IDENTIFICATION AND FUNCTIONAL ANALYSIS OF PROTEINS INVOLVED IN AUXIN REGULATED PLANT GROWTH

By

Jie Li

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Biochemistry and Molecular Biology - Doctor of Philosophy

2016

ABSTRACT

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF PROTEINS INVOLVED IN AUXIN REGULATED PLANT GROWTH

By

Jie Li

During growth, plants integrate cell division with cell elongation and cell differentiation. We used proteomics to identify proteins from auxin (indole-3-acetic acid; IAA) -induced rapidly growing corn (*Zea mays*) coleoptiles to find candidates controlling this growth as well as the underlying cell wall and cuticle biosynthesis. Of 86 proteins identified, 15 showed a predicted association with cell wall/cuticle biosynthesis or trafficking machinery; the analysis also revealed four proteins of unknown function. Parallel real-time PCR indicated that the steady-state mRNA levels of genes with a known or predicted role in cell-wall biosynthesis increase upon auxin-treatment. Importantly, genes encoding two of the hypothetical proteins (ARRP1 and Hypothetical protein 3, a putative endoglucanase) also showed higher levels of mRNA; their gene expression was directly correlated with coleoptile and leaf growth. This suggested a role of these two novel proteins in the regulation of processes related to cell and organ expansion and thus plant growth. These data are described in chapter 2.

Chapter 3 focuses on ARRP1 (Auxin rapid response protein 1), one of the proteins of unknown function associated with cell and organ growth. Upon auxin treatment *ARRP1* mRNA and protein increased within 30 minutes – prior to measurable growth and increased expression of genes encoding cell wall biosynthetic enzymes, suggesting an intracellular signaling function. This was further supported by the localization of ARRP1 at the cell periphery as well as in the nucleus. *Arabidopsis*

mutants lacking the *ARRP1* homologue grew normally under standard conditions, but showed delayed growth and delayed flowering when germinated in the dark and transferred to light. This phenotype that could be reversed by expressing *ZmARRP1* in the *Arabidopsis* mutant. *ZmARRP1* expression was also induced by red and blue light, indicating a possible role in phytochrome and cryptochrome signaling at the interface with the auxin response. *In situ* hybridization demonstrated that *ARRP1* mRNA is enriched in the vasculature of growing corn tissues. In addition, the corn ARRP1 interacted with the cytoplasmic domain of both *Arabidopsis* and *Zea mays* VH1/BRL2 which plays a role in the signaling of vascular development. Based on the fact that ARRP1 protein is involved in light-induced growth responses as well as with components of auxin signaling, we propose that it may integrate several environmental and hormonal signals affecting plant development.

Chapter 4 describes the initiation of a series of experiments investigating addtional proteins that interact with ARRP1 to elucidate other components of the signaling pathway that use ARRP1. Using *in vitro* pull-down assays and mass spectrometry, I identified 32 putative ARRP1-interacting proteins in the developing corn leaf. Some of these proteins appear to be involved in cell wall biosynthesis, including a beta-glucosidase homologue. Others are predicted to be signal molecules, including a tetratricopeptide repeat domain containing protein and a CobW/P47K family protein. Using semiquantitative RT-PCR, I showed that several of these genes have a similar expression pattern compared to *ARRP1*, suggesting a function in the same signaling/response path.

Acknowledgement

Since I first came to the US in the summer of 2009, I have had the most productive and pleasant six years so far in my life and have met some of the most important people to me here in MSU.

I was very lucky to be a graduate student with my advisor Prof. Susanne Hoffmann-Benning. She is a role model as an enthusiastic scientist. She trusted me and gave me plenty of freedom to explore the project according to my own pace and interest. She supported me during numerous hard times when I encountered failure and guide me through these darkness. She taught me to work independently and to think critically. I have learned so much from her in the past six years, and works cannot express my appreciation towards her as a guide and a mentor.

I want to thank my committee members: Drs. Thomas Sharkey, Robert Last, Michael Garavito, and Jonathon Walton. They provided a lot of constructive suggestions to my research that better refined my projects and my writing. They also encouraged me and offer useful resources.

I also want to thank my lab mates, especially Banita Tamot, who taught me many techniques and offer numerous resources. Lastly, I want to thank my wife, my parents and my friends for years of support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 INTRODUCTION: AUXIN INDUCED PLANT GROWTH AND THE INTEGRATION OF AUXIN WITH LIGHT AND OTHER HORMONES

With the increase in the world population comes the need for improved efficiency in the use of arable land. When faced with similar problems in the mid- and late- 20th century the first green revolution led by Norman Borlaug dramatically changed the field of agriculture (McKersie, 2015). During that time period, new chemical fertilizers and synthetic herbicides and pesticides were created and utilized. In addition, the generation of high-yielding crop varieties and the improvement of agricultural management techniques increased the agricultural yield and the nutritional intake of billions and has reduced child mortality and malnourishment of infants around the world (Khush, 1999). It also supported overall economic growth in many Asia countries over the years. However, due to the current climate change, population growth, and changing food preferences, global crop production now encounters a new crisis. To solve these problems and provide food security for the foreseeable future, we need crops that have greater yield or are more tolerant to biotic and abiotic stresses.

1.1 Phytohormones as essential regulators of plant growth

To adapt to the continuously changing environment, plants evolved a complex phytohormone network. Phytohormones are small signaling molecules that are essential in the regulation of plant growth, development, reproduction, and survival. Their concentrations are very low, but control a broad variety of biological mechanisms. They not only orchestrate intrinsic developmental programs but also convey environmental inputs and drive adaptive responses to a wide variety of biotic and abiotic stresses (Kende and Zeevaart, 1997; Santner *et al*., 2009; Santner and Estelle, 2009; Wolters and Jurgens, 2009; Depuydt and Hardtke, 2011).

Auxin, gibberellin (GA), cytokinin, abscisic acid (ABA) and ethylene are generally referred to as the five 'classic' plant hormones (Kende and Zeevaart, 1997). More recently, several additional compounds have been recognized as hormones, including brassinosteroids, jasmonate, salicylic acid, nitric oxide and strigolactones (Santner and Estelle, 2009) (Fig. 1.1a). Moreover, we now know that plants also use peptide hormones, such as Flowering locus T, CLAVATA3, or Phytosulfokine to regulate various growth responses (Corbesier *et al*., 2007; Lin *et al*., 2007; Tamaki *et al*., 2007; Jun *et al*., 2008; Sauter, 2015).

During growth, the plant integrates cell division with cell elongation and cell differentiation. Based on the phenotype of mutants with disrupted hormone biosynthesis or perception, auxin, gibberellins, cytokinin and brassinosteroids are considered essential for growth (Depuydt and Hardtke, 2011). Cytokinins regulate cell proliferation while gibberellins promote cell elongation; auxin is involved in both processes (Werner *et al*., 2001; Sauer *et al*., 2013; Claeys *et al*., 2014). Brassinostoids promote cell expansion, yet their role in cell division remains unclear (Nakaya *et al*., 2002; Hardtke *et al*., 2007).

1.1.1 The structure and function of auxin

Auxins were the first of the major phytohormones to be discovered. The term 'auxin' originates from the Greek word 'auxein', which means to enlarge/grow. In 1880, the first paper about the effect of auxin, 'The Power of Movement in Plants', was published by Charles and Francis Darwin (1880). They noted a messenger that is transmitted in the downstream direction from the tip of the coleoptile and causes its bending towards the light. In 1934, this messenger, auxin, was first isolated from *Avena*

sativa and structurally identified as indole-3-acetic acid (IAA) (Kögl F, 1934). IAA is the most abundant endogenous auxin and is able to fulfill most of the auxin functions. Now auxin is the generic name for a group of important molecules in plants. In addition to IAA, there are three more natural compounds with auxin activity, indole-3-butyric acid (IBA) (Ludwig-Muller and Epstein, 1991), 4-chloroindole-3-acetic acid (4-Cl-IAA) (Engvild, 1985), and phenylacetic acid (PAA) (Okamoto I, 1967; Fig. 1.1a). At high concentrations, auxins are toxic affect mainly dicots over monocot species, such as grasses and cereal crops. Because of these properties many synthetic compounds with auxin-like activity were developed and used as herbicides; one example, 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the world's most widely used herbicide (Grossmann, 2010) (Fig. 1.1b).

Auxin was classically defined as the stimulator to promote elongation in coleoptile and stem sections, but also rooting (Went, 1934). Since then, auxin was shown to be essential for multiple aspects of plant development. It controls cell division by directly or indirectly influencing both transcriptional and posttranscriptional regulation of cell cycle machinery components such as *CDKA , CYCD3, KRP1* and *KRP2* (Hemerly *et al*., 1993; Himanen *et al*., 2002; Menges *et al*., 2006; Perrot-Rechenmann, 2010). It also regulates cell elongation by altering cell wall plasticity. It controls cell differentiation as it affects root and shoot architecture (Gallavotti, 2013), leaf abscission (Wetmore and Jacobs, 1953), leaf venation, and fruit formation (de Jong *et al*., 2009). It mediates plant responses to pathogens but also to light, gravity and other abiotic factors (Woodward and Bartel, 2005; Kazan and Manners, 2009; Vanneste and Friml, 2009; Wang *et al*., 2010). Auxin function requires several components: gradients of auxin

concentration between tissues (I) and different cells (II) both of which are based on auxin biosynthesis and polar transport; and (III) auxin perception and signaling within the cell (Grones and Friml, 2015).

1.1.2 Auxin biosynthesis

Auxin biosynthesis can occur via either the tryptophan (Trp)-independent or Trp-dependent pathways (Fig. 1.2) (Korasick *et al*., 2013). The Trp-dependent pathways are better characterized and more important for plant development (Sugawara *et al*., 2009; Korasick *et al*., 2013). Based on major intermediates, the Trp-dependent pathway can be separated into four pathways, which synthesize auxin via the intermediates indole-3-acetaldoxime (IAOx), indole-3-acetamide (IAM), tryptamine (TAM), or indol-3-ylpyruvic acid (IPA), respectively. The IPA pathway appears to be the main route for the IAA biosynthesis in both maize and *Arabidopsis*. It consists of two steps: a Trp aminotransferase (TRYPTOPHAN AMINOTRANSFERASE OF *ARABIDOPSIS*: TAA) converts Trp to IPA, followed by the conversion of IPA to IAA by a flavin monooxygenase of the YUCCA (YUC) family (Korasick *et al*., 2013). Overexpression of YUC family genes in *Arabidopsis* leads to auxin overproduction phenotypes, but plants overexpressing TAA genes are indistinguishable from wild type, suggesting that YUC is probably the rate-limiting step of IPA pathway.

The Trp-deficient mutant of *Arabidopsis*, exhibits no difference in free IAA levels, but accumulates amide- and ester- linked IAA conjugate, when compared to wild type, indicating that the Trp-independent pathway also contributes to IAA biosynthesis (Wright *et al*., 1991; Normanly, 2010). In addition, the use of labeled Trp-precursors in feeding assays showed that the percent isotopic incorporation into IAA is greater than

that of tryptophan, and supports the concept of Trp-independent auxin biosynthesis (Normanly *et al*., 1993). However no important intermediates or enzymes associated for this pathway have been identified so far. This route is postulated to originate from either indole or indole-3-glycerol phosphate (Ouyang *et al*., 2000).

1.1.3 Auxin transport

Auxin is synthesized mainly in the shoot apex, the tip of the coleoptile, and young leaves and then translocated to other plant tissues or cells. This movement occurs via polar auxin transport, employing specific carriers for movement across the membrane as well as through bulk flow in the phloem (Goldsmith *et al*., 1974; Hoad, 1995). The polar transport and establishment of localized auxin maxima are crucial for plant development. This process regulates embryonic development, stem cell maintenance, root and shoot architecture, and tropic growth responses (Peer *et al*., 2011). IAA moves among plant tissues through a combination of membrane diffusion and carrier-mediated transport (Friml, 2003). Three classes of carrier proteins, which facilitate auxin uptake and efflux, have been identified in plants: AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) uptake permeases, ATP Binding Cassette subfamily B (ABCB/PGP) transporters, and PIN-FORMED (PIN) carrier proteins (Fig. 1.3) (Benjamins and Scheres, 2008; Petrasek and Friml, 2009; Zazimalova *et al*., 2010). IAA can enter cells via lipophilic diffusion in its protonated form (IAAH) or through proton symporters of the AUX1/LAX family in its anionic form (IAA-) (Ugartechea-Chirino *et al*., 2010; Della Rovere *et al*., 2015). ABCB4 appears to contribute to auxin uptake in root epidermal cells when auxin levels are low (Santelia *et al*., 2005; Terasaka *et al*., 2005). Once inside the cell, IAA exists predominantly in its anionic form, necessitating carrier mediated transport to exit cell.

PIN proteins and a subset of ABCB/PGP transporters mediate this tissue-specific cellular auxin export (Zazimalova *et al*., 2010). PIN proteins are integral membrane proteins essential for polarized auxin movement. Each member of the PIN family displays a unique tissue-specific expression pattern, and *pin* mutants generally exhibit growth phenotypes that are consistent with the loss of directional auxin transport in the corresponding tissue (Chen *et al*., 1998; Galweiler *et al*., 1998; Muller *et al*., 1998; Friml *et al*., 2002; Friml *et al*., 2002). Among them, PIN3 has a lateral localization in the cells of the shoot endodermis, in the root columella, and in pericycle cells and appears to function in the lateral redistribution of auxin that is involved in photo- and gravitropic growth (Friml *et al*., 2002). Dramatic progress in understanding the mechanism and regulation of auxin transport has made it clear that this process is essential in almost all plant growth stages and environmental responses (Peer *et al*., 2011).

1.1.4 Auxin perception

To trigger a biological process, auxin must be perceived by the plant. To date at least two auxin receptors are known. The most well-studied auxin receptor is the F-box protein TRANSPORT INHIBITOR RESISTANT1 (TIR1), which was identified as a subunit of the ubiquitin E3 ligase (Ruegger *et al*., 1998; Dharmasiri *et al*., 2005; Kepinski and Leyser, 2005) (Fig. 1.3). Crystallographic studies showed that IAA fits into the core of a ring-like structure formed by TIR1 (Tan *et al*., 2007). Binding of IAA promotes the interaction between TIR1 and proteins of the AUXIN/INDOLE ACETIC ACID (Aux/IAA) family, a group of proteins that regulates auxin-induced gene expression and development (Reed, 2001). The interaction with TIR1 triggers the ubiquitination of Aux/IAA proteins, which designates them for degradation by the 26S

proteasome (Kepinski and Leyser, 2004, 2005): At low, basal auxin concentration, Aux/IAA protein form an inhibitory complex with AUXIN RESPONSE FACTOR (ARF) family transcription factors that bind to the AUXIN RESPONSIVE ELEMENT (ARE) in the promotors of auxin response genes and repress their transcription. An increase in auxin levels leads to TIR1-Aux-IAA interaction and the proteasome-mediated degradation of Aux/IAAs, which in turn allows for a gradually increasing number of functionally active ARF proteins and the transcriptional activation of auxin regulons (Quint and Gray, 2006; Weijers and Friml, 2009; Sauer *et al*., 2013; Salehin *et al*., 2015).

Although conceptually straightforward, this system of auxin perception is functionally quite complex. *Arabidopsis thaliana* has five TIR1 members, termed AFB1-AFB5, which all bind auxin (Calderon Villalobos *et al*., 2012). There are also 29 members of Aux/IAA family that share a common structure of four domains. Since Aux/IAAs directly participate in the receptor–ligand interaction, they can be seen as auxin co-receptors (Tan *et al*., 2007). Several experiments suggest that plants form different TIR/AFB–Aux/IAA co-receptor pairs with distinct auxin affinities. In addition, plants also have 23 ARF genes, which generate additional receptor-transcription factor combinations (Wilmoth *et al*., 2005; Korasick *et al*., 2014). This diversity contributes to the great variety of auxin functions (Vernoux *et al*., 2011; Enders and Strader, 2015).

In addition to the chain of events initiated by the binding of auxin to TIR1 in the nucleus, two more auxin perception modes have been proposed. AUXIN BINDING PROTEIN1 (ABP1) is the longest known putative auxin receptor. It was first isolated form maize, but remains an enigma (Jones and Venis, 1989). ABP1 is mainly localized

in the endoplasmic reticulum (ER). However, it is secreted to some extent into the extracellular space and appears to be active as an auxin receptor there as well (Napier *et al*., 2002). Experimental evidence suggests that ABP1 mediates the rapid auxin response close to the plasma membrane, such as the rapid cell elongation response (Sauer and Kleine-Vehn, 2011). Several papers also indicated that ABP1 is also involved in the coleoptile elongation as a component of the auxin-light-signaling network (David *et al*., 2007; Ljung, 2013). However, a recent study suggests that ABP1 is not required for auxin signaling and plant development (Gao *et al*., 2015).

An additional recently identified candidate for an auxin receptor is the S-Phase Kinase-Associated Protein 2A (SKP2A), which is the regulator of the cell cycle and is involved with the degradation of at least two cell cycle factors, DPB and E2FC (Jurado *et al*., 2010). Further studies are needed to reveal more details of the auxin-SKP2A pathway.

1.2 The corn coleoptile as a model to examine auxin-induced growth

Maize (*Zea mays L*.) plays an important agronomic role as feed, food, and source of bioethanol. Auxin-induced cell elongation of Maize coleoptiles is one of the fastest and longest-known phytohormone responses known. Frits Went employed the famous experiments using excised oat coleoptiles and auxin-containing agar blocks as a tool to study the “auxin-hypothesis” of coleoptile elongation (Went, 1927). Since then corn coleoptiles have been widely used as a model system to study the regulation of cell growth and tropisms (Haga and Iino, 1998).

1.2.1 Auxin induced acid growth

Although auxin-induced coleoptile growth has been studied for almost one century, the biochemical basis behind this response is still not fully understood. Several studies suggested that one of the first steps is an auxin-induced pH change in the apoplastic space via rapid activation of proton secreting plasma membrane H^+-ATPase (Rayle and Cleland, 1970; Hager *et al*., 1971; Cosgrove, 1997; Hager, 2003; Cosgrove, 2005; Takahashi *et al*., 2012). This “Acid-growth theory” was first proposed by Hager (1971). In his theory, he suggests that the auxin-induced acidification of the apoplast is the prerequisite for cell elongation. The lower pH will activate so-called “wall-loosening proteins”, thus mediating a shifting of cellulose microfibrils and subsequent expansion growth. However, several publications showed that the pH established by IAA is only 4.8-5.0 which causes almost no enhancement of growth compared with the water control. Growth that is mediated by the fungal phytotoxin fusicoccin (FC) can be inhibited by neutral buffers infiltrated into the outer epidermal wall, but a substantial growth response occurs after addition of IAA under identical conditions, suggesting IAA and H^+ act via separate mechanisms (Kutschera, 1994, 2001, 2006; Niklas and Kutschera, 2012; Visnovitz *et al*., 2012; Burdach *et al*., 2014; Rudnicka *et al*., 2014). The details of the “Acid-growth theory” still need further investigation, but it is clear that auxin-induced proton secretion is insufficient to cause the rapid growth phenotype.

Wall-loosening proteins include expansins, xyloglucan endotransglucosylases/ hydrolases (XTH). Expansins are able to loosen bonds between cellulose and hemicellulose fibrils in the cell wall (Cosgrove *et al*., 2002; Cosgrove, 2015). By regulating xyloglucan turnover, XTHs also cause an enhancement of the cell wall

plasticity (Brummell and Hall, 1987; Cosgrove, 1997; Takahashi *et al*., 2012). Together, these changes lead to the initiation of turgor-driven cell elongation.

1.2.2 Auxin activation of ion channels

To study auxin induced rapid growth, coleoptiles were pulled off the primary leaf and, after removal of the tips, shaken in distilled water to remove residual auxin. Upon application of auxin the coleoptile growth rate is constant during the first four hours. But two hours later, the elongation rate slows down and completely ceases after one day. In 1981, Stevenson and Cleland showed that this cessation of coleoptile elongation in water can be prevented by the addition of absorbable solutes, such as sucrose or potassium chloride (KCl), suggesting that the uptake of osmolytes which maintain the turgor pressure is also important for auxin-induced growth (Stevenson and Cleland, 1981; Cosgrove and Cleland, 1983). Moreover, by importing K^+ ions, the most abundant cation in plants, cells can easily balance the charge of the secreted protons. This importance of K^+ for auxin-induced H^+ pumping had already been suggested in the 1970s (Haschke and Luttge, 1975; Nelles, 1977). In maize coleoptiles, auxin-induced cell elongation strongly depends on the availability of K^+ in the bathing medium and their uptake through potassium-selective ion channels: auxin-induced plant growth could be inhibited by addition of K^+ channel blockers or removal of K^+ ions from the medium (Claussen *et al*., 1997). Thus, studies of K^+ channels in maize coleoptiles were initiated (Philippar *et al*., 1999). Maize has at least two K^+ channels, ZMK1 and ZMK2 (Bauer *et al*., 2000; Deeken *et al*., 2000; Deeken *et al*., 2002). ZMK1 is mainly localized in cortex and epidermal cells. Its transcription is induced by auxin treatment. In addition, electrophysiological studies suggest that the auxin-induced acidification activates K^+

transport via ZMK1 (Philippar *et al*., 1999; Philippar *et al*., 2006). Using patch clamp studies, K^+ channel activation can be seen 10 min after auxin stimulation, which strongly suggests the involvement of K^+ uptake via ZMK1 in cell elongation (Philippar *et al*., 1999; Thiel, 1999; Philippar *et al*., 2006). One possible explanation to the 10 min delay is that the initial rise in growth rate is due to acidification of the apoplast and subsequent cell wall loosening. K^+ uptake via ZMK1 might thus be required for sustained growth, characterized by the need to import osmotically active substances.

1.2.3 Protein synthesis is important for maintaining cell elongation

In 1963, it was demonstrated that several antibiotics, including chloramphenicol, puromycin, and p-fluorophenylalanine, which suppress protein biosynthesis can also inhibit the elongation of coleoptiles and mesocotyls (Noodén and Thimann, 1963). In addition, using dactinomycin, a specific inhibitor of RNA synthesis, Key and Ingle showed that IAA-induced growth is significantly diminished when transcription is inhibited (Key and Ingle, 1964). As a result, the concept of growth-limiting proteins (GLPs) emerged and was corroborated by several subsequent experiments. Parallel to the suppression of protein biosynthesis, cell wall loosening and cell elongation are both inhibited by cycloheximide (CHX), a protein synthesis inhibitor (Cleland, 1971; Cleland and Haughton, 1971). Time course studies further suggested that the biosynthesis of several GLPs plays a key role in auxin-mediated elongation.

Using electron microscopy, osmiophilic particles (OPs) were found between the plasma membrane and the outer epidermal wall of corn coleoptiles (OEW; Kutschera *et al*., 1987). These particles were only seen in the auxin-induced growth tissues, not in FC or acid mediated ones. Closer investigation revealed that these particles are

secreted only to the outer wall of expanding epidermal cells, and were seen in a variety growing tissues/plants regardless of the growth-inducing signal (Kutschera and Kende, 1989; Hoffmann-Benning *et al*., 1994). The inhibition of protein synthesis by CHX diminished not only the appearance of OPs but also plant elongation. Monensin, is an ionophore that inhibits the secretory pathway. It causes the same growth phenotype as CHX treatment and prevents the formation of OPs (Hoffmann-Benning *et al*., 1994). In addition, enzyme-gold labeling confirmed that they are, at least in part, proteinateous. However despite the importance of the secretion of OPs for cell elongation, their exact composition remains unclear. Their localization and appearance suggested that they might transport cell wall or cuticle components or biosynthetic enzymes.

1.3 Integration of auxin signaling with other hormones and light

1.3.1 Crosstalk between auxin and other hormones

Plant development is largely postembryonic, which allows for adaptive responses to different environmental stimuli, such as light, nutrition, and temperature. Mounting evidence suggests that these external cues target the metabolism, distribution, or perception of plant hormones (Kende and Zeevaart, 1997; Hardtke *et al*., 2007; Santner *et al*., 2009; Depuydt and Hardtke, 2011; Enders and Strader, 2015; Singh and Savaldi-Goldstein, 2015). Plant hormones are very important endogenous mediators that control and coordinate numerous plant developmental processes. The final developmental output is determined by a complex network in which different hormonal pathways interact with each other as direct, indirect, and co-regulated cross talk. For direct cross talk, multiple hormonal pathways regulate the same target and jointly control the expression of a common gene or modulate the activity of the same protein. During

indirect cross talk, one hormone affects the perception, sensitivity, or availability of another hormone. If hormones regulate the same process via independent pathways that is called co-regulation (Chandler, 2009).

Peng *et al*. (2009) examined direct auxin crosstalk, by analyzing the *Arabidopsis* Hormone Database and found that 17% of all hormone-regulated genes were under control of more than one hormone. For example, in the *brevix radix* mutant (*brx*), both, brassinosteroids levels in roots, as well as the transcription of auxin-responsive genes are repressed, suggesting that several BR-responsive genes are coregulated by auxin (Mouchel *et al*., 2006). In addition, application of exogenous auxin affects the expression of 25% of BR-upregulated genes.

Indirect auxin crosstalk is the most common interaction mechanism between auxin and other hormones. For example, auxin enhances ethylene biosynthesis by stimulating the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. Auxin also regulates the metabolism of GA through the induction of several GA oxidase genes. In addition, several genes in the auxin signaling pathway are also regulated by other hormones. One example is the *AUX/IAA* gene *SHORT HYPOCOTYL2* (*SHY2*), whose transcriptional level in the root meristem transition zone is controlled by B-type *Arabidopsis* response regulator (ARR) transcription factors ARR1 and ARR2. Both also act downstream of cytokinin signaling (Dello Ioio *et al*., 2007; Dello Ioio *et al*., 2008). Furthermore, auxin distribution is affected by gibberellin, brassinosteroids, and cytokinin (Depuydt and Hardtke, 2011). In *Arabidopsis,* brassinosteroids induce the expression of *PIN1* and *PIN2* in the root and repress the transcription of *PIN3, PIN4* and *PIN7* in seedlings (Li *et al*., 2005; Mouchel *et al*., 2006). In contrast, cytokinins inhibit PIN

transcription through activation of SHY2, which is a potential ARFs repressor (Vieten *et al*., 2005; Pernisova *et al*., 2009).

1.3.2 Crosstalk between auxin signaling and light

Light, which provides energy for photosynthesis as well as information about seasonal timing and local habitat conditions, is vital for plant growth and development. Plants have developed sophisticated light signaling networks that detect and respond to changes in light duration, intensity and spectral quality. There are four major groups of light receptors, including the red and far-red light absorbing phytochromes (phy), the blue-light absorbing cryptochromes (cry), phototropins, and a ZTL-like F-box protein (Somers *et al*., 2004; Halliday *et al*., 2009). These receptors collectively control important aspects of plant development such as flowering and germination, tuberization and bud dormancy, and adaptive responses to microclimate and local habitat. Many of these light responses are achieved by linking to auxin signaling.

Photomorphogenesis is a complex process that refers to various developmental responses of plants to light signals (Whitelam *et al*., 1998). It depends on the action of several photoreceptors that interact with endogenous developmental programs. One key player is the phytochrome system. Phytochromes are a family of red/far-red responsive photoreceptors that constitute the most important regulator of photomorphogenesis, including de-etiolation, germination, shade avoidance, circadian rhythm and flowering (Yun-Jeong Han, 2007). *Arabidopsis* seedlings with an abundance of auxin show longer hypocotyls and reduced cotyledon expansion, similar to the phenotype which can be seen in dark-grown, etiolated seedlings and phytochrome deficient mutants (Vanderhoef and Briggs, 1978; Sawers *et al*., 2002;

Hoecker *et al*., 2004; Nagashima *et al*., 2008; Halliday *et al*., 2009). In contrast, plants with deficiencies in auxin biosynthesis display shorter hypocotyls that mirror enhanced photomorphogenesis/de-etiolation. Corn seedling development is similarly regulated by auxin and light. Light inhibits mesocotyl and coleoptile elongation by controlling the auxin supply from the coleoptiles (Vanderhoef and Briggs, 1978; Barker-Bridgers M, 1998). Together these phenomena suggest that light signals are closely tied to auxin homeostasis. In fact, through linking to the auxin system, light is able to control plant growth and development in response to the frequent changes in the light conditions.

By regulating both SUR2, a suppressor, and TAA1, an enhancer of IAA biosynthesis, light controls auxin levels *in planta* (Mikkelsen *et al*., 2004; Lau *et al*., 2008; Tao *et al*., 2008) (Fig. 1.2). SUR2 is a cytochrome P450 monooxygenase CYP83B1, which catalyzes the first step of indole glucosinolate biosynthesis thus converting IAOx to an inactive metabolite rather than IAA (Fig. 1.2). Disruption of SUR2 function in *Arabidopsis* mutants blocks the metabolic route from indole-3-acetaldoxime to glucosinolate, enhancing IAA production (Hoecker *et al*., 2004). TAA1, a tryptophan aminotransferase, converts tryptophan to IAA. Active phyB reduces IAA levels by coordinated activation of SUR2, and repression of the TAA1 transcription level (Stepanova *et al*., 2008; Tao *et al*., 2008). In the *taa1* mutant, plants are unable to respond to low R:FR ratios, due to the reduced IAA levels.

Phytochromes control not only auxin biosynthesis but also auxin availability. Auxin levels can be modulated by the *GH3* gene family of enzymes, which catalyze the conjugation of IAAs to amino acids for storage or degradation. By regulating the transcription of several GH3-type genes, phyA and phyB impose a strong influence on

active auxin levels. Plants with suppressed GH3 gene transcription show a dark specific elongated hypocotyl phenotype, while plants with elevated GH3 levels display an enhanced photomorphogenic seedling phenotype. Via these mechanisms, environmental signals can exert the fine-tuning of auxin availability (Nakazawa *et al*., 2001;Tanaka *et al*., 2002; Takase *et al*., 2003; Takase *et al*., 2004).

Phytochrome photoreceptors may also directly control auxin signaling. For instance, it has been shown that a recombinant oat phyA can interact with, and phosphorylate, the recombinant *Arabidopsis* auxin receptor SHY2/IAA3, as well as AXR3/IAA17, IAA1, IAA9 *in vitro* (Colon-Carmona *et al*., 2000). Native phyB was also shown to interact with recombinant SHY2/IAA3 and AXR3/IAA17. These studies provide a model explaining how light could exert direct control on Aux/IAA protein activity.

1.3.2.2 Light affects auxin transport

Auxin is a mobile morphogen. Reed showed that both shoot and root development can be significantly changed by N-1-naphylphtalamic acid (NPA), a polar auxin transporter inhibitor (Benning, 1986; Heyn *et al*., 1987; Reed *et al*., 1998), which alters the auxin distribution between shoot and root. This mechanism seems to be used by light to control seedling growth and development. In phytochrome and cryptochrome receptor mutants, the inhibition of hypocotyl growth caused by NPA was reduced, suggesting that auxin polar transport requires phytochrome and cryptochrome action (Salisbury *et al*., 2007). In addition, loss of phyA and phyB leads to a reduction of auxin transport between shoot and root, an accumulation of auxin in the shoot, and causes simultaneous elongation of the hypocotyl, reduction of primary root growth, and lateral root production (Bhalerao *et al*., 2002; Canamero *et al*., 2006; Salisbury *et al*., 2007).

Light regulates auxin movement by modifying the abundance and distribution of auxin transporters (PGPs and PINs). Several recent studies showed that the PIN3 transcription is highly correlated with phytochrome Pfr levels. phyB activation reduces PIN3 mRNA level, and phyB inactivation or loss lead to an increase of *PIN3* transcripts (Friml *et al*., 2002). In addition, the phyA, phyB, cry1, and cry2 light receptors regulate the PGP19 (P-glycoprotein 19) protein level within the upper portion of the hypocotyl collectively (Nagashima *et al*., 2008). This interferes directly with the auxin-transport activity of PGP-1.

At the same time, light signaling can also moderate auxin distribution by directly regulating PIN1, PIN2 and PIN7 protein function (Laxmi *et al*., 2008). Light controls the intracellular localization of PIN2 via the ELONGATED HYPOCPTYL 5 (HY5) branch of blue-light receptor pathway, thus maintaining its plasma membrane localization, and reducing vacuolar targeting for protein turnover (Oyama *et al*., 1997; Osterlund *et al*., 2000). Together, these results indicate that light regulates plant growth partially by changing the intracellular distribution of PIN proteins and, as a consequence, the auxin distribution within the plant.

1.3.2.3 Light and auxin signaling pathways have common gene targets

Light not only controls auxin levels and distribution but also directly integrates with auxin signal transduction. Upon perception of auxin, the transcription of three gene families, *Aux/IAA, SAUR,* and *GH3*, is rapidly induced. Aux/IAA proteins are repressors of auxin response factors. They have been reported to be involved with a range of processes, such as cotyledon expansion, hypocotyl and primary root elongation, and lateral root formation (Reed, 2001; Sauer *et al*., 2013). Although the function of SAUR

proteins still remains unknown, they have been implicated in a calmodulin (CaM) -auxin signal transduction pathway (Yang and Poovaiah, 2000; Li *et al*., 2015). By controlling the auxin levels, the GH3 proteins, a group of enzymes that conjugate free IAA with amino acids, have a strong effect on the auxin signaling pathway (Staswick *et al*., 2005; Park *et al*., 2007). Using transcriptomic analysis, Tepperman has shown that during seedling de-etiolation these gene families are regulated by phytochrome. In addition, genes with altered expression under low R:FR-ratio are highly correlated with auxin-related genes (Tepperman *et al*., 2001; Tepperman *et al*., 2006). Together these results suggest that phytochrome signaling operates closely with auxin perception.

These transcriptional links between light- and auxin-regulated gene expression were reinforced by mutant analysis. The *shy2/iaa3* mutant, which was isolated as a suppressor of the phytochrome chromophore-deficient mutant *hy2*, showed a constitutively photomorphogenic phenotype (Tian *et al*., 2002). A mild photomorphogenic phenotype was also seen in a dominant mutation of AXR2/IAA7 that stabilizes IAA7 by reducing the affinity for TIR1. This suggests that turnover of Aux/IAA is important for photomorphogenesis .

In plants, the shade avoidance syndrome demonstrates the strongest connection between auxin and light signaling. By investigating the biochemical principles behind this phenomenon, a number of *PHYTOCHROME RAPIDLY REGULATED* or *PAR* genes, which play a key role in coordinating light and auxin signaling, have been identified. Some of the *PAR* genes are members of the bHLH family of transcription factors, such as *PIL1, PAR1, PAR2*, and *HFR1* (Salter *et al*., 2003; Sessa *et al*., 2005; Roig-Villanova *et al*., 2006; Roig-Villanova *et al*., 2007). PAR1 and PAR2 suppress

auxin-induced expression of *Small Auxin-Up RNA gene15 (SAUR15*) and *SAUR68*. *PAR1, PAR2*, and *HFR1* have been demonstrated to play an important role in linking auxin signaling and the physiological response to low R:FR-ratio light.

Other *PAR* genes, including *ATHB2, ATHB4*, and *HAT1, 2, 3*, belong to the HD-zip class-II subfamily of transcription factors (Steindler *et al*., 1999; Sawa *et al*., 2002; Ciarbelli *et al*., 2008). Recently, ATHB4 protein has been shown to participate in shade avoidance as well. It can suppress the transcription of *SAUR15* and *SAUR68*. In addition, elevated ATHB4 activities reduce the hypocotyl response to exogenous auxin (Sorin *et al*., 2009). In addition, the expression of *HAT2* is significantly induced not only by low R:FR ratio light but also by auxin application (Sawa *et al*., 2002).

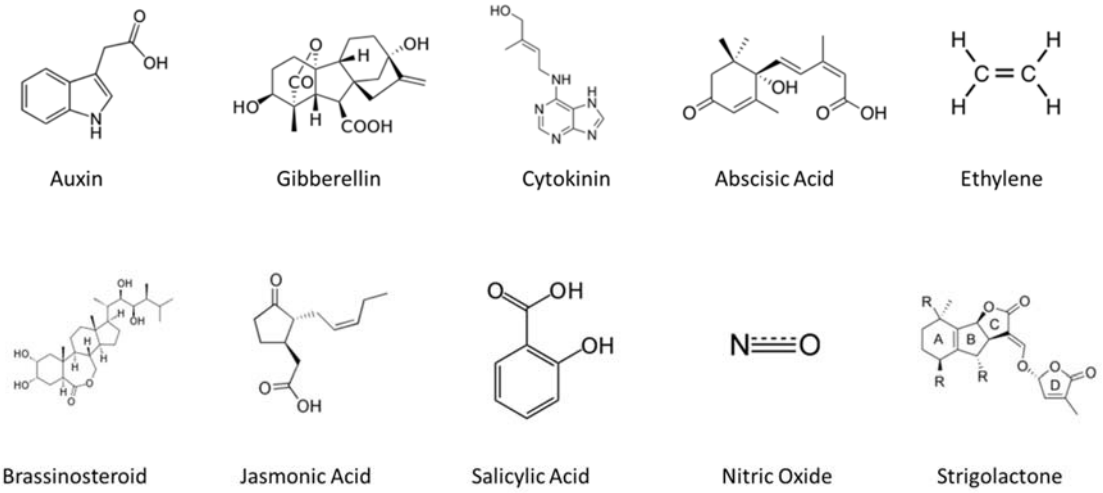
The bZip transcription factors HY5 (LONG HYPOCOTYL 5) and HYH (HY5 HOMOLOG) play prominent roles in light and auxin signaling (Holm *et al*., 2002). Light abolishes the activity of the COP1 E3 ligase, which targets HY5 for degradation, and leads to an accumulation of HY5 protein (Sibout *et al*., 2006). By binding to G-box motifs in promoter sequences, HY5 and HYH induce the transcription of light-related genes (Ang *et al*., 1998; Chattopadhyay *et al*., 1998; Holm *et al*., 2002); HY5 and HYH are also proposed as negative regulators of auxin signaling pathway. They directly regulate *AXR2/IAA7* and *SRL/IAA14* gene expression. In addition, loss of *HY5* causes a repression of the transcription of both genes (Cluis *et al*., 2004; Sibout *et al*., 2006). Therefore, it seems that light modulates the expression of both auxin-signaling components and modulators of auxin responsiveness.

In this thesis I compared proteins from either the intact corn coleoptile or the peeled outer epidermis of both, auxin-treated rapidly growing or non-treated slow

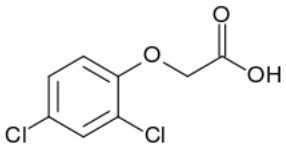
growing coleoptiles, using one-dimensional SDS-Polyacrylamide gel-electrophoresis (SDS-PAGE) followed by LC-ESI-MS/MS to identify proteins involved in expansion growth. Of 86 proteins identified, four were novel proteins of unknown function. I showed that one of the proteins of unknown function, ARRP1, with a DUF538 domain, is expressed in above ground tissues and enriched in the area where cell division and cell elongation growth take place. Upon auxin-treatment *ARRP1* mRNA and protein increase within 30 minutes – prior to measurable growth and to the expression of genes encoding cell wall biosynthetic enzymes. *ARRP1* expression could be induced by red and, to a lesser extent, blue light, indicating a possible role in phytochrome and cryptochrome signaling. *In situ* hybridization showed that *ARRP1* was expressed in the vasculature of growing tissues. This is consistent with our finding that corn ARRP1 interacts with the cytoplasmic domain of *Arabidopsis* VH1/BRL2, which plays a role in vascular development. *Arabidopsis* plants lacking the *ARRP1* homologue will grow normally under standard conditions, but show delayed growth, delayed flowering, and reduced yield, when germinated in the dark and transferred to light, a process that can be reversed by complementing the *Arabidopsis* mutant with the maize gene. These findings together with the localization of ARRP1 in cytoplasm and nucleus suggest a possible function in the light/auxin signaling path.

Figure 1.1 Chemical structure of the most common phytohormones (a) and 2,4-Dichlorophenoxyacetic acid (b), a synthetic auxin used as herbicide (Kende and Zeevaart, 1997; Santner *et al*., 2009; Santner and Estelle, 2009; Wolters and Jurgens, 2009; Depuydt and Hardtke, 2011).

(a)



(b)



2,4-Dichlorophenoxyacetic acid

Figure 1.2 Potential IAA biosynthetic pathways are integrated with the effect of the R:FR light ratio on IAA biosynthesis.

Pathways for which the enzymes have been identified are indicated by solid arrows; dashed arrows indicate pathways for which enzymes have not yet been identified and that may consist ofsingle or multiple steps. As phyB-Pfr enhances SUR2 and represses TTA1 transcript levels, it triggers the reciprocal control, leading to an increase in IAA production (Korasick et al., 2013).

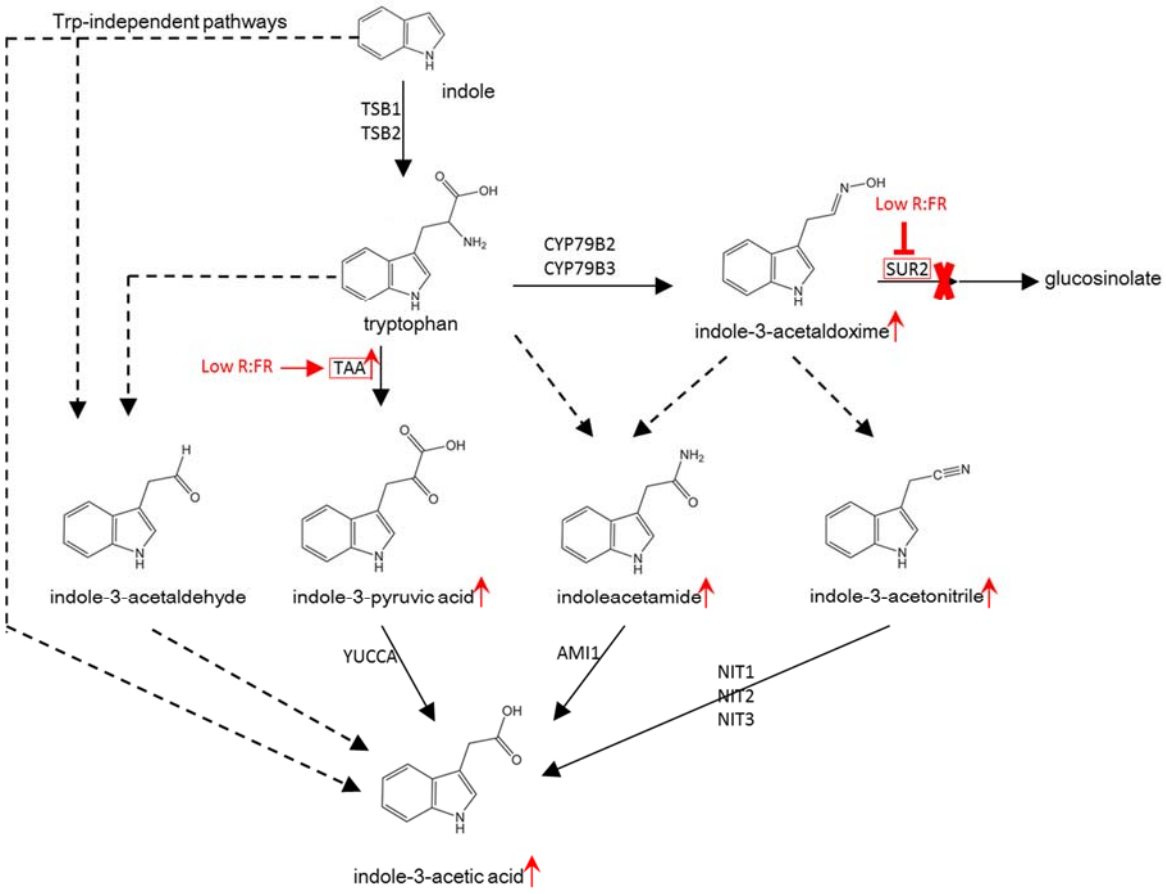
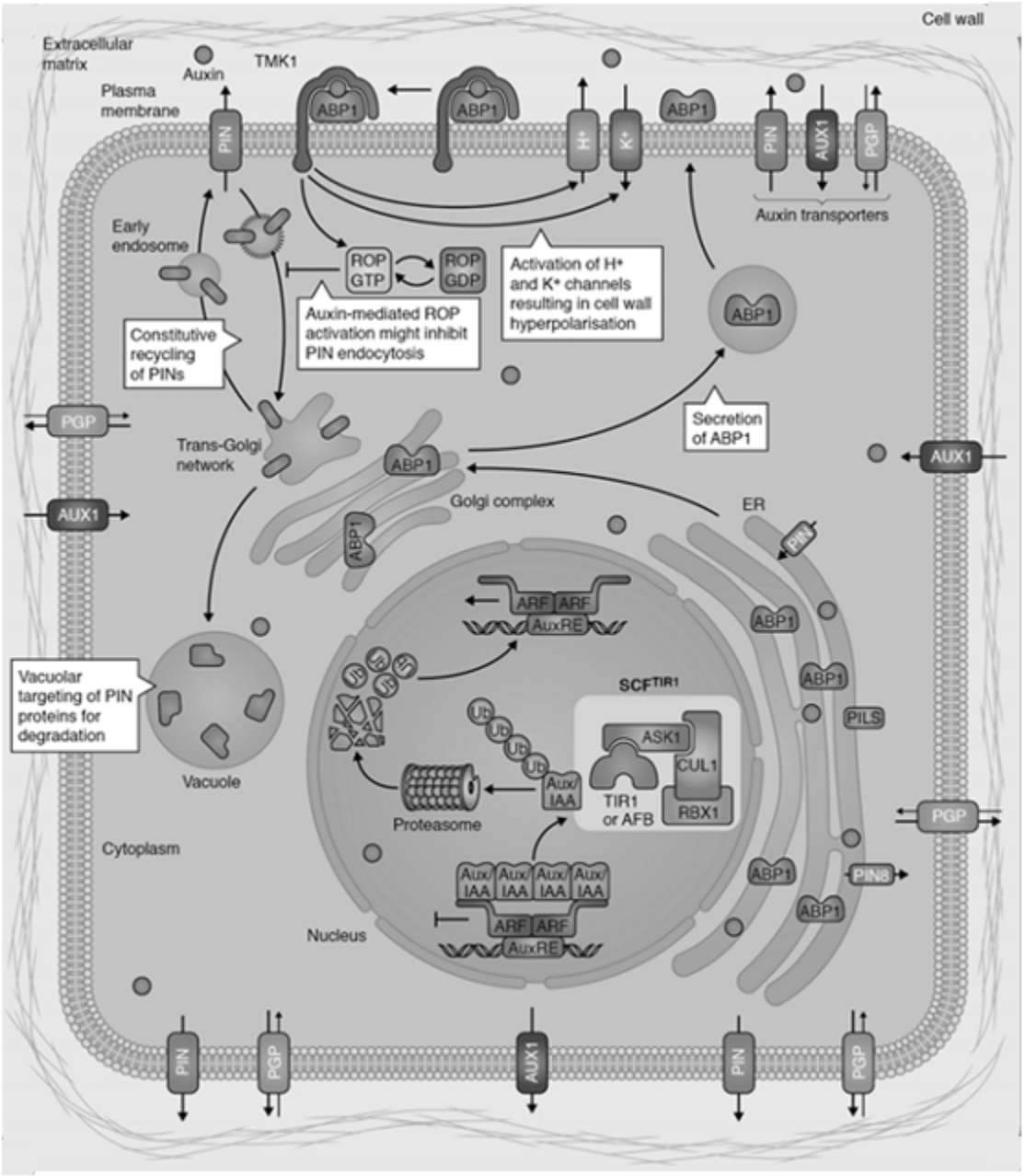


Figure 1.3 Overview of the cellular auxin machinery (Grones and Friml, 2015).



