IDENTIFICATION AND CHARACTERIZATION OF NOVEL AUXIN-CYTOKININ CROSS-TALK COMPONENTS

by

Andrej Hurný

January, 2018

A thesis presented to the
Graduate School
of the
Institute of Science and Technology Austria, Klosterneuburg, Austria
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

I hereby declare that this dissertation is my own work and that it does not contain other people's work without this being so stated; this thesis does not contain my previous work without this being stated, and the bibliography contains all the literature that I used in writing the dissertation.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee, and that this thesis has not been submitted for a higher degree to any other university or institution.

I certify that any republication of materials presented in this thesis has been approved by the relevant publishers and co-authors.

Signature: _____

Andrej Hurný

January, 2018

Abstract

The whole life cycle of plants as well as their responses to environmental stimuli is governed by a complex network of hormonal regulations. A number of studies have demonstrated an essential role of both auxin and cytokinin in the regulation of many aspects of plant growth and development including embryogenesis, postembryonic organogenic processes such as root, and shoot branching, root and shoot apical meristem activity and phyllotaxis. Over the last decades essential knowledge on the key molecular factors and pathways that spatio-temporally define auxin and cytokinin activities in the plant body has accumulated. However, how both hormonal pathways are interconnected by a complex network of interactions and feedback circuits that determines the final outcome of the individual hormone actions is still largely unknown. Root system architecture establishment and in particular formation of lateral organs is prime example of developmental process at whose regulation both auxin and cytokinin pathways converge. To dissect convergence points and pathways that tightly balance auxin - cytokinin antagonistic activities that determine the root branching pattern transcriptome profiling was applied. Genome wide expression analyses of the xylem pole pericycle, a tissue giving rise to lateral roots, led to identification of genes that are highly responsive to combinatorial auxin and cytokinin treatments and play an essential function in the auxin-cytokinin regulated root branching.

SYNERGISTIC AUXIN CYTOKININ 1 (SYAC1) gene, which encodes for a protein of unknown function, was detected among the top candidate genes of which expression was synergistically up-regulated by simultaneous hormonal treatment. Plants with modulated SYAC1 activity exhibit severe defects in the root system establishment and attenuate developmental responses to both auxin and cytokinin. To explore the biological function of the SYAC1, we employed different strategies including expression pattern analysis, subcellular localization and phenotypic analyses of the *syac1* loss-of-function and gain-of-function transgenic lines along with the identification of the SYAC1 interaction partners. Detailed functional characterization revealed that SYAC1 acts as a developmentally specific regulator of the secretory pathway to control deposition of cell wall components and thereby rapidly fine tune elongation growth.

Acknowledgments

First I would like to thank Eva for giving me the opportunity to join her research group, for her guidance, ability to motivate and willingness to be always there when I needed help. I really appreciate that.
I also want to thank to all my colleagues either from Belgium or Austria for nice and friendly atmosphere at work. Having you around made my days in the lab very enjoyable.
Special thanks go to my family and friends who were supporting me during all these years. Thanks for being there for me!

About the Author

Andrej Hurný completed a BSc in Natural Sciences at Comenius University in Bratislava (Slovakia) and an MSc in Molecular Biology, Genetics and Virology at Charles University in Prague (Czech Republic), before joining the research group of Prof. Eva Benková in September 2012 at Ghent University (Belgium). In April 2013 he moved with the whole research group to Austria where he helped with the establishment of the new laboratory at IST Austria.

List of Publications Appearing in Thesis

Hurný A., Benková E. (2017) Methodological Advances in Auxin and Cytokinin Biology. In: Dandekar T., Naseem M. (eds) Auxins and Cytokinins in Plant Biology. Methods in Molecular Biology, vol 1569. Humana Press, New York, NY

Table of Contents

1. Chapter 1

Methodological Advances in Auxin and Cytokinin Biology

Adapted and modified from

Hurný A., Benková E. (2017) Methodological Advances in Auxin and Cytokinin Biology. In: Dandekar T., Naseem M. (eds) Auxins and Cytokinins in Plant Biology. *Methods in Molecular Biology*, vol 1569. Humana Press, New York, NY

Methodological Advances in Auxin and Cytokinin Biology

Andrej Hurný[1] and Eva Benková[1]

[1]*Institute of Science and Technology, Austria*, *Klosterneuburg*

1.1 Introduction

The concept of plant hormones as chemical messengers that control plant growth and development is not a new one. Already in 1758, Duhamel du Monceau's experiments suggested communication between plant organs and showed that sap moving from the leaves controls root growth (du Monceau, 1758). More than a century later Julius von Sachs proposed that plants produce “organ-forming substances” - molecules moving to different parts of the plant where they control initiation and development of specific plant organs (von Sachs, 1880). Finally, Charles and Francis Darwin, with their experiments on phototropism of coleoptiles (described in "The Power of Movement in Plants" (Darwin, 1880)) that later led to the discovery of auxin by Went (1928), fully launched the modern research in plant growth substances.

The first note about cytokinin comes from 1913 when Gottlieb Haberlandt observed that compounds from phloem could stimulate cell division in potato parenchyma cells (Haberlandt, 1913). In the 1950s, kinetin, an active compound stimulating cell division, was isolated from herring sperm (Miller et al., 1956). The first naturally occurring cytokinin in plants named zeatin was isolated from immature maize endosperm (Letham, 1973).

Since these initial discoveries, a great number of studies have demonstrated an essential role of both auxin and cytokinin in the regulation of many aspects of plant growth and development including embryogenesis (Friml et al., 2003; Müller and Sheen, 2008), postembryonic organogenic processes such as root (Fukaki et al., 2002; Benková et al., 2003; De Smet et al., 2007; Laplaze et al., 2007; Bielach et al., 2012), and shoot branching (Leyser, 2009; Shimizu-Sato et al., 2009; Müller et al., 2015), root (Friml et al., 2002; Blilou et al., 2005; Dello Ioio et al., 2008; Růžička et al., 2009) and shoot apical meristem activity and phyllotaxis (Reinhardt et al., 2003; Zhao et al., 2010; Yoshida et al., 2011; Chickarmane et al., 2012) vasculature development (Mähönen et al., 2006a; Hejátko et al., 2009; Bishopp et

al., 2011b) as well as tropic responses (Rouse et al., 1998; Müller et al., 1998; Luschning et al., 1998). Importantly, a classic series of experiments by Skoog and Miller (1957) demonstrated that the ratio of cytokinin to auxin profoundly influences the morphogenesis of roots and shoots in plant tissue culture. This was one of the first studies revealing auxin and cytokinin interaction in the differentiation of plant organs and pointed at hormonal cross-talk as an important aspect of auxin and cytokinin regulatory functions (reviewed in Moubayidin et al., 2009; Depuydt and Hardtke 2011; Schaller et al., 2015).

Nevertheless, it has been primarily the recent boom of modern technologies and approaches including analytical chemistry, biochemistry, molecular biology, genetics, cell and developmental biology that have enabled rapid progress in deciphering the auxin and cytokinin activities at the molecular level. Due to ongoing improvements and development of new methods, we are gaining deeper insights into mechanisms that control auxin and cytokinin biosynthesis, distribution, perception and signal transduction as well as insights into their functions in the regulation of plant growth and development. In this review, we shall briefly discuss the major recent progress made in this area, and highlight the importance of continuous methodological improvements.

1.2 Discovery of auxin and cytokinin

Discovery of auxin is tightly linked with Darwin's early studies on coleoptiles. Based on the bending of coleoptiles toward unilateral light, the existence of a messenger molecule named auxein (from the Greek "auxein" meaning ''to grow'') was predicted, which was apparently transported from the site of light perception at the tip of coleoptile towards the site of response where bending occurs (Darwin 1880). Later, it was demonstrated that an asymmetric accumulation of auxin at the non-illuminated side compared to the illuminated side correlated with differential cell growth and organ bending (Boysen-Jensen, 1911). A model implementing a role for auxin and its asymmetric distribution in the regulation of plant tropic responses was proposed (Cholodny, 1927, 1928; Went, 1928). Although the existence of auxin as a molecule controlling plant growth had been predicted already by Darwin in 1880, its chemical identity remained unknown for a long time. In 1928 Went succeeded in capturing this growth substance from coleoptile tips into agar blocks and demonstrated its biological activity (Went, 1928). However, due to insufficient analytical methods for detecting low amounts of the hormone, the first auxin (indole-3-acetic acid, IAA) was purified from human urine and culture filtrates of several fungi, both of which are rich

sources of substances with auxin activity when tested in the bioassays (Kögl et al. 1934; Thimann and Koepfli 1935). A decade later IAA was eventually discovered in a plant (*Zea mays*) (Haagen-Smit et al. 1946).

The first experimental indication of the existence of cytokinins was reported by Gottlieb Haberlandt (1913), who observed that phloem sap can stimulate division of potato parenchyma cells. Further studies showed that compounds which trigger cell division are present in various other plant species (van Overbeek, 1941; Jablonski and Skoog, 1954). The first molecule with the ability to promote cell division was purified from autoclaved herring sperm DNA. The compound 6-(furfurylamino) purine was named kinetin, and although it is one of the most biologically active cytokinins, it is formed as a DNA degradation product and is not detected in plant tissues (Miller et al., 1955; Hall and de Ropp, 1955). The first naturally occurring cytokinin, zeatin, was almost simultaneously isolated from *Zea mays* by Miller (1961) and Letham (1963). Since then, many naturally occurring cytokinins have been isolated and found to be ubiquitous to all plant species (Salisbury and Ross, 1992).

The discovery and identification of auxin and cytokinins triggered the interest of researchers, who then diversified to explore pathways that underlie auxin and cytokinin biosynthesis and metabolism, their distribution, as well as perception and signal transduction of these two plant hormones. The establishment of *Arabidopsis thaliana* as a model organism for plant molecular biology was one of the important milestones in hormone molecular biology. The use of *Arabidopsis* for mutant screens based on sensitivities to auxin and cytokinin enabled the identification of genes and pathways controlling their metabolism, transport, perception and signaling. These in combination with novel technologies and approaches, such as large scale transcriptome profiling, proteomics, chemical genomics, and most recently mathematical modelling, resulted in major breakthroughs in our understanding of auxin and cytokinin biology.

1.3 Auxin and cytokinin: insights into biosynthesis

Although IAA had been recognized as the main native auxin already in 1935 (Thimann, and Koepfli), the question as to how auxin is synthesized remained unanswered for more than 70 years afterwards. Using genetic and biochemical tools, it has been found that IAA is mainly synthesized from L-tryptophan (Trp) via indole-3-pyruvate (IPA) in a two-step reaction catalysed by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC) (Figure 1a). The TAA family of amino

transferases which mediate the first step of the pathway was isolated from independent genetic screens for mutants affected in shade, ethylene, and responses to the auxin transport inhibitor NPA (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009). Severe auxin deficient phenotypes (in developmental processes such as embryogenesis, seedling growth, flower development, vascular patterning, root branching, tropisms, and shade avoidance) as well as reduced endogenous auxin levels were observed in mutants lacking activity of TAA1 and the homologous TAR1 and TAR2, which indicated their function in auxin homeostasis maintenance (Stepanova et al., 2008). The phenotypic defects observed in TAA1/TAR deficient mutants were partially rescued by auxin, whereas induction of TAA1 led to the accumulation of endogenous IPA. Importantly, the recombinant TAA1 protein has been found to catalyse the conversion of Trp into IPA *in vitro* thus providing evidence for its direct involvement in auxin biosynthesis (Stepanova et al., 2008; Tao et al., 2008).

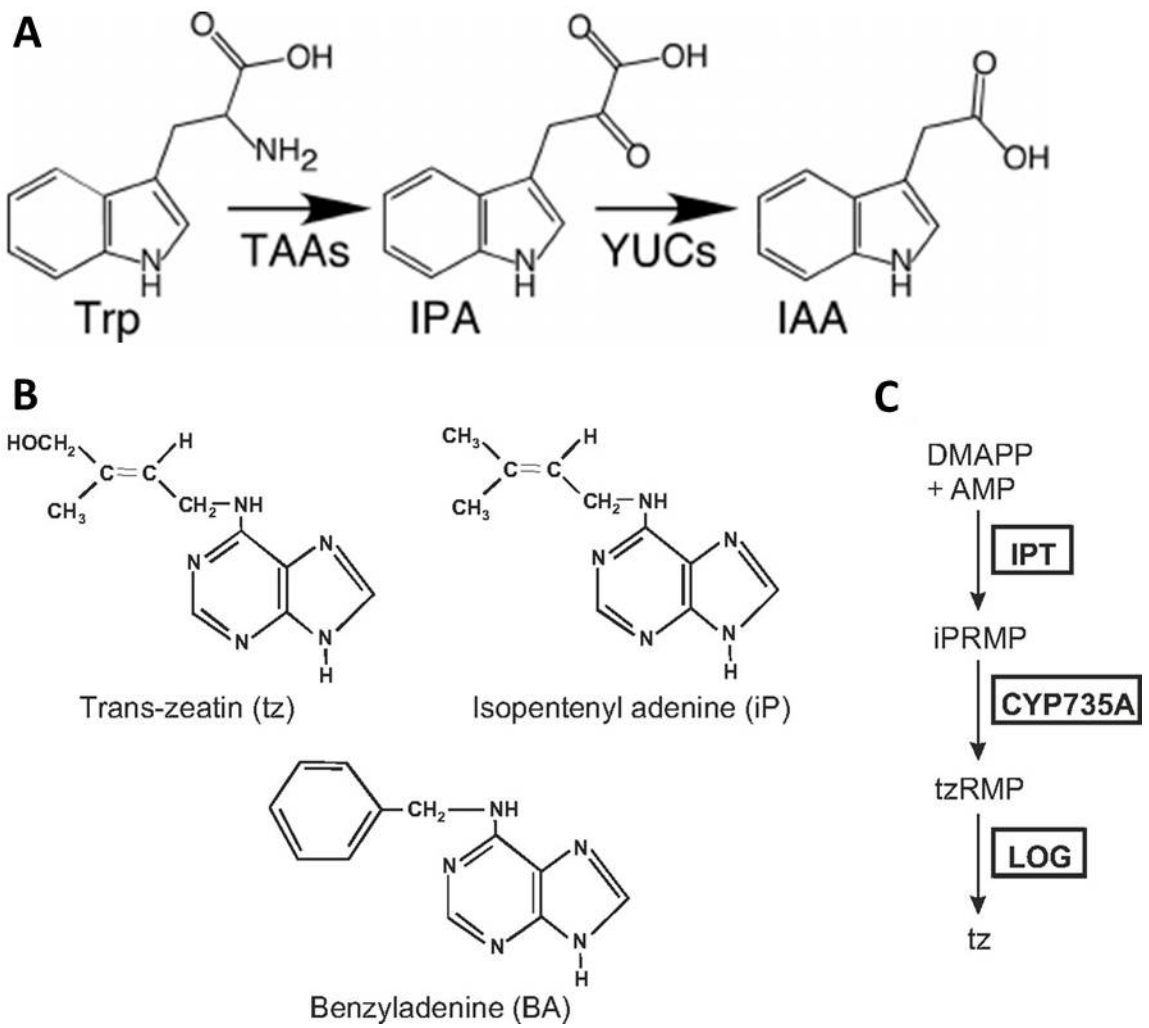


Figure 1 Biosynthesis pathways of auxin and cytokinin. (a) Auxin is synthesized from tryptophan (Trp), which is firstly converted by the TAA transaminase to IPA and then undergoes oxidative decarboxylation catalyzed by the YUC flavin monooxygenase. (b) Common cytokinins found in plants include the isoprenoid cytokinins trans-zeatin (tz) and isopentenyl-adenine (iP). Benzyladenine (BA) is a commonly used synthetic aromatic cytokinin. (c) The cytokinin biosynthesis pathway, highlighting the key enzymes involved. The initial step, in which dimethylallyl diphosphate (DMAPP) reacts with adenosine monophosphate (AMP) to form iP riboside 5′-monophosphate (iPRMP), is catalysed by the IPT gene family. Subsequently, cytochrome P450 mono-oxygenase (CYP735A) converts iPRMP into the tz nucleotide tz riboside 5′monophosphate (tzRMP). Finally, enzymes encoded by the LOG gene family catalyse the conversion of tzRMP into an active cytokinin form, in this case tz. Adapted from Dai et al., (2013) and El-Showk et al., (2013)

Similarly to *TAA1, YUC* genes were originally identified by a genetic screen in *Arabidopsis*. Using an activation-tagged mutant library, a flavin-containing monooxygenase *YUC1* was isolated. The *YUC1* (*yuc1D*) gain-of-function mutant exhibits increase in endogenous IAA and phenotypic alterations mimicking high auxin activity. Disruption of several *YUC* genes in *Arabidopsis* leads to defects in embryogenesis, seedling growth, flower development, and vascular pattern formation (Cheng et al., 2006, 2007). The developmental defects of the loss-of-function *yuc* mutants are rescued by the bacterial auxin biosynthesis gene *iaaM*, supporting *YUC* genes function in auxin biosynthesis (Cheng et al., 2006).

Although previously proposed to act in two independent pathways, recent genetic and biochemical studies showed that the TAAs and YUCs catalyse two consecutive reactions in the same pathway that converts Trp to IAA. Multiple lines of evidence support this model including similarities of both *taa* and *yuc* mutants phenotypes (Won et al., 2011) and enhancement of the auxin related phenotypes when both *YUC* and *TAA* are overexpressed in the same plants (Mashiguchi et al., 2011). Additionally, the YUC auxin overproduction phenotypes are suppressed in the *taa* mutant backgrounds, indicating that TAA acts upstream of YUC-mediated auxin biosynthesis (Won et al., 2011). Direct measurement of IPA levels reveals that *yuc* mutants accumulate IPA whereas *taa* mutants are partially IPA deficient, suggesting that TAAs catalyses synthesis of IPA which is converted by YUCs to IAA (Mashiguchi et al., 2011; Won et al., 2011). Finally, *in vitro* biochemical assays have demonstrated that TAA can convert Trp to IPA and that YUCs produce IAA using IPA as a substrate (Mashiguchi et al., 2011).

Early physiological studies on auxin biosynthesis suggested that auxin is primarily synthesized in the young developing organs such as leaves, shoot apical meristems, and developing fruits and seeds (Bartel, 1997; Ljung et al., 2001). The expression pattern of *TAA* and *YUC* genes modifies this established view on auxin biosynthesis. Local auxin production seems to take place in very distinct cell types, including root and apical embryo meristems, the root cap, quiescent centre (QC), root proximal meristem, vasculature of hypocotyls, as

well as apical hooks, thus hinting at the spatio-temporal control of the IAA biosynthesis throughout plant growth and development (Chent et al., 2006, 2007; Stepanova et al., 2008; Tao et al., 2008). Several transcription factors which control *TAA* and *YUC* genes expression have been identified and thus might determine spatio-temporal pattern of the IAA biosynthesis. *LEAFY COTYLEDON2* (*LEC2*) (Stone et al., 2008) *SHORT INTERNODES/ STYLISH* (*SHI/STY*) (Eklund et al., 2010), *PHYTOCHROME-INTERACTING FACTORs* (*PIFs*) (Franklin et al., 2011; Sun et al., 2012), *INDETERMINATE DOMAIN* (*IDD*) (Cui et al., 2012) and *PLETHORA* family members (Pinon et al., 2013) have been reported as transcriptional activators of *YUC* and *TAA1* genes. In contrast, the *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*) transcription factor, has been shown to negatively regulate some of *YUC* genes (Li et al., 2008).

Chemical biology-based studies provided additional support for the central role of the IPA pathway in IAA production. Chemical screens for auxin inhibitors uncovered L-kynurenine and L-amino-oxyphenylpropionic acid (L-AOPP) as TAA inhibitors and yucasin as a YUC inhibitor. Application of these compounds reduces endogenous IAA levels and results in phenotype alterations mimicking mutants deficient in auxin biosynthesis (Soeno et al., 2010; He et al., 2011; Nishimura et al., 2014).

Overall, genetic and biochemical analyses support the YUCs/TAAs mediated auxin biosynthesis as the major pathway used to produce auxin during plant development, whereas other pathways catalysed by CYP79B2/B3, nitrilases, aldehyde oxidases, and pyruvate decarboxylases might not be the main pathways in auxin biosynthesis (Zhao, 2012).

The great progress in elucidation of the cytokinin biosynthesis pathway occurred almost 20 years after identification of the chemical nature of cytokinins by Miller (1961) and Letham (1963). In 1978 Taya and co-workers reported biosynthesis of free cytokinins *in vitro* and demonstrated that cell-free extracts of the slime mold *Dictyostelium discoideum* converts adenosine monophosphate (AMP) and dimethylallyl pyrophosphate (DMAPP) to the active cytokinin iPMP (N6-(D2-isopentenyl)adenosine-5'-monophosphate (Taya et al., 1978). Subsequently, the *ISOPENTENYLTRANSFERASE* (*IPT*) gene from *Agrobacterium tumefaciens* was shown to encode an enzyme with similar activity (Akiyoshi et al., 1984). Later, nine *IPT*-homologues genes were identified by an *in silico* search in the *A.thaliana* genome. The expression of *IPT* genes (except *AtIPT2* and *AtIPT9*) in *E. coli* resulted in the secretion of the cytokinins isopentenyladenine (iP) and zeatin, confirming their function as cytokinin biosynthetic enzymes (Takei et al., 2001). *IPT* genes display distinct, tissue-

specific patterns of expression, indicative of cytokinin production sites (Miyawaki et al., 2004; Takei et al., 2004a).

Free iP-riboside generated via the IPT pathway, as well as the corresponding base, are further stereospecifically hydroxylated to trans-zeatin forms. The *CYP735A1* and *CYP735A2* encoding cytochrome P450 monooxygenases with cytokinin trans-hydroxylase enzymatic activity were identified in *A.thaliana* by a screen employing an (*AtIPT4*)/*P450* co-expression system in *Saccharomyces cerevisiae* (Takei et al., 2004b).

The final step in cytokinin biosynthesis, conversion of the cytokinin ribotides to their active, free base forms is catalyzed by the cytokinin nucleoside 5′-monophosphate phosphoribohydrolase LONELY GUY (LOG). These were first identified in rice by a genetic screen for defects in the maintenance of shoot meristems (Kurakawa et al., 2007). In *A.thaliana*, seven homologous genes that encode active LOG enzymes were detected. The *LOG* genes are differentially expressed in various tissues during plant development. (Kuroha et al., 2009). In accordance with their predicted function the conditional overexpression of *LOG*s in *Arabidopsis* reduced the content of iP riboside 5′-phosphates and increased the levels of iP and the glucosides (Kuroha et al., 2009) Alternatively, the cytokinin ribotides are dephosphorylated to the ribosides and subsequently converted to free-base cytokinins (Chen and Kristopeit 1981a, 1981b), however the corresponding genes have not yet been identified (Figure 1b and c).

Levels of active cytokinins in plant cells are tightly controlled. They might be either converted to storage forms through conjugation to glucose (Martine et al., 1998; Hou et al., 2004) or inactivated through irreversible cleavage by cytokinin oxidases (Werner et al., 2001; Werner et al., 2003), (Figure 1c). Development of highly sensitive analytical methods were instrumental in the detection of numerous cytokinins metabolites and in deciphering complex cytokinin metabolism, followed by identification of the corresponding metabolic enzymes and genes (Letham and Palni 1983; Mok and Mok 2001; Tarkowski et al., 2009).

1.4 Transport of auxin and cytokinin

By definition, hormones are chemical messengers that are transported to distant tissues and organs to regulate their physiology and development. Darwin's early experiments on coleoptiles had already indicated that controlled transport of auxin from the tip of coleoptile to the bending region might be an essential part of the mechanism through which auxin executes its regulatory function. Later, based on the transport studies, it was proposed

that cytokinins and auxin are synthesized only in root tips and shoot apices, respectively, and translocated to target tissues. Although the recent detailed investigations of expression patterns of auxin and cytokinin biosynthesis genes questions this over-simplified model, the tight control of hormone distribution through organs and tissues is considered to be the crucial component of their regulatory mechanisms. Nowadays, the broadly accepted concept is that both hormones are synthesized and act at various sites in a plant body and that they have coordinated functions as long-distance messengers as well as local signals.

The classical transport assays using radioactively labeled auxins outlined main routes of auxin movement in plants (Morris and Thomas, 1978). To transport auxin, plants use two distinct pathways: a non-polar passive distribution through phloem and an active cell-to-cell polar auxin transport (PAT). In the first pathway, most of the auxin and auxin derivatives are rapidly transported via unregulated flow in the mature phloem over long distances in both basipetal and acropetal directions (Nowacki and Bandurski, 1980). The second pathway is slower and acts over shorter distances, transporting auxin in a cell-to-cell manner from the shoot towards the root. In contrast to phloem transport, PAT is specific for active free auxins, occurs in a cell-to-cell manner and is strictly unidirectional. The main PAT stream from the apex towards the root occurs in the cambium and the adjacent partially differentiated xylem elements (Morris and Thomas, 1978; Lomax et al., 1995). In roots, the auxin stream continues acropetally towards the root tip, where part of the auxin is redirected backwards and transported through the root epidermis to the elongation zone (Rashotte et al., 2000).

Based on the chemical nature of auxin and the physiology of PAT, the model of cell-to-cell auxin of transport has been proposed, known as the chemiosmotic hypothesis (Rubery and Sheldrake, 1974; Raven, 1975). As a weak acid, a fraction of IAA exists in the acidic environment of the apoplast as the protonated, neutral form (IAAH), which may diffuse through the plasma membrane. In the more basic cytosol, auxin becomes deprotonated (IAA^-) and is unable to pass passively through the plasma membrane. The chemiosmotic hypothesis predicted that the exit of auxin anions from the cell is mediated by active efflux carriers and that the passive diffusion of auxin can be further facilitated by influx carriers. The polar membrane localization of the auxin efflux carriers in a file of adjacent cells would determine directionality of the auxin flow (Figure 2a).

It has been primarily genetic studies that led to discovery of genes required for auxin influx and efflux (Bennett et al., 1996; Gälweiler et al., 1998; Luschnig et al., 1998; Geisler et al., 2005; Cho et al., 2007). An auxin influx transporter *AUXIN RESISTANT1 (AUX1),*

encoding an amino acid permease-like protein, was found in a screen for auxin resistant plants (Pickett et al., 1990). Strong insensitivity to membrane-impermeable auxin (2,4-D) suggested that the *aux1* mutation interferes with auxin uptake (Bennett et al., 1996), which was confined by the transport assays using a *Xenopus* oocyte expression system (Yang et al., 2006). The *A.thaliana* genome encodes four auxin influx transporters: *AUXIN RESISTANT1* (*AUX1)* and three *Like AUX1 (LAX1, LAX2, LAX3)* (Parry et al., 2001; Swarup et al., 2008; Péret et al., 2012). Thorough exploration of mutants lacking AUX1/LAX activity revealed the essential role of the auxin uptake in the regulation of gravitropism, phototropism, root branching, phyllotaxis, and root hair development (Bennett et al., 1996; Bainbridge et al.,2008; Stone et al., 2008; Swarup et al., 2008; Jones et al., 2009; Péret et al, 2012).

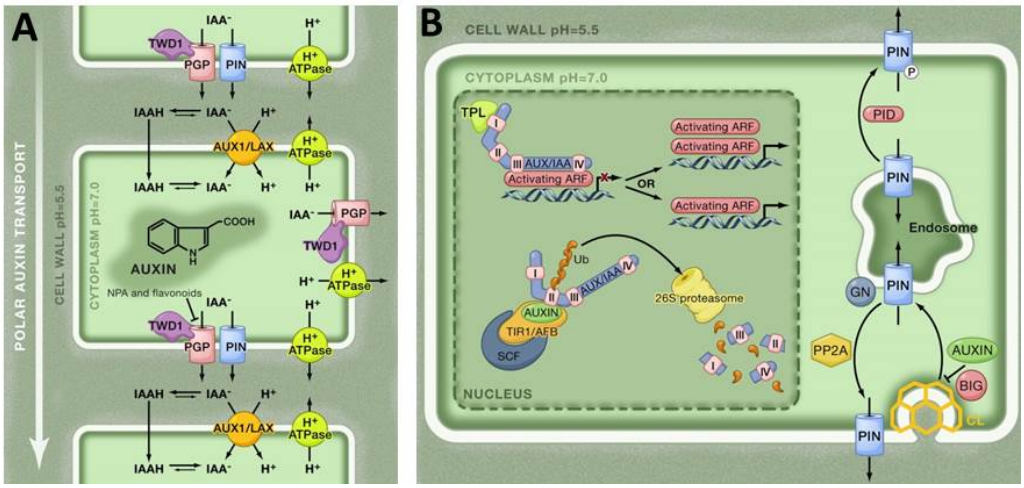


Figure 2 Model of auxin transport and signaling. The low pH in the apoplast (cell wall) is maintained through the activity of plasma membrane H^+ATPases. In the relatively acidic environment, a fraction of the weak acid, indole-3-acetic acid (IAA), the major form of auxin, becomes protonated. The protonated (IAAH) form is more lipophilic and can diffuse freely through the plasma membrane into the cell. Besides passive diffusion, auxin is also actively taken up from the apoplast by H^+/IAA^- symport mediated by AUX1/LAX influx carriers. Once inside the neutral cytosol, auxin is deprotonated and becomes trapped inside the cell. Auxin can leave the cell by auxin efflux carriers such as PIN-FORMED (PIN) proteins and P-glycoproteins (PGP) of the ATP-Binding Cassette family B (ABCB) transporter family. ABCB activity can be modulated by 1-naphthylphthalamic acid (NPA) and flavonoids that interfere with the interaction of ABCB and a protein that regulates it, TWISTED DWARF 1 (TWD1). The polar subcellular localization of PINs determines the direction of auxin flow out of the cell and thus the unidirectional auxin flow within tissues. (b) At low auxin concentrations, Aux/IAA transcriptional repressors are more stable and dimerize through their domains III and IV with auxin response factor (ARF) transcription factors. Through their binding to ARFs, Aux/IAA transcriptional repressors recruit the transcriptional corepressor TOPLESS (TPL) to activating ARFs, by which they are rendered transcriptionally inactive. At higher concentrations, auxin serves as molecular glue between domain II of Aux/IAA transcriptional repressors and TIR1/AFB F box proteins, thereby stimulating Aux/IAA ubiquitination by $SCF^{TIR1/AFB}$ E3 ligase and causing subsequent targeting for proteolysis mediated by the 26S proteasome. Degradation of Aux/IAAs derepresses the ARF activity on transcription. It is not clear whether ARFs act as monomers, dimers, or both. Outside the nucleus, PIN auxin efflux carriers cycle continuously between endosomal compartments and the plasma membrane. The exocytotic step requires the activity of GNOM, an ADP-ribosylation factor GTPase guanine nucleotide exchange factor (ARF-GEF), whereas endocytosis occurs in a clathrin-dependent manner. The PIN phosphorylation status, controlled by counterbalancing activities of PINOID kinase (PID) and protein phosphatase 2A (PP2A), affects the affinity for the apical or basal targeting pathways. Auxin inhibits PIN endocytosis through an unknown mechanism that requires BIG protein, the function of which is unclear. Adapted from Vanneste and Friml (2009).

Genetic screens were also instrumental in identifying molecular components of auxin efflux. In the early nineties, the *A.thaliana* mutant, *pin-formed1* (*pin1*) with needle-like inflorescence was described. The characteristic phenotype similar to wild type plants treated with chemical inhibitors of auxin efflux indicated defects in auxin transport. Auxin transport assays in *pin1* stem segments confirmed severe reduction of the basipetal flow of auxin and pointed to a function for PIN1 in auxin efflux (Okada et al., 1991). Indeed, identification of the mutant locus revealed that *PIN1* encodes a putative transmembrane protein with a predicted topology of transporter proteins (Gälweiler et al., 1998). Auxin transport assays in *Arabidopsis* and tobacco cell suspension culture as well as in heterologous non-plant systems including yeast, mammalian HeLa cells and *Xenopus* oocytes have provided evidence for an auxin efflux capacity of PIN proteins (Petrášek et al., 2006; Yang and Murphy, 2009; Barbez et al., 2013; Zourelidou et al., 2014). The *Arabidopsis PIN* gene family consists of eight members (Zažímalová et al., 2007; Adamovski and Friml 2015). Based on the localization and domain organization, these were divided into two groups. The first group consists of PIN1, PIN2, PIN3, PIN4 and PIN7 and is located at the plasma membrane. The second group comprising PIN5, PIN6, and PIN8 have a reduced middle hydrophilic loop and are located at the endoplasmic reticulum (ER), where they presumably control auxin flow between the cytosol and ER lumen, thus possibly affecting subcellular auxin homeostasis (Mravec et al., 2009; Ding et al., 2012). Similarly, PIN-LIKES proteins (PILS) are located in the ER and might play a role in regulation of intracellular auxin homeostasis (Barbez et al., 2012).

In addition to the PIN family of plant-specific auxin transporters, plant orthologs of the mammalian ATP-binding cassette subfamily B (ABCB)-type transporters of the multidrug resistance/phosphoglycoprotein (ABCB/MDR/PGP) protein family (Noh et al., 2001; Verrier et al., 2008) have been implicated in auxin transport. Biochemical evidence for the ABCB proteins auxin transport activity has been demonstrated both in plant and non-plant systems. In contrast to polar localization of PINs, which corresponds with known direction of auxin flow, the ABCBs presumably act in nondirectional long-distance auxin transport controlling amount of auxin in these streams (Noh et al., 2001; Verrier et al., 2008; Peer et al., 2011).

The chemiosmotic hypothesis predicted that the polar membrane localization of auxin transporters determines the directionality of the auxin flow. This concept was supported by observations of a polar subcellular localization for PIN proteins (Gälweiler et al., 1998; Luschnig et al., 1998) and a tight correlation between PIN polarity and directions of auxin

flow (Wisniewska et al., 2006). Phosphorylation of PINs controlled by a set of kinases and phosphatases (Benjamins et al., 2001; Friml et al., 2004; Michniewicz et al., 2007; Zhang et al., 2010; Huang et al., 2010; Zourelidou et al., 2014), Ca^{2+} signaling (Zhang et al., 2011), cell wall (Feraru et al., 2011) or mechanical signals orienting the plant microtubule network (Heisler et al., 2010) were found to determine PIN protein activity and polarity. Cell-biological studies revealed that PIN auxin efflux transporters may not solely reside at the plasma membrane since they undergo constitutive cycles of endocytosis and recycling back to the plasma membrane (Geldner et al., 2001; Dhonukshe et al., 2007) (Figure 2b). The constitutive endocytosis and recycling of PIN proteins depends on complex subcellular trafficking machinery including the coat protein clathrin (Dhonukshe et al., 2007; Kitakura et al., 2011; Wang et al., 2013), ADP-ribosylation factor guanine-nucleotide exchange factors ARF - GEFs (Geldner et al., 2001, 2003; Kleine-Vehn et al., 2008a,b; Naramoto et al., 2014); ARF-GTPase-activating protein VASCULAR NETWORK DEFECTIVE3 (Naramoto et al., 2010), the related ARF-GEF GNOM-LIKE1 (Teh and Moore, 2007) and small GTPase Rab1b (Feraru et al., 2012). Downstream of endocytosis, the early endosomal trafficking of PINs is controlled by another ARF-GEF, BFA-visualized endocytic trafficking defective1, and the Sec1/Munc18 family protein BEN2 (Tanaka et al., 2009, 2013). The endocytosis and constitutive recycling of PIN proteins has been implicated in the maintenance of PIN polar localization and as a mechanism for rapid modifications of PIN polarity during various developmental processes including embryogenesis (Friml et al., 2003; Robert et al., 2013), lateral root organogenesis (Benkova et al., 2003; Dubrovsky et al., 2008) or tropic responses (Friml et al., 2002, Kleine Vehn et al., 2010; Ding et al., 2011; Rakusova et al., 2011).

Like auxin, cytokinins are highly mobile molecules. However, in contrast to the well characterized transport machinery of auxin, the nature of cytokinin transport is less clear. Long-distance transport of cytokinin is supported by the discovery of cytokinins in xylem and phloem sap (Gillissen et al., 2000; Burkle et al., 2003; Bishopp et al., 2011a). In xylem sap, the major form of cytokinin is tZ-riboside (tZR) (Beveridge et al. 1997; Takei et al. 2001; Hirose et al. 2008), while in phloem sap iP-type cytokinins, such as iP-ribosides and iP-ribotides are detected (Corbesier et al. 2003; Hirose et al. 2008). Accordingly, grafting experiments between wild-type plants and cytokinin biosynthesis mutants showed preferential transport of different cytokinins; trans-zeatin tZ-type cytokinins were transported from the root to the shoot, while iP-type cytokinins moved from the shoot to the root (Matsumoto-Kitano et al., 2008). Thus, plants might use tZ- type as an acropetal messenger and iP-type cytokinins as basipetal messengers (Kudo et al., 2010). Recently, transport assays

using radiolabeled cytokinins confirmed basipetal movement of cytokinin through the phloem and revealed that basipetal transport of cytokinin occurs through symplastic connections in the phloem (Bishopp et al. 2011b). Reverse genetics approaches applied to systematically characterize the ATP-binding cassette transporter proteins in *A. thaliana* yielded the identification of ABCG14 as a transporter involved in the long-distance acropetal (root to shoot) translocation of the root-synthesized cytokinin. Plasma membrane-located ABCG14 is expressed primarily in the central cylinder of roots and loss of ABCG14 activity interferes with the translocation of tZ-type cytokinins from roots to shoots. *In planta* feeding of radiolabeled tZ suggests that ABCG14 acts as an efflux pump (Zhang et al., 2014).

Mechanisms of cytokinin uptake into cells have been studied using radiolabeled cytokinins in *Arabidopsis* cell cultures. Experiments predicted the presence of proton-coupled high-, medium-, and low affinity cytokinin transport systems (Burkle et al., 2003; Cedzich et al., 2008). So far, the equilibrative nucleoside transporter (ENT) family and the purine permease (PUP) family have been found to facilitate cytokinin transport (Burkle et al. 2003, Li et al., 2003; Hirose et al. 2005). Among *Arabidopsis* PUP family proteins (Gillissen et al., 2000), active uptake of free cytokinin bases and several adenine derivatives by PUP1 and PUP2 was demonstrated using a yeast system (Burkle et al., 2003). Expression of PUP2 in the phloem of *Arabidopsis* leaves suggested a role for PUP2 in phloem loading and unloading for long-distance transport of adenine and possibly cytokinins (Burkle et al., 2003). Among the plant ENT transporters, competitive uptake studies in yeast cells showed that *Arabidopsis* ENT3, ENT6, ENT7 and rice ENT2 can facilitate uptake of iP-riboside and tZ-riboside (Li et al., 2003; Hirose et al., 2005). Furthermore, mutants lacking either ENT3 or ENT8 exhibit reduced cytokinin uptake efficiency (Sun et al., 2005). Distinct expression patterns of *ENT* genes detected in root, leaf, and flower vasculature suggest that they may act differently during plant growth and development (Li et al., 2003; Sun at al., 2005; Hirose et al., 2008), however their function as cytokinin transporters *in planta* needs to be experimentally supported. In summary, in contrast to high substrate specificity of the auxin transport system, translocation of cytokinins *in planta* seems to be mediated through transporters with affinities to a broader spectrum of molecules such as purine derivatives and nucleosides.

1.5 Perception and signal transduction of auxin and cytokinin

Solving the puzzle of auxin and cytokinin perception mechanism has been undoubtedly one of the biggest challenges of the last years. Establishment of the *Arabidopsis* genetic model has provided excellent tools to address this long standing question and it has been forward genetic screens in *Arabidopsis* that have led to the identification of backbone elements of both auxin and cytokinin signal transduction cascades. Genetics in combination with advanced molecular and biochemical approaches enabled the achievement of a comprehensive view on the molecular principles of auxin and cytokinin perception and signal transduction.

Several independent forward genetic screens for mutants insensitive to auxin (Rouse et al., 1998; Ruegger et al., 1997, 1998) and expression profiling to isolate auxin inducible genes (Abel et al., 1995; Hagen and Guilfoyle 2002; Abel and Theologis 1996; Ulmasov et al., 1997) led to identification of all key molecular components required for auxin response such as TIR1 (encoding for F-box component of the E3 ubiquitin ligase $SCF^{TIR1/AFBs}$), the auxin early inducible *Aux/IAA* genes as well as the ARF transcription factors that recognise auxin response elements in the promoters of the *Aux/IAAs* (Gray et al., 1999; Abel and Theologis 2010). However, how these genes might constitute the pathway sensing and transducing hormonal signal was not obvious. Using advanced genetic and biochemical approaches the auxin signalling circuit has been resolved and TIR1 identified as the auxin receptor. It has been shown that auxin mediates interaction between TIR1/AFBs and Aux/IAA proteins which stimulates Aux/IAAs ubiquitination by $SCF^{TIR1/AFBs}$ E3-ubiquitin ligases for subsequent degradation by the proteasome. This leads to de-repression of ARFs, and transcriptional regulation of downstream response genes. At low auxin concentration, Aux/IAAs form a complex with ARF transcription factors and the transcriptional corepressor TOPLESS (TPL), thus preventing the ARFs from regulating target genes (Gray et al., 2001; Dharmasiri et al., 2005a,b; Kepinski and Leyser 2005; Tan et al., 2007; Szemenyei et al., 2008) (Figue 2b).

Although the framework which outlines the core molecular mechanism of auxin perception and signal transduction has been recognised, the question as to how *TIR1/AFB*, *Aux/IAAs* and *ARF* families, each comprising many homologous members, mediate specific developmental output remains to be answered. As indicated by recent studies, multiple levels of control appear to exist, including spatio-temporal specific expression of individual auxin signalling pathway components (Overvoorde et al., 2005; Okushima et al., 2005), as well as

differences in affinities of the TIR1/AFB auxin receptors for the Aux/IAA repressors (Calderón-Villalobos et al., 2012; Moss et al., 2015), of Aux/IAA repressors for the ARFs transcription factors (Vernoux et al., 2011; Lee et al., 2014; Korasick et al., 2014; Nanao et al., 2014; Shimizu-Mitao and Kakimoto, 2014), and of ARFs for their binding motifs in promoters of the target genes (Boer et al., 2014), which may allow fine-tuning of auxin responses.

After a period of biochemical attempts in the early 1970s to identify the cytokinin receptors, the forward genetic screens turned out to be successful strategies. In a screen of the activation tagged *Arabidopsis* mutants for cytokinin independent growth, the sensor histidine kinase CKI1 was recovered. This finding suggested that the multi-step phosphorelay similar to bacterial two-component signalling system might underlie the cytokinin signal transduction (Kakimoto, 1996). Another screen for cytokinin insensitive mutants led to identification of the *CRE1* (*CYTOKININ RESISTANT 1*) encoding a sensor histidine kinase related to CKI1 (Inoue et al., 2001). At about the same time, the *WOODEN LEG (WOL)* mutant allele of the AHK4/CRE1 gene (exhibiting severe defects in the vasculature differentiation; Mähönen et al., 2000) was identified, along with the AHK2 and AHK3 homologues required for cytokinin response (Hwang and Sheen et al., 2001; Ueguchi et al 2001; Higuchi et al., 2004; Nishimura et al., 2004). Elegant experiments in yeast and bacteria provided first evidence that CRE1/AHK4 functions as a cytokinin receptor (Inoue et al., 2001; Ueguchi et al., 2001, Suzuki et al., 2001); later corroborated by direct binding assays with radiolabeled cytokinins (Romanov et al., 2005, 2006; Stolz et al., 2011).

Subsequent studies focusing on the downstream signaling cascade revealed that genes with high similarity to molecular elements of the multi-step phosphorelay pathway including sensor histidine kinases (AHKs), histidine phosphotransfer proteins (AHPs) and response regulators (ARRs) are present in the *Arabidopsis* genome (Mizuno, 2005; Schaller et al., 2008). Genetic and biochemical characterization of their functions in the cytokinin response yielded the current model of the cytokinin signalling pathway. In brief, a cascade of auto- and transphosphorylation events triggered by cytokinin leads to activation of AHK receptors and transduction of the signal to downstream components. Downstream of the AHK receptors, the AHPs continuously translocate between cytosol and nucleus to mediate signalling by activating type-B ARABIDOPSIS RESPONSE REGULATORS (ARRs), transcription factors which then trigger the transcription of specific genes. A negative feed-back loop is provided by type-A ARRs, which inhibit the activity of type-B ARRs by an unknown

mechanism (Hwang and Sheen, 2001; Sakai et al., 2001; Mason et al., 2005; Hutchison et al., 2006; To et al., 2007, Argyros et al., 2008; Kieber and Schaller, 2014). Furthermore, a family of F-box proteins, called the KISS ME DEADLY (KMD) family, targets type-B ARR proteins for degradation and attenuates cytokinin pathway activity (Kim et al., 2013) (Figure 3). The large majority of cytokinin receptors localize to the ER, suggesting a central role of this compartment in cytokinin signaling (Caesar et al., 2011; Wulfetange et al., 2011); nevertheless, a small part of the cytokinin receptors might perceive a signal from the plasma membrane (Wulfetange et al., 2011).

