTTCCAGACTCGTGGTACTT
AT1G48410F8	GTTCCCATTGTCTCTTTGCTA
AT1G48410R8	AAGACTCACCATGCCAGATAC
AT1G48410F9	GGTGGAACGGTGAATAATTGG
AT1G48410R9	AGATAGAGCATCAACAAGCAC
AT1G48410F10	TCGGCATAGTCTCTCAATGTT
AT1G48410R10	GGTTTATGCCCAGTTGATCTA
AT1G48410F11	TGATTGGGTTTTAGCTTTAGC
AT1G48410R11	AGATTTTAGAGTCCACAACAG
AT1G48410F12	CGGTGGACAGAAGTGGGAATA
AT1G48410R12	TGCAGGGGGAACTACAATATC
AT1G48410F13	CAAGATGCACACGCTCAGTTT
AT1G48410R13	AGTTAAAGAAAGGATCAAAGT

Appendix 2: Primers used for mapping of AGO1 gene

no.	Name	Marker	Bac	Forward Primer	Reverse Primer	Position	Col	Ler
1	RM1	T20H2	T20H2	GTCCTTACTTGCTTACCAGAA	TTCAATGTTGAAGAGTGGCGG	6.945 mb	up	Down
2	RM2	F5J5	F5J5	GGAAGCAAAAATGTTAAAGA	AATCCTTAAACATTCATAAC	13.44 mb	up	Down
3	RM3	F22G10	F22G10	GACCAAGTTATCATTTATGAA	GATTCTTGGCATTACTGGCAC	20.075 mb	up	Down
4	RM4	LL45	F3L12	TCCAATCAAACATAAATTAGTCACTC	GTAAGTTTAAGGTTTTCACACACG	1.362 mb	up	down
5	RM5	LL46.50	T26C19	ATCATTTTGAAGAGGCATGTTAGG	TTTACATGGTAACGTAAGGTTCAG	9.490 mb	up	down
6	RM6	T7F6	T7F6	AAAGTTGAGGCTATGTGATCT	TTTAGCCAGGAACACAAGCGT	16.25 mb	up	down
7	RM7	T19F11	T19F11	TGGAAATGCAGATAAACGAGA	ATTTGTTGGTGTCTGGCTTAA	3.678 mb	up	down
8	RM8	MUO22	MUO22	CCCTGCTCTTCTTGTTGTCA	TGCAGCAGGATAGGTTGGT	11.151 mb	up	down
9	RM9	T8P19	T8P19	GCATGAGAACACGCAAAAGGA	TCCAGCGATAAATGTGCTGTA	17.981 mb	up	down
10	RM10	LL51	F4C21	CAAAACTCAAAACTCTTATCTATGTACA	TGCAATTGAAATTTGAGCGAGGA	1.394 mb	down	up
11	RM11	FCA1	FCAAL	GAATGAGAACCAAGTGAGTGC	ACCTATCAAACTCTTCACTGC	8.358 mb	up	down
12	RM12	F6I18	F6I18	TAAATCTACACAACACAGGAA	CCATACAAATTCACTCTACAA	15.079 mb	up	down
13	RM13	MHF15	MHF15	CCCATCAAAGGAGGCCATGTG	GGTCATGAAACCTAGAACTAGGGG	1.968 mb	up	down
14	RM14	F2P16	F2P16	CTTCACAACTTTATCATCTTC	TATAGTCCTGTCTTGTGTCAG	9.445 mb	up	down
15	RM15	MGI19	MGI19	GCTTGACATGAAGTGCTAAAC	TCTGTGTGATTCTCTCCAAGG	25.577 mb	up	down