REFERENCES

REFERENCES

Ang LH, Chattopadhyay S, Wei N, Oyama T, Okada K, Batschauer A, Deng XW (1998) Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of *Arabidopsis* development. Mol Cell **1:** 213-222

Barker-Bridgers M RD, Cohen JD, Jones AM (1998) Red-lightregulated growth: changes in the abundance of indoleacetic acid in the maize (*Zea mays L*.) mesocotyl. Planta **204:** 5

Bauer CS, Hoth S, Haga K, Philippar K, Aoki N, Hedrich R (2000) Differential expression and regulation of K(+) channels in the maize coleoptile: molecular and biophysical analysis of cells isolated from cortex and vasculature. Plant J **24:** 139-145

Benjamins R, Scheres B (2008) Auxin: the looping star in plant development. Annu Rev Plant Biol **59:** 443-465

Benning C (1986) Evidence supporting a model of voltage-dependent uptake of auxin into Cucurbita vesicles. Planta **169:** 228-237

Bhalerao RP, Eklof J, Ljung K, Marchant A, Bennett M, Sandberg G (2002) Shoot-derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. Plant J **29:** 325-332

Brummell DA, Hall JL (1987) Rapid cellular-responses to auxin and the regulation of growth. Plant Cell and Environment **10:** 523-543

Burdach Z, Kurtyka R, Siemieniuk A, Karcz A (2014) Role of chloride ions in the promotion of auxin-induced growth of maize coleoptile segments. Annals of Botany **114:** 1023-1034

Calderon Villalobos LI, Lee S, De Oliveira C, Ivetac A, Brandt W, Armitage L, Sheard LB, Tan X, Parry G, Mao H, Zheng N, Napier R, Kepinski S, Estelle M (2012) A combinatorial TIR1/AFB-Aux/IAA co-receptor system for differential sensing of auxin. Nat Chem Biol **8:** 477-485

Canamero RC, Bakrim N, Bouly JP, Garay A, Dudkin EE, Habricot Y, Ahmad M (2006) Cryptochrome photoreceptors cry1 and cry2 antagonistically regulate primary root elongation in *Arabidopsis thaliana*. Planta **224:** 995-1003

Chandler JW (2009) Auxin as compere in plant hormone crosstalk. Planta **231:** 1-12

Chattopadhyay S, Ang LH, Puente P, Deng XW, Wei N (1998) *Arabidopsis* bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. Plant Cell **10:** 673-683

Chen R, Hilson P, Sedbrook J, Rosen E, Caspar T, Masson PH (1998) The *Arabidopsis thaliana AGRAVITROPIC 1* gene encodes a component of the polar-auxin-transport efflux carrier. Proc Natl Acad Sci U S A **95:** 15112-15117

Ciarbelli AR, Ciolfi A, Salvucci S, Ruzza V, Possenti M, Carabelli M, Fruscalzo A, Sessa G, Morelli G, Ruberti I (2008) The *Arabidopsis* homeodomain-leucine zipper II gene family: diversity and redundancy. Plant Mol Biol **68:** 465-478

Claeys H, De Bodt S, Inze D (2014) Gibberellins and DELLAs: central nodes in growth regulatory networks. Trends Plant Sci **19:** 231-239

Claussen M, Luthen H, Blatt M, Bottger M (1997) Auxin-induced growth and its linkage to potassium channels. Planta **201:** 227-234

Cleland R (1971) The mechanical behavior of isolated *Avena* coleoptile walls subjected to constant stress: properties and relation to cell elongation. Plant Physiol **47:** 805-811

Cleland R, Haughton PM (1971) The effect of auxin on stress relaxation in isolated *Avena* coleoptiles. Plant Physiol **47:** 812-815

Cluis CP, Mouchel CF, Hardtke CS (2004) The *Arabidopsis* transcription factor HY5 integrates light and hormone signaling pathways. Plant J **38:** 332-347

Colon-Carmona A, Chen DL, Yeh KC, Abel S (2000) Aux/IAA proteins are phosphorylated by phytochrome *in vitro*. Plant Physiol **124:** 1728-1738

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science **316:** 1030-1033

Cosgrove DJ (1997) Relaxation in a high-stress environment: The molecular bases of extensible cell walls and cell enlargement. Plant Cell **9:** 1031-1041

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol **6:** 850-861

Cosgrove DJ (2015) Plant expansins: diversity and interactions with plant cell walls. Curr Opin Plant Biol **25:** 162-172

Cosgrove DJ, Cleland RE (1983) Osmotic properties of pea internodes in relation to growth and auxin action. Plant Physiol **72:** 332-338

Cosgrove DJ, Li LC, Cho HT, Hoffmann-Benning S, Moore RC, Blecker D (2002) The growing world of expansins. Plant Cell Physiol **43:** 1436-1444

David KM, Couch D, Braun N, Brown S, Grosclaude J, Perrot-Rechenmann C (2007) The auxin-binding protein 1 is essential for the control of cell cycle. Plant Journal **50:** 197-206

de Jong M, Mariani C, Vriezen WH (2009) The role of auxin and gibberellin in tomato fruit set. J Exp Bot **60:** 1523-1532

Deeken R, Geiger D, Fromm J, Koroleva O, Ache P, Langenfeld-Heyser R, Sauer N, May ST, Hedrich R (2002) Loss of the AKT2/3 potassium channel affects sugar loading into the phloem of *Arabidopsis*. Planta **216:** 334-344

Deeken R, Sanders C, Ache P, Hedrich R (2000) Developmental and light-dependent regulation of a phloem-localised K^+ channel of *Arabidopsis thaliana*. Plant J **23:** 285-290

Della Rovere F, Fattorini L, D'Angeli S, Veloccia A, Del Duca S, Cai G, Falasca G, Altamura MM (2015) *Arabidopsis* SHR and SCR transcription factors and AUX1 auxin influx carrier control the switch between adventitious rooting and xylogenesis in planta and in *in vitro* cultured thin cell layers. Ann Bot **115:** 617-628

Dello Ioio R, Linhares FS, Scacchi E, Casamitjana-Martinez E, Heidstra R, Costantino P, Sabatini S (2007) Cytokinins determine *Arabidopsis* root-meristem size by controlling cell differentiation. Curr Biol **17:** 678-682

Dello Ioio R, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Morita MT, Aoyama T, Costantino P, Sabatini S (2008) A genetic framework for the control of cell division and differentiation in the root meristem. Science **322:** 1380-1384

Depuydt S, Hardtke CS (2011) Hormone signalling crosstalk in plant growth regulation. Curr Biol **21:** R365-373

Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. Nature **435:** 441-445

Enders TA, Strader LC (2015) Auxin activity: past, present, and future. Am J Bot **102:** 180-196

Engvild KC (1985) Pollen irradiation and possible gene transfer in *Nicotiana* species. Theor Appl Genet **69:** 457-461

Friml J (2003) Auxin transport - shaping the plant. Curr Opin Plant Biol **6:** 7-12

Friml J, Benkova E, Blilou I, Wisniewska J, Hamann T, Ljung K, Woody S, Sandberg G, Scheres B, Jurgens G, Palme K (2002) AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. Cell **108:** 661-673

Friml J, Wisniewska J, Benkova E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature **415:** 806-809

Gallavotti A (2013) The role of auxin in shaping shoot architecture. J Exp Bot **64:** 2593-2608

Galweiler L, Guan C, Muller A, Wisman E, Mendgen K, Yephremov A, Palme K (1998) Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science **282:** 2226-2230

Gao Y, Zhang Y, Zhang D, Dai X, Estelle M, Zhao Y (2015) Auxin binding protein 1 (ABP1) is not required for either auxin signaling or *Arabidopsis* development. Proc Natl Acad Sci U S A **112:** 2275-2280

Goldsmith MH, Cataldo DA, Karn J, Brenneman T, Trip P (1974) The rapid non-polar transport of auxin in the phloem of intact *Coleus* plants. Planta **116:** 301-317

Grones P, Friml J (2015) Auxin transporters and binding proteins at a glance. J Cell Sci **128:** 1-7

Grossmann K (2010) Auxin herbicides: current status of mechanism and mode of action. Pest Manag Sci **66:** 113-120

Haga K, Iino M (1998) Auxin-growth relationships in maize coleoptiles and pea internodes and control by auxin of the tissue sensitivity to auxin. Plant Physiol **117:** 1473-1486

Hager A (2003) Role of the plasma membrane H+-ATPase in auxin-induced elongation growth: historical and new aspects. J Plant Res **116:** 483-505

Hager A, Menzel H, Krauss A (1971) Experiments and hypothesis concerning primary action of auxin in elongation growth. Planta **100:** 47-75

Halliday KJ, Martinez-Garcia JF, Josse EM (2009) Integration of light and auxin signaling. Cold Spring Harb Perspect Biol **1:** a001586

Hardtke CS, Dorcey E, Osmont KS, Sibout R (2007) Phytohormone collaboration: zooming in on auxin-brassinosteroid interactions. Trends Cell Biol **17:** 485-492

Haschke HP, Luttge U (1975) Stoichiometric correlation of malate accumulation with auxin-dependent K-H exchange and growth in *Avena* coleoptile segments. Plant Physiol **56:** 696-698

Hemerly AS, Ferreira P, de Almeida Engler J, Van Montagu M, Engler G, Inze D (1993) *cdc2a* expression in *Arabidopsis* is linked with competence for cell division. Plant Cell **5:** 1711-1723

Heyn A, Hoffmann S, Hertel R (1987) *In-vitro* auxin transport in membrane vesicles from maize coleoptiles. Planta **172:** 285-287

Himanen K, Boucheron E, Vanneste S, de Almeida Engler J, Inze D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell **14:** 2339-2351

Hoad GV (1995) Transport of hormones in the phloem of higher-plants. Plant Growth Regulation **16:** 173-182

Hoecker U, Toledo-Ortiz G, Bender J, Quail PH (2004) The photomorphogenesis-related mutant red1 is defective in CYP83B1, a red light-induced gene encoding a cytochrome P450 required for normal auxin homeostasis. Planta **219:** 195-200

Hoffmann-Benning S, Klomparens KL, Kende H (1994) Characterization of growth-related osmiophilic particles in corn coleoptiles and deep-water rice internodes. Annals of Botany **74:** 563-572

Holm M, Ma LG, Qu LJ, Deng XW (2002) Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in *Arabidopsis*. Genes Dev **16:** 1247-1259

Jones AM, Venis MA (1989) Photoaffinity labeling of indole-3-acetic acid-binding proteins in maize. Proc Natl Acad Sci U S A **86:** 6153-6156

Jun JH, Fiume E, Fletcher JC (2008) The CLE family of plant polypeptide signaling molecules. Cell Mol Life Sci **65:** 743-755

Jurado S, Abraham Z, Manzano C, Lopez-Torrejon G, Pacios LF, Del Pozo JC (2010) The *Arabidopsis* cell cycle F-box protein SKP2A binds to auxin. Plant Cell **22:** 3891-3904

Kazan K, Manners JM (2009) Linking development to defense: auxin in plant-pathogen interactions. Trends Plant Sci **14:** 373-382

Kende H, Zeevaart J (1997) The five "classical" plant hormones. Plant Cell **9:** 1197-1210

Kepinski S, Leyser O (2004) Auxin-induced SCF^{TIR1}-Aux/IAA interaction involves stable modification of the SCF^{TIR1} complex. Proc Natl Acad Sci U S A **101:** 12381-12386

Kepinski S, Leyser O (2005) The *Arabidopsis* F-box protein TIR1 is an auxin receptor. Nature **435:** 446-451

Key JL, Ingle J (1964) Requirement for the synthesis of DNA-Like RNA for growth of excised Plant Tissue. Proc Natl Acad Sci U S A **52:** 1382-1388

Khush GS (1999) Green revolution: preparing for the 21st century. Genome **42:** 646-655

Kögl F EH, Haagen-Smit AJ. (1934) Über die Isolierung der Auxine a und b aus pflanzlichen Materialien. IX. Mitteilung. Zeitschrift für Physiologische Chemie **243:** 209-226

Korasick DA, Enders TA, Strader LC (2013) Auxin biosynthesis and storage forms. J Exp Bot **64:** 2541-2555

Korasick DA, Westfall CS, Lee SG, Nanao MH, Dumas R, Hagen G, Guilfoyle TJ, Jez JM, Strader LC (2014) Molecular basis for AUXIN RESPONSE FACTOR protein interaction and the control of auxin response repression. Proc Natl Acad Sci U S A **111:** 5427-5432

Kutschera U (1994) The current status of the acid-growth hypothesis. New Phytologist **126:** 549-569

Kutschera U (2001) Gravitropism of axial organs in multicellular plants. Adv Space Res **27:** 851-860

Kutschera U (2006) Acid growth and plant development. Science **311:** 952-954; author reply 952-954

Kutschera U, Kende H (1989) Particles associated with the outer epidermal wall in internodes of deep-water rice. Annals of Botany **63:** 385-388

Lau S, Jurgens G, De Smet I (2008) The evolving complexity of the auxin pathway. Plant Cell **20:** 1738-1746

Laxmi A, Pan J, Morsy M, Chen R (2008) Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in *Arabidopsis thaliana*. PLoS One **3:** e1510

Li L, Xu J, Xu ZH, Xue HW (2005) Brassinosteroids stimulate plant tropisms through modulation of polar auxin transport in *Brassica* and *Arabidopsis*. Plant Cell **17:** 2738-2753

Li ZG, Chen HW, Li QT, Tao JJ, Bian XH, Ma B, Zhang WK, Chen SY, Zhang JS (2015) Three SAUR proteins SAUR76, SAUR77 and SAUR78 promote plant growth in *Arabidopsis*. Sci Rep **5:** 12477

Lin MK, Belanger H, Lee YJ, Varkonyi-Gasic E, Taoka K, Miura E, Xoconostle-Cazares B, Gendler K, Jorgensen RA, Phinney B, Lough TJ, Lucas WJ (2007) FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. Plant Cell **19:** 1488-1506

Ljung K (2013) Auxin metabolism and homeostasis during plant development. Development **140:** 943-950

Ludwig-Muller J, Epstein E (1991) Occurrence and *in vivo* biosynthesis of indole-3-butyric acid in corn (*Zea mays L.*). Plant Physiol **97:** 765-770

McKersie B (2015) Planning for food security in a changing climate. J Exp Bot **66:** 3435-3450

Menges M, Samland AK, Planchais S, Murray JA (2006) The D-type cyclin CYCD3;1 is limiting for the G1-to-S-phase transition in *Arabidopsis*. Plant Cell **18:** 893-906

Mikkelsen MD, Naur P, Halkier BA (2004) *Arabidopsis* mutants in the C-S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. Plant J **37:** 770-777

Mouchel CF, Osmont KS, Hardtke CS (2006) BRX mediates feedback between brassinosteroid levels and auxin signalling in root growth. Nature **443:** 458-461

Muller A, Guan C, Galweiler L, Tanzler P, Huijser P, Marchant A, Parry G, Bennett M, Wisman E, Palme K (1998) *AtPIN2* defines a locus of *Arabidopsis* for root gravitropism control. EMBO J **17:** 6903-6911

Nagashima A, Suzuki G, Uehara Y, Saji K, Furukawa T, Koshiba T, Sekimoto M, Fujioka S, Kuroha T, Kojima M, Sakakibara H, Fujisawa N, Okada K, Sakai T (2008) Phytochromes and cryptochromes regulate the differential growth of *Arabidopsis* hypocotyls in both a PGP19-dependent and a PGP19-independent manner. Plant J **53:** 516-529

Nakaya M, Tsukaya H, Murakami N, Kato M (2002) Brassinosteroids control the proliferation of leaf cells of *Arabidopsis thaliana*. Plant Cell Physiol **43:** 239-244

Nakazawa M, Yabe N, Ichikawa T, Yamamoto YY, Yoshizumi T, Hasunuma K, Matsui M (2001) *DFL1*, an auxin-responsive GH3 gene homologue, negatively regulates shoot cell elongation and lateral root formation, and positively regulates the light response of hypocotyl length. Plant J **25:** 213-221

Napier RM, David KM, Perrot-Rechenmann C (2002) A short history of auxin-binding proteins. Plant Mol Biol **49:** 339-348

Nelles A (1977) Short-term effects of plant hormones on membrane potential and membrane permeability of dwarf maize coleoptile cells (*Zea mays L.*) in comparison with growth responses. Planta **137:** 293-298

Niklas KJ, Kutschera U (2012) Plant development, auxin, and the subsystem incompleteness theorem. Front Plant Sci **3:** 37

Noodén LD, Thimann KV (1963) Evidence for a requirement for protein synthesis for auxin-induced cell enlargement. Proc Natl Acad Sci U S A **50:** 194-200

Normanly J (2010) Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. Cold Spring Harb Perspect Biol **2:** a001594

Normanly J, Cohen JD, Fink GR (1993) *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. Proc Natl Acad Sci U S A **90:** 10355-10359

Osterlund MT, Hardtke CS, Wei N, Deng XW (2000) Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*. Nature **405:** 462-466

Ouyang J, Shao X, Li J (2000) Indole-3-glycerol phosphate, a branchpoint of indole-3-acetic acid biosynthesis from the tryptophan biosynthetic pathway in *Arabidopsis thaliana*. Plant J **24:** 327-333

Oyama T, Shimura Y, Okada K (1997) The *Arabidopsis* HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes Dev **11:** 2983-2995

Park JE, Park JY, Kim YS, Staswick PE, Jeon J, Yun J, Kim SY, Kim J, Lee YH, Park CM (2007) GH3-mediated auxin homeostasis links growth regulation with stress adaptation response in *Arabidopsis*. J Biol Chem **282:** 10036-10046

Peer WA, Blakeslee JJ, Yang H, Murphy AS (2011) Seven things we think we know about auxin transport. Mol Plant **4:** 487-504

Pernisova M, Klima P, Horak J, Valkova M, Malbeck J, Soucek P, Reichman P, Hoyerova K, Dubova J, Friml J, Zazimalova E, Hejatko J (2009) Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. Proc Natl Acad Sci U S A **106:** 3609-3614

Perrot-Rechenmann C (2010) Cellular responses to auxin: division versus expansion. Cold Spring Harb Perspect Biol **2:** a001446

Petrasek J, Friml J (2009) Auxin transport routes in plant development. Development **136:** 2675-2688

Philippar K, Buchsenschutz K, Edwards D, Loffler J, Luthen H, Kranz E, Edwards KJ, Hedrich R (2006) The auxin-induced K^+ channel gene *Zmk1* in maize functions in coleoptile growth and is required for embryo development. Plant Mol Biol **61:** 757-768

Philippar K, Fuchs I, Luthen H, Hoth S, Bauer CS, Haga K, Thiel G, Ljung K, Sandberg G, Bottger M, Becker D, Hedrich R (1999) Auxin-induced K^+ channel expression represents an essential step in coleoptile growth and gravitropism. Proc Natl Acad Sci U S A **96:** 12186-12191

Quint M, Gray WM (2006) Auxin signaling. Curr Opin Plant Biol **9:** 448-453

Rayle DL, Cleland R (1970) Enhancement of wall loosening and elongation by Acid solutions. Plant Physiol **46:** 250-253

Reed JW (2001) Roles and activities of Aux/IAA proteins in *Arabidopsis*. Trends Plant Sci **6:** 420-425

Reed RC, Brady SR, Muday GK (1998) Inhibition of auxin movement from the shoot into the root inhibits lateral root development in *Arabidopsis*. Plant Physiol **118:** 1369-1378

Roig-Villanova I, Bou-Torrent J, Galstyan A, Carretero-Paulet L, Portoles S, Rodriguez-Concepcion M, Martinez-Garcia JF (2007) Interaction of shade avoidance and auxin responses: a role for two novel atypical bHLH proteins. EMBO J **26:** 4756-4767

Roig-Villanova I, Bou J, Sorin C, Devlin PF, Martinez-Garcia JF (2006) Identification of primary target genes of phytochrome signaling. Early transcriptional control during shade avoidance responses in *Arabidopsis*. Plant Physiol **141:** 85-96

Rudnicka M, Polak M, Karcz W (2014) Cellular responses to naphthoquinones: juglone as a case study. Plant Growth Regulation **72:** 239-248

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast grr1p. Genes Dev **12:** 198-207

Salehin M, Bagchi R, Estelle M (2015) SCF^{TIR1}/AFB-based auxin perception: mechanism and role in plant growth and development. Plant Cell **27:** 9-19

Salisbury FJ, Hall A, Grierson CS, Halliday KJ (2007) Phytochrome coordinates *Arabidopsis* shoot and root development. Plant J **50:** 429-438

Salter MG, Franklin KA, Whitelam GC (2003) Gating of the rapid shade-avoidance response by the circadian clock in plants. Nature **426:** 680-683

Santelia D, Vincenzetti V, Azzarello E, Bovet L, Fukao Y, Duchtig P, Mancuso S, Martinoia E, Geisler M (2005) MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development. FEBS Lett **579:** 5399-5406

Santner A, Calderon-Villalobos LI, Estelle M (2009) Plant hormones are versatile chemical regulators of plant growth. Nat Chem Biol **5:** 301-307

Santner A, Estelle M (2009) Recent advances and emerging trends in plant hormone signalling. Nature **459:** 1071-1078

Sauer M, Kleine-Vehn J (2011) AUXIN BINDING PROTEIN1: the outsider. Plant Cell **23:** 2033-2043

Sauer M, Robert S, Kleine-Vehn J (2013) Auxin: simply complicated. J Exp Bot **64:** 2565-2577

Sauter M (2015) Phytosulfokine peptide signalling. J Exp Bot **66:** 5161-5169

Sawa S, Ohgishi M, Goda H, Higuchi K, Shimada Y, Yoshida S, Koshiba T (2002) The *HAT2* gene, a member of the HD-Zip gene family, isolated as an auxin inducible gene by DNA microarray screening, affects auxin response in *Arabidopsis*. Plant J **32:** 1011-1022

Sawers RJ, Linley PJ, Farmer PR, Hanley NP, Costich DE, Terry MJ, Brutnell TP (2002) Elongated mesocotyl1, a phytochrome-deficient mutant of maize. Plant Physiol **130:** 155-163

Sessa G, Carabelli M, Sassi M, Ciolfi A, Possenti M, Mittempergher F, Becker J, Morelli G, Ruberti I (2005) A dynamic balance between gene activation and repression regulates the shade avoidance response in *Arabidopsis*. Genes Dev **19:** 2811-2815

Sibout R, Sukumar P, Hettiarachchi C, Holm M, Muday GK, Hardtke CS (2006) Opposite root growth phenotypes of *hy5* versus *hy5 hyh* mutants correlate with increased constitutive auxin signaling. PLoS Genet **2:** e202

Singh AP, Savaldi-Goldstein S (2015) Growth control: brassinosteroid activity gets context. J Exp Bot **66:** 1123-1132

Somers DE, Kim WY, Geng R (2004) The F-box protein ZEITLUPE confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. Plant Cell **16:** 769-782

Sorin C, Salla-Martret M, Bou-Torrent J, Roig-Villanova I, Martinez-Garcia JF (2009) ATHB4, a regulator of shade avoidance, modulates hormone response in *Arabidopsis* seedlings. Plant J **59:** 266-277

Staswick PE, Serban B, Rowe M, Tiryaki I, Maldonado MT, Maldonado MC, Suza W (2005) Characterization of an *Arabidopsis* enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell **17:** 616-627

Steindler C, Matteucci A, Sessa G, Weimar T, Ohgishi M, Aoyama T, Morelli G, Ruberti I (1999) Shade avoidance responses are mediated by the ATHB-2 HD-zip protein, a negative regulator of gene expression. Development **126:** 4235-4245

Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, DoleZal K, Schlereth A, Jurgens G, Alonso JM (2008) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell **133:** 177-191

Stevenson TT, Cleland RE (1981) Osmoregulation in the *Avena* coleoptile in relation to auxin and growth. Plant Physiol **67:** 749-753

Sugawara S, Hishiyama S, Jikumaru Y, Hanada A, Nishimura T, Koshiba T, Zhao Y, Kamiya Y, Kasahara H (2009) Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in *Arabidopsis*. Proc Natl Acad Sci U S A **106:** 5430-5435

Takahashi K, Hayashi K, Kinoshita T (2012) Auxin activates the plasma membrane H^+-ATPase by phosphorylation during hypocotyl elongation in *Arabidopsis*. Plant Physiol **159:** 632-641

Takase T, Nakazawa M, Ishikawa A, Kawashima M, Ichikawa T, Takahashi N, Shimada H, Manabe K, Matsui M (2004) *ydk1-D*, an auxin-responsive *GH3* mutant that is involved in hypocotyl and root elongation. Plant Journal **37:** 471-483

Takase T, Nakazawa M, Ishikawa A, Manabe K, Matsui M (2003) *DFL2*, a new member of the *Arabidopsis GH3* gene family, is involved in red light-specific hypocotyl elongation. Plant and Cell Physiology **44:** 1071-1080

Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. Science **316:** 1033-1036

Tan X, Calderon-Villalobos LI, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature **446:** 640-645

Tanaka S, Mochizuki N, Nagatani A (2002) Expression of the *AtGH3a* gene, an *Arabidopsis* homologue of the soybean *GH3* gene, is regulated by phytochrome B. Plant and Cell Physiology **43:** 281-289

Tao Y, Ferrer JL, Ljung K, Pojer F, Hong F, Long JA, Li L, Moreno JE, Bowman ME, Ivans LJ, Cheng Y, Lim J, Zhao Y, Ballare CL, Sandberg G, Noel JP, Chory J (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell **133:** 164-176

Tepperman JM, Hwang YS, Quail PH (2006) phyA dominates in transduction of red-light signals to rapidly responding genes at the initiation of *Arabidopsis* seedling de-etiolation. Plant J **48:** 728-742

Tepperman JM, Zhu T, Chang HS, Wang X, Quail PH (2001) Multiple transcription-factor genes are early targets of phytochrome A signaling. Proc Natl Acad Sci U S A **98:** 9437-9442

Terasaka K, Blakeslee JJ, Titapiwatanakun B, Peer WA, Bandyopadhyay A, Makam SN, Lee OR, Richards EL, Murphy AS, Sato F, Yazaki K (2005) PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in *Arabidopsis thaliana* roots. Plant Cell **17:** 2922-2939

Thiel GaW, R. (1999) Auxin augments conductance of K^+ inward rectifier in maize coleoptile protoplasts. Planta **208:** 38-45

Tian Q, Uhlir NJ, Reed JW (2002) *Arabidopsis* SHY2/IAA3 inhibits auxin-regulated gene expression. Plant Cell **14:** 301-319

Ugartechea-Chirino Y, Swarup R, Swarup K, Peret B, Whitworth M, Bennett M, Bougourd S (2010) The AUX1/LAX family of auxin influx carriers is required for the establishment of embryonic root cell organization in *Arabidopsis thaliana*. Ann Bot **105:** 277-289

Vanderhoef LN, Briggs WR (1978) Red light-inhibited mesocotyl elongation in maize seedlings: I. the auxin hypothesis. Plant Physiol **61:** 534-537

Vanneste S, Friml J (2009) Auxin: a trigger for change in plant development. Cell **136:** 1005-1016

Vernoux T, Brunoud G, Farcot E, Morin V, Van den Daele H, Legrand J, Oliva M, Das P, Larrieu A, Wells D, Guedon Y, Armitage L, Picard F, Guyomarc'h S, Cellier C, Parry G, Koumproglou R, Doonan JH, Estelle M, Godin C, Kepinski S, Bennett M, De Veylder L, Traas J (2011) The auxin signalling network translates dynamic input into robust patterning at the shoot apex. Mol Syst Biol **7:** 508

Vieten A, Vanneste S, Wisniewska J, Benkova E, Benjamins R, Beeckman T, Luschnig C, Friml J (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development **132:** 4521-4531

Visnovitz T, Solti A, Csikos G, Fricke W (2012) Plasma membrane H^+ -ATPase gene expression, protein level and activity in growing and non-growing regions of barley (*Hordeum vulgare*) leaves. Physiol Plant **144:** 382-393

Wang S, Bai Y, Shen C, Wu Y, Zhang S, Jiang D, Guilfoyle TJ, Chen M, Qi Y (2010) Auxin-related gene families in abiotic stress response in *Sorghum bicolor.* Funct Integr Genomics **10:** 533-546

Weijers D, Friml J (2009) SnapShot: Auxin signaling and transport. Cell **136:** 1172, 1172 e1171

Went FW (1927) On growth-accelerating substances in the coleoptile of *Avena sativa.* Proceedings of the Koninklijke Akademie Van Wetenschappen Te Amsterdam **30:** 10-19

Went FW (1934) A test method tor rhizocaline the root forming substance. Proceedings of the Koninklijke Akademie Van Wetenschappen Te Amsterdam **37:** 445-455

Werner T, Motyka V, Strnad M, Schmulling T (2001) Regulation of plant growth by cytokinin. Proc Natl Acad Sci U S A **98:** 10487-10492

Wetmore RH, Jacobs WP (1953) Studies on abscission - the inhibiting effect of auxin. American Journal of Botany **40:** 272-276

Whitelam GC, Patel S, Devlin PF (1998) Phytochromes and photomorphogenesis in *Arabidopsis*. Philos Trans R Soc Lond B Biol Sci **353:** 1445-1453

Wilmoth JC, Wang S, Tiwari SB, Joshi AD, Hagen G, Guilfoyle TJ, Alonso JM, Ecker JR, Reed JW (2005) NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. Plant J **43:** 118-130

Wolters H, Jurgens G (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. Nat Rev Genet **10:** 305-317

Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Ann Bot **95:** 707-735

Wright AD, Sampson MB, Neuffer MG, Michalczuk L, Slovin JP, Cohen JD (1991) Indole-3-acetic acid biosynthesis in the mutant maize orange pericarp, a tryptophan auxotroph. Science **254:** 998-1000

Yang T, Poovaiah BW (2000) Molecular and biochemical evidence for the involvement of calcium/calmodulin in auxin action. J Biol Chem **275:** 3137-3143

Yun-Jeong Han P-SS, and Jeong-Il Kim (2007) Phytochrome-mediated photomorphogenesis in plants. Journal of Plant Biology **50:** 230-240

Zazimalova E, Murphy AS, Yang H, Hoyerova K, Hosek P (2010) Auxin transporters--why so many? Cold Spring Harb Perspect Biol **2:** a001552

CHAPTER 2: CONTRIBUTION OF PROTEOMICS IN THE IDENTIFICATION OF NOVEL PROTEINS ASSOCIATED WITH PLANT GROWTH

This chapter was published in Journal of Proteome Research (2013):

Contribution of proteomics in the identification of novel proteins associated with plant growth
J Li, TJ Dickerson, S Hoffmann-Benning
Journal of proteome research 12 (11), 4882-4891

2.1 Abstract

The need to produce sufficient food for an increasing world population during today's climate changes puts urgency into our understanding of how plants grow under adverse environmental conditions. Since the epidermis is not only the interface between the plant and the environment, but also a growth limiting tissue, understanding the initiation and regulation of its expansion growth is essential for addressing this issue. We used mass spectrometry to identify proteins from auxin-induced rapidly-growing corn (*Zea mays*) coleoptiles to find possible candidates controlling this growth as well as the underlying cell wall and cuticle biosynthesis. Of 86 proteins identified, fifteen showed a predicted association with cell wall/cuticle biosynthesis or trafficking machinery; four were proteins of unknown function. As expected, real-time PCR indicated that the steady-state mRNA levels of genes with a known or predicted role in cell wall biosynthesis increases upon treatment with auxin. Importantly, genes encoding two of the hypothetical proteins also show higher levels of mRNA; additionally, their gene expression is down regulated as coleoptile growth ceases and up regulated in expanding leaves. This suggests a major role of those novel proteins in the regulation of processes related to cell and organ expansion and thus plant growth.

2.2 Introduction

As the world population grows, our need for food and fuel increases drastically. The green revolution, occurring from the 1930s to 1960s, addressed these issues through the development of high-yielding varieties, the distribution of hybridized seeds, synthetic fertilizers and pesticides, and a modernization of management techniques and irrigation systems (Borlaug, 2000). Today, the amount of arable land is limited and often there is competition between food and fuel crops. In addition, changes in the global climate may reduce future yields. To continue to provide sufficient food and fuel, we need plants that show either accelerated growth or have a higher grain or cell wall yield or quality and are more resistant to biotic and abiotic stresses. One proposed approach towards the solution of this problem is to better understand the regulation and the processes necessary for plant growth. The corn coleoptile is a good model system for these studies (Haga and Iino, 1998). It is a protective sheath surrounding the first leaf of monocotyledons as the mesocotyl grows and pushes the seedling through and out of the soil. At the same time the coleoptile also elongates though cell expansion. Once it reaches the surface and perceives light, coleoptile growth ceases and the leaves emerge. This expansion growth can be mimicked by treating the coleoptile with the growth hormone auxin which leads to rapid cell expansion and elongation of the entire organ. If this rapidly growing tissue is split longitudinally it will bend outward, suggesting that it is the outer epidermis, which mechanically limits the growth (Schopfer, 2006). Whether this is indeed the case has been debated for decades (Peters and Tomos, 2000; for reviews see Schopfer, 2006; Kutschera and Niklas, 2007; Savaldi-Goldstein, 2007).

Examination of the cell ultrastructure of rapidly growing tissues has shown the appearance of osmiophilic particles (OPs; Fig. 2.1) between the plasma membrane and the outer epidermal wall of rapidly growing tissues in a variety of plants (Robards, 1969; Oleson, 1980; Kutschera *et al*., 1987; Kutschera and Kende, 1989; Hoffmann-Benning *et al*., 1994a). Mostly, they are thought to be connected with cell-wall biosynthesis or loosening. Vaughn (2002) had shown a strong correlation of osmiophilic particles with an increased pectin deposition during weed dodder attachment to the host. Yet, while he could identify the pectin content of the newly formed wall, he reported no labeling of the particles themselves. Closer investigation of the osmiophilic particles has revealed that they are in part proteinaceous and are secreted only to the outer wall of expanding epidermal cells. Inhibition of the secretory pathway with monensin reduces not only the appearance of the osmiophilic particles but also organ growth and cutin synthesis (Hoffmann-Benning *et al*., 1994a, Hoffmann-Benning and Kende, 1994b). Upon inhibition of o-glycosylation the appearance of osmiophilic particles, synthesis and transport of proteins into cell wall, and concomitant growth were inhibited, suggesting that glycosylation and secretion are essential steps in auxin-induced expansion growth (Edelmann, 1995).

The key differences between cells of the outer epidermis as compared to cells of the inner parenchyma are the larger diameter of the outer epidermal wall and its superimposition with the cuticle. The localization and appearance of the OPs suggests that they could transport cell wall or cuticle components or enzymes for cell-wall or cuticle biosynthesis.

The plant cell wall and the cuticle play an important role in plant growth and development and form a barrier against pathogen infection. The cuticle provides the outermost barrier between a land plant and its aerial environment. It limits non-stomatal water loss and gas exchange (Baker *et al*., 1982; Riederer and Schreiber, 2001; Ristic and Jenks, 2002; Vogg *et al*., 2004; Aharoni *et al*., 2004), protects the plant from biotic and non-biotic stressors (Schweizer *et al*., 1996; Cameron *et al*., 2006; Bessire *et al*., 2007; Chassot *et al*., 2007), and controls proper organ fusion and development (Lolle *et al*., 1998; Sieber *et al*., 2000; Kurdyukov *et al*., 2006). The cutin matrix is a polymer which is mostly composed of C16 and C18 hydroxy and epoxy fatty acids, but also contains small amounts of phenolic acids and other aromatic monomers (Riley and Kolattukudy, 1975; Kolattukudy, 1996; Nawrath, 2002, 2006; Heredia, 2003). Depending on the composition of the epicuticular wax, the cuticle can be spiky to discourage feeding or slippery to protect from small herbivores or to catch insects in carnivorous plants (Riedel *et al*., 2003; Riedel *et al*., 2007). As the plant cell expands the cuticle has to grow with it. Earlier publications had shown that the rate of cutin biosynthesis increased during gibberellic acid-induced stem extension in peas while the composition did not appear to vary significantly in pea (Bowen and Walton, 1988). Yet in rapidly growing deepwater rice internodes, both the rate of cutin synthesis as well as its composition changed (Hoffmann-Benning and Kende, 1994b). Likewise, the composition of the cuticular wax in Kalanchoe changed during leaf expansion (Van Maarseveen *et al*., 2009). Several attempts are in progress to better characterize cutin biosynthesis and deposition and its relation to organ expansion (Suh *et al*., 2005; Kunst and Samuels, 2006; Samuels *et al*., 2008; Matas *et al*., 2011).

The cell wall is a dynamic structure which gives the cell and the complete organism its structure and stability but is also essential for cell division and differentiation, cell and organ expansion, and for the response to environmental stimuli and pathogen infection. (Cosgrove *et al*., 2002; Cosgrove, 2005; Huckelhoven, 2007; Anderson *et al*., 2010). It consists of an intertwined network of polysaccharides, lignin, and proteins (Carpita and Gibeaut, 1993). Up to 95% of its mass is composed of polysaccharides in the form of cellulose, hemicellulose and pectin. Only 5-10% of the cell wall mass is composed of proteins (Cassab and Varner, 1988). These cell wall proteins (CWPs) can be (i) structural components of the cell wall, (ii) enzymes involved in cell wall biosynthesis, modification or turnover, or (iii) they can play a role in generating signals within and transmitting them through the cell wall, or they can have as of yet unknown functions. While cell wall polysaccharides lend strength to the cell wall, they also confer rigidity and thus constrain cell expansion. Thus, cell expansion must consist of cell wall loosening as well as synthesis. This wall loosening can be accomplished through the activites of proteins such as expansins, endo-(1,4)-β-D-glucanases, and xyloglucan endoglucosylases/hydrolases (Cosgrove, 2005; Humphrey *et al*., 2007). Consequently, cells have to retain the ability to monitor changes in cell wall composition and integrity as well as signal those changes and regulate the biosynthesis machinery (Steinwand and Kieber, 2010).

Of the estimated number of CWPs, approximately one fourth, or four hundred, have been identified in *Arabidopsis*. Many contain posttranslational modifications, like hydroxylation, glycosylation or phosphorylation. These CWPs have to be transported to the apoplast and, most likely, pass through the secretory system. However, some

CWPs seem to be lacking predicted signal peptides and may use alternative pathways (for a review see Jamet *et al*., 2008). In addition, while the polysaccharide composition of the cell wall of grasses is well known, it is different from that of dicotyledonous plants in that it contains less pectin and xyloglucan and more heteroxylan and compounds like mixed-linked glucans (MLGs), which are of high nutritional importance. The majority of CWPs related to cell wall synthesis have been characterized in dicotyledonous plants, while their nature in grasses remains largely speculative (Burton *et al*., 2005, 2008; for a review see Fincher, 2009).

Our objective here was to better understand the changes that occur during expansion growth as it relates to regulation of biosynthetic processes in cereal grasses. Using corn coleoptiles and auxin-induced growth as a model system, we used a proteomics approach to identify proteins present in the epidermis of rapidly-growing corn coleoptiles and analyzed their expression pattern. Our approach allowed for the identification and characterization of two novel proteins, whose gene expression is correlated with growth and who have a possible role in cell wall or cutin biosynthesis in maize. Based on homology searches one could function in the early regulation/initiation of cell expansion growth while the second protein may be directly involved in the synthesis of mixed-linked glucans. As a result, they could be important for the production of biomass in agriculture.

2.3 Materials and Methods

2.3.1 Plant material and growth conditions

Zea mays Great Lakes 4758 hybrid seeds were rinsed thoroughly to remove fungicides and shaken in water for up to one hour to speed germination. Kernels were planted in a standard soil mixture containing equal parts of Bacto Soil (Michigan Pear Company, Houston), medium vermiculite and perlite. They were grown in the dark at room temperature. Unless indicated otherwise, coleoptiles were harvested 4.5 days after planting.

2.3.2 Sample harvest

Coleoptiles were pulled off the primary leaf and cut to a length of 10 mm after removal of the tips. All coleoptiles (40 total per experiment) were shaken in the dark in distilled water for 1 hour to remove residual auxin. Half were transferred into fresh water (control) and half into an auxin ($3x10^{-5}$ M indole acetic acid - IAA; Sigma-Aldrich; rapidly-growing sample). The samples were covered while shaking for 4-5 hours. Coleoptile length was measured at the end of the incubation period to confirm auxin action and induced growth. To collect the epidermis, twenty coleoptiles were carefully peeled, the resulting epidermis immediately dipped into liquid nitrogen and stored at -80°C (Fig. 2.2 & 2.3) for further experiments.

2.3.3 Protein collection and identification

Tissue from 20 coleoptiles per treatment was ground in 50 mM Tris/HCL (pH 8), 5% glycerol V/V, 0.1 M KCl and centrifuged at 10,000g for 2 min. Identical amounts of proteins from the supernatant were loaded onto a Bio-Rad Polyacrylamide15% Tris-HCl Ready Gel. Protein bands (indicated with asterisks and arrows in Fig. 2.4a) were

excised and digested with trypsin (Shevchenko *et al*., 1996). Each protein band was analyzed from at least two different gels from independent sample preparations according to Guelette *et al*. (2012): The digested peptides were extracted into 60% acetonitrile / 1% TFA. Liquid chromatography / mass spectrometry was performed on a Capillary LC system (Waters, Milford, MA) coupled to a LCQ DECA ion trap mass spectrometer (Thermofinnigan, San Jose, CA) equipped with a nanospray ionization source. The sample was trapped onto a Peptide Cap Trap (Michrom BioResources, Auburn, CA) and flushed onto a 5 cm x 75 µm ID picofrit column packed with 5 µm ProteoPep C18 material (New Objective, Woburn, MA). Peptides were eluted with a gradient of 2% to 95% ACN in 0.1% formic acid at a flow rate of 200 nl per minute for 60 minutes.

Peak lists for MS2 were generated by Bioworks and spectra searched using SEQUEST or MASCOT against *Zea mays* and *Arabidopsis* databases. Carbamidomethyl cysteine was set as fixed modification (+57.0215 Da), and oxidation of Met (+15.9949 Da) and phosphorylation of Ser, Thr, and Tyr (+79.96633 Da) was allowed. Up to two missed tryptic sites were permitted. Peptide tolerance was set to 2.5 Da, and MS/MS tolerance was set to 0.8 Da. Positive identifications were confirmed by individually comparing MS/MS spectra. Positive identification required at least two unique peptides per protein, counting only peptides with significant scores (95% confidence per peptide; >2.0 for SEQUEST). Database searches using individual tryptic fragments were performed using the BLAST searches at NCBI (http://www.ncbi.nlm.nih.gov/blast).

2.3.4 RNA extraction, quantitative & qualitative analysis

RNA was extracted from equal amounts of tissue using a Qiagen RNeasy Mini-Kit. The RNA was treated with 1 µl/100 ml of 10 Units/µl Roche RNase-free DNase I for 1 hour at 37°C followed by RNA cleanup using the same kit. Quantification was determined using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., now Thermo Scientific; Wilmington, DE). RNA quality was confirmed using 260/280 nm ratio.

Quantitative real-time PCR analysis was performed in 96-well plates on an ABI7000 instrument (Bio-Rad) using the 2×SYBR Green PCR core reagent (Applied Biosystems). Reactions were performed in a volume of 30 µl containing 2 µl cDNA, 0.1 mM forward and reverse primers, and 15 µl 2×SYBR Green master mix reagent. Each sample had three biological duplicates. The amplification conditions of all genes were: 95℃ for 3 min; 40 cycles of 95℃ for 10 s, 63℃ for 30 s, and 72℃ for 30 s; and a final extension at 72℃ for 5 min. At the end of the amplification, a dissociate stage was added to verify the amplification specificity, and the fluorescence was monitored. The threshold values were obtained using the automated setting of the instrument software, and the Cq (quantification cycle) value was also automatically calculated by the software. The data, expressed as Cq values, were imported into a Microsoft Excel applet for successive analysis. All the primers used were designed using the free Primer 3 software with the exception of the 18S primers and are shown in Table S2.1b. 18S was used to generate the stand curve and as a control. qPCR was performed with three biological replicates. Each biological replicate was repeated three times.

For RT-PCR 400 ng mRNA was reverse transcribed using Omniscript Reverse Transcriptase (Qiagen). All Primers used for RT-PCR are listed in the Table S2.1a. PCR products from 6-8 biological replicates were visualized with a 1% agarose gel and quantified using pixel counts. The 18S signal for each sample was used for normalization. Data were quantified using a Student's t test (Table S2.3). Using a linear regression analysis on the data confirmed that the average expression level of these genes is significantly decreased with time in all cases (p value <0.023)

The identity of the PCR products was confirmed by ligation into Promega's pGEMT-easy vector system followed by transformation into Stratagene's XL10-Gold Ultracompetent Cells. The Research Technology Support Facility at Michigan State University synthesized all oligonucleotides and performed all automated DNA sequencing.

2.4 Results and Discussion

2.4.1 Proteomics analysis

To identify proteins with a possible role in cell expansion growth corn coleoptiles were treated with water (control) or 3 x 10^{-5} M Indole acetic acid (IAA; rapidly growing) for four hours. Proteins from either the intact corn coleoptile or the peeled outer epidermis were extracted and separated using one-dimensional SDS-Polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 2.4a). The comparison between auxin-treated and control tissues was expected to reveal proteins involved in auxin-induced expansion growth. Comparison between coleoptile and epidermal tissue would indicate epidermis-specific roles. The comparison consistently revealed nine protein bands that were increased in the outer epidermis as compared to the total coleoptile (at. 75, 55, 50, 40, 25, 21, 19, 17, 9, 8 kDa, respectively; Fig. 2.4a as indicated with an asterisk). Quantification of the gel bands revealed increases of up to 30% above the band intensity in control lines. Several of these as well as six additional bands were increased in the auxin-induced rapidly growing sample (116, 56, 24, 17, 16, 9 kDa; indicated with arrows). Since the outer epidermis is discussed as the growth-limiting tissue (Peters and Tomos, 2000; Schopfer, 2006; Kutschera and Niklas, 2007; Savaldi-Goldstein, 2007) and is distinguished from the rest of the coleoptile tissue by containing the cuticle as well as a much thicker cell wall, these proteins could potentially play an important role in the regulation of organ growth as well as the associated cell wall and cutin biosynthesis. In-gel digestion (Shevchenko *et al*., 1996) followed by LC-ESI-MS/MS revealed 86 proteins, 66 of were derived from bands induced in the epidermis of rapidly growing coleoptiles (see table S2.2). The proteins were categorized by their predicted

function (Fig. 2.4b). Eighteen of these proteins are involved in protein biosynthesis (eight ribosomal proteins, five translation initiation or elongation factors) or are chaperones (five). The rise in this group of proteins is not surprising since an increase in protein synthesis and their proper folding will be necessary to synthesize building blocks and provide energy to maintain cell and organ expansion. One of them, cyclophilin, is known to be increased in germinating seedlings and growing tissues as well as in response to various stress factors and plant hormones (Marivet *et al*., 1994; Cho and Kim, 2008).

Fifteen proteins are involved in carbon metabolism and transport while others play a role in cell division and/or are part of the cytoskeleton (11). Eight proteins play a role in pathogen or stress response and an additional 19 proteins belong to no specific subgroup.

Of particular interest are proteins with a possible novel or not fully elucidated role in cell expansion and organ growth and the necessary cell wall and cutin biosynthesis: They could function in cell wall biosynthesis/ secretory pathway (I), lipid metabolism/cutin biosynthesis (II) or are previously uncharacterized hypothetical proteins with an either regulatory or synthetic function (III). Three groups of identified proteins fall into that category:

Proteins with a Possible Role in Secretory Pathway or Cell-Wall Biosynthesis. Not surprisingly, several of the identified proteins are possibly related to the cell wall or the secretory pathway (sec 15, calreticulin, translationally controlled tumor-protein like; see table 1): One protein which is secreted is the translationally controlled tumor protein like protein (TCTP). It was originally identified as a calcium-regulated gene involved in

the gravitropic bending of maize roots (Kim, J.-Y. and Poovaiah, B.W.; NCBI; submitted Sept. 2002) and belongs to the TCTP superfamily. *Arabidopsis* plants impaired in TCTP expression displayed a dwarf phenotype due to a reduced cell size and a reduced sensitivity to auxin (Berkowitz *et al*., 2008). This suggests that plant TCTPs may act in an early control of cell division and cell elongation rather than in cell wall synthesis.

The reversibly glycosylated polypeptide (RGP) is highly similar to the Golgi-associated se-wap41. RGPs are delivered to the cell wall/plasmodesmata via the Golgi apparatus (Sagi *et al*., 2005). They are now considered UDP-Ara mutases that catalyze the interconversion of UDP-L-arabinopyranose and UDP-L-arabinofuranose, which are important in heteroxylan biosynthesis.

Similarly, UDP-glucose pyrophosphorylase (UDPgp) and UDP-glucose dehydrogenase (UDPgdh), two enzymes with a function in the synthesis of cell-wall precursors, were found in our sample: UDPgp catalyzes the reversible conversion of UDP-glucose and pyrophosphate to glucose-1-phosphate and UTP. UDPgdh converts UDP-glucose to UDP-glucuronic acid, which is the precursor for UDP-galacturonic acid, UDP-xylose, UDP-arabinose, and other precursors for both the pectin and the hemicellulose components of cell walls.The identification of known cell-wall biosynthesis protein confirms the suitability of our approach for the identification of proteins associated with cell expansion and growth.

Proteins Associated with Lipid Metabolism. We detected several proteins associated with lipid metabolism and, thus a possible affiliation with cutin biosynthesis such as a lipoxygenase, an acyl transferase, phospholipase D, and several lipid transfer proteins (LTPs). The acyl transferase may function in either cutin or phytoalexin and

thus indirectly in lignin biosynthesis. Plant lipoxygenases belong to a large group of enzymes that catalyze the oxygenation of free polyunsaturated fatty acids, typically forming biologically active molecules, which are called oxylipins (Porta *et al*., 2002). Phospholipase D functions in the production of phosphatidic acid, a lipid involved in many aspects of stress signaling (Welti *et al*., 2002; McDermott *et al*., 2004; Testerink and Munnik, 2005; Lu *et al*., 2013). Rather than a biosynthetic, these proteins likely have a regulatory function (Feussner *et al*., 2002).

A second induced lipid-related protein is an acyltransferase homologue with a high similarity to N-hydroxycinnamoyl /benzoyltransferase and a possible function in phytoalexin biosynthesis and, indirectly, lignin biosynthesis (Marchler-Bauer *et al*., 2007).

Of the three identified lipid transfer proteins (LTPs), one is categorized as a non-specific lipid transfer protein, while the other two are grouped as two distinct phospholipid transfer proteins. Plant lipid transfer proteins are small cationic peptides of 7 to 10 kDa. They have the ability to bind phospholipids and other lipids and transfer them between a donor and a membrane *in vitro* (Kader, 1996). LTPs have a signal sequence and are typically transferred through the secretory pathway into the apoplast (Tsuboi *et al*., 1992; Thoma *et al*., 1993; Pyee *et al*., 1994; Carvalho *et al*., 2004). However, they have also been found in other cellular compartments and expanding hypocotyls (Irshad *et al*., 2008). Again, the finding of several proteins with a possible role in cell expansion confirms the usefulness of our approach for finding novel candidates:

Hypothetical Proteins with, As of yet, Unknown Function. We were able to identify four hypothetical proteins with, as of yet, unknown function. Hypothetical protein 1 (HP1) appears to be similar to an *Arabidopsis* protein which is associated with the mitochondria.

Hypothetical protein 2 (HP2) has similarity to MATH domain containing proteins. Proteins within this group include extracellular metallo proteases which are anchored to the membrane.