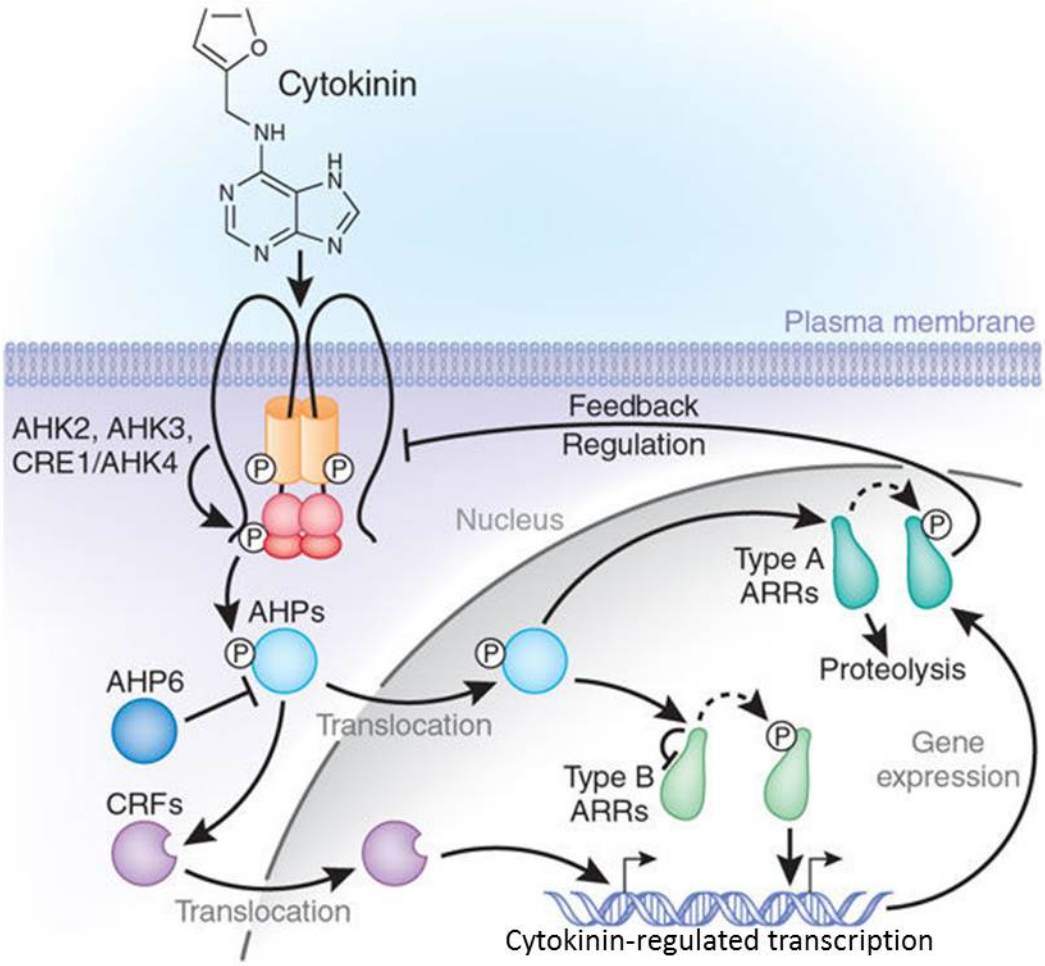


Figure 3 Model of cytokinin signaling pathway. Cytokinin is perceived by the AHK plasma membrane receptors. Cytokinin signal is further amplified by phosphorelay events starting from AHKs, which lead to the activation and subsequent nuclear translocation of AHP proteins. AHP proteins transfer the phosphoryl group to type A or type B ARR proteins. The former act as repressors of cytokinin signaling, whereas the latter act as positive transcriptional regulators of cytokinin-induced genes, including those encoding type A ARRs. CRF proteins are also activated by cytokinin, and after translocation to the nucleus they act as activators of cytokinin-regulated genes. Adapted from Santner et al., (2009).

Recently, a set of cytokinin-regulated transcription factors named cytokinin response factors (CRFs) have been described as a potential branch emerging from the classical multi-step phosphorelay parallel to that of type-B ARRs (Rashotte et al., 2006). CRFs are members of the AP2/EREBP family of transcription factors, containing a single AP2–DNA binding domain, distinct from both DREB and AP2 proteins. There are eight members of CRF family in *Arabidopsis* (CRF1-CRF8) with CRF7 and CRF8 being atypical as they lack C-terminal extensions (Sakuma et al., 2002; Nakano et al., 2006; Rashotte and Goertzen, 2010). The transcript abundance of certain *CRFs* (*CRF2*, CRF5 and *CRF6*) is rapidly upregulated by cytokinin (Rashotte et al., 2006). Protein-protein interaction analysis indicated that CRFs are able to interact with each other to form homo- and/or heterodimers as well as with components of the classical cytokinin signaling pathway. Transcriptome analysis has revealed a large overlap in CRFs and type B ARR targets, pointing at a close link between both branches of the cytokinin signaling pathway.

However, how the specificity of cytokinin response is achieved by the signalling cascade, where each step is supported by a gene family comprising several members, awaits further investigation. Importantly, elucidation of the molecular elements and mechanistic principles of auxin and cytokinin transduction pathways has enabled the development of specific sensors for monitoring auxin and cytokinin *in planta*. Nowadays, highly sensitive reporters such as *DR5* (Ulmasov et al., 1997); DII-VENUS (Band et al., 2012; Brunoud et al., 2012), and *TCS* (Müller and Sheen; 2008) are extensively used for mapping auxin and cytokinin activities, respectively, and demonstrate a great potential of these tools for better understanding of the roles of auxin and cytokinin in plant development.

1.6 Auxin and cytokinin interaction in regulation of plant development

Since the initial discovery of auxin and cytokinin, the number of reports supporting their regulatory role in various aspects of plant development has accumulated. Moreover, studies of auxin and cytokinin function in plant cell suspension growth provided the first evidence of hormonal interaction and its role in directing plant development. The experiments of Skoog and Miller (1957) demonstrated that both auxin and cytokinin are not only required to induce and maintain cell division and growth in plant tissue culture, but that the auxin:cytokinin ratio determines distinct organogenic pathways. A high ratio of cytokinin to auxin stimulated formation of shoots, whereas a low ratio induced root regeneration. Tight

communication between auxin and cytokinin is crucial for proper establishment of meristems in early embryogenesis (Muller and Sheen, 2008; Su et al., 2011), ovule development (Bencivenga et al., 2012), shoot apical meristem activity and phylotaxis (Reinhardt et al., 2003; Werner et al., 2003; Leibfried et al., 2005; Zhao et al., 2010), shoot and root branching (Domagalska and Leyser, 2011; Laskowski et al., 1995, 2008; Laplaze et al., 2007; Bielach, et al., 2012; Marhavý et al., 2011; 2014), root growth and meristem maintenance (Dello Ioio et al., 2008). Hence the deciphering of molecular and mechanistic bases of auxin and cytokinin interaction became one of the major themes in plant biology. Over the years, research on developmental processes in plants has uncovered genes and networks, giving first insights into molecular mechanisms of auxin and cytokinin cross-talk in the context of these complex developmental programs. Here, a few examples of auxin-cytokinin crosstalk mechanisms and their relevance in coordination of specific developmental processes are discussed.

It has been shown that specification of the root pole during the early phases of embryogenesis is dependent on the tightly balanced activity of auxin and cytokinin. Auxin was found to stimulate expression of the cytokinin signaling repressors *ARR7* and *ARR15* and thus to attenuate the output of the cytokinin pathway. Lack of this auxin-driven negative feedback loop resulted in the up-regulation of the cytokinin response and severe patterning defects at the embryonic root pole (Müller & Sheen, 2008). Interestingly, recent observations hint at another auxin-cytokinin regulatory module acting in the early embryogenesis. Among the transcriptional targets of AUXIN RESPONSE FACTOR (ARF5/MP), previously linked with embryonic root specification (Hardtke and Berleth, 1998; Hamann et al., 2002), TARGET OF MONOPTEROS (TMO3), coding for the CRF2 was identified (Schlereth et al., 2010). Expression of CRF2 and homologous genes is cytokinin responsive and interference with their functions leads to severe embryonic defects (Rashotte et al., 2006). Furthermore, two auxin efflux transporters (*PIN1* and *PIN7*), both shown to control distribution of auxin during early embryogenesis (Friml et al., 2003), were identified as CRF2 transcriptional targets (Šimášková et al., 2015). However, how these two regulatory circuits jointly coordinate early embryogenesis requires further investigation.

Auxin and cytokinin act in an antagonistic manner to define the root apical meristem size by promoting cell division and differentiation, respectively (Dello Ioio et al., 2007, Růžička et al., 2009). A complex network of auxin and cytokinin interactions has been implicated in the root meristem activity control. Cytokinin modulates the auxin pathway by affecting the expression of its signaling components. Cytokinin (through the AHK3 receptor

and ARR1 and ARR12 response regulators) was shown to directly activate transcription of the auxin repressor *IAA3/SHORT HYPOCOTYL 2* (*SHY2*). This leads to the attenuation of auxin responses and reduced expression of *PIN* auxin efflux transporters (Vieten et al., 2005; Dello Ioio et al., 2008, Pernisová et al., 2009, Růžička et al., 2009). Consequently, a decreased abundance of PINs limits the auxin supply to the root apical meristem, thereby restricting its meristematic activity (Dello Ioio et al., 2008; Růžička et al., 2009). Besides this transcription-based regulation of auxin activity and distribution, cytokinin was also found to modulate the endocytic trafficking of PIN1 by redirecting this membrane protein for lytic degradation in the vacuoles. (Zhang et al., 2011; Marhavý et al., 2011). This alternative mode of cytokinin action provides a mechanism for rapid control of auxin fluxes; and as recently suggested, the enhanced depletion of PIN1 at specific polar domains by cytokinin might also modulate direction of the auxin flow (Marhavý et al., 2014).

Another mechanism through which auxin and cytokinin balance each other's activities occurs by a crosstalk between their metabolic pathways. High cytokinin levels promote auxin biosynthesis (Jones et al., 2010) and auxin, in turn, gives feedback on the cytokinin metabolism by inducing CYTOKININ OXIDASE (CKX) thereby decreasing cytokinin levels (Eklöf et al., 1997, Nordström et al., 2004; Carabelli et al., 2007). On the other hand, in the root apical meristem, auxin enhances (in an IAA3/SHY2 dependent manner) the expression of ISOPENTENYL TRANSFERASE5 (IPT5), which encodes a rate limiting enzyme in the cytokinin biosynthesis, eventually resulting in the local up-regulation of cytokinin levels (Dello Ioio et al., 2008, Miyawaki et al., 2004).

Both auxin and cytokinin exhibit specific functions in the shoot apical meristem. High cytokinin promotes proliferation of undifferentiated cells, whereas auxin coordinates organogenesis in the peripheral zone (Schaller et al., 2015). Cytokinin participates in the WUSCHEL/WUS-CLAVATA/CLV, the core regulatory loop controlling shoot apical meristem activity, by stimulating WUS expression (Gordon et al., 2009). By direct repression of the ARR7 and ARR15 cytokinin signaling repressors, WUS further reinforces the cytokinin promoting effect on the WUS-mediated pathway (Leibfried et al., 2005). An important additional input in this cytokinin-driven regulation is provided by auxin. In mutants defective in auxin biosynthesis, transport and signaling, expression of *ARR7* and *ARR15* was found to be enhanced, and the *ARF5/MP* transcription factor was identified as a direct repressor of their transcription (Zhao et al., 2010). This constitutes a regulatory circuit in

which auxin enhances cytokinin response by attenuating the expression of the cytokinin signaling repressors, and consequently promoting WUS activity in the WUS-CLV loop.

At the peripheral zone of the shoot apical meristem, new organ formation is triggered by auxin (Reinhardt et al., 2003). Studies following pathways regulated by auxin transport and response revealed that initiation of the lateral organs is accompanied by modulations in the polarity of PIN1 and redirection of the auxin towards incipient primordia (Heisler et al., 2005). The accumulation of auxin correlates with a decrease in *SHOOT MERISTEMLESS* (*STM*) expression, which eventually results in lower cytokinin at the peripheral zone (Hamant et al., 2002). How PIN1 polarization throughout the shoot apical meristem is coordinated and whether cytokinin contributes to the regulation of polar auxin transport through mechanisms analogous to these detected in root is unknown. Nevertheless, a reduced level of PIN1 in the maize *ARR* repressor ortholog mutant *abphyl 1* supports such a scenario (Lee et al., 2009). Recently, Besnard et al. (2014) provide further evidence for cytokinin function in the peripheral zone and coordination of lateral organ initiation. Analysis of *AHP6* expression patterns along with monitoring of auxin and cytokinin sensitive reporters indicates that AHP6, which acts as a repressor of cytokinin signalling (Mähönen et al., 2006), regulates the spatiotemporal pattern of cytokinin activity at the shoot apical meristem periphery. The cytokinin inhibitory fields generated downstream of auxin by AHP6 might stabilize auxin fields, thereby increasing robustness of the phyllotactic patterning (Besnard et al., 2014).

Studies of auxin-cytokinin cross-talk directing other developmental process (including initiation and organogenesis of ovules; vasculature differentiation, shoot and root meristem activity and lateral branching (reviewed in Moubayidin et al., 2009; Depuydt and Hardtke 2011; Schaller et al., 2015) point towards specific as well as common aspects of mechanisms mediating mutual communication between these two hormonal pathways.

With increasing amounts of confirmed molecular interactions and circuits that determine hormone activity at the level of metabolism, transport, perception, and signaling, the prediction of hormone regulatory network behavior and output becomes unfeasible Modelling and mathematical simulations provide a novel means to address these issues and help to achieve better understanding of the complexity and dynamics of hormone action (Voß et al., 2014). .

For example, studies of the transcription factor *PHABULOSA* (*PHB*) and cytokinin in controlling the root meristem size showed that cytokinin regulates *microRNA165/166* and that both cytokinin and *microRNA165/166* jointly regulate *PHB*. In return, PHB promotes cytokinin biosynthesis by stimulation *IPT7* expression (Dello Ioio et al., 2012). One-

dimensional model and mathematical simulations provided insights into the functioning of such a complicated molecular network, showing that this regulatory loop restrains the reduction and accelerates the recovery of PHB levels thus providing robustness against cytokinin fluctuations (Dello Ioio et al., 2012).

A combination of experimental and modelling approaches has also been applied to integrate auxin and cytokinin pathways in the specification of vascular patterning. A two-dimensional multicellular model of Muraro et al., 2014 incorporated previous findings of a mutually inhibitory interaction between auxin and cytokinin, mediated through the auxin inducible repressor of the cytokinin signaling AHP6; cytokinin feedback on the PIN auxin efflux carriers and SHORT ROOT (SHR) promoted expression of the mobile *microRNA165/166* which silences *PHB* to form a gradient of *PHB* mRNA that controls the specification of xylem and inhibits *AHP6* expression (Bishopp et al., 2011b; Carlsbecker et al., 2010). Mathematical simulations revealed that this gene regulatory network is not sufficient to establish proper expression patterns of key marker genes as observed experimentally, and predicted additional negative regulators of cytokinin signaling and the mutual degradation of both *microRNA165*/6 and *PHB* mRNA (Muraro et al., 2014).

A genetic network tested in the model simulation of De Rybel et al., 2014 integrated two incoherent feed-forward loops and evaluated their impact on the patterning of vascular tissues. One of the feed-forward loops implements auxin-cytokinin antagonistic regulations of PIN mediated auxin efflux (Bishopp et al., 2011b; Mähönen et al., 2006). A second loop is based on the experimental identification of interaction between MONOPTEROS/ARF5 and TARGET OF MONOPTEROS5 /LONESOME HIGHWAY (TMO5)/LHW) and LONELY GUY4 (LOG4) which mediates auxin-dependent control of the cytokinin biosynthesis (De Rybel et al., 2013). The authors show that the individual subnetworks provide specific regulatory inputs, one generating a high-auxin domain whereas a second defines sharp boundaries between the high auxin domain and the neighboring cytokinin response domain. Integration of both regulatory circuits is sufficient to generate distinct hormonal zones and establishment of stable patterns within a vascular tissue (De Rybel et al., 2014).

1.7 Conclusion

History of auxin and cytokinin from the initial discoveries by brothers Darwin's (1880) and Gottlieb Haberlandt (1919) is a beautiful demonstration of unceasing continuity of research.

Novel findings are integrated into existing hypotheses and models and deepen our understanding of biological principles. At the same time new questions are triggered and hand to hand with this new methodologies are developed to address these new challenges.

1.8 References

Abel, S., Nguyen, M.D., Theologis, A. (1995) The PS-IAA4/5-like family of early auxin-inducible mRNAs in *Arabidopsis thaliana*. J Mol Biol. **251**:533-49.

Abel, S., and Theologis, A. (1996) Early genes and auxin action. Plant Physiol **111**: 9-17.

Abel, S., and Theologis, A. (2010) Odyssey of auxin. Cold Spring Harb Perspect Biol **2:** a004572.

Adamowski, M., Friml, J. (2015) PIN-dependent auxin transport: action, regulation, and evolution. Plant Cell. **27**: 20-32.

Akiyoshi, D.E., Klee, H., Amasino, R.M., Nester, E.W., Gordon, M.P. (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA **81**: 5994–98.

Argyros, R.D., Mathews, D.E., Chiang, Y.H., Palmer, C.M., Thibault, D.M., Etheridge, N., Argyros, D.A., Mason, M.G., Kieber, J.J., and Schaller, G.E. (2008). Type B response regulators of *Arabidopsis* play key roles in cytokinin signaling and plant development. Plant Cell **20:** 2102-2116.

Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T., and Kuhlemeier, C. (2008). Auxin influx carriers stabilize phyllotactic patterning. Genes Dev **22:** 810-823.

Band, L.R., Wells, D.M., Larrieu, A., Sun, J., Middleton, A.M., French, A.P., Brunoud, G., Sato, E.M., Wilson, M.H., Péret, B., Oliva, M., Swarup, R., Sairanen, I., Parry, G., Ljung, K., Beeckman, T., Garibaldi, J.M., Estelle, M., Owen, M.R., Vissenberg, K., Hodgman, T.C., Pridmore, T.P., King, J.R., Vernoux, T., Bennett, M.J. (2012). Root gravitropism is regulated by a transient lateral auxin gradient controlled by a tipping-point mechanism. Proc. Natl. Acad. Sci. U.S.A. **109**: 4668–4673.

Barbez, E., Kubeš M, Rolčík J, Béziat C, Pěnčík A, Wang B, Rosquete MR, Zhu J, Dobrev PI, Lee Y, Zažímalovà E, Petrášek J, Geisler M, Friml J, Kleine-Vehn J. (2012). A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. Nature **485**: 119–122.

Barbez, E., Lanková, M., Parezová, M., Maizel, A., Zazímalová, E., Petrášek, J., Friml, J., and Kleine-Vehn, J. (2013). Single-cellbased system to monitor carrier driven cellular auxin homeostasis. BMC Plant Biol. **13**: 20.

Bartel, B. (1997) Auxin Biosynthesis. Annu Rev Plant Physiol Plant Mol Biol. **48**: 51–66.

Bencivenga, S., Simonini, S., Benková, E., Colombo, L. (2012) The transcription factors BEL1 and SPL are required for cytokinin and auxin signaling during ovule development in *Arabidopsis*. Plant Cell. **24**:2886-97.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P., and Offringa, R. (2001) The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. Development **128**: 4057-4067.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115**:591-602.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A. (1996) *Arabidopsis AUX1* gene: a permease-like regulator of root gravitropism. Science **273**: 948-950.

Beveridge, C.A., Murfet, I.C., Kerhoas, L., Sotta, B., Miginiac, E., and Rameau, C. (1997) The shoot controls zeatin riboside export from pea roots. Evidence from the branching mutant *rms4*. Plant J. **11**: 339-345.

Besnard, F., Refahi, Y., Morin, V., Marteaux, B., Brunoud, G., Chambrier, P., Rozier, F., Mirabet, V., Legrand, J., Lainé, S., Thévenon, E., Farcot, E., Cellier, C., Das, P., Bishopp, A., Dumas, R., Parcy, F., Helariutta, Y., Boudaoud, A., Godin, C., Traas, J., Guédon, Y., Vernoux, T. (2014) Cytokinin signalling inhibitory fields provide robustness to phyllotaxis. Nature. **16**:417-421.

Bielach, A., Podlešáková, K., Marhavý, P., Duclercq, J., Cuesta, C., Müller, B., Grunewald, W., Tarkowski, P., and Benková, E. (2012). Spatiotemporal regulation of lateral root organogenesis in *Arabidopsis* by cytokinin. Plant Cell **24**: 3967-3981.

Bishopp, A., Benkova, E., and Helariutta, Y. (2011a) Sending mixed messages: auxin-cytokinin crosstalk in roots. Curr Opin Plant Biol **14**:10-16.

Bishopp, A., Lehesranta, S., Vaten, A., Help, H., El-Showk, S., Scheres, B., Helariutta, K., Mahonen, A.P., Sakakibara, H., and Helariutta, Y. (2011b) Phloem-transported cytokinin regulates polar auxin transport and maintains vascular pattern in the root meristem. Curr Biol **21**: 927-932.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005) The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature **433**: 39-44.

Boer, D.R., Freire-Rios, A., van den Berg, W.A., Saaki, T., Manfield, I.W., Kepinski, S., López-Vidrieo, I., Franco-Zorrilla, J.M., de Vries, S.C., Solano, R., Weijers, D., Coll, M. (2014) Structural basis for DNA binding specificity by the auxin-dependent ARF transcription factors. Cell **156**:577-89.

Boysen-Jensen, P. (1911) La transmission de l'irritation phototropique dans l'avena. Bulletin Academie Des Sciences et Lettres de Montpellier **3**: 1–24.

Brunoud, G., Wells, D.M., Oliva, M., Larrieu, A., Mirabet, V., Burrow, A.H., Beeckman, T., Kepinski, S., Traas, J., Bennett, M.J., Vernoux, T. (2012) A novel sensor to map auxin response and distribution at high spatio-temporal resolution. Nature **482**: 103–106.

Burkle, L., Cedzich, A., Dopke, C., Stransky, H., Okumoto, S., Gillissen, B., Kuhn, C., and Frommer, W.B. (2003) Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of Arabidopsis. Plant J **34**: 13-26.

Caesar, K., Thamm, A.M., Witthoft, J., Elgass, K., Huppenberger, P., Grefen, C., Horak, J., and Harter, K. (2011) Evidence for the localization of the *Arabidopsis* cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. J Exp Bot.62:5571-5580.

Calderón Villalobos, L.I., Lee, S., De Oliveira, C., Ivetac, A., Brandt, W., Armitage, L., Sheard, L.B., 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. Nature Chemical Biology **8**: 477 – 485.

Carabelli, M., Possenti, M., Sessa, G., Ciolfi, A., Sassi, M., Morelli, G., Ruberti, I. (2007) Canopy shade causes a rapid and transient arrest in leaf development through auxin-induced cytokinin oxidase activity. Genes & Development **21**: 1863-1868.

Carlsbecker, A., Lee, J.Y., Robertsm, C.J., Dettmer, J., Lehesranta, S., Zhou, J., Lindgren, O., Moreno-Risueno, M.A., Vatén, A., Thitamadee, S., Campilho, A.,

Sebastian, J., Bowman, J.L., Helariutta, Y., Benfey, P.N. (2010) Cell signalling by *microRNA165/6* directs gene dose-dependent root cell fate. Nature. **465**: 316-321.

C**edzich, A., Stransky, H., Schulz, B., Frommer, W.B.** (2008) Characterization of cytokinin and adenine transport in *Arabidopsis* cell cultures. Plant Physiol. **148**: 1857-1867.

Chen CM, Kristopeit SM (1981a) Metabolism of cytokinin: dephosphorylation of cytokinin ribonucleotide by 5_-nucleotidases from wheat germ cytosol. *Plant Physiol.* **67**: 494–498.

Chen CM, Kristopeit SM (1981b) Metabolism of cytokinin: deribosylation of cytokinin ribonucleoside by adenine nucleosidase from wheat germ cells. *Plant Physiol.* **68**, 1020–1023.

Cheng, Y., Dai, X., and Zhao, Y. (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Genes Dev. **20**:1790–1799.

Cheng, Y., Dai, X., and Zhao, Y. (2007) Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsi*s. Plant Cell. **19**: 2430–2439.

Chickarmane, V.S., Gordon, S.P., Tarr, P.T., Heisler, M.G., and Meyerowitz, E.M. (2012) Cytokinin signaling as a positional cue for patterning the apical–basal axis of the growing *Arabidopsis* shoot meristem. Proc. Natl. Acad. Sci. USA **109**: 4002-4007.

Cho, M., Lee, S.H., and Cho, H.T. (2007) P-glycoprotein4 displays auxin efflux transporter-like action in *Arabidopsis* root hair cells and tobacco cells. Plant Cell **19:** 3930-3943.

Cholodny, N. (1927) Wuchshormone und Tropismen bei den Pflanzen. Biologisches Zentralblatt **47**: 604–626.

Cholodny, N. (1928) Beiträge zur hormonalen Theorie von Tropismen. Planta **6**: 118–134.

Corbesier, L., Prinsen, E., Jacqmard, A., Lejeune, P., Van Onckelen, H., Périlleux, C., and Bernier, G. (2003) Cytokinin levels in leaves, leaf exudate and shoot apical meristem of *Arabidopsis thaliana* during floral transition. J. Exp. Bot. **54**: 2511-2517.

Cui, D., Zhao, J., Jing, Y., Fan, M., Liu, J., Wang, Z., Xin, W., Hu, Y. (2013) The *Arabidopsis* IDD14, IDD15, and IDD16 cooperatively regulate lateral organ morphogenesis and gravitropism by promoting auxin biosynthesis and transport. PLoS Genet. **9**:e1003759.

Darwin, C. (1880) The power of movement in plants. John Murray, London.

Dello Ioio, R., Galinha, C., Fletcher, A.G., Grigg, S.P., Molnar, A., Willemsen, V., Scheres, B., Sabatini, S., Baulcombe, D., Maini, P.K., Tsiantis, M. (2012) A PHABULOSA/cytokinin feedback loop controls root growth in *Arabidopsis*. Curr Biol. **22**:1699-1704.

Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and 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, M.T., Aoyama, T., Costantino, P., and Sabatini, S. (2008) A genetic framework for the control of cell division and differentiation in the root meristem. Science **322**: 1380-1384.

De Rybel, B., Adibi, M., Breda, A.S., Wendrich, J.R., Smit, M.E., Novák, O., Yamaguchi, N., Yoshida, S., Van Isterdael, G., Palovaara, J., Nijsse, B., Boekschoten, M.V., Hooiveld, G., Beeckman, T., Wagner, D., Ljung, K., Fleck, C., Weijers, D. (2014) Plant development. Integration of growth and patterning during vascular tissue formation in **Arabidopsis**. Science **345**:1255215.

De Rybel, B., Möller, B., Yoshida, S., Grabowicz, I., Barbier de Reuille, P., Boeren, S., Smith, R.S., Borst, J.W., Weijers, D. (2013) A bHLH complex controls embryonic vascular tissue establishment and indeterminate growth in *Arabidopsis*. Dev Cell. **24**:426-437.

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

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N.F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inze, D., Bennett, M.J., and Beeckman, T. (2007) Auxin-dependent regulation of lateral root positioning in the basal meristem of *Arabidopsis*. Development **134**: 681-690.

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

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

Dhonukshe, P., Aniento, F., Hwang, I., Robinson, D.G., Mravec, J., Stierhof, Y.D., and Friml, J. (2007) Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in Arabidopsis. Curr Biol **17**: 520-527.

Ding, Z., Galván-Ampudia, C.S., Demarsy, E., Łangowski, Ł., Kleine-Vehn, J., Fan, Y., Morita, M.T., Tasaka, M., Fankhauser, C., Offringa, R., and Friml, J. (2011) Light-mediated polarization of the PIN3 auxin transporter for the phototropic response in *Arabidopsis*. Nat. Cell Biol. **13**: 447–452.

Ding, Z., Wang, B., Moreno, I., Dupláková, N., Simon, S., Carraro, N., Reemmer, J., Pěnčík, A., Chen, X., Tejos, R., Skůpa, P., Pollmann, S., Mravec, J., Petrášek, J., Zažímalová, E., Honys, D., Rolčík, J., Murphy, A., Orellana, A., Geisler, M., Friml, J. (2012) ER-localized auxin transporter PIN8 regulates auxin homeostasis and male gametophyte development in *Arabidopsis*. Nat Commun. **3**: 941.

Domagalska, M.A., and Leyser, O. (2011) Signal integration in the control of shoot branching. Nat Rev Mol Cell Biol **12**: 211-221.

Dubrovsky, J.G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M.G., Friml, J., Shishkova. S., Celenza, J., Benková, E. (2008) Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. Proc Natl Acad Sci U S A.**105**: 8790-8794.

Du Monceau, D.,1758. La physique des arbres, Vol. I. Paris: HL Guérin & LF Delatour.

Eklöf, S., Åstot, C., Blackwell, J., Moritz, T., Olsson, O., Sandberg, G. 1997. Auxin-cytokinin interactions in wild-type and transgenic tobacco. Plant and Cell Physiology **38**: 225-235.

Eklund, D.M., Ståldal, V., Valsecchi, I., Cierlik, I., Eriksson, C., Hiratsu, K., Ohme-Takagi, M., Sundström, J.F., Thelander, M., Ezcurra, I., Sundberg, E. (2010) The *Arabidopsis thaliana* STYLISH1 protein acts as a transcriptional activator regulating auxin biosynthesis. Plant Cell. **22**: 349–363.

Feraru, E., Feraru, M.I., Kleine-Vehn, J., Martinière, A., Mouille, G., Vanneste, S., Vernhettes, S., Runions, J., Friml J. (2011) PIN polarity maintenance by the cell wall in *Arabidopsis*. Curr Biol. **21**:338-343.

Feraru, E., Paciorek, T., Feraru, M.I., Zwiewka, M., De Groodt, R., De Rycke, R., Kleine-Vehn, J., and Friml, J. (2010) The AP-3 b adaptin mediates the biogenesis and function of lytic vacuoles in *Arabidopsis*. Plant Cell **22**: 2812–2824.

Franklin, K.A., Lee, S.H., Patel, D., Kumar, S.V., Spartz, A.K., Gu, C., Ye, S., Yu, P., Breen, G., Cohen, J.D., Wigge, P.A., Gray, W.M. (2011) Phytochrome-interacting

factor 4 (PIF4) regulates auxin biosynthesis at high temperature. Proc. Nat. Acad. Sci. USA. **108**:20231–20235.

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

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003) Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature **426:** 147-153.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., Sandberg, G., Hooykaas, P.J., Palme, K., and Offringa, R. (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science **306**: 862-865.

Fukaki, H., Tameda, S., Masuda, H., and Tasaka, M. (2002) Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of *Arabidopsis*. Plant J **29:** 153-168.

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

Geisler, M., Blakeslee, J.J., Bouchard, R., Lee, O.R., Vincenzetti, V., Bandyopadhyay, A., Titapiwatanakun, B., Peer, W.A., Bailly, A., Richards, E.L., Ejendal, K.F., Smith, A.P., Baroux, C., Grossniklaus, U., Muller, A., Hrycyna, C.A., Dudler, R., Murphy, A.S., and Martinoia, E. (2005) Cellular efflux of auxin catalyzed by the *Arabidopsis* MDR/PGP transporter AtPGP1. Plant J **44**: 179-194.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., and Jurgens, G. (2003) The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell **112**: 219-230.

Geldner, N., Friml, J., Stierhof, Y.D., Jurgens, G., and Palme, K. (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature **413**: 425-428.

Gillissen, B., Burkle, L., Andre, B., Kuhn, C., Rentsch, D., Brandl, B., and Frommer, W.B. (2000) A new family of high-affinity transporters for adenine, cytosine, and purine derivatives in *Arabidopsis*. Plant Cell **12**: 291-300.

Gordon, S.P., Chickarmane, V.S., Ohno, C., Meyerowitz, E.M. (2009) Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. Proceedings of the National Academy of Sciences of the United States of America **106**: 16529-16534.

Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M. (1999) Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. Genes Dev **13**: 1678-1691.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. (2001) Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. Nature **414**: 271-276.

Haagen-Smit, A.J., Dandliker, W.B., Wittwer, S.H., Murneek, A.E. (1946) Isolation of 3-indoleacetic acid from immature corn kernels. Am J Bot **33**: 118–120

Haberlandt, G. (1913) Zur physiologie der zellteilung. Sitzungsber Akad Wiss Berlin Phys Math C **1**: 318-345.

Hagen, G., and Guilfoyle, T. (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol **49**: 373-385.

Hall, R.H., De Ropp, R.S. (1955) Formation of 6-furfurylaminopurine from DNA breakdown products. J Am Chem Soc **77**: 6400.

Hamann, T., Benkova, E., Baurle, I., Kientz, M., and Jurgens, G. (2002) The *Arabidopsis* BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. Genes Dev **16**: 1610-1615.

Hamant, O., Nogué, F., Belles-Boix, E., Jublot, D., Grandjean, O., Traas, J., Pautot, V. (2002) The KNAT2 homeodomain protein interacts with ethylene and cytokinin signaling. Plant Physiology **130**: 657-665.

Hardtke, C.S., Berleth, T. (1998) The *Arabidopsis* gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development EMBO J, **17**: 1405–1411.

He, W., Brumos, J., Li, H., Ji, Y., Ke, M., Gong, X., Zeng, Q., Li, W., Zhang, X., An, F., Wen, X., Li, P., Chu, J., Sun, X., Yan, C., Yan, N., Xie, D.Y., Raikhel, N., Yang, Z., Stepanova, A.N., Alonso, J.M., Guo, H. (2011) A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in *Arabidopsis*. Plant Cell. **23**: 3944–3960.

Heisler, M.G., Hamant, O., Krupinski, P., Uyttewaal, M., Ohno, C., Jönsson, H., Traas, J., Meyerowitz, E.M. (2010) Alignment between PIN1 polarity and microtubule orientation in the shoot apical meristem reveals a tight coupling between morphogenesis and auxin transport. PLoS Biol. **8**:e1000516.

Heisler, M.G., Ohno, C., Das, P., Sieber, P., Reddy, G.V., Long, J.A., and Meyerowitz, E.M. (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. Curr Biol **15**: 1899-1911.

Hejátko, J., Ryu, H., Kim, G.T., Dobesová, R., Choi, S., Choi, S.M., Soucek, P., Horák, J., Pekárová, B., Palme, K., Brzobohaty, B., and Hwang, I. (2009) The histidine kinases CYTOKININ-INDEPENDENT1 and ARABIDOPSIS HISTIDINE KINASE2 and 3 regulate vascular tissue development in *Arabidopsis* shoots. Plant Cell **21**: 2008-2021.

Higuchi, M., Pischke, M.S., Mahonen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Helariutta, Y., Sussman, M.R., and Kakimoto, T. (2004) In planta functions of the *Arabidopsis* cytokinin receptor family. Proc Natl Acad Sci U S A **101**: 8821-8826.

Hirose, N., Makita, N., Yamaya, T., Sakakibara, H. (2005) Functional characterization and expression analysis of a gene, OsENT2, encoding an equilibrative nucleoside transporter in rice suggest a function in cytokinin transport. Plant Physiol. **138**:196-206.

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H., and Sakakibara, H. (2008) Regulation of cytokinin biosynthesis, compartmentalization and translocation. J. Exp. Bot. **59**: 75-83.

Hou, B., Lim, E.K., Higgins, G., and Bowles, D.J. (2004) N-Glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. J. Biol. Chem. **279:** 47822-47832.

Huang, F., Zago, M.K., Abas, L., van Marion, A., Galvan-Ampudia, C.S., and Offringa, R. (2010) Phosphorylation of conserved PIN motifs directs *Arabidopsis* PIN1 polarity and auxin transport. Plant Cell **22**: 1129-1142.

Hutchison, C.E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M.W., Maxwell, B.B., Perdue, T.D., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2006) The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. Plant Cell **18**: 3073-3087.

Hwang, I., and Sheen, J. (2001) Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature **413**: 383-389.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001) Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. Nature **409**: 1060-1063.

Jablonski, J.R., and Skoog, F. (1954) Cell enlargement and cell division in excised tobacco pith tissue. Physiol Plant **7**:16–24.

Jones, A.R., Kramer, E.M., Knox, K., Swarup, R., Bennett, M.J., Lazarus, C.M., Leyser, H.M., and Grierson, C.S. (2009) Auxin transport through non-hair cells sustains root-hair development. Nat Cell Biol **11**: 78-84.

Jones, B., Gunneras, S.A., Petersson, S.V., Tarkowski, P., Graham, N., May, S., Dolezal, K., Sandberg, G., and Ljung, K. (2010) Cytokinin regulation of auxin synthesis in *Arabidopsis* involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction. Plant Cell **22:** 2956-2969.

Kakimoto, T. (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science **274**: 982-985.

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

Kieber, J.J. and Schaller G.E.(2014) Cytokinins. The Arabidopsis Book: e0168.

Kim, H.J., Chiang, Y.H., Kieber, J.J., and Schaller, G.E. (2013) SCF(KMD) controls cytokinin signaling by regulating the degradation of type-B response regulators. Proc. Natl. Acad. Sci. USA **110**: 10028-10033.

Kitakura, S., Vanneste, S., Robert, S., Löfke, C., Teichmann, T., Tanaka, H., and Friml, J. (2011) Clathrin mediates endocytosis and polar distribution of PIN auxin transporters in *Arabidopsis*. Plant Cell **23**: 1920–1931.

Kleine-Vehn, J., Dhonukshe, P., Sauer, M., Brewer, P.B., Wisniewska, J., Paciorek, T., Benkova, E., and Friml, J. (2008) ARF GEF-dependent transcytosis and polar delivery of PIN auxin carriers in *Arabidopsis*. Curr Biol **18**: 526-531.

Kleine-Vehn, J., Ding, Z., Jones, A.R., Tasaka, M., Morita, M.T., and Friml, J. (2010). Gravity-induced PIN transcytosis for polarization of auxin fluxes in gravity-sensing root cells. Proc. Natl. Acad. Sci. USA **107**: 22344–22349.

Kleine-Vehn, J., and Friml, J. (2008) Polar targeting and endocytic recycling in auxin-dependent plant development. Annu Rev Cell Dev Biol **24**: 447-473.

Korasick, D.A., Westfall, C.S., Lee, S.G., Nanao, M.H., Dumas, R., Hagen, G., Guilfoyle, T.J., Jez, J.M., Strader, L.C. (2014) Molecular basis for AUXIN RESPONSE FACTOR protein interaction and the control of auxin response repression. Proceedings of the National Academy of Sciences, USA **111**: 5427 – 5432.

Kögl, F. , Erxleben, H., and Haagen-Smit, A. J. (1934) Über die Isolierung der Auxine a und b aus pfl anzlichen Materialien. 9. Mitteilung über pfl anzliche Wachstumsstoffe. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie* **225**: 215 – 229.

Kudo, T., Kiba, T., and Sakakibara, H. (2010) Metabolism and long-distance translocation of cytokinins. J Integr Plant Biol **52**: 53-60.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., and Kyozuka, J. (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445**: 652-655.

Kuroha, T., Tokunaga, H., Kojima, M., Ueda, N., Ishida, T., Nagawa, S., Fukuda, H., Sugimoto, K., and Sakakibara, H. (2009) Functional analyses of LONELY GUY cytokinin-activating enzymes reveal the importance of the direct activation pathway in Arabidopsis. Plant Cell **21**: 3152-3169.

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M.B., Offringa, R., Graham, N., Doumas, P., Friml, J., Bogusz, D., Beeckman, T., and Bennett, M. (2007)

Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell **19**: 3889-3900.

Laskowski, M., Grieneisen, V.A., Hofhuis, H., Hove, C.A., Hogeweg, P., Maree, A.F., and Scheres, B. (2008) Root system architecture from coupling cell shape to auxin transport. PLoS Biol **6**: e307.

Laskowski, M.J., Williams, M.E., Nusbaum, H.C., and Sussex, I.M. (1995) Formation of lateral root meristems is a two-stage process. Development **121**: 3303-3310.

Lee, B.H., Johnston, R., Yang, Y., Gallavotti, A., Kojima, M., Travençolo, B.A., Costa Lda F., Sakakibara, H., Jackson, D. (2009) Studies of aberrant *phyllotaxy1* mutants of maize indicate complex interactions between auxin and cytokinin signaling in the shoot apical meristem. Plant Physiology **150**: 205-216.

Lee, S., Sundaram, S., Armitage, L., Evans, J.P., Hawkes, T., Kepinski, S., Ferro, N., Napier, R.M. (2014) Defining binding effi ciency and specifi city of auxins for SCF(TIR1/AFB)-Aux/IAA co-receptor complex formation. ACS Chemical Biology **9**: 673 – 682.

Leibfried, A., To, J.P., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438**: 1172-1175.

Letham, D.S., Palni, L.M.S. (1983) The biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol **34**:163–197.

Letham, D.S. (1963) Zeatin, a factor inducing cell division isolated from Zea mays. Life Sci **8**: 569–573.

Leyser, O. (2009) The control of shoot branching: an example of plant information processing. Plant Cell Environ. **32**: 694-703.

Li, G., Liu, K., Baldwin, S.A., Wang, D. (2003) Equilibrative nucleoside transporters of *Arabidopsis thaliana*. cDNA cloning, expression pattern, and analysis of transport activities. J Biol Chem. Sep **278**: 35732-35742.

Li, L.C., Qin, G.J., Tsuge, T., Hou, X.H., Ding, M.Y., Aoyama, T., Oka, A., Chen, Z., Gu, H., Zhao, Y., Qu, L.J. (2008) SPOROCYTELESS modulates YUCCA expression to regulate the development of lateral organs in *Arabidopsis*. New Phytol. **179**:751–764.

Ljung, K., Bhalerao, R.P., and Sandberg, G. (2001) Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. Plant J **28**: 465-474.

Lomax, T.L., Muday, G.K. and Rubery, P.H. (1995) Auxin transport. In: P.J. Davies (Ed.) Plant Hormones: Physiology, Biochemistry and Molecular Biology, Kluwer, Dordrecht, Netherlands, pp. 509–530.

Luschnig, C., Gaxiola, R.A., Grisafi, P., and Fink, G.R. (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. Genes Dev **12**: 2175-2187.

Marhavý, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J. Schwechheimer, C., Murphy, A., and Benková, E. (2014) Cytokinin controls polarity of PIN1-dependent auxin transport during lateral root organogenesis. Curr. Biol. **24**: 1031-1037.

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., and Benková, E. (2011) Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis. Dev. Cell. **21:** 796-804.

Martin R.C. , Mok M.C., and Mok D.W.S. (1999) Isolation of a cytokinin gene, ZOG1, encoding zeatin O-glucosyltransferase from *Phaseolus lunatus* Proc. Natl. Acad. Sci. USA **96**: 284-289.

Mashiguchi, K,, Tanaka, K., Sakai, T., Sugawara, S., Kawaide, H., Natsume, M., Hanada, A., Yaeno, T., Shirasu, K., Yao, H., McSteen, P., Zhao, Y., Hayashi, K., Kamiya, Y., Kasahara, H.. (2011) The main auxin biosynthesis pathway in Arabidopsis. Proc. Natl Acad. Sci. U S A. **108**: 18512–18517.

Mason, M.G., Mathews, D.E., Argyros, D.A., Maxwell, B.B., Kieber, J.J., Alonso, J.M., Ecker, J.R., and Schaller, G.E. (2005) Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell **17**: 3007-3018.

Matsumoto-Kitano, M., Kusumoto, T., Tarkowski, P., Kinoshita-Tsujimura, K., Václavíková, K., Miyawaki, K., and Kakimoto, T. (2008) Cytokinins are central regulators of cambial activity. Proc. Natl. Acad. Sci. USA **105**: 20027-20031.

Mähönen, A.P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P.N., and Helariutta, Y. (2000) A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev **14**: 2938-2943.

Mähönen, A.P., Bishopp, A., Higuchi, M., Nieminen, K.M., Kinoshita, K., Tormakangas, K., Ikeda, Y., Oka, A., Kakimoto, T., and Helariutta, Y. (2006a) Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science **311**: 94-98.

Michniewicz, M., Zago, M.K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M.G., Ohno, C., Zhang, J., Huang, F., Schwab, R., Weigel, D., Meyerowitz, E.M., Luschnig, C., Offringa, R., and Friml, J. (2007): Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell **130**: 1044-1056.

Miller, C.O. (1961) Kinetin-like compound in maize. Proc Natl Acad Sci USA **47**:170–174.

Miller, C.O., Skoog, F., von Saltza, M., Strong, F.M. (1955) Kinetin, a cell division factor from deoxyribonucleic acid. J Am Chem Soc **77**:1392.

Miyawaki, K., Matsumoto-Kitano, M., Kakimoto, T. (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J. **37**:128–138.

Mok, D.W., and Mok, M.C. (2001) Cytokinin Metabolism and Action. Annu Rev Plant Physiol Plant Mol Biol **52**: 89-118.

Morris, D.A. and Thomas, (1978) A microautoradiographic study of auxin transport in the stem of intact pea seedlings (*Pisum sativum L.*). J. Exp. Bot. **29**: 147–157.

Moss, B.L., Mao, H., Guseman, J.M., Hinds, T.R., Hellmuth, A., Kovenock, M., Noorassa, A., Lanctot, A., Villalobos, L.I., Zheng, N., Nemhauser, J.L. (2015) Rate Motifs Tune Auxin/Indole-3-Acetic Acid Degradation Dynamics. Plant Physiol. **169**: 803-813.

Moubayidin, L., Di Mambro, R., and Sabatini, S. (2009) Cytokinin-auxin crosstalk. Trends Plant Sci **14**: 557-562.

Mravec, J., Skůpa, P., Bailly, A, Hoyerová, K., Krecek, P., Bielach, A., Petrásek, J., Zhang, J., Gaykova, V., Stierhof, Y.D., Dobrev, P.I., Schwarzerová, K., Rolcík, J., Seifertová, D., Luschnig, C., Benková, E., Zazímalová, E., Geisler, M., Friml, J., (2009) Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. Nature **459**:1136-1140.

Muraro, D., Mellor, N., Pound, M.P., Help, H., Lucas, M., Chopard, J., Byrne, H.M., Godin, C., Hodgman, T.C., King, J.R., Pridmore, T.P., Helariutta, Y., Bennett, M.J., Bishopp, A. (2014) Integration of hormonal signaling networks and mobile microRNAs is required for vascular patterning in *Arabidopsis* roots. Proc Natl Acad Sci USA. **111**: 857-862.

Müller, D., Waldie, T., Miyawaki, K., To, J.P., Melnyk, C.W., Kieber, J.J., Kakimoto, T., Leyser, O. (2015) Cytokinin is required for escape but not release from auxin mediated apical dominance. Plant J. **82**:874-886.

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

Müller, B., and Sheen, J. (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature **453**: 1094-1097.

Nakano, T., K. Suzuki, T. Fujimura and H. Shinshi (2006) Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. Plant Physiol **140**: 411-432.

Nanao, M.H., Vinos-Poyo, T., Brunoud, G., Thévenon, E., Mazzoleni, M., Mast, D., Lainé, S., Wang, S., Hagen, G., Li, H., Guilfoyle, T.J., Parcy F, Vernoux T, Dumas R. (2014) Structural basis for oligomerization of auxin transcriptional regulators. Nat Commun. **7**: 3617.

Naramoto, S., Kleine-Vehn, J., Robert, S., Fujimoto, M., Dainobu, T., Paciorek, T., Ueda, T., Nakano, A., Van Montagu, M.C.E., Fukuda, H., and Friml, J. (2010) ADP-ribosylation factor machinery mediates endocytosis in plant cells. Proc. Natl. Acad. Sci. USA **107**: 21890–21895.

Naramoto, S., Otegui, M.S., Kutsuna, N., de Rycke, R., Dainobu, T., Karampelias, M., Fujimoto, M., Feraru, E., Miki, D., Fukuda, H., Nakano, A., Friml J. (2014) Insights into the localization and function of the membrane trafficking regulator GNOM ARF-GEF at the Golgi apparatus in *Arabidopsis.* Plant Cell. **26**:3062-3076.

Nishimura, T., Hayashi, K., Suzuki, H., Gyohda, A., Takaoka, C., Sakaguchi, Y., Matsumoto, S., Kasahara, H., Sakai, T., Kato, J., Kamiya, Y., Koshiba, T. (2014) Yucasin is a potent inhibitor of YUCCA, a key enzyme in auxin biosynthesis. Plant J.**77**: 352–366.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis*. Plant Cell **16**: 1365-1377.

Noh, B., Murphy, A.S., and Spalding, E.P. (2001) Multidrug resistance-like genes of *Arabidopsis* required for auxin transport and auxin-mediated development. Plant Cell **13**: 2441-2454.

Nordström, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Astot, C., Dolezal, K., Sandberg, G. (2004) Auxin regulation of cytokinin biosynthesis in Arabidopsis thaliana: A factor of potential importance for auxin-cytokinin-regulated development. Proc. Natl. Acad. Sci. USA **101**: 8039-8044.

Nowacki, J. and Bandurski, R.S. (1980) Myo-inositol esters of indole-3-acetic acid as seed auxin precursors of Zea mays L. Plant Physiol. **65**: 422–427.

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J. and Shimura, Y. (1991) Requirement of the auxin polar transport system in early stages of Arabidopsis floral bud formation. Plant Cell **3**: 677– 684.

Okushima, Y., Overvoorde, P.J., Arima, K., Alonso, J.M., Chan, A., Chang, C., Ecker, J.R., Hughes, B., Lui, A., Nguyen, D., Onodera, C., Quach, H., Smith, A., Yu, G., and Theologis, A. (2005) Functional genomic analysis of the *AUXIN RESPONSE FACTOR* gene family members in *Arabidopsis thaliana*: unique and overlapping functions of ARF7 and ARF19. Plant Cell **17**: 444-463.

Overvoorde, P.J., Okushima, Y., Alonso, J.M., Chan, A., Chang, C., Ecker, J.R., Hughes, B., Liu, A., Onodera, C., Quach, H., Smith, A., Yu, G., Theologis, A.

(2005) Functional genomic analysis of the *AUXIN/INDOLE-3-ACETIC ACID* gene family members in *Arabidopsis thaliana*. Plant Cell **17**: 3282-3300.

Parry, G., Delbarre, A., Marchant, A., Swarup, R., Napier, R., Perrot-Rechenmann, C., and Bennett, M.J. (2001) Novel auxin transport inhibitors phenocopy the auxin influx carrier mutation *aux1*. Plant J **25**: 399-406.

Peer, W.A., Blakeslee, J.J., Yang, H., Murphy, A.S. (2011) Seven things we think we know about auxin transport. Mol Plant. **4**:487-504.

Péret, B., Swarup, K., Ferguson, A., Seth, M., Yang, Y., Dhondt, S., James, N., Casimiro, I., Perry, P., Syed, A., Yang, H., Reemmer, J., Venison, E., Howells, C., Perez-Amador, M.A., Yun, J., Alonso, J., Beemster, G.T., Laplaze, L., Murphy, A., Bennett, M.J., Nielsen, E., Swarup, R. (2012) *AUX/LAX* genes encode a family of auxin influx transporters that perform distinct functions during *Arabidopsis* development. Plant Cell **24**: 2874-2885.

Pernisová, M., Klima, P., Horak, J., Valkova, M., Malbeck, J., Soucek, P., Reichman, P., Hoyerova, K., Dubova, J., Friml, J., Zazimalova, E., and 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.

Petrášek, J., Mravec, J., Bouchard, R., Blakeslee, J.J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M., Dhonukshe, P., Skupa, P., Benkova, E., Perry, L., Krecek, P., Lee, O.R., Fink, G.R., Geisler, M., Murphy, A.S., Luschnig, C., Zazimalova, E., and Friml, J. (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. Science **312**: 914-918.

Pickett, F.B., Wilson, A.K., and Estelle, M. (1990) The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiol **94**: 1462-1466.

Pinon, V., Prasad, K., Grigg, S.P., Sanchez-Perez, G.F., Scheres, B. (2013) Local auxin biosynthesis regulation by PLETHORA transcription factors controls phyllotaxis in *Arabidopsis*. Proc. Nat. Acad. Sci. USA. **110**:1107–1112.

Rakusová, H., Gallego-Bartolomé, J., Vanstraelen, M., Robert, H.S., Alabadí, D., Blázquez, M.A., Benková, E., and Friml, J. (2011) Polarization of PIN3-dependent auxin transport for hypocotyl gravitropic response in *Arabidopsis thaliana*. Plant J. **67**: 817–826.

Raven, J.A. (1975) Transport of indoleacetic acid in plant cells in relation to ph and electrical potential gradients, and its significance for polar iaa transport. New Phytologist **74**: 163–172.

Rashotte, A.M., Brady, S., Reed, R., Ante, S. and Muday, G.K. (2000) Basipetal auxin transport is required for gravitropism in roots of *Arabidopsis*. Plant Physiol. **122**: 481–490.

Rashotte, A.M., Mason, M.G., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2006) A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proc. Natl. Acad. Sci. USA **103**: 11081-11085.

Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003) Regulation of phyllotaxis by polar auxin transport. Nature **426**: 255-260.

Robert, H.S., Grones, P., Stepanova, A.N., Robles, L.M., Lokerse, A.S., Alonso, J.M., Weijers, D., Friml, J. (2013) Local auxin sources orient the apical-basal axis in *Arabidopsis* embryos. Curr Biol. **23**: 2506-2512.

Romanov, G.A., Lomin, S.N., and Schmülling, T. (2006) Biochemical characteristics and ligand-binding properties of *Arabidopsis* cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J. Exp. Bot. **57**: 4051-4058.

Romanov, G.A., Spíchal, L., Lomin, S.N., Strnad, M., and Schmülling, T. (2005) A live cell hormone-binding assay on transgenic bacteria expressing a eukaryotic receptor protein. Anal. Biochem. **347**: 129-134.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M., Leyser, O. (1998) Changes in auxin response from mutations in an *AUX/IAA* gene. Science **279**: 1371–1373.

Rubery, P.H., and Sheldrake, A.R. (1974) Carrier-mediated auxin transport Planta **118**: 101-121.

Ruegger, M., Dewey, E., Gray, W.M., 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.

Ruegger, M., Dewey, E., Hobbie, L., Brown, D., Bernasconi, P., Turner, J., Muday, G., and Estelle, M. (1997) Reduced naphthylphthalamic acid binding in the *tir3* mutant of *Arabidopsis* is associated with a reduction in polar auxin transport and diverse morphological defects. Plant Cell **9**:745-757.

Růžička, K., Simaskova, M., Duclercq, J., Petrasek, J., Zazimalova, E., Simon, S., Friml, J., Van Montagu, M.C., and Benkova, E. (2009) Cytokinin regulates root meristem activity via modulation of the polar auxin transport. Proc Natl Acad Sci USA **106**: 4284-4289.

Sachs, J. (1880) Arb Bot Inst Würzburg **3**:452-488.

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., and Oka, A. (2001) ARR1, a transcription factor for genes immediately responsive to cytokinins. Science **294**: 1519-1521.

Sakuma, Y., Q. Liu, J. G. Dubouzet, H. Abe, K. Shinozaki and K. Yamaguchi-Shinozaki (2002) "DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression." Biochem Biophys Res Commun **290**: 998-1009.

Schaller, G.E., Bishopp, A., Kieber, J.J. (2015) The yin-yang of hormones: cytokinin and auxin interactions in plant development. Plant Cell. **27**:44-63.

Schaller, G.E., Kieber, J.J., and Shiu, S.-H. (2008) Two-component signaling elements and histidyl-aspartyl phosphorelays. in The Arabidopsis Book, C. Somerville and E. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists).

Schlereth A, Möller B, Liu W, Kientz M, Flipse J, Rademacher EH, Schmid M, Jürgens G, Weijers D. (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature **464**: 913-916.

Shimizu-Mitao, Y., and T. Kakimoto. (2014) Auxin sensitivities of all *Arabidopsis* Aux/IAAs for degradation in the presence of every TIR1/AFB. Plant & Cell Physiology **55**: 1405– 1459 .

Šimášková, M., O'Brien, J.A., Khan, M., Van Noorden, G., Ötvös, K., Vieten, A., De Clercq, I., Van Haperen, J.M., Cuesta, C., Hoyerová, K., Vanneste, S., Marhavý, P., Wabnik, K., Van Breusegem, F., Nowack, M., Murphy, A., Friml, J., Weijers, D., Beeckman, T., Benková, E. (2015) Cytokinin response factors regulate PIN-FORMED auxin transporters. Nat Commun. **6**: 8717.

Shimizu-Sato, S., Tanaka, M., and Mori, H. (2009) Auxin-cytokinin interactions in the control of shoot branching. Plant Mol Biol **69:** 429-435.

Skoog, F., and Miller, C. (1957). Chemical regulation of growth and organ formation in plant tissue cultured in vitro. Symp. Soc. Exp. Biol. **11:** 118-131.

Soeno, K., Goda, H., Ishii, T., Ogura T, Tachikawa T, Sasaki E, Yoshida S, Fujioka S, Asami T, Shimada Y. (2010) Auxin biosynthesis inhibitors, identified by a genomics-based approach, provide insights into auxin biosynthesis. Plant Cell Physiol. **51**: 524–536.

Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.Y., Dolezal, K., Schlereth, A., Jurgens, G., and Alonso, J.M. (2008) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell **133**: 177-191.

Stolz, A., Riefler, M., Lomin, S.N., Achazi, K., Romanov, G.A., and Schmülling, T. (2011) The specificity of cytokinin signalling in *Arabidopsis thaliana* is mediated by differing ligand affinities and expression profiles of the receptors. Plant J. **67**: 157-168.

Stone, S.L., Braybrook, S.A., Paula, S.L., Kwong, L.W., Meuser, J., Pelletier, J., Hsieh, T.F., Fischer, R.L., Goldberg, R.B., Harada, J. (2008) *Arabidopsis* LEAFY COTYLEDON2 induces maturation traits and auxin activity: implications for somatic embryogenesis. Proc. Nat. Acad. Sci. USA. **105**: 3151–3156.

Stone, B.B., Stowe-Evans, E.L., Harper, R.M., Celaya, R.B., Ljung, K., Sandberg, G., and Liscum, E. (2008) Disruptions in AUX1-dependent auxin influx alter hypocotyl phototropism in *Arabidopsis*. Mol Plant **1**: 129-144.

Sun, J., Hirose, N., Wang, X., Wen, P., Xue, L., Sakakibara, H., and Zuo, J. (2005) *Arabidopsis SOI33/AtENT8* gene encodes a putative equilibrative nucleoside transporter that is involved in cytokinin transport in planta. J. Integr. Plant Biol. **47**: 588-603.

Sun, J., Qi, L., Li, Y., Chu, J., Li, C. (2012) PIF4-mediated activation of *YUCCA8* expression integrates temperature into the auxin pathway in regulating *Arabidopsis* hypocotyl growth. PLoS Genet. **8**: e1002594.

Su, Y.H., Liu, Y.B., and Zhang, X.S. (2011) Auxin-cytokinin interaction regulates meristem development. Mol Plant **4**: 616-625.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H., and Mizuno, T. (2001) The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. Plant Cell Physiol **42**: 107-113.

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S., Levesque, M.P., Carrier, D., James, N., Calvo, V., Ljung, K., Kramer, E., Roberts, R., Graham, N., Marillonnet, S., Patel, K., Jones, J.D., Taylor, C.G., Schachtman, D.P., May, S., Sandberg, G., Benfey, P., Friml, J., Kerr, I., Beeckman, T., Laplaze, L., and Bennett, M.J. (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol **10**: 946-954.

Szemenyei, H., Hannon, M., Long, J.A. (2008) TOPLESS mediates auxin-dependent transcriptional repression during *Arabidopsi*s embryogenesis. Science **319**: 1384-1386.

Takei, K., Sakakibara, H., Sugiyama, T. (2001) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. J. Biol. Chem. **276**: 26405–26410.

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., Yamaya, T., Sakakibara, H. (2004a). *AtIPT*3, an Arabidopsis isopentenyltransferase gene, is a key determinant of macronutrientresponsive cytokinin biosynthesis. Plant Cell Physiol. **45**:1053–1062.

Takei, K., Yamaya, T., Sakakibara, H. (2004b). *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of trans-zeatin. J. Biol. Chem. **279**: 41866–41872.

Tanaka, H., Kitakura, S., De Rycke, R., De Groodt, R., and Friml, J. (2009) Fluorescence imaging-based screen identifies ARF GEF component of early endosomal trafficking. Curr. Biol. **19**: 391–397.

Tanaka, H., Kitakura, S., Rakusová, H., Uemura, T., Feraru, M.I., De Rycke, R., Robert, S., Kakimoto, T., and Friml, J. (2013) Cell polarity and patterning by PIN trafficking through early endosomal compartments in *Arabidopsis thaliana*. PLoS Genet. **9**: e1003540.

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

Tao, Y., Ferrer, J.L., Ljung, K., Pojer, F., Hong, F., Long, J.A., Li, L., Moreno, J.E., Bowman, M.E., Ivans, L.J., Cheng, Y., Lim, J., Zhao, Y., Ballaré, C.L., Sandberg, G., Noel, J.P., Chory, J. (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell. **133**: 164–176.

Taya, Y., Tanaka, Y., Nishimura S. (1978) 5'-AMP is a direct precursor of cytokinin in *Dictyostelium discoideum*. Nature **271**:545–547.

Tarkowski, P., Ge, L., Wan Hong Yong, J., Swee Ngin Tan. (2009) Analytical methods for cytokinins Trends in Analytical Chemistry, Vol. 28, No. 3.

Teh, O.K., and Moore, I. (2007) An ARF-GEF acting at the Golgi and in selective endocytosis in polarized plant cells. Nature **448**: 493–496.

Thimann, K.V., Koepfli, J.B. (1935) Identity of the growth-promoting and root-forming substances of plants. Nature **135**: 101.

To, J.P., Deruere, J., Maxwell, B.B., Morris, V.F., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2007) Cytokinin regulates type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay. Plant Cell **19**: 3901-3914.

Ueguchi, C., Sato, S., Kato, T., and Tabata, S. (2001) The AHK4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in *Arabidopsis thaliana*. Plant Cell Physiol **42**: 751-755.

Ulmasov, T., Hagen, G., Guilfoyle T.J. (1997) ARF1, a transcription factor that binds auxin response elements. Science **276**: 1865–1868.

Ulmasov, T., Murfett, J., Hagen, G., Guilfoyle, T.J. (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9**: 1963-1971.

Van Overbeek, J., Conklin, M.E., Blakeslee, A.F. (1941) Factors in coconut milk essential for growth and development of very young datura embryos. Science **94**: 350–351.

Vernoux, T., Brunoud, G., Farcot, E., Morin, V., Van den Daele, H., Legrand, J., Oliva, M., Das, P., Larrieu, A., Wells, D., Guédon, Y., Armitage, L., Picard, F., Guyomarch, S., Cellier, C., Parry, G., Koumproglou, R., Doonan, J.H., 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

Verrier, P.J., Bird, D., Burla, B., Dassa, E., Forestier, C., Geisler, M., Klein, M., Kolukisaoglu, U., Lee, Y., Martinoia, E., Murphy, A., Rea, P.A., Samuels, L., Schulz, B., Spalding, E.J., Yazaki, K., and Theodoulou, F.L. (2008) Plant ABC proteins--a unified nomenclature and updated inventory. Trends Plant Sci **13**: 151-159.

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

Voß, U., Bishopp, A., Farcot, E., Bennett, M.J. (2014) Modelling hormonal response and development. Trends Plant Sci. **19**:311-319.

Wang, C., Yan, X., Chen, Q., Jiang, N., Fu, W., Ma, B., Liu, J., Li, C.,Bednarek, S.Y., and Pan, J. (2013) Clathrin light chains regulate clathrin-mediated trafficking, auxin signaling, and development in *Arabidopsis*. Plant Cell **25**: 499–516.

Went, F. (1928) Wuchsstoff und Wachstum. Receuil Des Travaux Botaniques Neerlandais **25**: 1–116.

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

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmulling, T. (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15**: 2532-2550.

Wisniewska, J., Xu, J., Seifertova, D., Brewer, P.B., Ruzicka, K., Blilou, I., Rouquie, D., Benkova, E., Scheres, B., and Friml, J. (2006) Polar PIN localization directs auxin flow in plants. Science **312**: 883.

Won, C., Shen, X., Mashiguchi, K., Zheng, Z., Dai, X., Cheng, Y., Kasahara, H., Kamiya, Y., Chory, J., Zhao, Y. (2011) Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF ARABIDOPSIS and YUCCAs in *Arabidopsis*. Proc. Natl Acad. Sci. USA. **108**: 18518–18523.

Wulfetange, K., Lomin, S.N., Romanov, G.A., Stolz, A., Heyl, A., and Schmulling, T. (2011) The cytokinin receptors of *Arabidopsis* are located mainly to the endoplasmic reticulum. Plant Physiol **156**: 1808-1818.

Yamada, M., Greenham, K., Prigge, M.J., Jensen, P.J., and Estelle, M. (2009) The *TRANSPORT INHIBITOR RESPONSE2* gene is required for auxin synthesis and diverse aspects of plant development. Plant Physiol. **151**: 168–179.

Yang, H., and Murphy, A.S. (2009) Functional expression and characterization of *Arabidopsis* ABCB, AUX 1 and PIN auxin transporters in *Schizosaccharomyces pombe*. Plant J. **59**: 179–191.

Yang, Y., Hammes, U.Z., Taylor, C.G., Schachtman, D.P., and Nielsen, E. (2006) High-affinity auxin transport by the AUX1 influx carrier protein. Curr Biol **16**: 1123-1127.

Yoshida, S., Mandel, T., and Kuhlemeier, C. (2011) Stem cell activation by light guides plant organogenesis. Genes Dev **25**: 1439-1450.

Zažímalová, E., Krecek, P., Skupa, P., Hoyerova, K., and Petrasek, J. (2007) Polar transport of the plant hormone auxin - the role of PIN-FORMED (PIN) proteins. Cell Mol Life Sci **64**: 1621-1637.

Zhang, J., Nodzynski, T., Pencik, A., Rolcik, J., and Friml, J. (2010) PIN phosphorylation is sufficient to mediate PIN polarity and direct auxin transport. Proc Natl Acad Sci U S A **107**: 918-922.

Zhang, J., Vanneste, S., Brewer, P.B., Michniewicz, M., Grones, P., Kleine-Vehn, J., Löfke, C., Teichmann, T., Bielach, A., Cannoot, B., Hoyerová, K., Chen, X., Xue, H.W., Benková, E., Zažímalová, E., Friml, J. (2011). Inositol trisphosphate-induced Ca^{2+} signaling modulates auxin transport and PIN polarity. Dev. Cell **20**: 855–866**.**

Zhang, K., Novak, O., Wei, Z., Gou, M., Zhang, X., Yu, Y., Yang, H., Cai, Y., Strnad, M., Liu, C.J. (2014) *Arabidopsis* ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. Nat Commun.**5**: 3274.

Zhang, W., To, J.P., Cheng, C.Y., Eric Schaller, G., and Kieber, J.J. (2011). Type-A response regulators are required for proper root apical meristem function through the post-transcriptional regulation of PIN auxin efflux carriers. Plant J.

Zhao, Z., Andersen, S.U., Ljung, K., Dolezal, K., Miotk, A., Schultheiss, S.J., and Lohmann, J.U. (2010) Hormonal control of the shoot stem-cell niche. Nature **465**: 1089-1092.

Zourelidou, M., Absmanner, B., Weller, B., Barbosa, I.C., Willige, B.C., Fastner, A., Streit, V., Port, S.A., Colcombet, J., de la Fuente van Bentem, S., Hirt, H., Kuster, B., Schulze, W.X., Hammes, U.Z., Schwechheimer, C. (2014) Auxin efflux by PIN-FORMED proteins is activated by two different protein kinases, D6 PROTEIN KINASE and PINOID. Elife: 3.

2. Chapter 2

Transcriptome profiling reveals novel components of the auxin and cytokinin cross-talk at pericycle

Manuscript in preparation

Author contributions:
A.H. analyzed impact of auxin and cytokinin signaling components on the root system architecture, and contributed to functional characterization of genes synergistically regulated by auxin and cytokinin.

Transcriptome profiling reveals novel components of the auxin and cytokinin cross-talk at pericycle

Jerome Duclerque[1]*, Candela Cuesta[2]*, Andrej Hurný[2], Jakub Rolcik[3], Boris Parizot[1], Nicola Cavallari[2], Tom Beeckmann[1], Eva Benková[2]

[1]*Department of Plant Systems Biology, VIB, 9052 Gent, Belgium*
[2]*Institute of Science and Technology, Austria*, *Klosterneuburg*
[3]*Laboratory of Growth Regulators, Faculty of Science, Palacký University, and Institute of Experimental Botany, Academy of Sciences of the Czech Republic, CZ-78371 Olomouc, Czech Republic*

2.1 Introduction

The root as an underground organ is of vital importance for plant life. It anchors the plant body in the soil, responds to abiotic and biotic stresses, and is responsible for water and nutrients uptake. Exposed to a myriad of external signals, the root system constantly adjusts its development by modulation of root meristem activity, root elongation growth as well as branching pattern in order to optimize water and nutrient provision to the plant body (Lopez-Bucio et al., 2003). In particular, formation of lateral roots (LRs) is one of the key determinants of root architecture with an eminent impact on the efficiency of soil exploitation.

In *Arabidopsis thaliana*, LRs initiate from selected pericycle cells that acquire attributes of founder cells, subsequently undergo a series of anticlinal divisions, and produce a few short initial cells. After initiation, coordinated cell division and differentiation give rise to lateral root primordia (LRP) that continue to grow and emerge through the cortex and epidermal layers of the primary root; finally, the lateral root apical meristems take over growth control (Malamy and Benfey, 1997; Ötvös and Benková E., 2017).

Auxins and cytokinins are two classes of plant hormones that are key in the regulation of lateral root organogenesis. Auxin promotes the earliest events related to lateral root organogenesis, including priming, an accumulation of auxin in protoxylem cells (De Smet et al., 2007; Moreno Risueno et al., 2010), founder cells specification (Dubrovsky et al., 2008), initiation (Benková et al., 2003; Dubrovsky et al., 2008), as well as the later phases of primordia formation and emergence (Benková et al., 2003; Swarup et al., 2008). Major components of auxin signal transduction including the auxin receptors TIR1/AFBs, an F-box

proteins of a ubiquitin ligase complex, which upon auxin perception target the Aux/IAAs transcriptional repressors for degradation, thereby de-repressing AUXIN RESPONSE FACTORS (ARFs) transcription factors, have been implicated in lateral root organogenesis control (Dharmasiri et al., 2005; Kepinski and Leyser, 2005, Casimiro et al., 2001; Benkova et al., 2003; Peret et al., 2009). Mutations in genes encoding auxin receptors TIR1/AFBs, transcriptional regulators ARF7 and ARF19 as well as inhibition of auxin signaling as result of auxin repressors accumulation such as SLR/IAA14, IAA28, SHY2/IAA3, BDL/IAA12, MSG2/IAA19 and CRANE/IAA18 cause dramatic defects in LR initiation and development (Tian and Reed, 1999; Rogg and Bartel, 2001; Tatematsu et al., 2004; Uehara et al., 2008; De Rybel et al., 2010; De Smet et al., 2010). Critical for the auxin activity is the dynamic control of its distribution mediated through the polar auxin transport machinery. Families of AUXIN RESISTANT1 (AUX1)-like AUX1 (LAX) auxin influx carriers (Marchant et al., 1999), PIN-FORMED (PIN) auxin efflux carriers (Galweiller et al., 1998; Luschnig et al., 1998), and members of the multi-drug-resistant/ P-glycoprotein subfamily of ATP-binding cassette proteins are major components of polar auxin transport (Noh et al., 2001) and chemical or genetic interference with their activities dramatically compromises normal LR initiation and development (Vanneste and Friml, 2009; Casimiro et al., 2001; Benkova et al., 2003; Peret et al., 2009).

Besides auxin, several other plant hormones have been found to regulate LR organogenesis, among which cytokinin exhibits one of the strongest inhibitory effects (Li et al., 2006; Dello Ioio et al., 2007; Laplaze et al., 2007; Skylar and Wu, 2011). Increase in cytokinin activity, either by exogenous manipulation of cytokinin levels or by endogenous modulation of the activity of genes involved in cytokinin biosynthesis suppresses LR initiation and development (Li et al., 2006; Laplaze et al.,2007; Bielach et al., 2010). In contrast, transgenic plants overexpressing the C*YTOKININ OXIDASE/DEHYDROGENASE* (*CKX*), a gene coding for the enzyme that degrades cytokinin, exhibit increased LR number (Werner et al., 2003; Laplaze et al., 2007). Likewise, suppression of the cytokinin signaling pathway either by interfering with the receptor ARABIDOPSIS HISTIDINE KINASE4 (AHK4)/CYTOKININ RESISTANT1 (CRE1) and its homologues AHK2 and AHK3, or the positive components, the B-type ARABIDOPSIS RESPONSE REGULATORs (ARRs), typically enhance LR organogenesis (Higuchi et al., 2004; Riefler et al., 2006; Schaller et al., 2015; Mason et al., 2005).

The antagonistic roles of auxin and cytokinin in the control of lateral root organogenesis imply that their activities must be mutually tightly controlled and put special demands on the mechanisms that mediate their interaction. After a highly successful period of molecular explorations of individual signal transduction pathways (Kakimoto et al., 1996; Inoue et al., 2001; Kepinsky et al., 2005; Dharmasiri et al., 2005; To et al., 2007), the molecular bases of their interaction are studied intensively.

Recent works on how the auxin and cytokinin regulatory network is established and orchestrated revealed different types of molecular connections. Cytokinin has been found to stimulate auxin biosynthesis (Nordström et al., 2004; Jones et al., 2010), whereas auxin feedback on cytokinin biosynthesis might be mediated through direct transcriptional control of *ISOPENTENYL TRANSFERASE* (*IPT*) genes by the auxin response factor ARF19 (Yang et al., 2017). Auxin control over the transcription of cytokinin signaling repressors ARR7 and ARR15 appears to be critical for an establishment of the embryonic root pole (Müller and Sheen, 2008) and both auxin and cytokinin signaling pathways are interconnected via ARF5 mediated direct transcriptional control of CYTOKININ RESPONSE FACTOR 2 (CRF2) (Schlereth et al., 2010). Furthermore, cytokinin fine tunes auxin distribution via modulation of the polar auxin transport machinery. In the root apical meristem, the auxin-cytokinin circuit was found to be mediated through the AHK3 receptor and the downstream transcription factor ARR1 that adjusts the expression of the IAA3/SHORT HYPOCOTYL 2 (SHY2) auxin signaling repressor and attenuates the expression of several *PIN* genes as a consequence (Dello Ioio et al., 2008). Furthermore, CYTOKININ RESPONSE FACTORs (CRFs), transcription factors acting downstream of cytokinin perception, have been identified as direct transcriptional regulators of genes that encode *PIN* auxin transporters, thus revealing another molecular link between these two hormonal pathways (Šimášková et al., 2015). In addition to the transcriptional control, cytokinin selectively enhances the PIN vacuolar trafficking at specific polar domains, thereby directing auxin flow. This cytokinin-mediated polarization mechanism has been proposed to operate in developmental processes that depend on the rapid redirection of the auxin stream, including LR organogenesis (Marhavý et al., 2011, 2014).

Although there are several hints at the molecular nature of the auxin-cytokinin cross-talk (Chandler and Werr 2015; Schaller et al., 2015), our knowledge on the key players is still very limited. Here we used transcriptome profiling to uncover novel convergence points and molecular links between auxin and cytokinin that orchestrate establishment of root system architecture. Genome-wide monitoring of xylem pole pericycle gene expression patterns in

response to auxin, cytokinin and simultaneous auxin and cytokinin treatments led to the identification of potential cross-talk factors between these two hormonal pathways. We found that specific components of auxin and cytokinin metabolic, signaling and transport pathways are under tight control of their hormonal counterpart and thus mutually coordinate proper auxin – cytokinin readout at the xylem pole pericycle. Interestingly, despite well-established antagonistic functions of auxin and cytokinin in LR organogenesis, our study reveals a set of genes of which expression at the xylem pole pericycle is regulated by auxin and cytokinin in a synergistic manner and strictly depends on both hormones. Functional analysis confirmed the important role of this group of synergistically regulated genes in auxin – cytokinin regulated root system establishment.

2.2 Results

2.2.1 Cytokinin modulates oscillatory auxin responses at the priming zone

The root system architecture is a result of coordinated growth of the primary root that concurrently branches and forms new lateral organs. The pattern of branching (frequency of initiations of new lateral roots) is controlled at multiple levels including priming, founder cell specification, primordia formation and interaction with adjacent tissues (Ötvös and Benkova 2017). Among the earliest cues that define positioning of the incipient branching site, the regular oscillatory response of auxin in the root priming zone (basal meristem) has been recognized (De Smet et al, 2006; Moreno Risueno et al., 2010). In contrast, an increase of cytokinin in the basal meristem has been found to suppress frequency of lateral root initiation (Bielach et al., 2012). To explore whether and how cytokinin modulates the oscillating auxin response in the priming zone we monitored the dynamics of the auxin reporter *DR5:LUCIFERASE* expression in growing roots. Accordingly to previous reports we observed a pulses of the *DR5:LUCIFERASE* response with a period of 6h08 ± 0h35, which corresponds to 2.4 ± 0.5 pulses over a 20 hours (Fig. 1A and B, Table S1). A high exogenous cytokinin doses (50 to 100 nM N6-benzyladenine) led to the absence of DR5 pulses accompanied with a significant reduction of the luciferase signal (68 ± 4.22 and 53.14± 3.12 ADU $6min^{-1}$, respectively) compared to that of controls (122.45 ± 3.02 ADU $6min^{-1}$) (Fig. 1G and H, Table S1). Similarly, roots overexpressing the cytokinin biosynthetic enzyme ISOPENTENYL TRANSFERASE3 (*35S::IPT3*) displayed a weak luciferase signal (38.63 ± 1.45 ADU $6min^{-1}$, Fig. S1A and B, Table S1) with a dramatically reduced frequency of

DR5::LUCIFERASE pulses (0.21 ± 0.14 pulses/20h). To explore impact of reduced cytokinins levels on auxin response in the oscillatory zone we overexpressed the cytokinin-degrading enzyme *CK OXIDASE 3* (*35S::CKX3*) in wild-type plants. We observed a higher frequency of the *DR5::LUCIFERASE* pulses (5.84 ± 0.56 pulses /20h, Fig. S1C and D; Table S1) as well as an increased luciferase signal (146.98±3.99) when compared to control. Interestingly, treatments with low (0.1 and 1nM) cytokinin doses displayed a higher frequency of the *DR5::LUCIFERASE* pulses (period 1h57±0h22 and 3h±0h27, respectively), which corresponds to 6.67 ± 0.9 and 5.62 ± 0.48 pulses/20h, respectively (Fig. 1C and D, Table S1). A further increase of exogenous cytokinin levels to 10nM resulted in an overall decrease of the *DR5::LUCIFERASE* signal (75.86 ± 2.21, Table S1), and reduction of pulse frequency to 2.13 ± 0.35 pulses over 20-hours (Fig. 1E and F; Table S1).

These results suggest that cytokinin modulates oscillatory pattern of auxin response in a dosage dependent manner. Reduction of endogenous cytokinin levels increases both frequency and strength of the auxin response maximum. Low concentrations of cytokinin increase frequency of auxin maxima establishment, but reduce their amplitude, whereas high cytokinin attenuates both frequency as well strength of auxin maxima (Table S1).

To correlate observed cytokinin driven changes of the auxin oscillatory pattern with an impact on the root branching we scored LRP density in roots with varying cytokinin levels. We found that a higher frequency of oscillations caused either by reduced levels of endogenous cytokinin in *35S::CKX3* roots or by exogenous supply of low cytokinin dosage (1nM and 10nM) correlated with increased LRP density (Table S1). In contrast higher cytokinin concentrations, which suppressed both frequency and amplitude of auxin oscillations, correlated with a reduced LRP density, in accordance with the known role of cytokinin as an inhibitor of LRP initiation (Laplaze et al., 2007; Bielach et al., 2012). Noteworthy, despite dramatically reduced *DR5::LUC* signal in roots either exposed to 50 nM cytokinin or overexpressing *IPT3*, LRP could be detected, suggesting that very low levels of auxin might still be sufficient for LRP initiation.

These results point out that cytokinin might exhibit dual effects on LRP initiation, whereas at high levels cytokinin represses root branching, at low concentration it might promote LR initiation.

2.2.2 Identification of auxin and cytokinin regulated genes at the root pericycle

To uncover genes implied in the auxin-cytokinin cross-talk coordinating root branching, genome-wide transcriptome profiling was applied. We aimed to identify genes at whose transcriptional regulation auxin and cytokinin pathways converge during early lateral root organogenesis. For these purposes, we used conditions under which auxin (1μM 1-naphthaleneacetic acid; NAA) alone stimulated lateral root initiation, and when applied simultaneously with cytokinin (10μM), this stimulatory effect of auxin would be fully inhibited (Fig. 2A and S2A). To determine the most suitable duration of hormone treatment, quantitative (q)RT-PCR expression analyses on early auxin and cytokinin response genes of the *type-A ARR* and *AUX/IAA* families, respectively, were performed. Within 3 hours of treatment cytokinin significantly increased expression of *ARR3*, *ARR4* and *ARR5* cytokinin markers and this stimulatory effect was counteracted by simultaneous auxin application. Likewise, auxin stimulated transcription of *IAA3*, *IAA14* and *IAA19* was attenuated by cytokinin provision (Fig. 2B). In order to restrict the expression profiling to the xylem pole pericycle tissue, where LRP initiation takes place, we performed fluorescence activated cell sorting (FACS) using the xylem pole pericycle specific Gal4-GFP enhancer trap line J0121 (Laplaze et al., 2005, Fig. S2A). The labeled RNA was hybridized to the Arabidopsis Tiling 1.0R array (Affymetrix), which offers whole-genome coverage. We found that hormonal treatment affected 5313 genes representing approximately 19% of the genes present on the array. Precisely, 3173, 1454 and 3340 were significantly differentially regulated (Fold Change, FC>1.5 or <-1.5 and P<0.01) by auxin, cytokinin and both treatments, respectively (Fig. 2C). Furthermore, none of them could be rejected based on the calculated q-values (q≤0.05; http://genomics.princeton.edu/storeylab/qvalue/; Storey and Tibshirani, 2003). To get more information on the involvement of these gene pools, we used the ChipEnrich software which could identify statistically significant enriched Gene Ontology (GO) categories and biological processes from microarray analysis (http://www.arexdb.org/software.jsp; Orlando *et al*., 2008). The set of auxin-regulated genes displayed a strong enrichment in auxin signaling (P=7.86E^{-10}), in root morphogenesis (P=3.95E^{-06}), in lateral root formation (P=2.48E^{-47}) with a stronger enrichment in lateral root initiation process (P=1.58E^{-71}, Fig. 2G). The genes affected by cytokinin are also indicated to be involved in LR initiation (P=2.88E^{-07}), root morphogenesis (P=2.36E^{-06}) and in cytokinin signaling (P=8.31E^{-06}, Fig. 2G). When auxin and cytokinin are applied simultaneously, the

previous processes such as auxin signaling (P=6.38E^{-05}), cytokinin signaling (P=4.85E^{-06}) and LR initiation (P=6.18E^{-26}, Fig. 2G) are enriched but at a lower level. In contrast the double treatment affects more genes involved in root morphogenesis (P=3.46E^{-12}).

To validate the accuracy of the transcriptomic profiling obtained, we compared our list of auxin-regulated genes in pericycle cells to existing LR related microarray datasets using the VisuaLRTC spreadsheet (Parizot *et al*., 2010, Table S2, S3). Although the original set ups were different, we observed an overlap of 700 genes and 697 genes between our list and respectively the genes induced by auxin in the root segment (Vanneste et al., 2005) and those specifically involved in asymmetric cell division process which is crucial for proper LR organogenesis (De Smet et al., 2008). Interestingly, 391 genes overlapped between the three datasets with 98.21% of similar transcriptionally change (Fig. 2D, Table S2). In addition, the tilling array provided 649 genes affected by auxin (including 170 unknown proteins) as potentially new LRI components which are absent in the ATH1 microarray (Fig. 2D). Recently a list of 226 cytokinin-responsive genes was generated in a microarray meta-analysis performed through 13 ATH1 Affymetrix array experiments on several varieties of cytokinin-treated samples (Bhargava *et al*., 2013) and 573 cytokinin-regulated genes was identified by RNA-seq experiment (Bhargava *et al*., 2013). We compared these lists of cytokinin-regulated genes with our list of genes affected by cytokinin in the pericycle. We identify 63 and 119 genes overlapped, respectively, with the cytokinin-regulated genes identified by Arabidopsis Tiling 1.0R array. From them 49 genes were overlapped between the three datasets (Fig. 1E). As auxin and cytokinin co-treatment are usually used in callus induction of the shoot regeneration process which shares a common genetic control with LR initiation (Sugimoto *et al*., 2010; Duclercq *et al*., 2011), we compared our list of genes affected during the double treatment with genes affected during the early step of callus induction (Che et al., 2006; Xu et al., 2012). Interestingly, almost 50% of affected genes by double treatment (1760 genes) are also affected during callus induction (Fig. 1F). In addition due to the use of the Arabidopsis Tiling 1.0R array we identified 623 genes not present in the ATH1 platform and regulated when auxin and cytokinin were add simultaneously.

2.2.3 Auxin and cytokinin mutually modulate their activities in the pericycle.

Auxin and cytokinin metabolic, transport and signaling pathways are interconnected at multiple levels (Schaller et al., 2015). To identify convergence points of the complex auxin – cytokinin network that are specific to the xylem pole pericycle a genome wide profiling dataset was thoroughly inspected. The large scale comparison of expression patterns revealed that many genes implicated in auxin activity control are highly sensitive to cytokinin application and vice versa auxin significantly altered cytokinin regulatory genes when compared to untreated control (Figure S2B and S2C).

2.2.4 AHK2 and AHK3 mediated signaling regulates production of biologically active auxin.

To explore sensitivity of pathways that determine production of auxin and cytokinin hormonal fluctuations, expression profiles of genes acting in the major metabolic pathways were thoroughly scanned (Table S4, Ljung 2013; Kasahara 2016). Expression of genes involved in the biosynthesis of L-Tryptophan (L-Trp), a major precursor of auxin biosynthesis in plants, was largely unchanged by auxin and cytokinin (Fig. 3A). Several components of the indole-3-pyruvic acid (IPyA) pathway such as *TAR2* and *YUC5*, *YUC6*, *YUC8* and *YUC9* were strongly attenuated by auxin, indicating negative feedback triggered by excess of auxin, whereas upregulation of *YUC7* expression by cytokinin might contribute to increase of auxin production (Fig. 3A, Table S4).

When compared to the IPyA pathway expression of genes involved in the indole-3-acetaldoxime (IAOx) pathway, which mediates conversion of IAOx to auxin were more sensitive to hormonal perturbations. Expression of the cytochrome P450 monooxygenases *CYP79B2* and *CYP79B3*, which catalyze conversion of L-Trp to the indole-3-acetaldoxime (IAOx), the common precursor for camalexin (CAM), indole glucosinolates (IGs) and IAA were significantly enhanced by cytokinin, but attenuated by auxin (Fig. 3A; S3A; Table S4). Expression of downstream components involved in IAOx conversion to either IGs or indole-3-acetonitrile (IAN) and further to CAM and IAA was also differentially regulated by auxin and cytokinin (Fig. 3A, Table S4). Unlike auxin, cytokinin promoted expression of genes involved in IAOx to IGs, and IAN to IAA conversion, but attenuated those involved in conversion of IAN to CAM (Fig. 3A, Table S4).

Essential negative regulatory feedback of auxin on its own activity is linked with pathways controlling auxin inactivation via conjugation with amino acids. Expression of several *GH3* genes, which encode IAA–amino synthase catalyzing conversion of free IAA to IAA-amino acid conjugates, was strongly upregulated by auxin (e.g. *GH3.3* induced with a fold change of 9.06), whereas specific IAA-amino acid conjugate hydrolases which release free, active IAA were repressed by auxin (e.g. ILL6 decreased with a fold change of -2.70; Fig. 3A, Table S4). In contrast, cytokinin attenuated expression of several genes involved in auxin deactivation either via methyl-esterification or conjugation of IAA with amino acids (Fig. 3A, Table S4).

Altogether this thorough analysis of auxin metabolic pathways and their sensitivity to hormonal treatments strongly suggests that cytokinin contributes to the maintenance of biologically active auxin levels in the root. On one side cytokinin upregulates several genes of IPyA and IAAox pathways, two major branches of auxin biosynthesis, and on the other it significantly attenuates expression of genes acting in auxin conjugation pathways.