Hypothetical protein 3 (Hyp3) is a 22 kDa protein with a similarity to endo-1,3-1,4-beta-D-glucanases. It could potentially be involved in the extension of mixed-linked glucans, a nutritionally important group of unusual cell wall polysaccharides mostly limited to cereal grasses (Rieder and Samuelsen, 2012). They contain stretches of (1,4)-β-D-glucans with interspersed (1,3)-β-glycosyl groups, which introduce “kinks” in the polysaccharide chains that lead to an increased solubility and flexibility (Fincher, 2009). Their exact composition varies between different species and tissues and increases during phases of rapid cell elongation (Carpita *et al*., 2001; Gibeaut *et al*., 2005; McCann *et al*., 2007). While enzymes involved in the depolymerisation of (1,3;1,4)-β-D-glucans are well characterized, scientists are just starting to identify some of the genes including members of the CslF and H gene families that may contribute to (1,3;1,4)-β-D-glucan synthesis (Burton and Fincher, 2012). These genes largely control the synthesis of polymers in the Golgi apparatus (Carpita and McCann, 2010), however, the enzymes necessary for linkage and expansion of larger chains are yet to be identified. Expression and enzymatic activity of the barley homologue are induced in response to auxin (Kotake *et al*., 2002). It was hypothesized that endo-1,3-1,4-β-D-

glucanases act via simultaneous hydrolysis and incorporation of new glucans into the cell wall during plant growth (Hoson *et al*., 1992; Thomas *et al*., 2000). The exact function of hypothetical protein 3 remains to be determined. Initial experiments are described in the appendix of this thesis.

Hypothetical protein 4 (Hyp4) contains a DUF538 domain. Its *Arabidopsis* homologue has been found to interact with VIT, a receptor modulator protein that is known to interact with brassinosteroid-receptor-like (BRL) proteins and predicted to play a role in signaling.70 However, the role of Hyp 4-like/DUF538 proteins in the signaling path remains unknown. The two latter hypothetical proteins are of particular interest because they may be new factors in the regulation or synthesis of cell wall or cuticle as they relate to growth.

Several studies looking at gene expression in growing *Arabidopsis* stems (Suh *et al*., 2005), corn epidermis (Nakazono *et al*., 2003), and CW proteins in *Arabidopsis* hypocotyls (Irshad *et al*., 2008) or epidermal membranes of rye (Deng *et al*., 2012) have identified a large number of genes/proteins with a possible role in CW synthesis or growth, yet, of the 12 proteins we investigated further (see Table 1), only lipoxygenase, wall associated kinase, UDPgdh, and LTPs have been found in one or more of those studies. The homologues to the newly identified hypothetical proteins Hyp3 and Hyp4 have not been described. This shows that despite the 1-D gel electrophoresis, the selection of bands that are intensified in the growing epidermis, led to the discovery of additional proteins.

2.4.2 Expression studies

Protein identification of 1D SDS-PAGE bands often yields more than one protein per band. Hence it is difficult to quantitatively assess their individual abundance. In addition, some less abundant proteins may escape discovery. Despite these shortfalls, our proteomics data still lead to the identification of four novel proteins of unknown function. To examine the differential expression of the genes encoding our proteins of interest, quantitative RT-PCR analysis was performed for lipoxygenase, UDPgdh, UDPgp, TCTP, PLTP, ATH, RGP, two hypothetical proteins (Hyp3 & 4) as well as two proteins that were identified with only one tryptic fragment. The latter were included because a) the fragment had been identified with a good score in two independent experiments, b) the mass of the protein band corresponded to the molecular weight of the protein band, and c) the literature suggests that they are involved in cell wall biosynthesis (sec15-homologue: S15L; fiber dTDP-glucose 4-6 dehydratase: FDG). Except for Hyp 3 and Hyp 4, these proteins are expected to be involved in cell expansion growth and, as a result, their gene expression should be increase also. Hence, they could serve as positive controls. Steady-state mRNA levels in epidermal peels from slow versus rapidly growing coleoptiles were determined using quantitative PCR (Fig. 2.5; Fig. S2.1 visualizes abundance of RT-PCR products):

2.4.3 Genes encoding proteins with a possible role in cutin biosynthesis:

We had detected several proteins associated with lipid metabolism: A lipoxygenase, an acyltransferase homologue (ATH), and three distinct lipid transfer proteins. While the expression of genes encoding ATH and PLTP was induced slightly to 150-200%, expression of the lipoxygenase gene was reduced to ca 50%, of the

expression in control samples. The reduction in lipoxygenase gene expression in rapidly growing coleoptiles correlates with the findings of Kutschera *et al*. (2010) who had observed an increase in gene expression during cessation of rye coleoptile growth. Thus, expression of the lipoxygenase gene appears to be inversely correlated with growth.

The function of the corn *ATH* is currently not known. Li *et al*. (2008) had identified an acyl-CoA: diacylglycerol acyltransferase, which is expressed in the *Arabidopsis* stem and is necessary for stem wax ester biosynthesis. However, the corn protein identified here shows little similarity to the acyltransferase found in *Arabidopsis* and, hence, may have a very different function in plant growth.

LTPs have long been candidates for wax transport through the cell wall. They are abundant in the epidermis, are secreted into the apoplast and can bind fatty acids (Samuels *et al*., 2008). Yet, the large number of LTPs in plants has made it difficult to identify their individual roles. Experimental evidence points to a wide array of possible functions for lipid transfer proteins: cutin synthesis (Pyee *et al*., 1994; Han *et al*., 2001; Kunst and Samuel, 2003; Samuels *et al*., 2008; Lee *et al*., 2009), β-oxidation, embryogenesis, allergenics, pollen adherence, plant signaling and plant defense (for a review see Carvalho and Gomes 2007). Several of their functions appear to be epidermis specific (Tchang *et al*. 1988, Arondel *et al*. 1991, Clark and Bohnert 1999). Auxin-mediated regulation of gene expression of acyltransferase (a,b), lipoxygenase (b), and several LTP homologs (a, b, c), has been previously shown in the epidermis of corn coleoptiles (a; Nakazono *et al*., 2003) and *Arabidopsis* stems (b; Suh *et al*., 2005) using microarray analysis and in a cell wall proteomics experiment in *Arabidopsis*

hypocotyls (c; Irshad *et al*., 2008). We only see a small up regulation of gene expression for PLTP (1.7 fold) and ATH (1.8 fold). To further investigate and confirm the role of PLTP in cell expansion growth, we determined its gene expression in coleoptiles that were moved from dark to light. When coleoptiles are exposed to light their growth ceases within a few days. PLTP gene expression was reduced in parallel to coleoptile growth cessation further indicating a role in cell expansion (Fig 2.6).

2.4.4 Genes encoding proteins with a possible role in cell wall biosynthesis

Since cell wall synthesis is necessary to maintain cell expansion growth, it would be expected that expression of genes encoding cell wall biosynthesis proteins is increased. We monitored the expression of several putative cell-wall biosynthesis genes in response to auxin as well as during the light-induced cessation of coleoptile growth. Indeed, quantitative RT- PCR showed that expressionof *UDPgp* and *UDPgdh*, was increased approximately two-fold, while *FDG* was increased almost threefold and *RGP* was increased fivefold upon treatment with auxin (Fig. 2.5; Table 2.1; sup. Fig. 2.1).

RGP is highly similar to the Golgi-associated se-wap41. It was previously isolated from maize mesocotyl cell wall and found to localize to plasmodesmata (Epel *et al*., 2005). RGPs have been shown to auto-glycosylate reversibly (Dhugga *et al*., 1991; Dhugga *et al*., 1997; Delgado *et al*., 1998; Testasecca *et al*., 2004). They are delivered to the cell wall/ plasmodesmata via the Golgi apparatus (Sagi *et al*., 2005) and have been shown to be involved in pollen development (Drakakaki *et al*., 2006) and cell wall (xyloglucan/ hemicellulose) biosynthesis in pea, cotton, and *Arabidopsis* (Delgado *et al*., 1998; Zhao and Liu, 2002). They are homologues to UDP-glucose:protein transglucosylases, a group of proteins also involved in cell wall biosynthesis (UPTGs;

Bocca *et al*., 1999). Several *Arabidopsis* RGPs function as UDP-Ara mutases with a role in heteroxylan synthesis (Rautengarten *et al*., 2011).

UDPgps catalyze the conversion of UTP and glucose-1-phosphate to UDP-glucose and pyrophosphate. UDPgp acts at the intersection between multiple pathways: it can provide the substrate for sucrose biosynthesis or participate in sucrose breakdown, it can be involved in the synthesis for the carbohydrate moiety of glycolipids and glycoproteins and it provides a substrate (UDP-glucose), which is directly or indirectly used for cell wall biosynthesis (for a review see Kleczkowski *et al*. 2004). *Arabidopsis ugp1/ugp2* double mutants showed little to no change in growth and cell wall composition but displayed reduced seed formation (Meng *et al*. 2009). In poplar, on the other hand, it was found up regulated in late cell expansion and secondary cell wall formation (Hertzberg *et al*. 2001).

A third protein from our list, UDPgdh, converts UDP-glucose to UDP-glucuronic acid, which is then converted to other precursors for both the pectin and the hemicellulose components of cell walls (Mohnen 2002; Seifert 2004). Localization studies in *Arabidopsis* led to the suggestion of an alternative UDP-glucuronic acid synthesis pathway via inositol in rapidly expanding hypocotyls (Seitz *et al*. 2000). However, Kärkönen *et al*. (2005) showed that while a reduction of the enzyme activity in transposon-tagged mutants showed a reduced pentose content in the cell wall, it had no impact on plant growth and development. Yet, it appears that at least one of the two isoforms of this enzyme in maize is necessary for polysaccharide biosynthesis. Quantitative RT-PCR showed that expression of all four cell wall related genes was increased upon treatment with auxin (Fig. 2.5).

To further confirm the correlation with growth, the expression of all three genes was monitored during the growth cessation of coleoptiles in response to light. *UDPgdh* and *RGP* showed a 50% reduction in gene expression within five days (Fig. 2.6). This finding was confirmed when the expression of the *Arabidopsis* homologue for the maize *RGP* (At5g16510) was studied in silico using the Bio-Array Resource for *Arabidopsis* Functional Genomics (http://bar.utoronto.ca/) - it indicated co-expression with UDP-glucose dehydrogenase (UDPgdh).

Finding UDPgp, UDPgdh and RGP increased in our rapidly growing samples and the fact that their expression is correlated with coleoptile growth confirms the known importance of these proteins for cell wall synthesis and as factors controlling growth.

2.4.5 Hypothetical proteins

The question remained whether the gene expression for two of the hypothetical proteins is associated with growth. Quantitative PCR showed that the *Hyp3* and *Hyp4* gene expression in response to auxin treatment was increased 2.5 and 1.7 fold, respectively (Fig. 2.5). To confirm the relevance of the two hypothetical proteins for cell and organ expansion we investigated their expression levels in several growing versus non-growing plant tissues using semi-quantitative PCR (Fig. 2.6 and 2.7). *Hyp 3* expression was higher in the epidermis than in total coleoptile tissue (Fig. 2.7a), suggesting the epidermis as predominant locus of action. As seedlings were moved to the light, the coleoptile stopped expanding while leaf expansion increased. In parallel, *Hyp3* expression was reduced to 68% of the dark expression within two days of the transition to light and to 40% of the dark expression levels after 5 days in the light (Fig. 2.6 & 2.7a; $p<0.02$). In leaves exposed to light, *Hyp3* expression increased almost two

fold as the leaf expanded (Fig. 2.7a). This suggests that it plays a role in the growth-controlling processes within the epidermis.

Hyp4 expression is induced 1.7-fold in auxin-treated coleoptiles and reduced by 50% in light-exposed, slow growing coleoptiles (Fig. 2.6 & 2.7b). It, too, showed reduced expression as the coleoptile growth ceased and increased expression levels in expanding leaves. Together with the observation that its expression is higher in coleoptile and leaves than in the coleoptile epidermis, this suggests that it may participate in more general, possibly regulatory or signaling processes during growth.

Both, *Hyp3* and *Hyp4* expression is increased in response to auxin. More importantly, their expression levels are reduced as coleoptile growth ceases and increased during leaf expansion. This suggests that they, indeed, play a role in cell expansion growth, possibly with Hyp3 in a synthetic and Hyp4 a regulatory function.

2.5 Conclusions

Our goal was the identification of novel proteins with a role in cell and organ growth. Using rapidly growing corn coleoptiles as a model system and a combination of proteomics and PCR analysis, we have been able to identify 15 proteins with known or suggested roles in trafficking or cutin- or cell wall- biosynthesis, including four novel proteins of unknown function. For nine of those, the up-regulation is apparent at the mRNA level. The expression of the genes encoding two of the novel and uncharacterized proteins, Hyp 3 and Hyp 4 is increased in rapidly growing coleoptiles and expanding leaves but reduced in coleoptiles that have ceased growing. Our findings suggest they are indeed associated with cell (and plant) growth. They provide new and additional tools for the understanding of whole plant growth and can now be studied for their effect on whole plant growth and their usefulness in the increased production of biomass.

Figure 2.1 Transmission electron micrograph of a cross section through a corn coleoptile epidermis cell treated with 30 µM IAA for 1 hour.

The osmiophilic particles (arrows) are present between the plasma membrane and the cell wall and within a secretory vesicle. The size bar corresponds to 200 nm.

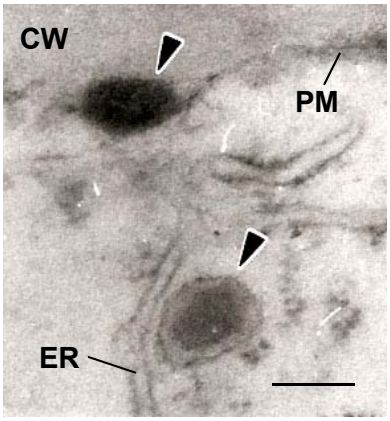


Figure 2.2 Flow chart of experimental setup for coleoptile treatment and harvest.

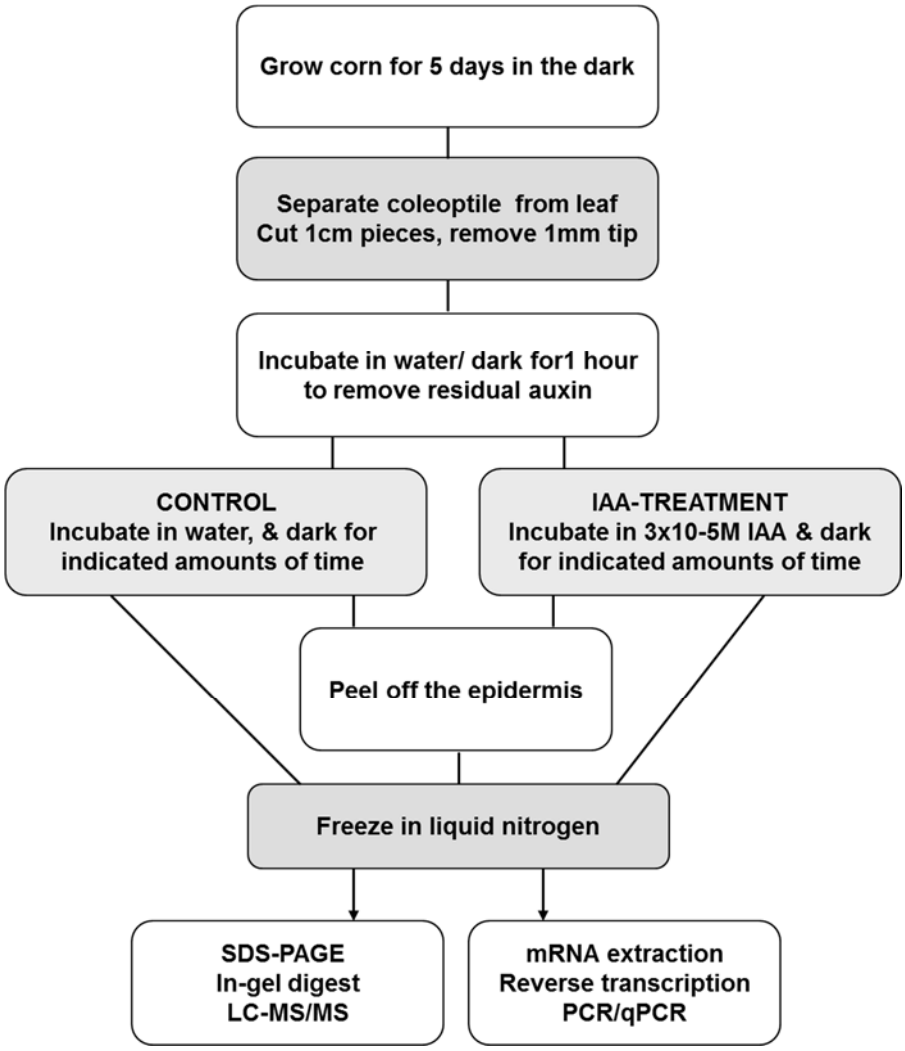
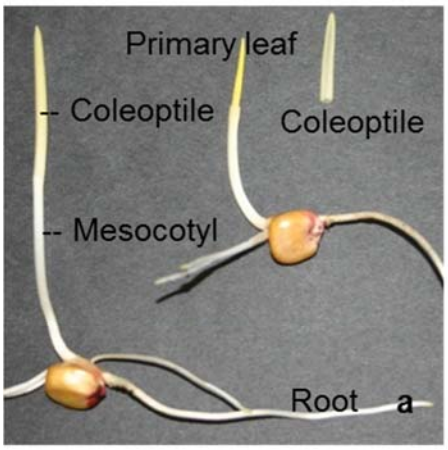
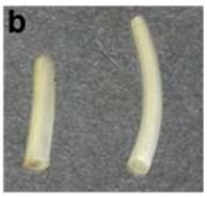
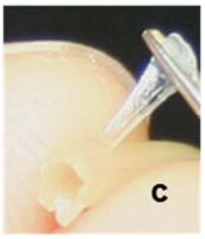


Figure 2.3 Auxin induced corn coleoptile rapidly growth.

Corn seedlings consist of a coleoptile, mesocotyl, primary leaf, and root (a). Size difference of auxin-depleted coleoptiles after incubation in water (slow-growing, left) or auxin (rapidly-growing, right) for four hours (b). Measurements indicate coleoptile length after four hours in either water (control) or 30μM IAA. Data are mean and SE obtained from 45 coleoptiles. c) Preparation of epidermal peel as used in our experiments.







Segment Length (mm):

IAA: 13.0 ± 0.9

Co: 10.8 ± 0.5

Figure 2.4a SDS-PAGE gel comparison of banding patterns.

The marked bands were selected for LC-ESI-MS because they showed differences between the treatments (Co-Control, IAA-auxin; arrow) and/or the sample site (coleoptile, epidermis; asterisk)

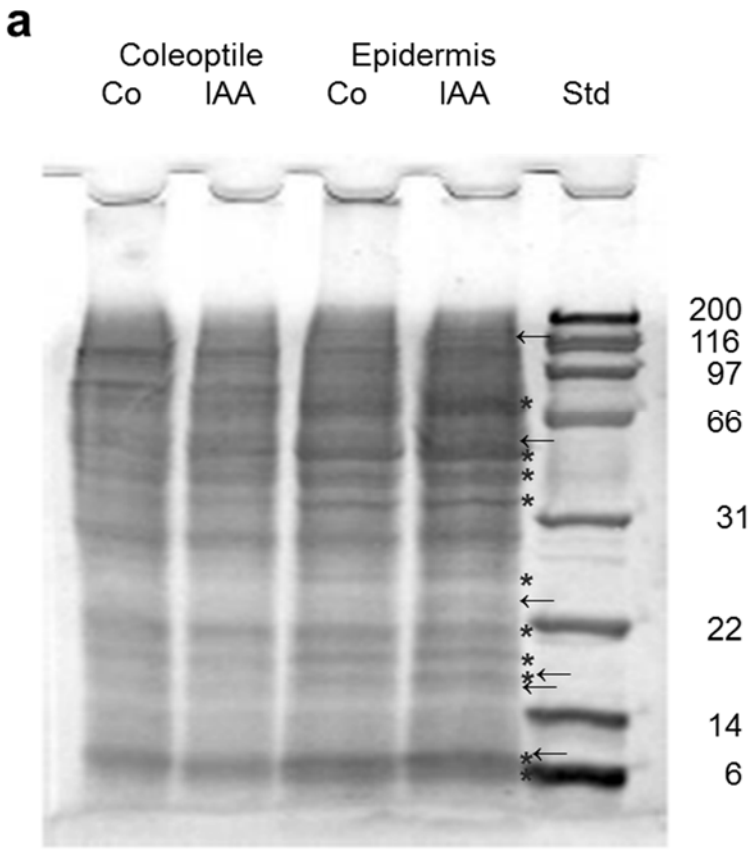


Figure 2.4b Grouping of proteins enhanced in the epidermis as compared to the coleoptile and upon auxin-induction based on their predicted function/ involvement in cellular processes:
(A) 11% Secretory pathway, cell wall and cuticle associated. (B) 13% Carbon metabolism/ETC. (C) 7% Structural/cell division. (D) 7% Others-oxidoreducatase, mutases, etc. (E) 7% Unknown/hypothetical proteins. (F) 23% Protein synthesis/degradation. (G) 16% Transcription/translation, splicing

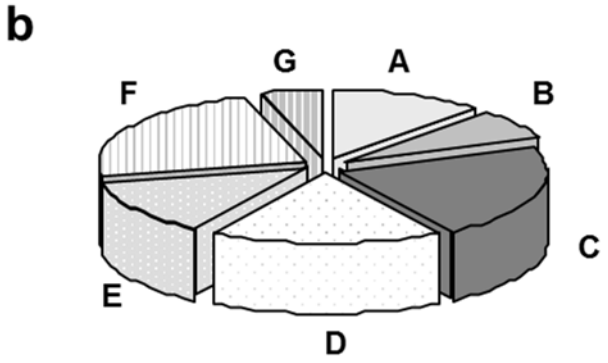


Figure 2.5 Differential expression of auxin-induced genes in the corn coleoptile using quantitative RT-PCR.

"Induced": coleoptiles were incubated in 30μM IAA for four hours; "Control": coleoptiles incubated in water for four hours. STD:18S mRNA; Lipo: Lipoxygenase; UPDgd: UDP-glucose dehydrogenase; UDPgP: UDP-glucose pyrophosphorylase; TCTP: translationally-controlled tumor protein; PLTP: phospholipid transfer protein; RGP: reversibly glycosylated protein; ATH: Acyl transferase; HP3/4: hypothetical proteins 3 and 4; S15L: sec 15-like protein; FDG: Fiber dTDP-glucose 4-6 dehydratase; Values are the mean and standard error for 9 replicates (three biological, three technical replicates, each).

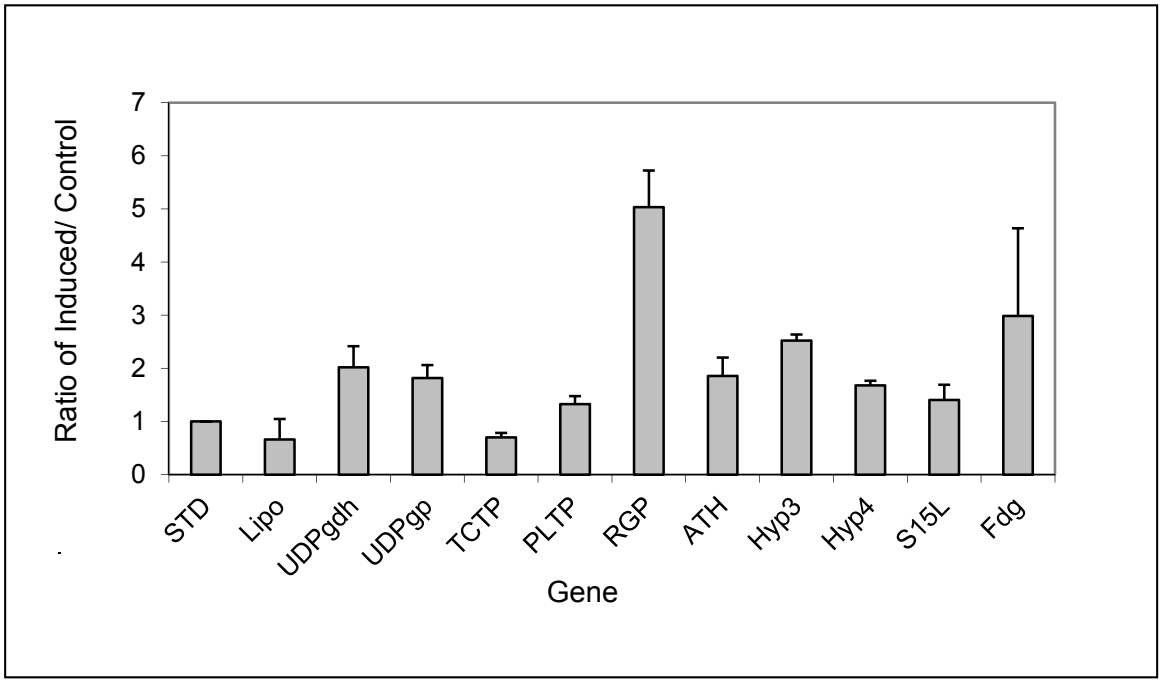


Figure 2.6 Semiquantitative analysis of the expression levels of genes encoding UDPgdh, UDPgp, Hyp 4, Hyp 3, PLTP, and RGP in corn coleoptiles (a) and leaf (b) after the transition of dark to light.

Bars represent the mean ± S.E. of three biological replicates. Statistical analysis (t test) revealed significant reduction compared with the day 0 time point ($p < 0.1$: *, $p < 0.01$:**).

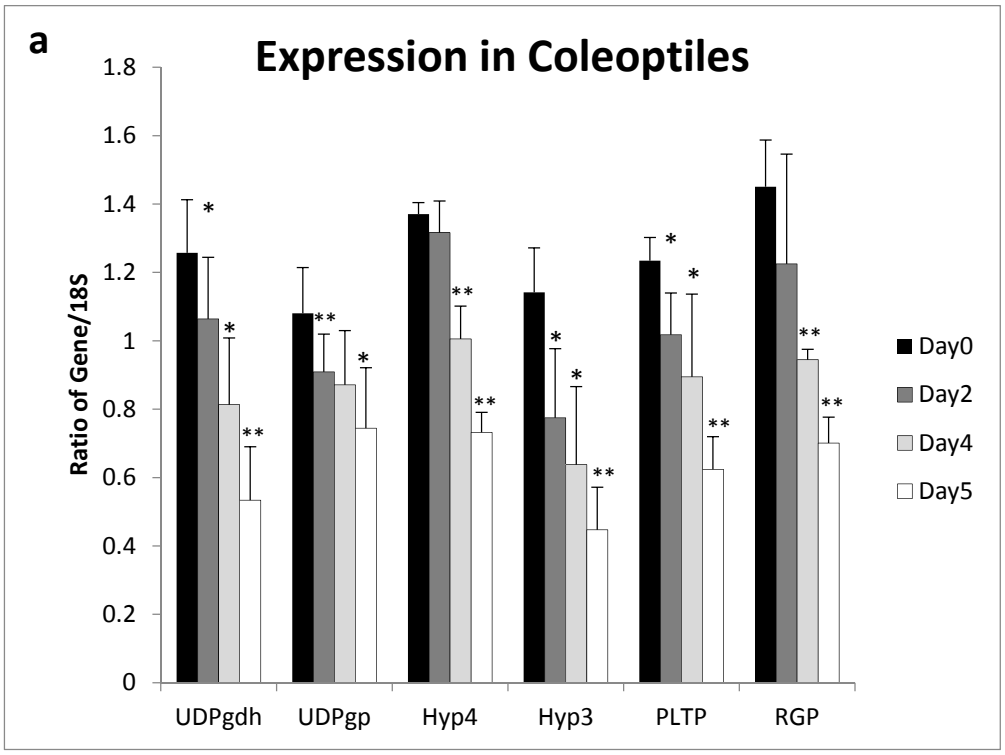


Figure 2.6 (cont'd)

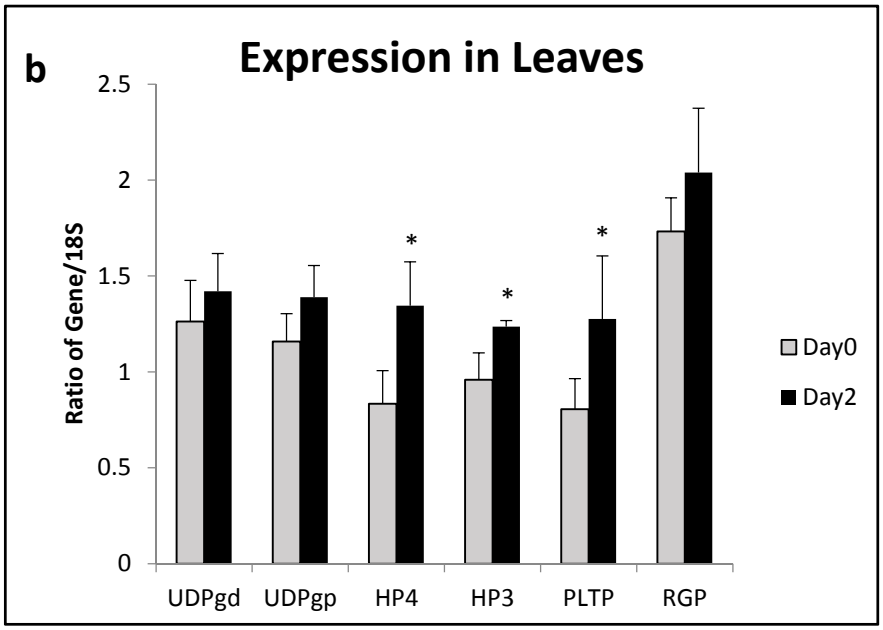


Figure 2.7 Expression levels of genes encoding Hyp 3 (a) and Hyp 4 (b) in various corn seedling organs as determined by RT-PCR.

“Epidermis” corresponds to the epidermis of coleoptiles of seedlings germinated and grown in the dark for five days; Material for coleoptiles was harvested either after five days in the dark (dark) or 1, 2, or 5 days after transition of the 5-day-old, dark-grown seedling to light. Leaf samples were taken either from the leaf within the coleoptile of the 5-day-old, dark-grown seedling (undeveloped leaf) or two days after transition to light (2 days light). Bars represent the mean +/- S.E. of 3 biological replicates.

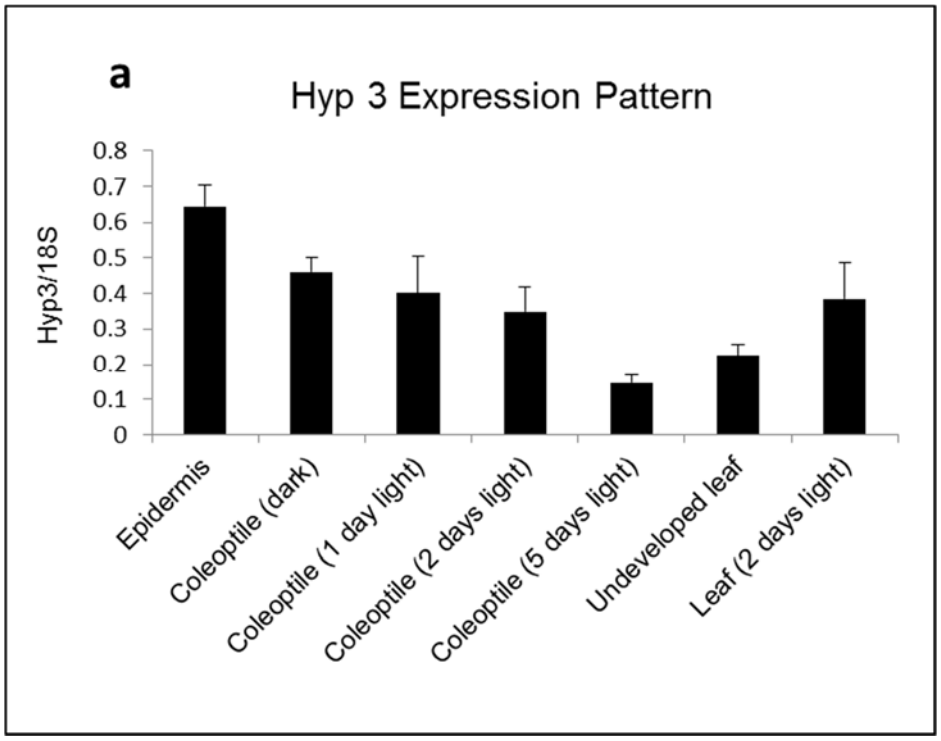


Figure 2.7 (cont'd)

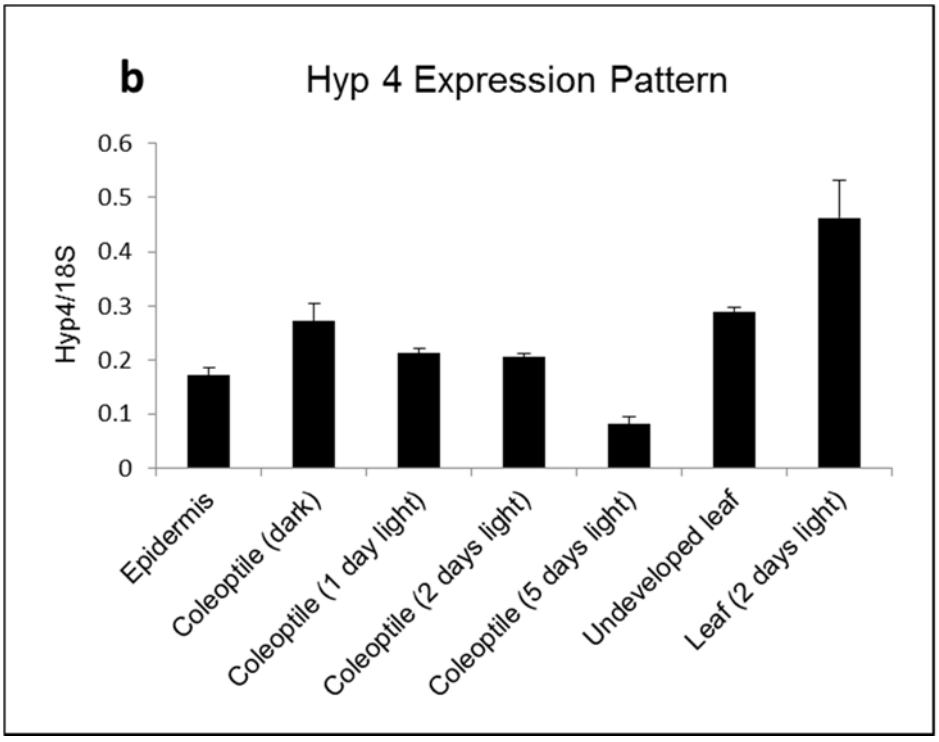


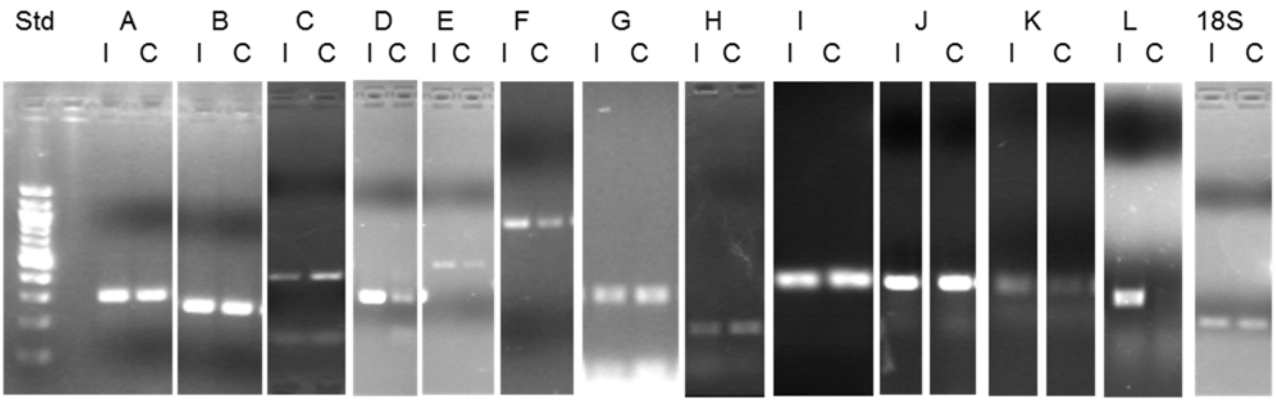
Table 2.1: Auxin-regulated epidermal proteins with functions related to secretory pathway, cell wall or cuticle metabolism with comparison of RT-PCR to microarray data.

Arrows indicate induction or reduction; U: no significant difference to control; NA: not analyzed: no oligonucleotides present on the microarray chip. Similar proteins which are up-regulated in the epidermis of top stems in Arabidopsis (Suh et al., 2005).

Protein	Access #	MW	qPCR	Possible Function
Sec15-like	BAB93424	105	↑	Vesicle trafficking
Lipoxygenase[1]	AAF76207	98	↓	Regulator of lipid-based biochemical and signal transduction pathways
Wall-associated kinase 4 like[1]	AAF18509	74	↑	ATP binding / calcium ion binding protein kinase
UDP-glucose dehydrogenase	AAK16194	65	↑	Conversion of UDP-glucose to UDP-glucuronic acid
UDP-glucose pyrophosphorylase	AAP86317	58	↑	Reversible production of UDPglucose and pyrophosphate (PPi) from Glc-1-P and UTP
Acyltransferase homolog	BAA93453	56	↑	Possibly lignin biosynthesis
Reversibly glycosylated polypeptide	AAM65020	41	↑	Biosynthesis of hemicellulose, may use UDP-glucose as substrate
Hypothetical protein #3	CAC21955	29	↑	possible (1,3;1,4) -β-D-endo glucanase
Hypothetical protein #4	BU571995	22	↑	Unknown; similar to rice NP_00106738 with DUF538
Fiber dTDP-glc 4-6 dehydratase	AAR07660	21	↑	Unknown
TCTP-like	AAN40686	19	↓	Ca binding, present in secretions
Phospholipid transfer protein A	AAB06443	12	↑	Lipid transfer

Figure S2.1 PCR products obtained from induced (I) and control (C) coleoptile epidermis.
The table (b) displays mean and standard error of the pixilation intensity comparisons between the gene of interest and the control 18S for 6-8 replicates [(IAA/18S)/ (Co/18S)]. A: RGP/ reversibly glycosylated protein; B: Hyp 4; C: Lipoxygenase; D: PLTP ; E: TCTP/ translationally-controlled tumor protein; F: UDPgp/ UDP-glucose pyrophosphorylase ; G: WAK/ wall associated kinase; H: Sec15-like; I: FDP/ Fiber dTDP-glucose 4-6 dehydratase; J: UDPgdh/ UDP-glucose dehydrogenase; K: ATH/ Acyl transferase; L: Hyp 3; 18S

a



b Protein name	Accession #	(IAA/18S) (Co/18S)
A RGP	AAM65020	1.09 + 0.32
B Hyp 4	BU571995	0.92 + 0.31
C Lipoxygenase	AAF76207	0.58 + 0.07
D PLTP	AAB06443	1.74 + 0.21
E TCTP	AAN40686	0.71 + 0.04
F UDP-gp	AAP86317	1.54 + 0.21
G WAK	AAF18509	1.12 + 0.36
H Sec15-like	BAB93424	0.87 + 0.45
I FDG	AAR07660	1.01 + 0.58
J UDP-gdh	AAK16194	1.31 + 0.53
K Acyltransferase	BAA93453	1.35 + 0.77
L Hyp 3	CAC 21955	2.47 + 0.95

Table S2.1: Primers used for quantitative (a) and semiquantitative (b) RT-PCR;

a Protein	Primers	Access # CDS
Reversibly glycosylated polypeptide	F' ATCTCATTGGCCCTGCTATG R' GTCTTGACTCCCAGGCTCAG	AY087476
Translationally controlled tumor-like protein	F' GCCGTGAAGGTGGTTGATAT R' GCTTTCTCTGGCTCCAACAC	AF548024
Lipoxygenase	F' CAACACCTCCGACACGAAGG R' GGGGAAACGCAAACAATCTA	AF271894
Phospholipid transfer protein	F' CCTGCAACTGCCTCAAGAAC R' AGTCGGTGGAGGTGCTGAT	ZMU66105
UDP-Glucose-pyrophosporylase	F' GCTCAAGGTTTCTCCCAGTG R' TGAAGGGTTAGCTGGATTCG	AY260746
Sec 15	F' TGAATGTCCTTGCATTTGGA R' GTGCCATGAAGCTCACAGAA	AP003764
Acyltransferase	F' CTACTTCGGCAACCTCATCC R' TACTCCTCCAGCCTCCTGGT	AB026495
UDP-Glucose dehydrogenase	F' GATCCTCACCACCAACCTGT R' CGTAAGCCACCTCAGTCACA	AC079887
Fiber dTDP-glucose 4-6-dehydratase	F' GTTCAAAGTCTCCGCTCTGC R' ACCCTACGATGTTCGACTGG	AY378100
Wall-associated kinase-like 4	F' CAGACTCACCTCTTCAGCTACG R' GTTGTTGTACAGCCGCTTC	T24D18
Hypothetical Protein 2	F' GGAGCTTCTCATCTGGGTCA R' GTTCCACTTCCTCTGGGACA	DQ246015 (mRNA)
Hypothetical Protein 3	F' CACGAGGTTCCCTACTACGC R' TCAGTTGAGGTGCTTGTTGAA	AX053124
Hypothetical Protein 4	F' GGGTAAGCTCCACTGCAAGG R' GTTCTTGTCCCCCTCCTCCT	BU571995

b Protein	Primers	Amplicon size
Reversibly glycosylated polypeptide	F' GGTGCTGACTTTGTGCGTGG R' CCCACATGTCGTCGTAGCG	~303 bps
Translationally controlled tumor-like protein	F' CCTATCTCCTATCTGGCG R' GGTTGCACCCTCGACACCC	~393 bps
Lipoxygenase	F' CATCGGCGAGTTCCTCGG R' GGGAGCTCCTTGGTGCCG	~530 bps
Phospholipid transfer protein	F' CGGCCATCAGCTGCGGGC R' GGAGGTGCTGATGGTGTAGG	~ 260 bps
UDP-Glucose-pyrophosporylase	F' CGCTGGCTGACGTTAAGGG R' GGATGGACTTGAACCGAGC	~ 490 bps
Sec 15	F' CTTCTCACGGATCACTGG R' GCCCACATCTCTGAAAGG	~250 bps
Acyltransferase	F' GCAGCAGGCTTATGCTGC R' GATCTGGAATGGGGGCAG	~154 bps
UDP-Glucose dehydrogenase	F' GAAGAGTAGCCACACACCG R' GCTGTTATGGGCGTTGGTC	~300 bps
Fiber dTDP-glucose 4-6-dehydratase	F' GCTCCCACCCTAGAACTTCC R' GAGCTTCTGCTATGTCGCCG	~235 bps
Wall-associated kinase-like 4	F' GTTGTTGTACAGCCGCTTC R' CCATGGTGTTGTTCACTGC	~456 bps
Hypothetical Protein 4	F' GCAAGGTGGACCACTACTTCG R' CGTCCCTGGGAACGACACC	~279 bps

Table S2.2: Proteins identified in the outer epidermis of corn coleoptiles; samples in bold were from bands which appeared induced upon treatment with auxin.