To assess the impact of cytokinin on auxin metabolism, levels of free IAA and two major auxin conjugates IAA-glu and IAA-asp were measured in roots of cytokinin receptor mutants including *ahk2*, *ahk3*, *cre1* and higher order *ahk2ahk3*, *ahk2cre1*, *ahk3cre1* mutants. Lack of the AHK3 receptor activity in *ahk3* single as well as *ahk2ahk3* and *ahk3cre1* multiple mutants correlated with significant reduction of auxin levels in roots supporting a positive role of cytokinin in auxin biosynthesis (Fig. 3B). On the other hand lack of the AHK2 activity in single *ahk2* single as well as *ahk2ahk3* and *ahk2cre1* multiple mutants led to significant increases of IAA-asp and IAA-glu conjugates (Fig. 3C, D). Unlike AHK2 and AHK3, loss of the CRE1/AHK4 activity did not significantly affect levels of either free auxin or its conjugates. Moreover, no recovery of auxin could be detected in *ahk3cre1* and *ahk2cre1*, when compared to *ahk3* and *ahk2*, respectively, suggesting that AHK4/CRE1 might not act as an antagonist of either AHK2 or AHK3 in the regulation of auxin metabolism by cytokinin (Fig. 3B-D). Altogether these results strongly support a role of cytokinins in auxin metabolism control and suggest that specific branches of the signaling might fine tune particular auxin metabolic pathways. The AHK3 mediated branch of cytokinin signaling contributes to the regulation of auxin biosynthesis and maintaining overall levels of auxin, whereas AHK2 dependent signaling seems to balance the ratio between free and conjugated IAA derivatives.

2.2.5 Auxin targets cytokinin biosynthesis and deactivation pathways to fine tune cytokinin levels in the pericycle.

In *Arabidopsis*, cytokinin is biosynthesized through the activity of adenosine phosphate-isopentenyl transferase (IPT) (Sakakibara, 2006). Several *IPT* genes, such as *IPT1*, *IPT5* and *IPT7* were downregulated either by cytokinin or auxin, in accordance with previous observations (Miyawaki *et al*., 2004) (Fig. S3C, Table S5). In contrast, auxin and cytokinin differentially regulate expression of *CYP735A1* and *CYP735A2*, which encode enzymes converting iP-nucleotide to tZ-nucleotide (Sakakibara, 2006). Whereas cytokinin enhances, auxin suppresses expression of these genes (Fig. S3C, Table S5). Previously, this antagonistic regulation has been observed in roots (Takei et al., 2004) and indicates that also in pericycle auxin and cytokinin activities converge on fine tuning levels of specific cytokinin derivatives. Expression of two homologues of *LONELY GUY* (*LOG*) family, *LOG1* and *LOG9*, encoding cytokinin-activating enzymes (Kuroha et al., 2009) was significantly reduced by auxin (Fig. S3C, Table S5). CYTOKININ OXIDASE (CKX)-mediated degradation is essential catabolic pathway for irreversible inactivation of cytokinins (Werner et al., 2003). Our transcriptome profiling data reveal that both auxin and cytokinin modulate expression of these genes in the pericycle. Whereas cytokinin enhances expression of *CKX3* and *CKX5*, thereby strengthening negative regulatory feedback on its own production (Fig. S3B, S3C, Table S5), *CKX4,* another family member, is downregulated by both auxin and cytokinin (Fig. 3B, Table S5). Furthermore, in the pericycle both hormones might adjust the level of active cytokinin via control of *ADENINE PHOSPHORIBOSYL TRANSFERASE* (*APT2*, *APT4, APT5*) gene expression, encoding for enzymes deactivating cytokinins by their conversion from free bases to nucleotide (Zhang et al., 2013, Fig. S3C, Table S5).

Detailed analysis of the transcriptome shows that in the xylem pole pericycle auxin and cytokinin metabolic pathways rapidly react to hormonal fluctuations. As expected, under excess of either auxin or cytokinins homeostatic mechanisms including suppression of their own biosynthesis and upregulation of pathways that deactivate hormone activity via degradation and conjugation are triggered (Nordström et al., 2004; Jones et al, 2010). Besides activation of mechanisms that maintain levels of respective hormones at physiological levels, both auxin and cytokinin metabolic pathways are tightly interconnected and rapidly sense availability of their hormonal counterpart. Cytokinin via enhanced expression of biosynthetic genes and suppression of these controlling auxin conjugations might effectively modulate levels of biologically active auxin in the pericycle. Measurements of auxin metabolites in

roots of three AHK receptor mutants support this positive role of cytokinin in auxin metabolism and suggest that specific branches of cytokinin signaling might fine tune distinct auxin metabolic pathways. In contrast, auxin via attenuation of several *IPTs* and *LOG* genes can reduce production of active cytokinin derivatives, although the stimulatory effect on genes involved in cytokinin conjugation/deactivation provides support also for its feedback on pathways that control balance between biologically active and non-active cytokinin metabolites.

2.2.6 Expression of negative feedback-loop components of auxin and cytokinin signaling is sensitive to hormonal perturbations.

To dissect components of auxin and cytokinin signaling that might mediate interaction between the two pathways in the xylem pole pericycle, expression of the core signaling cascade genes in response to hormonal treatments was evaluated. Expression of auxin (*TIR1*/*AFBs*) and cytokinin (*AHKs*) receptors was largely insensitive to any hormonal perturbations (Table S4, S5). Likewise, transcription of *ARFs* and *type-B ARRs* transcription factors, which mediate signaling downstream of the respective receptors were mostly unresponsive to treatments with their hormonal counterparts (Table S4, S5).

In contrast, genes that provide negative feedback on signal transduction cascades such as *Aux/IAAs* for auxin and *type-A ARR* and *AHP6* for cytokinin pathway, sensitively responded to the hormones. In accordance with previous reports, auxin and cytokinin enhanced expression of number of *Aux/IAAs* and *type-A ARR* genes, respectively (Abel et al, 1996; Kieber and Schaller 2014). In the pericycle, *IAA6*, *IAA29*, *IAA5*, *IAA30*, *IAA20* and *IAA1* were among the most responsive to auxin treatment, whereas *ARR16*, *ARR7*, *ARR15* and *ARR17* exhibited the highest responsiveness to cytokinin (Table S4, S5). Interestingly, several *Aux/IAAs* and *type-A ARR* genes showed also significant sensitivity to the treatment with their hormonal counterparts. Cytokinin enhanced expression of *IAA5*, *IAA31*, *IAA3*, whereas that of *IAA14* was attenuated (Table S4). Transcriptome profile of auxin treated pericycle confirmed previous finding on the auxin sensitive expression of *AHP6* (Mähönen et al., 2006) and revealed that several type-A response regulators including *ARR6*, *ARR17*, *ARR5* were down- whereas *ARR7* was up-regulated by auxin (Table S5). To validate these transcriptome based expression profiles *GUS* reporters for selected *Aux/IAA* and *ARR* genes were employed. Expression of *IAA14::GUS*, *IAA19::GUS*, *IAA28::GUS*, *ARR7::GUS*, *ARR5::GUS*, *ARR16::GUS*, *ARR15::GUS* and *ARR12::GUS* in response to hormonal

treatments largely confirmed patterns detected by transcriptome profiling (Fig. 4A, Fig. S4A, Table S4 and S5).

Altogether these results suggest that in the xylem pole pericycle, auxin and cytokinin signaling pathways might mutually modulate their activities via rapid transcriptional regulation of components that provide negative feedback on the respective signal transduction cascades.

2.2.7 Interplay of auxin and cytokinin signaling components in the pericycle determines root branching pattern.

To assess contribution of the auxin and cytokinin signaling components to the lateral root organogenesis, in particular of these whose expressions in the pericycle is under control of their hormonal counterparts, we analyzed the respective mutant lines. In the selected *Aux/IAAs* (*iaa5, iaa30, iaa6, iaa29, iaa12, iaa14, iaa17, and iaa19*) and *type-A ARR* loss of function mutants (*arr3*, *arr4*, *arr5*, *arr6*, *arr7*, *arr9*, *arr15*, *arr16*) neither LRP density nor root growth were significantly affected, presumably due to their functional redundancies (Fig. 4B, S4B). Higher order *type-A arr3,4,5,6,8,9* mutant exhibited reduced LRP density, which is an expected output of an enhanced cytokinin signaling due to attenuated activity of multiple *type-A ARR* repressors (Fig. 4B and Fig. S4B).

To examine function of the cytokinin and auxin regulated *AUX/IAAs* and *ARRs* in the cytokinin regulated root branching, respective mutants were grown on media supplemented with cytokinin. Whereas the LRP density in most of tested *type-A ARR* mutants was not significantly altered (Fig. 4C), a higher order *arr3,4,5,6,8,9* and *arr5* mutants exhibited enhanced sensitivity to cytokinin inhibition in line with their function of cytokinin signaling repressors (Fig. 4C, S4C). In contrast, *iaa5*, *iaa30, iaa14*, *iaa29* and *iaa6, iaa19* loss of function mutants initiated more LRP at inhibitory concentrations of cytokinin compared to the wild type control, suggesting that attenuation of the negative feedback on auxin signaling is able to counterbalance cytokinin effect on the LRP initiation (Fig. 4C, S4C).

As expected, interference with some type-B ARR transcriptional regulators, namely *ARR1*2 and *ARR14*, reduced sensitivity of LRP initiation to cytokinin, in line with their positive role in the signal transfer (Fig. 4C, S4C). Surprisingly, lack of the ARR1, ARR10 and ARR11 function resulted in oversensitivity of the LRP initiation to cytokinin (Fig. 4C and S4C), suggesting that downstream components of the cytokinin signaling cascade might

have distinct functions in the regulation of the LR initiation. To explore whether observed cytokinin sensitivity differences between two subgroups of *type-B arr* mutants (type-B1 including ARR1, ARR10 and ARR11 and type-B2 - ARR2, ARR12, ARR14) might result from perturbed interaction with auxin signaling we analyzed expression of *AUX/IAAs* in respective mutants. QRT-PCR analysis revealed that in the cytokinin oversensitive *type-B1 arr1*, and *arr10* mutants an expression of multiple *AUX/IAA* genes was reduced in roots exposed to cytokinin when compared to wild type control (Fig. 5A, 5B). On the other hand expression of tested *Aux/IAAs* was either not changed or enhanced in subgroup of *type-B2 arr* (*arr12* and *arr14*) mutants that exhibit reduced cytokinin sensitivity (Fig. 5A, 5B). To confirm observed *AUX/IAAs* expression differences between two mutant subgroups we examined expression of *IAA14*, a potent repressor of the LRP initiation (Fukaki et al., 2002) using *IAA14::GUS* reporter introduced into *arr10* and *arr12* (Fig. S5A). In accordance with transcriptome profiling data, cytokinin reduced expression of *IAA14::GUS* in the central cylinder of wild-type roots, and at the root tip a modest increase of *GUS* signal was detected (Table S4, Fig. 4A and S5A, S5B). Loss of the ARR10 function interfered with cytokinin induced expression of *IAA14::GUS,* whereas in the *arr12* overall increase of the *IAA14* reporter signal was observed irrespective of the cytokinin provision (Fig. S5A, S5B). Hence, expression analysis of the IAA14 reporter supports our findings that individual type-B ARRs might differ in interaction with auxin pathway.

Typically accumulation of Aux/IAAs including IAA14 leads to strong suppression of the LRP initiation (Fukaki et al., 2002; Vanneste at al., 2005; De Smet et al., 2010). However, oversensitivity of LRP initiation to cytokinin in the subgroup of *type-B1 arr* mutants correlates with reduced expression *Aux/IAAs* (Fig. 5A, 5B; S5A-C). To examine developmental relevance of the crosstalk between ARR10/ARR12 and IAA14, multiple *iaa14arr10; iaa14arr1arr10* and *iaa14arr12* mutants were analyzed. Lack of the *IAA14* auxin repressor in *arr10*, *arr1arr10* as well as *arr12* reduced sensitivity of LRP initiation to cytokinin (Fig. 4C).

Although both type-B1 and -B2 ARR subgroups differ in levels of *Aux/IAAs* expression, attenuation of the auxin negative feedback loop e.g. by limiting *IAA14* expression, can reduce sensitivity to cytokinin in both these subgroups. Hence, oversensitivity of the LRP initiation to cytokinin in the *type-B1 arr* mutants does not seem to be a consequence of the accumulation of specific AUX/IAAs, but might be result of the overall drop in auxin activity, for example via reduction of auxin production, which is manifested by low expression of *AUX/IAAs.*

2.2.8 Transcriptome profiling supports a role of cytokinin in the regulation of polar auxin transport.

Biologically important aspect of auxin and cytokinin cross-talk involves cytokinin regulation of the polar auxin transport (Adamovski and Friml, 2015). In the pericycle, exogenous auxin application enhanced expression of some auxin efflux carriers such as *PIN1* (FC=2.63) and *PIN7* (FC=1.43) whereas expression of *ABCBs/PGPs*, *PILS* or auxin influx carriers such as *AUX1* or *LAX3* were not dramatically affected (Table S4). In response to cytokinin no significant changes in the expression of auxin transport related genes could be detected. However, unlike modest impact of auxin and cytokinin on transcription of auxin transporters, both hormones altered significantly expression of factors controlling subcellular trafficking and membrane localization of PIN auxin transporters such as *PINOID* (*PID1*), *WAG1* (Friml et al., 2004), *NPY1*, *NPY4*, *NPY5* (Chen et al., 2007, 2008), *PBP1* (Benjamins et al., 2003) (Table S4). This is largely in accordance with recent findings demonstrating that important part of auxin and cytokinin feedback on polar auxin transport might involve regulation of the PIN subcellular trafficking (Paciorek et al., 2005; Marhavy et al., 2011; 2014).

2.2.9 Transcriptome profiling reveals common targets of auxin and cytokinin in the pericycle.

Besides genes related to the auxin and cytokinin pathways thorough analysis of the transcriptome profile led to identification of unknown potential targets of both hormonal pathways. Among 5313 genes perturbed by at least one of the hormonal applications (fold change (FC) >1.5 or <-1.5 and P<0.01) we found 1205, 347 and 896 genes responsive specifically to auxin, cytokinin or combinatorial treatment, respectively. Expression of 1,868 genes was altered by both auxin and combinatorial hormonal treatment, and 1005 genes changed their expression after cytokinin as well as the double treatment (Fig. 2C). Comparison of the gene expression profiles in response to individual (either auxin or cytokinin) versus combinatorial treatment by both hormones led to identification of two main gene clusters – genes regulated in an additive and a non-additive manner. As an additive we considered genes whose transcriptional change by simultaneous hormonal treatment is equal to the sum of changes caused by individual hormones. Non-additive regulation is considered when the change in gene expression by combined application of hormones differs

significantly from additive (sum) effects of the individual hormonal treatments. Our transcriptome profiling revealed 1199 genes, which were regulated by auxin and cytokinin in a non-additive manner (ratio between expected and observed additive values >1.5 or <0.5), thus being strong candidates for molecular components of the auxin and cytokinin interaction (Table S6). To examine a developmental relevance of genes at whose transcriptional regulation both hormonal pathways might converge, we selected candidate genes for detailed functional analyses. We focused on two specific subgroups of non - additively controlled genes exhibiting either synergistic positive or synergistic negative expression pattern in response to simultaneous auxin and cytokinin treatment. In these groups the genes are significantly up-/down-regulated by simultaneous hormonal treatment when compared to the expected additive effect of both hormones separately (Fig. 6A, Fig. S6A). The expression pattern of selected candidates was validated using qRT-PCR (Fig. 6A and S6A). Expression of two highly synergistically regulated genes (*At1G15590* and *At1G15600*) was evaluated using transcriptional reporter constructs. In roots of both *At1G15600p::GUS* well as well as *AtG15590p::GUS* transgenic seedlings strong staining of GUS reporter was detected after simultaneous hormonal treatment when compared to control, auxin or cytokinin treatments (Fig. 6B, 6C). Importantly, the expression analysis of candidate genes in planta confirmed that activity of both hormones auxin and cytokinin is strictly required to trigger transcription in the same tissues and synergism is not output of hormonal effects in the distinct cell types.

To gain more insights into the function of these genes in the auxin-cytokinin crosstalk and in regulation of the root system architecture, we assessed a root growth and branching in the respective mutants. Whereas in most of the mutants primary roots growth on media with and without hormonal supply was largely unaffected (Fig. 6D, S6B) root branching particularly in roots exposed to hormones were significantly altered in large proportion of tested mutants when compared to wild type seedlings (Fig. 6E, S6C). Mutations in several genes synergistically up-regulated by auxin and cytokinin led to reduced sensitivity of LR initiation to cytokinin inhibition (*AT1G15600*; *AT1g02389*; *AT4G32300*; *AT2G19410*; *AT3G13080*) and mutations in genes including *AT1G49560*, *At1G18870*, *AT1G17430*, *ATG243140* altered sensitivity of LR initiation to combinatorial auxin and cytokinin supply (Fig. 6E, S6C). Altogether these results indicate that auxin cytokinin regulated root system architecture at molecular levels might not necessarily be outcome of mutual antagonisms of these two hormonal pathways. Striking alterations of root system architecture caused by loss of functions in genes controlled by both hormones, in particular when exposed to hormonal

perturbations, indicate that these genes might balance inputs from both auxin and cytokinin to ensure their proper developmental readout.

2.3 Discussion

Iterative formation of lateral root organs from the primary root is under the tight control of auxin and cytokinin pathways which are wired via multiple levels of interactions (Vanstraelen and Benkova, 2012). Although their mutual antagonism in control of lateral root organogenesis is well established, the molecular mechanisms underlying auxin – cytokinin interactions are scarcely understood so far. To uncover genes implied in the auxin – cytokinin cross-talk, which determine the architecture of the root system, a transcriptome profiling approach was applied. Genome wide expression profiling revealed a number of xylem pole pericycle genes responsive to auxin and cytokinin perturbations. Among genes regulated by auxin and cytokinin core components of pathways that determine their own outputs at the level of metabolism, transport and signaling, as well as genes that might be important integrators of hormonal signals from both pathways were recognized.

Our transcriptome profiling confirmed that some previously recognized general regulatory mechanisms activated by auxin and cytokinin might also act at the pericycle. In agreement with other studies, excess of auxin as well as that of cytokinins was found to trigger a negative feedback to suppress their own production (Nordström et al., 2004; Jones et al, 2010). Accordingly, auxin and cytokinin impact on components, which control posttranslational modifications and polarity establishment of PIN auxin efflux transporters such as PID1 and WAG, in line with recent findings on the role of both hormones in fine tuning polar auxin transport (Fiml et al., 2004; Paciorek et al., 2005; Marhavy et al, 2011). In addition, detailed analysis of core components of auxin and cytokinin pathways and their sensitivities to their respective hormonal counterparts hint at several specific convergence points that balance auxin – cytokinin readouts at the pericycle. Our data indicate that cytokinin is involved in maintaining levels of auxin via distinct steps of its metabolism. Cytokinin promotes expression of *YUCCA7* acting in the indole-3-pyruvic acid (IPyA) pathway, a major tryptophan-dependent IAA biosynthesis pathway (Ljung et al., 2013) and in agreement with previous report (Jones et al., 2010) genes of IAOx pathway cytochrome P450 monooxygenases *(CYP79B2* and *CYP79B3),* which catalyze the conversion of L-Trp to indole 3-acetaldoxime (IAOx) as well as several *NITRILASE* (*NIT*) gene family members,

which downstream of IAOx convert indole-3-acetonitrile (IAN) to auxin are enhanced by cytokinin. Furthermore, downregulation of several *GH3* family members points out that cytokinin might also manage a pool of active auxin by attenuating its conjugation with amino acids. Reduced levels of free auxin and accumulation of auxin conjugates in cytokinin receptor mutants strongly corroborate the role of cytokinin in auxin production via fine tuning activities of both auxin biosynthesis as well as auxin-conjugation pathways. Noteworthy, reduced levels of free auxin and accumulation of auxin conjugates, which correlates with lack of the AHK3 and AHK2 cytokinin receptor activities, respectively, suggest that distinct branches of cytokinin signaling might target specific pathways of auxin metabolism. In addition, analysis of the type-B ARRs, which transduce cytokinin signal downstream of the receptors, supports regulation of auxin activity by distinct branches of cytokinin signaling. Two subgroups - type-B1 and -B2 ARRs were recognized based on their inputs into the cytokinin regulated lateral root initiation. Whereas lack of the type-B1 ARRs (including ARR1, ARR10 and ARR11) correlated with increased sensitivity of root branching to cytokinin inhibition, mutations in type-B2 ARRs (including ARR12 and ARR14) attenuated this cytokinin inhibitory effect. Unexpectedly, cytokinin oversensitive type-B1 *arr* mutants are characterized by overall reduction of *Aux/IAAs* expression, unlike type-B2 ARR mutants in which higher levels of *Aux/IAAs* transcripts were detected. As typically accumulation of Aux/IAA auxin repressors correlates with inhibition of lateral root initiation (Fukaki et al., 2002; Vanneste et al., 2005), we hypothesize that low *Aux/IAAs* expression detected in *type-B1 arrs* mutants might be related to the feedback on the auxin activity that presumably involves regulation of auxin metabolism. In such a scenario type-B1 ARRs, together with AHK2 and AHK3, would be involved in maintaining auxin levels, and lack of their activity is manifested by overall reduction of the *Aux/IAAs* expression due to reduced auxin production. On the other hand previous studies have shown that AHK4/CRE1 along with ARR2 and ARR12 are involved in cytokinin triggered lytic degradation of PIN1 auxin efflux carrier, and that in *ahk4/cre*, *arr2* and *arr12* mutants PIN1 is largely insensitive to this cytokinin effect (Marhavy et al., 2011). In contrast, cytokinin promoted PIN1 lytic degradation is unaffected in *ahk2*, *ahk3* as well as type-B1 mutants (Marhavy et al., 2011). Altogether our and previously published data suggest that distinct branches of cytokinin signaling might regulate specific auxin pathways. We propose that AHK2 and AHK3 along with type-B1 ARRs fine tune levels of the biologically active auxin via modulation of auxin biosynthesis and its conjugation, whereas the AHK4 mediated branch of cytokinin signaling

together with type-B2 ARRs determines the capacity of polar auxin transport and thereby auxin distribution.

We hypothesize that the concerted action of these distinct cytokinin transduction pathways might contribute to the dosage dependent effects of cytokinin on the auxin oscillatory pattern and consequently on the LR initiation pattern. At low cytokinin concentrations AHK2 and AHK3 dependent pathways might be activated and promote auxin biosynthesis thereby enhancing LR initiation. With increasing cytokinin levels AHK4 and type-B2 ARRs mediated signaling attenuates auxin transport and consequently LR initiation is reduced.

Aside from genes of auxin and cytokinin pathways which might mutually balance each other's readouts, our transcriptome profiling revealed novel potential components of auxin and cytokinin cross-talk. Subgroups of genes whose expression either requires input from both auxin and cytokinin pathways, or in response to auxin plus cytokinin supply their expression excesses additive effects of individual hormonal treatments might represent important convergence points acting downstream of auxin and cytokinin signaling. Importantly, as confirmed by expression analysis in tissues using *GUS* reporters, the non-additive response is a result of auxin and cytokinin activity interplay in the same tissue and not an output of spatially distinct events. Among genes non-additively regulated by auxin and cytokinin, synergistically up- and down-regulated gene clusters have been identified. Several candidates from each cluster encompassing genes of unknown function (*AT1G15600*, *AT1G15590*, *AT1G02380*, *AT4G32300*, *AT1G17430*), transcriptional regulators and chromatin modifiers (*AT1G49560*, *AT2G43140*, *AT2G35270, AT2G28200*), kinases (*AT1G64080*, *AT2G19410*), genes involved in cell wall modifications (*AT1G02460*, *AT4G14130*, *AT5G23210*), transport (*AT4G25640*, *AT3G13080*), plant metabolism or other functions (*AT1G18870*, *AT3G47470*) have been selected for the functional analyses. Detailed phenotype analysis of candidates revealed that mutations interfering with their function significantly affect establishment of the root system architecture under hormonal perturbations. Interestingly, some of the synergistically regulated genes tested by auxin and cytokinin have been recently linked with function in root adaptive responses to nutrient availability (*AT1G49560;* Nagarajan et al., 2016; Medici et al., 2015), environmental stresses caused by heavy metals including cadmium (*AT3G13080*, Brunetti et al., 2015), aluminum (*AT4G1413,* Zhu et al., 2013) and other environmental stresses (*AT4G25640*, Zhang et al., 2017), as well as in the regulation of hormonal pathways involved in stress responses such as

salicylic acid (*AT1G18870*, Garcion et al., 2008) or abscisic acid (*AT2G43140,* Tian et al., 2015). Hence we hypothesize, that auxin and cytokinin pathways converging at the common downstream targets might represent important regulatory modules for rapid integration of environmental signals and flexible adaption of the root system architecture.

2.4 Material and methods

Plant material and growth conditions

The transgenic *Arabidopsis thaliana* (L.) Heynh. lines have been described elsewhere: *pIAA14::GUS* (Vanneste et al.,2005), *IAA19::GUS* (Tatematsu et al., 2004), *IAA28::GUS* (De Rybel et al., 2010), *DR5:LUCIFERASE* (Moreno-Risueno et al., 2010), *CYC1;1B::GUS* (Ferreira et al., 1994) *arr1-2*, *arr1-4*, *arr2-4*, *arr10-1*, *arr11-1*, *arr11-2*, *arr12-1* (Mason et al., 2005), *arr2-1* (Marhavy et al., 2011), *arr3-1*, *arr4-1*, *arr5-1*, *arr6-1*, *arr9-1, arr3,4,5,6,8,9* (To et al., 2004), *axr2-1/iaa7* (Nagpal et al., 2000), *cre1-12*; *ahk2-2*; *ahk3-3*; *cre1-12ahk2-2*; *cre1-12ahk3-3*; *ahk2-2,ahk3-3* (Higuchi et al., 2004); *axr3-1/iaa17* (Leyeser et al., 1996), *axr5-1/iaa1* (Yang et al., 2004), *bdl-1* (De Smet et al., 2010), *slr- 1*(Fukaki et al., 2002), *msg2-1* (Tatematsu et al., 2004), *iaa4-1*, *iaa5-1*, *iaa6-1*(Overvoorde et al., 2005), *iaa28-1*(De Rybel et al., 2010) and *arr7* (N858131), *arr10-5* (N39989), *arr13-1* (N655053), *arr14-1* (N875481), *arr15-1* (N411750), *arr16-1* (N873779), *iaa12-1* (N25213), *iaa14-1* (N25214), *iaa17-1* (SALK_065697C), *iaa19-1* (N655206), *iaa20-1* (SALK_017453), *iaa28-2* (N669043), *iaa29-1* (SALK_152235), *AT1G02380* (Salk_129654), *AT1G02460* (Salk_093618), *AT1G15600* (Salk_151420), *AT1G17430* (Salk_042510), *AT1G18870* (Salk_073287), *AT1G49560* (Salk_095775), *AT1G64080* (Salk_070770), *AT2G19410* (Salk_140776), *AT2G28200* (Salk_137213), *AT2G35270* (Salk_094394), *AT2G43140* (Salk_123812), *AT3G13080* (Salk_044022), *AT3G47470* (Salk_138555), *AT4G14130* (Salk_039464), *AT4G25640* (Salk_057798), *AT4G32300* (Salk_105027), *AT5G23210* (Salk_053542) *ARR7::GUS*, *ARR16::GUS*, *ARR5::GUS*, *ARR12::GUS* were obtained from the European Arabidopsis Stock Centre (NASC). Primers used for the genotyping and to quantify gene expression levels are listed in Table S8 – S9. Knock out lines of *arr* mutants were confirmed by RT-PCR (Table S10). Seeds were sterilized with chloral gas, sown in Petri dishes on 0.8 per cent agar with 1 per cent sucrose-containing 0.5 Murashige and Skoog (MS) medium, stored for 2 days at 4°C, and grown on vertically oriented plates in growth chambers under a 16-h-light/8-h-dark photoperiod at 18°C. Seven days after germination,

seedlings were harvested and processed. All cloning procedure was conducted by using Gateway™ (Invitrogen) technology; with the sequences of all used vectors available online (https://gateway.psb.ugent.be/).

For promoter analyses of *At1G15600* and *AT1G15590,* an upstream sequence of 2522bp and 876bp, respectively, were amplified by PCR and introduced into the pDONRP4-P1R entry vector.

Luciferase activity imaging and data processing

7 days old seedlings were sprayed with a 5mM potassium luciferine (Sigma, CAS Number: 50227, D-Luciferin potassium salt) and then imaged using the Lumazone platform from Photometrics (Dark Box Type 4). Images of seedlings were taken with a CCD camera (PIXIS: 1024B Digital Charge-Coupled-Device (CCD) Camera System) from Princeton Instruments plus imaging lens of 50 mm, which we complemented with 1 close-up lens. Luciferase expression movies were made by acquiring consecutive chemiluminescence images (6 minute exposure) for 20 hours and then combining the frames into a movie using WinView software. Luciferase was measured by selecting the region of interest and quantifying the analog-digital units (ADU) per pixel using the IMAGEJ software (http://rsbweb.nih.gov/ij/).

Analyses of LRP initiation cytokinin sensitivity

Mutants and control seedlings were grown on 0.5 MS medium without or supplemented with cytokinin - 0.1 nM to 0.1 mM 6-Benzyladenine, and auxin - 1μM 1-naphthaleneacetic acid (NAA). Seven days after germination, the plant material was cleared as described (Malamy and Benfey et al., 1999) and total number of lateral root primordia per cm of root length evaluated. Root lengths were measured with the IMAGEJ software (http://rsbweb.nih.gov/ij/). Lateral root primordia were counted with a differential interference contrast microscope BX51 (Olympus). At least 20 seedlings were analysed and the experiments were repeated twice independently. For the statistical evaluation, the *t*-test was done with the EXCEL statistical package.

Plant Protoplast Fluorescence Activated Cell Sorting

Plant protoplast fluorescence activated cell sorting protocol adapted from Birnbaum et al. 2005. Approximately 5,000 seeds (per replicate) of each GFP line used in the experiments

were sterilized and plated on high growth rate media (0.087% Murashige and Skoog medium, 4.5% sucrose) in 16 hrs of light. To allow rapid harvesting, seeds were arranged in rows on square plates at a density of approximately 500 seeds per row on top of nylon mesh (Nitex 03 100/47, Sefar America, Bricarcliff Manor, New York). Six days after plants were placed into a growth chamber (approximately 4 to 5 days after germination) roots were cut off about 1 cm from their tip. Dissected roots were placed in protoplasting solution B inside 70 μm cell strainers placed in small Petri dishes and incubated for one hour at room temperature with agitation. Protoplasted cells were collected from Petri dishes and concentrated by spinning down (at approximately 800 RCF). The supernatant was aspirated and the cell pellet was resuspended in 1.5 ml of Solution A. The cell suspension was then filtered through a 40 μm cell strainer. GFP expressing cells were isolated on a fluorescence activated cell sorter (either a Cytomation MoFlo or a Becton Dickinson FACSVantage) fit with a 100 μm nozzle at a rate of 2,000 to 5,000 events per second. We mainly used a fluid pressure of 30 psi. Protoplasts from non-GFP expressing Columbia wild-type plants were used as a negative control for establishing sorting criteria based on the following cell properties: A) a cluster of live protoplasts with intact membranes was selected based on a high forward to side scatter ratio. B) GFP positive cells were selected by their emission intensity in the green channel (~530 nm) above negative controls. Cells were sorted directly into lysis buffer (Qiagen RLT buffer), mixed and immediately frozen at -80° C for later RNA extraction. An autofluorescence filter was established by eliminating cells that fluoresced at equal intensity in the green and orange (~575 nm) channels. Standard Affymetrix protocols were then used for amplifying, labeling and hybridizing RNA samples. Birnbaum et al. 2005

Transcriptome profiling

RNA was extracted from pericycle cell sorted using the RNeasy Plant Mini Kit (Qiagen). A DNase treatment with the RNase-free DNase Set (Qiagen) was carried out for 15 min at 25°C. Total RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer. All RNA samples were rejected if they did not reach a minimum concentration of 100 ng μl^{-1}, a 260 nm/280 nm ratio between 1.8 and 2.0 and a RNA integrity number superior to 7.5, measured with an Agilent 2100 Bioanalyzer (Agilent, USA). Arabidopsis Tiling 1.0R arrays (Affymetrix) were hybridized at the VIB Nucleomics Core (www.nucleomics.be) according to the manufacturer's instructions. Data were normalized from CEL files using the robust multiarray average (RMA) algorithm (Irizarry et al., 2003). The probe annotation was obtained from athtiling1.0rcdf (Naouar et al., 2009). Differential

expression analysis was determined using the *eBayes* function from the *limma* package in R (Smyth, 2004). *P* values were calculated and then transformed into false-discovery rates (FDR), or *Q* values according to the method described by Storey and Tibshirani (2003), as implemented in the R package *qvalue*. The ChipEnrich software was used for the gene ontology (GO) enrichment analysis (Orlando et al., 2009). Visualization and hierarchical clustering of the microarray data were performed in the Multiexperiment Viewer MeV 4.9.0 (http://www.tm4.org; Saeed et al., 2003) using the Pearson correlation coefficient. Venn diagram were generated with BioVenn (Hulsen et al., 2008).

Analysis of gene expression

GUS activity was detected as described[15]. For quantitative RT-PCR RNA was extracted with the RNeasy kit (QIAGEN) from 7-day old roots of Arabidopsis (without root tip). Expression levels were normalized to UBQ10. A DNase treatment with the RNase-free DNase Set (Qiagen) was carried out for 15 min at 25°C. Poly(dT) cDNA was prepared from 1 µg total RNA with iScript™cDNA Synthesis Kit (Bio-Rad) and quantified with a LightCycler 480 (Roche) and SYBR GREEN I Master (Roche) according to the manufacturer's instructions. PCR was carried out in 384-well optical reaction plates heated for 10 min to 95°C to activate hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 60 sec at 95°C and annealing/extension for 60 s at 58°C. Targets were quantified with specific primer pairs designed with the Beacon Designer 4.0 (Premier Biosoft International). All RT-PCR experiments were done at least in triplicate.

Auxin Measurements

Approximately 30 mg (fresh weight) of 7 days old whole root material frozen in liquid nitrogen was ground with pestle and mortar and extracted for 5 min with 1 ml of cold phosphate buffer (50 mM; pH 7.0) containing 0.02% sodium diethyldithiocarbamate and the following ^{15}N- and/or $^{2}H_5$-labeled internal standards: [$^{2}H_5$]IAA, [^{15}N,$^{2}H_5$]IAAsp, [^{15}N,$^{2}H_5$]IAGlu. The samples were put into a freezer (−20°C) and centrifuged at 36,000 × *g* after 24 h. Supernatants were transferred into glass tubes, evaporated to dryness, and methylated with ethereal diazomethanol (Pencik et al., 2009). Further processing by immunopurification was performed as described (Pencik et al., 2009) and final analysis was done with a UHPLC coupled to a Waters Xevo TQ MS detector.

2.5 *Figures and tables*

Figure 1

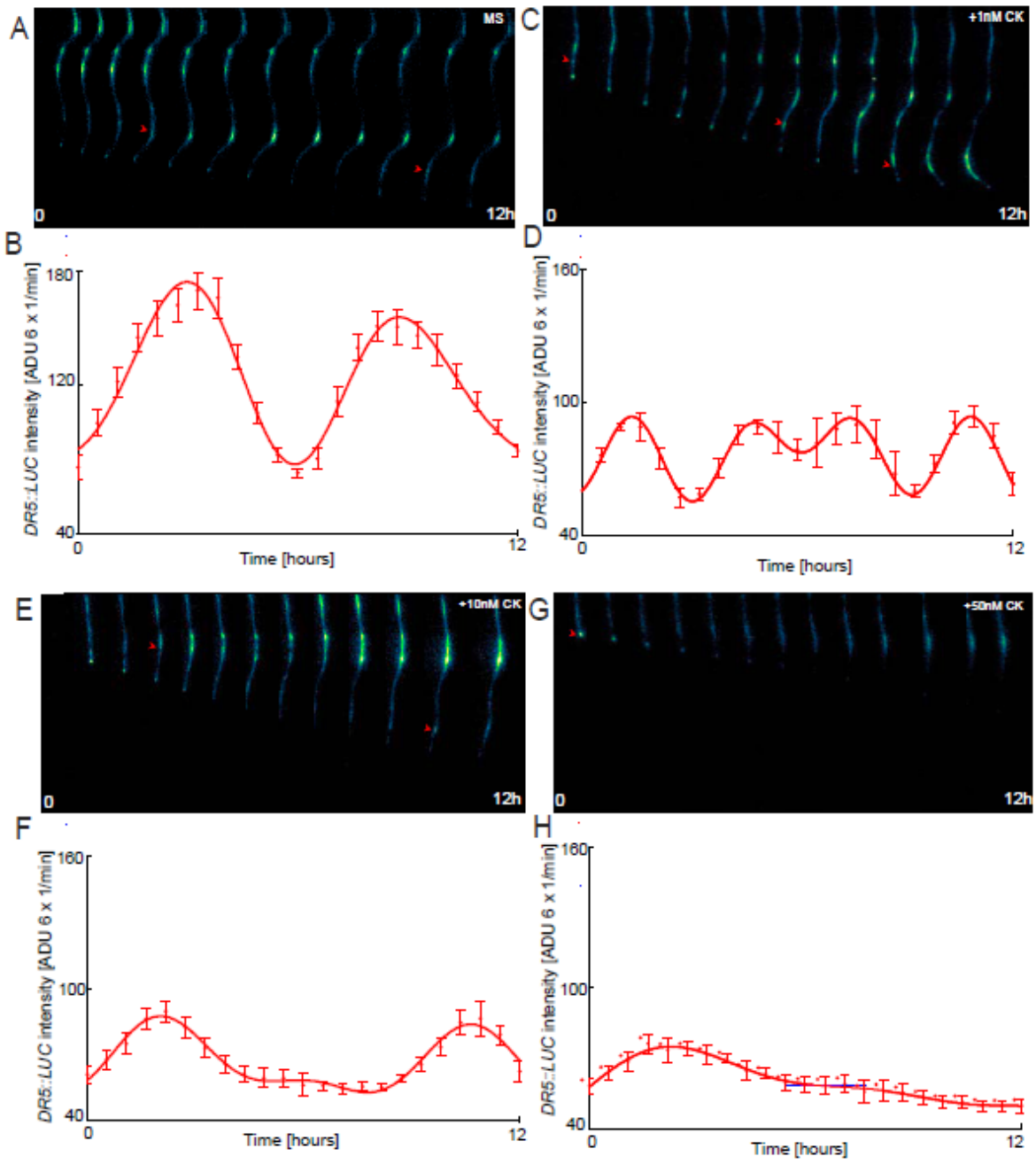


Figure 1. Cytokinins modulate oscillation of the auxin response in a dose-dependent manner.
(A, C, E, G) Real time monitoring of *DR5:LUCIFERASE* expression and (B, D, F, H) quantification of the luminescence signal in the basal meristem and the elongation zone of the main root observed in 7-day-old plants growing on medium supplemented with cytokinin (CK) - 0 (A,B), 1 (C, D), 10 (E, F) or 50 (G, H) nM 6-Benzyladenine. (A, C, E, G) Time series of the *DR5::LUCIFERASE* signal in the root monitored for 12 hours. Red arrows indicate consecutive pulses of *DR5::LUCIFERASE*.

Figure 2

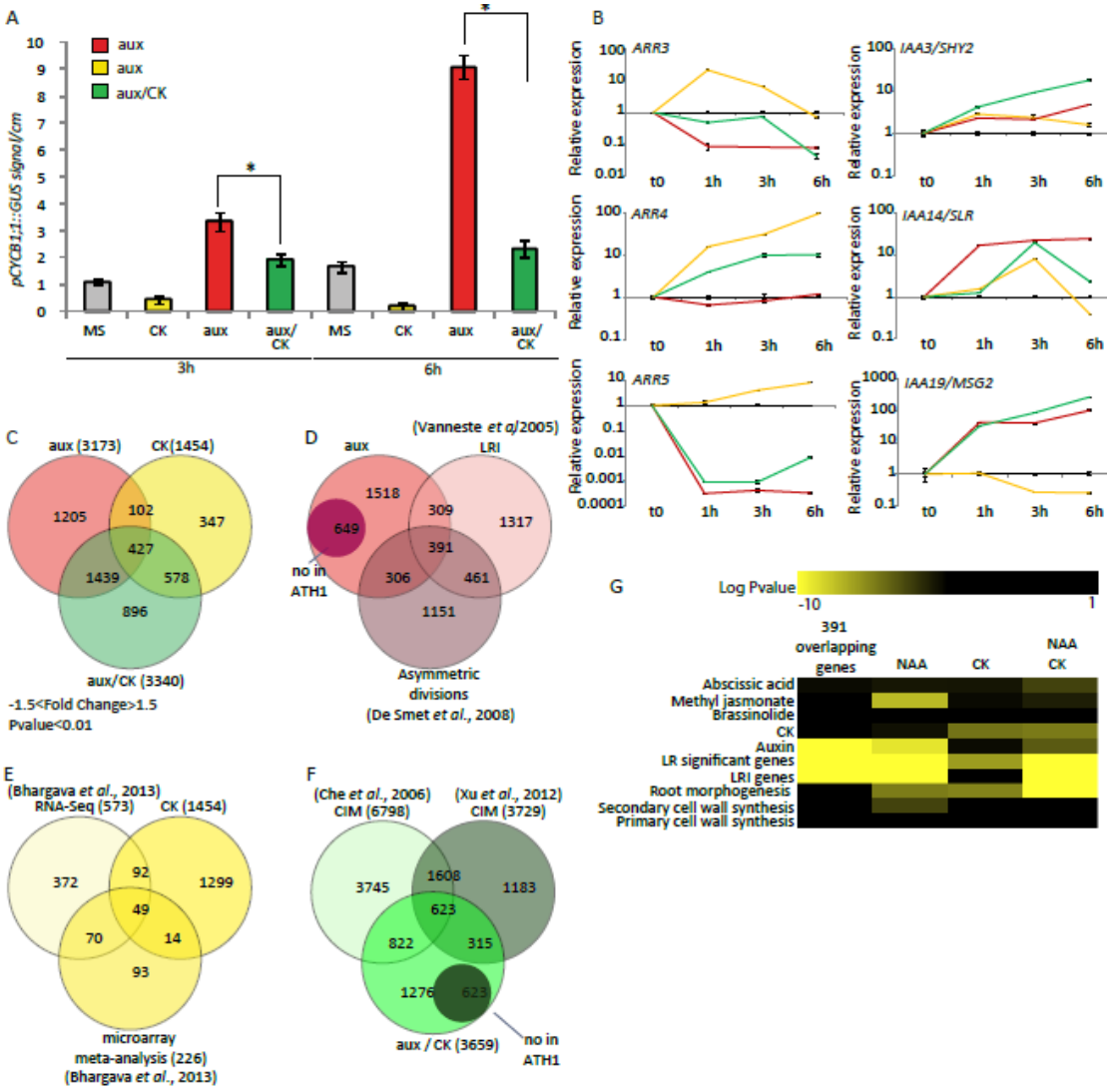


Figure 2. Identification of auxin and cytokinin regulated genes at the root pericycle using transcriptome profiling.

(**A**) Auxin (1µM 1-naphthaleneacetic acid; NAA) stimulates lateral root initiation when compared to untreated roots on Murashige Skoog (MS) medium, and simultaneous application of cytokinin (10µM 6-Benzyladenine) counteracts this auxin effect. Hormones applied on 5 days old *Arabidopsis* seedlings, lateral root primordia initiation scored 3 and 6 hour after treatments. *CYC1;1B::GUS* reporter used to visualize lateral root initiation events. (B) Quantitative (q)RT-PCR expression analyses of early auxin and cytokinin response genes of the *type-A ARR* and *AUX/IAA* families, respectively. Within 3 hours of treatment cytokinin significantly increased expression of *ARR3*, *ARR4* and *ARR5* cytokinin markers observed and this stimulatory effect was counteracted by simultaneous auxin application. Likewise, auxin stimulated transcription of *IAA3/SHY2*, *IAA14/SLR* and *IAA19/MSG* was attenuated by cytokinin provision. Auxin (1µM 1-naphthaleneacetic acid; NAA) red, cytokinin (10µM 6-Benzyladenine), green and auxin together with cytokinin yellow applied on 5 days old *Arabidopsis* seedlings. C. Xylem pole specific genes responsive to auxin, cytokinin and simultaneous treatments with both hormones. (D – F) Venn diagrams of the overlap between hormone regulated genes identified in this study when compared to other microarray datasets including auxin regulated genes in root (Vanneste et al., 2005) and xylem pole pericycle (De Smet et al., 2008) (D), genes regulated by cytokinin (Bhargava et al., 2013) (E) and genes regulated by callus induction medium CIM (Che et al., 2006 and Xu et al., 2012) (F). (G) Gene Ontology (GO) categories and biological processes significantly enriched in the transcriptome profiling dataset by auxin and cytokinins.

Figure 3

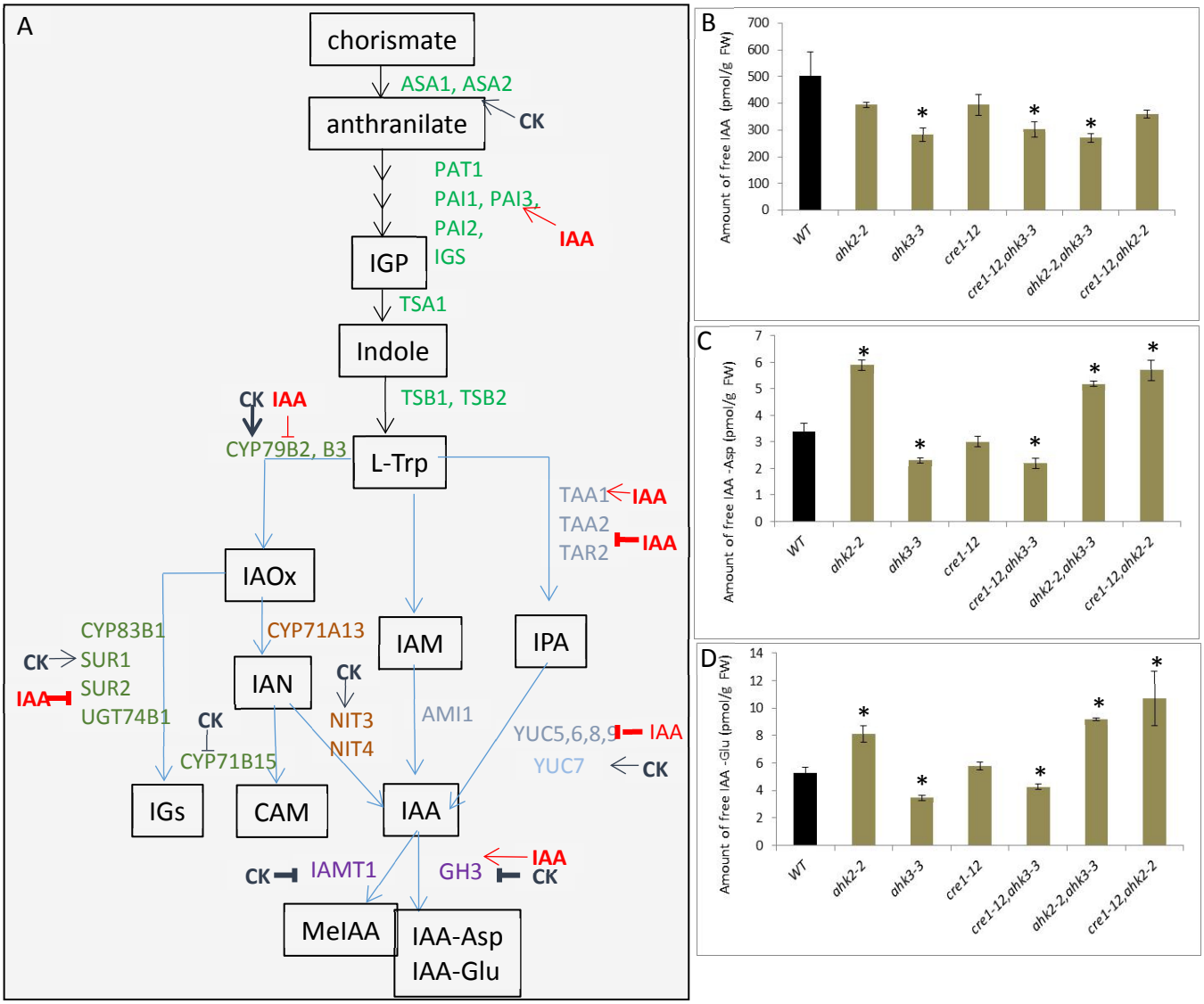


Figure 3. Auxin biosynthesis pathways in *Arabidopsis* regulated by cytokinin and auxin.
(**A**) Major auxin biosynthesis pathways in Arabidopsis, with genes shown to be differentially regulated by auxin (red) and cytokinin (blue) in the microarray dataset. L-tryptophan biosynthesis pathway – green, IPyA pathway – blue, IAAox pathway – orange, auxin deactivation pathway – purple (**B** - **D**). Levels of free IAA (B), auxin amid conjugates IAA-Asp (C) and IAA-Glu (D) in cytokinin receptor mutants. Error bars mark standard errors (n=3).

Figure 4

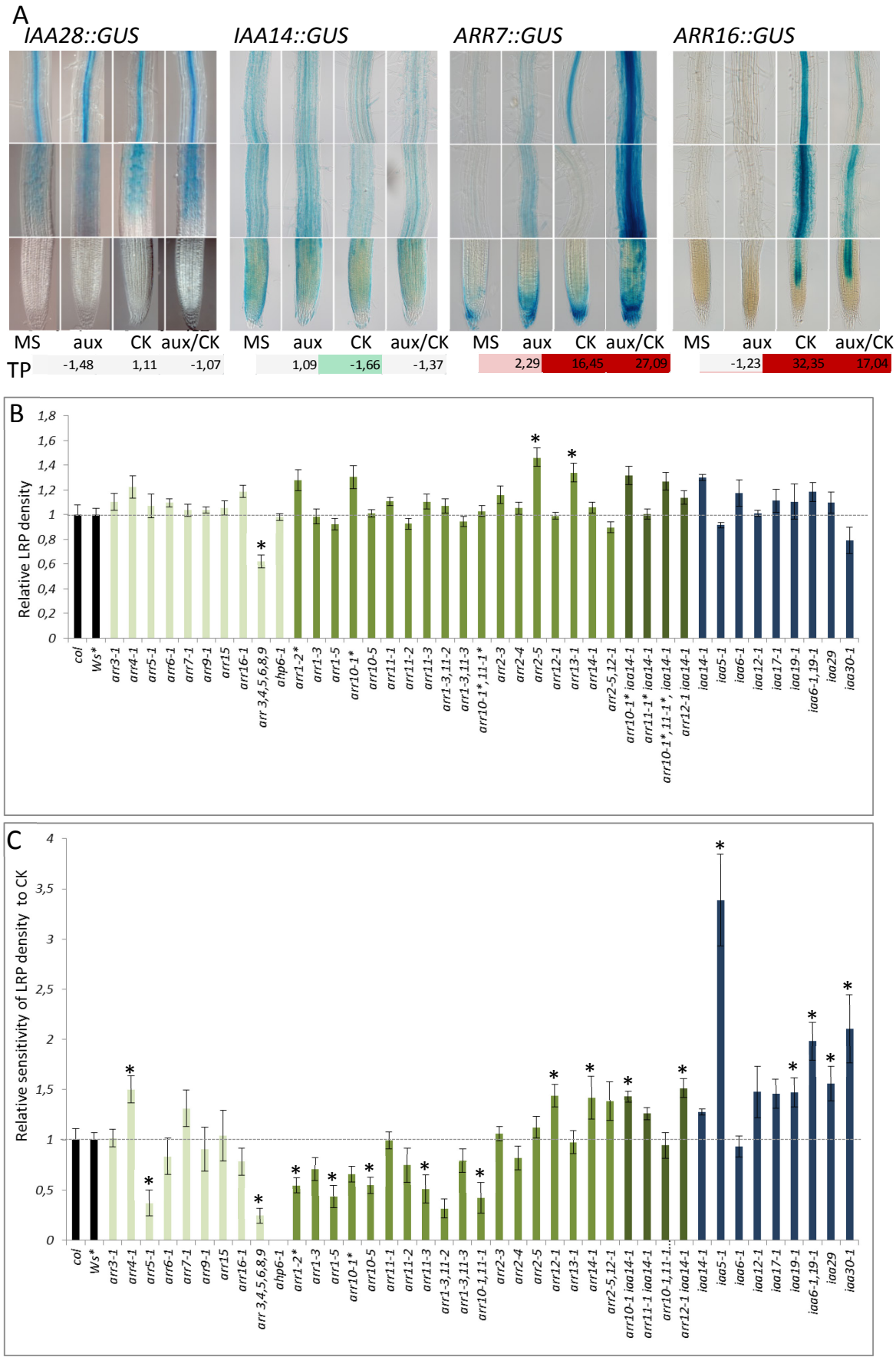

A
IAA28::GUS
IAA14::GUS
ARR7::GUS
ARR16::GUS
MS aux CK aux/CK MS aux CK aux/CK MS aux CK aux/CK MS aux CK aux/CK
TP -1,48 1,11 -1,07 1,09 -1,66 -1,37 2,29 16,45 27,09 -1,23 32,35 17,04
B
Relative LRP density
C
Relative sensitivity of LRP density to CK
col
Ws*
arr3-1
arr4-1
arr5-1
arr6-1
arr7-1
arr9-1
arr15
arr16-1
arr 3,4,5,6,8,9
ahp6-1
arr1-2*
arr1-3
arr1-5
arr10-1*
arr10-5
arr11-1
arr11-2
arr11-3
arr1-3,11-2
arr1-3,11-3
arr10-1*,11-1*
arr2-3
arr2-4
arr2-5
arr12-1
arr13-1
arr14-1
arr2-5,12-1
arr10-1* iaa14-1
arr11-1* iaa14-1
arr10-1*,11-1*, iaa14-1
arr12-1 iaa14-1
iaa14-1
iaa5-1
iaa6-1
iaa12-1
iaa17-1
iaa19-1
iaa6-1,19-1
iaa29
iaa30-1

Figure 4. Perturbations in cytokinin and auxin signaling affect cytokinin sensitivity of LR initiation.
(A) Expression of auxin and cytokinin signaling components *IAA28*, *IAA14*, *ARR7* and *ARR16* monitored using transcriptional fusion with *GUS* reporter confirms transcriptome profiling results. Auxin (1μM 1-naphthaleneacetic acid; NAA), cytokinin (10μM 6-Benzyladenine), and auxin together with cytokinin applied on 5 days old *Arabidopsis* seedlings for 5 hours. TP indicates transcriptome profiling expression profile.
(B, C) LRP initiation in *type-A* and *-B arr* and *aux/iaa* mutants growing on control medium (B) or on medium supplemented with cytokinin (C). Error bars represent SE (n = 20). *, statistically significant differences for values compared with wild-type as determined by Student's t-test ($P < 0.05$). MS - Murashige and Skoog medium, cytokinin – 100 nM cytokinin derivate N6-benzyladenine, LRP/cm – total number of initiated LR primordia and LR per cm of root length in 7 days old seedlings.

Figure 5

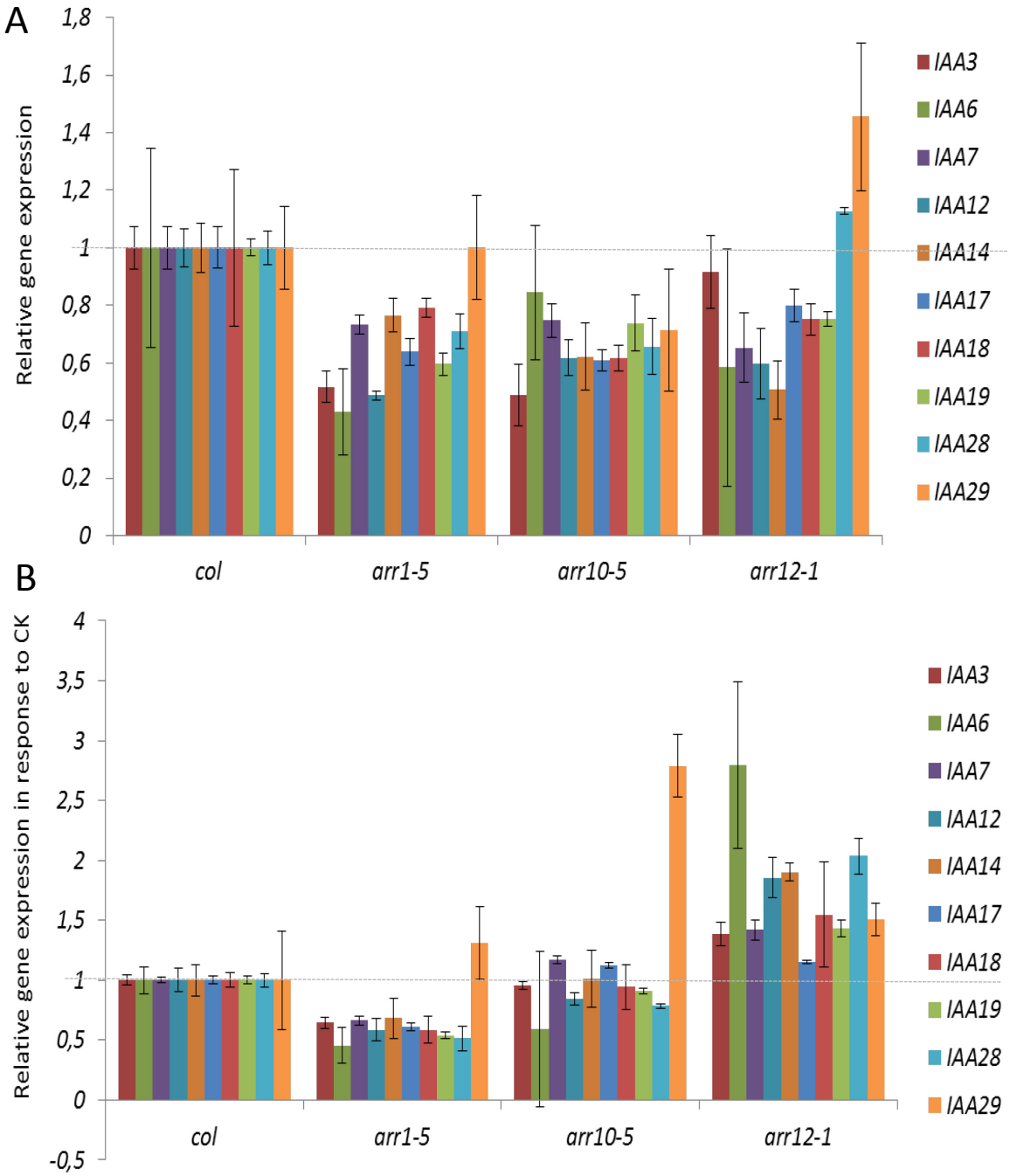


Figure 5. Type-B response regulators ARR1, ARR10 and ARR12 differentially control expression of *Aux/IAAs*.

(A, B) Relative *Aux/IAA* expression levels in type-B *arr* mutants compared to their respective wild-type after growing 7 days on MS (A) or cytokinin containing medium (B). MS - Murashige and Skoog medium, cytokinin – 100 nM cytokinin derivate N6-benzyladenine,

Figure 6.

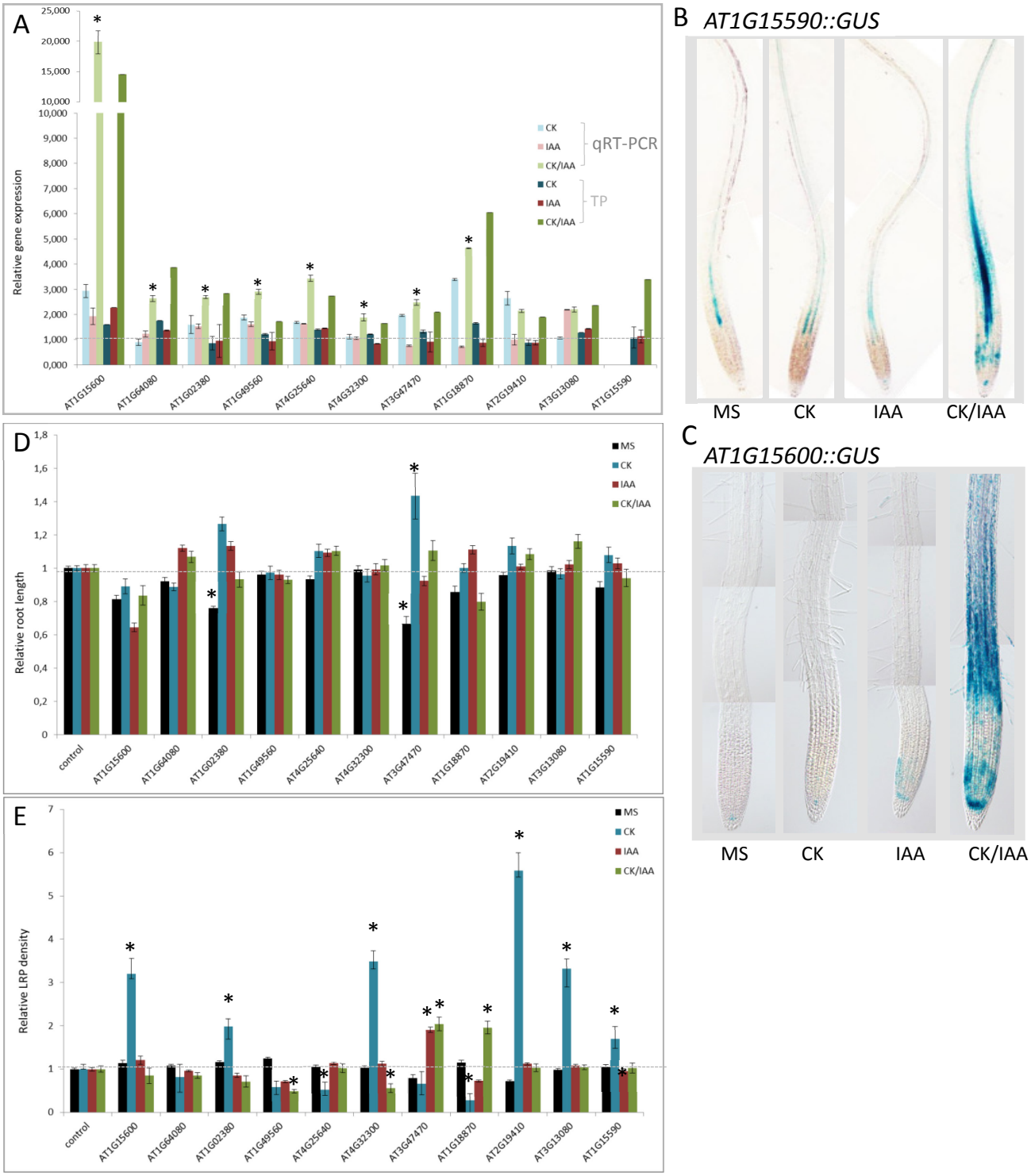
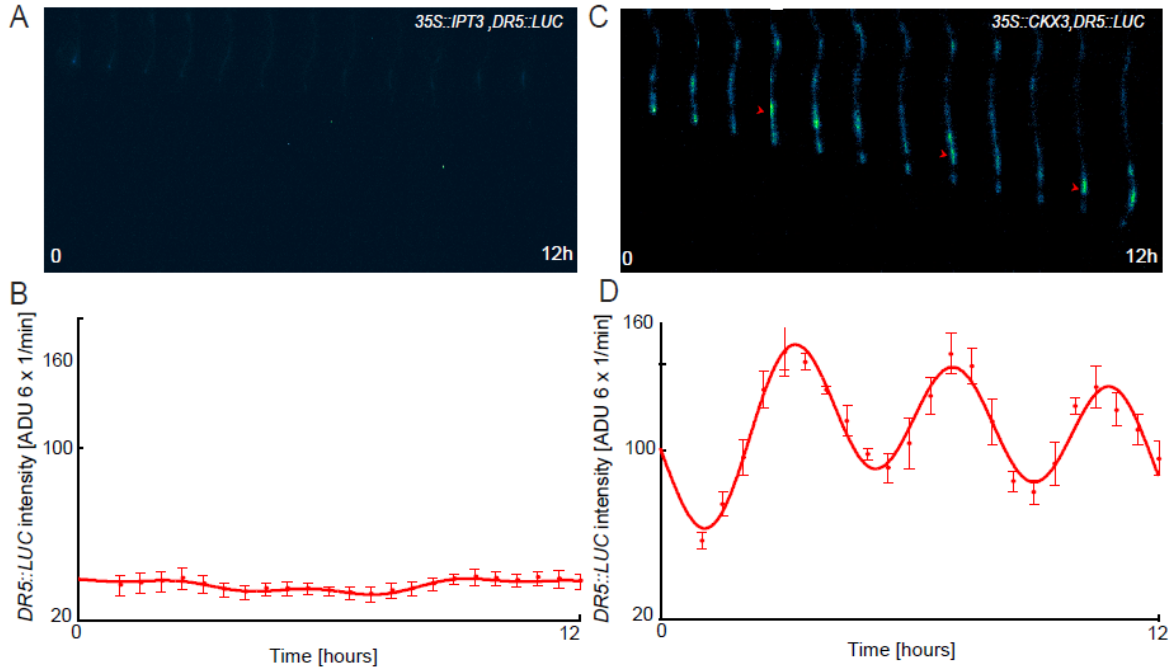


Figure 6. Genes synergistically up-regulated by auxin and cytokinin impact on the root system establishment.

(A) Relative expression levels of genes synergistically up-regulated by auxin and cytokinin analysed by qRTPCR when compared to transcriptome profiling data (TP). Auxin (1μM 1-naphthaleneacetic acid; NAA), cytokinin (10μM 6-Benzyladenine), and auxin together with cytokinin applied on 5 days old *Arabidopsis* seedlings for 3 hours. (B, C) Synergism of auxin and cytokinin in control of *AT1G15590 and At1G15600* monitored using transcriptional fusion with *GUS* reporter confirms transcriptome profiling results. (D, E) Root growth and LRI initiation density in mutants of auxin and cytokinin synergistically regulated genes. Error bars

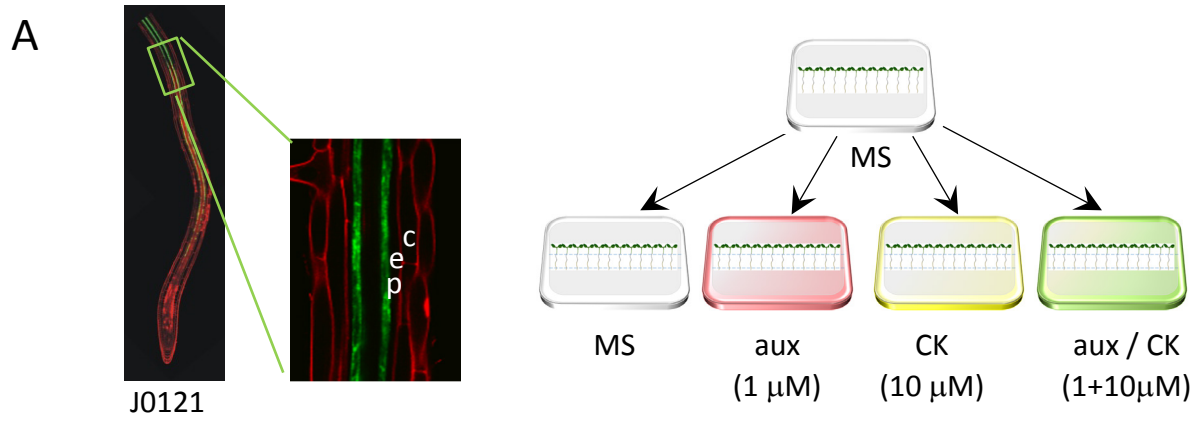
represent SE (n = 20). (*) statistically significant differences for values compared with wild-type as determined by Student's t-test ($P < 0.05$). MS - Murashige and Skoog medium, cytokinin – 100 nM cytokinin derivate N6-benzyladenine, Auxin (1μM 1-naphthaleneacetic acid; NAA), LRP/cm – total number of initiated LR primordia and LR per cm of root length in 7 days old seedlings.

Supplementary Figure 1



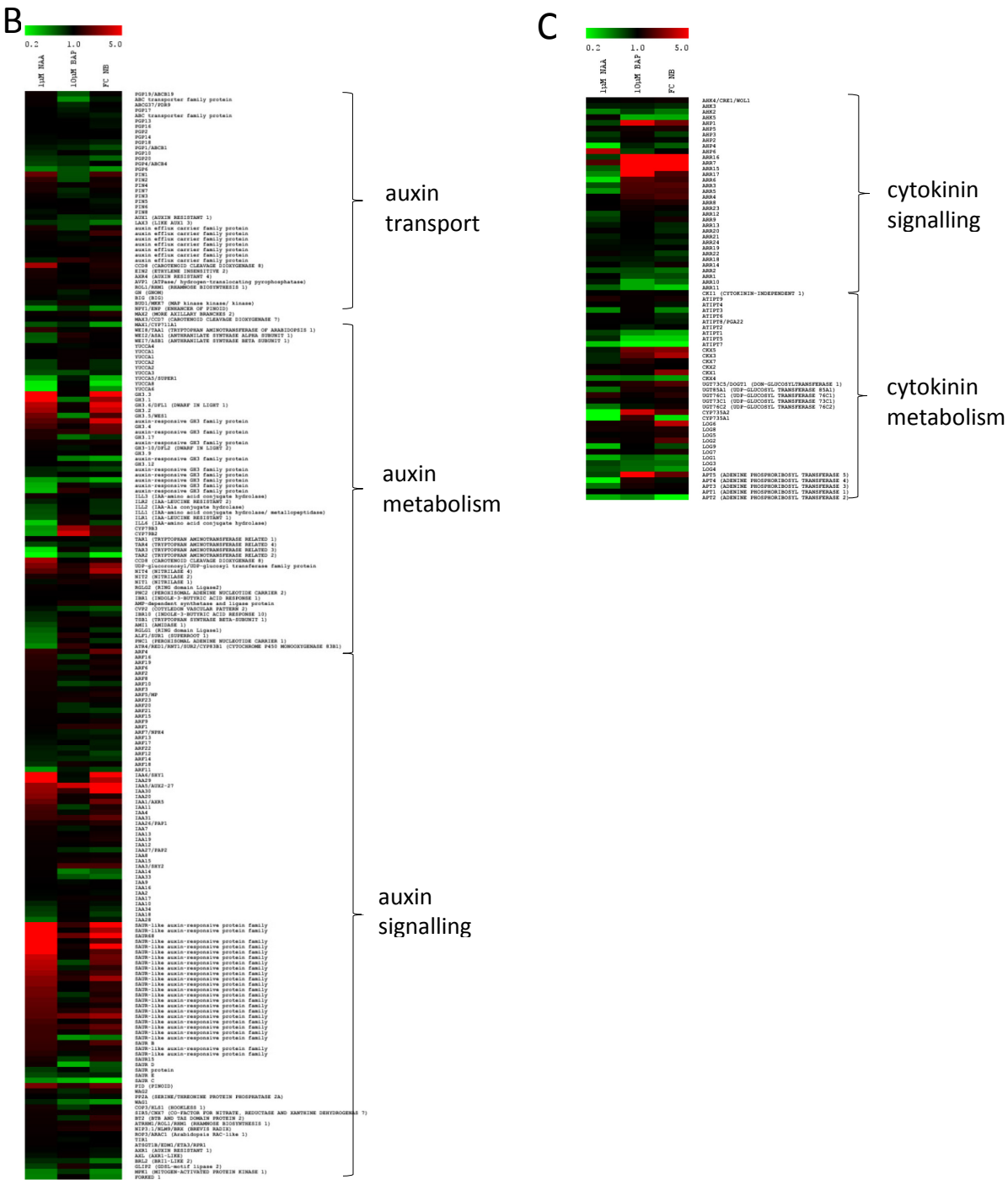
Supplementary Figure 1. Manipulations of endogenous cytokinin levels induce a change in the oscillatory behavior of *DR5::LUCIFERASE*. (A, C) Profile of DR5 expression and (B, D) quantification of the luminescence signal in the basal meristem and the elongation zone of the roots displaying a low (*35S::CKX3*) (A, B) or high (*35S::IPT3*) (C, D) content of cytokinin. Time series of the *DR5::LUCIFERASE* signal in *35S::CKX3* (b) or *35S::IPT3* (c) roots monitored for 12 hours. Red arrowheads indicate the peak of the DR5 pulse.

Supplementary Figure 2A



Supplementary Figure 2A. Genes of auxin and cytokinin regulatory pathways are sensitive to hormonal perturbations.
(A) Scheme of the transcriptome profiling experimental set up. 5 days old Arabidopsis seedlings were treated with auxin, cytokinin or auxin and cytokinin for three hours. Roots without root tips collected for fluorescence activation cell sorting (FACS) and transcriptome profiling.

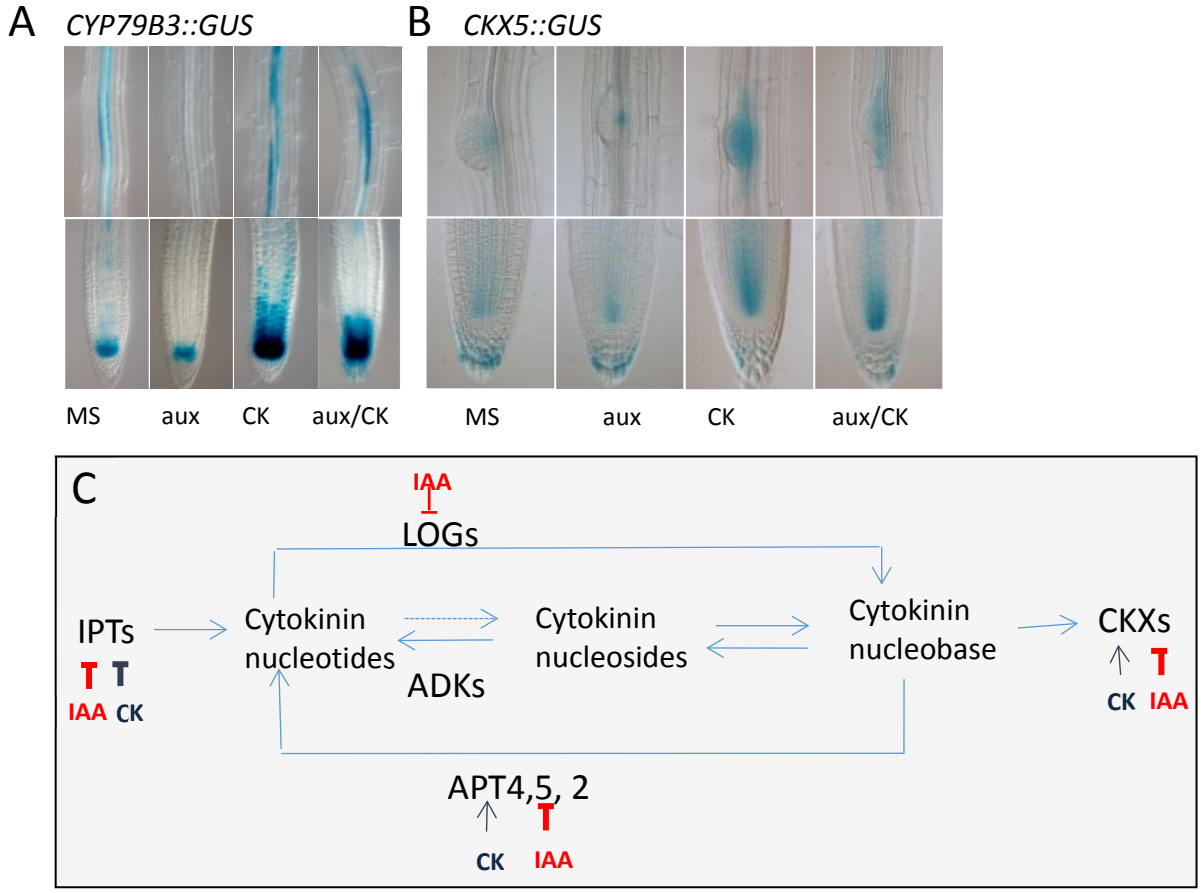
Supplementary Figure 2B, C



Supplementary Figure 2B, C. Genes of auxin and cytokinin regulatory pathways are sensitive to hormonal perturbations.

(B, C) Heat map of expression profiles of auxin and cytokinin regulatory genes acting in metabolism, transport and signaling and their sensitivity to auxin, cytokinin and combinatorial treatments.

Supplementary Figure 3

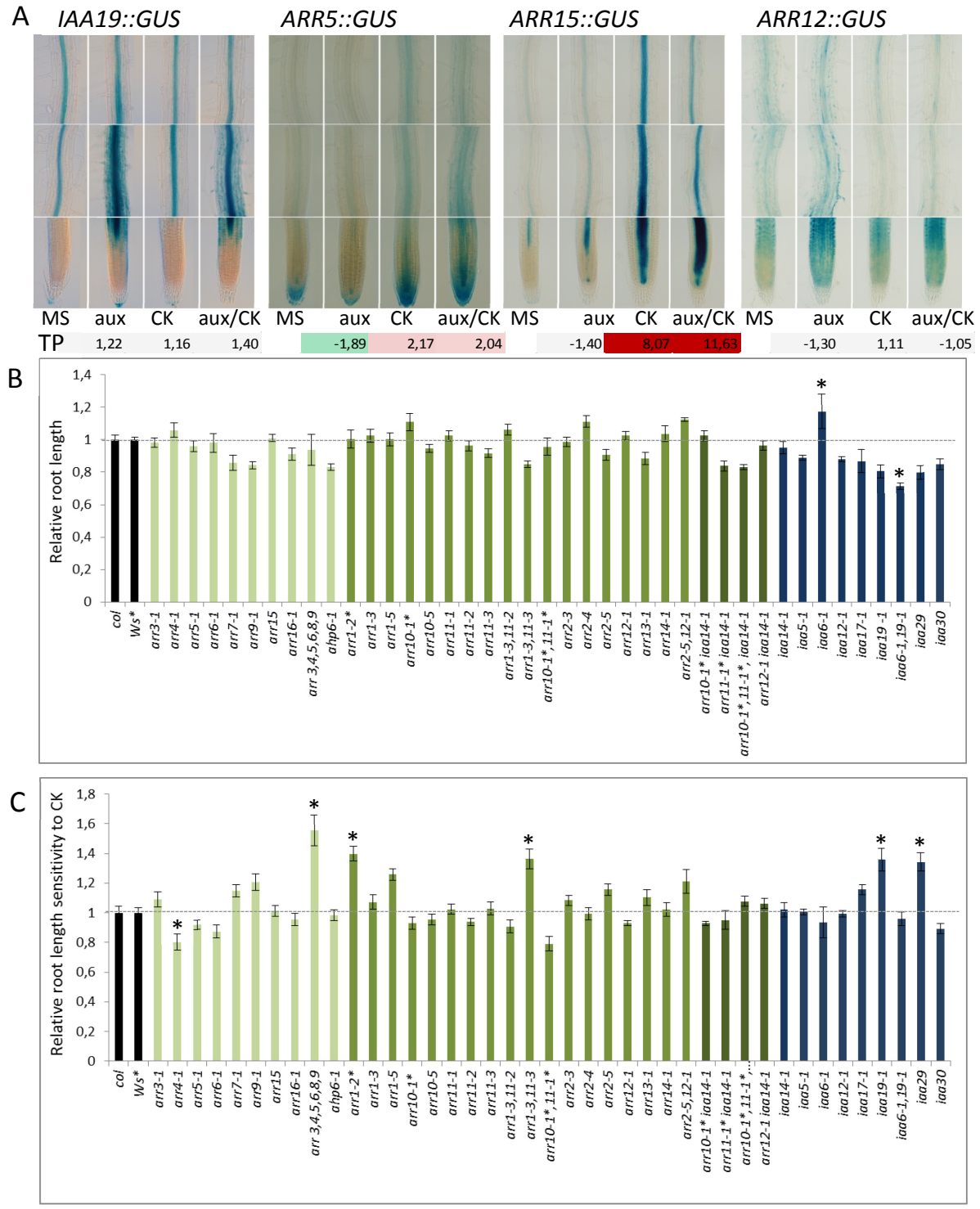


Supplementary Figure 3: Cytokinin biosynthesis pathways in Arabidopsis regulated by cytokinin and auxin.

(A, B) Expression of *CYP79B3::GUS* and *CKX5::GUS* reporters in response to auxin, cytokinin and combinatorial auxin and cytokinin treatments confirms transcriptome profiling. Auxin (1µM 1-naphthaleneacetic acid; NAA), cytokinin (10µM 6-Benzyladenine), and auxin together with cytokinin applied on 5 days old *Arabidopsis* seedlings for 5 hours.

(C) Major cytokinin biosynthesis pathways in *Arabidopsis*, with genes shown to be differentially regulated by auxin (red) and cytokinin (blue) in the microarray dataset.

Supplementary Figure 4



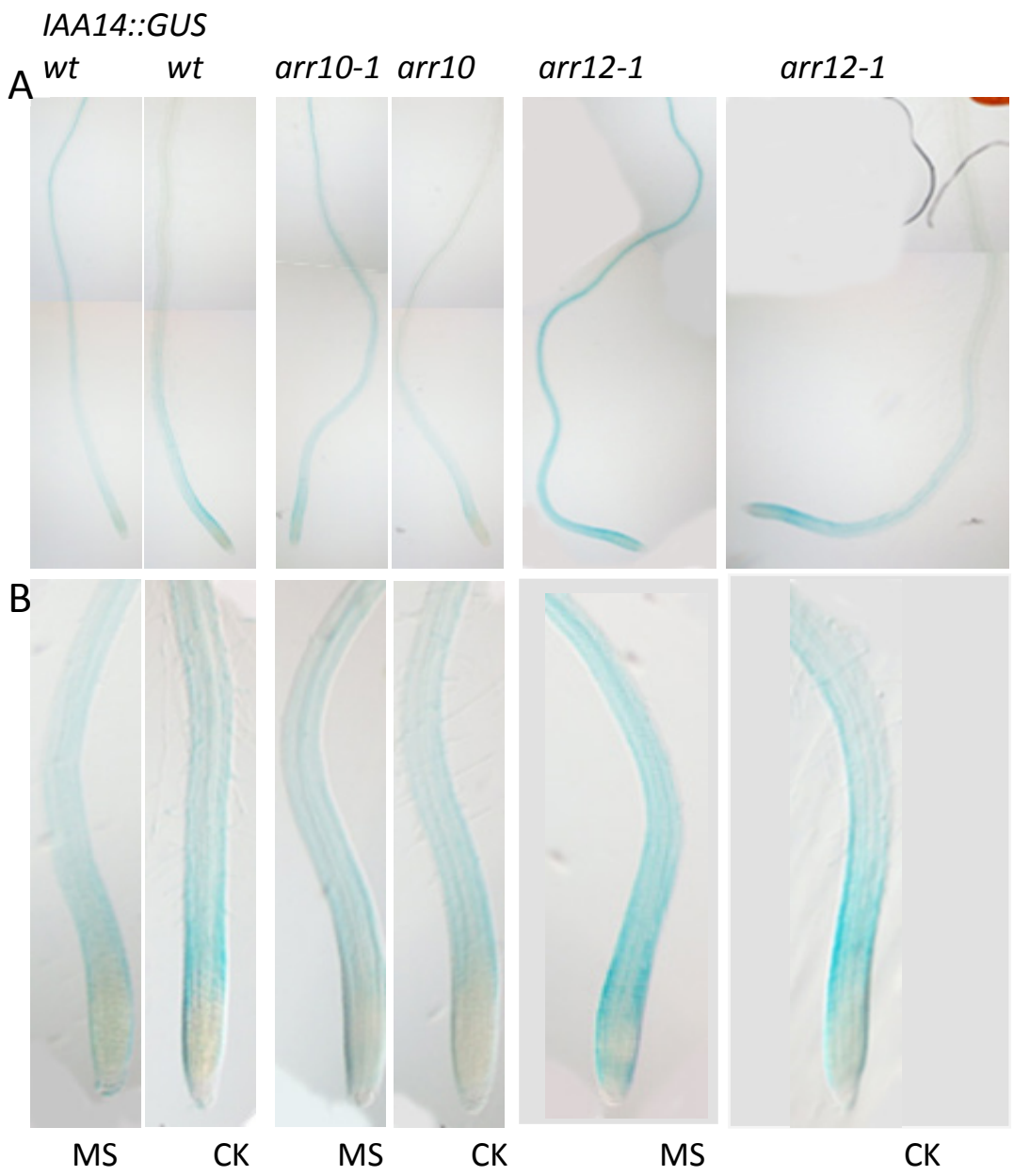
Supplementary Figure 4: Perturbations in cytokinin and auxin signaling affect root length cytokinin sensitivity.

(A) Expression of auxin and cytokinin signaling components *IAA19*, *ARR5*, *ARR15* and *ARR12* monitored using transcriptional fusion with *GUS* reporter confirms transcriptome profiling results. Auxin (1μM 1-naphthaleneacetic acid; NAA), cytokinin (10μM 6-Benzyladenine), and auxin together with cytokinin applied on 5 days old *Arabidopsis* seedlings for 5 hours. TP indicates transcriptome profiling expression profile.

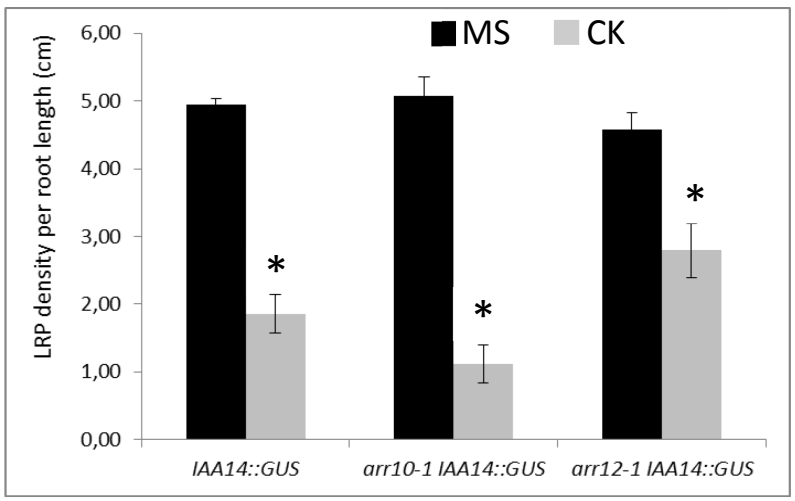
(B, C) Root growth in *type-A* and *-B arr* and *aux/iaa* mutants growing on control medium (B) or on medium supplemented with cytokinin (C). Error bars represent SE (n = 20). (*, statistically significant differences for

values compared with wild-type as determined by Student's t-test ($P < 0.05$). MS - Murashige and Skoog medium, cytokinin – 100 nM cytokinin derivate N6-benzyladenine, RL - root length in 7 days old seedlings.