Protein Identification	Access #	Trp frag.	MW	Localization/Function Sequest/ Sequence Charge state (Z)
ASR1	AAG28426	2	98	Carbon-oxygen lyase; cytosol; hydrolase *3.43 (R)SGVTATDLVLTVTQM*LR Z=2* *3.25 (K)AFEDADSLGLTGHER Z=2*
Elongation factor 2 (putative) rice	NP_001046972 NP_001052057 (At1g45332)	2	9 4	Elongation factor; GTP-binding; protein biosynthesis; *3.23 (K)AFLPVIESFGFSSQLR Z=2* *3.03 (R)LYMEARPLEEGLAEAIDDGR Z=2*
Phospholipase D maize	NP_001105686 =Q43270 (At3g15730)	5	9 2	Phosphoric diester hydrolase; Ca-binding; *3.04 (K)VTLYQDAHVPDNFVPR Z=2* *2.91 (R)TLDTVHHDDFHQPNFEGGSIK Z=3* *2.76 (R)DSEIAM*GAYQPYHLATR Z=2* *2.48 (R)NPDDSGSFVQDLQISTM*FTHHQK Z=3* *2.30 (R)YDTQYHSLFR Z=2*
Lipoxygenase **maize**	**NP_001105003** **=AAF76207** **=LOX** **(At1g55020** **)**	**9**	**9** **8**	**Key regulator of lipid-based biochemical and signal transduction pathways** *4.26 (R)GDGTLVPVAIELSLPELR Z=2* *3.09 (K)LYEGGIQLPK Z=2* *3.06 (K)LEGLTVQQALHGNR Z=2* *2.90 (K)YGDHTSTITAEHIEK Z=2* *2.59 (R)GMAVADPSSPYK Z=2* *2.31 (R)VNSLEGNFIYATR Z=2* *70 (K)DWNFTEQALPDDLIK Z=2* *63 (K)YGDHTSTIR Z=2*
Methionine synthase **maize**	**AAL33589** **=LOC54194** **2**	**1** **6**	**8** **4**	**Cytosol, catalyze the formation of methionine by the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine** *3.63 (K)YGAGIGPGVYDIHSPR Z=2* *3.54 (K)KLNLPILPTTTIGSFPQTVELR Z=2* *3.30 (R)GTQTLGLVTSAGFPAGK Z=2* *3.17 (R)YLFAGVVDGR Z=2* *3.13 (K)YIPSNTFSYYDQVLDTTAMALGAVPER Z=2* *2.88 (K)GMLTGPVTILNWSFVR Z=2* *2.84 (K)ALAGQKDEAYFAANAAAQASR Z=2* *2.73 (K)YTEVKPALTNMVSAAK Z=2* *2.70 (K)ISEEEYVTAIKEEINK Z=2* *2.68 (R)KYTEVKPALTNMVSAAK Z=2* *2.63 (R)IPSAEEIADR Z=2* *2.57 (K)ALGVDTVPVLVGPVSYLLLSKPAK Z=3*

Table S2.2 (cont'd)

					2.21	*(R)VLNTGSPITVPVGR*	*Z=2*	
					2.18	*(R)IINVIGEPIDEK*	*Z=2*	
UDP-glucose pyrophosphoryl ase poplar		**AAP86317 (At5g17310)**	**4**	**5 8**	**Stress-responsive, provides substrate for the reversibly glycosylated polypeptide**			
					3.93	*(K)VLQLETAAGAAIR*	*Z=2*	
					2.77	*(R)ANPANPSIELGPEFK*	*Z=2*	
					2.25	*(K)LDILLAQGK*	*Z=1*	
					2.06	*(K)YSNSNIEIHTFNQSQYPR*	*Z=2*	
Acyltransferase homolog petunia		**BAA93453**	**3**	**5 6**	**Phospholipid biosynthesis**			
					3.06	*(R)RPLLAVQFTK*	*Z=2*	
					2.56	*(R)VRLELPASAEAHEK*	*Z=2*	
					2.06	*(R)GVPLSLQPIHDR*	*Z=2*	
Adenosylhomocysteinase-like p r		**CD439905**	**4**	**5 3**	**Linked to the cyclin-mediated regulation of the cell cycle**			
					3.01	*(K)IPDPEST DNAEFK*	*Z=2*	
					2.69	*(K)VAVVCGYGDVGK*	*Z=2*	
					2.63	*(R)LVGVSEETTTGVK*	*Z=2*	
					3.30	*(R)WVFPETNTGILVLAEGR*	*Z=2*	
Glyceraldehyde 3-P-dehydrogenase maize		**XP_479895 CAA336 20 P0873 5**	**8**	**5 3**	**Oxidoreductase**			
					5.11	*(K)VIHDNFGIIEGLMTTVHAITATQK*	*Z=3*	
					4.11	*(K)VIHDNFGIIEGLM*TTVHAITATQK*	*Z=3*	
					3.17	*(R)VPTVDVSVVDLTVR*	*Z=2*	
					3.15	*(R)NPEEIPWGEAGAEYVVESTGVFTDKDK*	*Z=2*	
					3.00	*(K)TLLFGEKPVTVFGIR*	*Z=2*	
					2.92	*(K)AGIALNDHFVK*	*Z=2*	
					2.66	*(K)YDTVHGQWK*	*Z=2*	
Acid invertase maize		**CAD91358**	**2**	**5 3**	**Sucrose cleavage**			
					3.27	*(R)VLVDHSIVESFAQGGR*	*Z=2*	
					2.51	*(K)GLDGSLATHFCQDESR*	*Z=2*	
Beta-7 tubulin maize		**AAA19708 (At2g29550)**	**1 6**	**5 1**	**Structural protein/involved in cell divison**			
					133	*(K)GHYTEGAELIDSVLDVVR*	*Z=2*	
					92	*(R)AVLMDLEPGTMDSVR*	*Z=2*	
					80	*(K)EVDEQM*INVQNK*	*Z=2*	
					78	*(K)YAGDSDLQLER*	*Z=2*	*
					76	*(R)VSEQFTAMFR*	*Z=2*	
					72	*(R)KLAVNLIPFPR*	*Z=2*	
					64	*(R)YLTASAMFR*	*Z=2*	
					64	*(K)LAVNLIPFPR*	*Z=2*	
					60	*(R)LHFFMVGFAPLTSR*	*Z=2*	
					57	*(R)FPGQLNSDLR*	*Z=2*	
					52	*(K)NMMCAADPR*	*Z=2*	
					51	*(R)RVSEQFTAMFR*	*Z=2*	
					40	*(K)SSVCDIPPIGLK*	*Z=2*	*
					40	*(K)NSSYFVEWIPNNVK*	*Z=2*	
					37	*(K)IREEYPDR*	*Z=2*	

Table S2.2 (cont'd)

Beta-5 tubulin **maize**	**CAA52720**	**17**	**5** **0**	**Structural protein/involved in cell divison** *133 (K)GHYTEGAELIDSVLDVVR Z=2* *105 (R)ALTVPELTQQMWDAK Z=2* *92 (R)AVLMDLEPGTMDSVR Z=2* *80 (K)EVDEQM*INVQNK Z=2* *76 (R)VSEQFTAMFR Z=2* *72 (R)KLAVNLIPFPR Z=2* *69 (R)YVGTSDLQLER Z=2 ** *64 (R)YLTASAMFR Z=2* *64 (K)LAVNLIPFPR Z=2* *60 (R)LHFFMVGFAPLTSR Z=2* *57 (R)FPGQLNSDLR Z=2* *52 (K)NMMCAADPR Z=2* *51 (R)RVSEQFTAMFR Z=2* *46 (K)LTTPSFGDLNHLISATMSGVTCCLR Z=3*
Beta-6 tubulin **maize**	**AAA20186** **(At5g23860)**	**18**	**5** **0**	**Structural protein/involved in cell divison** *133 (K)GHYTEGAELIDSVLDVVR Z=2* *105 (R)ALTVPELTQQMWDAK Z=2* *92 (R)AVLMDLEPGTMDSVR Z=2* *80 (K)EVDEQM*INVQNK Z=2* *76 (R)VSEQFTAMFR Z=2* *72 (R)KLAVNLIPFPR Z=2* *65 (R)EILHIQGGQCGNQIGAK Z=2 ** *64 (R)YLTASAMFR Z=2* *64 (K)LAVNLIPFPR Z=2* *60 (R)LHFFMVGFAPLTSR Z=2* *57 (R)FPGQLNSDLR Z=2* *52 (K)NMMCAADPR Z=2* *51 (R)RVSEQFTAMFR Z=2* *50 (K)SSVCDIPPR Z=2* *46 (K)LTTPSFGDLNHLISATMSGVTCCLR Z=3* *43 (R)YTGNSDLQLER Z=2 ** *40 (K)NSSYFVEWIPNNVK Z=2* *37 (K)IREEYPDR Z=2 **
Elongation factor 1α **maize**	**BAA08249** **(At1g07920)**	**4**	**5** **0**	**Translation elongation factor; protein synthesis** *72 (K)IGGIGTVPVGR Z=2* *60 (K)YYCTVIDAPGHR Z=2* *58 (R)VETGVIKPMVVTFGPTGLTTEVK Z=3* *43 (K)STTTGHLIYK Z=2*
Alpha1-tubulin **maize**	**CAA33734** **At1g63740**	**11**	**5** **0**	**Structural protein** *123 (R)FDGALNVDVNEFQTNLVPYPR Z=2* *100 (K)TIGGGDDAFNTFFSETGAGK Z=2* *98 (R)LVSQVISSLTASLR Z=2*

Table S2.2 (cont'd)

				89	*(K)CGINYQPPSVVPGGDLAK*	*Z=2*	*
				88	*(R)AVFVDLEPTVIDEVR*	*Z=2*	*
				79	*(R)TIQFVDWCPTGFK*	*Z=2*	
				75	*(K)DVNAAVATIK*	*Z=2*	
				74	*(R)IHFMLSSYAPVISAEK*	*Z=2*	
				43	*(R)QLFHPEQLISGK*	*Z=2*	
				42	*(K)AYHEQLSVAEITNSAFEPSSMMAK*	*Z=2*	
Alpha-tubulin #3 **Maize**	**CAA44861 (At5g19970)**	**7**	**50**	**Structural protein**			
				123	*(R)FDGALNVDVNEFQTNLVPYPR*	*Z=2*	
				98	*(R)LVSQVISSLTASLR*	*Z=2*	
				98	*(R)AIFVDLEPTVIDEVR*	*Z=2*	
				79	*(R)TIQFVDWCPTGFK*	*Z=2*	
				75	*(K)DVNAAVATIK*	*Z=2*	
				43	*(R)QLFHPEQLISGK*	*Z=2*	
Alpha-tubulin #5 **Maize**	**CAA44862 At5g19780**	**6**	**50**	**Structural protein**			
				105	*(R)LISQIISSLTTSLR*	*Z=2*	*
				98	*(R)AIFVDLEPTVIDEVR*	*Z=2*	*
				89	*(K)CGINYQPPSVVPGGDLAK*	*Z=2*	
				75	*(K)DVNAAVATIK*	*Z=2*	
				74	*(R)IHFMLSSYAPVISAEK*	*Z=2*	
				43	*(R)QLFHPEQLISGK*	*Z=2*	
Tublin alpha-2/alpha-4 chain ***Arabidopsis***	**At1g04820**	**6**	**50**	**Structural protein**			
				98	*(R)LVSQVISSLTASLR*	*Z=2*	*similar to maize α-tubulin 1*
				88	*(R)AVFVDLEPTVIDEVR*	*Z=2*	
				79	*(R)TIQFVDWCPTGFK*	*Z=2*	
				74	*(R)IHFMLSSYAPVISAEK*	*Z=2*	
				43	*(R)QLFHPQLISGK*	*Z=2*	
UDP-glucosyltransferase BX9 **Maize**	**AAL57038 (At5g05800)**	**4**	**50**	**Detoxification of benzoxazinoids in maize; signal transduction and carbohydrate transport and metabolism in *Arabidopsis***			
				99	*(K)LVPTATASLHGVVQADR*	*Z=2*	
				82	*(R)GFESGALPDGVEDEVR*	*Z=2*	
				45	*(R)TDLTDLVDLIK*	*Z=2*	
				40	*(K)ALSVPVFAVAPLNK*	*Z=2*	
UDP-glucosyltransferase BX8 **Maize**	**AAL57037 (At5g05900)**	**3**	**49**	**Detoxification of benzoxazinoids in maize; signal transduction and carbohydrate transport and metabolism in *Arabidopsis***			
				116	*(R)LSALLSAADGEAGEAGGR*	*Z=2*	
				65	*(R)GVGITVFHTAGAR*	*Z=2*	
				63	*(K)VGTEVAGDQLER*	*Z=2*	
Enolase **Maize**	**CAA39454 (At2g36530)**	**7**	**48**	**2-phospho-D-glycerate hydrolase; activity increase twice its initial level after 48 hours of anaerobiosis;** Catalyzes the conversion of phosphoglercerate to phosphopyruvate, only dehydration step in glycolytic pathway			
				2.63/ 92	*(K)VNQIGSVTESIEAVR*	*Z=2*	
				2.25	*(R)GNPTVEVDVGLSDGSYAR*	*Z=2*	

Table S2.2 (cont'd)

				2.16/ 119 (R)GAVPSGASTGIYEALELR Z=2 *2.02 (K)IPLYQHIANLAGNK Z=2* *133 (R)IEEELGDAAVYAGAK Z=2* *69 (K)AVSNVNNIIGPAIVGK Z=2*
Translation EF-TuM	AAG32661 (At4g02930)	3	4 8	Elongation factor; mitochondrial protein synthesis *79 (R)GSALSALQGNNDEIGK Z=2* *62 (K)TGEDVEILGLAQTGPLK Z=2* *60 (K)AVAFDEIDKAPEEK Z=2*
Translation initiation factor eIF-4A **Maize**	**AAB67607** **(At3g13920)**	**8**	**4** **7**	**Protein synthesis** *95 (K)FYNVLIEELPANVADLL Z=2* *87 (R)GIYAYGFEKPSAIQQR Z=2* *77 (R)KGVAINFVTR Z=2* *74 (K)GLDVIQQAQSGTGK Z=2* *61 (K)RDELTLEGIK Z=2* *57 (K)VHACVGGTSVR Z=2* *55 (R)DHTVSATHGDMDQNTR Z=2* *50 (R)SRDHTVSATHGDMDQNTR Z=2*
S-adenosylmethionine synthetase	**At1g02500** **At3g17390**	**3**	**4** **3**	**Synthesis of AdoMet by donating a methyl group** *71 (R)FVIGGPHGDAGLTGR Z=2* *52 (K)VLVNIEQQSPDIAQGVHGHFTK Z=3*
Pyruvate dehydrogenase E1 alpha subunit maize	AAC72195 (At1g59900)	4	4 3	Oxidative decarboxylation of pyruvate *81 (R)DVTTTPAELVTFFR Z=2* *75 (K)ESSMPDTSELFTNVYK Z=2* *43 (R)TRDEISGVR Z=2* *38 (R)YHGHSM*SDPGSTYR Z=3*
Calreticulin **maize**	**CAA54975**	**3**	**4** **3**	**Ca-binding protein (coats vesicles)** *2.98 (K)SGTLFDNIIITDDPALAK Z=2* *2.29 IGIELWQVK Z=2*
Hypothetical protein 1	At3g15000	2	43	Mitochondrial; unknown function; similar to At1g53260 *74 (K)TLAQVVGSEEEAR Z=2*
Adenine nucleotide translocat **or maize**	**CAA33743** **(At4g28390)** **(At3g08580)**	**11**	**4** **2**	**Mitochondrial ADP/ATP carrier protein 1 or 2** *93 (R)AIAGAGVLSGYDQLQILFFGK Z=2* *70 (R)TIKDEGFSSLWR Z=2* *59 (R)QFDGLVDVYR Z=2* *56 (K)SSLDAFKQILK Z=2* *54 (R)MMMTSGEAVK Z=2* *51 (R)MMM*TSGEAVK Z=2* *50 (R)YFPTQALNFAFK Z=2* *47 (K)LLIQNQDEMIK Z=2* *38 (R)MM*M*TSGEAVK Z=2* *32 (K)LLIQNQDEM*IK Z=2*
Phytase	**CAA11391**	**4**	**4**	**Phosphoric monoester hydrolase in developing seed**

Table S2.2 (cont'd)

maize				*5.48 (R)AHYDALAVADPAANVEGLAAEASEYK Z=3* *3.83 (K)VADETTKPAIQEDGAESK Z=2* *2.84 (K)GEEFEDLDDTQKLEVYNSIIVESGR Z=2* *2.18 (K)LLDDSMAAR Z=2*
Reversibly glycosylated polpepti de maize	**AAB49896 (At5g15650)**	**4**	**4 1**	**Role in biosynthesis of hemicullulosic polysaccharides(cell wall), may use UDP-glucose as substrate; high similarity to Golgi-associated proteins se- wap41:** Isolated from maize mesocotyl cell wall; immunolocalizes to plasmodesmata; α-1,4- glucan-protein synthase *2.25 (R)EGAHTAVSHGLWLNIPDYDAPTQLVKPK Z=2* *2.16 (K)NAAYIGTPGK Z=1*
Herbicide safener binding protein/o-methyltransferase maize	**AAC12715 (At4g35160)**	**15**	**4 0**	**Protects against injury from chloroacetabulude & thiocarbamate herbicides** *7.15 (132) (R)HGGAASAAELVTALSLPSTK Z=2* *4.00 (67) (K)LVLHHLTDEECVK Z=2* *3.47 (R)CAVELGIPTAIYR Z=2* *2.83 (R)YIEAGIGLAEWFK Z=1* *3.59 (R)QRDEKEWSELFTK Z=2* *2.99 (76) (K)LLAASGVFTVDK Z=2* *2.94 (R)ISPVSYLLVDGIPHEDKMNKTALVLTCTSTR Z=2* *2.92 (R)YIEAGIGLAEWFK* *2.39 (K)IIATKPADGAKMINYVEGDMFSFIPPAQTVVLK Z=2* *2.27 (K)CTVLAPPK Z=2* *2.23 (K)AQADIWR Z=2* *2.12 (R)DEKEWSELFTK Z=1* *2.05 (K)IIATKPADGAK Z=2* *42 (K)LLAQCR Z=2*
Fructose bisphosphate aldolase maize	**CAA31366 (At2g36460)**	**6**	**3 9**	**Gluconeogensis, glycolysis, pentose phosphate pathway** *3.25 (K)ALNEHHVLLEGTLLKPNMVTPGSDSK Z=3* *2.59 (K)GTIEVVGTDKETTTQGHDDLGKR Z=2* *2.48 (K)VDKGTIEVVGTDKETTTQGHDDLGKR Z=3* *2.27 (K)GILAADESTGTIGKR Z=2* *56 (K)GDAADTESLHVK Z=2*
Actin 2/7 maize	**AAB40106 (At5g09810)**	**3**	**3 7**	**Protein binding/structural protein** *2.89 (R)VAPEEHPVLLTEAPLNPK Z=2* *101 (K)SYELPDGQVITIGAER Z=2* *68 (R)TTGIVLDSGDGVSHTVPIYEGYALPHAILR Z=3*
Probable Glyceraldehyde-3-phosphate dehydrogenase maize	**CAA30151 (At3g04120)**	**9**	**3 7**	**Probable Glyceraldehyde-3-phosphate dehydrogenase C1; Glycolysis/ gluconeogenesis** *104 (R)VPTVDVSVVDLTVR Z=2* *71 (K)TLLFGDKPVTVFGIR Z=2* *68 (K)YIHDNFGIVEGLMTTVHAITATQK Z=3 ** *58 (K)HSDITLK Z=2 ** *53 (K)VIHDNFGIIEGLMTTVHAITATQK Z=3 **

Table S2.2 (cont'd)

				*48 (K)KVISAPSK Z=2 ** *47 (K)AGIALNDHFVK Z=2 ** *39 (K)YDTVHGHWK Z=2* *42 (K)GASYEDIKK Z=2*
Glyceraldehyde-3-phosphate dehydrogenase GAPC3 **maize**	**AAA87579 (At1g13440)**	**12**	**3** **6**	**Glycolysis/ gluconeogenesis** *127 (K)GIMGYVEEDLVSTDFTGDSRZ=2* *113 (R)VPTVDVSVVDLTVR Z=2* *98 (K)GIMGYVEEDLVSTDFTGDSRZ=2* *78 (K)AGIALNDHFIK Z=2* *58 (K)TLLFGEKPVTVFGIR Z=2* *56 (R)SSIFDAK Z=2 ** *55 (K)TLLFGEKPVTVFGIR Z=2* *51 (K)YDTVHGQWK Z=2* *46 (K)KVVISAPSK Z=2* *40 (R)FGIVEGLMTTVHSITATQK Z=3 ** *40 (K)GASYEEIKK Z=2*
Adenosine kinase **Maize**	**CAB40376 (At5g03300)**	**6**	**3** **6**	**Phosphate transfer using ATP or GTP as donor** *4.19 (90) (R)IAVITQGADPVVVAEDGK Z=2* *2.36 (82) (K)VLPYADYIFGNETEAK Z=1(2)* *2.05 (R)GWETENIEEIALK Z=1* *77 (K)LNNAILAEEK Z=2* *55 (K)LVDTNGAGDAFVGGFLSQLVLGK Z=3* *43 (K)DKFGEEMKK Z=2*
Malate dehydrogenase [NAD]	**At1g53240**	**7**	**3** **6**	**Mitochondrial; carbon fixation, carbon metabolism** *84 (R)TQDGGTEVVEAK Z=2* *69 (K)LFGVTTLDVVR Z=2* *64 (R)DDLNFNINAGIVK Z=2* *64 (K)KLFGVTTLDRVVR Z=2* *60 (K)R TQDGGTEVVEAK Z=2* *59 (K)VAILGAAGGIGQPLALLMK Z=2* *43 (K)RTQDGGTEVVEAK Z=2*
Malate dehydrogenase **maize**	**AAB64290 (At5g43330) (At1g04410)**	**10**	**3** **6**	**NADP; oxidoreductase, cytoplasmic** *3.89 (K)SQASALEAHAAPNCK Z=2* *3.50 (K)VAILGAAGGIGQPLSLLMK Z=2* *96 (K)MELVDAAFPLLK Z=2* *94 (K)VLVVANPANTNALILK Z=2* *86 (K)MDATAQELTEEK Z=2* *65 (R)KKMDATAQELTEEK Z=2* *57 (K)KM*DATAQELTEEK Z=2* *51 (R)ALGQISER Z=2* *44 (K)IVQGLPIDEFSR Z=2*
Hypothetical protein 2	**At3g58290**	2	3 2	**MATH-domain-containing protein**s: extracellular metalloproteases which are anchored to the membrane and are capable of cleaving growth factors, extracellular matrix proteins, and biologically active peptides

Table S2.2 (cont'd)

				43 (R)LKLNRALEK Z=2 *39 (K)LIIGAR Z=2*
40S ribosomal protein S4 **maize**	**AAB66899 (At5g58420)**	**5**	**3 0**	**Protein synthesis** *101 (K)FDVGNVVM*VTGGR Z=2* *77 (K)GSFETIHVEDSLGHQFATR Z=3* *62 (R)EVISILM*QR Z=2* *44 (R)LHPIRDEDAK Z=2*
40S ribosomal protein S3A	**At3g04840** **At1g43170**	**2**	**3 0**	**Protein synthesis** *55 (R)LRAEDVQGR Z=2* *46 (R)VLAHTQIR Z=2*
Voltage-dependent anion channel protein 1a maize	**AAD56651 (At3g01280)**	**2**	**3 0**	**Mitochondrial potassium transporter; porin** *87 (K)KADLILGEIQSQIK Z=2* *45 (K)GDNLTGAYYHK Z=2*
Voltage-dependent anion channel protein 1b maize	**AAD56652 (At3g01280)**	**2**	**3 0**	**Mitochondrial potassium transporter; porin** *45 (K)GDNLTGAYYHK Z=2* *41 (K)KADLIFSEIHSQIK Z=2*
Voltage-dependent anion channel protein 2 **maize**	**AAD56653 (At5g15090)**	**3**	**2 9**	**Mitochondrial anion channel; porin** *144 (K)AIAVGADAAFDTSSGDLTK Z=2* *90 (R)KDEAIFNEIQSQLK Z=2* *49 (K)HNNVTVDVK Z=2*
Putative 14-3-3 protein	**AAU93690 (At3g02520)**	**4**	**2 9**	**Regulatory processes and signal transduction** *107 (R)GKIEAELSNIC*DGILK Z=2* *99 (K)QAFDEAISELDTLGEESYK Z=2 ** *70 (K)DSTLIM*QLLR Z=2* *45 (K)TVDVEELTVEER Z=2*
60S ribosomal protein L7	At2g44120	2	28	Protein synthesis *57 (R)NHYVEGGDAGNR Z=2* *49 (K)NEDKLEFSK Z=2*
14-3-3-like protein	**AAT06575 (At3g02520)**	**8**	**2 9**	**Regulatory processes and signal transduction; different from AAU93690** *143 (K)QAFDEAISELDSLGEESYK Z=2 ** *107 (R)GKIEAELSNIC*DGILK Z=2* *79 (K)AAQDIALAELAPTHPIR Z=2* *70 (K)DSTLIM*QLLR Z=2* *64 (K)LLDSHLVPSSTAAESK Z=2* *56 (R)KNEEHVNLIK Z=2* *48 (K)ESAESTM*VAYK Z=2*
Hypothetical protein 3 **Maize**	**CAC21955 (At3g23560)**	**2**	**2 9**	**Unknown; low similarity to dienelactone hydrolases** *88 (R)AYVSGAASSSR Z=2* *36 (R)HEVPYYAK Z=2*
Ribosomal protein S6 RPS6-1 **maize**	**AAB51304 (At5g10360)**	**5**	**2 8**	**Ribosome** *122 (R)ISQEVSGDALGEEEFK Z=2* *89 (K)KGENDLPGLTDTEKPR Z=3* *48 (K)KLEIDDDQK Z=2* *38 (R)LLLHR Z=2*

Table S2.2 (cont'd)

				35 (K)QGVLTSGR Z=2
Putative thioredoxin peroxidase rice	**BAD27915 (At5g06290)**	**3**	**28**	**Electron transport, reduces H_2O_2** *3.18 (K)EGVIQHSTINNLAIGR Z=2* *1.95 (R)AGGVDDLPLVGNK Z=2* *1.85 (R)ASVVDDLPLVGNK Z=2*
Putative ascorbate peroxidase rice	CB816056 (At1g07890)	2	27	H_2O_2 detoxification *37 (R)EDKPQPPPEGR Z=2* *2.94 (K)ELLSGDKEGLLQLPSDK Z=2*
Nucleoside diphosphate kinase 3 rice	AAV59386 (At4g11010)	2	26	Cytosolic, Required for Coleoptile Elongation in Rice;similar to 18kDa sugarcane NPK1 *2.87 (R)KIIGATNPLASEPGTIR Z=2* *2.44 (R)TFIAIKPDGVQR Z=2*
Mn superoxide dismutase maize	**AAA72022 (At3g10920)**	**2**	**25**	**Associated w/ increased mitochondrial activity during plant growth & development** *2.96 (K)GDASAVVQLQGAIK Z=2* *2.92 (K)KLSVETTANQDPLVTK Z=2*
40S Ribosomal protein S8 maize	**AAB06330**	**2**	**25**	**Ribosome** *64 (R)TLDPHIEEQFSSGR Z=2* *63 (K)SAIVQVDAAPFK Z=2*
Hypothetical protein 4 maize	**BU571995 (At1g09310)**	**3**	**22**	**Unknown ; similar to rice NP_00106738 with DUF538** *3.30 (R)AKAEVYVGDAAGQEK Z=2* *3.19 (R)HVTYGAEVSAVADK Z=2* *2.04 (K)AEVYVGDAAGQEK Z=2*
Formate dehydrogenase rice	AAL33598 (At5g14780)	3	21	Methane metabolism; oxidoreductase activity; response to wounding *51 (R)LQIDPELEK Z=2* *39 (R)LLLQR Z=2* *36 (R)AYDLFGK Z=2*
Ribosomal protein L18	NP_001054680 (At3g05590)	2	21	Cytoplasmic protein biosynthesis; ribosome *2.49 (K)NIAVIVGTVTDDKR Z=2* *1.88 (A)PLGENTVLLR Z=2*
Glyoxylase I Cicer arientinum	**CAA12028 (At1g08110)**	**2**	**21**	**Calmodulin binding, found in etiolated hypocotyls, carbon-metabolism** *3.18 (K)EAPANNPGLQTEVDPATK Z=2* *2.49 (R)GFGHIGVTVDDVHK Z=2*
Translationally controlled tumor-protein-like maize	**AAN40686 (At3g16640)**	**5**	**19**	**Ca binding, present in secretions** *3.09 (K)NLTAVLEPEKADEFKK Z=2* *2.27 (R)LQEQPPFDKK Z=2* *2.24 (K)VVDIVDTFR Z=2* *115 (-)M*LVYQDLLSGDELLSDSFTYK Z=2*
Cyclophilin maize	**CAA48638 (At2g21130)**	**6**	**18**	**Cyclosporin; isomerase; rotamase; increases in germinating seedlings and growing tissue; involved in protein folding/modification; chaperone** *3.75 (K)HVVFGQVVEGM*DVVK Z=2* *3.19 (R)IVM*ELYANEVPK Z=2* *2.96 (K)HVVFGQVVEGMDVVK Z=2* *2.69 (R)GNGTGGESIYGEKFPDEK Z=2* *2.60 (K)SGARGVRLEGAGRGGR Z=2* *2.55 (R)IVMELYANEVPK Z=2*

Table S2.2 (cont'd)

Translation initiator factor 5A **maize**	**CAA69225 (At1g13950)**	**4**	**1 8**	**Protein biosynthesis** *2.80 (R)LPTDETLVAQIK Z=2* *2.69 (K)DDLRLPTDETLVAQIK Z=2* *92 (R)LPTDETLVAQIK Z=2*
Nucleoside diphosphate kinase 1 sugarcane	AAB40609 (AT4g09320)	3	1 8	Synthesis of NTPs other than ATP; kinase activity towards histone H1 *3.34 (R)NVIHGSDSIESANK Z=2* *2.87 (K)IIGATNPLASEPGTIR Z=2* *2.87 (R)KIIGATNPLASEPGTIR Z=2*
Microtubule-associated protein	**AT5g44610**	**2**	**1 8**	**Epidermal cell morphogenesis** *3.12 DMGQQTQGKTRVGSRVRNRKT Z=2* *>2.5 DMGQQTQGKTRVGSRVRNRKTLSSK Z=2*
Heat-shock protein 17.2 **maize**	**CAA46641 (At1g53540)**	**2**	**1 6**	**Heat shock protein for low molecular weight molecules; chaperone** *2.99 (K)VEVEDGNVLLISGQR Z=2* *1.95 (R)AALENGVLTVTVPK Z=1*
Probable ribosomal protein S16 **rice**	**AAA33916 (At2g09990)**	**2**	**1 7**	**Protein biosynthesis; ribosome** *2.60 (K)AFEPILLAGR Z=2* *2.22 (K)TAVAVAYTKPGR Z=2*
Ribosomal protein S14	**B30097**	**2**	**1 6**	**Protein biosynthesis; ribosome** *2.20 (R)IEDVTPVPTDSTR Z=2* *2.13 (K)ELGITALHIK Z=2*
Histone H2B **maize**	**CAA40565 (At3g45980)**	**3**	**1 6**	**Blocked amino end; chromosomal protein; DNA binding nucleosome core** *(2.66 (K)AM*SIM*NSFINDIFEK Z=2)* *90 (K)AM*SIM*NSFINDIFEK Z=2* *51 (K)QVHPDIGISSK Z=3* *49 (K)KPAEEEPAAEK Z=2*
Actin depolymerizin factor **maize**	**CAA66311 (At5g52360)**	**3**	**1 6**	**Enhancer of actin turnover** *3.98 (R)YAIYDFDFVTAEDVQK Z=2* *3.11 (K)EIVVDQVGDR Z=2* *2.76 (R)SGVSVDECM*LK Z=2*
Putative ribosomal protein L34 **rice**	**BAC99505 (At1g26880)**	**2**	**1 4**	**Protein biosynthesis; ribosome** *2.77 (R)TVNRPYGGVLSGTAVR Z=2* *2.24 (R)CVLAGGPNDVLERR Z=2*
Profilin **maize**	**AAB86960 (At2g19770)**	**2**	**1 4**	**Actin binding protein with complex effects on actin organization** *81 (K)DFDEPGTLAPTGLFVGGTK Z=2* *69 (K)YMVIQGEPGVVIR Z=2*
Phospholipid transfer protein **maize**	**AAB06443 (At2g38540)**	**3**	**1 2**	**Lipid transfer** *2.98 (R)GVSGLNAGNAASIPSK Z=2* *3.24 (K)NAAAGVSGLNAGNAAASIPSK Z=2*
Lipid transfer protein(non-specific) rice	BAD87070 (At5g01870)	3	1 2	Lipid transfer *3.46 (R)SLLQQANNTPDR Z=2* *2.14 (K)NVANGANGSGTYISR Z=2 Edman AMSVSTVYSTLMPCLL/PFVQM*
Immunophilin maize	AAV28625 (At5g64350)	2	1 2	Peptidyl-prolyl cis-trans isomerase *91 (K)DPGQQPFSFSIGQGSVIK Z=2*

Table S2.2 (cont'd)

				46 (K)VTVHCTGYGK Z=2
Histone H4	**AAA33474** **At1g07660**	**2**	**1** **1**	**Histone** *65 (R)DNIQGITKPAIR Z=2* *57 (R)DAVTYTEHAR Z=2*
Phospholipid transfer protein **maize**	**P19656** **(At2g38540)**	**6**	**1** **1**	**Lipid transfer** *3.81 (K) NAAAGVSGLNAGNAASIPSK Z=2 (6B6)* *3.41 (R)GVSGLNAGNAASIPSK Z=2* *2.87 (K)CGVSIPYTISTSTDCSR Z=2* *2.55 (K)GQGSGPSAGCCSGVR Z=2* *2.11 (R)GTGSAPSASCCSGVR Z=2* *Edman CGQVASAIAPCISYARGQGSGPSAGXXSG*
Cyclotide family protein **(Umi11)**	**NP_001105812** **AY679129**	**1E**	**1** **0**	Induced during Ustilago infection and subsequent tumor formation *Edman sequence ATLCYTGETCKYIGCLTPAASDNY*

Table S2.3: Statistical analysis of RT-PCR data.

					Average	**Dev**				**Average**	**Dev**
		gd			UDPgd		UDPgp			UDPgp	
Coleoptile	Day0	1.386559	1.300126	1.084463	1.257049	0.155587	1.087187	1.210668	0.942753	1.080203	0.134094
	Day2	1.346444	1.060704	0.785593	1.064247	0.280442	0.942671	0.998766	0.786073	0.90917	0.110233
	Day4	1.009865	0.810279	0.618863	0.813002	0.195515	0.983527	0.94045	0.6891	0.871026	0.159018
	Day5	0.670928	0.567189	0.36404	0.534052	0.156104	0.900801	0.779744	0.552322	0.744289	0.176924
Leaf	Day0	1.410264	1.360329	1.016697	1.26243	0.21427	1.325095	1.09192	1.056968	1.157994	0.145764
	Day2	1.55384	1.512203	1.193627	1.41989	0.197053	1.516766	1.202487	1.447729	1.388994	0.165167

					Average	**Dev**				**Average**	**Dev**
		HP3			HP3		PLTP			PLTP	
Coleoptile	Day0	1.2278	1.205214	0.992077	1.141697	0.130066	1.158458	1.256325	1.288578	1.234454	0.067761
	Day2	0.970019	0.788685	0.566476	0.77506	0.202116	1.1583	0.940886	0.9532	1.017462	0.122125
	Day4	0.805419	0.729809	0.377402	0.637543	0.228439	1.168437	0.710021	0.805522	0.89466	0.241858
	Day5	0.578698	0.432497	0.330643	0.447279	0.124686	0.617171	0.722773	0.532711	0.624218	0.095227
Leaf	Day0	0.981603	1.086588	0.80856	0.958917	0.140396	0.89177	0.621016	0.903512	0.805433	0.159818
	Day2	1.258019	1.249659	1.199224	1.235634	0.031808	1.306338	1.588016	0.933154	1.275836	0.328495

Table S2.3 (cont'd)

			Average	**Dev**				
HP4			HP4		p Val	UDPgd	UDPgp	HP4
1.408349	1.343831	1.35993	1.370703	0.033581	day0-day2	0.066351	0.00719	0.127303
1.422694	1.252318	1.273769	1.31626	0.092796	day2-day4	0.018021	0.22542	0.010711
1.08592	1.031535	0.899456	1.005637	0.095892	day4-day5	0.005753	0.01578	0.027237
0.683225	0.796933	0.717244	0.732467	0.058362	day0-day4	0.019752	0.079276	0.007133
0.68306	1.022729	0.795573	0.833787	0.173029	day0-day2	0.201104	0.072303	0.020158
1.150783	1.287325	1.597098	1.345069	0.228692				

			Average	**Dev**				
RGP			RGP		p Val	HP3	PLTP	RGP
1.58276	1.459258	1.309534	1.450517	0.136823	day0-day2	0.010692	0.091844	0.106188
1.595246	1.049891	1.029831	1.224989	0.320809	day2-day4	0.037511	0.112208	0.119305
0.977501	0.939082	0.917588	0.944724	0.030352	day4-day5	0.062711	0.119301	0.009396
0.77991	0.629235	0.693705	0.70095	0.075598	day0-day4	0.020711	0.063343	0.009755
1.538514	1.878294	1.780981	1.732596	0.174981	day0-day2	0.034797	0.057569	0.126772
1.720895	2.388568	2.009379	2.039614	0.334862				

REFERENCES

REFERENCES

Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004) The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. Plant Cell 16:2463-2480

Anderson CT, Carroll A, Akhmetova L, Somerville C (2010) Real-time imaging of cellulose reorientation during cell wall expansion in *Arabidopsis* roots. Plant Physiol 152:787-796.

Arondel V, Tchang F, Baillet B, Vignols F, Grellet F, Delseny M, Kader J-C, Puigdomenech P (1991) Multiple mRNA coding for phospholipid-transfer protein form *Zea mays* arise from alternative splicing. Gene 99:133–136

Baker EA (1982) Chemistry and morphology of leaf epicuticular waxes. In: DJ Cutler, KL Alvin, and CE Price, eds, The Plant Cuticle, Academic Press, London, pp. 139-165

Berkowitz O, Jost R, Pollmann S, Masle J (2008) Characterization of TCTP, the translationally controlled tumor protein, from *Arabidopsis thaliana*. Plant Cell 20: 3430-3447.

Bessire M, Chassot C, Jacquat A-C, Humphry M, Borel S, MacDonald-Comber P, Metraux J-P, Nawrath C (2007) A permeable cuticle in *Arabidopsis* leads to a strong resistance to *Botrytis cinerea*. EMBO J 26:2158 – 2168

Bocca SN, Kissen R, Rojas-Beltran JA, Noel F, Gebhardt C, Moreno S, du Jardin P, Tandecarz, JS (1999) Molecular cloning and characterization of the enzyme UDP-glucose: protein transglucosylase from potato. Plant Physiol and Biochemistry 37: 809-819

Borlaug, N.E. (2000) The green revolution revisited and the road ahead. Anniversary Nobel Lecture, Norwegian Nobel Institute in Oslo, Norway. September 8, 2000.

Burton RA, Farrokhi N, Bacic A, Fincher GB (2005) Plant cell wall polysaccharide biosynthesis: real progress in the identification of participating genes. Planta 221: 309-312

Burton RA, Jobling SA, Harvey AJ, Shirley NJ, Mather DE, Bacic A, Fincher GB (2008) The genetics and transcriptional profiles of the cellulose synthase-like HvCslF gene family in barley (*Hordeum vulgare L*.). Plant Physiol 146:1821–1833

Burton and Fincher (2012) (1,3;1,4)-β-D-Glucans in cell walls of the poaceae, lower plants, and fungi: A tale of two linkages. Molecular Plant 2: 873-882.

Bowen DJ, Walton TJ (1988) Cutin composition and biosynthesis during gibberellic-acid induced stem extension of *Pisum sativum var. Meteor*. Plant Sci 55:115–127

Cameron KD, Teece MA, Smart LB (2006) Increased accumulation of cuticular wax and expression of lipid transfer protein in response to drought stress in leaves of *Nicotiana glauca*. Plant Physiol 140:176-183

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. Plant J 3:1-30

Carpita NC, Defernez M, Findlay K, Wells B, Shoue DA, Catchpole G, Wilson RH, McCann MC (2001) Cell wall architecture of the elongating maize coleoptile. Plant Physiol 127: 551-565

Carpita NC, McCann MC (2010) The maize mixed-linkage (1→3),(1→4)-β-D-glucan polysaccharide is synthesized at the golgi membrane. Plant Physiology 153:1362-1371

Carvalho OA, Gomes VM (2007) Role of plant lipid transfer proteins in plant cell physiology – a concise review. Peptides 38:1144-1153

Carvalho AO, Teodoro CES, Da Cunha M, Okorokova-Façanha AL, Okorokov LA, Fernandes KVS, Gomes VM (2004) Intracellular localization of a lipid transfer protein in *Vigna unguiculata* seeds. Physiologia Plantarum 122:328–336

Cassab GI, Varner JE (1988) Cell wall proteins. Ann Rev Plant Physiol Plant Molecular Biol 39:321-353

Ceserani T, Trofka A, Gandotra N, Nelson T (2009) VH1/BRL2 receptor-like kinase interacts with vascular-specificadaptor proteins VIT and VIK to influence leaf venation. Plant Journal 57:1000-1014.

Chassot C, Metraux JP (2005) The cuticle as source of signals for plant defense. Plant Biosystems 139:28–31

Chassot C, Nawrath C, Metraux JP (2007) Cuticular defects lead to full immunity to a major plant pathogen. Plant J 49:972-980

Clark AM, Bohnert HJ (1999) Cell-specific expression of genes of the lipid transfer protein family from *Arabidopsis thaliana*. Plant Cell Physiol 40:69-76

Cho EK, Kim M (2008) A red algal cyclophilin has an effect on development and growth in *Nicotiana tabacum*. Plant Physiol Biochem 46:868-874

Cosgrove DJ, Li LC, Cho H-T, Hoffmann-Benning S, Moore RC, Blecker D (2002) The growing world of expansins. Plant Cell Physiol 43:1436-1444

Cosgrove DJ (2005) Growth of the plant cell wall. Nature Rev Mol Cell Biol 6:850—861

Delgado IJ, Wang Z, de Rocher A, Keegstra K, Raikhel NV (1998) Cloning and characterization of *AtRGP1* . A reversibly autoglycosylated *Arabidopsis* protein implicated in cell wall biosynthesis. Plant Physiol 116: 1339-1350

Deng, Z.; Xu, S.; Chalkley, R. J.; Oses-Prieto, J. A.; Burlingame, A. L.; Wang, Z.-Y.; Kutschera, U. (2012) Rapid auxin-mediated changes in the proteome of the epidermal cells in rye coleoptiles: Implications for the initiation of growth. Plant Biol. 2012, 14, 420−427.

Dhugga KS, Tiwari SC, Ray PM (1997) A reversibly glycosylated polypeptide (RGP1) possibly involved in plant cell wall synthesis: Purification, gene cloning, and trans-Golgi localization. PNAS 94:7679-7684

Dhugga KS, Ulvskov P, Gallagher SR, Ray PM (1991) Plant polypeptides reversibly glycosylated by UDP-glucose. Possible components of Golgi beta-glucan synthase in pea cells. J Biol Chem 266:21977-21984

Drakakaki G, Zabotina O, Delgado I, Robert S, Keegstra K, Raikhel NV (2006) *Arabidopsis* reversibly glycosylated polypeptides 1 and 2 are essential for pollen development. Plant Physiol 142:1480-1492

Epel BL, van Lent JWM, Cohen L, Kotlizky G, Katz A, Yahalom A (1996) A 41 kDa protein isolated from maize mesocotyl cell walls immunolocalizes to plasmodesmata. Protoplasma 191:70-78.

Feussner I, Wasternack C (2002) The lipoxygenase pathway. Ann Rev Plant Biol 53: 275-297

Fincher GB (2009) Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. Plant Physiol 149:27-37

Gao X, Stumpe M, Feussner I, Kolomiets M (2008) A novel plastidial lipoxygenase of maize (*Zea mays*) ZmLOX6 encodes for a fatty acid hydroperoxide lyase and is uniquely regulated by phytohormones and pathogen infection. Planta 227:491-503

Gibeaut DM, Pauly M, Bacic A, Fincher GB (2005) Changes in cell wall polysaccharides in developing barley (*Hordeum vulgare*) coleoptiles. Planta 221: 729- 738.

Guelette BS, Benning UF, Chamberlin B., and Hoffmann-Benning, S (2012) Identification of lipids and lipid-binding proteins in phloem exudates from *Arabidopsis thaliana*. J Exp Bot. doi:10.1093/jxb/ERS028

Haga K, Iino M(1998) Auxin-growth relationships in maize coleoptiles and pea internodes and control by auxin of the tissue sensitivity to auxin. Plant Physiol 117: 1473-1486

Han GW, Lee JY, Song HK, Chang C, Min K, Moon J, Shin DH, Kopka ML, Sawaya MR, Yuan HS, Kim TD, Choe J, Lim D, Moon HJ, Suh SW (2001) Structural basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution X-ray crystallography. J Mol Biol 308:263–278

Heredia A (2003) Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer. Biochim Biophys Acta 1620:1–7

Hertzberg M, Aspeborg H, Schrader J, Andersson A, Erlandsson R, Blomqvist K, Bhalerao R, Uhlén M, Teeri TT, Lundeberg J, Sundberg B, Nilsson P, Sandberg G (2001) A transcriptional roadmap to wood formation. PNAS 98:14732-14737

Hoffmann-Benning S, Klomparens KL, Kende H (1994a) Characterization of growth-related osmiophilic particles in corn coleoptiles or deep water rice internodes. Annals Bot 74:563-572

Hoffmann-Benning S, Kende H (1994b) Cuticle biosynthesis in rapidly growing internodes of deepwater rice. Plant Physiol 104:719–723

Hoson T, Masuda Y, Nevins DJ 1992. Comparison of the outer and inner epidermis: Inhibition of auxin-induced elongation of maize coleoptiles by glucan antibodies. Plant Physiol 98:1298-1303

Huckelhoven R (2007) Cell wall associated mechanisms of disease resistance and susceptibility. Ann Rev Phytopathol 45:101-127

Inouhe M, Hayashi K, Thomas BR, Nevins DJ (2000) Exo- and endoglucanases of maize coleoptile cell walls: their interaction and possible regulation. International Journal of Biological Macromolecules 27: 157-162.

Irshad M, Canut H, Borderies G, Pont-Lezica R, Jamet E (2008) A new picture of cell wall protein dynamics in elongating cells of *Arabidopsis thaliana*: Confirmed actors and newcomers. BMC Plant Biol 8:94-109

Jamet E, Albenne C, Boudart G, Irshad M, Canut H, Pont-Lezica R (2008) Recent advances in cell wall proteomics. Proteomics 8:893-908.

Kader J-C (1996) Lipid-transfer proteins in plants. Ann Rev Plant Physiol Plant Mol Biol 47:627–654

Kärkönen A, Murigneux A, Martinant J-P, Pepey E, Tatout C, Dudley BJ, Fry SC (2005) UDP-glucose dehydrogenases of maize, a role in cell wall pentose biosynthesis. Biochem J 391:409–415

Kleczkowski LA, Geisler M, Ciereszko I, Johansson H (2004) UDP-glucose pyrophosphorylase: An old protein with new tricks. Plant Physiol 134:912-918

Kolattukudy PE (1996) Biosynthetic pathways of cutin and waxes, and their sensitivity to environmental stresses. In: G. Kerstiens, Editor, Plant Cuticles, BIOS Scientific Publishing Ltd., Oxford, pp. 83–108

Kotake T, Nakagawa N, Takeda K, Sakurai N (2000) Auxin-induced elongation growth and expressions of cell wall-bound exo-and endo-beta-glucanases in barley coleoptiles. Plant Cell Physiol 41: 1272-1278

Kunst L, Samuels AL (2003) Biosynthesis and secretion of plant cuticular wax. Prog Lipid Res 42: 51–80

Kurdyukov S, Faust A, Nawrath C, Bär S, Voisin D, Franke R, Schreiber L, Saedler H, Métraux J-P, Yephremov A (2006) The epidermis-specific extracellular β-hydrolase BODYGUARD, an *Arabidopsis* homologue of fungal cutinases, controls cuticle development and morphogenesis in plants. Plant Cell 18:321–339

Kutschera U, Bergfeld R, Schopfer P (1987) Cooperation of epidermis and inner tissues in auxin-mediated growth of maize coleoptiles. Planta 170:68-180

Kutschera U, Kende H (1989) Particles associated with the outer epidermal wall in internodes of deepwater rice. Ann Bot 63:385-388

Kutschera U, Niklas J (2007) The epidermal-growth-control theory of stem elongation: an old and new perspective. J Plant Physiol 164:1395–1409

Kutschera U, Deng Z, Oses-Prieto JA, Burlingame AL, Wang Z-Y (2010) Cessation of coleoptile elongation and loss of auxin sensitivity in developing rye seedlings. A quantitative proteomic analysis. Plant Signaling and Behavior 5: 509-517

Lee SB, Go YS, Bae HJ, Park JH, Cho SH, Cho HJ, Lee DS, Park OK, Hwang I, Suh MC (2009) Disruption of glycosylphosphatidylinositol-anchored lipid transfer protein gene altered cuticular lipid composition, increased plastoglobules, and enhanced susceptibility to infection by the fungal pathogen *Alternaria brassicicola*. Plant Physiol 150:42-54

Li F, Wu X, Lam P, Bird D, Zheng H, Samuels L, Jetter R, Kunst L (2008) Identification of the wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in *Arabidopsis*. Plant Physiol 148:97-107

Lolle SJ, Hsu W, Pruitt RE (1998) Genetic analysis of organ fusion in *Arabidopsis thaliana*. Genetics 149:607–619

Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Zhang D, Bryant SH (2007) CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res 35(D):237-240

Marivet J, Margis-Pinheiro M, Pierre Frendo P, Burkard G (1994) Bean cyclophilin gene expression during plant development and stress conditions. Plant Mol Biol 26: 1181-1189

Matas AJ, Yeatsa TH, Buda, GJ, Zheng Y, Chatterjee S, Tohge T, Ponnal L, l Adato A, Aharoni A, Stark R, Fernie AR, Fei Z,. Giovannoni JJ, and Rose JKC (2011) Tissue- and cell-type specific transcriptome profiling of expanding tomato fruit provides insights into metabolic and regulatory specialization and cuticle formation. The Plant Cell 23: 3893–3910,

Meng M, Geisler M, Johansson H, Harholt J, Scheller HV, Mellerowicz EJ, Kleczkowski LA (2009). UDP-glucose pyrophosphorylase is not rate limiting, but is essential in *Arabidopsis*. Plant Cell Physiol 50:998-1011.

Mohnen D (2002) Biosynthesis of pectins. In: GB Seymour, JP Knox, eds Pectins and their Manipulation. Blackwell Publishing and CRC Press, Oxford, pp 52-98

Nakazono M, Qiu F, Borsuk LA, Schnable PS (2003) Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: identification of genes expressed differentially in epidermal cells or vascular tissues of maize. Plant Cell 15:583-596

Nawrath C (2002) The biopolymers cutin and suberin. In: C.R. Somerville and M.E. Meyerowitz, eds, The *Arabidopsis* Book, American Society of Plant Biologists kio

Nawrath C (2006) Unraveling the complex network of cuticular structure and function. Curr Opin Plant Biol 9:281-287

Oleson P (1980) The visualization of wall-associated granules in thin sections of higher plants: occurrence, distribution, morphology and possible role in cell wall biogenesis. Z Pflanzenphysiologie 96:35-48

Peters WS, Thomos AD (2000) The mechanic state of "inner tissue" in the growing zone of sunflower hypocotyls and the regulation of its growth rate following excision. Plant Physiol 123:605-612

Pyee J, Yu H, Kolattukudy PE (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (Brassica oleracea) leaves. Arch Biochem Biophys 311:460–468

Rautengarten C, Ebert B, Herter T, Petzold CJ, Ishii T, Mukhopadhyay A, Usadel B, Scheller HV (2011) The interconversion of UDP-arabinopyranose and UDP-arabinofuranose is indispensable for plant development in *Arabidopsis.* The Plant Cell 23: 1373-1390

Riedel M, Eichner H, Meimberg H, Jetter R (2007) Chemical composition of epicuticular wax crystals on the slippery zone in pitchers of five Nepenthes species and hybrids. Planta 225:1517-1534

Riedel M, Eichner A, Jetter R (2003) Slippery surfaces of carnivorous plants: composition of epicuticular wax crystals in *Nepenthes alata* Blanco pitchers. Planta 218:87–97

Riederer M, Schreiber L (2001) Protecting against water loss: analysis of the barrier properties of plant cuticles. J Exp Bot 52:2023–2032

Riley RG, Kolattukudy PE (1975) Evidence for covalently attached p-coumaric acid and ferulic acid in cutins and suberins. Plant Physiol 56:650–654

Ristic Z, Jenks MA (2002) Leaf cuticle and water loss in maize lines differing in dehydration avoidance. J Plant Physiol 159:645-651

Robards AW (1969) Particles associated with developing cell walls. Planta 88:376

Sagi G, Katz A, Guenoune-Gelbart D, Epel BL (2005) Class 1 reversibly glycosylated polypeptides are plasmodesmal-associated proteins delivered to plasmodesmata via the golgi apparatus. Plant Cell 17:1788-1800

Samuels L, Kunst L, Jetter R (2008) Sealing plant surfaces: Cuticular wax formation by epidermal cells. Ann Rev Plant Biol 59:683-707

Savaldi-Goldstein S (2007) The epidermis both drives and restricts plant shoot growth. Nature 446:199-202

Schopfer P (2006) Biomechanics of plant growth. Am J Bot 93:1255-1265

Schweizer P, Felix G, Buchala A, Muller C, Metraux JP (1996) Perception of free cutin monomers by plant cells. Plant J 10:331–341

Seifert, GJ (2004) Nucleotide sugar interconversions and cell wall biosynthesis: how to bring the inside to the outside. Curr Opin Plant Biol 7:277-284

Seitz B, Klos C, Wurm M, Tenhaken R (2000) Matrix polysaccharide precursors in *Arabidopsis* cell walls are synthesised by alternate pathways with organ specific expression patterns. Plant J 21:537-546

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Analyt Chem 68:850-858

Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Metraux JP, Nawrath C (2000) Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell 12: 721–737

Suh MC, Samuels AL, Jetter R, Kunst L, Pollard M, Ohlrogge J, Beisson F (2005) Cuticular lipid composition, surface structure, and gene expression in *Arabidopsis* stem epidermis. Plant Physiol 139:1649–1665

Tchang F, This P, Stiefel V, Arondel V, Morch MD, Pages M, Puigdomenech P, Grellet F, Delseny M, Bouillon P (1988) Phospholipid transfer protein: full-length cDNA and amino acid sequence in maize. J Biol Chem 263:16849-16855

Testasecca P, Wald FA, Cozzarín ME, Moreno S (2004) Regulation of self-glycosylation of reversibly glycosylated polypeptides from *Solanum tuberosum*. Physiol Plant121:27-34

Thoma SL, Kaneko Y, Somerville CR (1993) An *Arabidopsis* lipid transfer protein is a cell wall protein. Plant J 3:427–437

Thomas BR, Inouhe M, Simmons CR, Nevins,DJ (2000) Endo-1,3;1,4-beta-glucanase from coleoptiles of rice and maize: role in the regulation of plant growth. Internat J Biol Macromol 27:145-149

Tsuboi S, Osafune T, Tsugeki R, Nishimura M, Yamada M (1992) Nonspecific lipid transfer protein in castor bean cotyledons cells: subcellular localization and a possible role in lipid metabolism. J Biochem 111:500–508

Van Maarseveen C, Han, H, Jetter, R (2009) Development of the cuticular wax during growth of *Kalanchoe daigremontiana* (*Hamet et Perr. de la Bathie*) leaves. Plant Cell Envir 32:78-81

Vaughn KC (2002) Attachment of the parasitic weed dodder to the host. Protoplasma 219:227-237

Vogg G, Fischer S, Leide J, Emmanuel E, Jetter R, Levy AA, Riederer M (2004) Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid ß-ketoacyl-CoA synthase. J Exp Bot 55:1401-1410

Zhao GR, Liu JY (2002) Isolation of a cotton *RGP* gene: a homolog of reversibly glycosylated polypeptide highly expressed during fiber development. Biochim Biophys Acta-Gene structure and expression 1574:370-374

Edelmann HGB, R.; Schopfer, P. (1995) Effect of inhibition of protein glycosylation on auxin-induced growth and the occurrence of osmiophilic particles in maize (*Zea mays L.*) coleoptiles. J. Exp. Bot. **46:** 1745-1752

CHAPTER 3: ARRP1 IN THE REGULATION OF CELL EXPANSION GROWTH

3.1 Abstract

During growth, plants integrate cell division with cell elongation and differentiation. To better understand these processes we previously identified proteins from auxin-treated rapidly-growing corn coleoptiles (Li *et al.,* 2013). One of these proteins, ARRP1 (Auxin rapid response protein 1), is closely associated with cell and organ expansion growth in above ground tissues. Here we show that upon auxin-treatment *ARRP1* mRNA and protein increase within 30 minutes – prior to measurable growth and increased expression of genes encoding cell wall biosynthetic enzymes, suggesting an intracellular signaling function. This is further supported by the localization of ARRP1 in both the cytoplasm and the nucleus. *ARRP1* expression is induced by red and blue light, suggesting a possible role in phytochrome and cryptochrome signaling at the interface with the auxin response. *Arabidopsis* plants lacking the *ARRP1* homologue grew normally under standard conditions, but showed delayed growth and delayed flowering when germinated in the dark and transferred to light, a phenotype that can be reversed by expression of ZmARRP1 in the *Arabidopsis* mutant.