Supplementary Figure 5



C

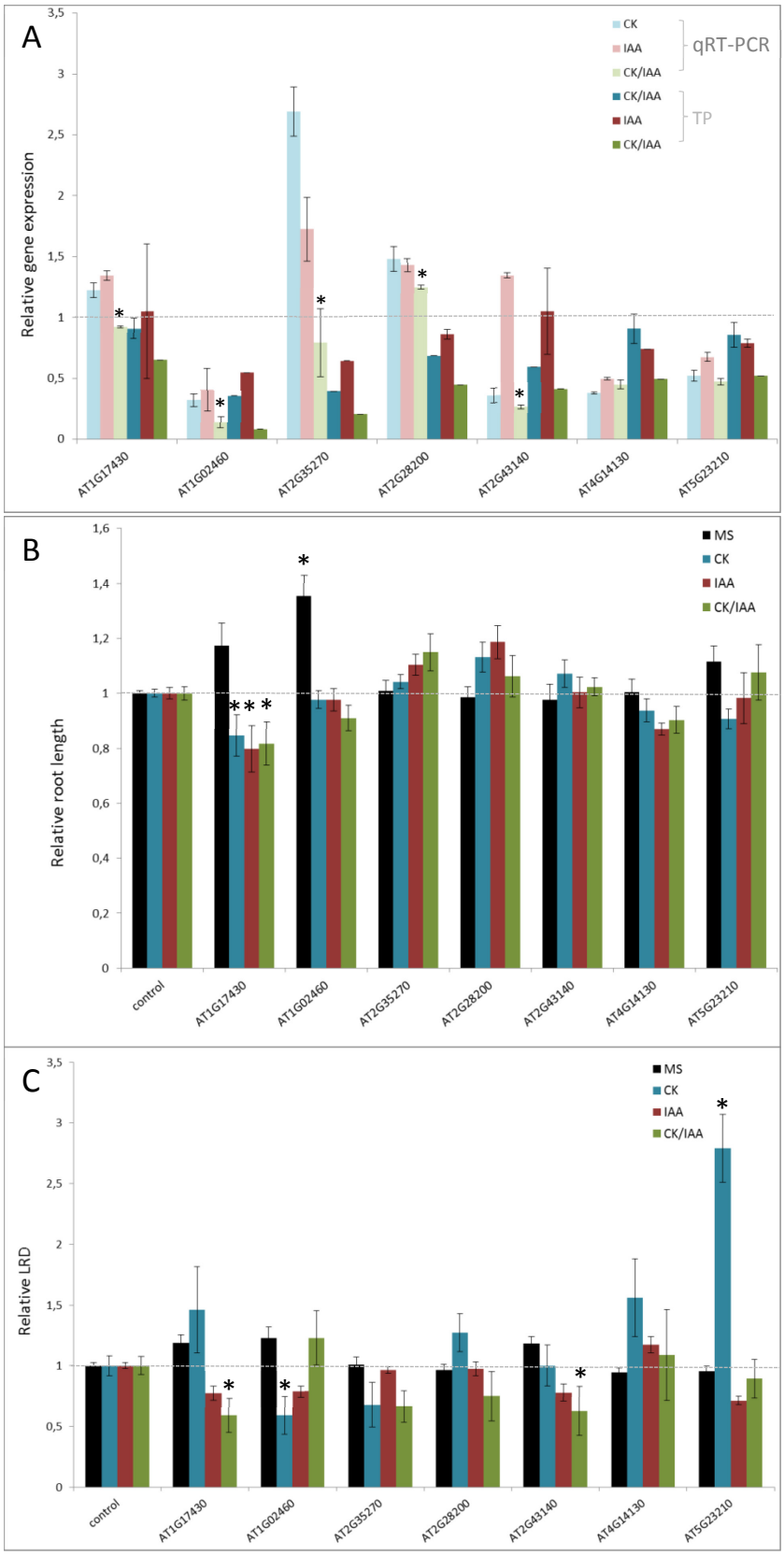


Supplementary Figure 5: Type-B response regulators ARR10 and ARR12 differentially control expression of *IAA14.*

(A) *IAA14::GUS* expression levels in *type-B arr10* and *arr12* mutants compared to their respective wild-type after growing 7 days on MS (A) or cytokinin containing medium (B).

(C) LRP initiation in type-*B arr10* and *arr12* mutants growing on control medium or on medium supplemented with cytokinin. (*) statistically significant differences for values compared with wild-type as determined by Student's t-test ($P < 0.05$,). MS - Murashige and Skoog medium, cytokinin – 100 nM cytokinin derivate N6-benzyladenine.

Supplementary Figure 6



Supplementary Figure 6: Genes synergistically down-regulated by auxin and cytokinin impact on the root system establishment.

(A) Relative expression levels of genes synergistically down-regulated by auxin and cytokinin analysed by qRTPCR when compared to transcriptome profiling data (TP). Auxin (1μM 1-naphthaleneacetic acid; NAA), cytokinin (10μM 6-Benzyladenine), and auxin together with cytokinin applied on 5 days old Arabidopsis seedlings for 3 hours. (B,C) Root growth and LRI initiation in mutants of auxin and cytokinin synergistically regulated genes. Error bars represent SE (n = 20).(*) statistically significant differences for values compared with wild-type as determined by Student's t-test ($P < 0.05$). MS - Murashige and Skoog medium, cytokinin – 100 nM cytokinin derivate N6-benzyladenine, Auxin (0.1μM 1-naphthaleneacetic acid; NAA), LRP/cm – total number of initiated LR primordia and LR per cm of root length in 7 days old seedlings.

Table S1

	LRP density (LRP/cm)	Number of oscillations /20h	Oscillation period	Peak period	Valley period	Average luminescence (ADU 6min-1)
MS	5.65 ± 0.15	2.4 ± 0.5	6h08 ± 0h35	4h49 ± 0h22	1h25 ± 0h05	122.45 ± 3.02
0.1nM BA	8.01 ± 0.16	6.67 ± 0.9	1h57 ± 0h22	1h48 ± 13	0h12 ± 0h8	147.23 ± 9.24
1nM BA	7.47 ± 0.48	5.62 ± 0.48	3h ± 0h27	2h33 ± 0h10	0h40 ± 0h21	84.72 ± 3.42
10nM BA	5.65 ± 0.67	2.13 ± 0.35	7h45 ± 0h29	2h33 ± 0h12	5h39 ± 0h4	75.86 ± 2.21
50nM BA	4.64 ± 0.66	0.22 ± 0.46	-	-	-	68 ± 4.22
100nM BA	0.92 ± 0.94	-	-	-	-	53.14 ± 3.12
1µM BA	0.27 ± 0.49	-	-	-	-	13.08 ± 0.5
35S::CKX3	8.04 ± 0.23	5.84 ± 0.56	3h42 ± 0h16	2h58 ± 26	0h30 ± 0h12	146.98 ± 3.99
35S::IPT3	3.43 ± 0.20	0.21 ± 0.14	-	-	-	38.63 ± 1.45

Table S1: **Cytokinins change oscillatory behavior of the auxin response in a dose-dependent manner**. Exogenous applications or changes in endogenous cytokinin content affect *DR5::LUCIFERASE* oscillations (number of pulses over 20h, wavelength, duration and amplitude of oscillations) and LRP density.

Table S2 (Supplementary Excel File) VisuaLRTC

Table S3 (Supplementary Excel File) Expression profiles of genes in response to treatments with auxin, cytokinin (CK) and simultaneous treatments

Table S4

Table S4	Expression profile of genes involved in regulation of auxin activity								
ID	Tair10 2011	auxin	Pvalue	CK	Pvalue	aux/CK	Pvalue	Pvalue aux/CK	Pvalue aux/CK
Auxin metabolism									
Tryptophan pathway									
AT5G05730	WEI2/ASA1 (ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1)	-1,37	0	1,53	2E-04	1,07	0,24	0	2E-04
AT2G29690	ATHANSYNAB__ASA2 (ANTHRANILATE SYNTHASE 2); anthranilate synthase	1,14	0,12	1,08	0,376	1,07	0,41	0,4	0,597
AT1G25220	WEI7/ASB1 (ANTHRANILATE SYNTHASE BETA SUBUNIT 1)	-1,41	0,01	1,26	0,018	1,07	0,3	0,01	0,037
AT5G17990	TRP1 (tryptophan biosynthesis 1)	-1,34	0	1,35	0,003	1,09	0,16	0	0,009
AT5G05590	PAI2 (PHOSPHORIBOSYLANTHRANILATE ISOMERASE 2)	1,97	0	1,21	0,223	2,15	0	0,39	0,002
AT1G29410	PAI3 (phosphoribosylanthranilate isomerase 3); phosphoribosylanthranilate isomerase	1,42	0	1,13	0,067	1,53	0	0,52	0,002
AT3G54640	TRP3__TSA1 (TRYPTOPHAN SYNTHASE ALPHA CHAIN); tryptophan synthase	-1,13	0,07	1,17	0,029	-1,01	0,87	0,06	0,024
AT5G54810	ATTSB1_TRP2_TRPB__TSB1 (TRYPTOPHAN SYNTHASE BETA-SUBUNIT 1); tryptophan synthase	-1,17	0,01	1,24	0,011	1,15	0,03	0	0,23
AT4G27070	TSB2 (TRYPTOPHAN SYNTHASE BETA-SUBUNIT 2); tryptophan synthase	-1,11	0,15	1,22	0,015	1,01	0,84	0,04	0,015
Indole-3-pyruvic acid (IPyA) pathway									
AT1G70560	WEI8/TAA1 (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1)	1,76	0	-1,03	0,747	1,21	0,02	0,01	0,01
AT1G23320	TAR1 (TRYPTOPHAN AMINOTRANSFERASE RELATED 1); L-tryptophan:2-oxoglutarate aminotransferase/ L-tryptophan:pyruvate amino	1,12	0,1	1,09	0,375	-1,08	0,42	0,06	0,017
AT4G24670	TAR2 (TRYPTOPHAN AMINOTRANSFERASE RELATED 2)	-5,64	0	-1,41	1E-03	-4,10	0	0,08	4E-05
AT5G11320	YUCCA4	1,11	0,3	-1,03	0,705	1,03	0,6	0,84	0,886
AT4G32540	YUCCA1	1,04	0,6	1,15	0,09	1,13	0,1	0,13	0,632
AT1G21430	YUCCA11	1,03	0,75	1,13	0,156	-1,10	0,16	0,56	0,029
AT1G48910	YUCCA10	-1,01	0,95	1,17	0,12	1,07	0,59	0,77	0,414
AT2G33230	YUCCA 7	-1,11	0,38	2,29	0,008	1,97	0,01	0	0,552
AT4G13260	YUCCA2	-1,23	0,09	1,16	0,205	-1,15	0,08	0,58	0,011
AT1G04610	YUCCA3	-1,32	0,05	-1,33	0,277	-1,09	0,48	0,33	0,503
AT1G04180	YUCCA 9	-1,61	0	1,03	0,703	-1,94	0	0,15	0,001
AT5G43890	YUCCA5/SUPER1	-2,26	0	-1,08	0,292	-2,27	0	0,77	3E-04
AT4G28720	YUCCA8	-3,85	0	-1,01	0,868	-3,46	0	0,53	2E-03
AT5G25620	YUCCA6	-4,07	0	1,09	0,525	-1,85	0	0	0,002
AT5G20960	AAO1 (ARABIDOPSIS ALDEHYDE OXIDASE 1)	-1,25	0,05	-1,14	0,147	-1,40	0	0,16	0,039
AT1G08980	AMI1 (AMIDASE 1)	-1,30	0,02	1,28	0,033	1,14	0,06	0	0,273
IAAox pathway (IAOx to IAA conversion)									
AT4G39950	CYP79B2	-2,01	0	4,32	1E-05	2,06	0	0	0,001
AT2G22330	CYP79B3	-1,84	0,07	3,41	0,002	1,92	0,02	0	0,009
IAOx to IAN conversion									
AT2G30770	CYP71A13	1,12	0,1	1,01	0,877	1,31	0,12	0,2	0,04
IAN to auxin conversion									
AT5G22300	NIT4 (NITRILASE 4)	2,42	0	1,63	4E-04	3,76	0	0,01	4E-05
AT3G44300	NIT2 (nitrilase 2)	1,46	0,01	1,21	0,014	1,68	0	0,25	9E-04
AT3G44320	NIT3 (NITRILASE 3)	-1,02	0,81	1,69	8E-05	1,43	0	0	0,014
IAAox to IG									
AT4G31500	ATR4/RED1/RNT1/SUR2/CYP83B1 (CYTOCHROME P450 MONOOXYGENASE 83B1)	-1,80	0	1,80	9E-05	1,02	0,71	0	3E-05
AT4G13770	CYP83A1	-1,18	0,3	-1,05	0,874	1,55	0,22	0,05	0,13
AT2G20610	ALF1/SUR1 (SUPERROOT 1)	-1,52	0	1,62	2E-04	1,05	0,4	0	4E-04
AT1G24100	UGT74B1 (UDP-glucosyl transferase 74B1)	-2,15	0	1,25	0,006	-1,45	0	0	4E-05
IG to IAN conversion									
AT5G26000	BGLU38__TGG1 (THIOGLUCOSIDE GLUCOHYDROLASE 1); hydrolase, hydrolyzing O-glycosyl compounds / thioglucosidase	1,05	0,7	1,16	0,183	1,42	0,12	0,17	0,517
AT5G25980	TGG2 (GLUCOSIDE GLUCOHYDROLASE 2)	1,14	0,13	1,08	0,336	-1,06	0,41	0,13	0,458
AT3G14210	ESM1 (epithiospecifier modifier 1)	-1,25	0,02	-1,07	0,351	-1,15	0,05	0,56	0,121
IAN to camalexin									
AT3G26830	CYP71B15/PAD3 (PHYTOALEXIN DEFICIENT 3)	-1,25	0,41	-2,73	0,026	-1,04	0,87	0,14	0,007
Auxin deactivation									
GH3 family IAA conjugation									
AT2G23170	GH3.3	9,06	0	1,07	0,728	8,41	0	0,19	2E-05
AT2G14960	GH3.1	7,68	0	-1,55	0,002	3,40	0	0	1E-07
AT5G54510	GH3.6/DFL1 (DWARF IN LIGHT 1)	3,90	0	1,24	0,174	4,66	0	0,01	6E-05
AT4G37390	GH3.2	3,58	0	1,16	0,513	4,11	0	0,33	9E-04
AT4G27260	GH3.5/WES1	2,79	0	-1,72	0,013	2,18	0	0	1E-04
AT5G13370	auxin-responsive GH3 family protein	2,41	0	1,72	0,002	4,41	0	0	1E-05
AT1G59500	GH3.4	2,08	0	1,17	0,322	2,58	0	0,22	6E-04
AT5G13360	auxin-responsive GH3 family protein	1,52	0,12	1,65	0,08	2,51	0	0,09	0,144
AT1G28130	GH3.17	1,51	0,1	-1,54	0,097	-1,15	0,49	0	0,091
AT5G49160	DDM2/MET1 (METHYLTRANSFERASE 1)	1,29	0,01	-1,06	0,532	1,41	0	0,45	0,004
AT5G55250	IAMT1 (IAA CARBOXYLMETHYLTRANSFERASE 1)	1,25	0,56	-4,11	0,006	-3,06	0,01	0	0,039
AT5G13350	auxin-responsive GH3 family protein	1,22	0,04	1,23	0,041	1,30	0,01	0,03	0,036
AT4G03400	GH3-10/DFL2 (DWARF IN LIGHT 2)	1,17	0,18	-1,07	0,492	1,09	0,28	0,32	0,015
AT2G47750	GH3.9	1,07	0,44	-1,01	0,962	1,17	0,07	0,3	0,27
AT1G48670	auxin-responsive GH3 family protein	1,05	0,83	-1,63	0,015	-2,07	0	0,02	0,254
AT5G13320	GH3.12	1,00	0,97	1,09	0,185	-1,09	0,3	0,82	0,035
AT1G48660	auxin-responsive GH3 family protein	-1,12	0,26	-1,18	0,033	-1,29	0,01	0,23	0,008
AT5G13380	auxin-responsive GH3 family protein	-1,12	0,17	1,05	0,501	1,06	0,62	0	0,071
AT1G19890	ATMGH3__MGH3 (MALE-GAMETE-SPECIFIC HISTONE H3); DNA binding	-1,22	0,13	-1,14	0,314	-1,16	0,24	0,65	0,73
AT1G78670	ATGGH3 (gamma-glutamyl hydrolase 3)	-1,23	0,23	1,01	0,881	1,05	0,59	0,14	0,609
AT1G48690	auxin-responsive GH3 family protein	-1,94	0	-1,58	0,037	-2,25	0	0,23	0,058
AT5G51470	auxin-responsive GH3 family protein	-2,22	0,1	1,27	0,564	-1,13	0,73	0,04	0,129
AT1G23160	auxin-responsive GH3 family protein	-2,38	0,04	1,74	0,11	1,14	0,66	0	0,027
AT2G23260	UGT84B1 (UDP-glucosyl transferase 84B1); UDP-glycosyltransferase/ abscisic acid glucosyltransferase/ indole-3-acetate beta-glu	-1,00	0,96	1,05	0,598	-1,17	0,13	0,45	0,16
AT1G51760	JR3__IAR3 (IAA-ALANINE RESISTANT 3); IAA-Ala conjugate hydrolase/ metallopeptidase	-1,20	0,07	1,20	0,144	1,05	0,57	0,02	0,29
AT5G54140	ILL3 (IAA-amino acid conjugate hydrolase)	1,36	0,02	-1,01	0,953	1,22	0,03	0,43	0,088
AT3G02875	ILR1 (IAA-LEUCINE RESISTANT 1)	-1,37	0,02	1,38	0,01	1,12	0,2	0,01	0,055
AT1G51780	ILL5 (IAA-amino acid conjugate hydrolase/ metallopeptidase)	-1,21	0,1	1,19	0,178	1,02	0,85	0,06	0,156
AT1G44350	ILL6 (IAA-amino acid conjugate hydrolase)	-2,70	0,01	1,40	0,069	-1,27	0,2	0,03	0,017

AUXIN TRANSPORT									
auxin influx									
AT2G38120	AUX1 (AUXIN RESISTANT 1)	-1,02	0,77	-1,20	0,01	-1,16	0,02	0,0	0,66
AT1G77690	LAX3 (LIKE AUX1 3)	-1,18	0,03	-1,24	0,013	-1,56	0	0,01	0,014
auxin efflux									
AT1G73590	PIN1	2,63	0	-1,33	0,066	2,13	0	0	4E-05
AT2G34570	MEE21 (maternal effect embryo arrest 21)	1,79	0	-1,05	0,633	1,53	0,01	0,04	1E-04
AT5G57090	PIN2	1,76	0,05	-1,31	0,346	1,27	0,23	0,14	0,059
AT2G01420	PIN4	1,43	0,01	1,14	0,106	1,39	0,01	0,89	0,044
AT1G23080	PIN7	1,43	0	-1,15	0,05	1,11	0,11	0	0,002
AT1G70940	PIN3	1,16	0,09	-1,10	0,314	-1,03	0,73	0,01	0,362
AT5G16530	PIN5	1,07	0,61	-1,00	0,976	-1,07	0,59	0,71	0,354
AT1G77110	PIN6	1,01	0,9	1,05	0,65	-1,03	0,7	0,2	0,3
AT1G77110	PIN6	1,01	0,9	1,05	0,65	-1,03	0,77	0,29	0,34
AT5G15100	PIN8	-1,05	0,57	1,09	0,253	-1,01	0,83	0,8	0,745
AT1G26530	PIN domain-like family protein	1,69	0,03	-1,03	0,845	1,77	0	0,3	0,021
AT2G46230	PIN domain-like family protein	1,38	0,01	1,08	0,469	1,31	0,02	0,8	0,001
Regulators of auxin transport									
AT2G34650	PID (PINOID)	2,92	0	1,67	2E-04	2,67	0	0,27	4E-05
AT5G54490	PBP1 (PINOID-BINDING PROTEIN 1)	2,67	0	2,11	8E-04	3,23	0	0,01	5E-04
AT2G26700	PID2 (PINOID2)	2,18	0	-1,23	0,186	1,77	0	0,09	0,002
AT1G53700	WAG1 (WAG 1)	-1,10	0,46	-1,53	0,005	-1,90	0	0	0,032
AT2G23050	NPY4 (NAKED PINS IN YUC MUTANTS 4)	2,04	0,03	1,21	0,548	2,84	0,01	0,05	0,004
AT4G37590	NPY5 (NAKED PINS IN YUC MUTANTS 5)	1,89	0	-1,16	0,033	1,60	0	0,0	7E-06
AT5G67440	NPY3 (NAKED PINS IN YUC MUTANTS 3)	-1,07	0,42	-1,18	0,075	-1,09	0,18	0,77	0,164
AT4G31820	NPY1/ENP (ENHANCER OF PINOID)	-1,85	0	-1,19	0,163	-1,72	0	0,4	0,023
AT3G14370	WAG2 (kinase/ protein serine/threonine kinase)	1,29	0,06	-1,16	0,21	1,59	0	0,1	8E-04
AT1G25490	EER1/RCN1 (ROOTS CURL IN NPA)	1,01	0,84	-1,05	0,439	-1,11	0,09	0,01	0,2
AT5G55910	D6PK (D6 PROTEIN KINASE)	1,19	0,02	-1,18	0,018	-1,01	0,8	0,01	0,014
AT2G18040	PIN1AT (PEPTIDYLPROLYL CIS/TRANS ISOMERASE, NIMA-INTERACTING 1); peptidyl-prolyl cis-trans isomerase	1,09	0,15	1,01	0,916	1,08	0,15	0,94	0,174
AT1G18350	BUD1/MKK7 (MAP kinase kinase/ kinase)	-1,15	0,15	1,36	0,011	-1,04	0,55	0,08	7E-05
AT2G43790	MAPK6_MPK6__ATMPK6 (ARABIDOPSIS THALIANA MAP KINASE 6); MAP kinase/ kinase	1,01	0,78	1,02	0,759	-1,02	0,61	0,53	0,389
AT1G13980	GN (GNOM)	1,18	0,03	-1,11	0,108	1,01	0,82	0,04	0,017
PGPs									
AT3G28860	PGP19/ABCB19	1,23	0,01	-1,36	0,001	1,14	0,03	0,22	3E-04
AT3G55030	PGPS2 (phosphatidylglycerolphosphate synthase 2); CDP-alcohol phosphatidyltransferase/ CDP-diacylglycerol-glycerol-3-phosp	1,22	0,01	-1,05	0,464	1,11	0,13	0,07	0,01
AT3G28380	PGP17 (P-GLYCOPROTEIN 17)	1,04	0,45	-1,09	0,209	-1,02	0,78	0,3	0,336
AT1G27940	PGP13 (P-GLYCOPROTEIN 13)	1,03	0,54	1,04	0,536	-1,04	0,39	0,19	0,138
AT3G28360	PGP16 (P-GLYCOPROTEIN 16)	1,03	0,7	-1,00	0,957	-1,05	0,41	0,6	0,323
AT4G25960	PGP2 (P-GLYCOPROTEIN 2)	1,01	0,83	1,02	0,785	1,03	0,56	0,67	0,968
AT1G02530	PGP12 (P-GLYCOPROTEIN 12)	-1,01	0,86	-1,11	0,207	1,00	0,96	0,3	0,143
AT4G01830	PGP5 (P-GLYCOPROTEIN 5)	-1,01	0,88	1,05	0,646	1,18	0,12	0,05	0,305
AT1G28010	PGP14 (P-GLYCOPROTEIN 14)	-1,02	0,75	1,06	0,323	1,01	0,89	0,96	0,185
AT3G28390	PGP18 (P-GLYCOPROTEIN 18)	-1,03	0,62	-1,06	0,344	-1,11	0,13	0,23	0,207
AT2G36910	PGP1/ABCB1 (ATP BINDING CASSETTE SUBFAMILY B1)	-1,08	0,29	-1,12	0,106	-1,32	0	0,04	0,017
AT5G46540	PGP7 (P-GLYCOPROTEIN 7)	-1,08	0,34	1,04	0,558	1,01	0,91	0,4	0,22
AT1G02520	PGP11 (P-GLYCOPROTEIN 11)	-1,10	0,25	-1,25	0,024	-1,11	0,12	0,72	0,116
AT4G01820	MDR3__PGP3 (P-GLYCOPROTEIN 3); ATPase, coupled to transmembrane movement of substances	-1,10	0,11	-1,00	0,93	-1,05	0,54	0,65	0,832
AT1G10680	PGP10 (P-GLYCOPROTEIN 10)	-1,12	0,13	1,09	0,222	-1,07	0,31	0,8	0,028
AT3G55320	PGP20 (P-GLYCOPROTEIN 20)	-1,21	0,01	-1,18	0,03	-1,47	0	0,01	0,01
AT2G47000	PGP4/ABCB4 (ATP BINDING CASSETTE SUBFAMILY B4)	-1,26	0,01	-1,18	0,034	1,01	0,9	0	0,02
AT3G62150	PGP21 (P-GLYCOPROTEIN 21)	-1,31	0	1,02	0,83	-1,20	0,03	0,2	0,023
AT4G18050	PGP9 (P-GLYCOPROTEIN 9)	-1,54	0,02	1,12	0,493	-1,08	0,54	0	0,251
AT2G39480	PGP6 (P-GLYCOPROTEIN 6)	-1,64	0	-1,32	0,002	-1,86	0	0,1	8E-04
Auxin signaling									
AT3G62980	TIR1 (TRANSPORT INHIBITOR RESPONSE 1)	1,11	0,19	-1,03	0,658	1,03	0,63	0,25	0,301
AT4G03190	AFB1 (AUXIN SIGNALING F-BOX 1)	1,13	0,27	-1,25	0,103	-1,16	0,07	0,01	0,45
AT3G26810	AFB2 (AUXIN SIGNALING F-BOX 2)	-1,05	0,42	-1,09	0,16	-1,10	0,11	0,25	0,679
AT1G12820	AFB3 (AUXIN SIGNALING F-BOX 3)	1,04	0,56	-1,07	0,296	-1,16	0,03	0,01	0,147
AT4G02570	CUL1 (CULLIN 1)	1,07	0,21	-1,02	0,722	1,05	0,32	0,57	0,241
AT2G02560	CAND1 (CULLIN-ASSOCIATED AND NEDDYLATION DISSOCIATED)	1,20	0,02	-1,00	0,943	1,05	0,4	0,06	0,228
AT3G28970	Domain of unknown function (DUF298)	1,00	0,95	1,02	0,813	1,03	0,48	0,9	0,653
AT5G20570	RBX1 (RING-BOX 1)	1,08	0,24	-1,01	0,824	-1,04	0,49	0,21	0,715
AT4G36800	RCE1 (RUB1 CONJUGATING ENZYME 1)	-1,24	0,02	1,02	0,715	-1,14	0,07	0,18	0,029
AT5G60450	ARF4	1,84	0	1,27	0,014	2,62	0	0	9E-05
AT4G30080	ARF16	1,65	0,15	-1,26	0,434	1,20	0,49	0,12	0,062
AT1G19220	ARF19	1,59	0	1,17	0,19	1,45	0	0,49	0,066
AT1G30330	ARF6	1,56	0	-1,12	0,342	1,23	0,05	0,01	0,002
AT5G62000	ARF2	1,36	0	1,19	0,044	1,43	0	0,74	0,019
AT5G37020	ARF8	1,35	0	-1,05	0,542	1,07	0,43	0,02	0,247
AT2G28350	ARF10	1,33	0,04	-1,27	0,067	-1,18	0,15	0	0,554
AT2G33860	ARF3	1,30	0,04	-1,03	0,78	1,20	0,05	0,24	0,069
AT1G19850	ARF5/MP (MONOPTEROS)	1,23	0,05	1,22	0,056	1,47	0	0,05	0,047
AT1G43950	ARF23	1,22	0,15	1,35	0,147	1,17	0,44	0,69	0,673
AT1G35240	ARF20	1,20	0,13	-1,15	0,27	-1,02	0,76	0,07	0,454
AT1G34410	ARF21	1,13	0,44	-1,15	0,447	-1,21	0,31	0,55	0,93
AT1G35520	ARF15	1,12	0,56	1,11	0,378	-1,04	0,81	0,80	0,540
AT4G23980	ARF9	1,10	0,14	1,06	0,571	1,28	0	0,03	0,066
AT1G59750	ARF1	1,08	0,31	1,62	1E-03	1,63	0	0	0,87
AT3G17185	TAS3/TASIR-ARF (TRANS-ACTING SIRNA3); other RNA	1,00	0,98	-1,39	0,005	-1,75	0	0	0,036
AT5G20730	ARF7/NPH4 (NON-PHOTOTROPHIC HYPOCOTYL)	-1,01	0,89	-1,09	0,24	-1,12	0,08	0,1	0,81
AT1G34170	ARF13	-1,03	0,66	-1,06	0,443	-1,05	0,51	0,45	0,594
AT1G77850	ARF17	-1,06	0,35	1,03	0,614	-1,10	0,06	0,31	0,073
AT1G34390	ARF22	-1,09	0,22	-1,14	0,048	-1,07	0,3	0,75	0,044
AT1G34310	ARF12	-1,11	0,43	-1,05	0,722	-1,25	0,12	0,33	0,15
AT1G35540	ARF14	-1,11	0,26	-1,14	0,079	-1,17	0,29	0,32	0,311
AT3G61830	ARF18	-1,17	0,26	1,31	0,047	1,16	0,03	0,05	0,379
AT5G57735	tasiR-ARF; other RNA	-1,18	0,02	-1,17	0,088	-1,25	0,26	0,72	0,449
AT2G46530	ARF11	-1,85	0	-1,07	0,322	-1,27	0,01	0	0,007

AT4G32280	IAA29	5,71	0	-1,06	0,624	3,79	0	0	2E-06
AT1G15580	IAA5/AUX2-27	3,42	0	4,34	3E-05	6,95	0	0	0,000
AT3G62100	IAA30	3,35	0	1,95	0,013	5,84	0	0	0,001
AT2G46990	IAA20	3,13	0	1,22	0,029	1,49	0	0	0,007
AT4G14560	IAA1/AXR5	2,65	0	1,21	0,321	2,74	0	0,45	8E-07
AT4G28640	IAA11	2,16	0	-1,23	0,149	1,47	0	0	0,002
AT5G43700	IAA4	1,99	0	-1,04	0,644	1,68	0	0,03	1E-04
AT3G17600	IAA31	1,94	0	1,83	0,003	2,46	0	0,05	0,047
AT3G16500	IAA26/PAP1 (PHYTOCHROME-ASSOCIATED PROTEIN 1)	1,44	0,02	1,27	0,042	1,61	0	0,35	0,024
AT3G23050	IAA7	1,28	0,01	-1,09	0,215	1,15	0,03	0,13	0,007
AT2G33310	IAA13	1,26	0,01	1,04	0,573	1,29	0	0,67	0,017
AT3G15540	IAA19	1,22	0,01	1,16	0,097	1,40	0	0,32	0,012
AT1G04550	IAA12	1,17	0,13	1,05	0,539	1,17	0,05	0,62	0,093
AT4G29080	IAA27/PAP2 (PHYTOCHROME-ASSOCIATED PROTEIN 2)	1,15	0,3	-1,14	0,372	-1,25	0,02	0,1	0,541
AT2G22670	IAA8	1,15	0,03	1,13	0,086	1,15	0,02	0,99	0,522
AT1G80390	IAA15	1,12	0,48	1,25	0,226	1,37	0,07	0,04	0,089
AT1G04240	IAA3/SHY2	1,10	0,19	2,18	1E-04	2,13	0	0	0,927
AT4G14550	IAA14	1,09	0,41	-1,66	0,003	-1,37	0	0,01	0,062
AT5G57420	IAA33	1,09	0,76	-1,35	0,279	-1,51	0,11	0,01	0,603
AT5G65670	IAA9	1,06	0,27	-1,03	0,546	1,06	0,24	0,9	0,074
AT3G04730	IAA16	1,03	0,64	1,08	0,322	1,06	0,32	0,32	0,729
AT3G23030	IAA2	-1,01	0,86	-1,00	0,952	-1,03	0,62	0,28	0,345
AT1G04250	IAA17	-1,02	0,71	1,19	0,022	1,18	0,02	0,01	0,724
AT1G04100	IAA10	-1,12	0,4	1,10	0,499	-1,05	0,55	0,24	0,286
AT1G15050	IAA34	-1,21	0,03	1,10	0,222	-1,12	0,25	0,31	0,036
AT1G51950	IAA18	-1,25	0,07	1,05	0,513	-1,24	0,06	0,9	0,025
AT5G25890	IAA28	-1,48	0	1,11	0,107	-1,07	0,3	0	0,008
AT1G29500	SAUR-like auxin-responsive protein family	8,66	0	1,85	0,123	7,03	0	0,01	2E-04
AT1G29490	SAUR-like auxin-responsive protein family	8,25	0	1,07	0,303	3,63	0	0,01	6E-05
AT4G13790	auxin-responsive protein, putative	6,24	0	-1,06	0,54	-1,23	0,1	0	0,524
AT1G29510	SAUR68	6,15	0	2,63	0,011	6,79	0	0,5	5E-05
AT1G29450	SAUR-like auxin-responsive protein family	6,11	0	1,02	0,937	2,37	0	0,01	3E-05
AT3G03820	SAUR-like auxin-responsive protein family	5,35	0	1,34	0,263	6,33	0	0,64	4E-04
AT1G29460	SAUR-like auxin-responsive protein family	5,15	0	1,12	0,378	2,34	0	0,01	3E-05
AT1G29440	SAUR-like auxin-responsive protein family	4,19	0	1,13	0,48	2,71	0	0,2	0,002
AT5G18080	SAUR-like auxin-responsive protein family	3,92	0	-1,28	0,114	2,57	0	0,02	1E-04
AT1G29430	SAUR-like auxin-responsive protein family	3,77	0	-1,09	0,818	1,74	0	0,01	0,059
AT5G18030	SAUR-like auxin-responsive protein family	3,49	0	1,42	0,026	2,17	0	0,06	0,002
AT4G12410	SAUR-like auxin-responsive protein family	3,14	0	1,63	0,003	3,49	0	0,33	2E-04
AT3G03850	SAUR-like auxin-responsive protein family	3,08	0	1,06	0,567	1,66	0	0,01	9E-04
AT3G03830	SAUR-like auxin-responsive protein family	2,80	0	1,04	0,796	1,72	0,01	0	0,01
AT5G18020	SAUR-like auxin-responsive protein family	2,76	0	-1,17	0,456	1,47	0,07	0,01	0,029
AT5G18060	SAUR-like auxin-responsive protein family	2,57	0	-1,05	0,804	1,86	0,01	0,01	0,011
AT5G27780	SAUR-like auxin-responsive protein family	2,52	0,05	1,14	0,536	1,33	0,18	0,03	0,409
AT5G20820	SAUR-like auxin-responsive protein family	2,42	0	1,43	0,02	2,20	0	0,03	0,059
AT4G36110	SAUR-like auxin-responsive protein family	2,37	0	2,78	0,002	3,45	0	0,01	0,224
AT3G03840	SAUR-like auxin-responsive protein family	2,11	0	1,06	0,58	1,55	0,02	0,02	0,010
AT2G45210	SAUR-like auxin-responsive protein family	1,97	0	1,81	0,015	2,54	0	0,15	0,101
AT4G38840	SAUR-like auxin-responsive protein family	1,93	0,07	1,14	0,639	1,90	0,07	0,71	0,006
AT5G50760	SAUR-like auxin-responsive protein family	1,91	0,03	-1,85	0,032	-1,59	0,06	0	0,207
AT5G20810	SAUR B	1,85	0	1,26	0,06	2,26	0	0,1	0,002
AT5G18050	SAUR-like auxin-responsive protein family	1,79	0,07	1,30	0,204	1,30	0,19	0,02	0,289
AT1G29420	SAUR-like auxin-responsive protein family	1,63	0,01	-1,03	0,805	1,50	0	0,42	0,015
AT3G12955	auxin-responsive protein-related	1,59	0,05	-1,09	0,717	2,14	0	0,2	0,01
AT4G38850	SAUR15	1,39	0,09	-1,25	0,149	1,71	0,01	0,26	1E-04
AT2G18010	auxin-responsive family protein	1,32	0,14	1,30	0,024	1,35	0,21	0,57	0,384
AT4G34760	auxin-responsive family protein	1,31	0,11	1,07	0,728	1,51	0,01	0,27	0,101
AT3G03847	auxin-responsive family protein	1,29	0,14	1,26	0,158	1,28	0,18	0,85	0,368
AT2G24400	SAUR D	1,26	0,26	-2,31	0,008	-1,34	0,02	0,04	0,055
AT4G38860	auxin-responsive protein, putative	1,25	0,31	-2,08	0,002	-1,45	0	0,01	0,002
AT3G51200	auxin-responsive family protein	1,18	0,11	1,15	0,148	1,16	0,09	0,56	0,555
AT3G20220	auxin-responsive protein, putative	1,14	0,06	-1,02	0,879	-1,45	0,12	0,3	0,101
AT5G10990	auxin-responsive family protein	1,13	0,27	1,90	2E-04	2,27	0	0	0,049
AT4G34770	auxin-responsive family protein	1,11	0,59	1,45	0,101	1,31	0,11	0,29	0,856
AT4G09530	auxin-responsive family protein	1,07	0,54	1,12	0,155	1,03	0,83	0,33	0,01
AT2G21200	auxin-responsive protein, putative	1,07	0,51	1,21	0,033	-1,13	0,64	0,43	0,707
AT3G60690	auxin-responsive family protein	1,06	0,29	1,51	2E-04	1,34	0	0,01	0,097
AT3G53250	auxin-responsive family protein	1,04	0,85	1,36	0,111	1,45	0,03	0,26	0,944
AT2G28085	auxin-responsive family protein	1,03	0,84	1,15	0,306	1,01	0,95	0,91	0,162
AT1G19830	auxin-responsive protein, putative	1,02	0,75	1,06	0,404	1,13	0,23	0,67	0,435
AT2G21210	auxin-responsive protein, putative	-1,01	0,91	1,18	0,16	1,12	0,16	0,12	0,949
AT4G34780	auxin-responsive family protein	-1,05	0,71	-1,03	0,85	-1,04	0,79	0,35	0,019
AT2G37030	auxin-responsive family protein	-1,07	0,65	-1,35	0,041	1,05	0,8	0,54	0,12
AT5G66260	auxin-responsive protein, putative	-1,08	0,7	1,04	0,83	-1,32	0,23	0,47	0,018
AT3G61900	auxin-responsive family protein	-1,12	0,21	-1,26	0,03	-1,39	0	0,02	0,206
AT1G79130	auxin-responsive protein	-1,12	0,4	1,24	0,033	-1,09	0,36	0,36	0,015
AT2G21220	auxin-responsive protein, putative	-1,13	0,36	1,18	0,389	-1,07	0,72	0,05	0,402
AT1G16510	auxin-responsive family protein	-1,14	0,56	1,12	0,412	1,09	0,46	0,32	0,779
AT1G56150	auxin-responsive family protein	-1,19	0,24	1,18	0,029	-1,06	0,53	0,2	0,01
AT3G43120	SAUR protein	-1,20	0,14	-1,02	0,706	-1,30	0,02	0,95	0,05
AT4G34750	SAUR E	-1,29	0,01	-1,17	0,18	-1,12	0,09	0,04	0,971
AT1G72430	auxin-responsive protein-related	-1,32	0	-1,20	0,015	-1,72	0	0	6E-04
AT4G22620	auxin-responsive family protein	-1,33	0,05	1,76	0,006	1,40	0,02	0	0,026
AT2G46690	auxin-responsive family protein	-1,33	0,29	-1,53	0,024	-1,42	0,02	0,3	0,445
AT1G20470	auxin-responsive family protein	-1,38	0,07	1,11	0,323	-1,11	0,25	0,05	0,045
AT5G42410	auxin-responsive family protein	-1,42	0,05	-1,22	0,207	-1,34	0,09	0,85	0,298
AT3G09870	auxin-responsive family protein	-1,44	0	1,15	0,054	1,13	0,17	0	0,499
AT1G19840	auxin-responsive protein	-1,45	0,01	-1,11	0,297	-2,11	0	0,02	2E-04
AT1G75580	auxin-responsive protein, putative	-1,47	0	1,98	1E-04	1,41	0	0	0,004
AT1G75590	auxin-responsive family protein	-1,51	0	-1,32	0,012	-2,76	0	0	4E-04
AT2G16580	auxin-responsive protein, putative	-1,53	0,04	-1,22	0,093	-2,06	0	0,19	0,004
AT4G34790	auxin-responsive family protein	-1,55	0,06	-1,17	0,202	-1,70	0	0,88	0,041
AT3G12830	auxin-responsive family protein	-1,62	0	-1,52	0,002	-3,11	0	0	1E-04
AT4G31320	SAUR C	-1,76	0	-2,34	3E-04	-3,67	0	0	0,003
AT5G53590	auxin-responsive family protein	-1,79	0	1,03	0,725	-1,26	0,02	0,02	0,025
AT5G03310	auxin-responsive family protein	-1,83	0	1,14	0,163	1,24	0,1	0	0,011
AT1G76190	auxin-responsive family protein	-1,92	0,01	-1,56	0,001	-2,37	0	0,35	0,014
AT1G43040	auxin-responsive protein, putative	-1,97	0,01	-1,10	0,654	-1,59	0,03	0,31	0,017
AT2G36210	auxin-responsive family protein	-2,39	0	-1,73	0,007	-1,11	0,54	0	0,058
AT1G17345	auxin-responsive family protein	-2,43	0	2,78	3E-04	1,14	0,26	0	0,001
AT4G34810	auxin-responsive protein	-2,44	0	-1,20	0,28	-3,14	0	0,25	3E-04
AT4G00880	auxin-responsive family protein	-2,60	0	-2,04	0,002	-2,50	0	0,03	0,516
AT4G34800	auxin-responsive family protein	-2,77	0	1,03	0,853	-2,36	0	0,53	0,002

Table S4: **Expression profile of genes involved in regulation of auxin activity**

Table S5

Table S5.	Expression profile of genes involved in regulation of cytokinin activity								
ID	Tair10 2011	auxin	Pvalue	CK	Pvalue	Aux/CK	Pvalue	Pvalue aux/CK vs aux	Pvalue aux/CK vs CK
Cytokinin biosynthesis									
IPT pathway									
AT5G20040	ATIPT9	1,49	0,0098	1,12	0,2776	1,34	0,0215	0,1597	0,0315
AT2G27760	ATIPT2	1,03	0,6007	-1,10	0,2217	-1,01	0,8767	0,1059	0,5808
AT4G24650	ATIPT4	1,01	0,8988	1,11	0,2389	-1,05	0,5269	0,1705	0,1513
AT3G19160	ATIPT8/PGA22	-1,00	0,9449	1,04	0,5464	-1,09	0,1742	0,3619	0,3468
AT1G25410	ATIPT6	1,08	0,4994	1,07	0,4468	-1,13	0,2182	0,1569	0,1825
AT3G63110	ATIPT3	-1,77	0,0097	1,10	0,5763	-1,69	0,0067	0,8875	0,0025
AT1G68460	ATIPT1	-1,25	0,0203	-1,82	9E-05	-2,21	0,0001	0,0007	0,0182
AT5G19040	ATIPT5	-1,13	0,1823	-2,92	8E-06	-2,92	2E-06	3E-04	0,9113
AT3G23630	ATIPT7	-2,65	0,0005	-8,33	2E-06	-14,25	3E-08	4E-05	0,0051
iPT to tZ conversion									
AT1G67110	CYP735A2	-6,19	1E-07	4,27	2E-06	2,42	2E-05	1E-07	0,0015
AT5G38450	CYP735A1	-6,58	8E-07	1,91	0,0025	-3,43	5E-05	0,0056	3E-05
LOG pathway									
AT2G35990	LOG2	1,25	0,1144	1,12	0,4193	2,33	0,0007	0,0069	0,0034
AT5G11950	LOG8	1,32	0,0039	1,24	0,0085	1,51	5E-05	0,1085	0,0062
AT5G06300	LOG7	1,18	0,073	-1,01	0,899	1,42	0,0007	0,0813	0,0004
AT4G35190	LOG5	-1,01	0,9562	1,12	0,3051	1,40	0,0227	0,0761	0,1576
AT5G26140	LOG9	-2,49	0,0009	1,01	0,9306	-1,28	0,2175	0,0375	0,0218
AT2G28305	LOG1	-2,24	0,0678	-1,39	0,2077	-1,70	0,0135	0,4318	0,3711
AT3G53450	LOG4	-1,36	0,0697	-1,50	0,0033	-1,86	0,0008	0,0999	0,1866
glucosylation									
AT5G05870	UGT76C1 (UDP-glucosyl transferase 76C1); UDP-glycosyltransferase/ cis-zeatin O-beta-D-glucosy	1,74	0,0037	1,10	0,5033	1,62	0,004	0,4244	0,004
AT5G05860	UGT76C2; UDP-glycosyltransferase/ cis-zeatin O-beta-D-glucosyltransferase/ cytokinin 7-beta-gl	-1,57	0,0189	-1,06	0,6035	1,28	0,0288	0,0009	0,0027
degradation									
AT5G56970	CKX3	-1,12	0,4679	2,39	0,0006	3,67	2E-05	1E-05	0,0047
AT1G75450	CKX5	-1,08	0,6372	2,79	9E-05	3,12	5E-06	0,0004	0,5491
AT2G41510	CKX1 (cytokinin oxidase/dehydrogenase 1)	1,45	0,0011	1,15	0,0755	3,10	4E-06	5E-05	1E-05
AT5G21482	CKX7	-1,13	0,7339	1,60	0,0431	1,07	0,5829	0,6043	0,0561
AT2G19500	CKX2	1,09	0,1272	1,16	0,0663	1,02	0,6195	0,0969	0,0311
AT4G29740	CKX4	-1,66	0,0021	-1,56	0,0015	-1,95	0,0001	0,0184	0,0745
Cytokinin deactivation									
AT5G11160	APT5 (adenine phosphoribosyl transferase 5)	-1,27	0,048	7,86	5E-07	2,59	6E-06	4E-05	1E-05
AT4G12440	APT4 (adenine phosphoribosyl transferase 4)	-4,43	1E-05	1,55	0,0559	-1,04	0,7729	7E-06	0,0422
AT1G80050	APT2 (adenine phosphoribosyl transferase 2)	-2,26	0,001	-1,73	0,002	-4,69	4E-06	0,0026	8E-05
AT2G46790	APRR9 (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR 9)	-1,07	0,6238	-1,60	0,0077	-1,10	0,179	0,7373	0,0072
AT3G09820	ADK1 (adenosine kinase 1)	1,00	0,9472	1,05	0,442	1,04	0,4521	0,4576	0,9082
AT5G03300	ADK2 (ADENOSINE KINASE 2)	-1,09	0,1525	1,04	0,4781	-1,02	0,6995	0,1471	0,4201
Cytokinin transport									
AT1G19770	ATPUP14	-1,39	0,0564	1,08	0,3815	-1,16	0,0966	0,2698	0,0444
AT1G57990	ATPUP18	-1,30	0,097	-1,15	0,1793	-1,25	0,0591	0,824	0,2576
AT1G28220	ATPUP3	1,18	0,1085	1,03	0,5767	1,25	0,0757	0,6265	0,4241
AT1G44750	ATPUP11	1,19	0,0158	1,14	0,0556	1,19	0,019	0,9507	0,3992
AT4G18197	Member of a family of proteins related to PUP1	1,23	0,0258	-1,17	0,1926	1,11	0,3838	0,0927	0,0684
AT5G41160	ATPUP12	1,29	0,0336	1,01	0,9205	1,09	0,4795	0,0499	0,347
AT4G18195	Member of a family of proteins related to PUP1	1,07	0,3986	1,10	0,3333	1,09	0,2501	0,4382	0,9018
AT4G18190	Member of a family of proteins related to PUP1	-1,05	0,6055	1,11	0,2328	1,01	0,9361	0,4744	0,1696
AT1G75470	ATPUP15	1,01	0,8875	1,14	0,1662	-1,02	0,8834	0,1599	0,0857
AT2G24220	ATPUP5	-1,21	0,051	1,11	0,1991	-1,06	0,352	0,1758	0,0168
AT4G18210	ATPUP10	1,06	0,6633	-1,04	0,6973	-1,07	0,2945	0,3252	0,5718
AT1G19770	ATPUP14	-1,39	0,0564	1,08	0,3815	-1,16	0,0966	0,2698	0,0444
AT1G57943	ATPUP17	-1,23	0,1754	1,01	0,9305	-1,24	0,1014	0,3184	0,1405
AT1G28230	ATPUP1	-1,03	0,6644	1,14	0,2073	-1,25	0,0251	0,1506	0,005
AT1G57990	ATPUP18	-1,30	0,097	-1,15	0,1793	-1,25	0,0591	0,824	0,2576
AT2G33750	Member of a family of proteins related to PUP1	-1,25	0,0352	1,11	0,4124	-1,26	0,0502	0,1941	0,1105
AT1G09860	Member of a family of proteins related to PUP1	-1,27	0,0534	-1,21	0,0239	-1,42	0,0094	0,0249	0,102
AT1G47603	Member of a family of proteins related to PUP1	-2,56	0,0005	1,14	0,3242	-1,74	0,0041	0,0016	0,0007
AT1G30840	ATPUP4	-2,57	0,0028	1,49	0,0247	-1,89	0,0028	0,2048	3E-06
AT1G02630	ENT8 (Equilibrative Nucleoside Transporter)	-1,10	0,3424	-1,22	0,0266	-1,08	0,4248	0,7419	0,2164
AT1G61630	equilibrative nucleoside transporter 7	-1,00	0,9595	1,07	0,4567	-1,15	0,1615	0,1622	0,0737
AT4G05130	equilibrative nucleoside transporter 4	-1,37	0,0643	-1,10	0,403	-1,27	0,0806	0,9172	0,0344
AT4G05140	equilibrative nucleoside transporter family protein	-1,16	0,0977	-1,05	0,6667	-1,28	0,0207	0,237	0,2472
AT3G09990	Nucleoside transporter family protein	-1,13	0,1379	-1,02	0,805	-1,31	0,0021	0,1383	0,1109
AT4G05110	equilibrative nucleoside transporter 6	-1,96	2E-05	-1,12	0,4432	-2,05	3E-05	0,9351	0,0031