In situ hybridization demonstrated that *ARRP1* mRNA is enriched in the vasculature of growing corn tissues. In addition, corn ARRP1 interacts with the cytoplasmic domain of both *Arabidopsis* and *Zea mays* VH1/BRL2, a receptor-like kinase, which plays a role in the signaling of vascular development. Based on the fact that ARRP1 is involved in light- and auxin-mediated growth responses, we propose that it may act downstream of BRL2 at the intersection of several environmental and hormonal signals affecting plant development and the transition to light.

3.2 Introduction

To adapt to the continuously changing environment, plants evolved a complex phytohormone network. Phytohormones are a group of small signaling molecules that are essential for plant survival, growth, development, and reproduction (Kende and Zeevaart, 1997; Wolters and Jurgens, 2009; Depuydt and Hardtke, 2011). Auxins were the first of the major phytohormones to be discovered. Auxin regulates cell division and cell elongation. It controls cell differentiation, root and shoot architecture, leaf abscission and fruit formation. In addition, it mediates plant responses to light, gravity, pathogen, and abiotic stress (Woodward and Bartel, 2005; Kazan and Manners, 2009; Vanneste and Friml, 2009; Weijers and Friml, 2009; Wang *et al*., 2010; Sauer *et al*., 2013). Auxin is mainly synthesized in the tips of shoot and young leaves.

Perceiving auxin signal is the first step for an auxin triggered response. In the past decades at least three auxin receptors have been identified. The F-box protein TRANSPORT INHIBITOR RESISTANT1 (TIR1) is the best-studied auxin receptor. Binding of IAA into the ring-like structure of TIR1 promotes the interaction between TIR1 and proteins of the AUXIN/INDOLE ACETIC ACID (Aux/IAA) family (Tan *et al*., 2007), a group of proteins that regulates auxin-induced gene expression and development (Reed, 2001). *Arabidopsis thaliana* has 5 members of TIR1 (Calderon Villalobos *et al.*, 2012), 29 members of Aux/IAA genes (Tan *et al*., 2007), and 23 members of ARF genes (Wilmoth *et al*., 2005; Korasick *et al*., 2014). Experimental evidence suggests that plants form different TIR/AFB-Aux/IAA co-receptor pairs with different auxin affinities, which contribute to the great variety of auxin functions (Vernoux *et al*., 2011; Korasick *et al*., 2014; Enders and Strader, 2015).

Auxin is not only acting in a linear pathway, but also in conjunction with other hormones, such as brassinosteroid and cytokinin (Depuydt and Hardtke, 2011; Mouchel *et al*., 2006; Scacchi *et al*., 2010). In addition, there is crosstalk between auxin and light signaling. Light conveys environmental information such as local habitat conditions and seasonal change. The four major light receptors, the red and far-red light absorbing phytochromes (phy), the blue-light absorbing cryptochromes (cry), phototropins, and a ZTL-like F-box protein (Somers *et al*., 2004; Halliday *et al*., 2009) collectively control important aspects of plant development such as flowering and germination, tuberization, and bud dormancy, and also drive the plant towards an adaptive response to their local habitat. Much of this light response is achieved by linking to auxin system.

Light controls auxin availability in plants by regulating both TAA1, an enhancer, and SUR2, a suppressor of IAA biosynthesis (Mikkelsen *et al*., 2004; Lau *et al*., 2008; Halliday *et al*., 2009). TAA1 encodes a tryptophan aminotransferase, which converts tryptophan to IPA in a parallel branch to the YUCCA metabolic pathway (Stepanova *et al*., 2008; Tao *et al*., 2008). SUR2, a cytochrome P450 monooxygenase CYP83B1, catalyzes the first step of indole glucosinolate biosynthesis. Disruption of SUR2 function in *Arabidopsis* mutants blocks the metabolic route from indole-3-acetaldoxime to glucosinolate, enhancing IAA production (Hoecker *et al*., 2004). By inducing the transcription level of *SUR2* and suppressing TAA1, activated phyB controls the IAA homeostasis in plants (Halliday *et al*., 2009).

Light also regulates auxin flow by modifying the abundance and function of PGPs and PINs, which are auxin polar transport carrier proteins. Activated phyB suppresses the transcription of *PIN3*, while phyB inactivation or loss leads to a dramatic increase of

PIN3 mRNA levels (Friml *et al*., 2002). Within the upper portion of the hypocotyl, the PGP19 protein level is regulated by the phyA, phyB, cry1, and cry2 light receptors collectively (Nagashima *et al*., 2008). In addition, light signaling can modulate auxin distribution by directly modifying PIN1, PIN2 and PIN7 protein activity. The blue-light receptor pathway maintains PIN2's plasma membrane localization, and reduces its protein turnover in the vacuole (Laxmi *et al*., 2008).

Light not only controls auxin levels and distribution, but also directly integrates with auxin signal transduction. A number of transcription factors, *PHYTOCHROME RAPIDLY REGULATED or PAR* genes, play a key role in coordinating light and auxin signaling (Salter *et al*., 2003; Sessa *et al*., 2005; Roig-Villanova *et al*., 2006; Roig-Villanova *et al*., 2007*)*. Among them, PAR1 and PAR2, members of the bHLH family of transcription factors, suppress auxin-induced expression of Small Auxin-Up RNA gene15 (SAUR15) and SAUR68. Plants with upregulated PAR1 or PAR2 activity show a diminished response to exogenous auxins (Roig-Villanova *et al*., 2007). Plants with reduced expression of many SAUR genes either through increased expression of PAR1/2 or artificial mircroRNA show reduced hypocotyl growth, similarly, overexpression of SAUR63, led to increased hypocotyl elongation (Chae *et al*., 2012). In addition, phyA may directly control auxin signaling. Recombinant oat phyA has been shown to phosphorylate recombinant SHY2/IAA3, and other AUX/IAA proteins *in vitro* (Colon-Carmona *et al*., 2000). These studies provide a model for the connection between light and auxin signaling system. However details about the integration of light and auxin signaling remain unknown.

Maize (*Zea mays L*.) is an important agronomic crop. It provides food and a source of biofuel. Auxin-induced cell elongation of maize coleoptiles is one of the fastest phytohormonal responses known and has been widely used as a model system to study regulation of cell growth and tropisms (Haga and Iino, 1998). Auxin-induced cell elongation is mediated by several biochemical changes, including apoplastic space acidification, ion uptake, and *de novo* protein synthesis.

In former study, we used a proteomics approach and identified four novel growth-related proteins in corn coleoptiles (Li *et al*., 2013). Here we show that a newly identified protein, Auxin-Response Protein1 (ARRP1) is induced rapidly and transiently by auxin within 30 minutes – and prior to known cell wall biosynthesis enzymes. In addition to auxin, its expression is also controlled by red and blue light, suggesting that it might also be involved in light signaling. However, its expression is unaffected by brassinosteroids or cytokinins. *Arabidopsis* mutants, deficient in *ARRP1* expression, show a delayed growth phenotype when germinated in the dark and transferred to light. Overexpression of the corn gene in *atarrp1-1/-2* double mutants can rescue this phenotype. Our findings suggest that ARRP1 is required during early seedling growth and during transition to light. ARRP1 interacts with BRL2, a receptor-like kinase with a function in vascular bundle development. *In situ* hybridization demonstrates that *ARRP1* is enriched in the vasculature of growing tissues, overlapping with BRL2 expression pattern. Based on these findings, we propose that ARRP1 acts in conjunction with BRL2 at the intersection of environmental (light) and hormonal (auxin) signals affecting plant and vascular development in early plant development.

3.3 Method and Materials

3.3.1 Plant material and growth conditions

Zea mays Great Lakes 4758 hybrid seeds (Great Lakes Hybrids, Ovid, MI) were shaken in water for 0.5-1 h to remove fungicides and speed germination. Seeds were germinated in a standard soil mixture containing equal parts of Bacto Soil (Michigan Pear Company, Houston), medium vermiculite, and perlite. Seeds were germinated in the dark at 22 ℃. For auxin treatment, coleoptiles were harvested after 4.5 days. To examine gene expression throughout the plant, four-day old seedlings were moved to a growth chamber at 22 ℃ with 16 h day/8 h night (LD). For expression studies in response to light, three-day old seedlings were moved to different light conditions as described below.

Arabidopsis thaliana var. Columbia-0 seedlings were incubated for 20 min in 20% bleach/0.1% Triton X-100 for sterilization, washed several times with sterilized water, and plated on a Petri dish containing 1% sucrose and half-strength Murashige and Skoog Basal medium (Sigma-Aldrich). They were stored in darkness at 4℃ for 2 days, then transferred to the growth chamber at 22℃ with 16 h day/8 h night (LD) for two weeks. *Arabidopsis* seedlings were then planted in soil and grown in the growth chamber at same conditions.

3.3.2 Sequence analysis and bioinformatics analysis

Homologues to ARRP1 and VH1/BRL2 were determined using the National Center for Biotechnology website (http://www.ncbi.nlm.nih.gov). Using ClustalW2, homologous sequence alignments were performed (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Predicted gene expression patterns were

obtained through the Bio-Analytic Resource for Plant Biology (http://bar.utoronto.ca/). The following gene names/accession numbers were used: *ZmARRP1*: GRMZM2g139786; *AtARRP1-1*: At1g56580; *AtARRP1-2*: At1g09310; *ZmBRL2*: GRMZM2g002515; *AtBRL2*: At2g01950.

3.3.3 Hormone and light response analyses

To investigate the *ARRP1* response to auxin, brassinosteroids, and cytokinin treatment, coleoptiles were pulled off the primary leaf of maize seedlings and cut to a length of 10 mm after removal of the tips as described in Li *et al*., 2013. All coleoptiles were first shaken in distilled water for 1 h in the dark, transferred to fresh water as control or to 30 μM indole acetic acid, 20 μM benzyladenine, or 1 μM 24-epibrassinolide (Sigma-Aldrich) for up to five hours. The samples were collected every half hour in liquid nitrogen, and stored at -80°C.

To understand the *ARRP1* response to different light conditions, *Zea mays* seeds were germinated in the dark at room temperature for 3 days, and then transferred to growth chambers with white light, blue light (420 nm), red light (660 nm), or far red light (735 nm) at a photoperiod of 16h light and 8h dark at 22°C. The amount of light was set to 40 μmol m^{-2} s^{-1} for all chambers. Leaves were collected every 12 hours, frozen in liquid nitrogen, and stored at -80°C.

RNA and protein extraction and quantification are described below. Experiments were performed with three biological replicates.

3.3.4 Total RNA isolation and quantification

Equal amounts of plant tissue were harvested and ground to powder in liquid nitrogen. Using the Qiagen RNeasy Plant Mini-Kit, total RNA was extracted and treated

with 10 units/100 mL Roche RNase-free DNase I for 1 h at 37°C. After incubation, RNA was cleaned up using the same kit. The quantification of total RNA amount was performed by a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Using Omniscript reverse transcriptase, 1µg of total RNA was reverse transcribed to cDNA (Qiagen).

For quantitative PCR: Using the 2 X SYBR Green PCR core reagent (Applied Biosystems), real-time PCR analysis was performed on an ABI7000 instrument in 96-well plates (Bio-Rad). Each sample had three technical replicates and three biological replicates for a total of nine determinations. 18S was used to generate the standard curve and for normalization.

For semiquantitative PCR: PCR products were separated in a 1% agarose gel and quantified using ImageJ. Each sample had three biological replicates. The 18S signal for each sample was used for normalization. PCR primers and conditions are listed in table 3.1. Student's t test was used to determine significance.

3.3.5 Recombinant ARRP1 and VH1 kinase-domain construct for protein-interaction essays, purification, and antibody production.

To generate C-terminal His-tagged fusion proteins of ARRP1, AtBRL2 (*Arabidopsis*) or ZmBRL2 (maize), the respective cDNAs were isolated using RT-PCR from RNA extracts of five-day-old corn coleoptiles. Fragments were amplified by PCR with BamHI and NdeI sites at either end and cloned into digested pET15b expression vector using High Fidelity Taq (Roche). To create a fusion of His-tag with AtBRL2 or ZmBRL2, only the intracellular C-terminus of AtBRL2 or ZmBRL2 containing the kinase domain was used. All constructs were confirmed by sequencing.

Protein expression was induced by 0.1 mM IPTG. Proteins were extracted from *E.coli.* culture and purified with Ni-NTA agarose (QIAGEN) according to the manufacturer's instructions. Purified ARRP1 was sent to Cocalico Biologicals, Inc. for polyclonal antibody production using mice. Specificity of the antibody is shown in figure S3.1b.

Purified AtBRL2 and ZmBRL2 were used to confirm the interaction between ARRP1 and these proteins. All primer sequences used are given in table S3.1a.

3.3.6 Protein extraction, and western blot analysis

Protein extraction was performed as described in He *et al.* (1996) with some modifications: 100 mg plant tissue was ground in liquid N_2, resuspended in 100 µl of 1 X tissue homogenization buffer (50 mM Tris-HCl, pH 6.8, 50 mM DTT, 0.1% Tween 20, 10% glycerol), and centrifuged at 12000 rpm for 20 min. The supernatant was transferred to a new 1.5 ml tube, boiled for 10 min, and resolved by a Bio-Rad 12% Tris-HCl SDS-PAGE gel.

Extraction of nuclear proteins was performed as described by Sikorskaite *et al.* (2013). One gram of tissue was ground in liquid N_2 into powder and resuspended in 10 ml of nuclei isolation buffer (10 mM MES-KOH (pH 5.4), 10 mM NaCl, 10 mM KCl, 2.5 mM EDTA, 250 mM sucrose, 1mM DTT). Cell debris was removed by filtration through two layers of cheesecloth twice and 10% Triton X-100 was added to the supernatant until a final concentration of 0.5%. Using Percoll/sucrose gradient centrifugation, nuclei were isolated and washed twice with NIB buffer (Sikorskaite *et al*., 2013).

Protein gels were electroblotted (100 V, 1 h) onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked with 2% bovine serum

albumin (BSA) in TBST buffer for 1 h, followed by incubation with the primary antibody (diluted 1:5000) overnight at 4 ℃, and washed with TBST buffer for 10 min each time, five times total. After the wash, membranes were incubated with secondary antibody, HRP conjugated goat anti-mouse antibody, (1:2000; Bio-RAD) for 1 h, then washed with TBST buffer five times as before. After reaction with Pierce ECL Western blotting substrate (Thermo), bands were visualized using HyBlot CL autoradiography film, and quantified with ImageJ.

3.3.7 Identification and verification of T-DNA insertion lines

The SALK T-DNA database (http://signal.salk.edu/cgi-bin/tdnaexpress) was searched for insertion lines in *ARRP1 Arabidopsis thaliana* homologue and seeds were obtained from the Arabidopsis Biological Resource Center (Alonso *et al*., 2003). Using PCR genotyping, plants that were homozygous mutants for the T-DNA insertion were identified. Mutant plants were confirmed using reverse transcriptase–mediated PCR.

3.3.8 Transient expression and stable transgenic transformation

To generate a Carboxy-terminal ARRP1-CFP fusion protein, the full-length *ARRP1* ORF was amplified from five day-old corn coleoptile cDNA by PCR and introduced into the binary vector pDONR-207. Using the GATEWAY *in vitro* site-specific recombination, the ORF was then transferred to pEARLY-G103, according to manufacturer's protocol (Invitrogen). Primers used are listed on table S3.1. The constitutive expression of the *ARRP1:CFP* was driven by a cauliflower mosaic virus 35S promoter; the construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

For transient expression of *ARRP1*, the cells of the transformed *Agrobacterium* overnight culture were pelleted by centrifugation in a 1.5 ml tube at room temperature. Using the infiltration buffer (50 mM MES, pH 5.6, 0.5% Glucose [w/v], 2 mM Na_3PO_4 and 100 mM acetosyringone) the pellet was washed three times and resuspended in 1 mL of the same buffer. The bacterial suspension was diluted with infiltration buffer to adjust the inoculum concentration to an optical density at 600 nm (OD_{600}) of 0.05 and inoculated into the lower epidermis of tobacco (*Nicotiana benthamiana*) for transient expression. After 48 hours, localization was determined using confocal microscopy (Olympus FV1000SP CLSM). For CFP, the excitation laser wavelength is 458 nm, emission fluorescence wave length is 480-500 nm, For YFP, excitation laser wavelength is 515 nm, emission fluorescence wavelength is 525-570 nm.

For stable expression, the constructs were transformed into *Arabidopsis thaliana*. Columbia-0 plants, using *Agrobacterium*-mediated floral dip, as described by Clough & Bent 1998. Kanamycin-resistant plants were selected on MS medium with 1% (w/v) sucrose, and 35 mg/L kanamycin (Sigma-Aldrich) at 21 °C and 16-h light/8-h dark photoperiod. Kanamycin-resistant 3-week-old plants were confirmed by genotyping PCR using GFP- specific primers (table S3.1).

3.3.9 *In vitro* co-immunoprecipitation

Using 10 units of protein kinase A, one ug of purified His6::AtBRL2 or His6::ZmBRL2 was phosphorylated in the phosphorylation buffer (50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 100 µM ATP and 5 µM cAMP). Phosphorylated and unphosphorylated protein was incubated with 2 mL of maize leaf protein extraction solution at 4℃ in 1 X PBS overnight in separate preparations. The

mixture was incubated with Ni-NTA agarose for 1 h and washed three times with the 1 X PBS containing 20 mM imidazole. The Ni-NTA agarose was resuspended in 1X SDS-sample buffer and boiled for 10 min. The protein mixture (BRL2-interacting proteins) was resolved by 12% Tris-HCl ready SDS-PAGE gel and ARRP1 presence examined using western blot using the anti-ARRP1 antibody.

3.3.10 Yeast two hybrid assay

The full length ORF of *ZmARRP1* was inserted into the yeast two hybrid vector pGBKT7, which contains the GAL4 binding domain. The intracellular domain of *AtBRL2* and *ZmBRL2* PCR fragments was inserted into the pGADT7 vector, which contains the GAL4 activity domain. *ZmARRP1* & *AtBRL2* vectors were co-transformed into yeast AH109 Strain. After the first selection on SD/-Leu/-Trp plates, the co-transformants were tested for the GAL4 expression on SD/-Leu/-Trp/-His plates.

3.3.11 *In situ* hybridization

Coleoptiles of 5-day-old *Zea mays* seedlings and two week old leaves were fixed in 4% paraformaldehyde and embedded in paraffin as described by Karlgren *et al*., 2009. A 150 bp fragment from DUF538 conserved domain of *ARRP1* was amplified using RT-PCR, and used as the RNA probe for hybridization was generated. The PCR product was inserted into the pGEM T-easy vector (Promega) in sense direction. *In vitro* transcription of the probes, using digoxigenin-labeled UTP was performed by the TP7 and SP6 polymerase (NEB) according to the manufacturer's protocol. RNA *in situ* hybridization was conducted according to a published protocol (Karlgren *et al.*, 2009). The DIG-labeled RNA probe was identified using the DIG Wash and Block Buffer set (Roche). A Nikon Eclipse microscope was used for image acquisition.

3.4 Results and Discussion

3.4.1 *ARRP1* encodes a protein with a DUF538 conserved domain

Using a proteomics approach, we had previously identified an auxin-induced protein, ARRP1, in rapidly growing corn coleoptiles (Chapter 2; Jie *et al*., 2013). The nucleotide sequence of ARRP1 revealed a single intron-less open reading frame of 675 bp that encodes a predicted protein of 224 amino acids with an estimated molecular mass of 24 kD (Fig. 3.1a). A typical TATA box sequence (TATATAA) is found upstream of the only ATG start site at -105. In addition to the TATA box, we also found an ARR1-binding element, which is known to be involved chloroplast development, leaf senescence, and vascular differentiation (table S3.2).

ARRP1 belongs to a superfamily of plant proteins containing a conserved domain of unknown function 538 (DUF538) (Fig. 3.1b). A search of public databases through the National Center for Biotechnology Information (NCBI) website revealed 50 *Zea mays* protein sequence entries with a predicted DUF538 domain. All encode small molecular weight proteins that range from 15 kD to 25 kD. My analysis of DUF538 gene family members revealed representatives of this superfamily in 69 plant species, all within the Embryophyta. However, DUF538 proteins have not been found in algae and cyanobacteria, indicating that DUF538 proteins are not essential for photosynthesis (Fig. 3.2).

Several DUF538 containing genes are induced by abiotic and biotic factors, such as mild drought stress, nutrient deficiency, crown gall, and mixed elicitors (Gholizadeh, 2011). It was suggested that DUF538 proteins may function in the regulation of various environmental signal transduction pathways (Gholizadeh and Baghban Kohnehrouz,

2010). Based on previous evidence in other plants that DUF538-containing proteins may have a regulatory function, we examined whether ARRP1 serves as a regulatory or signal-transduction factor.

3.4.2 Sequence alignment of *ZmARRP1* and its *Arabidopsis* homologues At1g56580 and At1g09310

Arabidopsis contains two *ARRP1* homologues: At1g09310 (45% protein identity) and At1g56580 (44% protein identity), both of which contain the DUF538 conserved domain (Fig. 3.1b). Within the DUF538 domain the protein identity is 56% to At1g09310 and 57% to At1g56580.

At1g56580, also called SVB (SMALLER WITH VARIABLE BRANCHES), was found highly expressed in the leaf trichomes of the *glabra3–shapeshifter* (*gl3–sst*) *siamese* (*sim*) double mutant that shows an arrest of trichome development, leading to smaller trichome branches of variable length (Marks *et al*., 2009).

At1g09310 was found to be upregulated in plants exposed to biotic and abiotic stress. Its protein level was tightly controlled by ubiquitinase and metacaspase (Saracco *et al*., 2009; Tsiatsiani *et al*., 2013). More recently a large-scale protein-interaction approach showed its possible interaction with BRL2, a BRL1-like receptor kinase, suggesting that ARRP1 may play an important role in the signaling pathway downstream of the BRL2 receptor (Ceserani *et al*., 2009). Interestingly, this study also showed that BRL2 regulates the venation pattern in leaves, a traitthat is also controlled by auxin, providing more evidence that ARRP1 acts in the signaling path of multiple abiotic signals.

3.4.3 *ARRP1* expression is correlated with cell division and cell elongation

To examine the expression of *ZmARRP1*, we determined the developmental profile of its expression (Fig. 3.3). Total RNA and protein from different tissues of five-day-old, dark-grown seedlings and at different times after the transfer to light were quantified using semi-quantitative RT-PCR and western blotting. *ARRP1* was strongly expressed in the above ground tissue while mRNA and protein were barely detectable in the root. Once coleoptiles are exposed to light their growth ceases. *ARRP1* showed reduced expression in parallel to this growth cessation: The mRNA level dropped more than 50% within five days. Similarly, a 60% decrease was observed in the protein levels (Fig. 3.3b). To further investigate the correlation between *ARRP1* expression and leaf development/tissue expansion, we monitored gene expression and protein abundance in expanding corn leaves. In this case, exposure to light enhanced growth. *ARRP1* expression levels increased about 30% in the developing leaf within two days of transition to light (Fig. 3.3a). Western blotting revealed a simultaneous increase in ARRP1 protein (Fig. 3.3b). Our findings suggest that both *ARRP1* gene expression and protein abundance are correlated with expansion growth in above-ground tissues.

In monocots, leaf differentiation proceeds basipetally (from tip to base) in a continuous manner. Young maize leaves can be divided into four distinct stages of leaf development, which include a basal zone, a transitional zone, a maturing zone, and mature zone (Li *et al*., 2010). Genes that encode enzymes for cell cycle regulation and chromatin structure, cell wall biosynthesis, auxin and brassinosteroid biosynthesis, and potential signaling proteins are highly expressed near the base of the leaf, corresponding to region I and II in Fig. 3.4a. Genes encoding enzymes for the Calvin

cycle, isoprenoid biosynthesis, redox regulation and the light reactions of photosynthesis are highly expressed the maturing zone. Our analysis of the different leaf zones showed that while *ARRP1* expression was seen in all tissues, both, mRNA level and protein abundance were highest in the basal zone (II; Fig. 3.4b & c), suggesting ARRP1 is correlated with the cell division and cell elongation growth.

Our data suggest (1) that the ARRP1 functions predominantly in the above-ground tissues; (2) that its expression is highly correlated with plant growth; and (3) that it is highly enriched in areas of the leaf where cell division and cell elongation take place. Because the expression pattern indicates a strong connection between ARRP1 and plant growth, ARRP1 might be involved in the regulation of plant signaling processes associated with growth-promoting hormones such as auxin, brassinosteroids, cytokinin or environmental factors such as light.

3.4.4 *ARRP1* expression is controlled by auxin and light

Because ARRP1 was originally identified in auxin-treated corn coleoptiles (Li *et al.*, 2013), I determined gene expression (Fig. 3.5a) and protein levels (Fig. 3.5b) at various times after addition of auxin. *ARRP1* mRNA was increased immediately after the addition of auxin, reaching the highest level within 30 min. This elevated transcript level is maintained for up to two hours, then decreases slowly to a basal level within the next two hours. In comparison, the expression of genes which are involved cell wall (CW) biosynthesis (UDP-glucose dehydrogenase: UDPgd (Karkonen *et al.*, 2005); a putative mixed-linked β-glucanase; Hyp 3) is induced slowly and gradually over five hours of auxin treatment and does not display the rapid and transient auxin response of *ARRP1*. The ARRP1 protein level is also upregulated by auxin, but reaches the highest

level two hours later, either due to the fact that translation is a time consuming process or due to an increased protein stability. Closer examination of the western blot revealed the presence of a doublet band suggesting possible post-translational modification. Analysis of the ARRP1 sequence indicates the presence of several putative phosphorylation sites (Fig. 3.1a). The fact that we only detect the protein doublet in some western blots is likely due to the resolution of the gel but could also be caused by selective phosphorylation only under certain conditions. The fact that the induction of *ARRP1* expression precedes the expression of other CW-related genes indicates that *ARRP1* is an early auxin-response gene with a possible signaling function which may regulate the expression of other genes including those related to CW biosynthesis. The fact that protein levels remain high for five hours suggests that it is necessary for the maintenance of the response as well.

In addition to auxin, cytokinins and brassinosteroid are also regulators of plant growth (Depuydt and Hardtke, 2011). Hence, we examined whether *ARRP1* expression was induced by application of these two hormones. Neither cytokinins nor 24-epibrassinolide induced expression of *ARRP1* (Fig. S3.2).

As previous data had shown that *ARRP1* expression is increased in leaves when seedlings were grown under white light, I examined whether *ARRP1* can be induced by specific wavelengths to understand, which photoreceptor is responsible for the induction of *ARRP1* expression. Three-day old, dark-grown corn seedlings were transferred to growth chambers with white, red, blue, or far red light. Protein and mRNA levels in leaves were examined every 12 hours for the next three days (Fig. 3.6 a/b). The results showed that white light has the strongest inductive effect on *ARRP1* expression and

protein abundance; Student's t-test showed that mRNA and protein levels are also induced significantly after 2.5 days by red ($p<0.005$) and blue light ($p<0.01$), but not by far red light. These observations that *ARRP1* not only responds to auxin, but also can be induced by blue and red light, suggest that ARRP1 might integrate auxin and light signals (phytochrome and possibly cryptochrome).

Light exerts influence on auxin signaling by regulating the biosynthesis and distribution of auxin (Teale *et al*., 2006; Halliday *et al*., 2009). We originally identified ARRP1 in auxin-treated dark-grown coleoptiles. Once these are exposed to light, coleoptile growth ceases while the growth of the developing leaf increases. This response is known to be regulated by phytochrome. Recent publications have shown a tight correlation between phytochrome sensing and auxin signaling: Phytochrome interacting factors (PIFs) regulate both auxin biosynthesis and signaling (Hornitschek *et al*., 2012). PIFs also interact with brassinosteroids (BRs) to regulate auxin biosynthesis and transport, which leads to an increase in cell expansion (de Lucas and Prat, 2014). Based on the *ARRP1* response to auxin and light and its correlation with growth, I propose that it functions in this pathway and integrates auxin and light signaling.

3.4.5 *At*ARRP1 is required in the initiation of plant growth and dark/light transition

Arabidopsis contains two close homologues to ZmARRP1. While overall sequence identity of the proteins is only 45%, it increases to over 55% within the DUF 538 domain. As with *ZmARRP1* in corn, *AtARRP1* and *AtARRP2* are highly expressed in expanding leaves and five day old dark-grown hypocotyls but barely expressed in the root. Their expression levels were significantly decreased in fully expanded leaves further indicating a correlation with plant growth (Fig. 3.7).

I obtained knock-out mutants of *AtARRP1* in *Arabidopsis* from The Arabidopsis Information Resource (TAIR; Arabidopsis.org) (Fig. 3.8a and 3.8b). I used scanning electron microscopy to examine whether the trichome growth was affected in At1g09310 mutants as had been shown for At1g56580. Both At1g56580 (*atarrp1-2*) and At1g09310 (*atarrp1-1*) single mutants produced trichomes with smaller length and variable number of branches similar to those seen by Marks *et al*. (2009) for At1g56580 (Fig. 3.8c). Except for this trichome branching phenotype, the two single mutants do not show any visible growth phenotype compared to wild type (Fig. 3.8b). This result suggests that there might be functional redundancy among these DUF538 proteins. To examine this possibility I generated At1g56580xAt1g09310 double mutants. Since *ARRP1* expression is regulated by auxin and light, we germinated seeds in the dark and examined the hypocotyl length of different mutants. While the hypocotyl length was indistinguishable from WT plants, the double mutant showed a slower growth upon transition to light after five days of growth in the dark, a difference that was especially obvious in the early development stages (Fig. 3.8d). The inflorescence emergence and flowering time are delayed by two weeks in the double mutant, but leaf number at the time of flowering and diameter of the rosette and stem are identical to the wild type control (Fig. 3.8e). These results suggest that At1g56580 and At1g09310 play a role in the early plant growth during transition into light.

I expressed *ZmARRP1*, in *Arabidopsis* wild type and double mutant to examine if ZmARRP1 and AtARRP1/2 execute the same function. *ZmARRP1* completely restores the wild type phenotype in the double mutant (Fig. 3.8d). Plant growth in the complementation line was restored to the wild-type level, and the delayed stem

emergence time and flowering time were also rescued. Because At1g090310 and At1g56580 are highly expressed in the juvenile leaf (Fig. 3.7), this suggests that they may only function in the early stage of leaf development. When the leaves enter a later developmental stage, other regulatory factors may have dominant effects on plant growth and causing differences in phenotype to fade. These data suggest that ZmARRP1 and its *Arabidopsis* homologues At1g090310 and At1g56580 serve identical functions in both plants. This suggests that ARRP1 may be a regulator universal to all land plants.

3.4.6 ZmARRP1 interacts with BRL2

My findings imply that ARRP1 fulfills the same function in *Arabidopsis* and corn. As AtARRP1-2 was previously identified as an AtBRL2-interacting protein (Cesarani *et al*., 2013), I examined whether ZmARRP1 also interacts with AtBRL2. The intracellular portion of AtBRL2 contains a canonical kinase motif at the carboxyl end, which is proposed to mediate specific protein-protein interactions (Clay and Nelson, 2002). Using both a phosphorylated and a non-phosphorylated C-terminal 998-amino acid fragment of AtBRL2 containing a His tag as bait, I performed pull down assays to identify interacting proteins from *Zea mays* leaf extracts. Presence of ARRP1 in the complex was detected using western blotting with the ZmARRP1 specific antibody (Fig. 3.9b). The non-ARRP1-binding protein Hyp3 was used as a negative control. ARRP1-BRL2-interaction could be seen when BRL2 was phosphorylated but not when it was unphosphorylated (Fig. 3.9b). The result showed that ZmARRP1 only interacts with phosphorylated AtBRL2, suggesting the phosphorylation is very important for protein-protein interaction. This interaction was confirmed using Y2H (Fig. S3.4).

A search of public databases through the National Center for Biotechnology Information (NCBI) website revealed one predicted BRL2 protein in *Zea mays* (ZmBRL2) with 60% identity to AtBRL2 protein(Fig. 3.9a). An pull down assay using the 1003-amino acid C-terminal ZmBRL2 fragment fused to a His-tag showed that ZmARRP1 also interacts with the phosphorylated intracellular domain ZmBRL2 (Fig. 3.9c). This cross-reactivity between ZmARRP1 and AtBRL2 suggests that similar BRL2 signaling pathways exist in both maize and *Arabidopsis*.

BRL2 is a receptor-like kinase. Proteins with a phosphorylated tyrosine or serine/threonine are often recognized by receptor like kinase protein interaction domains. Our western blots showed doublet bands for ZmARRP1, suggesting possible post-translational modifications. Based on a bioinformatics analysis (NetPhos 2.0) we found ARRP1 contains at least three serine and two tyrosine embedded in a phosphorylation target sequence (Fig. 3.1a), suggesting ARRP1 a good downstream candidate for the ZmBRL2 signal pathway.

Despite the fact that the AtBRL2 protein sequence is closely related to *Arabidopsis* BRL1, a brassinosteroid-responsive receptor kinase, and shares with it the presence of an amino acid island thought to contribute to ligand specificity (Li and Chory, 1997), AtBRL2 does not respond to brassinosteroids, but can be induced 2-fold by auxin-treatment (Clay, 2002). The same study showed that BRL2 most likely is involved in vascular differentiation and leaf venation patterning, which would suggest an interaction with the auxin or cytokinin signal pathway (Clay, 2002). The fact that both BRL2 and ARRP1 are induced by auxin but not brassinosteroids or cytokinins and the

fact that they show direct interaction, suggests that they act jointly in an auxin-mediated signaling pathway, perhaps as it relates to vascular differentiation.

3.4.7 *ARRP1* and *VH1/BRL2* are expressed in overlapping patterns

Interacting proteins often are encoded by genes expressed in the same cells and under the same conditions. To determine whether *ARRP1* is co-expressed with BRL2, we characterized the expression patterns of *ARRP1*, using *in situ* RNA hybridization. Expression of *ARRP1* is broad and diffuse in the early stage of developing leaves and coleoptiles. In mature leaves, transcripts for *ARRP1* decrease significantly suggesting *ARRP1* might only function in the early stage of leaf development. Further examination of young leaves and the coleoptile found that *ARRP1* is enhanced in the vascular bundles (Fig 3.11).

Vascular bundle development is regulated by auxin (Scarpella *et al*., 2006; Fabregas *et al*., 2015). In addition, studies showed that the transcription of BRL2 was restricted to provascular and procambial cells in organ primordia. In leaves, expression of BRL2 significantly decreased in the maturing vascular elements and ends in fully differentiated mature leaf (Clay, 2002). This expression pattern overlaps with our findings for *ZmARRP*1. Together with the finding that ZmARRP1 interacts with BRL2, we proposed that auxin modifies the ZmARRP1-BRL2 interaction, leading to a change in gene expression and leaf expansion and venation pattern.

3.4.8 Subcellular localization of ARRP1

To investigate the subcellular localization of ARRP1, a C-terminal ARRP1-CFP fusion protein was transiently expressed in tobacco leaves co-infiltrated with ER marker and Golgi markers. As shown (Fig. 3.10), ARRP1-CFP is localized in the cytosol.

Interestingly, it is also found in the nucleus. Colocalization with ER and Golgi markers is limited to the area surrounding the nucleus.

The CaMV 35S promoter used in the ARRP1-CFP expression construct is a very strong constitutive promoter which sometimes will cause non-targeted expression. To confirm that ARRP1 is indeed localized in the nucleus, we isolated the nuclei from the leaves of *Zea mays*, using differential centrifugation (Fig. 3.11b). Western blot analysis showed that while ARRP1 is localized mostly in the total cell extract, it is also present in significant amounts in the nucleus (Fig. 3.11c). Histone H3, a known nuclear protein, is only found in the nuclear fraction. These data suggest that ARRP1 moves to the nucleus. Because ARRP1 does not contain any nuclear targeting signal, translocation to the nucleus is likely to require additional proteins.

3.5 Conclusions:

In this paper we showed that ARRP1, an auxin-response protein is induced by auxin as well as by red light and that its expression is associated predominantly with expanding above-ground tissues in maize and *Arabidopsis*. It is localized in the cytoplasm as well as in the nucleus and, thus, could regulate gene expression. ARRP1 interacts with BRL2, a receptor-like kinase that plays a role in the venation pattern of plants. These observations suggest that ARRP1 participates in auxin- and light-related signaling. We propose that ARRP1 integrates the auxin- and red-light response as they relate to the expansion of leaves and the development of the vasculature. In this function it could interact with auxin directly, with an auxin binding protein/receptor, and/or with an additional signal that is transmitted through the BRL2 kinase to ARRP1. We propose three possible mechanisms for its function: in response to a stimulus, (I) ARRP1 moves from cytosol to the nucleus where it binds additional activators and regulates gene expression; (II) it moves to the nucleus where it binds additional protein and regulates cell division; or (III) it binds and modifies other, possibly cytoplasmic proteins, thus affecting their function (Fig. 3.12).

Figure 3.1 The ARRP1 protein belongs to a DUF538 superfamily.

(a) The ARRP1 protein sequence contains a DUF538 conserved domain (amino acids 25 to 140; highlighted in gray). Predicted phosphorylation (Netphos 2.0) sites are indicated in red. (b) Alignment of the complete sequence of ZmARRP1, AtARRP1-1 and AtARRP1-2. Amino acids that are conserved among all of the compared sequences are labeled by asterisks, and those that are conserved among at least two sequences are labeled by a dot.

a

```
MTLTIPDEVRAKAEVYVGDAAGQEKTRLLLQETGLPSGLL   40
PLRDIIECGYVEETGFVWLRQRRKVDHYFAKAGRHVSYGA   80
EVSAVADKGRLKKITGVKAKEMLLWVTLHEICVDDPPTGK  120
LHCKAIGGLSRSFPVEAFEAEEPAPPGAGGVVPRDAEEEE  160
EVEKEEGEGDKNPEQEAAAEKDGDAPAAAPAPEEAESKAE  200
EKADKEVSSADPAVVHAEALAAKN                  224
```

b

```
AtARRP1-1       MGLVT-DEVRARAEKYTGDEICREKTKEFLKEVSMPNGLLPLKDIEEVGYDRETGIVWLK 59
AtARRP1-2       MGLVT-EEVRAKAEMYTGDEICREKTKCFLKEISMPNGLLPLKDIEEVGYDRESGVVWLK 59
ZmARRP1         MTLTIPDEVRAKAEVYVGDAAGQEKTRLLLQETGLPSGLLPLRDIIECGYVEETGFVWLR 60
                * *.  :****:** *.**   :***: :*:* .:*.*****:** * ** .*:*.***:

AtARRP1-1       QKKSITHKFEAIGKLVSYATEVIAQVEVGKIKKLTGVKAKELLIWVTLNELVLEQPTSSG 119
AtARRP1-2       QKKSITHKFTEIDKLVSYGTEVTAIVETGKIKKLTGVKAKELLIWVTINEIYTEEPPT-- 117
ZmARRP1         QRRKVDHYFAKAGRHVSYGAEVSAVADKGRLKKITGVKAKEMLLWVTLHEICVDDPPTG- 119
                *::.: * *   .: ***.:** * .: *::**:*******:*:***::*:  ::*.:

AtARRP1-2       KITFKTPTTLSRTFPVTAFIVPEEPAK------EEPAKEEPAKEKSSE---ATEAKEAVA 168
AtARRP1-1       KINFRTPTGLSRTFPVSAFVVPEV--------------EKPATEKNNG---TTEVKEAVA 162
ZmARRP1         KLHCKAIGGLSRSFPVEAFEAEEPAPPGAGGVVPRDAEEEEEVEKEEGEGDKNPEQEAAA 179
                *:  ::   ***:*** ** . *              *:   **..     .  :**.*

AtARRP1-2       IKEAVAVKEAA---------------------------------- 179
AtARRP1-1       VTDA---------------------------------------- 166
ZmARRP1         EKDGDAPAAAPAPEEAESKAEEKADKEVSSADPAVVHAEALAAKN 224
```

Figure 3.2 Illustration of the distribution of the DUF538 conserved domain in plants.

Red numbers indicate the number of DUF538-containing genes identified in each individual species so far. Blue numbers indicate the number of species that possess genes that belong to the DUF538 superfamily. Only species above the blue line contain representative of DUF538-containing proteins

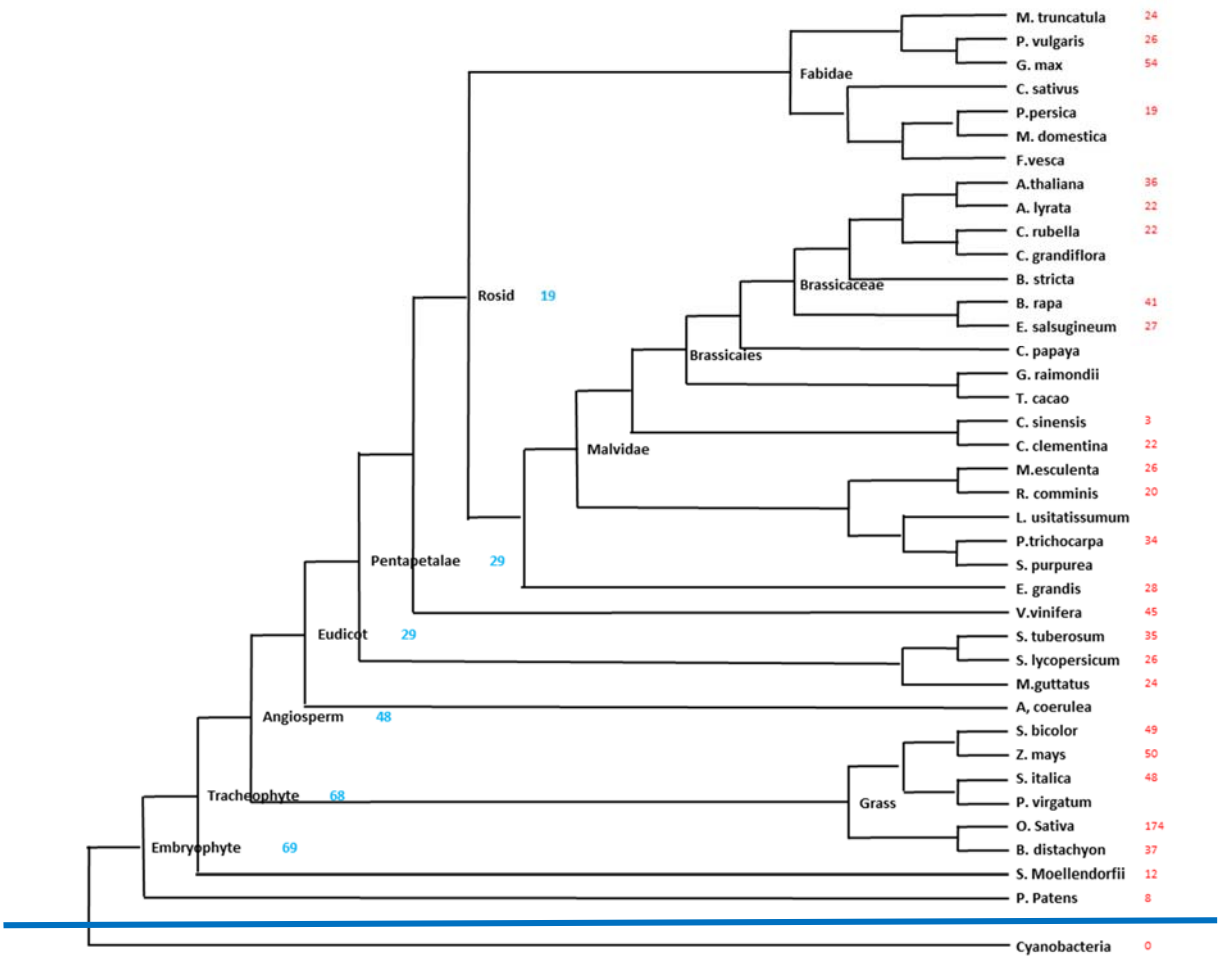
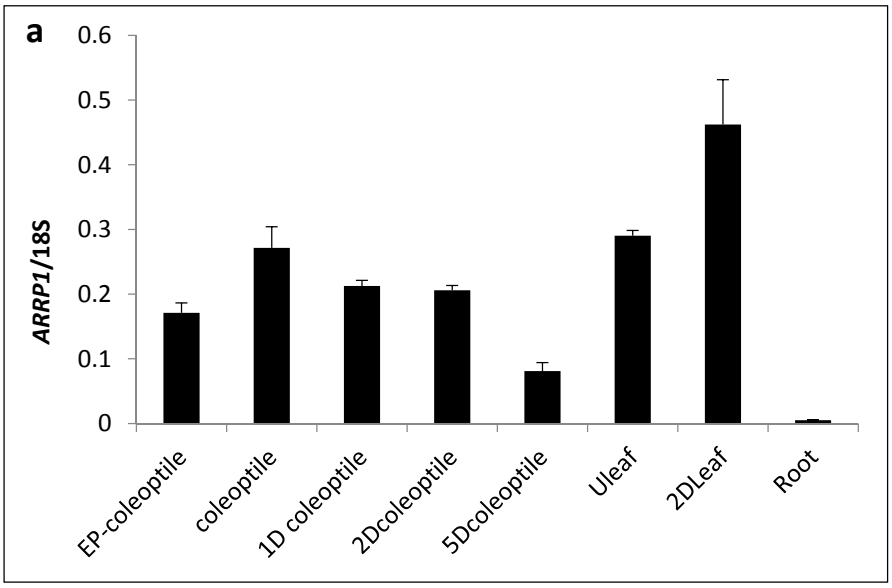


Figure 3.3 ARRP1 levels throughout the corn seedlings.

(a) *ARRP1* expression relative to 18S RNA in different parts of the corn seedlings (modified from Li *et al*. 2013) Values are mean and S.E. of three biological replicates. (b) Quantification of ARRP1 protein throughout the seedling as determined by western blotting using an ARRP1-specific antibody.



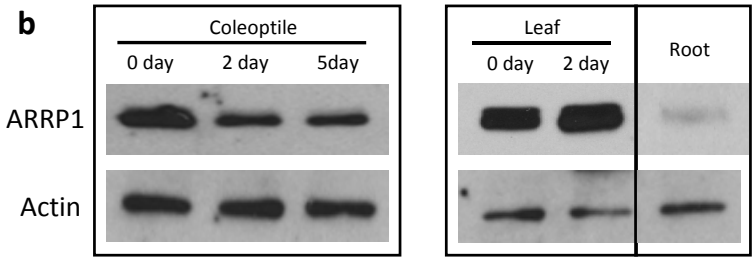
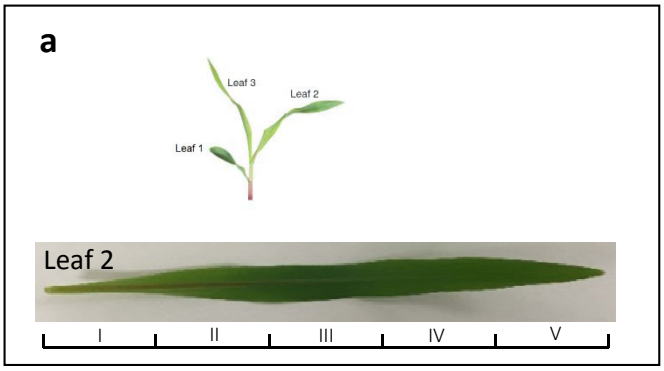
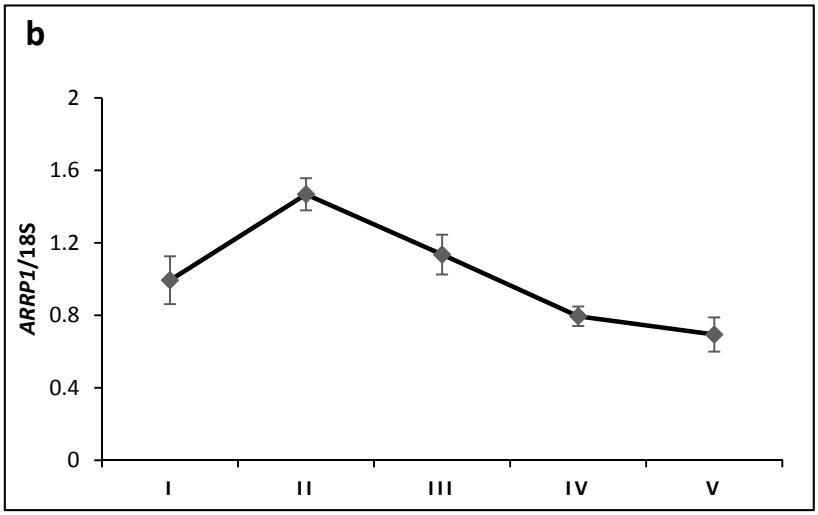


Figure 3.4 *ARRP1* expression in different leaf zones.