Cytokinin signalling									
AT2G01830	AHK4/CRE1/WOL1	-1,01	0,9075	1,13	0,0428	1,24	0,0055	0,0166	0,0842
AT5G35750	AHK2	-1,65	0,0003	-1,23	0,0178	-1,59	4E-05	0,9573	0,0019
AT1G27320	AHK3	-1,09	0,235	-1,08	0,5126	-1,14	0,0869	0,5118	0,3866
AT2G47430	CKI1 (CYTOKININ-INDEPENDENT 1)	1,04	0,4794	1,15	0,1271	1,08	0,2731	0,14	0,7574
AT3G21510	AHP1	-1,26	0,0234	4,63	2E-06	2,84	1E-05	1E-06	0,0007
AT5G19710	FUNCTIONS IN: unknown molecular functions unknown; INVOLVED IN: biological_process unkno	1,07	0,6459	1,60	0,0029	1,22	0,2957	0,2866	0,0264
AT1G03430	AHP5	1,16	0,1219	1,32	0,0103	1,46	0,0022	0,0121	0,1317
AT5G39340	AHP3	-1,21	0,0314	1,20	0,0607	-1,23	0,0204	0,4381	0,0025
AT3G29350	AHP2	-1,11	0,127	1,10	0,1437	-1,03	0,6905	0,3985	0,0189
AT3G16360	AHP4	-3,93	0,0003	-1,11	0,4453	-1,40	0,1894	0,0077	0,1365
AT1G80100	AHP6	3,79	0,0002	-1,24	0,1172	-1,02	0,8759	0,0011	0,7372
AT2G40670	ARR16	-1,23	0,0406	32,35	8E-09	17,04	3E-08	2E-07	9E-05
AT1G19050	ARR7	2,29	0,02	16,45	1E-05	27,09	3E-06	1E-06	0,008
AT1G74890	ARR15	-1,40	0,0863	8,07	1E-05	11,63	3E-06	1E-05	0,0009
AT3G56380	ARR17	-1,76	0,0332	7,00	0,0001	2,19	0,033	0,0028	0,0024
AT5G62920	ARR6	-3,33	3E-05	2,38	0,0004	1,93	0,0004	2E-07	0,0753
AT1G59940	ARR3	-1,35	0,0302	2,22	5E-05	2,31	2E-05	2E-05	0,7265
AT3G48100	ARR5	-1,89	0,001	2,17	0,0003	2,04	0,0003	2E-06	0,2782
AT1G10470	ARR4	1,05	0,5544	1,91	3E-05	2,22	2E-06	6E-07	0,0165
AT2G41310	ARR8	1,02	0,7685	1,40	0,0024	1,54	0,0001	0,0002	0,03
AT5G62120	ARR23	1,05	0,5569	1,26	0,0112	1,11	0,1969	0,8597	0,0031
AT3G57040	ARR9	-1,21	0,045	1,07	0,0427	1,34	0,0036	6E-05	0,0093
AT3G04280	ARR22	1,15	0,326	-1,04	0,7729	-1,23	0,1895	0,0031	0,3259
AT2G01760	ARR14	1,04	0,7332	-1,12	0,4524	1,26	0,158	0,0997	0,0089
AT2G25180	ARR12	-1,30	0,0031	1,11	0,0969	-1,05	0,2871	0,0041	0,0095
AT5G49240	APRR4 (PSEUDO-RESPONSE REGULATOR 4)	-1,03	0,7157	1,09	0,3476	-1,02	0,7571	0,3535	0,8657
AT2G27070	ARR13	-1,08	0,2728	1,04	0,6328	-1,13	0,211	0,1277	0,1481
AT3G62670	ARR20	1,07	0,4849	1,03	0,7755	-1,15	0,213	0,0555	0,2684
AT5G07210	ARR21	-1,10	0,1622	1,01	0,9083	-1,03	0,7364	0,9264	0,6936
AT1G49190	ARR19	-1,03	0,6369	-1,02	0,7215	-1,01	0,8768	0,8853	0,7379
AT4G00760	APRR8 (PSEUDO-RESPONSE REGULATOR 8)	-1,17	0,1897	-1,03	0,7636	-1,16	0,1846	0,469	0,0879
AT5G58080	ARR18	-1,08	0,3711	-1,06	0,5011	-1,11	0,1751	0,3541	0,4502
AT2G01760	ARR14	1,04	0,7332	-1,12	0,4524	1,26	0,158	0,0997	0,0089
AT4G16110	ARR2	-1,32	0,0217	-1,29	0,009	-1,51	0,0003	0,1253	0,0065
AT3G16857	ARR1	1,01	0,893	-1,35	0,0016	-1,31	0,0008	0,0009	0,5929
AT4G31920	ARR10	-1,06	0,3469	-2,13	3E-05	-1,49	0,0004	0,0002	0,0006
AT1G67710	ARR11	-1,44	0,0194	-2,14	0,0002	-2,78	7E-06	0,0034	0,0717

Table S5: **Expression profile of genes involved in regulation of cytokinin activity**

Table S6 (Supplementary Excel File): Expression profile of genes regulated by auxin and cytokinin in a non-additive manner

Table S7

	Name	Primer
Genotyping	arr1-2_LP	GAGCAGCAGAGTGTTACCGAC
	arr1-2_RP	TTGCTTTGATTTCACGTTGTG
	arr2-1_LP	TTATTAAATGCCAGTGGCAGC
	arr2-1_RP	CGACAAGAACTCGAAGATTCG
	arr3-1_LP	ACATGGTCATGGTTTAGCGAC
	arr3-1_RP	TTTTTGATTCCGATTTTGGTG
	arr4-1_LP	TTTATGTGCGACACGTTGATGACTACTTT
	arr4-1_RP	GGAGGCGCGAGAGATTAAAGGGACATCTAT
	arr5-1_LP	TCTCTCTGTGGTACATTTCTTGAAAAATGGG
	arr5-1_RP	CTTGGGGAAATTTCTAAGAAAAGCCATGTA
	arr6-1_LP	AAATCTTGCATCCCATTCCA
	arr6-1_RP	TCCTGAAGCACAAATCACGA
	arr7-1_LP	TCATATCCTGAAAGTCCTGGC
	arr7-1_RP	TAATTGAGCAATAACCACCGG
	arr9-1_LP	GGATCCCAGACTCTTTATTTCTCTTCCTC
	arr9-1_RP	CCCACATACAACATCATCATCATATTCC
	arr10-1_LP	TATCGGCATTAGCCATTATTGGTCGTTAC
	arr10-1_RP	ATGCGGTCTGTGCCTGATTCGTTGTTGTA
	arr11-1_LP	GCTAAATTATTGGAAGAATATGGG
	arr11-1_RP	TTCACCACCTCCAAGAAAATG
	arr12-1_LP	CGGTACAATATGCGGATTTTGATTCGGTAT
	arr12-1_RP	TAATAGCTTGCTGATTAGCCACACCACTGA
	arr13-1_LP	TGTGATGATCAAGGATGGAAC
	arr13-1_RP	TCAATCAAACTATCGTTTCAATGTC
	arr14-1_LP	CACAAGCTCCATGGTTGATTC
	arr14-1_RP	TATCTCCAACATCGCCATTTC

	arr15-1_TP	ATATTGACCATCATACTCATTGC
	arr15-1_LP	AAACCAAACAAAAGAAAAGCAGAA
	arr15-1_RP	TGTGAACTTTCAATTGATTCACC
	arr16-1_LP	AGACCTTTCTTGCTGCACAAG
	arr16-1_RP	ATTGGAGTATTTGGGTTTGGG
	iaa12-1_TP	GGCAATCAGCTGTTGCCCGTCTCACTGGTG
	iaa12-1_LP	cagTCAAGTGGTAGGATGGCCACCAATT
	iaa12-1_RP	CTTCTGAGGTTCCCATGATCCGAAGCCT
	iaa14-1_TP	GGCAATCAGCTGTTGCCCGTCTCACTGGTG
	iaa14-1_LP	CTTAAAGACCCTTCTAAGCCTCCTG
	iaa14-1_RP	GCATGACTCGACAAACATCCTG
	Iaa17-1_LP	CGATTTTCCTCAAGTACGGTG
	Iaa17-1_RP	TTTCCTTCACTTGTGCTTTCG
	iaa19-1_LP	ATACCCCCAAGGTACATCACC
	iaa19-1_RP	AGATGAATATGACGTCGTCGG
	Iaa20-1_LP	TGGAACTCCTTCCATGTTCAC
	Iaa20-1_RP	CCGTTTTAGACCGATTATGGC
	iaa28-2_LP	TTTGTCTCATGAGTCACGGTG
	iaa28-2_RP	CACCACTGGAGCTACCTCAAC
	Iaa29-1_LP	GTAGCCAGTCACCCTCTTTCC
	Iaa29-1_RP	CGAACACAACCTTTTCCAAAG
	LBb1	GCGTGGACCGCTTGCTGCAACT
	LBa1	TGGTTCACGTAGTGGGCCATCG
	LBb1_3	ATTTTGCCGATTTCGGAAC
RT-PCR	qARR1_for	TTGAAGAAACCGCGTGTCGTCT
	qARR1_rev	CCTTCTCAACGCCGAGCTGATTAA
	qARR2_for	GGAATGATGTTGCCAGTAGC

	qARR2_rev	GTTATTGAAGACCGAGTGAGTAG
	qARR3_for	CCGTTGATGACAGCCTAGTTGA
	qARR3_rev	CGTGACTTTGCAGGATGTGATT
	qARR4_for	CTGTATGCCTGGAATGACT
	qARR4_rev	AATAAGAAATCTTGAGCACCT
	qARR5_for	ACACTTCTTCATTAGCATCACCG
	qARR5_rev	CTCCTTCTTCAAGACATCTATCGA
	qARR6_for	GTCTACCCTGTTCACTCG
	qARR6_rev	AGAATCATCAGTGTAGGCT
	qARR7_for	GCATTCAGAGAAGTACCAGTAGTG
	qARR7_rev	GCTAAGGTCTTGGCCTCTATAC
	qARR9_for	TTCCTGCAAGAATCAGCAGATGTT
	qARR9_rev	AGTTGTCTCAATCTCCTCCAGCTT
	qARR10_for	TCAGAAATTCCGCGTTGCTCTGAA
	qARR10_rev	TGTGAGTCAATAGCCGCCCTGTTA
	qARR11_for	TAATGATGTCGGTGGACGGCGAAA
	qARR11_rev	AAGCTCCGTGTTGCACTCCCTTCA
	qARR12_for	GGCCAGTCATCTTCAGAAATTCCG
	qARR12_rev	TGATTAGCCACACCACTGATCCTC
	qARR13-LP	CCCTAATCAGAATCAGGGACAAGC
	qARR13-RP	ACTTGAACCCGAAATACCCGATCC
	qARR14-LP	TCCTGGAAACTCGAAGAAGTCACG
	qARR14-RP	AGAATCCGCTTTGGTACAGCTTTG
	qARR15_for	CTTCAGCACTCAGAGAAATCC
	qARR15_rev	GTCTCTAGATTAACCCCTAGACTCT
	qARR16_for	ATCACCGATTACTGTATGCC
	qARR16_rev	GCTTCTGCAGTTCATGAGAT
	qIAA1_for	TTGGGATTACCCGGAGCACAAG

qIAA1_rev	GCGCTTGTTGTTGCTTCTGACG
qIAA4_for	GTTGGTGATGTTCCTTGGGAGATG
qIAA4_rev	GGTTTGTTAAAGACCACCACAACC
qIAA5_for	TCCGCTCTGCAAATTCTGTTCG
qIAA5_rev	ACGATCCAAGGAACATTTCCCAAG
qIAA6_for	TGCCAAGGTACATCTCCGACGA
qIAA6_rev	CATAGGAGTGGCGAAGGAGGGTAA
qIAA7_for	TCTGCTGTTCCCAAGGAGAAGACT
qIAA7_rev	GCCATCCCACCACTTGTGCTTTAG
qIAA12_for	TGGGTCTAAACGCTCTGCTGAATC
qIAA12_rev	ACCACTTGACTTGAACGAGGAGGA
qIAA14_for	ACGAGGACAAAGATGGTGACTGGA
qIAA14_rev	ATGACTCGACAAACATCGGCCAGG
qIAA17_for	GCCAAGGCACAAGTTGTGGGAT
qIAA17_rev	TTTGGCAGGAAACCATCACGTTCT
qIAA19_for	TCGGTGTGGCCTTGAAAGATGG
qIAA19_rev	TGCATGACTCTAGAAACATCCCCC
qIAA20_for	TGTTCAACGCATCCATTCTCTGG
qIAA20_rev	GCACGTGACTCTTCTCATTGCAC
qIAA28_for	GCTCCTCCTTGTCACCAATTCACT
qIAA28_rev	ACTGGAGCTACCTCAACCCTGTTA
qIAA29_for	GCGACGTTTGGGTTAGGGAATG
qIAA29_rev	GCCATTCAAGGCAAACAAACGC
qUBQ10_for	CACACTCCACTTGGTCTTGCGT
qUBQ10_rev	TGGTCTTTCCGGTGAGAGTCTTCA

Table S7: **Primers used for the genotyping and quantification of gene expression levels.**

Table S8

Gene	Forward	Reverse
AT1G02380	AGACGTCACCATCATCTCCATGC	ACTGCCTGTGGAGAATCCTTGG
AT1G02460	ACCGATTCAGTGTTGCTTGTTCC	TGTTACAATGGTCCCATCCACTTG
AT1G15600	ACTTCTGGTTATGTTTGGCTCTCC	ACACATATGACCACAGGCGTAAG
AT1G17430	CATGCTTATCGGCTGCAAAGGC	TGTTAACAGCGTGTCCTGTTTCC
AT1G18870	GCCTAGAGGTGATTCAAAGGTTCG	ACTCAAGATCGTCTTTGGGACTGG
AT1G49560	CCAAAGGTGGAAACTGGTTTAGGC	AGCCACATGAGGCAACAATCCC
AT1G64080	AACAGCGACTACACCGTCGTCTAC	AGTCGTCGCGTCTTCTTGACTC
AT2G19410	TGGATCCATTAATCAACCGAGAGC	AGGATCGGGTAACGTTGGAGAC
AT2G28200	TGACGCAACCCAAGTCTAGTGC	TAGGGCTGAGCCGGAAACTTTG
AT2G35270	TTCTCGCTACGTCCACAACGTC	ACCGTCATCTTCGTGGTGGTTG
AT2G43140	GAGGATAAGCGGGAAGCTGAAG	ATCTGCGTAGCTCGTTTGCTTG
AT3G13080	ATGGTTCTGCTTCTAAGCAATGGG	TTGAGGTGTACTCAGCCACAAGC
AT3G47470	CCGCTTAACTTTGCTCCTACGC	AACATCGCCAACCTCCCGTTTG
AT4G14130	GGCACCGTCACTGCTTACTACTTG	TGTTGCTCTCTGTCTCCTTTCCC
AT4G25640	CTCCGCCGTCTCTATCTCTCTTTC	AGAGCACTTCCCATACCAAGCAAG
AT4G32300	ATCTGCACTTGGTGCGGCTAAG	ACTCGTATGCGAGCAGCCTATG
AT5G23210	CCCGTCTCATCTCTCACAATGAAG	CATTAGCGTGAAGTGCCTCCTG

Table S8: Primers used for qRTPCR analysis of auxin and cytokinin synergistically regulated gene expression levels.

Table S9

AGI code	salk line	LP	RP
AT1G02380	Salk_129654	TCATGTTCTAGGAAGTTGCGAG	ATTCGCTTCCTCGTGTATCTG
AT1G02460	Salk_093618	TTTGAGACACATGCTCGTGAG	ACAAAAGTGGTGGGATCTTCC
AT1G15600	Salk_151420	GTGTTCTCCTTGCTGTGGAAG	ACATAAAGCCCTCTCCGATTC
AT1G17430	Salk_042510	TTCCCAATTCATCGCTGTAAG	CTTTGATGACTCAGCAGTTGATG
AT1G18870	Salk_073287	ACAATCCCAGTTCGATCTTCC	TCGGATTGATCTCCAGTCATC
AT1G49560	Salk_095775	TGGTCCAACATTAAATGCAATAG	AAACTTTCAGCAACCCAAATG
AT1G64080	Salk_070770	GGAGGAGCCAAGTTTTGTTTC	ACATCAACATTCCAGCTGGTC
AT2G19410	Salk_140776	TGGATCCATTAATCAACCGAG	TCATTCTTCAAACGTTCGACC
AT2G28200	Salk_137213	AATCCTATAAACACCGGCCAC	ACTCCCAACCTCATCTTGACC
AT2G35270	Salk_094394	TCACCAACTACGTTACCTCCG	AATCCCATTTTAGTCCGTGTTG
AT2G43140	Salk_123812	TTTTGTCATGGTCTGCCTTTC	AACGAAAAAGTTACCGTTGGG
AT3G13080	Salk_044022	AGGGAGCCTTTTTATGCTCAC	AAGTCAGCAATTGCTTTGGAG
AT3G47470	Salk_138555	GTTCTTCCGGTAGGTTGAACC	AATAGTGTTGTGCCATGGGTC
AT4G14130	Salk_039464	GGCCTGATCATGAGAATAAAGG	TCCTCATGGGTTGACTCTTTG
AT4G25640	Salk_057798	CTCAAGCCCATCAGCTACATC	TGGCTTGAGACAGTGATCATG
AT4G32300	Salk_105027	TCCATCTTTGAATTCCACCTG	TCAAAGATGATGAAATTCCGG
AT5G23210	Salk_053542	ATTTACCACACACGCTTTTGG	CCCCAGTACAAGTCACACGAC

Table S9: **Primers used for genotyping of auxin and cytokinin synergistically regulated gene expression levels.**

Table S10

Gene	Line	Relative expressio	stdev
ARR1	Ws	1	0.0098
	arr1-2	0.018	0.0016
ARR2	Col	1	0.0490
	arr2-1	0.101	0.0207
ARR10	Ws	1	0.0060
	arr10-1	0.059	0.0003
ARR11	Ws	1	0.0533
	arr11-1	0.009	3.67E-05
ARR12	Col	1	0.0490
	arr12-1	0.003	0.0001
ARR13	Col	1	0.0717
	arr13-1	0.078	0.0057
ARR14	Col	1	0.0930
	arr14-1	0.097	0.0120

Gene	Line	Relative expressio	stdev
ARR3	Col	1	0.0683
	arr3-1	0.015	0.0068
ARR4	Col	1	0.0687
	arr4-1	0.080	0.0084
ARR5	Col	1	0.0330
	arr5-1	0.025	5.96E-18
ARR6	Col	1	0.1214
	arr6-1	0.058	0.0056
ARR7	Col	1	0.0112
	arr7-1	0.067	1.21E-17
ARR15	Col	1	0.0303
	arr15-1	0.004	0.0004
ARR16	Col	1	0.0446
	arr16-1	0.102	1.85E-07

Table S10: **Relative expression level of *ARR* genes to confirm loss of their expression in mutant alleles.**

2.6 References

Abel, S., and Theologis, A. (1996) Early genes and auxin action. *Plant Physiol* 111, 9-17.

Adamowski, M., Friml, J. (2015) PIN-dependent auxin transport, action, regulation, and evolution. *Plant Cell* 27, 20-32.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115, 591-602.

Benjamins R, Ampudia CS, Hooykaas PJ, Offringa R. (2003) PINOID-mediated signaling involves calcium-binding proteins. *Plant Physiol*. 132:1623-30.

Bielach, A., Podlešáková, K., Marhavý, P., Duclercq, J., Cuesta, C., Müller, B., Grunewald, W., Tarkowski, P., and Benková, E. (2012). Spatiotemporal regulation of lateral root organogenesis in *Arabidopsis* by cytokinin. *Plant Cell* 24, 3967-3981.

Birnbaum K, Jung JW, Wang JY, Lambert GM, Hirst JA, Galbraith DW, Benfey PN. (2005)Cell type-specific expression profiling in plants via cell sorting of protoplasts from fluorescent reporter lines. *Nat Methods.* 2:615-9.

Bhargava A, Clabaugh I, To JP, Maxwell BB, Chiang YH, Schaller GE, Loraine A, Kieber JJ. (2013) Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-Seq in Arabidopsis. *Plant Physiol.* 162:272-94

Brunetti P, Zanella L, De Paolis A, Di Litta D, Cecchetti V, Falasca G, Barbieri M5, Altamura MM, Costantino P, Cardarelli M (2015) Cadmium-inducible expression of the ABC-type transporter AtABCC3 increases phytochelatin-mediated cadmium tolerance in Arabidopsis. *J. Exp Bot*. 66:3815-29.

Casimiro, I., Marchant, A., Bhalerao, R.P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inze, D., Sandberg, G., Casero, P.J., and Bennett, M. (2001). Auxin transport promotes Arabidopsis lateral root initiation. Plant Cell 13, 843-852.

Chandler JW, Werr W. (2015) Cytokinin-auxin crosstalk in cell type specification. *Trends Plant Sci.* 20:291-300.

Che P, Lall S, Nettleton D, Howell SH. (2006) Gene expression programs during shoot, root, and callus development in Arabidopsis tissue culture. *Plant Physiol*. 141:620-37

Cheng Y, Qin G, Dai X, Zhao Y. (2007) NPY1, a BTB-NPH3-like protein, plays a critical role in auxin-regulated organogenesis in Arabidopsis. *Proc Natl Acad Sci U S A*. 104: 18825-9.

Cheng Y, Qin G, Dai X, Zhao Y. (2008) NPY genes and AGC kinases define two key steps in auxin-mediated organogenesis in Arabidopsis. *Proc Natl Acad Sci U S A*. 105:21017-22.

De Rybel, B., Vassileva, V., Parizot, B., Demeulenaere, M., Grunewald, W., Audenaert, D., Van Campenhout, J., Overvoorde, P., Jansen, L., Vanneste, S., Moller, B., Wilson, M., Holman, T., Van Isterdael, G., Brunoud, G., Vuylsteke, M., Vernoux, T., De Veylder, L., Inze, D., Weijers, D., Bennett, M.J., and Beeckman, T. (2010). A novel aux/IAA28 signaling cascade activates GATA23-dependent specification of lateral root founder cell identity. *Curr Biol* 20, 1697-1706.

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N.F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inze, D., Bennett, M.J., and Beeckman, T. (2007). Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. Development 134, 681-690.

De Smet I, Vassileva V, De Rybel B, Levesque MP, Grunewald W, Van Damme D, Van Noorden G, Naudts M, Van Isterdael G, De Clercq R, Wang JY, Meuli N, Vanneste S, Friml J, Hilson P, Jürgens G, Ingram GC, Inzé D, Benfey PN, Beeckman T. (2008) Receptor-like kinase ACR4 restricts formative cell divisions in the *Arabidopsis* root. 322:594-7.

De Smet, I., Lau, S., Voss, U., Vanneste, S., Benjamins, R., Rademacher, E.H., Schlereth, A., De Rybel, B., Vassileva, V., Grunewald, W., Naudts, M., Levesque, M.P., Ehrismann, J.S., Inze, D., Luschnig, C., Benfey, P.N., Weijers, D., Van Montagu, M.C., Bennett, M.J., Jurgens, G., and Beeckman, T. (2010). Bimodular auxin response controls organogenesis in Arabidopsis. *Proc Natl Acad Sci U S A* 107, 2705-2710.

Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and 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, M.T., Aoyama, T., Costantino, P., and Sabatini, S. (2008) A genetic framework for the control of cell division and differentiation in the root meristem. *Science* 322, 1380-1384.

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

Dubrovsky, J. G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M. G., Friml, J., Shishkova, S., Celenza, J., and Benková, E. (2008). Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. *Proc. Natl. Acad. Sci. USA* 105, 8790–8794.

Duclercq J, Sangwan-Norreel B, Catterou M, Sangwan RS. (2011) De novo shoot organogenesis: from art to science. *Trends Plant Sci.* 16:597-606.

Ferreira, P.C., Hemerly, A.S., Engler, J.D., van Montagu, M., Engler, G., and Inze, D. (1994). Developmental expression of the arabidopsis cyclin gene cyc1At. Plant Cell 6, 1763-1774.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., Sandberg, G., Hooykaas, P.J., Palme, K., and Offringa, R. (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science* 306, 862-865.

Fukaki, H., Tameda, S., Masuda, H., and Tasaka, M. (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. Plant J 29, 153-168.

Garcion C1, Lohmann A, Lamodière E, Catinot J, Buchala A, Doermann P, Métraux JP (2008) Characterization and biological function of the ISOCHORISMATE SYNTHASE2 gene of Arabidopsis. *Plant Physio*l. 147:1279-87.

Gälweiler L, Guan C, Müller A, Wisman E, Mendgen K, Yephremov A, Palme K. (1998) Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science.* Dec 18;282(5397):2226-30.

Higuchi, M., Pischke, M.S., Mahonen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Helariutta, Y., Sussman, M.R., and Kakimoto, T. (2004). In planta functions of the Arabidopsis cytokinin receptor family. *Proc Natl Acad Sci U S A* 101, 8821-8826.

Hulsen, T., de Vlieg, J., & Alkema, W. (2008). BioVenn–a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC genomics*, *9*(1), 488.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001) Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* 409, 1060-1063.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U. and Speed, T.P. (2003) Exploration, normalization, and summaries of high density **oligonucleotide array probe level data. *Biostatistics*, 4, 249–264.**

Jones, B., Gunneras, S.A., Petersson, S.V., Tarkowski, P., Graham, N., May, S., Dolezal, K., Sandberg, G., and Ljung, K. (2010) Cytokinin regulation of auxin synthesis in *Arabidopsis* involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction. *Plant Cell* 22, 2956-2969.

Kakimoto, T. (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274, 982-985.

Kasahara, H., (2016) Current aspects of auxin biosynthesis in plants. *Bioscience, Biotechnology, and Biochemistry*, 80:1, 34-42,

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

Kieber, J.J. and Schaller G.E.(2014) Cytokinins. The Arabidopsis Book, e0168.

Kuroha T, Tokunaga H, Kojima M, Ueda N, Ishida T, Nagawa S, Fukuda H, Sugimoto K, Sakakibara H. (2009) Functional analyses of LONELY GUY cytokinin-activating enzymes reveal the importance of the direct activation pathway in Arabidopsis. *Plant Cell* 21:3152-69.

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M.B., Offringa, R., Graham, N., Doumas, P., Friml, J., Bogusz, D., Beeckman, T., and Bennett, M. (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. *Plant Cell* 19, 3889-3900.

Laplaze L, Parizot B, Baker A, Ricaud L, Martinière A, Auguy F, Franche C, Nussaume L, Bogusz D, Haseloff J. (2005) GAL4-GFP enhancer trap lines for genetic manipulation of lateral root development in Arabidopsis thaliana. *J Exp Bot.* 56:2433-42

Leyser, H. M., Pickett, F. B., Dharmasiri, S. & Estelle, M. (1996). Mutations in the AXR3 gene of Arabidopsis result in altered auxin response including ectopic expression from the SAUR-AC1 promoter. *Plant J.* 10, 403–413

Li, X., Mo, X., Shou, H., and Wu, P. (2006). Cytokinin-mediated cell cycling arrest of pericycle founder cells in lateral root initiation of Arabidopsis. *Plant Cell Physiol* 47, 1112-1123.

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

Luschnig, C., Gaxiola, R. A., Grisafi, P., and Fink, G. R. (1998). EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in Arabidopsis thaliana. *Genes Dev.* 12, 2175–2187.

López-Bucio, J., Cruz-Ramírez, A., and Herrera-Estrella, L. (2003). The role of nutrient availability in regulating root architecture. *Curr. Opin. Plant Biol*. 6, 280–287.

Mähönen AP, Bishopp A, Higuchi M, Nieminen KM, Kinoshita K, Törmäkangas K, Ikeda Y, Oka A, Kakimoto T, Helariutta Y. (2006) Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. *Science.* 311:94-8.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. *Development* 124, 33-44.

Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ. (1999) AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. *EMBO J.* Apr 15;18(8):2066-73.

Mason, M.G., Mathews, D.E., Argyros, D.A., Maxwell, B.B., Kieber, J.J., Alonso, J.M., Ecker, J.R., and Schaller, G.E. (2005). Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. *Plant Cell* 17, 3007-3018.

Moreno-Risueno, M.A., Van Norman, J.M., Moreno, A., Zhang, J., Ahnert, S.E., and Benfey, P.N. (2010). Oscillating gene expression determines competence for periodic Arabidopsis root branching. *Science* 329, 1306-1311.

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., and Benková, E. (2011) Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis. *Dev Cell* 21, 796-804.

Marhavý P, Duclercq J, Weller B, Feraru E, Bielach A, Offringa R, Friml J, Schwechheimer C, Murphy A, Benková E: Cytokinin Controls Polarity of PIN1-Dependent Auxin Transport during Lateral Root Organogenesis. *Curr. Biol.* 2014, **24**:1031–1037.

Medici A, Marshall-Colon A, Ronzier E, Szponarski W, Wang R, Gojon A, Crawford NM, Ruffel S, Coruzzi GM, Krouk G. (2015) AtNIGT1/HRS1 integrates nitrate and phosphate signals at the Arabidopsis root tip. *Nat Commun.* 6:6274.

Müller, B., and Sheen, J. (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453, 1094-1097.

Nagpal, P. et al. AXR2 (2000). Encodes a Member of the Aux/IAA Protein Family. *Plant Physiol*. 123, 563–574

Nagarajan VK, Satheesh V, Poling MD, Raghothama KG, Jain A. (2016). Arabidopsis MYB-related HHO2 Exerts Regulatory Influence on a Subset of Root Traits and Genes Governing Phosphate Homeostasis. *Plant Cell Physiol*. 57:1142-52.

Naouar, N., Vandepoele, K., Lammens, T., Casneuf, T., Zeller, G., Van Hummelen, P., & De Veylder, L. (2009). Quantitative RNA expression analysis with Affymetrix Tiling 1.0 R arrays identifies new E2F target genes. *The Plant Journal*, *57*(1), 184-194

Noh, B., Murphy, A.S., and Spalding, E.P. (2001) Multidrug resistance-like genes of *Arabidopsis* required for auxin transport and auxin-mediated development. *Plant Cell* 13, 2441-2454.

Nordström, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Astot, C., Dolezal, K., Sandberg, G. (2004) Auxin regulation of cytokinin biosynthesis in Arabidopsis thaliana, A factor of potential importance for auxin-cytokinin-regulated development. *Proc Natl Acad Sci USA* 101, 8039-8044.

Overvoorde PJ, Okushima Y, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Liu A, Onodera C, Quach H, Smith A, Yu G, Theologis A. (2005).Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in Arabidopsis thaliana. *Plant Cell* 17, 3282–3300

Ötvös K, Benková E. (2017) Spatiotemporal mechanisms of root branching. *Curr Opin Genet Dev*. 2017 Aug;45:82-89.

Orlando DA, Brady SM, Koch JD, Dinneny JR, Benfey PN (2009) Manipulating large-scale Arabidopsis microarray expression data: identifying dominant expression patterns and biological process enrichment. *Methods Mol Biol* 553: 57–77

Paciorek T, Zazímalová E, Ruthardt N, Petrásek J, Stierhof YD, Kleine-Vehn J, Morris DA, Emans N, Jürgens G, Geldner N, Friml J. (2005) Auxin inhibits endocytosis and promotes its own efflux from cells. *Nature* 435:1251-6.

Parizot B, De Rybel B, Beeckman T. (2010) VisuaLRTC: a new view on lateral root initiation by combining specific transcriptome data sets. *Plant Physiol.* 153:34-40.

Pencik, A., Rolcik, J., Novak, O., Magnus, V., Bartak, P., Buchtik, R., Salopek-Sondi, B., and Strnad, M. (2009). Isolation of novel indole-3-acetic acid conjugates by immunoaffinity extraction. Talanta 80, 651-655.

Peret, B., De Rybel, B., Casimiro, I., Benkova, E., Swarup, R., Laplaze, L., Beeckman, T., and Bennett, M.J. (2009). Arabidopsis lateral root development: an emerging story. *Trends Plant Sci* 14, 399-408.

Riefler, M., Novak, O., Strnad, M., and Schmulling, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18, 40-54.

Rogg, L.E., and Bartel, B. (2001). Auxin signaling: derepression through regulated proteolysis. *Dev Cell* 1, 595-604.

Sakakibara H.(2006) Cytokinins: activity, biosynthesis, and translocation. *Annu Rev Plant Biol.* 57:431-49

Saeed, A.I., Sharov, V., White, J. et al. (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques,* 34, 374–378.

Schaller, G.E., Bishopp, A., Kieber, J.J. (2015) The yin-yang of hormones, cytokinin and auxin interactions in plant development. *Plant Cell*. 27, 44-63.

Schlereth A, Möller B, Liu W, Kientz M, Flipse J, Rademacher EH, Schmid M, Jürgens G, Weijers D. (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. *Nature* 464, 913-916.

Skylar, A., and Wu, X. (2011). Regulation of meristem size by cytokinin signaling. *J Integr Plant Biol* 53, 446-454.

Smyth, G.K. (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat. App. Genet. Mol. Biol*. 3.

Storey JD, Tibshirani R. (2003) Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A.* 100:9440-5.

Sugimoto K, Jiao Y, Meyerowitz EM. (2010) Arabidopsis regeneration from multiple tissues occurs via a root development pathway. *Dev Cell.* 18:463-71

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S., Levesque, M.P., Carrier, D., James, N., Calvo, V., Ljung, K., Kramer, E., Roberts, R., Graham, N., Marillonnet, S., Patel, K., Jones, J.D., Taylor, C.G., Schachtman, D.P., May, S., Sandberg, G., Benfey, P., Friml, J., Kerr, I., Beeckman, T., Laplaze, L., and Bennett, M.J. (2008). The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol 10, 946-954.

Šimášková M, O'Brien JA, Khan M, Van Noorden G, Ötvös K, Vieten A, De Clercq I, Van Haperen JMA, Cuesta C, Hoyerová K, et al.: Cytokinin response factors regulate PIN-FORMED auxin transporters. *Nat. Commun.* 2015, 6:8717.

Takei K, Yamaya T, Sakakibara H. (2004) Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-Zeatin. *J Biol Chem.* 279:41866-72.

Tanaka M, Takei K, Kojima M, Sakakibara H, Mori H. (2006) Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance *Plant J.* 45:1028-36.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M.K., Harper, R.M., Liscum, E., and Yamamoto, K.T. (2004). MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. *Plant Cell* 16, 379-393.

Tian H, Guo H, Dai X, Cheng Y, Zheng K, Wang X, Wang S (2015). An ABA down-regulated bHLH transcription repressor gene, bHLH129 regulates root elongation and ABA response when overexpressed in Arabidopsis. *Sci Rep.* 5:17587.

Tian, Q., and Reed, J.W. (1999). Control of auxin-regulated root development by the Arabidopsis thaliana SHY2/IAA3 gene. *Development* 126, 711-721.

To, J.P., Deruere, J., Maxwell, B.B., Morris, V.F., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2007). Cytokinin regulates type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay. *Plant Cell* 19, 3901-3914.

Uehara, T., Okushima, Y., Mimura, T., Tasaka, M., and Fukaki, H. (2008). Domain II mutations in CRANE/IAA18 suppress lateral root formation and affect shoot development in Arabidopsis thaliana. *Plant Cell Physiol* 49, 1025-1038.

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

Vanneste, S., De Rybel, B., Beemster, G.T., Ljung, K., De Smet, I., Van Isterdael, G., Naudts, M., Iida, R., Gruissem, W., Tasaka, M., Inze, D., Fukaki, H., and Beeckman, T. (2005). Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in Arabidopsis thaliana. *Plant Cell* 17, 3035-3050.

Vanstraelen M, Benková E. (2012) Hormonal interactions in the regulation of plant development. *Annu Rev Cell Dev Biol.* 28:463-87

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

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmulling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* 15, 2532-2550.

Xu K, Liu J, Fan M, Xin W, Hu Y, Xu C.A (2012) A genome-wide transcriptome profiling reveals the early molecular events during callus initiation in Arabidopsis multiple organs. *Genomics.* 100:116-24

Yang ZB, Liu G, Liu J, Zhang B, Meng W, Müller B, Hayashi KI, Zhang X, Zhao Z, De Smet I, Ding Z. (2017) Synergistic action of auxin and cytokinin mediates aluminum-induced root growth inhibition in Arabidopsis. *EMBO Rep.* 18:1213-1230

Yang X, Lee S, So JH, Dharmasiri S, Dharmasiri N, Ge L, Jensen C, Hangarter R, Hobbie L, Estelle M. (2004) The IAA1 protein is encoded by AXR5 and is a substrate of SCF(TIR1). *Plant J.* 40, 772–782

Zhang H, Zhao FG, Tang RJ, Yu Y, Song J, Wang Y, Li L, Luan S. (2017) Two tonoplast MATE proteins function as turgor-regulating chloride channels in Arabidopsis. *Proc Natl Acad Sci U S A*. 114:E2036-E2045.

Zhang X, Chen Y, Lin X, Hong X, Zhu Y, Li W, He W, An F, Guo H. (2013) Adenine phosphoribosyl transferase 1 is a key enzyme catalyzing cytokinin conversion from nucleobases to nucleotides in Arabidopsis. *Mol Plant.* 6:1661-72.

Zhu XF1, Lei GJ, Wang ZW, Shi YZ, Braam J, Li GX, Zheng SJ. (2013) Coordination between apoplastic and symplastic detoxification confers plant aluminum resistance. *Plant Physiol.* 162:1947-55.

3. Chapter 3

Auxin and cytokinin converge at SYAC1 to rapidly control plant elongation growth.

Manuscript in preparation

Author contributions:
E.B. and A.H. conceived and designed the experiments; A.H performed all the experiments; A.H. prepared the figures; and A.H. and E.B. wrote the manuscript.

Auxin and cytokinin converge at *SYAC1* to rapidly control plant elongation growth.

Andrej Hurný[1], Candela Cuesta[1], Krisztina Ötvös[1], Nicola Cavallari[1], Juan Carlos Montesinos Lopez[1], Marcal Galemi Rovira[1], András Gorzsás[2] and Eva Benková[1]

[1]*Institute of Science and Technology, Austria*, *Klosterneuburg*
[2]*Department of Chemistry, Umeå University, Linnaeus väg 6, SE-901 87 Umeå, Sweden*

3.1 Introduction

Every stage of the life cycle of plants is governed by small signaling molecules called hormones. Auxin and cytokinin are among the principal hormonal regulators of plant growth and development including embryogenesis (Friml et al., 2003; Müller and Sheen, 2008), root (Dello Ioio et al., 2008; Růžička et al., 2009) and shoot apical meristem activity (Zhao et al., 2010), root (Benková et al., 2003; Bielach et al., 2012; Laplaze et al., 2007) and shoot branching (Leyser, 2009), vascular tissue development (Bishopp et al., 2011) and phyllotaxis (Reinhardt et al., 2003). Although the main pathways that manage their metabolism, transport, perception and signaling have been identified (Dharmasiri et al., 2005; Hwang and Sheen, 2001; Inoue et al., 2001; Kepinski and Leyser, 2005), how both hormonal pathways are interconnected to form a complex regulatory network enabling rapid adjustment of plant growth and development to the ever changing environmental conditions is largely unknown. Tightly balanced activities of auxin and cytokinin are essential for root system establishment. Developmental processes shaping root organ architecture including specification of stem cell niche during early embryogenesis, root meristem maintenance requiring fine coordination between cell division and differentiation, rapid cell elongation growth as well as root branching are all dependent on the perfect interplay of auxin and cytokinin pathways (Dello Ioio et al., 2008; Müller and Sheen, 2008; Ruzicka et al., 2007). Recent studies have provided the first insights into the molecular mechanisms underlying their cross-talk. It has been shown that auxin mediated attenuation of cytokinin signalling output by upregulation type-A ARR7 and ARR15 repressors is critical for specifying the root stem-cell niche (Müller and Sheen, 2008). The maintenance of root apical meristem size requires tight communication

between cytokinin and auxin signaling pathways. The cytokinin signal, perceived by AHK3 and downstream acting ARR1 and ARR12 response regulators, was found to directly control expression of the auxin repressor IAA3/SHY2-2. This leads to the attenuation of auxin responses and reduced expression of PIN auxin efflux transporters. Consequently, a decreased abundance of PINs limits the auxin supply to the root apical meristem, thereby restricting its meristematic activity and promoting cell differentiation and elongation (Dello Ioio et al., 2007; 2008; Pernisová et al., 2009; Růžička et al., 2009).

In addition, rapid elongation of the differentiated cells at the periphery of the root apical meristem, one of the important determinants of root growth, is under extensive hormonal control from auxin, cytokinin and ethylene. Whereas an increase in auxin severely limits root elongation growth, mutants in auxin perception and signal transduction such as *tir1*, *axr2/iaa7*, *axr3/iaa17*, (Ruegger et al. 1998, Leyser et al. 1996, Wilson et al. 1990) are largely resistant to this auxin inhibitory effect. Inhibition of root growth by cytokinin and ethylene is closely linked with auxin activity control in cells undergoing rapid expansion. Ethylene has been shown to stimulate auxin biosynthesis and basipetal auxin transport toward the elongation zone, where it activates a local auxin response leading to inhibition of cell elongation. Consistently, in mutants affected in auxin perception or basipetal auxin transport, ethylene cannot activate the auxin response nor repress root growth (Lewis et al., 2011; Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007). An essential part of the cytokinin repressing effects on cell expansion result from its interaction with the ethylene pathway (Beemster and Baskin, 2000). Cytokinin has been found to stabilize the ACS5 and ACS9 rate-limiting enzymes in ethylene biosynthesis, thereby contributing to the ethylene-auxin regulatory circuit that controls root cell elongation (Chae et al., 2003; Street et al., 2016).