(a) Illustration of a nine-day-old corn seedling. Leaf 2 was used to monitor the *ARRP1* expression level. (b) Semiquantitative analysis of the *ARRP1* mRNA levels in different corn leaf zones. *ARRP1* was quantified relative to 18S RNA. (c) Protein levels of ARRP1 in the same leaf zones as determined using western blot. Actin was used as a control. Experiments were performed in triplicate. Zone I: predominantly cell division with initiation of cell elongation; Zone II: declining cell division, high rate of cell elongation; Zone III: transition zone; some cell division; increase of transcripts associated with photosynthetic machinery; Zone IV: maturation zone; high expression of genes involved with the photosynthetic machinery Zone V: mature, fully developed zone; initiation of apoptosis





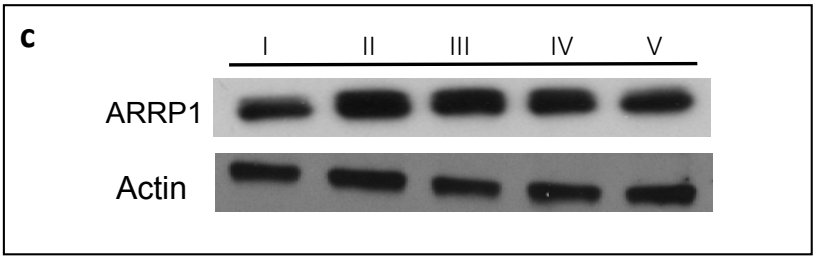
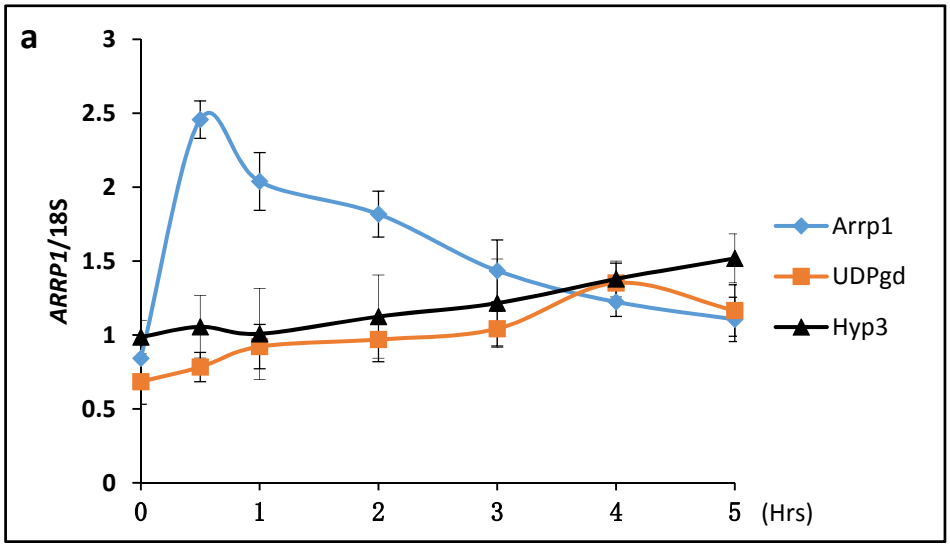


Figure 3.5 Response of ARRP1 in five days old corn coleoptiles to Auxin treatment.

(a) Semiquantitative analysis of mRNA level changes of genes encoding ARRP1, UDPgd and Hyp3 in corn coleoptiles within five hrs Auxin-treatment. UDPgd: UDP-glucose dehydrogenase; Hyp3: a putative endoglucanase. All genes were quantified relative to 18S RNA. Bars represent the mean ± S.E. of three biological replicates. (b) Protein level changes of ARRP1 within 5 hrs Auxin-treatment by western blotting analysis. Actin was used as a control.



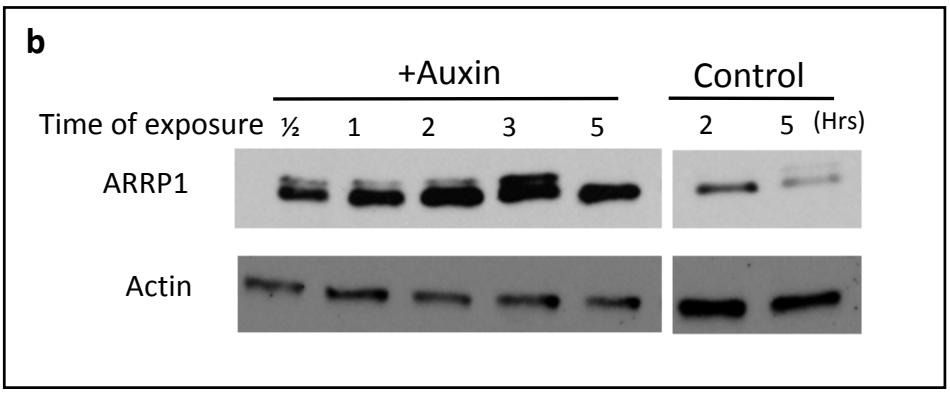


Figure 3.6 *ARRP1* expression in corn leaves in response to different light conditions.

(a) Semiquantitative analysis of *ZmARRP1* expression in corn leaves at various days after transfer of the seedlings to different light conditions. R : red light at 660 nm; FR: far red light at 735 nm; Blue: blue light at 420 nm; white: regular light condition. Values are mean and S.E. of three independent biological replicates. Asterisks indicate a significant difference ($p<0.01$; student's t-test) as compared to leaves kept in the dark. (b) Protein level changes of ARRP1 response to different light conditions by western blotting analysis. Values are mean and S.E. of three independent biological replicates.

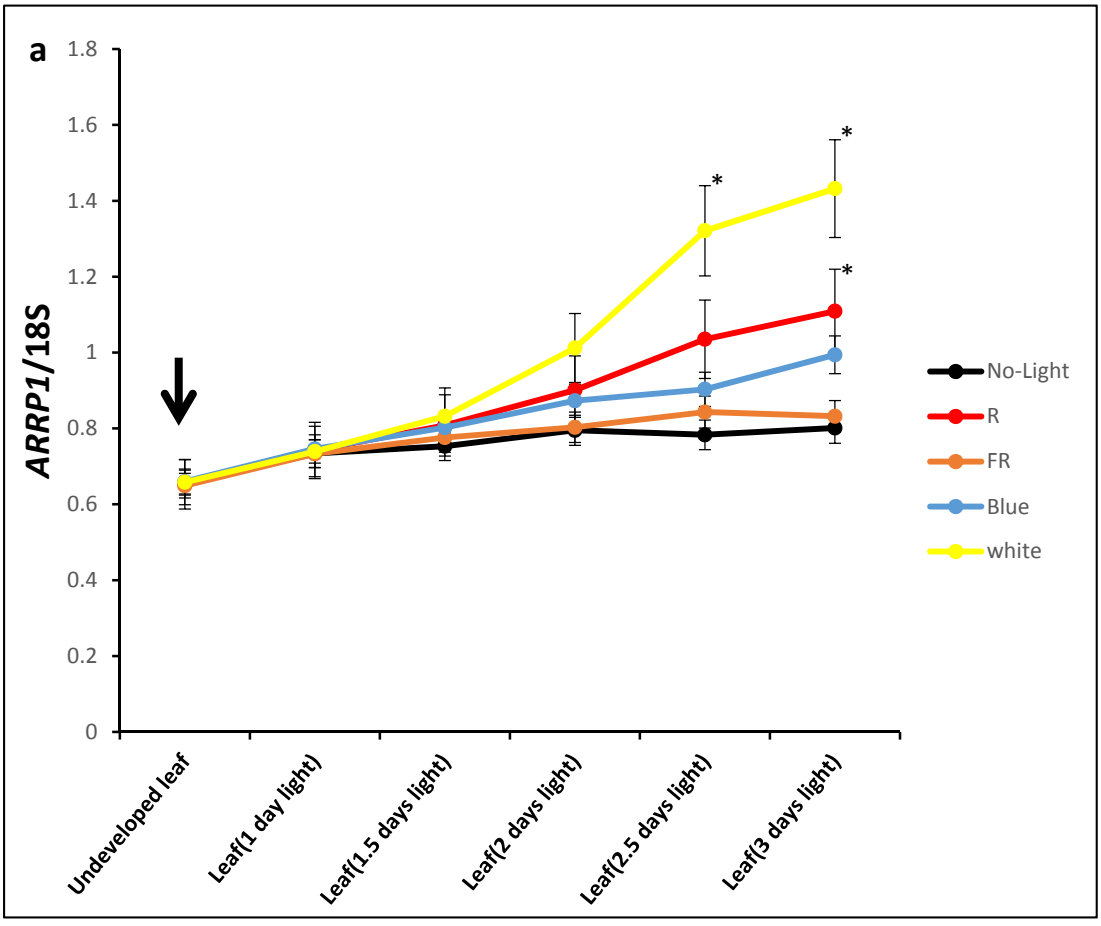


Figure 3.6 (cont'd)

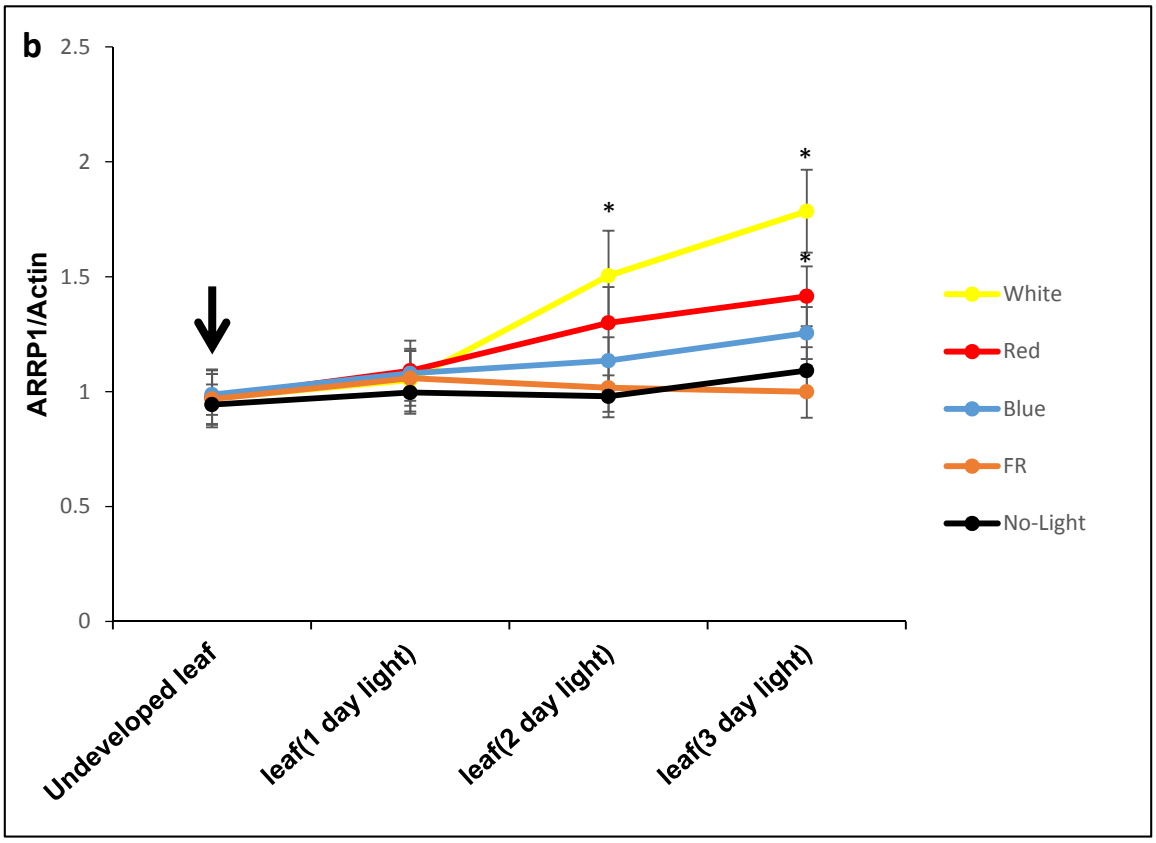


Figure 3.7 Expression levels of *AtARRP1-1* (a) and *AtARRP1-2* (b) in various *Arabidopsis thaliana* organs, as determined by RT-PCR.

Material for root was harvested from 7 day old seedlings germinated on MS plate. Developing leaf corresponds to 7 day old young leaf, while mature leaf corresponds to 1 month old fully developed leaf. Hypocotyl material was harvested from 5 day old dark-grown seedling germinated on MS plate. Values are mean and S.E. of three independent biological replicates.

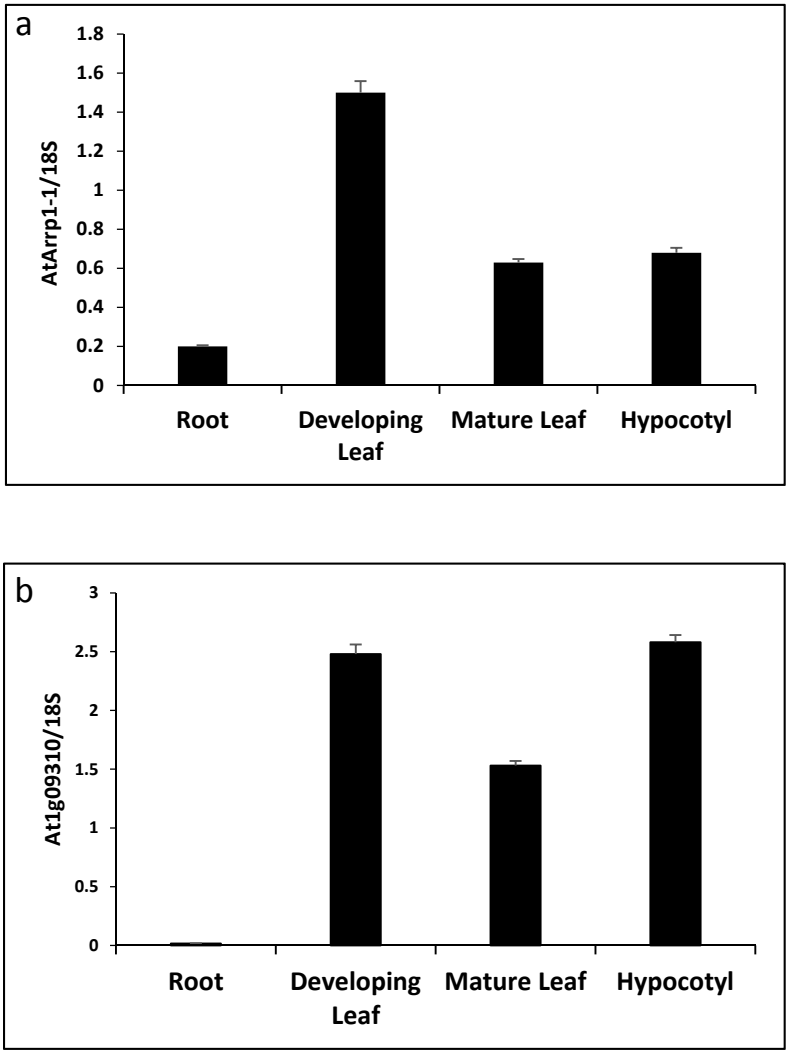


Figure 3.8 Single knock out mutants of *atarrp1-1* and *atarrp1-2* show trichomes with smaller branch length and variable number of branches.

(a) Using RT-PCR, we confirmed that transcription of *atarrp1-1* and *atarrp1-2* was completely abolished in the knockout mutant. (b) Comparing to wild type, the knockout mutants*atarrp1-1* and *atarrp1-2* did not show any morphological vegetative phenotype. (c) Both knockout mutants show changes in trichome development. The trichomes of mutants showed higher branch numbers and shorter branch length which is consist with a previous study (Marks *et al*., 2009). (d) The double mutant showed a slower growth phenotype upon transition to light after five days germination in dark, which can be complemented by overexpression of *ZmARRP1* in *Arabidopsis* mutant. Overexpression of *ZmARRP1* in WT plants did not show any significantly phenotype. (e) The inflorescence emergence time and flowering time are delayed by two weeks in the double mutant, but leaf number at the time of flowering, and diameter of the rosette and stem are identical to the wild type control

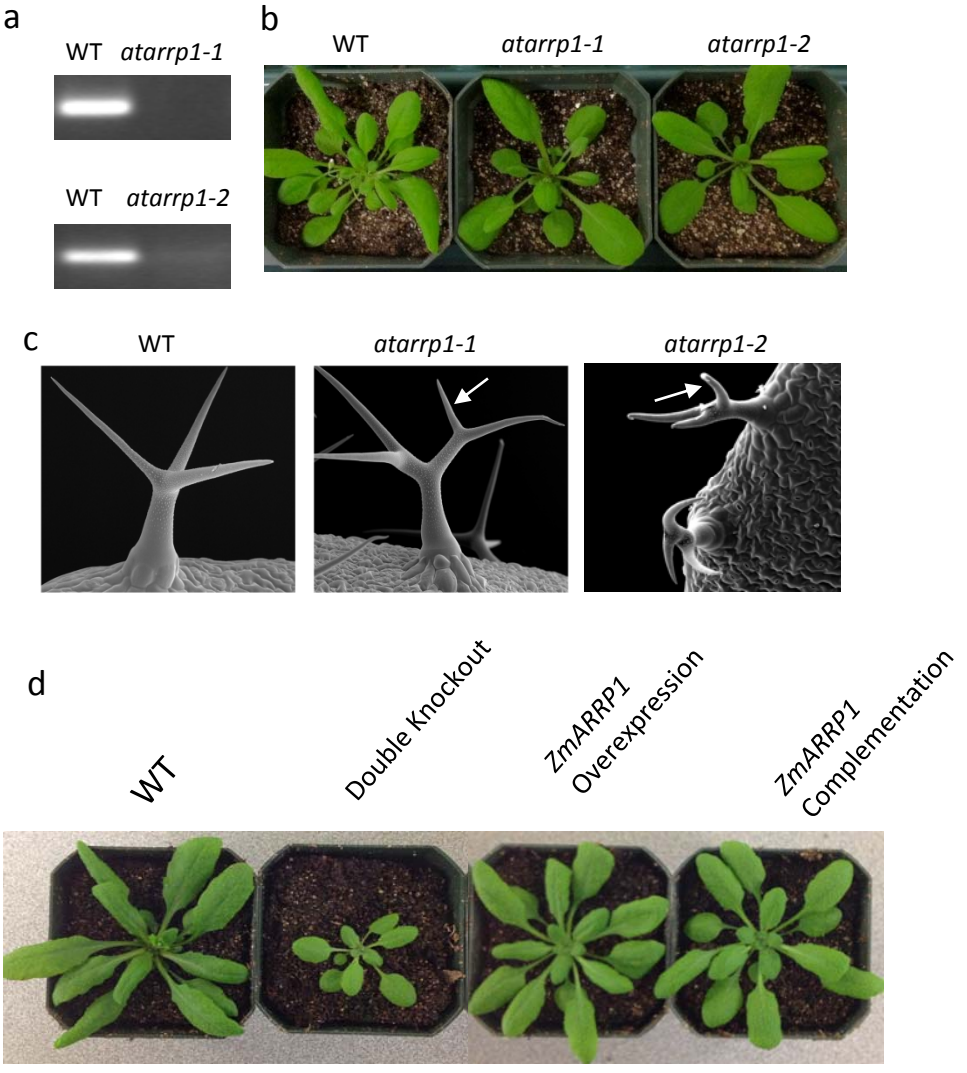


Figure 3.8 (cont'd)

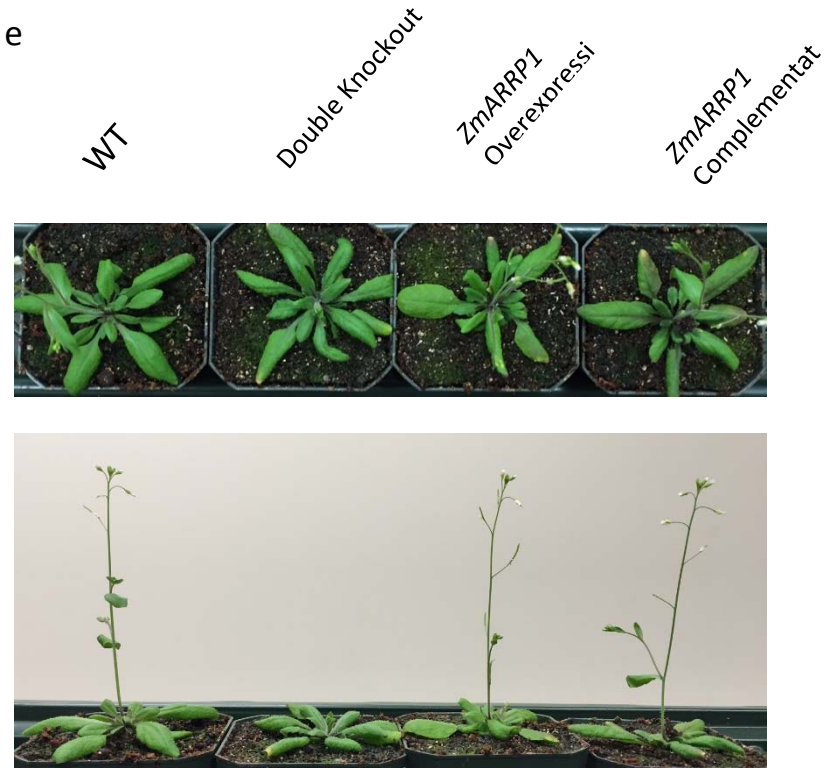


Figure 3.9 ARRP1 interacts with BRL2 homologues from *Arabidopsis* and maize.

(a) Alignment of the carboxyl terminal region of intracellular domain of ZmBRL2 and AtBRL2. Amino acids that are conserved between the two sequences are labeled by asterisks. (b) The carboxy-terminal region of the intracellular domain of *Arabidopsis* BRL2 was expressed, purified and then mixed with the protein extract of maize leaf for five hours. A Ni-Column was used to pull-down the AtBRL2 protein complex. Using an anti ZmARRP1 antibody, we confirmed the presence of ZmARRP1 in the AtBRL2 protein complex, indicating the interaction between ZmARRP1 and AtBRL2. (c) A parallel *in vitro* pull-down assay showed ZmARRP1-ZmBRL2 interaction. Endo-glucanase was used as a negative control.

a

```
AtBRL2    RARRRDADDAKMLHSLQAVN-SATTWKIEK-EKEPLSINVATFQRQLRKLKFSQLIEATN 58
ZmBRL2    RARRKEAREARMLSSLQDGTRTATTWKLGKAEKEALSINVATFQRQLRRLTFTQLIEATN 60
           ****::* :*:** ***  . :*****: * ***.*************:*.*:*******

AtBRL2    GFSAASMIGHGGFGEVFKATLKDGSSVAIKKLIRLSCQGDREFMETAEMETETLGKIKHR 118
ZmBRL2    GFSAGSLVGSGGFGEVFKATLKDGSCVAIKKLIHLSYQGDREFTA----EMETLGKIKHR 116
          ****.*::* ***************.*******:** ******      * *********

AtBRL2    NLVPLLGYCKIGEERLLVYEFMQYGSLEEVLHGPRTGEKRRILGWEERKKIAKGAAKGLC 178
ZmBRL2    NLVPLLGYCKIGEERLLVYEYMSNGSLEDGLHG-----RALRLPWERRKRVARGAARGLC 171
          ********************:*. ****: ***     :   * **.**::*:***:***

AtBRL2    FLHHNCIPHIIHRDMETKSSNVLLDQDMEARVSDFGMARLISALDTHLSVSTLAGTPGYV 238
ZmBRL2    FLHHNCIPHIIHRDMK--SSNVLLDGDMEARVADFGMARLISALDTHLSVSTLAGTPGYV 229
          ***************:  ******* ******:**************************

AtBRL2    PPEYYQSFRCTAKGDVYSIGVVMLEILSGKRPTDKEEFGDTNLVGWSKMKAREGKHMEVI 298
ZmBRL2    PPEYYQSFRCTAKGDVYSLGVVFLELLTGRRPTDKEDFGDTNLVGWVKMKVREGTGKEVV 289
          ******************:***:**:*:*:******:********* ***.***.  **:

AtBRL2    DEDLLKEGSSESLNEKEGFEGGVIVKEMLRYLEIALRCVDDFPSKRPNMLQVVASLRELR 358
ZmBRL2    DPELV-------IAAVDGEE-----KEMARFLELSLQCVDDFPSKRPNMLQVVATLRELD 342
          * :*:       :   :* *     *** *:**::*:******************:****

AtBRL2    GSENNSHSHSNSL-- 371
ZmBRL2    DAPPSHQQAPASACD 362
          .:  . :. . *
```

b

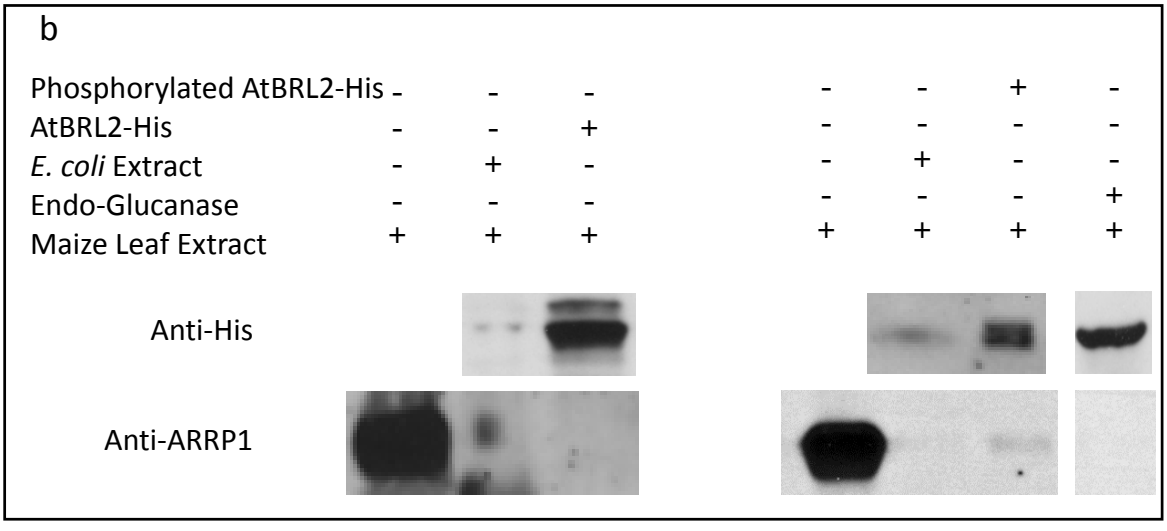


Figure 3.9 (cont'd)

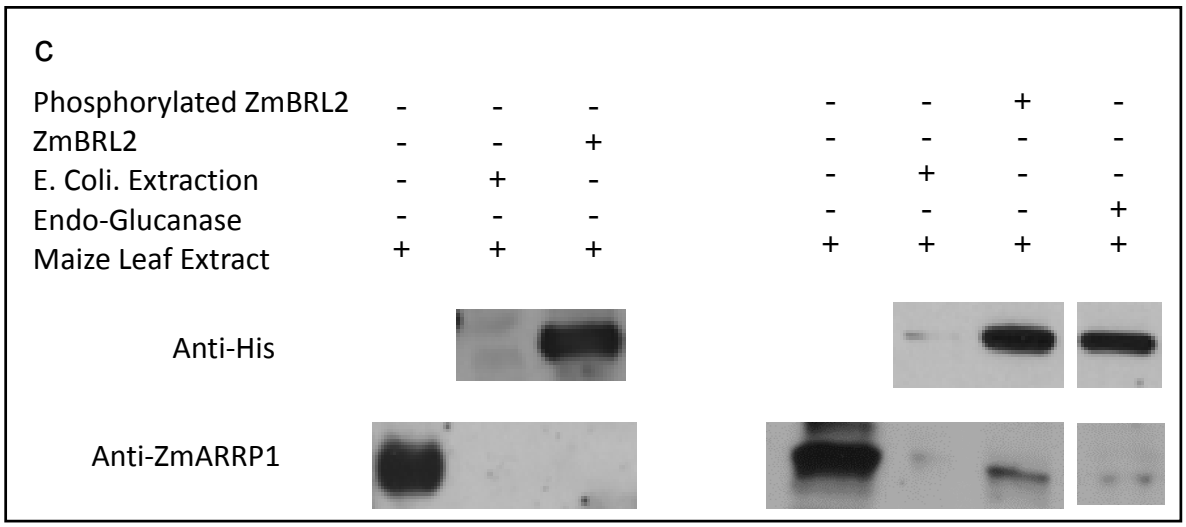


Figure 3.10 Subcellular localization of ARRP1

(a) Cyan fluorescent protein was fused to the C-terminus of ARRP1. Using confocal microscopy, ARRP1-CFP is visible mainly in the periphery of cell and in the nuclei. (b) Gradient differential centrifuge was used to purify the nuclei from the cell fraction. (c) Western blotting analysis confirmed the presence of ARRP1 in both cytosol and nuclei fraction, while the nuclear protein histone H3 was only detected in the nucleus.

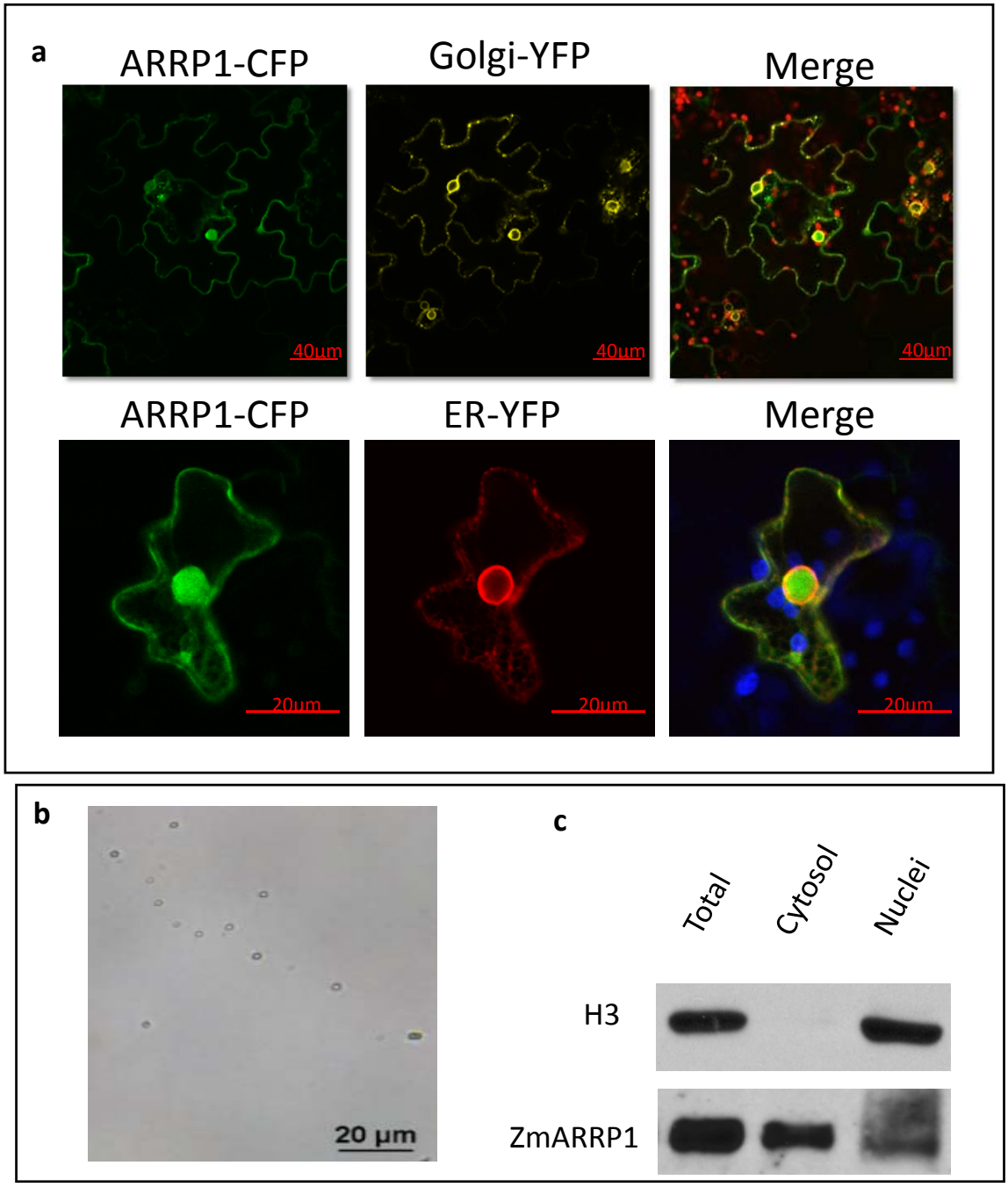


Figure 3.11 *In situ* hybridization shows that ZmARRP1 is expressed in the young leaves and enriched in the vascular bundles.

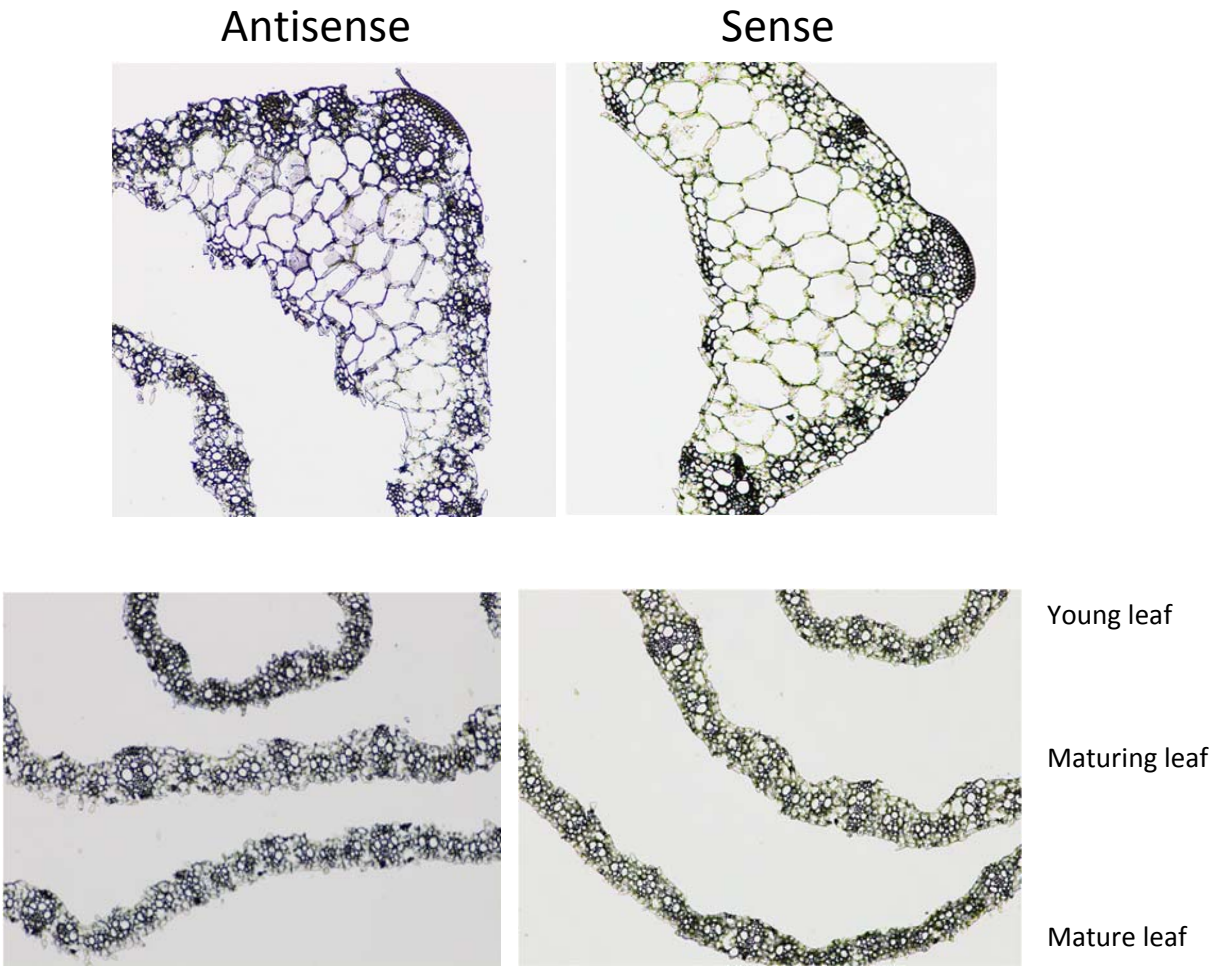


Figure 3.12 A model for proposed function of ARRP1.

ARRP1 interacts with BRL2, a receptor-like kinase. in response to a stimulus, (I) ARRP1 moves from cytosol to the nucleus where it binds additional activators and regulates gene expression; (II) it moves to the nucleus where it binds additional protein and regulates cell division; or (III) it binds and modifies other, possibly cytoplasmic proteins, thus affecting their function

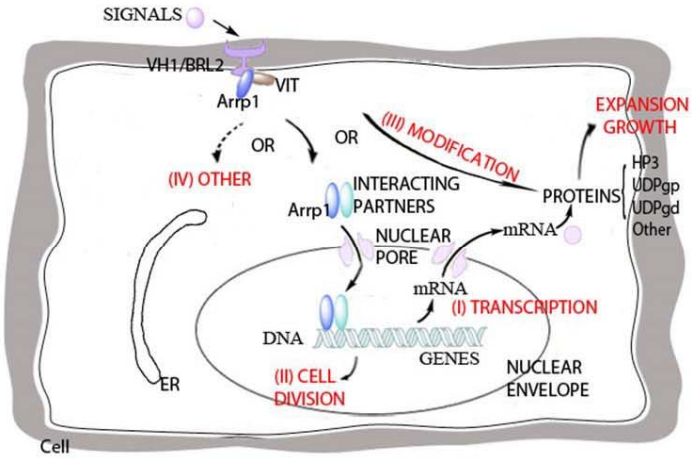


Table S3.1 Primers for the paper

Primer Name	Primer Sequence
pET15b-Arrp1-F	CATATGATGACGCTGACGATCCCGGAC
pET15B-Arrp1-R	GGATCCTCAGTTCTTGGCGGCCAGCGC
pDONR207-Arrp1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCATGACGCTGACGATCCCGGAC
pDONR207-Arrp1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTCTTGGCGGCCAGCGC
Arrp1-RT-PCR-F	GGGTAAGCTCCACTGCAAGG
Arrp1-RT-PCR-R	GTTCTTGTCCCCCTCCTCCT
At1g09310-LP	GGTGTTGTATGGCTGAAGCA
AT1G09310-RP	CTTCGCTGCTCTTCTCCTTG
AT1G56580-LP	TTGCCATTGAAGGACATTGA
AT1G56580-RP	GCAGGCTTCTCAACTTCAGG
LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
pET15b-VH1-K-Zea Mays-F	CATATGCGCGCGCGGCGGAAGGAGGCGCGCGAG
pET15b-VH1-K-Zea Mays-R	GGATCCTCAGTCACAGGCAGAGGCAGGCGCTTG
pET15b-VH1-K-*Arabidopsis*-F	CATATGCGTGCGAGGAGGAGAGATGCGG
pET15b-VH1-K-*Arabidopsis*-R	GGATCCTTACAAGCTGTTACTGTGACTGTG

Table S3.2 *Cis-regulatory element* within 1 Kb ARRP1 promotor

Cis-Regulatory element	site	Sequence	Cis-Regulatory element	site	Sequence
DOFCOREZM	-10	AAAG	NODCON2GM	-428	CTCTT
ANAERO2CONSENSUS	-16	AGCAGC	OSE2ROOTNODULE	-428	CTCTT
MYBPZM	-33	CCWACC	EBOXBNNAPA	-441	CANNTG
CACTFTPPCA1	-49	YACT	MYCCONSENSUSAT	-441	CANNTG
DPBFCOREDCDC3	-50	ACACNNG	GATABOX	-447	GATA
RAV1AAT	-52	CAACA	GT1CONSENSUS	-447	GRWAAW
GCAACREPEATZMZEIN	-58	GCAACGCAAC	IBOXCORE	-447	GATAA
2SSEEDPROTBANAPA	-65	CAAACAC	SEF4MOTIFGM7S	-454	RTTTTTR
CANBNNAPA	-65	CNAACAC	ARR1AT	-456	NGATT
DOFCOREZM	-74	AAAG	MYBCORE	-460	CNGTTR
TATABOX4	-105	TATATAA	DOFCOREZM	-472	AAAG
CANBNNAPA	-123	CNAACAC	TAAAGSTKST1	-473	TAAAG
CACTFTPPCA1	-125	YACT	POLASIG1	-475	AATAAA
CACTFTPPCA1	-140	YACT	PREATPRODH	-483	ACTCAT
DPBFCOREDCDC3	-141	ACACNNG	CACTFTPPCA1	-484	YACT
GTGANTG10	-161	GTGA	POLASIG1	-490	AATAAA
GT1CONSENSUS	-177	GRWAAW	POLASIG3	-493	AATAAT
GT1GMSCAM4	-177	GAAAAA	GATABOX	-504	GATA
POLLEN1LELAT52	-178	AGAAA	DOFCOREZM	-510	AAAG
DOFCOREZM	-180	AAAG	XYLAT	-512	ACAAAGAA
TAAAGSTKST1	-181	TAAAG	RAV1AAT	-514	CAACA
CAATBOX1	-191	CAAT	SORLIP1AT	-518	GCCAC
CCAATBOX1	-192	CCAAT	RAV1AAT	-524	CAACA
MYBCORE	-227	CNGTTR	DOFCOREZM	-531	AAAG
CACTFTPPCA1	-234	YACT	TAAAGSTKST1	-532	TAAAG
MYBPZM	-251	CCWACC	ARR1AT	-536	NGATT
CACTFTPPCA1	-284	YACT	DOFCOREZM	-553	AAAG
ACGTATERD1	-294	ACGT	-300ELEMENT	-557	TGHAAARK
ACGTABOX	-295	TACGTA	CACTFTPPCA1	-560	YACT
SEF3MOTIFGM	-319	AACCCA	CPBCSPOR	-573	TATTAG
CIACADIANLELHC	-321	CAANNNNATC	CAATBOX1	-578	CAAT
CARGCW8GAT	-363	CWWWWWWWWG	CACTFTPPCA1	-589	YACT
CACTFTPPCA1	-365	YACT	GTGANTG10	-608	GTGA
CACTFTPPCA1	-372	YACT	EBOXBNNAPA	-611	CANNTG
SEF3MOTIFGM	-376	AACCCA	RAV1AAT	-614	CAACA
INRNTPSADB	-424	YTCANTYY			

Figure S3.1 Expression and purification of ARRP1 and western blotting analysis.

(a) Expression and purification of ARRP1. Different fractions were separated by SDS-PAGE gel and stained by coomassie blue. Elution fraction 2 & 3 showed accumulation of a protein with the same size of ARRP1 (b) Identity of the band as ARRP1 was confirmed using Anti-ARRP1 antibody.

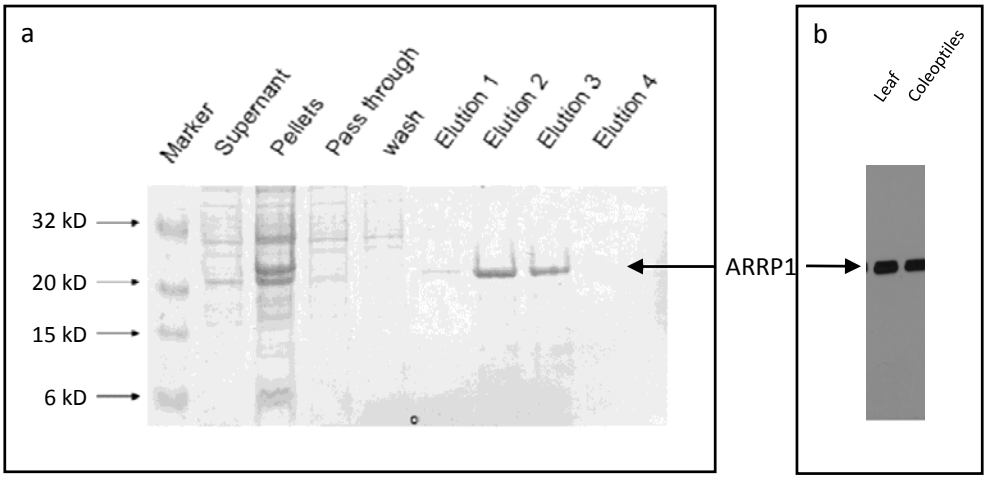


Figure S3.2 *ARRP1* mRNA levels in corn coleoptile are unchanged by cytokinin or brassinosteroid.

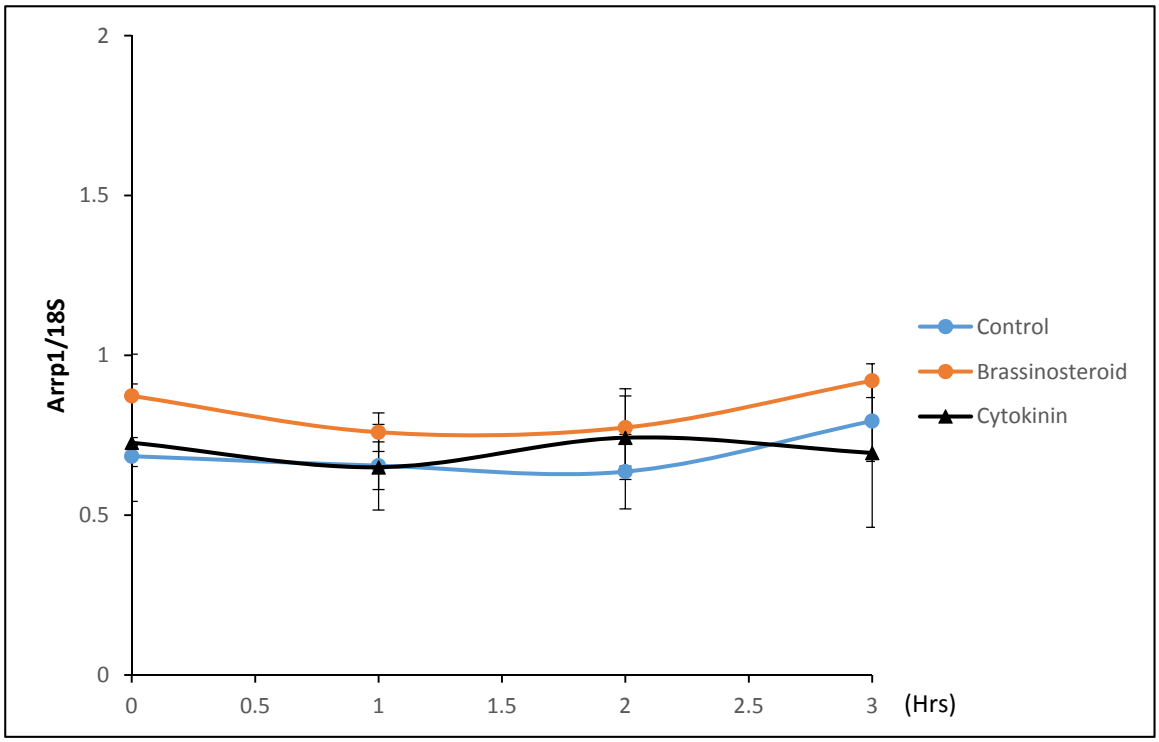
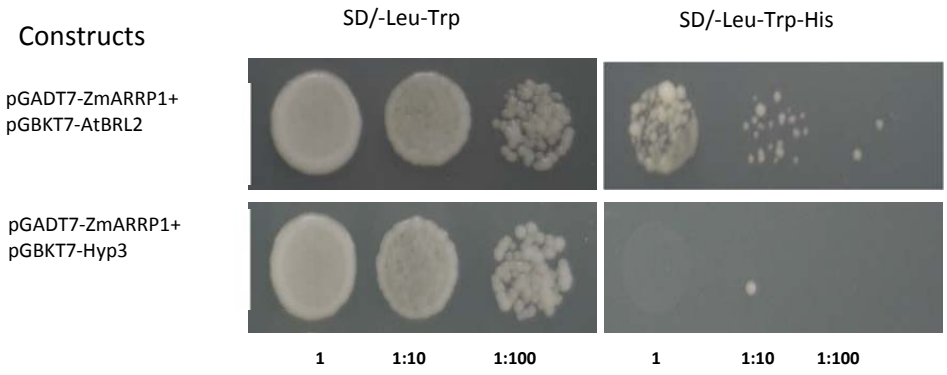


Figure S3.3 Y2H analysis of ZmARRP1 with AtBRL2.

AtBRL2 was insert into the pGBKt7 vector, while *ZmARRP1* was insert into the pGADT7 vector. The co-transformed yeast cells were tested on selection medium.



REFERENCES

REFERENCES

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653-657

Becker D, Hedrich R (2002) Channelling auxin action: modulation of ion transport by indole-3-acetic acid. Plant Molecular Biology **49:** 349-356

Benjamins R, Scheres B (2008) Auxin: the looping star in plant development. Annu Rev Plant Biol **59:** 443-465

Brummell DA, Hall JL (1987) Rapid cellular-responses to auxin and the regulation of growth. Plant Cell and Environment **10:** 523-543

Burdach Z, Kurtyka R, Siemieniuk A, Karcz A (2014) Role of chloride ions in the promotion of auxin-induced growth of maize coleoptile segments. Annals of Botany **114:** 1023-1034

Calderon Villalobos LI, Lee S, De Oliveira C, Ivetac A, Brandt W, Armitage L, Sheard LB, Tan X, Parry G, Mao H, Zheng N, Napier R, Kepinski S, Estelle M (2012) A combinatorial TIR1/AFB-Aux/IAA co-receptor system for differential sensing of auxin. Nat Chem Biol **8:** 477-485

Ceserani T, Trofka A, Gandotra N, Nelson T (2009) VH1/BRL2 receptor-like kinase interacts with vascular-specific adaptor proteins VIT and VIK to influence leaf venation. Plant J **57:** 1000-1014

Chae K, Isaacs CG, Reeves PH, Maloney GS, Muday GK, Nagpal P, Reed JW (2012) *Arabidopsis* SMALL AUXIN UP RNA63 promotes hypocotyl and stamen filament elongation. Plant J **71:** 684-697

Claeys H, De Bodt S, Inze D (2014) Gibberellins and DELLAs: central nodes in growth regulatory networks. Trends Plant Sci **19:** 231-239

Claussen M, Luthen H, Blatt M, Bottger M (1997) Auxin-induced growth and its linkage to potassium channels. Planta **201:** 227-234

Clay NK (2002) VH1, a provascular cell-specific receptor kinase that influences leaf cell patterns in *Arabidopsis*. The Plant Cell Online **14:** 2707-2722

Colon-Carmona A, Chen DL, Yeh KC, Abel S (2000) Aux/IAA proteins are phosphorylated by phytochrome *in vitro*. Plant Physiol **124:** 1728-1738

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science **316:** 1030-1033

Cosgrove DJ (1997) Relaxation in a high-stress environment: The molecular bases of extensible cell walls and cell enlargement. Plant Cell **9:** 1031-1041

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol **6:** 850-861

de Lucas M, Prat S (2014) PIFs get BRright: PHYTOCHROME INTERACTING FACTORs as integrators of light and hormonal signals. New Phytologist **202:** 1126-1141

Depuydt S, Hardtke CS (2011) Hormone signalling crosstalk in plant growth regulation. Curr Biol **21:** R365-373

Enders TA, Strader LC (2015) Auxin activity: Past, present, and future. Am J Bot **102:** 180-196

Engvild KC (1985) Pollen irradiation and possible gene transfer in Nicotiana species. Theor Appl Genet **69:** 457-461

Fabregas N, Formosa-Jordan P, Confraria A, Siligato R, Alonso JM, Swarup R, Bennett MJ, Mahonen AP, Cano-Delgado AI, Ibanes M (2015) Auxin influx carriers control vascular patterning and xylem differentiation in *Arabidopsis thaliana*. PLoS Genet **11:** e1005183

Friml J, Wisniewska J, Benkova E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature **415:** 806-809

Gao Y, Zhang Y, Zhang D, Dai X, Estelle M, Zhao Y (2015) Auxin binding protein 1 (ABP1) is not required for either auxin signaling or *Arabidopsis* development. Proc Natl Acad Sci U S A **112:** 2275-2280

Gholizadeh A (2011) Heterologous expression of stress-responsive DUF538 domain containing protein and its morpho-biochemical consequences. Protein J **30:** 351-358

Gholizadeh A, Baghban Kohnehrouz B (2010) Identification of DUF538 cDNA clone from *Celosia cristata* expressed sequences of nonstressed and stressed leaves. Russian Journal of Plant Physiology **57:** 247-252

Goldsmith MH, Cataldo DA, Karn J, Brenneman T, Trip P (1974) The rapid non-polar transport of auxin in the phloem of intact Coleus plants. Planta **116:** 301-317

Grones P, Friml J (2015) Auxin transporters and binding proteins at a glance. J Cell Sci **128:** 1-7

Haga K, Iino M (1998) Auxin-growth relationships in maize coleoptiles and pea internodes and control by auxin of the tissue sensitivity to auxin. Plant Physiol **117:** 1473-1486

Halliday KJ, Martinez-Garcia JF, Josse EM (2009) Integration of light and auxin signaling. Cold Spring Harb Perspect Biol **1:** a001586

Hardtke CS, Dorcey E, Osmont KS, Sibout R (2007) Phytohormone collaboration: zooming in on auxin-brassinosteroid interactions. Trends Cell Biol **17:** 485-492

Hoad GV (1995) Transport of hormones in the phloem of higher-plants. Plant Growth Regulation **16:** 173-182

Hoecker U, Toledo-Ortiz G, Bender J, Quail PH (2004) The photomorphogenesis-related mutant red1 is defective in CYP83B1, a red light-induced gene encoding a cytochrome P450 required for normal auxin homeostasis. Planta **219:** 195-200

Hoffmann-Benning S, Kende H (1994) Cuticle biosynthesis in rapidly growing internodes of deepwater rice. Plant Physiol **104:** 719-723

Hoffmann-Benning S, Klomparens KL, Kende H (1994) Characterization of growth-related osmiophilic particles in corn coleoptiles and deepwater rice internodes. Annals of Botany **74:** 563-572

Hornitschek P, Kohnen MV, Lorrain S, Rougemont J, Ljung K, Lopez-Vidriero I, Franco-Zorrilla JM, Solano R, Trevisan M, Pradervand S, Xenarios I, Fankhauser C (2012) Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. Plant J **71:** 699-711

Jones AM, Venis MA (1989) Photoaffinity labeling of indole-3-acetic acid-binding proteins in maize. Proc Natl Acad Sci U S A **86:** 6153-6156

Jun JH, Fiume E, Fletcher JC (2008) The CLE family of plant polypeptide signaling molecules. Cell Mol Life Sci **65:** 743-755

Karkonen A, Murigneux A, Martinant JP, Pepey E, Tatout C, Dudley BJ, Fry SC (2005) UDP-glucose dehydrogenases of maize: a role in cell wall pentose biosynthesis. Biochem J **391:** 409-415

Karlgren A, Carlsson J, Gyllenstrand N, Lagercrantz U, Sundstrom JF (2009) Non-radioactive *in situ* hybridization protocol applicable for Norway spruce and a range of plant species. J Vis Exp

Kazan K, Manners JM (2009) Linking development to defense: auxin in plant-pathogen interactions. Trends Plant Sci **14:** 373-382

Kende H, Zeevaart J (1997) The five "classical" plant hormones. Plant Cell **9:** 1197-1210

Korasick DA, Westfall CS, Lee SG, Nanao MH, Dumas R, Hagen G, Guilfoyle TJ, Jez JM, Strader LC (2014) Molecular basis for AUXIN RESPONSE FACTOR protein interaction and the control of auxin response repression. Proc Natl Acad Sci U S A **111:** 5427-5432

Kutschera U, Bergfeld R, Schopfer P (1987) Cooperation of epidermis and inner tissues in auxin-mediated growth of maize coleoptiles. Planta **170:** 168-180

Kutschera U, Kende H (1989) Particles associated with the outer epidermal wall in internodes of deepwater rice. Annals of Botany **63:** 385-388

Kutschera U, Wang ZY (2015) Growth-limiting proteins in maize coleoptiles and the auxin-brassinosteroid hypothesis of mesocotyl elongation. Protoplasma

Lau S, Jurgens G, De Smet I (2008) The evolving complexity of the auxin pathway. Plant Cell **20:** 1738-1746

Laxmi A, Pan J, Morsy M, Chen R (2008) Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in *Arabidopsis thaliana*. PLoS One **3:** e1510

Li J, Dickerson TJ, Hoffmann-Benning S (2013) Contribution of proteomics in the identification of novel proteins associated with plant growth. J Proteome Res **12:** 4882-4891

Li P, Ponnala L, Gandotra N, Wang L, Si Y, Tausta SL, Kebrom TH, Provart N, Patel R, Myers CR, Reidel EJ, Turgeon R, Liu P, Sun Q, Nelson T, Brutnell TP (2010) The developmental dynamics of the maize leaf transcriptome. Nat Genet **42:** 1060-1067

Lin MK, Belanger H, Lee YJ, Varkonyi-Gasic E, Taoka K, Miura E, Xoconostle-Cazares B, Gendler K, Jorgensen RA, Phinney B, Lough TJ, Lucas WJ (2007) FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. Plant Cell **19:** 1488-1506

Ludwig-Muller J, Epstein E (1991) Occurrence and *in vivo* biosynthesis of indole-3-butyric acid in corn *(Zea mays L*.). Plant Physiol **97:** 765-770

Marks MD, Wenger JP, Gilding E, Jilk R, Dixon RA (2009) Transcriptome analysis of *Arabidopsis* wild-type and gl3-sst sim trichomes identifies four additional genes required for trichome development. Mol Plant **2:** 803-822

Michalko J, Dravecka M, Bollenbach T, Friml J (2015) Embryo-lethal phenotypes in early mutants are due to disruption of the neighboring gene. F1000Res **4:** 1104

Mikkelsen MD, Naur P, Halkier BA (2004) *Arabidopsis* mutants in the C-S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. Plant J **37:** 770-777

Mouchel CF, Osmont KS, Hardtke CS (2006) BRX mediates feedback between brassinosteroid levels and auxin signalling in root growth. Nature **443:** 458-461

Nagashima A, Suzuki G, Uehara Y, Saji K, Furukawa T, Koshiba T, Sekimoto M, Fujioka S, Kuroha T, Kojima M, Sakakibara H, Fujisawa N, Okada K, Sakai T (2008) Phytochromes and cryptochromes regulate the differential growth of *Arabidopsis* hypocotyls in both a PGP19-dependent and a PGP19-independent manner. Plant J **53:** 516-529

Nakaya M, Tsukaya H, Murakami N, Kato M (2002) Brassinosteroids control the proliferation of leaf cells of *Arabidopsis thaliana*. Plant Cell Physiol **43:** 239-244

Nakazawa M, Yabe N, Ichikawa T, Yamamoto YY, Yoshizumi T, Hasunuma K, Matsui M (2001) *DFL1*, an auxin-responsive GH3 gene homologue, negatively regulates shoot cell elongation and lateral root formation, and positively regulates the light response of hypocotyl length. Plant J **25:** 213-221

Okamoto I IY, Koizumi T. (1967) Isolation of indole-3-acetic acid, phenylacetic acid and several plant growth inhibitors from etiolated seedlings of Phaseolus. Chemical and Pharmaceutical Bulletin **15:** 159-163

Olesen P (1980) Visualization of wall-associated granules in thin-sections of higher-plant cells - occurrence, distribution, morphology and possible role in cell-wall biogenesis. Zeitschrift Fur Pflanzenphysiologie **96:** 35-48

Peer WA, Blakeslee JJ, Yang H, Murphy AS (2011) Seven things we think we know about auxin transport. Mol Plant **4:** 487-504

Petrasek J, Friml J (2009) Auxin transport routes in plant development. Development **136:** 2675-2688

Reed JW (2001) Roles and activities of Aux/IAA proteins in *Arabidopsis*. Trends Plant Sci **6:** 420-425

Robards AW (1969) Particles associated with developing plant cell walls. Planta **88:** 376-379

Roig-Villanova I, Bou-Torrent J, Galstyan A, Carretero-Paulet L, Portoles S, Rodriguez-Concepcion M, Martinez-Garcia JF (2007) Interaction of shade avoidance and auxin responses: a role for two novel atypical bHLH proteins. EMBO J **26:** 4756-4767

Roig-Villanova I, Bou J, Sorin C, Devlin PF, Martinez-Garcia JF (2006) Identification of primary target genes of phytochrome signaling. Early transcriptional control during shade avoidance responses in *Arabidopsis*. Plant Physiol **141:** 85-96

Salter MG, Franklin KA, Whitelam GC (2003) Gating of the rapid shade-avoidance response by the circadian clock in plants. Nature **426:** 680-683

Santner A, Estelle M (2009) Recent advances and emerging trends in plant hormone signalling. Nature **459:** 1071-1078

Saracco SA, Hansson M, Scalf M, Walker JM, Smith LM, Vierstra RD (2009) Tandem affinity purification and mass spectrometric analysis of ubiquitylated proteins in *Arabidopsis*. Plant J **59:** 344-358

Sauer M, Robert S, Kleine-Vehn J (2013) Auxin: simply complicated. J Exp Bot **64:** 2565-2577

Sauter M (2015) Phytosulfokine peptide signalling. J Exp Bot **66:** 5161-5169

Scacchi E, Salinas P, Gujas B, Santuari L, Krogan N, Ragni L, Berleth T, Hardtke CS (2010) Spatio-temporal sequence of cross-regulatory events in root meristem growth. Proc Natl Acad Sci U S A **107:** 22734-22739

Scarpella E, Marcos D, Friml J, Berleth T (2006) Control of leaf vascular patterning by polar auxin transport. Genes Dev **20:** 1015-1027

Sessa G, Carabelli M, Sassi M, Ciolfi A, Possenti M, Mittempergher F, Becker J, Morelli G, Ruberti I (2005) A dynamic balance between gene activation and repression regulates the shade avoidance response in *Arabidopsis*. Genes Dev **19:** 2811-2815

Sikorskaite S, Rajamaki ML, Baniulis D, Stanys V, Valkonen JP (2013) Protocol: Optimised methodology for isolation of nuclei from leaves of species in the *Solanaceae* and *Rosaceae* families. Plant Methods **9:** 31

Somers DE, Kim WY, Geng R (2004) The F-box protein ZEITLUPE confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. Plant Cell **16:** 769-782

Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, DoleZal K, Schlereth A, Jurgens G, Alonso JM (2008) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell **133:** 177-191

Takahashi K, Hayashi K, Kinoshita T (2012) Auxin activates the plasma membrane H^+-ATPase by phosphorylation during hypocotyl elongation in *Arabidopsis*. Plant Physiol **159:** 632-641

Takase T, Nakazawa M, Ishikawa A, Kawashima M, Ichikawa T, Takahashi N, Shimada H, Manabe K, Matsui M (2004) *ydk1-D*, an auxin-responsive GH3 mutant that is involved in hypocotyl and root elongation. Plant Journal **37:** 471-483

Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. Science **316:** 1033-1036

Tan X, Calderon-Villalobos LI, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature **446:** 640-645

Tanaka S, Mochizuki N, Nagatani A (2002) Expression of the *AtGH3a* gene, an *Arabidopsis* homologue of the soybean GH3 gene, is regulated by phytochrome B. Plant and Cell Physiology **43:** 281-289

Tao Y, Ferrer JL, Ljung K, Pojer F, Hong F, Long JA, Li L, Moreno JE, Bowman ME, Ivans LJ, Cheng Y, Lim J, Zhao Y, Ballare CL, Sandberg G, Noel JP, Chory J (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell **133:** 164-176

Teale WD, Paponov IA, Palme K (2006) Auxin in action: signalling, transport and the control of plant growth and development. Nat Rev Mol Cell Biol **7:** 847-859

Thompson WF, Cleland R (1971) Auxin and ribonucleic Acid synthesis in pea stem tissue as studied by deoxyribonucleic Acid-ribonucleic Acid hybridization. Plant Physiol **48:** 663-670

Tsiatsiani L, Timmerman E, De Bock PJ, Vercammen D, Stael S, van de Cotte B, Staes A, Goethals M, Beunens T, Van Damme P, Gevaert K, Van Breusegem F (2013) The *Arabidopsis* metacaspase9 degradome. Plant Cell **25:** 2831-2847

Vanneste S, Friml J (2009) Auxin: a trigger for change in plant development. Cell **136:** 1005-1016

Vernoux T, Brunoud G, Farcot E, Morin V, Van den Daele H, Legrand J, Oliva M, Das P, Larrieu A, Wells D, Guedon Y, Armitage L, Picard F, Guyomarc'h S, Cellier C, Parry G, Koumproglou R, Doonan JH, Estelle M, Godin C, Kepinski S, Bennett M, De Veylder L, Traas J (2011) The auxin signalling network translates dynamic input into robust patterning at the shoot apex. Mol Syst Biol **7:** 508

Wang S, Bai Y, Shen C, Wu Y, Zhang S, Jiang D, Guilfoyle TJ, Chen M, Qi Y (2010) Auxin-related gene families in abiotic stress response in Sorghum bicolor. Funct Integr Genomics **10:** 533-546

Weijers D, Friml J (2009) SnapShot: Auxin signaling and transport. Cell **136:** 1172, 1172 e1171

Werner T, Motyka V, Strnad M, Schmulling T (2001) Regulation of plant growth by cytokinin. Proc Natl Acad Sci U S A **98:** 10487-10492

Wilmoth JC, Wang S, Tiwari SB, Joshi AD, Hagen G, Guilfoyle TJ, Alonso JM, Ecker JR, Reed JW (2005) NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. Plant J **43:** 118-130

Wolters H, Jurgens G (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. Nat Rev Genet **10:** 305-317

Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Ann Bot **95:** 707-735

Xinhua Dai YZ, Da Zhang, Jilin Chen, Xiuhua Gao, Mark Estelle & Yunde Zhao (2015) Embryonic lethality of *Arabidopsis abp1-1* is caused by deletion of the adjacent BSM gene. Nature Plants **1 Page number?**

Zazimalova E, Murphy AS, Yang H, Hoyerova K, Hosek P (2010) Auxin transporters-why so many? Cold Spring Harb Perspect Biol **2:** a001552

CHAPTER 4: CONCLUSIONS AND OUTLOOKS

4.1 Contribution of Proteomics in the Identification of Novel Proteins Associated with Plant Growth

My first objective (chapter two) was to further understand the changes during auxin-induced expansion growth and identify associated proteins. I used auxin (IAA)-induced rapidly growing corn (Zea mays) coleoptiles as a model system for rapid growth and water treated coleoptiles as a control. By comparing the proteomics changes between auxin-treated and control tissues, and between coleoptile and epidermal tissues, I identified 82 candidates involved in this growth as well as the underlying cell wall and cuticle biosynthesis and four proteins of unknown function.

Using quantitative RT-PCR, I could show that increases in the protein level are also reflected in the gene expression. In addition, expression of these genes was increased in rapidly growing coleoptiles, leaves and reduced in coleoptiles that have stopped growing, a finding which confirms their correlation with growth.

I focused on two hypothetical proteins, HYP 3, which is a putative mixed-linked glucanase, and HYP 4, a DUF538-containing protein. Their gene expression is increased 2.5 and 1.7-fold, respectively. In addition, *HYP4* expression is reduced by 50% in light-exposed, slow growing coleoptiles. It was also induced twofold in the developing leaves. Strong expression of *HYP4* in all parts of the coleoptile suggested that it has a universal, not an epidermis-specific function. On the other hand, *HYP* 3 expression was higher in the epidermis than in total coleoptile tissue suggesting the epidermis as predominant locus of action. *HYP3* expression levels decreased in coleoptiles and increased in leaves, suggesting that it plays a role in the growth-controlling processes within the epidermis.

4.2 ARRP1 in the regulation of cell expansion growth

The objective of the third chapter of my thesis was to elucidate the function of HYP4. HYP4, which we now call ARRP1 (Auxin rapid response protein1), is a novel protein with a DUF538 domain. Using pulldown assays and yeast two hybrid assays, I showed that like one of ARRP1's *Arabidopsis* homologues, corn ARRP1 interacts with both ZmBRL2 and AtBRL2, a receptor-like kinase that plays a role in the venation pattern of plants, suggesting ARRP1 might function downstream of BRL2 signal pathway.

RT-PCR and western blotting analysis, showed that *ARRP1* expression is closely associated with cell and organ expansion growth, and mainly in above ground tissues. Like the receptor BRL2, with which it interacts, *ARRP1* expression can be induced by the hormone auxin but shows no response to cytokinins and brassinosteroid treatment. In addition *ARRP1* transcription was induced immediately after the addition of auxin, reaching the highest level within 30 min and then decreasing slowly to a basal level within the next four hours, indicating that *ARRP1* is an early auxin-response gene with a possible signaling function. In addition, its expression is induced by red and blue light. These observations suggest that ARRP1 participates in auxin- and light-related signaling

This argument is further supported by the finding that ARRP1 is localized in the periphery of the cell as well as in the nucleus. In addition RNA *in situ* hybridization demonstrated that *ARRP1* is expressed in developing leaves and coleoptiles particularly in the vascular bundles. This expression pattern corresponds to the *BRL2* expression pattern.

Arabidopsis plants lacking the *ARRP1* homologue produced trichomes with smaller length and variable number of branches, but no significant growth phenotype compared to wild type. However, the double mutant showed slower growth upon transition to light after five days germination in darkness, a difference that was especially obvious in the early development stages. Inflorescence emergence and flowering were delayed by two weeks in the double mutant, but leaf number at the time of flowering and diameter of the rosette and stem are identical to the wild type control. By overexpressing *ZmARRP1* in the *Arabidopsis* double mutant, *ZmARRP1* completely complements the phenotype, indicating that ZmARRP1 and AtARRP1/2 execute the same function.

Taken together, these data suggest that ARRP1 acts downstream of the BRL2, a receptor-like kinase, integrates light and auxin signals, and affects plant development and the transition to light. Three pathways of ARRP1 action are possible (Figure 3.12):

- ARRP1 moves from cytosol to the nucleus where it binds additional activators and regulates gene expression;
- ARRP1moves to the nucleus where it binds additional protein and regulates cell division; or
- ARRP1 binds and modifies other, possibly cytoplasmic proteins, thus affecting their function.

Below I am proposing experiments to examine and distinguish between the respective ARRP1 functions

4.3 Future perspective

4.3.1 Questions remaining about ARRP1 function

In my dissertation research, I demonstrated that ARRP1 interacts with BRL2 and is expressed in overlapping patterns with BRL2. But whether ARRP1 and BRL2 are localized in the same subcellular compartment which would allow for their interaction remains unknown. To further test my hypothesis, a co-localization analysis should be performed. The YFP tag will be fused to the C-terminal BRL2, while the CFP tag would be fused to ARRP1. Both vectors will be transformed into *Agrobacterium* and inoculated into the epidermis of tobacco for transient expression. The fluorescence will be determined using confocal microscopy. In addition, we can also use fluorescence resonance energy transfer (FRET) technique to confirm not only BRL2 and ARRP1 co-localization but also the protein-protein interaction. As an alternative to FRET, BiFC (Bimolecular fluorescence complementation) can be used.

My localization study showed that ARRP1, which doesn't contain any signal peptide or nuclear targeting signal, is present in both cytosol and nucleus, indicating a possible translocation movement from the cytoplasm into the nucleus. To test this hypothesis, a stable *Arabidopsis* transformant that expresses *ARRP1-CFP* will be generated. Using the fluorescence recovery after photobleaching (FRAP) technique, we can photobleach the CFP signal inside the nucleus and monitor the trafficking of new unbleached protein into the nucleus and thus assess the mobility of ARRP1. If this translocation occurs, we may want to further test whether auxin can facilitate this movement. To achieve that, I will spray auxin onto the *Arabidopsis* leaves, use the same procedure to measure the recovery, and compared to the non-auxin treatment

leaves as a control. Alternatively, KAEDE, a photoactivatable fluorescent protein, can be used (Patterson and Lippincott-Schwartz, 2002; Lippincott-Schwartz *et al*., 2003).

Finally, although my data strongly suggest that ARRP1 functions downstream of the BRL2 receptor-like kinase, I still need functional data to support this conclusion. To better understand how ARRP1 and BRL2 work together, a homozygous T-DNA knockout mutant of BRL2 will be obtained, mRNA and protein level of ARRP1 should be measured in this mutant. In addition, I will cross the *brl2* and *atarrp1-1/1-2* to make a triple mutant. If the triple mutant shows a similar phenotype to *brl2*, it would suggest that BRL2 and ARRP1 are in the same pathway. Finally, I will also test whether overexpression of *ARRP1* can complement the phenotype of *brl2* mutant. The constitutive expression vector of *ARRP1* driven by 35S promoter will be introduced into the *brl2* mutant. The morphological phenotypes, leaf vein development and the secondary auxin response genes will be monitored and compared to the wild type. Together these experiments would provide insights into the ARRP1 function and further our understanding of the BRL2 signaling pathway.

4.3.2 Identification of ARRP1 interacting partners

As ARRP1 is a small protein that does not contain any other known functional domains, it may require partners to trigger the plant response or to move to the nucleus. To identify these interaction partners, I performed an *in vitro* pull down assay, using a ARRP1 specific antibody. Proteins were extracted from young maize leaves, which have the highest ARRP1 protein level, and then incubated overnight with the antibody. Mouse serum without ARRP1 antibody was used as control. The protein complex was

then purified using protein-A beads. The comparison revealed nine protein bands that were only showed in the pull down assay as compared to the control (Fig. 4.1a as indicated with an asterisk). In gel digestion followed by LC-ESI-MS/MS revealed 34 interaction candidates (Fig. 4.1b & Table S4.1). Based on the annotations of 28 of the 34 isolated proteins, they function in : (I) targeted protein modification/degradation; (II) regulation of cell division and cell elongation; (III) cell metabolic processes and (IV) signal transduction.

I focused on candidate partners with a possible novel or not fully elucidated role in downstream signal transduction. Among them, I found two tetratricopeptide repeat domain (TPR) containing proteins, one of which is the homologue of *Arabidopsis* VIT protein. The TPR domain is a structural motif present in a wide range of proteins that mediate protein-protein interactions and the assembly of multiprotein complexes. TPR-containing proteins often play roles in numerous vital cell processes, such as cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, and neurogenesis (Sikorski *et al*., 1991; Zachariae and Nasmyth, 1996; D'Andrea and Regan, 2003). Of particular interest is the ZmVIT, with 54% protein identity to AtVIT. *Arabidopsis* plants impaired in VIT expression results in vein pattern defects and in alterations in response to auxin. AtVIT had been shown to interact with both AtARRP1-1and AtBRL2 (Ceserani *et al*., 2009). Taken together, I propose that ZmVIT also interacts with both ZmBRL2 and ZmARRP1, and plays an important role in the early stage of plant growth, and integration of light- and auxin- signaling pathway.

The second identified protein involved in the signal transduction is Auxin signaling F box protein 2 (AFB2). AFB2 is a member of a small subclade consisting of

seven proteins, including TIR1, AFB1, 3, and COl1 (Gagne *et al*., 2002). Among them, AFB1,2,3 and TIR1 are most closely related. Like TIR1, AFB2 interacts with the Aux/IAA proteins in an auxin dependent manner and acts redundantly with the other three TIR1/AFB proteins to regulate diverse aspects of growth and development. Plants that are deficient in all four proteins are auxin insensitive and exhibit a severe embryonic phenotype (Dharmasiri *et al*., 2005). The quadruple mutant also forms rosettes with small, highly curled leaves and shows deficiency in leaf venation, which is similar to that of the *BRL2* mutants, but more severe. In addition, AFB2 is mainly localized in the nucleus which provides a possible explanation why ARRP1 is also found there.

I also identified two putative annexin proteins. Annexins are soluble proteins capable of Ca^{2+}-dependent or independent association with membrane phospholipids (Gerke *et al*., 2005). They play a role in coordinating development and response to environmental changes. Plant annexins are induced by abiotic stress including salinity, cold, oxidative and mechanic stress (Mortimer *et al*., 2008; Laohavisit and Davies, 2011). Pathogen attack or symbiotic interaction also induced the transcription of annexins (Niebel Fde *et al*., 1998; Vandeputte *et al*., 2007). More recently, several experiments showed that the expression of a number of *Arabidopsis*, alfalfa, and tobacco annexins was affected by both light and auxin, as well as abiotic stress (Kovacs *et al*., 1998; Cantero *et al*., 2006; Baucher *et al*., 2011). The multifunctionality and the similar response to auxin and light make annexins good candidates for the downstream of BRL2-ARRP1 signal pathway.

I am also interested in several novel proteins with unknown functions. Hypothetical protein 1 appears to be similar to a tomato ASR1 (abscisic acid stress

ripening protein), now called abscisic acid stress ripening like protein (ASRL1), which is a small plant-specific protein. It is one of numerous plant gene products with unknown biological roles that become upregulated under water- and salt-stress conditions (Kalifa *et al*., 2004; Goldgur *et al*., 2007).

Hypothetical protein 2 has similarity to other proteins of unknown function with a DUF250 domain which are also called triose-phosphate transporter (TPT) family. TPT Proteins mediate export of stromal triose-P into the cytosol to produce sucrose. In contrast to the aforementioned starch-storing plant species, maize uses sucrose as its major transitory storage in the leaves rather than starch. By affecting HYP2 activity, ARRP1 may exert significant influence on leaf sucrose level and the plant energy status, which then ultimately regulates plant growth.

4.3.3 Confirmation of protein-protein interaction

To test the interaction between ARRP1 and proteins listed in table 1, yeast two hybrid (Y2H) analysis was performed as described in chapter 3. Interaction between ARRP1 and TPR1, Annexin1, and ASRL1 was confirmed (Fig. 4.2). Because Y2H also has several limitations including interactions involving membrane proteins, self-activating proteins, or proteins requiring post-translational modifications, it may lead to false negatives (see chapter 3). In the future, I would like to use FRET as a backup technique to check the interaction of ARRP1 candidates that do not interact in the Y2H.

In addition, due to the low sensitivity of Coomassie blue staining, the list of protein interaction partners may not be complete. In the future, we can use the maize leaf cDNA library and for large scale screenings which may help us identify several important candidates that are in trace concentration.

4.3.4 Expression study of three confirmed ARRP1- binding partners

To determine whether the three confirmed candidates, *TPR1*, *Annexin1*, and *ASRL1,* are co-expressed with *ARRP1*, we characterized the expression of all three genes, using RT-PCR. My earlier study showed that *ARRP1* was strongly expressed in the above ground tissue while mRNA and protein were barely detectable in the root. *ARRP1* showed reduced expression in the coleoptiles when their growth ceased and significantly increased in the young leaves when their growth was enhanced by light. Along the length of maize leaf, *ARRP1* expression was seen in all tissues, but both, mRNA level and protein abundance were highest in the basal zone, suggesting ARRP1 is involved with the cell division and cell elongation growth.

ASRL1 exhibits a similar expression pattern as *ARRP1*. *ASRL1* is expressed in early stage embryos, in the shoot, and in emerging and expanding leaves. The transcription level of *ASRL1* increased about 50% in the developing leaf and dropped 60% in the growth ceased coleoptiles (Fig. 4.3 a/b). Like *ARRP1*, *ASRL1* mRNA is at its highest level in the basal zone and decreased along the maize leaf. A search of the Bioanalytic Source for Plant Biology (http://bar.utoronto.ca/efp_maize/cgi-bin/efpWeb.cgi) suggests that the expression of *ASRL1* is associated with the vascular cells.

The expression patterns of *TPR1* and *Annexin1* also overlapped with *ARRP1*, but shows several differences. Both genes are highly expressed not only in the shoot and leaves, but also in the root. Just like *ARRP1*, transcript levels are correlated with plant expansion growth. In leaves, they are also enriched in the basal zone but closer to the bottom of the leaf than *ARRP1*. They are not predicted to be restricted in the

vascular cells where *ARRP1* was highly expressed. In the future, we can perform *in situ* hybridization analysis to confirm the tissue specific expression.

In my dissertation research, I demonstrated that *ARRP1* transcription was induced immediately after the addition of auxin, reaching the highest level within 30 min, but showed no changes upon cytokinin and brassinosteroid treatment. *ARRP1* expression level increased significantly after 2.5 days in red and blue light conditions. As I proposed that these TPR1, Annexin1 and ASRL1 function in downstream of ARRP1, it would be necessary to check their response to different hormone and light conditions. Using quantitative RT-PCR, the mRNA level of each gene will be monitored and compared to the 18S. These experiments would consolidate my hypothesis of the BRL2-ARRP1 signal pathway.

4.3.5 Evaluation of the T-DNA knock-out mutants

I already confirmed that TPR1, Annexin1, and ASRL1 interact with ARRP1, and that their expression pattern is similar to ARRP1. To further explore their physiological function in plant growth, homozygous T-DNA mutants defective in these genes will be obtained. Morphological phenotypes of mutants will be monitored and compared to BRL2 and ARRP1 mutant. The mRNA level of secondary auxin inducible genes will also be measured, as will be the levels of these genes in ARRP1 overexpression and knock-down lines. Together these results will further our understanding of the function of BRL2-ARRP1 signaling pathway.

Figure 4.1a SDS-PAGE gel comparison of pull-down assay.

ARRP1 specific antibody was used to pull down the interacting partners form the leaf total protein extraction. The marked bands were selected for LC-ESI-MS

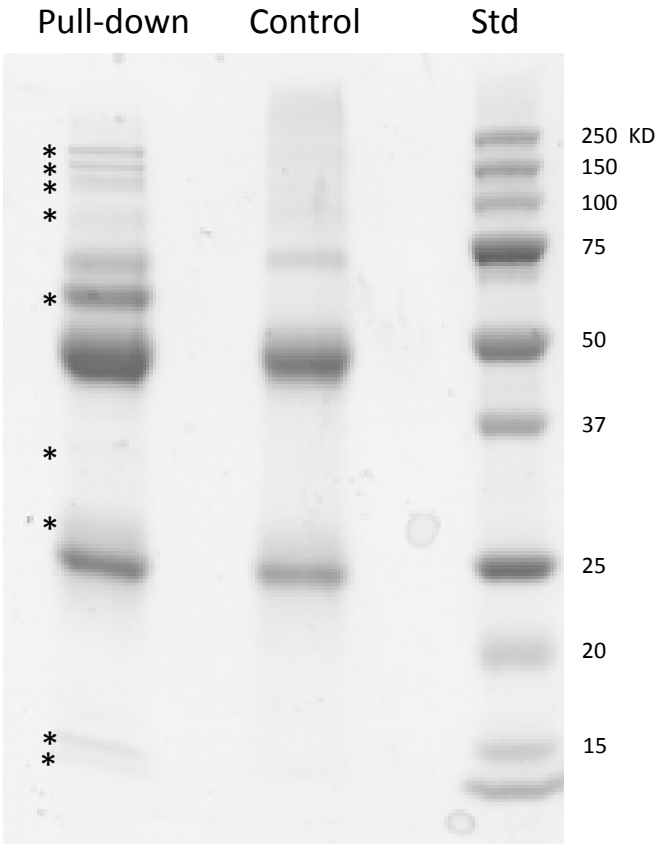


Figure 4.1b Grouping of proteins identified in the pull-down assay based on their predicted function/involvement in cellular processes.

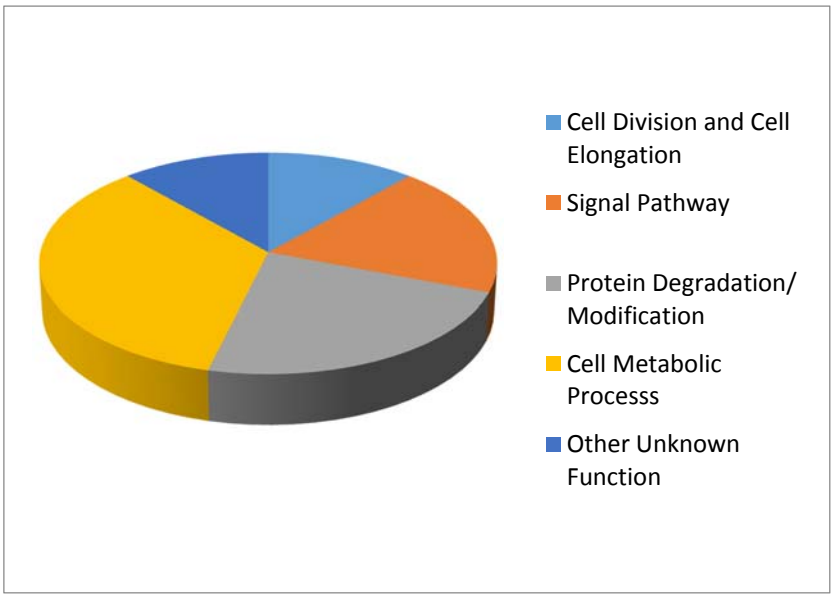


Figure 4.2 Yeast two hybrid analyses for confirming the protein-protein interaction of ARRP1 and its partners.

TPR1: TPR domain containing protein 1; AFB2: auxin signaling F box protein; ASRL1: abscisic acid stress ripening like protein 1; HYP2: hypothetical protein 2; Annexin1: Annexin like protein1.

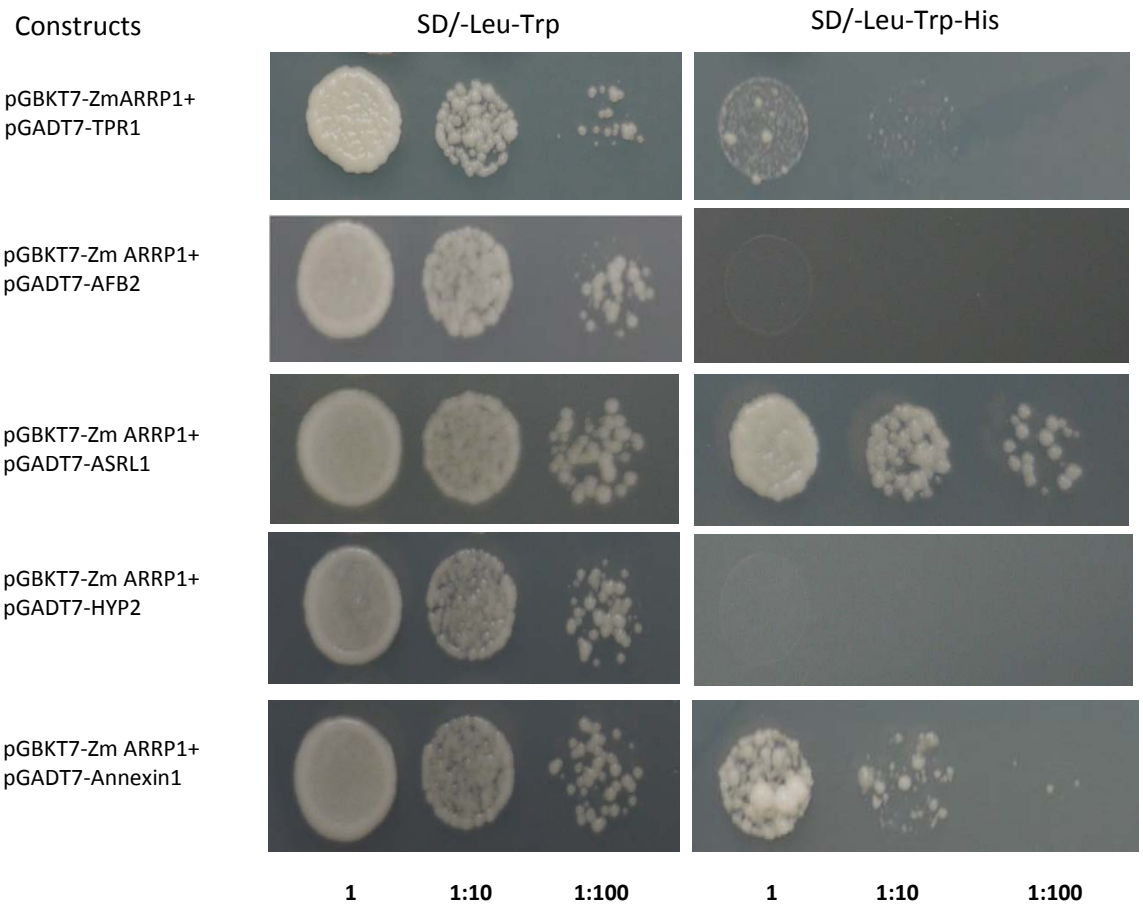


Figure 4.3 Semiquantitative analyses of transcription levels of genes encoding ASRL1 (a, b), Annexin1 (c, d) and TPR1 (e, f) in different corn seedling organs and along the young leaf. "Epidermis" corresponds to the epidermis of coleoptiles of seedlings germinated and grown in the dark for five days; Material for coleoptiles was harvested either after five days in the dark (dark) or 1, 2, or 5 days after transition of the 5-day-old, dark-grown seedling to light. Leaf samples were taken either from the leaf within the coleoptile of the 5-day-old, dark-grown seedling (undeveloped leaf) or two days after transition to light (2 days light). Young maize leaves were taken from 2 weeks old corn seedling and divided into five zones as we described in chapter 3 (Fig 3.4a). Bars represent the mean ± S.E. of 3 biological replicates

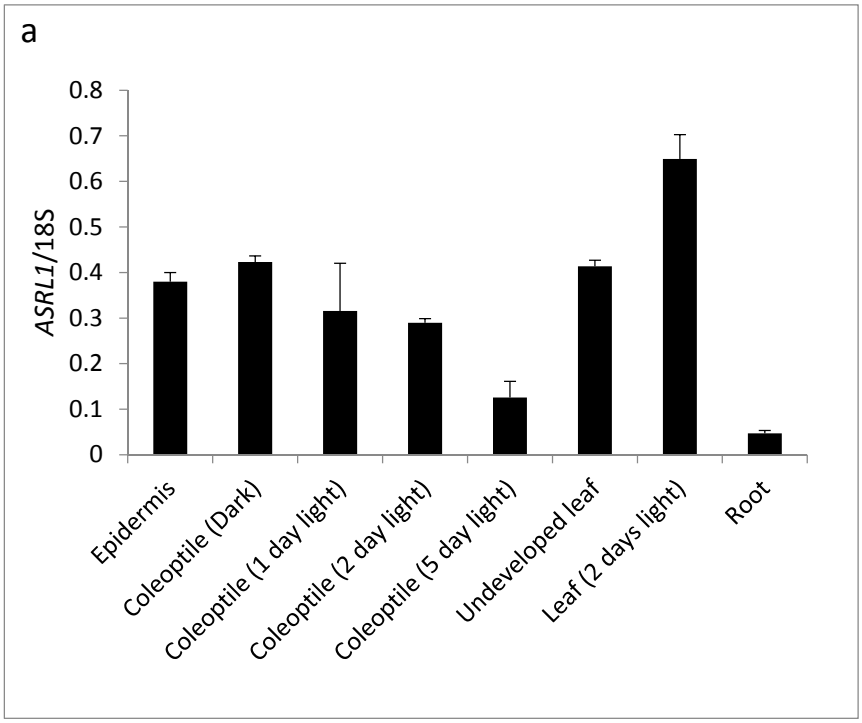


Figure 4.3 (cont'd)

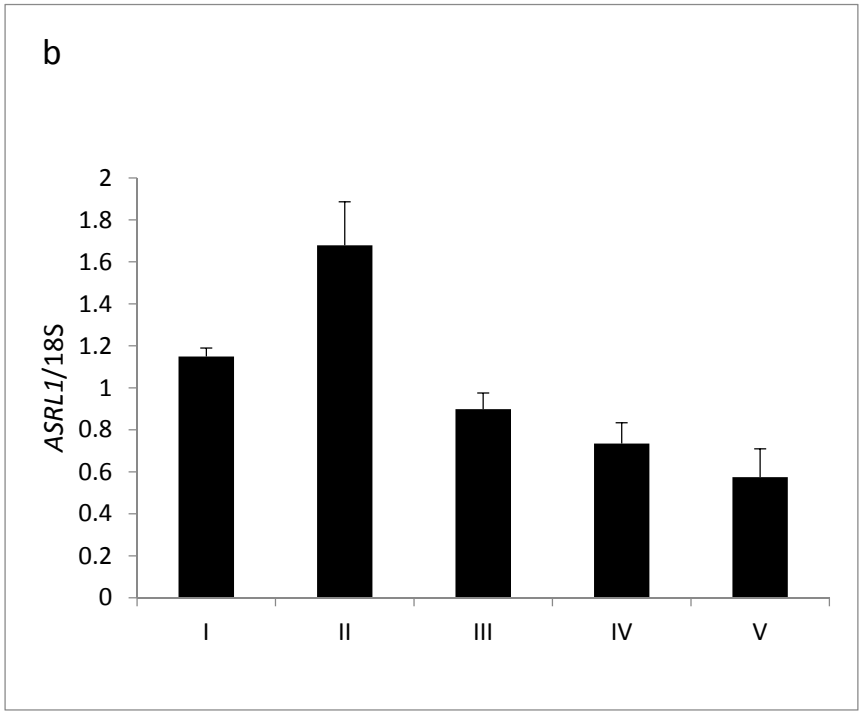
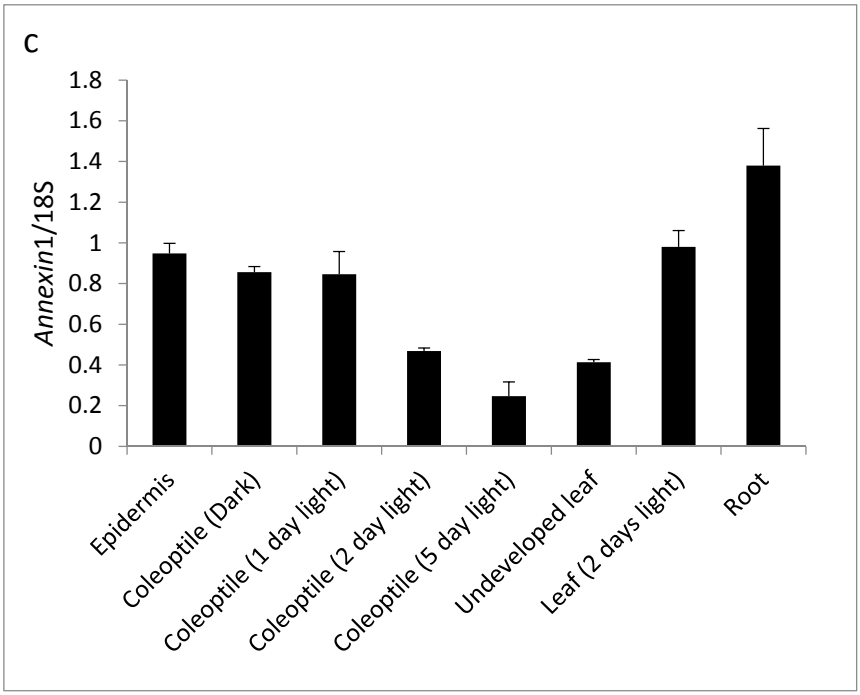


Figure 4.3 (cont'd)



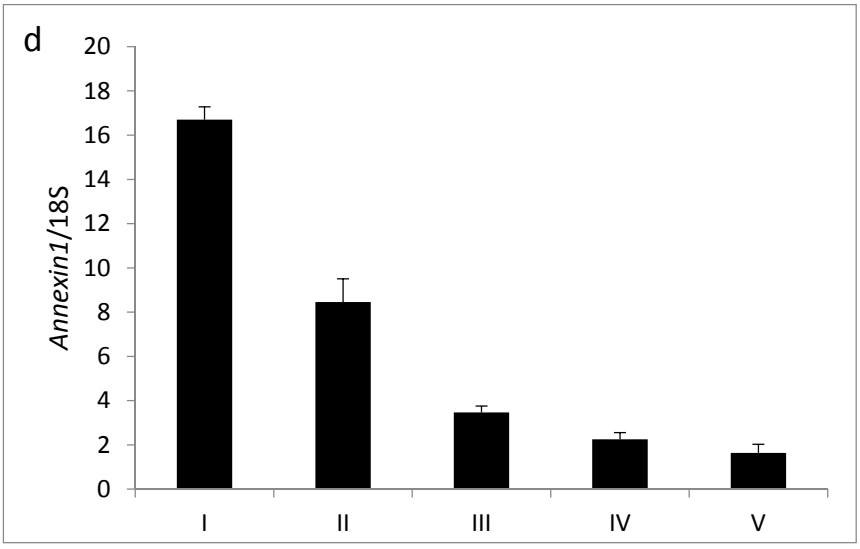
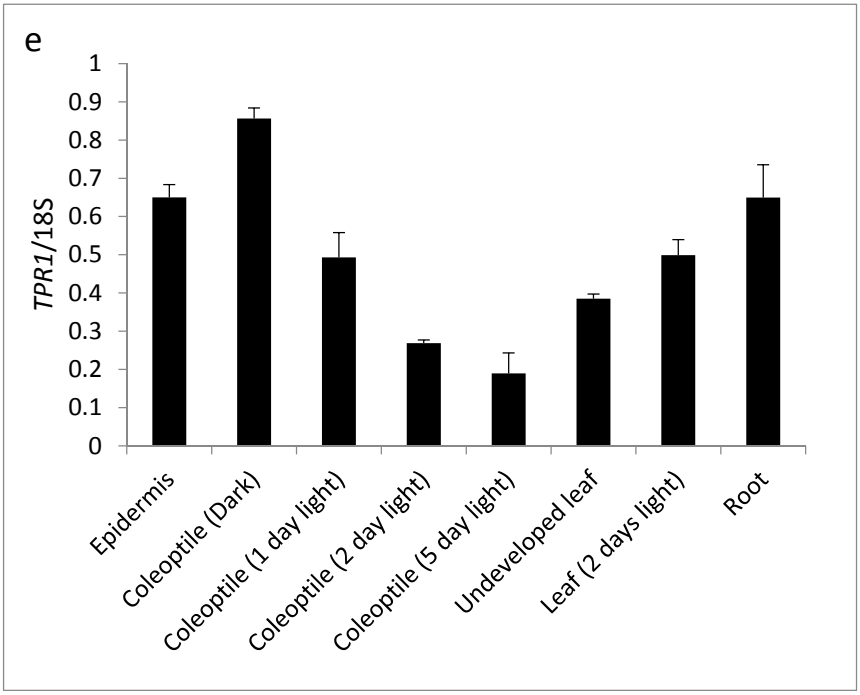


Figure 4.3 (cont'd)



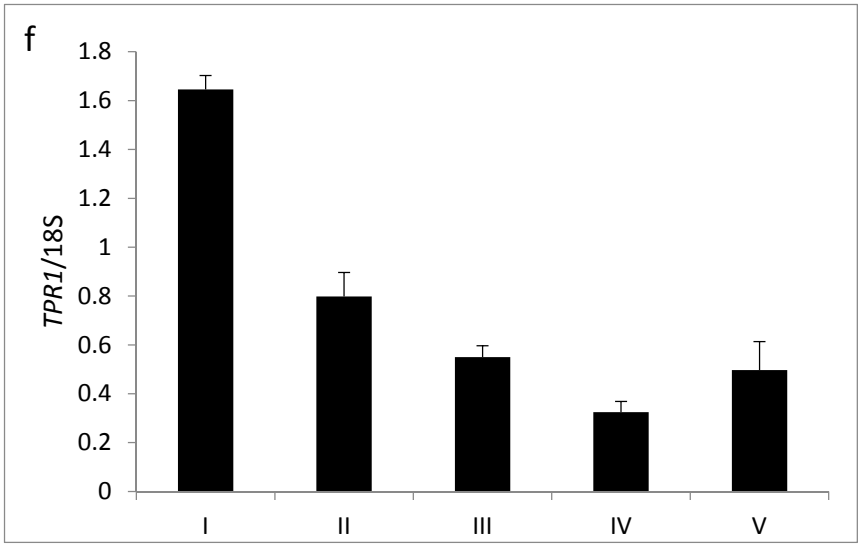


Table 4.1 ARRP1-interacting proteins with a possible novel or not fully elucidated role in downstream signal transduction

Protein	Access #	MW	Possible Function
Auxin Signaling F-box 2	GRMZM2G095786	66	Auxin signal pathway
ZmVIT	GRMZM2G047093	103	Homologue of AtVIT, function in the BRL2 signal pathway
TPR domain containing protein1	GRMZM2G392710	81	Possibly signal transduction molecule
Annexin 1 like	GRMZM2G061950	38	Coordinate development and response to environmental changes
Annexin 2 like	GRMZM2G064993	35	Coordinate development and response to environmental changes
Hypothetical protein #1 (ASRL1)	GRMZM2G136910	16	Response to abiotic stress
Hypothetical protein #2	GRMZM2G081105	41	DUF250 containing protein
Hypothetical protein #3	GRMZM2G381709	96	possible ubiquitin conjugating enzyme E2

Table S4.1: Proteins identified in the ARRP1 pull down assay

Protein Identification	Access #	Trp fra	MW	Localization/Function Sequence Charge state (Z)
ATP synthase, putative, expressed	GRMZM2G094497_T01	6	54	ATP synthase (R)TVSGVAGPLVILDK(V) Z=2 (R)GYPGYmYTDLATIYER(A) Z=2 (R)IALTTAEYLAYEcGK(H) Z=2 (K)IPLFSAAGLPHNEIAAQIcR(Q) Z=3 (R)TVSGVAGPLVILDK(V) Z=2 (R)VTLELNLANDPTIER(I) Z=2
Os6bglu24 - beta-glucosidase homologue,	GRMZM2G077015_T01	5	64	catalyzes the hydrolysis of the glycosidic bonds (K)ENGIEPYVTLFHWDTPQALVDSYGGFLDDR(I) Z=3 (K)VcFVHFGDVVK(N) Z=2 (K)NWFTFNEPQTFSSFSYGTGIcAPGR(C) Z=3 (K)LVGSYDIMGINYYTSR(F) Z=2 (K)VDICTGYTPVLNTDDAYAAQETK(G) Z=2
DnaK family protein, putative, expressed	GRMZM2G001500_T01	2	49	Involved in chromosomal DNA replication (R)QAVVNPENTFFSVK(R) Z=2 (R)IINEPTAASLAYGFEK(K) Z=2
tetratricopeptide repeat domain containing protein, expressed ZmVIT	GRMZM2G047093_T01	5	10 4	Signal transduction (K)ISLFEEcGMLDR(A) Z=2 (K)LLETVFGDQNVANR(W) Z=2 (K)ILQHAGNFTAAAALADEAR(S) Z=3 (K)LIQIEDPLAEATK(Y) Z=2 (K)VLLAFQAVK(Q) Z=2
annexin, putative, expressed	GRMZM2G061950_T01	2	35	Coordinate development and response to environmental change (R)TPAQLFAVK(Q) Z=2 (R)LALGGMGTDEDDLTR(V) Z=2
COBW domain containing protein, putative, expressed	GRMZM2G180418_T02	2	79	play a central role in zinc metabolism (K)LDGVVTLVDAK(H) Z=2 (K)GIVNEAVQQIAYADR(I) Z=2 (K)IDLVKEPEVLSLVER(I) Z=3
exo-beta-glucanase, expressed	GRMZM2G076946_T02	3	64	Cell wall biosynthesis (K)ENGIEPYVTLFHWDTPQALVDSYGGFLDDR(I) Z=3 (K)LVGSYDIMGINYYTSR(F) Z=2 (K)YSPVLNTDDAYAAQETK(G) Z=2

Table S4.1 (cont'd)

6-phosphofructokinase, putative, expressed	GRMZM2G004932_T01	1	63	one of the most important regulatory enzymes of glycolysis (K)SFGFDTAVEEAQR(A) Z=2
annexin, putative, expressed	GRMZM2G064993_T01	1	38	Coordinate development and response to environmental change (R)SITDEISGDFER(A) Z=2
AUXIN SIGNALING F-BOX 2, putative, expressed	GRMZM2G095786_T01	1	66	Auxin signal pathway (R)KLTRLSTSGQLTDR(A) Z=2
soluble starch synthase 3, putative, expressed	GRMZM2G121612_T01	1	134	pathway starch biosynthesis (K)VGGLGDVVTSLSR(A) Z=2
abscisic stress-ripening, putative, expressed	GRMZM2G136910_T01	3	15	Response to abiotic stress (K)QHLGEAGAIAAGAFALYEK(H) Z=2 (K)KDEEQPAGEYGYSETEVVTATGEGEYER(Y) Z=3 (K)ITEEVAAAAAVGAGGYVFHEHHEK(K) Z=3
ulp1 protease family, C-terminal catalytic domain containing protein, expressed	GRMZM2G348480_T01	1	32	catalyzes two essential functions in the SUMO pathway (K)EIEmTYAKQTDRYK(H) Z=2
HD domain containing protein 2, putative, expressed	GRMZM2G378040_T01	1	42	DNA replication (K)LVDPEQGVDAGAGR(A) Z=2
Hypothetical protein	GRMZM2G381709_T01	1	96	catalyze the attachment of ubiquitinto the substrate protein (R)cPRTAIVSDVDALERTVNVK(W) Z=2
tetratricopeptide repeat domain containing protein, expressed	GRMZM2G392710_T01	1	81	Signal transduction (R)NEEALEMMEK(A) Z=2
phenylalanine ammonia-lyase, putative, expressed	GRMZM2G029048_T01	1	77	enzyme that catalyzes a reaction converting L-phenylalanine to ammonia and trans-cinnamic acid (R)NTLLQGYSGIR(F) Z=2
phospholipase D, putative, expressed	GRMZM2G054559_T01	1	92	it hydrolyzes to produce the signal molecule phosphatidic acid (R)LEGPIAWDVLYNFEQR(W) Z=2
peroxidase precursor, putative, expressed	GRMZM2G394500_T01	3	38	biosynthesis and degradation of lignin, (K)TAPINIGLAAFEVIDEIK(A) Z=2 (R)LSAPPAQIVPAYR(N) Z=2 (R)DLPDSTFTVSELIR(N) Z=2

Table S4.1 (cont'd)

amine oxidase, flavin-containing, domain containing protein, expressed	GRMZM2G034152_T01	5	56	catalyzes the oxidative cleavage of alkylamines into aldehydes and ammonia (R)VIVVGAGMSGISAAK(R) Z=2 (K)RLSEAGITDLLILEATDHIGGR(M) Z=3 (R)LSEAGITDLLILEATDHIGGR(M) Z=3 (K)FDYEFAEPPR(V) Z=2 (R)AIYQFDmAVYTK(I) Z=2
phosphoenolpyruvate carboxylase, putative, expressed	GRMZM2G083841_T01	4	109	used for carbon fixation in CAM/ C4 plants (K)VSEDDKLIEYDALLVDR(F) Z=3 (R)FLNILQDLHGPSLR(E) Z=3 (K)AIADGSLLDLLR(Q) Z=2 (R)QVFTFGLSLVK(L) Z=2
elongation factor Tu, putative, expressed	GRMZM2G106061_T01	4	50	DNA replication (K)KYDEIDAAPEER(A) Z=3 (R)GITINTATVEYETETR(H) Z=2 (R)ELLSNYEYDGDEVPIVAGSALK(A) Z=2 (K)IGDTVDIVGIR(D) Z=2
Hypothetical protein	GRMZM2G081105_T01	2	41	Unknown function (R)SECDDIEEA(K) Z=2 (K)TSDVFDTLLQ(K) Z=2
glyceraldehyde-3-phosphate dehydrogenase, putative, expressed	GRMZM5G845611_T01	4	47	erves to break down glucose for energy and carbon molecules (K)IVDDTTISVDGKPITVVSSR(D) Z=3 (K)ILDEEFGIVK(G) Z=2 (R)AAALNIVPTSTGAAK(A) Z=2 (R)VPTPNVSVVDLVINTVK(T) Z=2
lipoxygenase, putative, expressed	GRMZM2G015419_T02	2	102	catalyze the dioxygenation of polyunsaturated fatty acids in lipids (K)LVEDTTDHVLR(F) Z=3 (R)YTmEINALAR(E) Z=2 (K)LDPEVYGPAESAITK(E) Z=2
von Willebrand factor type A domain containing protein, putative, expressed	GRMZM2G320152_T01	2	66	a large multimeric glycoprotein (R)AGFIVLISDGLDGQSK(W) Z=2 (K)EGGDDPVLQAIAASLLQR(K) Z=3
fructose-bisphospate aldolase isozyme, putative, expressed	GRMZM2G046284_T01	2	41	Calvin cycle is a carbon fixation pathwa (R)EAAYYQQGAR(F) Z=2 (R)ATPEQVAAYTLK(L) Z=2

Table S4.1 (cont'd)

starch synthase, putative, expressed	GRMZM2G008263_T01	2	66	Starch biosynthesis (K)ELTSGPDKGVELDGVLR(T) Z=3 (R)AGIDDAEEIAPLAK(E) Z=2
LTPL122 - Protease inhibitor/seed storage/LTP family protein precursor, expressed	GRMZM2G429000_T01	2	13	Protease inhibitor (K)FGVcADVLGLVK(G) Z=2 (K)LSALVNYcGK(C) Z=2
Hypothetical protein	GRMZM2G095082_T01	2	45	unknown function (R)LLEEPDAQLVDIRPLK(D) Z=3 (K)ELIDEIKEIGQALLPLPGDAK(S) Z=3
Cupin domain containing protein, expressed	GRMZM2G064096_T01	2	22	Signal transduction (K)SSVTANDFYFHGLAGQGK(I) Z=2 (K)VTFLDDAQVK(K) Z=2
DnaK family protein, putative, expressed	GRMZM2G056039_T01	2	71	DNA replication (R)IINEPTAAAIAYGLDKK(A) Z=3 (K)EQVFSTYSDNQPGVLIQVYEGER(T) Z=3
aquaporin protein, putative, expressed	GRMZM2G014914_T01	1	30	Integral membrane pore proteins (K)AFQSAYFDR(Y) Z=2
glycosyl hydrolase family 3 protein, putative, expressed	AC234091.1_FGT001	2	83	Cell wall biosynthesis (K)VSQLGDEAAGVPR(L) Z=2 (K)NDAGILPLDR(S) Z=2

REFERENCES

REFERENCES

Baucher M, Oukouomi Lowe Y, Vandeputte OM, Mukoko Bopopi J, Moussawi J, Vermeersch M, Mol A, El Jaziri M, Homble F, Perez-Morga D (2011) *Ntann12* annexin expression is induced by auxin in tobacco roots. J Exp Bot **62:** 4055-4065

Cantero A, Barthakur S, Bushart TJ, Chou S, Morgan RO, Fernandez MP, Clark GB, Roux SJ (2006) Expression profiling of the *Arabidopsis* annexin gene family during germination, de-etiolation and abiotic stress. Plant Physiol Biochem **44:** 13-24

Ceserani T, Trofka A, Gandotra N, Nelson T (2009) VH1/BRL2 receptor-like kinase interacts with vascular-specific adaptor proteins VIT and VIK to influence leaf venation. Plant J **57:** 1000-1014

D'Andrea LD, Regan L (2003) TPR proteins: the versatile helix. Trends Biochem Sci **28:** 655-662

Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jurgens G, Estelle M (2005) Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell **9:** 109-119

Gagne JM, Downes BP, Shiu SH, Durski AM, Vierstra RD (2002) The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*. Proc Natl Acad Sci U S A **99:** 11519-11524

Gerke V, Creutz CE, Moss SE (2005) Annexins: linking Ca^{2+} signalling to membrane dynamics. Nat Rev Mol Cell Biol **6:** 449-461

Goldgur Y, Rom S, Ghirlando R, Shkolnik D, Shadrin N, Konrad Z, Bar-Zvi D (2007) Desiccation and zinc binding induce transition of tomato abscisic acid stress ripening 1, a water stress- and salt stress-regulated plant-specific protein, from unfolded to folded state. Plant Physiol **143:** 617-628

Kalifa Y, Gilad A, Konrad Z, Zaccai M, Scolnik PA, Bar-Zvi D (2004) The water- and salt-stress-regulated *Asr1* (abscisic acid stress ripening) gene encodes a zinc-dependent DNA-binding protein. Biochem J **381:** 373-378

Kovacs I, Ayaydin F, Oberschall A, Ipacs I, Bottka S, Pongor S, Dudits D, Toth EC (1998) Immunolocalization of a novel annexin-like protein encoded by a stress and abscisic acid responsive gene in alfalfa. Plant J **15:** 185-197

Laohavisit A, Davies JM (2011) Annexins. New Phytol **189:** 40-53

Lippincott-Schwartz J, Altan-Bonnet N, Patterson GH (2003) Photobleaching and photoactivation: following protein dynamics in living cells. Nat Cell Biol **Suppl:** S7-14

Mortimer JC, Laohavisit A, Macpherson N, Webb A, Brownlee C, Battey NH, Davies JM (2008) Annexins: multifunctional components of growth and adaptation. J Exp Bot **59:** 533-544

Niebel Fde C, Lescure N, Cullimore JV, Gamas P (1998) The Medicago truncatula *MtAnn1* gene encoding an annexin is induced by Nod factors and during the symbiotic interaction with Rhizobium meliloti. Mol Plant Microbe Interact **11:** 504-513

Patterson GH, Lippincott-Schwartz J (2002) A photoactivatable GFP for selective photolabeling of proteins and cells. Science **297:** 1873-1877

Sikorski RS, Michaud WA, Wootton JC, Boguski MS, Connelly C, Hieter P (1991) TPR proteins as essential components of the yeast cell cycle. Cold Spring Harb Symp Quant Biol **56:** 663-673

Vandeputte O, Lowe YO, Burssens S, D VANR, Hutin D, Boniver D, Geelen D, El Jaziri M, Baucher M (2007) The tobacco *Ntann12* gene, encoding an annexin, is induced upon *Rhodoccocus fascians* infection and during leafy gall development. Mol Plant Pathol **8:** 185-194

Zachariae W, Nasmyth K (1996) TPR proteins required for anaphase progression mediate ubiquitination of mitotic B-type cyclins in yeast. Mol Biol Cell **7:** 791-801

APPENDICES

APPENDIX A:

STUDY OF A PUTATIVE ENDO-(1,3)(1,4)-B-GLUCANASE

5.1 Introduction

Plant cell walls not only provide mechanical support for cells, but also protect against dehydration, pathogens, and other environmental factors (Cosgrove, 1997). They consist of cellulose microfibrils embedded in a matrix of hemicelluloses, pectins, glycoproteins, and phenolics (Carpita and Gibeaut, 1993; Cosgrove, 2005). There are two types of cell wall, primary and secondary cell wall, that differ in the mobility, arrangement, and composition of matrix polymers, the higher-order organization of microfibrils, and their roles in the life of plant (Cosgrove and Jarvis, 2012). The variation of plant cell wall composition among cell types and species are significant. For instance, the walls of grass differ dramatically from dicot cell walls mainly in the relative abundance of non-cellulosic polysaccharides, their associations with cellulose, pectins, and proteins. (Carpita, 1996; Vogel, 2008).These variations contribute to the differences in mechanical properties that occur in different species and cell types (Cosgrove and Jarvis, 2012). It also influences the strength and flexibility of lumber and textiles, the pulping properties of wood fibers, and cell wall recalcitrance to deconstruction for biofuel production (Cosgrove, 2005; Kiemle *et al*., 2014).

Primary cell walls in dicots, noncommelinoid monocts, and gymnosperms are characterized by a significant abundance of xyloglucan (XyG), pectic polysaccharides and structure proteins (Fincher, 2009). In contrast, the predominant noncellulosic polysaccharides in primary cell walls of commelinoid monocots (e.g. grasses, sedges, rushes and gingers) are glucuronoarabinoxylan (GAX) and (1,3)(1,4)-β-D-glucan (MLG), with relatively minor proportions of XyG and pectic polysaccharides (Buckeridge *et al*., 2004; Vogel, 2008). The GAX, that take the place of XyG in dicots, play an important

structural role by crosslinking cellulose microfibrils. During the secondary cell wall formation, GAX also oxidatively cross-linked with each other and with lignin by phenolics (Ralph *et al*., 1995; Vogel, 2008).

MLGs are considered to be unique to the grass cell wall (Carpita, 1996; Trethewey *et al*., 2005). Recently, they have also been found in other species like lycophyte *Selaginella moellendorffii* and *Equisetum* (Sorensen *et al*., 2008; Harholt *et al*., 2012). The grass MLGs are homopolymers of unbranched and unsubstituted chains of β-glucopyranosyl linked through approximately 30% of β-(1,3) bonds and 70% of β-(1,4) bonds (Woodward, 1983). The unit structure was elucidated by digestion of MLGs with endo-(1,3)(1,4)-β-glucanase purified from *Bacillus subtilis*. This enzyme only cleaves a (1,4)-β-glucosyl linkage if it is next to a (1,3)-β-glucosyl linkage (Anderson and Stone, 1975). The result shows that MLGs of grasses are composed of a mixture of trisaccharide and tetrasaccharide units. Within each unit, two or three contiguous (1,4)-β-glucosyl residues are joined with a single (1,3)-β-glucosyl linkage (Woodward, 1983; Woodward, 1983). Up to 10% of the polymers contain more than three (1,4)-linked residues, however contiguous (1,3)-linked residue units have not been found. By creating an irregular shape within the linear polymer, the (1,3)-β-glucosyl linkages increase flexibility and solubility (Fincher, 2009).

In certain grains, including barley, wheat, and oat, MLG can accumulate to large amounts in the cell walls of the endosperm and act as a storage carbohydrate (Trethewey *et al*., 2005; Wilson *et al*., 2006; Guillon *et al*., 2011). They are part of our natural diet and have a cholesterol-lowering effect as well as possibly immune-regulating properties (Rieder and Samuelsen, 2012). In the context of my project, MLGs

have also been hypothesized to play a role in cell expansion (Luttenegger and Nevins, 1985; Buckeridge *et al*., 2004; Gibeaut *et al*., 2005). In the cell walls of coleoptiles and young leaves of grasses, MLG abundance peaked during the rapid elongation phases and dramatically dropped when cell expansion ceased (Carpita *et al*., 2001; Roulin *et al*., 2002). Several functional roles have been suggested for MLG, including providing a source of metabolizable energy (Wada, 1978; Roulin *et al*., 2002), coating cellulose microfibrils (Carpita *et al*., 2001), forming gel-like matrixes in the wall (Kozlova *et al*., 2014), and tethering cellulose microfibrils to form a load-bearing network (Kiemle *et al*., 2014).

Grasses not only provide the majority of calories consumed by humans; their cell walls are also becoming a significant source of renewable biofuel. It is important to better understand the regulation and the processes necessary for grass growth. Auxin-induced maize coleoptile elongation is one of the best-characterized phytohormonal responses. This growth enhancement by auxin includes several cellular processes, one of which is the specific adjustment in the mechanical properties of the cell wall (Taiz, 1984). This adjustment has been correlated with qualitative and quantitative changes in certain matrix polysaccharides in the cereal grasses (Loescher and Nevins, 1972; Darvill AG, 1978). Among these changes, the effect of auxin on the composition of β-glucans in cell wall appears to be the most significant one. IAA treatment of maize coleoptile segments caused a transient increase followed by a decrease in the β-glucan content of the cell walls, suggesting a link between glucan metabolism and auxin responses (Luttenegger and Nevins, 1985). A higher autohydrolytic activity, mediated by a wall associated exoglucanase and an endoglucanase for degradation of MLG, was

detected in cell wall extracts from maize coleoptiles (Inouhe and Nevins, 1991). Using the specific antisera against either of these glucanase enzymes will dramatically inhibit the coleoptile elongation growth, indicating a role of glucanases in control of plant elongation (Thomas *et al*., 2000). In addition, auxin also directly induces the transcription of endoglucanase, suggesting that the de novo synthesis of endoglucanase is necessary for sustained cell elongation (Kotake *et al*., 2000). Although it is clear that these glucanases are essential for auxin induced coleoptile elongation, how they function is still under debate. In the original theory, glucanases are thought to be involved with cell wall loosening (Inouhe and Nevins, 1991). However several recent experiments suggest that instead of increasing walls plasticity, glucanase might just provide a potential energy source, by degrading MLGs (Roulin *et al*., 2002; Takeda *et al*., 2010).

Endo-(1,3)(1,4)-β-glucanase is a carbohydrate-acting enzyme of class GH16b . Class GH16b enzymes contain not only endo-glucanases but also xyloglucan endotransglucosylase/hydrolase (XTHs) (Strohmeier *et al*., 2004). The sequence similarity between XTHs and endo-glucanase suggested the possibility that endo-glucanase might be able to use XyG as an alternative substrate. *In vitro* experiments demonstrated that corn endo-glucanase not only hydrolyses MLG but also XyG (Hatfield and Nevins, 1987). Taken together, these results make endo-glucanase a good candidate for an ancient and universal mechanism of growth control in cereal plants.

In our comparison of auxin-treated corn coleoptiles we have identified a protein of unknown function, called HYP3, which encodes a putative endo-(1,3)(1,4)-β-glucanase. In Chapter 2 (Jie *et al*., 2013) I showed that expression of HYP3 is correlated with cell

expansion growth in coleoptiles and leaves (Fig. 2.7a) (Li *et al*., 2013). *HYP3* cDNA was cloned from maize and the recombinant HYP3 protein expressed in *E. coli* and purified. However recombinant HYP3 did not exhibit any enzyme activity *in vitro* analysis.

5.2 Materials and Methods

5.2.1 Protein sequence analysis and bioinformatics analysis

Protein sequence and full cDNA sequence were obtained from the phytozome website (phytozome.jgi.doe.gov). Using the national center for biotechnology website, the conserved domains and two *Arabidopsis* homologues of HYP3 were determined and aligned. Potential signal peptide and glycosylation sites were predicted by SignalP 4.1 server and NetNGlyc 1.0 server, respectively.

5.2.2 Recombinant HYP3 expression and purification

To generate C-terminal His-tagged fusion proteins of HYP3, the full length ORF was isolated from RNA extracts of five-day-old corn coleoptiles, using RT-PCR, with BamHI and NdeI sites at either end. After the digestion, the ORF was inserted into the pET42b expression vector. The construct was confirmed by sequencing.

Protein expression was performed as described in chapter 3. However HYP3 was predominately enriched in the inclusion body. To obtain the protein for *in vitro* enzymatic analysis, the inclusion body was purified and dissolved in PBS buffer containing 8 M Urea. For protein refolding, the denatured protein was then dripped slowly into 50 volumes of PBS buffer with 10 mM DTT under constant stirring. The protein was purified by Ni-column and analyzed by SDS-PAGE.

5.2.3 *In vitro* enzymatic analysis

The enzyme activity and substrate specificity of the purified HYP3 were determined using the dinitrosalicylic acid (DNS) method (Miller, 1959). Different substrates were dissolved in 0.1 M sodium acetate buffer at pH 5.0 and incubated with 10 µg HYP3 protein solution for at 50°C for 10 min. The reaction was then stopped and the

absorbance of the supernatant was measured at 575 nm. Endo-glucanase (Sigma) and lichenase (megazyme) were used as control.

5.2.4 Identification and verification of T-DNA insertion lines

The SALK T-DNA database (http://signal.salk.edu/cgi-bin/tdnaexpress) was searched for insertion lines in *HYP3 Arabidopsis thaliana* homologue and seeds were obtained from the Arabidopsis Biological Resource Center (Alonso *et al*., 2003). Using PCR genotyping, homozygous mutants for the T-DNA insertion were identified. Mutant plants were confirmed using reverse transcriptase–mediated PCR.

5.2.5 Transient expression and stable transgenic transformation

Following the method described in chapter 3, a vector for expression of carboxy-terminal HYP3-GFP fusion protein was generated, transformed into *Agrobacterium* and inoculated into the lower epidermis of tobacco for transient expression. After 48 hours, localization was determined using confocal microscopy.

To generate the HYP3 overexpression line, the constructs were transformed into *Arabidopsis thaliana* Columbia-0 plants, using *Agrobacterium*-mediated floral dip as described by Clough & Bent 1998. Overexpression plants were confirmed by genotyping PCR and RT-PCR.

5.3 Results

5.3.1 Sequence analysis of the full-length *HYP3* gene

HYP3 (GRMZM2G073079) has an open reading frame of 795 bp that encodes a predicted protein of 264 amino acid residues with an estimated molecular weight of 28.6 kD and a pI of 6.3. It contains a potential 25 amino acid signal peptide sequence (SignalP 4.1) and two predicted N-linked glycosylation sites (Fig. 5.1a). Several endo-(1,3)(1,4)-β-glucanases in other species, including sorghum, *Setaria italica, Oryza sativa,* and brachypodium exhibit high protein sequence similarity, in the comparison with HYP3. HYP3 contains a dienelactone hydrolase domain (DLH domain), which can catalyze the hydrolysis of dienelactone to maleylacetate, a domain that is conserved among other endoglucanases as well (Fig. 1a). These results suggest that HYP3, a putative maize glucanase, might possess the same enzyme activity as other endo-(1,3)(1,4)-β-glucanase. I found two *HYP3* homologues in *Arabidopsis*: *AtHYP3-1* (At3g23570) and *AtHYP3-2* (At3g23600) (Fig. 1b). Both of them contain the dienelactone hydrolase domain and are predicted to be involved with plant response to abiotic stress (Gong *et al*., 2001).

5.3.2 Expression of HYP3 and enzymatic analysis

The full-length ORF of *HYP3* was cloned into the pET42b vector and transformed into the *E. coli*. Protein expression was induced by 0.1mM IPTG for 3 hours. After the ultra-sonication for disrupting the cell membrane, different fractions of cell lysis were then analyzed by SDS-PAGE. Comparison between the control and induced *E. coli*. protein bands revealed the presence of a highly expressed protein with 29 kD molecular weight, which corresponds to HYP3. However, this recombinant HYP3 was

predominantly located in the inclusion bodies. Low temperature, low IPTG concentration during the induction, or fusion of MBP domain to C-terminus of HYP3, could not improve the solubility of HYP3. Using the combination of a denature-renature method with the Ni-IDA affinity column (Fischer *et al*., 1993), I was finally able to obtain purified protein, with 90% purity.

Enzymatic analysis was performed to test the hydrolytic activity of HYP3. Endo-(1,3)(1,4)-β-glucanase (megazyme) and β-glucanase (Sigma) from *Bacillus subtilis* were used as positive controls. Using lichenin, one kind of MLGs, as substrate, HYP3 did not exhibit any enzyme activity while the two controls effectively hydrolyzed the substrate (Fig 2). I tested all three enzymes against various polysaccharides, including barley β-glucan, sodium carboxymethyl cellulose (CMC), and XyG. As expected, endo-(1,3)(1,4)-β-glucanase also hydrolyzed barley β-glucan, but with a lower activity compared to lichenin. β-glucanase showed no substrate specificity and hydrolyzes all three polysaccharides. However HYP3 still exhibited no hydrolytic activity. This may be either due to protein misfolding during the renaturing process or missing post transcriptional modifications.

5.3.3 Phenotyping of *athyp3-1* and *athyp3-2* knock-out mutants, and *ZmHYP3* overexpression lines.

I obtained *athyp3-1* and *athyp3-2* knock-out mutants in *Arabidopsis* from the Arabidopsis Information Resource. The two single mutants do not show any growth phenotype compared to wild type (Fig. 3a). By crossing single mutants, I was able produce the double knock-out mutant, which was also indistinguishable from wild type. In addition, overexpression of *ZmHYP3* in *Arabidopsis* also did not cause any

morphological phenotypes (Fig 3b). The lack of an effect of ZmHYP3 is not necessarily surprising since *Arabidopsis* is not expected to contain MLGs. The HYP3 homologues in *Arabidopsis* may hence serve an entirely different function or use other glucans as substrate.

5.3.4 Subcellular localization of HYP3

To investigate the subcellular localization of HYP3, a C-terminal HYP3-green fluorescent protein (GFP) fusion protein was transiently expressed in tobacco leaves. As shown (Fig. 5.4), HYP3-GFP is localized in the periphery of the cell.

5.3.5 Conclusions and outlook

In chapter 2, I had identified HYP3, a putative maize endo-(1,3)(1,4)-β-glucanase, from auxin induced rapidly-growing corn coleoptiles. Expression studies suggest that it plays an important role in cell elongation growth. However, recombinant HYP3 did not show any enzyme activity, which may be due to the misfolding or loss of post transcriptional modifications. To examine this possibility, I will attempt to express ZmHYP3 in wheat germ cell-free system (Olliver *et al*., 1998) and repeat the activity assay. An alternative method that we can try is to directly purifying the HYP3 protein from the *Arabidopsis* stable overexpression line, by using a GFP affinity column (ChromoTeck).

MLGs are unique to monocots, which might be the reason why the *Arabidopsis* double mutant and overexpression lines did not show any phenotypic differences. In the future, using RNAi or CRISPR/Cas9, we can generate the *HYP3* knock down mutant in maize. The morphological phenotype, auxin response and cell wall composition will be

monitored and compared to the wildtype. Together these results will shed light on the physiological function of HYP3.

Figure 5.1 Protein sequence alignment of HYP3 (a), AtHYP3 and AtHYP3-2 (b).

(a) The HYP3 protein sequence contains a DLH domain (amino acids 45 to 220; highlighted in yellow). Predicted signal peptide (SignalP 4.1) are labeled by asterisks. Two Asn amino acids, that are predicted to be N-glycosylated with high potential, are indicated in red.

a

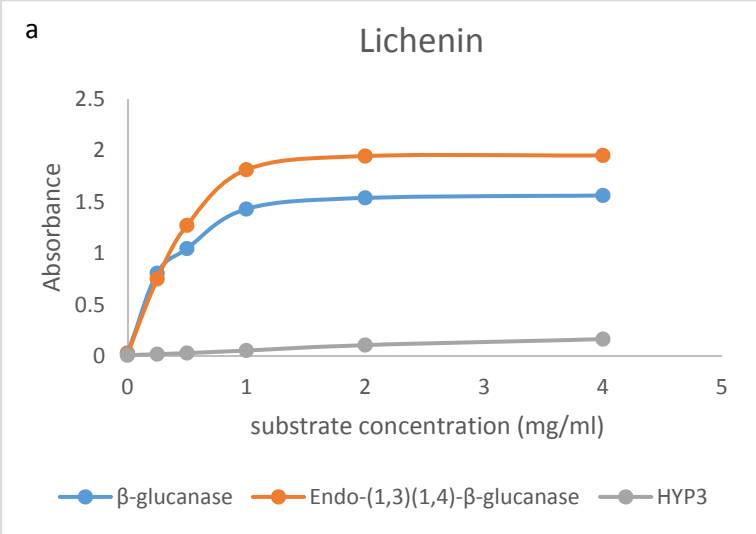
```
MPSSAQVLLCLAAVLAAAAATTAEAHSQCLDNPPDRSIHGRQLAEAGEVV      50
************************
HDLPGGLRAYVSGAASSSRAVVLASDVFGYEAPLLRQIADKVAKAGYFVV      100

VPDFLKGDYLDDKKNFTEWLEAHSPVKAAEDAKPLFAALKKEGKSVAVGG      150

YCWGGKLSVEVGKTSDVKAVCLSHPYSVTADDMKEVKWPIEILGAQNDTT      200

TPPKEVYRFVHVLRERHEVPYYAKIFQGVEHGFACRYNTTDPFAVKTAET      250

ALAYMVSWFNKHLN           264
```

b

```
HYP3          MPSSAQVLLCLAAVLAAAAATTAEAHSQCLDNPPDRSIHGRQLAEAGEVVHDLPGGLRAY
AtHYP3-1      ----------------------MSGHQCTENPPDLDPT------SGSGHVEKLGNLDTY
AtHYP3-2      ----------------------MSGPQCCENPPTLNPV------SGSGHVEKLGGLDAY
                                     :  ** :***  .        :*.   :  * * :*
HYP3          VSGAASSSRAVVLASDVFGYEAPLLRQIADKVAKAGYFVVVPDFLKGDYLD---DKKNFT
AtHYP3-1      VCGSTHSKLAVLLVPHVFGYETPNLRKLADKVAEAGFYAVVPDFFHGDPYNPENQDRPFP
AtHYP3-2      VSGSAESKLCVLLISDIFGFEAPNLRALADKVAASGFYVVVPDYFGGDPYNPSNQDRPIP
              *.*:: *. .*:*  .:**:*:* ** :***** :*::.****:: **  :   :.: :
HYP3          EWLEAHSPVKAAEDAKPLFAALKKEGK-SVAVGGYCWGGKLSVEVGKTSDVKAVCLSHPY
AtHYP3-1      IWMKDHELEKGFEESKPIVEALKNKGITSIGAAGFCWGAKVAVELAKEKLVDATVLLHPA
AtHYP3-2      VWIKDHGCDKGFENTKPVLETIKNKGITAIGAAGMCWGAKVVVELSKEELIQAAVLLHPS
               *:: *   *. *::**:. ::*::*  ::...* ***.*: **:.* . :.*. * **
HYP3          SVTADDMKEVKWPIEILGAQNDTTTPPKEVYRFVHVLRERHEVPYYAKIFQGVEHGFACR
AtHYP3-1      RVTVDDIKEVNLPIAVLGAEIDQVSPPELVRQFEDILASKPQVKSFVKIFPRCKHGWTVR
AtHYP3-2      FVNVDDIKGGKAPIAILGAEIDQMSPPALLKQFEEILSSKPEVNSYVKIHPKVSHGWTVR
               *..**:*  : ** :***: *  :**  : :* .:* .: :*  :.**.   .**:: *
HYP3          YNTTDPFAVKTAETALAYMVSWFNKHLN
AtHYP3-1      YNENDPSEVEAAMEAHKDMLAWLIDYLK
AtHYP3-2      YNIDEPEAVKAAEEAHKEMLDWFVTYIK
              **  :*  *::*  *   *: *:  :::
```

Figure 5.2 Enzymatic analysis of HYP3 (grey), Endo-(1,3)(1,4)-β-glucanase (red), and β-glucanase (blue) using lichenin (a), barley β-glucan (b), carboxymethyl cellulose (c), and xyloglucan (d) as substrates.

The absorbance were measured at 575 nm.



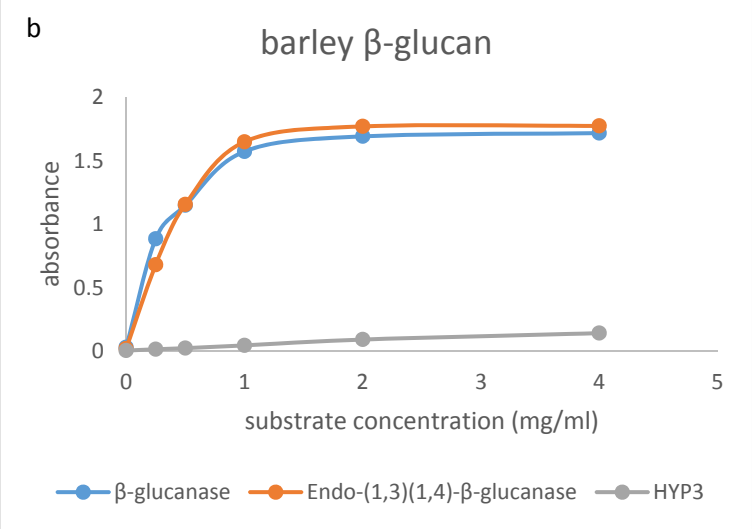


Figure 5.2 (cont'd)

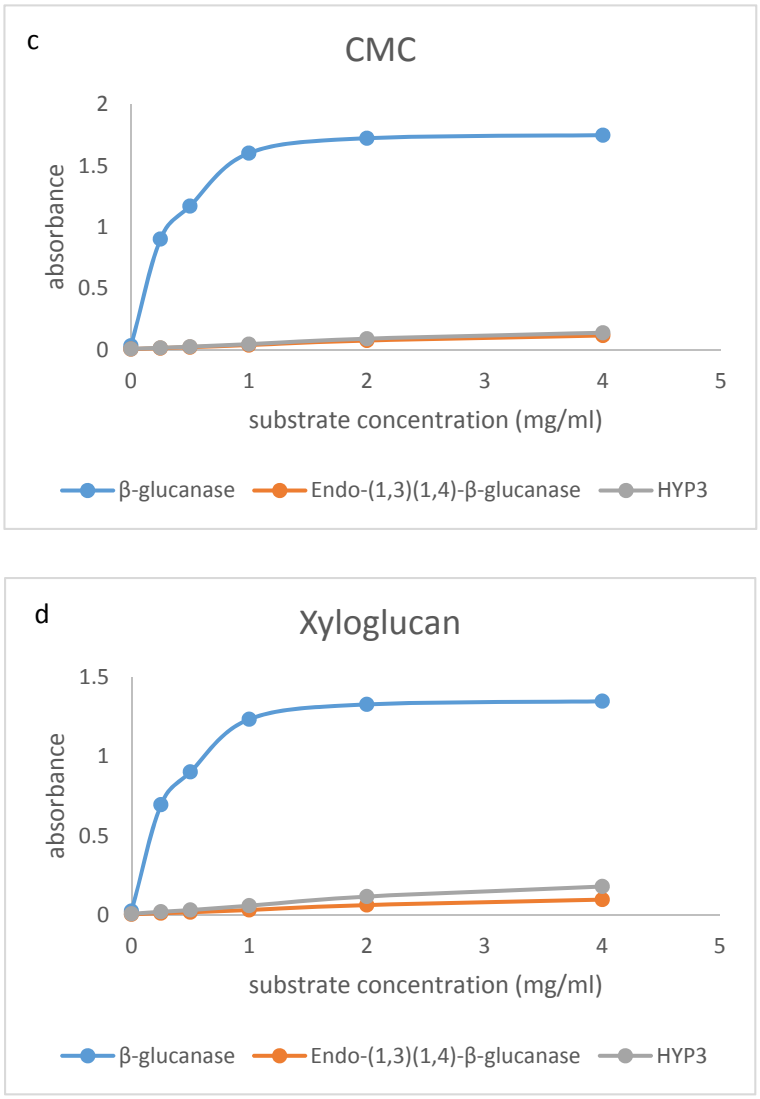


Figure 5.3 Phenotype of *athyp3-1*, *athyp3-2*, and *ZmHYP3* overexpression lines.

(a) Knock-out mutants *athyp3-1* and *athyp3-2* did not show any phenotype. (b) The double mutant and overexpression lines also are indistinguishable from wild type.

(a)

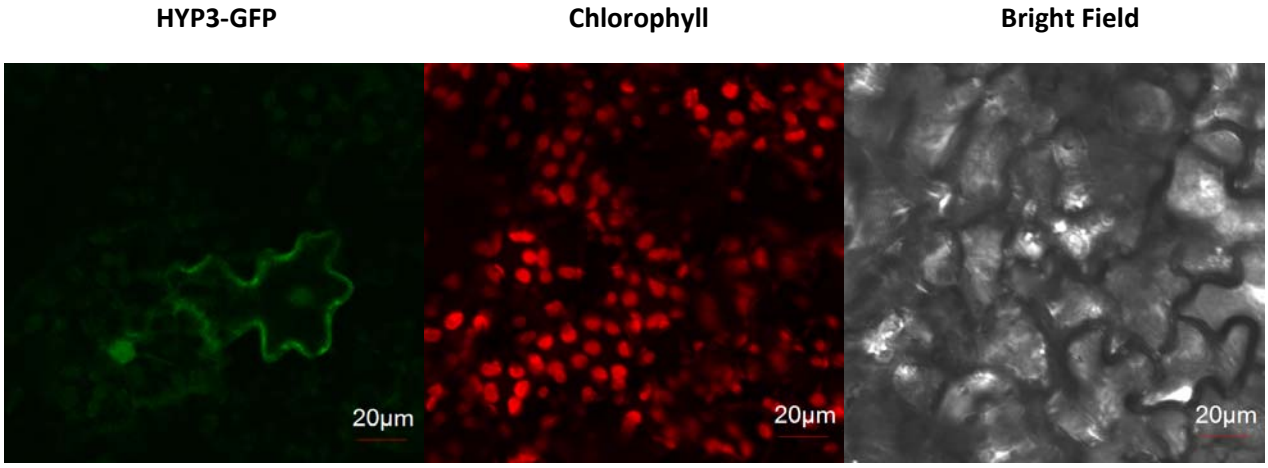
WT *athyp3-1* *athyp3-2*

(b)

WT *athyp3-1* X *athyp3-2* Overexpression

Figure 5.4 Subcellular localization of HYP3.

Green fluorescent protein was fused to the C-terminus of HYP3. Using confocal microscopy, HYP3-GFP is visible mainly in the periphery of cell.



APPENDIX B:

REFERENCES

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653-657

Anderson MA, Stone BA (1975) A new substrate for investigating the specificity of beta-glucan hydrolases. FEBS Lett **52:** 202-207

Buckeridge MS, Rayon C, Urbanowicz B, Tine MAS, Carpita NC (2004) Mixed linkage (1-> 3),(1-> 4)-beta-D-glucans of grasses. Cereal Chemistry **81:** 115-127

Carpita NC (1996) Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol **47:** 445-476

Carpita NC, Defernez M, Findlay K, Wells B, Shoue DA, Catchpole G, Wilson RH, McCann MC (2001) Cell wall architecture of the elongating maize coleoptile. Plant Physiol **127:** 551-565

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J **3:** 1-30

Cosgrove DJ (1997) Assembly and enlargement of the primary cell wall in plants. Annu Rev Cell Dev Biol **13:** 171-201

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol **6:** 850-861

Cosgrove DJ, Jarvis MC (2012) Comparative structure and biomechanics of plant primary and secondary cell walls. Front Plant Sci **3:** 204

Darvill AG SC, Hall MA (1978) Cell wall structure and elongation growth in Zea mays coleoptile tissue. New Phytologist**:** 503-516

Fincher GB (2009) Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. Plant Physiol **149:** 27-37

Fischer B, Sumner I, Goodenough P (1993) Isolation, renaturation, and formation of disulfide bonds of eukaryotic proteins expressed in Escherichia coli as inclusion bodies. Biotechnol Bioeng **41:** 3-13

Gibeaut DM, Pauly M, Bacic A, Fincher GB (2005) Changes in cell wall polysaccharides in developing barley (*Hordeum vulgare*) coleoptiles. Planta **221:** 729-738

Gong Z, Koiwa H, Cushman MA, Ray A, Bufford D, Kore-eda S, Matsumoto TK, Zhu J, Cushman JC, Bressan RA, Hasegawa PM (2001) Genes that are uniquely stress regulated in salt overly sensitive (*sos*) mutants. Plant Physiol **126:** 363-375

Guillon F, Bouchet B, Jamme F, Robert P, Quemener B, Barron C, Larre C, Dumas P, Saulnier L (2011) *Brachypodium distachyon* grain: characterization of endosperm cell walls. J Exp Bot **62:** 1001-1015

Harholt J, Sorensen I, Fangel J, Roberts A, Willats WG, Scheller HV, Petersen BL, Banks JA, Ulvskov P (2012) The glycosyltransferase repertoire of the spikemoss *Selaginella moellendorffi*i and a comparative study of its cell wall. PLoS One **7:** e35846

Hatfield RD, Nevins DJ (1987) Hydrolytic Activity and Substrate Specificity of an Endoglucanase from *Zea mays* Seedling Cell Walls. Plant Physiol **83:** 203-207

Inouhe M, Nevins DJ (1991) Auxin-enhanced glucan autohydrolysis in maize coleoptile cell walls. Plant Physiol **96:** 285-290

Kiemle SN, Zhang X, Esker AR, Toriz G, Gatenholm P, Cosgrove DJ (2014) Role of (1,3)(1,4)-beta-glucan in cell walls: interaction with cellulose. Biomacromolecules **15:** 1727-1736

Kotake T, Nakagawa N, Takeda K, Sakurai N (2000) Auxin-induced elongation growth and expressions of cell wall-bound exo- and endo-beta-glucanases in barley coleoptiles. Plant Cell Physiol **41:** 1272-1278

Kozlova LV, Ageeva MV, Ibragimova NN, Gorshkova TA (2014) Arrangement of mixed-linkage glucan and glucuronoarabinoxylan in the cell walls of growing maize roots. Ann Bot **114:** 1135-1145

Li J, Dickerson TJ, Hoffmann-Benning S (2013) Contribution of proteomics in the identification of novel proteins associated with plant growth. J Proteome Res **12:** 4882-4891

Loescher W, Nevins DJ (1972) Auxin-induced Changes in *Avena* Coleoptile Cell Wall Composition. Plant Physiol **50:** 556-563

Luttenegger DG, Nevins DJ (1985) Transient nature of a (1-> 3), (1-> 4)-beta-d-glucan in *Zea mays c*oleoptile cell walls. Plant Physiol **77:** 175-178

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem **31:** 426-428

Olliver L, Grobler-Rabie A, Boyd CD (1998) *In vitro* translation of messenger RNA in a wheat germ extract cell-free system. Methods Mol Biol **86:** 229-233

Ralph J, Grabber JH, Hatfield RD (1995) Lignin-ferulate cross-links in grasses-active incorporation of ferulate polysaccharide esters into ryegrass lignins. Carbohydrate Research **275:** 167-178

Rieder A, Samuelsen AB (2012) Do cereal mixed-linked beta-glucans possess immune-modulating activities? Mol Nutr Food Res **56:** 536-547

Roulin S, Buchala AJ, Fincher GB (2002) Induction of (1->3,1->4)-beta-D-glucan hydrolases in leaves of dark-incubated barley seedlings. Planta **215:** 51-59

Sorensen I, Pettolino FA, Wilson SM, Doblin MS, Johansen B, Bacic A, Willats WG (2008) Mixed-linkage (1->3),(1->4)-beta-D-glucan is not unique to the Poales and is an abundant component of *Equisetum arvense* cell walls. Plant J **54:** 510-521

Strohmeier M, Hrmova M, Fischer M, Harvey AJ, Fincher GB, Pleiss J (2004) Molecular modeling of family GH16 glycoside hydrolases: potential roles for xyloglucan transglucosylases/hydrolases in cell wall modification in the poaceae. Protein Sci **13:** 3200-3213

Taiz (1984) Plant cell expansion: regulation of cell wall mechanical properties. Annu Rev Plant Physiol**:** 587-657

Takeda H, Sugahara T, Kotake T, Nakagawa N, Sakurai N (2010) Sugar treatment inhibits IAA-induced expression of endo-1,3:1,4-beta-glucanase EI transcripts in barley coleoptile segments. Physiol Plant **139:** 413-420

Thomas BR, Inouhe M, Simmons CR, Nevins DJ (2000) Endo-1,3;1,4-beta-glucanase from coleoptiles of rice and maize: role in the regulation of plant growth. Int J Biol Macromol **27:** 145-149

Trethewey JA, Campbell LM, Harris PJ (2005) (1->3),(1->4)-beta-d-Glucans in the cell walls of the Poales (*sensu lato*): an immunogold labeling study using a monoclonal antibody. Am J Bot **92:** 1660-1674

Vogel J (2008) Unique aspects of the grass cell wall. Curr Opin Plant Biol **11:** 301-307

Wada SR, P. M. (1978) Matrix polysaccharides of oat coleoptile cell walls. Phytochemistry**:** 923–931

Wilson SM, Burton RA, Doblin MS, Stone BA, Newbigin EJ, Fincher GB, Bacic A (2006) Temporal and spatial appearance of wall polysaccharides during cellularization of barley (*Hordeum vulgare*) endosperm. Planta **224:** 655-667

Woodward JRF, G. B.; Stone, B. A. (1983) Water-soluble (1→3),(1→4)- β -D-glucans from barley (*Hordeum vulgare*) endosperm. II. Fine structure. Carbohydr. Polym.**:** 207-225

Woodward JRP, D. R.; Fincher, G. B. (1983) Water-soluble (1→3),(1→4)- β -D-glucans from barley (*Hordeum vulgare*) endosperm. I. Physicochemical properties. Carbohydr. Polym.**:** 143-156