Although the role of hormones in the regulation of root elongation growth is well established, the molecular pathways and mechanisms downstream of hormonal signals that control expansion of cells are largely unknown. Rapid cell elongation is tightly linked with simultaneous modulation of cell wall properties as well as synthesis and secretion of new building/remodeling materials. The plant cell wall consists of a complex structure of carbohydrates and proteins, and it confers mechanical strength to the plant during development and stress resistance. The major structural and functional components of the primary walls are hemicelluloses, cellulose and pectin (reviewed in Wolf and Greiner, 2012). While cellulose microfibrils are synthesized by plasma membrane (PM)–localized cellulose

synthase complexes, pectin and hemicelluloses are synthesized at the Golgi apparatus and delivered through the secretory pathway to the cell exterior (reviewed in Driouich et al., 2012).

The plant secretory pathway consists of numerous functionally interlinked organelles. The first organelle of the secretory pathway is the endoplasmic reticulum (ER) in which proteins are synthesized and assembled for export to the Golgi apparatus. It is conventionally accepted that the Golgi apparatus, which in plants is made up of numerous, motile and polarized stacks of membranous compartments called cisternae, collects membranes and lumenal content from the ER for further processing and sorting via endosomes to distal compartments which include the trans-Golgi network (TGN), vacuoles and the plasma membrane (Foresti and Denecke, 2008). The plant plasma membrane interfaces the cell content with the external environment, which is largely occupied by a cell wall. Our knowledge of TGN-mediated secretion has been recently extended mainly through the identification of ECHIDNA and YIP (for YPT/RAB GTPase Interacting Protein) proteins as a TGN localized complex, necessary for the secretion of cell wall polysaccharides and cell elongation (Gendre et al., 2013). Nevertheless, how the secretion of cell wall components during plant cell elongation is controlled and whether hormones to regulate cell expansion target secretory pathway remains poorly understood.

Here we identified unknown regulatory component of the secretory pathway that in response to auxin and cytokinin rapidly controls elongation growth. *SYNERGISTIC AUXIN CYTOKININ 1 (SYAC1)* gene was recovered by genome wide transcriptome profiling as a common target of auxin and cytokinin, whose expression in roots is strictly dependent on both auxin and cytokinin. Detailed functional characterization revealed that SYAC1 acts as a developmentally specific regulator of the secretory pathway to control deposition of cell wall components and thereby rapidly fine tune elongation growth.

3.2 Results

3.2.1 The *SYAC1* expression is synergistically upregulated by auxin and cytokinin.

To identify novel molecular components and mechanisms of auxin-cytokinin interaction, the genome wide transcriptome profiling of roots exposed to auxin, cytokinin and both hormones simultaneously was performed (Duclerque et al., in preparation). *SYNERGISTIC AUXIN CYTOKININ 1 (SYAC1)* gene, which encodes for a protein of unknown function, was detected among the top candidate genes of which expression was synergistically up-regulated by simultaneous hormonal treatment when compared to the expected additive effect of both hormones applied separately. Whereas treatment with either auxin or cytokinin increased *SYAC1* expression 2.27 ± 0.00081 and 1.60 ± 0.004, respectively, application of both hormones simultaneously resulted in 14.53 ± 6.12503E^{-07} higher expression when compared to untreated control. The *SYAC1* expression profile in roots was further validated by quantitative real-time PCR (Fig. 1A). To examine *SYAC1* expression pattern with tissue resolution transgenic lines expressing *pSYAC1:GUS* and *pSYAC1:nlsGFP* reporter constructs were generated. No *pSYAC1:GUS* signal could be detected in roots grown on the control medium. Exposure to cytokinin for 6 hours triggered *pSYAC1::GUS* expression in the quiescent center (QC) and columella initials (CI) (Fig. 1B), and in response to auxin additional patchy staining in the cells of the apical root meristem could be detected (Fig. 1B). Importantly, simultaneous application of both hormones dramatically enhanced expression of the *pSYAC1:GUS* and *pSYAC1::nlsGFP* in the stem cell niche, cells of the apical root meristem and in the root elongation zone (Fig. 1B, C and S1C), thus corroborating results of transcriptome profiling. To evaluate impact of long term exposure to hormones *pSYAC1:GUS* lines were grown on media supplemented with low concentration of auxin and cytokinins. Similarly to short-term hormonal treatments, in roots grown on medium supplemented with both hormones enhanced *pSYAC1:GUS* expression was detected when compared to non-treated or treated with cytokinin or auxin only roots (Fig. S1A and S1B). Interestingly, pattern of *SYAC1* expression in roots grown on auxin and cytokinin differs from that observed after short term hormonal application, suggesting that plants might activate effective homeostatic mechanism that balance endogenous levels of plant hormones. As application of cytokinin and auxin might lead to deregulation of other hormonal pathways in particularly that of ethylene, we examined sensitivity of *SYAC1* expression to this hormone.

To exclude the effect of ethylene on transcriptional regulation of *SYAC1*, *pSYAC1:GUS* line was treated for 6 hours with 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor of ethylene biosynthesis) alone or in combination with auxin and cytokinin. No enhancement of the *pSYAC1:GUS* expression was detected in plants treated with either ACC only or when applied with either cytokinin or auxin. Similarly, ACC did not interfere with auxin – cytokinin synergistic effect on *pSYAC1:GUS* expression, suggesting that ethylene alone cannot trigger *SYAC1* transcription, and neither it can substitute auxin or cytokinin to synergistically activate *SYAC1* expression (Fig. S1D). Taken together, expression analysis confirms *SYAC1* as a novel common target of auxin and cytokinin pathways acting in roots.

3.2.2 *SYAC1* expression in planta.

To further explore in which developmental processes *SYAC1* might be involved we monitored its expression pattern throughout the whole lifespan of *Arabidopsis thaliana*. Strong *SYAC1* expression was detected in the whole mature embryo except the embryonic root (Fig. 1D). In 2-days-old seedlings high expression of *SYAC1* in the cotyledons and upper part of hypocotyl was observed (Fig. 1E) and this gradually decreased in older seedlings (Fig. 1F,G). In the etiolated seedlings expression *of SYAC1* at the base of hypocotyl and in the concave side of apical hook was found (Fig. 1H). These observations suggest that *SYAC1* function might not be restricted to plant roots, but act also in other plant development processes.

3.2.3 *SYAC1* balances auxin and cytokinin inputs to coordinate lateral root organogenesis.

To gain more insights into the *SYAC1* developmental function detailed phenotype analysis of plants with modulated SYAC1 activity was performed. Characterization of the available mutant lines revealed that the T-DNA is inserted either in the middle of the 3' untranslated region (UTR) (*syac1-1*, *syac1-2*, *syac1-3*) or in the middle of second intron (*syac1-4*) (Fig. S2A), and thus it is not fully suppressing *SYAC1* expression (Fig. S2B). To obtain a *syac1* knock-out line, we used the CRISPR/Cas9 approach. In the *syac1-5* mutant allele the CRISPR/Cas9 cassette introduces an extra thymine at 90 bps after the ATG, which results in a STOP codon after 33 amino acids in SYAC1 coding sequence (Fig. S2A). To investigate impact of increased *SYAC1* activity on plant development, transgenic lines carrying *SYAC1* fused to either *GFP* reporter or *-HA tag* under control of *35S* (*SYAC1-GFPox*, *GFP-SYAC1ox*, *SYAC1-HAox*) were generated. Whereas no significant LR initiation defects could

be observed in *syac1-3, syac1-5* (Fig. 2A and S2C), both *syac1-3* as well as *syac1-5* mutant alleles exhibited significant resistance to cytokinin inhibitory and modest, but statistically significant oversensitivity to auxin promoting effects on LR initiation (Fig. 2A and S2C).Although no difference in LR density was observed in plants grown on MS media, the root system of both *syac1-3* and *syac1-5* alleles clearly showed a delay in LR development manifested by accumulation of LR primordia at stage I and decreased proportion of later developmental stages (Fig. 2B and S2D; classification according to Malamy and Benfey, 1997). To confirm the role of SYAC1 in LR organogenesis, we re-introduced *SYAC1* genomic DNA under its own promotor (*pSYAC1:gSYAC1-GFP*) into the *syac1-3* mutant background, which fully recovered sensitivity of LR initiation to cytokinin and LR development (Fig. 2B and 2C). Interestingly, overexpression of *SYAC1* caused a significant decrease in LR density (Fig. S2E) and severe defects in LR primordia development (Fig. S2F).These results demonstrate that interference with SYAC1 function causes severe defects in the root system establishment and attenuates developmental responses to both auxin and cytokinin.

3.2.4 Modulation of SYAC1 activity interferes with elongation growth.

Enrichment of *SYAC1*expression in tissues and organs with restricted cell expansion including hypocotyl and cotyledons in mature embryo, QC, CI, inner side of the apical hook, root elongation zone (Fig. 1D-H) motivated us to closely investigate root and hypocotyl elongation in plants with modulated SYAC1 activity. Whereas no significant alterations in root length of 5-day-old *syac1-3* and *syac1-5* seedlings compared to the Columbia control were observed, overexpression of *SYAC1* severely inhibited root growth (Fig. 2D, S2G). To test whether accumulation of SYAC1 might have a rapid effect on elongation growth a live tracking of roots with conditional expression of *SYAC1* induced by β-estradiol (*pEST:SYAC1-GFP*) was performed. After only 4 hours induction we observed a 35% reduction in root growth rate relative to Columbia control (Fig. 2E), indicating that alterations in SYAC1 activity might rapidly modulate root growth kinetics. Hypocotyls of 3-day-old dark-grown etiolated seedlings were significantly longer in both *syac1-3* and *syac1-5* alleles when compared to Columbia control. Contrary to the phenotypes observed in *syac1* mutants, *SYAC1*overexpression correlated with severe reduction of hypocotyl length (Fig. 2F). Since hypocotyl growth in darkness is largely driven by cell elongation rather than cell proliferation

(Gendreau et al., 1997), the hypocotyl growth defects observed in *syac1* mutant and *SYAC1ox* strongly suggest SYAC1 function in regulation of cell elongation.

3.2.5 SYAC1 is required for proper development of apical hook.

Specific expression of *SYAC1* in the concave side of the apical hook, prompted us to investigate its importance for this developmental process. In Columbia plants, shortly after germination (about 15-20h), the hypocotyl progressively bends to establish an apical hook with an angle around 180° (formation phase). This angle is stabilized during the maintenance phase. Subsequently, about 60 h after germination a progressive opening of the hook occurs to reach a hook value around 20° (opening phase) (Raz and Ecker, 1999; Žádníková et al., 2010). In *syac1-3* and *syac1-5*, the formation phase occurred at a similar rate to Columbia control, but the maintenance phase was shortened and the opening of the hook started already 35 hours after the germination (Fig. 2G). Introduction of *pSYAC1:gSYAC1-GFP* into the *syac1-3* background recovered this defect and prolonged the maintenance phase till 60 h after germination as normally observed in Columbia. The apical hook development is a result of tightly orchestrated differential growth along the apical-basal axis of the hypocotyl. During formation of the hook curvature the elongation rate of cells on the outer side exceeds that of those on the inner side of the hypocotyl. Once they have passed through the apex of the hook, the growth rate of cells on the inner side exceeds that of cells on the outer side of the hook, the hypocotyl straightens (Raz and Koornneef, 2001). Inability to form apical hook due to *SYAC1* overexpression and its premature opening caused by lack of the SYAC1 activity indicate that SYAC1 is required for coordination of differential growth and support its specific role in fine tuning cell elongation.

3.2.6 SYAC1 localizes to the Golgi/TGN/Endosomal/PVC compartments.

To better understand the function of SYAC1 protein, we next investigated its subcellular localization in Arabidopsis root cells by colocalization with cellular compartment specific reporters. In estradiol inducible line 5 hours after induction of SYAC1-GFP signal is restricted to the small compartments in the cell interior. Measurement of Pearson correlation coefficient revealed a high SYAC1 colocalization pattern with Golgi compartments labeled by the anti-SEC21 (0,57 ± 0,01) and with TGN labeled by anti-ECH (0,51 ± 0,02) antibody. This subcellular localization was further confirmed by anti-ARF1 (0,45 ± 0,02) and anti-SYP61 (0,49 ± 0,02) antibodies, which label both Golgi and TGN. A strong colocalization pattern was also observed with the prevacuolar/endosomal compartments (PVC) labeled with

a mixture of anti-ARA7 and anti-RHA1 (0,52 ± 0,02) antibodies. In contrast, almost no colocalization was observed between SYAC1 and anti-BIP2 (0,04 ± 0,04) and anti-PIN2 (0,03 ± 0,04) antibodies, which label ER and plasma membrane, respectively (Fig. 3A, B). Accordingly, SYAC1-GFP in *SYAC1-GFPox* line (Fig. S3A, B) exhibited strong colocalisation with markers for Golgi (anti-SEC21; 0,55 ± 0,02), TGN (anti-ECH 0,60 ± 0,02), for both of them together (anti-ARF1; 0,55 ± 0,02 and anti-SYP61; 0,40 ± 0,02) and PVC (anti-ARA7/anti-RHA1; 0,44 ± 0,02) but almost no colocalization with markers for ER (anti-BIP2; 0,01 ± 0,03) and PM (anti-PIN2; 0,02 ± 0,02). To further validate the immunocolocalization results, we crossed *GFP-SYAC1ox* line with the multicolor 'Wave' marker set (Geldner et al., 2009) for analysis of plant cell membrane compartments. We observed strong colocalization of SYAC1 signal with marker lines for Golgi (wave 18R; 0,53 ± 0,03 and wave 127R; 0,42 ± 0,02), markers for Golgi and endosomes (wave 25R; 0,69 ± 0,03 and wave 29R; 0,35 ± 0,03), for Golgi and TGN (*SYP61:SYP61-CFP*; 0,45 ± 0,02), for TGN and early endosomes (wave 13R; 0,27 ± 0,06) and for endosomes/recycling endosomes (wave 34R; 0,31 ± 0,05 and wave 129R; 0,33 ± 0,02). In agreement with immunocolocalization experiments, SYAC1 signal displayed only minor colocalization with marker for ER/PM (wave 6R; 0,06 ± 0,02), markers for PM (wave 131R; 0,02 ± 0,02 and wave 138R; 0,02 ± 0,03). Marker for vacuoles (wave 9R; 0,03 ± 0,02) showed also only a minimal colocalization pattern (Fig. S3C, D). These results strongly support that SYAC1 largely resides at Golgi/TGN/Endosomal/PVC compartments.

3.2.7 SYAC1 interacts with YIP5b, YIP4a, YIP4b and ECH.

To further assess the molecular function of SYAC1 we identified its molecular interactors using a tandem affinity purification assay with SYAC1 as bait. Several proteins including β-ketocyl reductase 1 (KCR1), an ubiquitin receptor protein (DSK2), Prohibitin 4 (PHB4) and Integral membrane YIP1 family protein (YIP5b) were recovered as potential interactors of SYAC1. As YIP5b is a member of the YIP (for YPT/RAB GTPase Interacting Protein) family in *Arabidopsis thaliana* that form a TGN-localized complex with YIP4a and YIP4b homologues and Echidna (ECH) integral membrane protein (Drakakaki et al., 2012; Gendre et al., 2013), we decided to include them in detailed interaction studies. In Yeast two-hybrid assay (Y2H), a strong interaction between SYAC1 and all three YIP family members has been recovered. SYAC1 interacted with ECH and KCR1, although this was weaker in comparison with YIPs and no interaction with DSK2 and PHB4 protein was observed (Fig.

4A). Y2H results were further validated in planta using Bimolecular fluorescence complementation (BiFC) assay. SYAC1 tagged with the C-terminus of EYFP, and YIP5b, YIP4a, YIP4b, ECH, KCR1, DSK2 and PHB4 tagged with the N-terminus of EYFP, were transiently expressed in Arabidopsis root suspension culture. Yellow fluorescence was detected in protoplasts overexpressing SYAC1 in combination with YIP5b, YIP4a, YIP4b and ECH, indicating the direct interaction of these proteins *in vivo*. No EYFP signal was detected in cells overexpressing SYAC1 with KCR1, DSK and PHB4 (Fig. 4B) in agreement with result of Y2H assay. Finally, the interaction between SYAC1 and YIP4a and between SYAC1 and ECH was also confirmed by coimmunoprecipitation (Co-IP) assay (Fig. 4C). Results from tandem affinity purification, BiFC and Co-IP assays revealed SYAC1 interaction with YIP5b, YIP4a, YIP4b and ECH protein, and indicate its function in protein complex involved in maintaining functionality of the secretory pathway (Gendre et al., 2013).

3.2.8 SYAC1 regulates secretory pathway

SYAC1 localization in Golgi/TGN/Endosomal/PVC compartments and identification of the interaction partners pointed at its potential function in the secretory pathway. The secretory pathway is of vital importance for all eukaryotic cells, since it manufactures, stores and distributes macromolecules, lipids and proteins as cargo to intracellular and extracellular locations (reviewed in Bassham et al., 2008). To assess the involvement of SYAC1 in the regulation of secretion, we performed a transient expression assays in Arabidopsis mesophyll protoplasts and evaluated impact of SYAC1-HAox or HA-SYAC1ox on the secretory index of the α-Amylase (Amy) reporter - a protein that is transported without any intrinsic sorting signal and can be detected by its endogenous enzymatic activity. As a control plasmid encoding only mCherry tag was used. The secretion index was determined by quantifying ratio of the α-Amylase activity in the medium and in the cells. Overexpression of the SYAC1 protein decreased the secretion index from 0,7 ± 0,04 in control sample to 0,55 ± 0,02 (SYAC1-HAox) and 0,45 ± 0,01 (HA-SYAC1ox), which hints at function of SYAC1 as a negative regulator of the anterograde secretory route to the cell surface. Because of SYAC1 colocalization with markers for PVC compartments, we decided to explore SYAC1 involvement in transport to the vacuoles. For that, an α-Amylase with a vacuolar sorting signal (Amy-Spo) was co-transfected with either SYAC1-HA or HA-SYAC1 encoding plasmids. Secretion index was increased from 0,07 ± 0,007 in control sample to 0,29 ± 0,01 (SYAC1-HAox) and 0,28 ± 0,03 (HA-SYAC1ox), which suggests that transport to vacuoles is impaired and more α-Amylase might be secreted out of the cells. Furthermore, SYAC1

effect on α-Amylase containing an ER retention signal (Amy-HDEL), which redirects the protein back to ER was tested. Co-transfection of SYAC1 significantly decreased the secretion index in protoplasts with leaky retention of α-Amylase from 0,34 ± 0,01 in control sample to 0,24 ± 0,004 (SYAC1-HAox) and 0,26 ± 0,04 (HA-SYAC1ox) (Fig. 5A). Altogether these results indicate that SYAC1 might modulate activity of secretory pathway, and coordinate trafficking of cargos towards extracellular space and vacuoles.

3.2.9 SYAC1 regulates secretion of soluble cell wall polysaccharides.

In plants, new cell wall components such as pectins and hemicellulose are proposed to be delivered to the cell exterior via the secretory pathway (reviewed in Wolf and Greiner, 2012). SYAC1 reduction of α-Amylase secretion, along with its Golgi/TGN/Endosomal localization and interaction with YIPs and Echidna proteins, prompted us to explore role of SYAC1 in control of soluble cell wall polysaccharides (pectin and hemicellulose) secretion. Investigating the seed coat epidermis, in which the TGN is highly specialized for pectic mucilage secretion (Young et al., 2008) using ruthenium red staining assay revealed that mucilage release from mature seeds was greatly reduced in *SYAC1-GFPox* seeds, relative to Columbia (Fig. S4A), which is in line with anticipated function of SYAC1 as a regulator of polysaccharide secretion. Hemicellulose components of cell wall, monitored using LM15 an anti-xyloglucan antibody, were enriched around QC in Columbia control and the *syac1-5* roots. In the *SYAC1-GFPox* line, enhanced staining of root epidermal, cortex and endodermal was detected, resembling defects in xyloglucan distribution in roots of *ech* and *yip4a yip4b* mutants (Fig. 5B). Taken together, these data support SYAC1 function in modulation of the cell wall matrix polysaccharides delivery, including both hemicelluloses and pectins. The defects in the secretion of cell wall components might lead to alterations in the cell wall structure and its physical properties. To assess whether SYAC1 triggered changes in delivery of cell wall components affect composition and physical properties of cell walls, hypocotyls of etiolated seedlings were inspected using Fourier transform-infrared (FT-IR) microspectroscopy (Mouille et al., 2003) and atomic force microscopy (AFM) (Peaucelle et al., 2015). FT-IR analysis revealed that enhanced SYAC1 activity in plant cells substantially alters composition of the cell walls, which is manifested by significantly reduced proportion of carbohydrates (Fig. 5C). Accordingly, increase in SYAC1 activity has a direct impact on the physical properties of cell walls and reduces their stiffness (Fig. S4B). Thus, these results indicate that SYAC1 is an essential regulator of the TGN-mediated secretion of cell wall

components such as pectins and xyloglucan and ultimately affects composition and physical properties of cell walls. Importantly, SYAC1 might regulate specific pathways of secretion machinery as localization of plasma membrane proteins such as PIN1 and PIN2 were not affected by SYAC1 (Fig. S4C).

3.2.10 Reduction of *syac1* activity partially recovers cell elongation defects in *yip4a yip4b* mutant.

It has been found that defects in YIP/ECH protein complex activity leads to ineffective pectin and hemicellulose secretion, which correlates with reduced root and etiolated hypocotyl growth (Gendre et al., 2013). Strong physical interaction observed between SYAC1 and YIP4a/YIP4b components of the YIP/ECH protein complex, as well as their functional overlap in control of secretory trafficking and elongation growth prompted us to examine closely their genetic interaction. To assess contributions of SYAC1 and YIP components of YIP/ECH complex to regulation of elongation growth a homozygote *yip4a-2 yip4b-1 syac1-3* triple mutant line was generated. Detailed analysis of root and hypocotyls growth kinetics revealed that defects in elongation growth caused by lack of *yip4a* and *yip4b* activity can be partially recovered when SYAC1 activity is simultaneously attenuated (Fig. 6A,B). These results indicate that SYAC1 and YIPs/ECH functions converge at the regulation of elongation growth. However, whereas YIPs together with ECH act as a positive factors required for secretion of cell wall components to control elongation growth, SYAC1 might act as developmentally specific regulator, which can through fine tuning activity of secretory pathway coordinates growth kinetics.

3.3 Discussion

The current accepted view is that auxin acts antagonistically with cytokinin to control root development (Dello Ioio et al., 2008; Müller and Sheen, 2008). This antagonism is based on the competition between auxin as a promotor of cell division, and cytokinin as a promotor of cell differentiation in regulation of root meristem size (Dello Ioio et al., 2007; Růžička et al., 2009). Indeed, to specify the root stem-cell niche during embryogenesis, auxin represses cytokinin action by activating expression of ARR7 and ARR15, which encode type-A ARR proteins that repress CK responses (Müller and Sheen, 2008). However, this antagonist interaction between auxin and cytokinin does not occur in all developmental contexts: for instance in the control of shoot apical meristems, auxin acts synergistically with CK through direct transcriptional repression of ARR7 and ARR15 (Zhao et al., 2010). Hence, as discussed in (Schaller et al., 2015), the concept of yin-yang is probably more accurate, as auxin and cytokinin act together dynamically, with roles that can be paradoxically antagonistic and supportive, to provide robustness to developmental processes. Here, we show that auxin and cytokinin can converge in a positive synergistic manner upon transcriptional regulation of *SYNERGISTIC AUXIN CYTOKININ 1 (SYAC1)* gene. Separate treatment with auxin or cytokinin is not sufficient to trigger high expression of *SYAC1*, which suggest that interaction between auxin and cytokinin transcription pathways is needed. Ethylene stimulated auxin biosynthesis, *PIN* gene expression and auxin transport, along with the ability of cytokinin to stimulate ethylene suggests close regulatory circuits between these three hormones (Chae et al., 2003; Ruzicka et al., 2007; Swarup et al., 2007). Since no transcriptional upregulation of *SYAC1* by treatment with ethylene alone, or in combination with auxin and cytokinin was observed, direct and indirect effects of ethylene on auxin-cytokinin synergism in *SYAC1* expression regulation can be excluded.

SYAC1 encodes for a protein of unknown function, therefore, various strategies including subcellular localization, expression pattern analysis, phenotypic analyses of the *syac1* loss-of-function and gain-of-function transgenic lines along with the identification of the SYAC1 interaction partners by tandem affinity purification were employed in order to elucidate its role in *Arabidopsis thaliana*. SYAC1 strong co-localization with Golgi markers (SEC21, Got1 and MEMB12), TGN marker (ECH), Golgi and TGN markers (ARF1, SYP61, VTI12, Rab D1 and Rab D2a), endosomal markers (Rab A1e and Rab A1g) and with PVC markers (ARA7 and RHA1) suggested SYAC1 potential function in the secretory pathway but also

possible involvement in transport to the vacuoles. No colocalization with ER markers (BIP2 and NIP1;1), vacuole marker (VAMP711) and PM markers (NPSN12 and PIP1;4) indicates that SYAC1 does not play a role in the early steps of the secretory pathway (transport from ER to Golgi) and that SYAC1 might be released prior vesicles fused with PM and vacuoles.

The secretory pathway plays an essential function in transport and distribution of proteins and other cargos between different compartments, which is achieved by shuttling small membrane-enclosed vesicles. This vesicle-mediated transport involves several key steps: formation of transport vesicles at the donor compartment; sorting of cargo protein into nascent transport vesicles; delivery of transport vesicles to the acceptor compartment; tethering followed by fusion of the vesicles with the acceptor compartment (Guo et al., 2017). Attempts to reveal in which of these steps SYAC1 could be involved and what its biological function is led us to focus on its interactors, identified by tandem affinity purification approach and confirmed by complementary assays including Y2H, CoIP and BIFC. The YIP protein family members were found among strongest interactors of SYAC1. Proteins of this family are found in all eukaryotic organisms, but have been studied most extensively in yeast and, more recently, in mammals (Soonthornsit et al., 2017). Their ability to bind RAB GTPases is making them attractive candidates for the recruitment of RABs onto target membranes (Chen et al., 2004; Heidtman et al., 2003; Kano et al., 2009; Tanimoto et al., 2011; Yang et al., 1998; Yoshida et al., 2008). Indeed, it has been shown that human Yip3 protein possesses GDF (GDI-displacement factors) activity and can release Rab proteins from GDI (GDP-dissociation inhibitor) at the late-Golgi and endosomal membranes (Sivars et al., 2003). *Arabidopsis* YIP4a and YIP4b proteins display the YIP domain topology (Shakoori et al., 2003) and are similar to yeast YIP4p which may function later in the secretory pathway (Inadome et al., 2007). Thus strong interaction observed between SYAC1 and YIP4a/b supports its function in vesicle trafficking. In which step of vesicular trafficking SYAC1 is involved and what its molecular function is in relation to the YIP/RAB complex is still unclear, but SYAC1 localization at Golgi/TGN/Endosomal/PVC compartments suggests its involvement rather in later stages of trafficking. It would be relevant to find out if SYAC1 is able to interfere with the ability of YIPs to interact with RABs and in this way regulate processes in the secretory pathway.

Important support for the role of SYAC1 in regulation of the secretory pathway provided α-amylase secretory assay. α-Amylase, an enzyme that catalyzes the hydrolysis of long chain polysaccharides, is substrate of secretory pathway released to extracellular matrix.

Significant decrease of the α-amylase secretion index (ratio between a-amylase activity in medium versus intracellular) indicated SYAC1 function as a negative regulator of the secretory pathway. However, modulation of secretory index of α-amylase fused to sporamin vacuolar targeting signal points out that SYAC1 might also interfere with transport of cargos to the vacuoles. In planta observations further supported role of SYAC1 as regulator of secretory pathways. Perturbation in pectic mucilage secretion from the seed coat in the *SYAC1* overexpressor line is in line with its proposed role as a negative regulator of secretion. On the other hand, increased levels of xyloglucans, a hemicellulose component of cell wall, in root cells of the *SYAC1ox* and no changes in accumulation of plasma membrane proteins point to SYAC1 function in fine tuning secretion of specific cargos such as pectins and hemicelluloses. Impact of SYAC1 on secretion of specific cell wall components was further confirmed by FT-IR analysis, which revealed aberrant cell wall composition in *SYAC1ox*, in particular reduced proportion of carbohydrates when compared to proteins and other cell wall components.

Secreted polysaccharides such as pectin and xyloglucan are important for cell elongation and play a key role in determining the mechanical properties of the cell walls (Hayashi and Kaida, 2011; Wolf and Greiner, 2012), and thus changes in their proportion could be associated with changes in plant stiffness. Accordingly, as observed with atomic force microscopy the increase of *SYAC1-GFP* activity significantly reduced stiffness of cell walls.

In line with SYAC1 function as a regulator of cell wall components delivery, its interacting partners including the YIP protein family has also been connected with the secretion of cell wall polysaccharides (Gendre et al., 2013). YIP4 proteins have been found to interact with ECHIDNA (ECH) and form a complex at TGN to mediate the secretion of the cell wall components. According to our CoIP experiment, both ECH and YIP4a proteins bind SYAC1, but Y2H experiments showed that the interaction between SYAC1 and YIPs is stronger as interaction between SYAC1 and ECH. This could indeed suggest a competition between SYAC1 and ECH in interaction with YIPs, which could lead to changes in secretion. In contrast to ECH and YIP proteins, which are ubiquitously expressed and needed for proper secretion of cell wall components in plant cells (Gendre et al., 2013), *SYAC1* expression is developmentally specific and by changing the availability of different cell wall components might directly impact on LR organogenesis, specific phases of the apical hook development as well as in rapidly fine tune an elongation growth of hypocotyls and roots. The rapid effect of SYAC1 activity on root growth was also proven by use of the *pEST:SYAC1-GFP* inducible

line, in which 4 hours after the induction of *SYAC1* expression 35% reduction in root growth rate relative to Columbia was observed. In contrast to the overexpression lines, *syac1* mutant plants showed developmentally specific phenotypes in accordance to SYAC1 expression pattern. The increased length of etiolated hypocotyls and shortened maintenance phase during apical hook development in *syac1* mutants could be caused by increased secretion of cell wall components in comparison to Columbia, which would lead to cell elongation. Although we were not able to detect SYAC1 expression in roots of *pSYAC1:GUS* line without external hormone application, some basal expression cannot be excluded and in that case, delay in LR development in *syac1* mutant or reduction of number of LRs in overexpressor lines could be explained by aberrant cell wall composition in cells of LRP or surrounding cells. Taken together, SYAC1 is a fast and effective player in regulation of the plant growth. Therefore, identification of the role of the SYAC1 in Golgi/TGN/Endosomal/PVC-mediated secretion of pectin and hemicellulose provides a foundation for dissecting the molecular mechanisms that underpin developmentally specific polysaccharide secretion to the plant cell wall. As measurements of α-Amylase suggest, secretion of cell wall modifying enzymes could be also mediated by SYAC1.

Noteworthy, the SYAC1 activity in root is largely dependent on synergistic interaction of auxin and cytokinin pathways. This hormone dependent expression of *SYAC1* prompted us to search environmental cues that would increase the levels of auxin and cytokinin or increase their signaling in the root. Indeed, a synergistic effect of auxin and cytokinin in regulation of root growth has been shown during environmental stresses cues such as pathogen infection and aluminum (Al) stress. Al stress has been shown to rewire the auxin–cytokinin cross talk, where auxin acts with cytokinin in a synergistic way in the root transition zone to regulate root growth (Yang et al., 2017). The fungal pathogen *Plasmodiophora brassicae*, the causal agent of the Brassicaceae clubroot disease, has been shown to downregulate the cytokinin degradation pathway and upregulate auxin signaling genes, which lead to cell elongation and consequent club growth (Siemens et al., 2006). Exact molecular mechanism of auxin and cytokinin synergistic interaction upon *SYAC1* transcription is still unknown but it would be an important component in our understanding of plant development through hormonal cross talk. Thus, identification of SYAC1 in mediating the secretion of cell wall polysaccharides provides an additional control point for the regulation of cell elongation by hormonal and developmental signals.

3.4 Material and Methods

Plant material and growth conditions.

The *syac1-1* (salk_151420C, Col-0, KAN^R) and *syac1-2* (salk_151662B, Col-0, KAN^R) T-DNA insertion lines were obtained from the Salk Institute. The *syac1-3* (GABI-KAT 760F05, Col-0, SUL^R) and *syac1-4* (GABI-KAT 961C03, Col-0, SUL^R) T-DNA insertion lines were obtained from the GABI KAT seed collection. Genotyping primers are listed in Supplementary Table 1. The *syac1-5* CRISPR line was prepared in collaboration with the VBCF Protein Technologies Facility (www.vbcf.ac.at) (see below). The transgenic fluorescent-protein marker lines in Col-0 background have been described elsewhere: mCherry tagged wave line 6, 9, 13, 18, 25, 29, 34, 127, 129, 131, 138 (Geldner et al., 2009) *SYP61::SYP61-CFP* (Drakakaki et al., 2012). The *echidna* mutant has been described in (Gendre et al., 2011)and *yip4a-2 yip4b-1* in (Gendre et al., 2013)).

Seeds of *Arabidopsis* were plated and grown on square plates with solid half strength Murashige and Skoog (MS) medium (Duchefa) supplemented with 0.5 g L^{-1} MES, 10 g L^{-1} Suc, 1% agar and pH adjusted to 5.9. The plates were incubated at 4 °C for 48 h to synchronize seed germination and then vertically grown under a 16:8 h day/night cycle photoperiod at 21 °C. Cytokinin and auxin treatments were performed with the N6-benzyladenine cytokinin derivative (Sigma) and Naphthaleneacetic acid (Sigma), respectively. Short treatments (6 hours) were performed with 10 µM cytokinin and 1 µM auxin and long treatments (5-7days) with 100 nM cytokinin and 100 nM auxin when treated separately and 50 nM when added together. Estradiol treatment was performed with 5uM β-Estradiol (Sigma).

Cloning and generation of transgenic lines.

All cloning procedure was conducted by using Gateway™ (Invitrogen) technology; with the sequences of all used vectors available online (https://gateway.psb.ugent.be/). For promoter analysis of SYAC1, an upstream sequence of 2522bp was amplified by PCR and introduced into the pDONRP4-P1R entry vector. Then transcriptional lines (pSYAC1:GUS, pSYAC1:nlsGFP) were created: for pSYAC1:GUS, an LR reaction with SYAC1 promoter in pDONORP4-P1R, pEN-L1-S-L2,0 and pK7m24GW,0 vectors was performed. For pSYAC1:nlsGFP line, an LR reaction with SYAC1 promoter in pDONORP4-P1R, pEN-L1-NF-L2,0 and pB7m24GW,0 was performed. To generate the overexpressor, inducible and translational lines (SYAC1-GFPox, SYAC1-HAox, HA-SYAC1ox, pEST:SYAC1-GFP,

pSYAC1:SYAC1-GFP), SYAC1 ORF sequence with or without STOP codon was amplified and fused through a linker (4 Glycines and 1 Alanine) to GFP or HA tag. The fragments were first introduced into pDONR221 and then into pB2GW7,0 (overexpressor lines), p2GW7,0 (protoplast expression assays), pMDC7 (estradiol inducible line), or in combination with SYAC1 promotor in pDONRP4-P1R into pB7m24GW,3 (translational line). For GFP-SYAC1ox transgenic line SYAC1 ORF was amplified, introduced to pDONR221 and to the pB7FWG2.0 destination vector. To generate translational fusion line pSYAC1:gSYAC1-GFP, SYAC1 promoter was amplified together with the genomic fragment of the SYAC1 gene, cloned into pDONRP4-P1R and together with pEN-L1-F-L2,0 introduced into pB7m24GW,3. Cloning primers are listed in Supplementary Table 1. All transgenic plants were generated by the floral dip method (Clough and Bent, 1998), and transformants were selected on plates with appropriate antibiotic.

Generation of CRISPR/Cas9 line.

Design of the gRNA for *SYAC1* gene, molecular cloning and plant transformation was done in collaboration with VBCF Protein Technologies Facility (www.vbcf.ac.at). Design, specificity and activity of gRNA: GATGGTCAGCAACCACACGA was performed using online available tools: http://cbi.hzau.edu.cn/cgi-bin/CRISPR and http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design. gRNA was cloned into pGGZ003 CRISPR/Cas9 destination vector. Transformants were selected, genomic sequence of *SYAC1* amplified and sequenced. Individual mutant lines with single base pair insertion in coding sequence (90 bps after the ATG -at the place of gRNA binding) were selected. Plants were propagated to obtain homozygote lines and CRISPR/Cas9 cassette was outcrossed.

Quantitative RT–PCR.

RNA was extracted (RNeasy kit (Qiagen)) from roots of 6-day-old plants under all conditions (untreated, 1 µM auxin, 10 µM cytokinin and both together for 6 h). A DNase treatment with the RNase-free DNase Set (Qiagen) was carried out for 15 min at 25 °C. Poly(dT) cDNA was prepared from 1 µg of total RNA with the iScript cDNA Synthesis Kit (Biorad) and analyzed on a LightCycler 480 (Roche Diagnostics) with the SYBR Green I Master kit (Roche Diagnostics) according to the manufacturer's instructions. *SYAC1* expression was quantified with specific primer pair Fw: ACTTCTGGTTATGTTTGGCTCTCC and Rv: ACACATATGACCACAGGCGTAAG. All PCRs were performed in triplicate. Expression levels were first normalized to CDKA1 expression levels and then to the respective expression levels in untreated plants.

Phenotypic analysis.

For root length analysis, seedlings were photographed and root lengths were measured with ImageJ software (https://imagej.nih.gov/ij/). About 20–30 seedlings were processed and 3 independent experiments were performed. To score LRP density, 5 to 7-day-old seedlings (n=10-15) were first processed by clearing (Malamy and Benfey, 1997). In brief, seedlings were incubated in a solution containing 4% HCl and 20% methanol for 10 min at 65 °C, followed by 10 min incubation in 7% NaOH/60% ethanol at room temperature. Next, seedlings were rehydrated by successive incubations in 60, 40, 20 and 10% ethanol for 15 min, followed by incubation (15 min up to 2 h) in a solution containing 25% glycerol and 5% ethanol. Finally, seedlings were mounted in 50% glycerol and root lengths were measured on scanned slides with ImageJ. LRP scoring was performed by using a DIC Olympus BX53 microscope.

Real-time analysis and statistics of the apical hook development.

Development of seedlings was recorded at 1-h intervals for 5 days at 21°C with an infrared light source (880 nm LED; Velleman, Belgium) by a spectrum-enhanced camera (EOS035 Canon Rebel Xti; 400DH) with built-in clear wideband-multicoated filter and standard accessories (Canon) and operated by EOS utility software. Angles between hypocotyl axis and cotyledons were measured by ImageJ software. At least 10 seedlings with synchronized germination were processed. For more details see (Zhu et al., 2017)).

Histochemical and histological analysis.

To detect β-Glucuronidase (GUS) activity, mature embryos and 2 to 5-day-old seedlings were incubated in reaction buffer containing 0.1M sodium phosphate buffer (pH 7), 1mM ferricyanide, 1mM ferrocyanide, 0.1% Triton X-100 and 1mg ml^{-1} X-Gluc for 24 h in dark at 37°C. Afterwards, chlorophyll was removed by destaining in 70% ethanol and seedlings were cleared as described above. GUS expression was monitored by differential interference contrast microscopy (Olympus BX53).

Immunolabeling in roots (4 to 5-day-old seedlings) was performed using an automated system (Intavis in situ pro) according to published protocol (Sauer et al., 2006). Roots were fixed in 4% paraformaldehyde for 1 h in vacuum at room temperature. Afterwards, seedlings were incubated for 30–45 min in PBS (2.7mM KCl, 137mM NaCl, 4.3mM Na_2HPO_4 $2H_2O$ and 1.47mM KH_2PO_4, pH 7.4) containing 2% driselase (Sigma), and then in PBS supplemented with 3% NP40 and 20% DMSO. After blocking with 3% BSA in PBS, samples

were incubated with primary antibody for 2 hours. Antibody dilutions were rabbit anti-BIP2 (1:200) (Agrisera AS09481), rabbit anti-SEC21 (1:800) (Agrisera AS08327), rabbit anti-ARF1 (1:600) (Agrisera AS08325), rabbit anti-SYP61 (1:200) (Sanderfoot et al., 2001), rabbit anti-ECH (1:600) (kindly provided by R.P.Bhalerao, Umea Plant Science Centre), rabbit anti-ARA7+RHA1 1:1 (1:100) (Haas et al., 2007), rabbit anti-PIN1 (1:1000) (Paciorek et al., 2005), rabbit anti-PIN2 (1:1000) (provided by C. Luschnig, University of Natural Resources and Life Sciences, Vienna), mouse anti-GFP (1:600) (Sigma G6539), and rat anti-LM15 (1:100) (http://plantprobes.net). Secondary antibody incubation was carried on for 2 h. Anti-mouse-Alexa 488 (Life Technologies, 1252783), Cy3-conjugated anti-rabbit antibody (Sigma, C2306) and Anti-rat-Alexa 633 (Thermo Fisher Scientific, A-21094) were diluted 1:600 in blocking solution. Samples were mounted in solution containing 25 mg mL^{-1} DABCO (Sigma) in 90% glycerol, 10% PBS, pH 8.5. Signal was monitored using a confocal laser scanning microscope (LSM 700, Zeiss). Images were analyzed by using ImageJ software.

Co-localization analysis.

Pearson's correlation coefficient (R) was used for co-localization analyses: the analysis is based on the pixel intensity correlation over space and was performed using Image J software. After splitting the two channels, region of interest (ROI) was identified. For our analysis, 1 cell was considered as 1 ROI; in every root approximately 5 cells (5 ROIs) were measured and a minimum of 10 roots were used. Co-localization plug-in using an automatic threshold was used to obtain Rcoloc value, which represent Pearson's correlation coefficient.

Confocal imaging and image analysis.

Zeiss LSM 700 confocal scanning microscope using either 20x or 40x (water immersion) objectives were employed to monitor expression of fluorescent reporters. GFP (YFP) and Cy3 signals were detected either at 488 nm excitation/507 nm emission or 550 nm excitation/570 nm emission wavelength, respectively. Quantification of immunodetected PIN1 and PIN2 expression in root meristems was performed by measurement of membrane signal in cortex and epidermal cells, respectively. Signal in approximately 10 cells in a minimum of 10 roots was measured using ImageJ software. Statistical significance was evaluated by Student's t-test.

Live tracking of vertically grown roots.

Arabidopsis seedling root growth was observed according to (von Wangenheim et al., 2017). Briefly, Columbia and the transgenic reporter line pEST:SYAC1-GFP were grown for 4 days in plant MS medium plates at 22°C. Arabidopsis seedlings were carefully transferred on a block of solid agar gel, supplemented with or without 5µM Estradiol, avoiding any air draft, and inserted in a split imaging chamber (ThermoFisher) with a spatula. The imaging chamber was mounted on an inverted vertical confocal microscope (Zeiss Axio Observer with LSM 700 scanhead) with custom illumination system. Root growth was monitored using a 10x objective and the TipTracker software over a period of 20 h with an imaging interval of 30 min. Root growth for eight plants (4 Columbia and 4 pEST:SYAC1-GFP) was followed in control agar medium and for another eight plants in Estradiol containing medium. A script for the open-source software Fiji (Schindelin et al., 2012) was used to convert multi-position time series image files into multiple hyperstacks, each containing a single root position. TipTracker output file with the coordinates of single root positions along the time series was used to measure the growth rate of the individual roots (µm/h).

Transient expression in root suspension culture protoplasts.

The transient expression assays were performed on 4-days-old Arabidopsis root suspension culture by PEG mediated transformation. Protoplasts were isolated in enzyme solution (1% cellulose (Serva), 0.2% Macerozyme (Yakult), in B5 - 0.34M glucose-mannitol solution (2.2 g MS with vitamins, 15.25 g glucose, 15.25 g mannitol, pH to 5.5 with KOH) with slight shaking for 3–4 h, and afterwards centrifuged at 800g for 5 min. The pellet was washed and resuspended in B5 glucose-mannitol solution to a final concentration of $4x10^6$ protoplasts /mL. DNAs were gently mixed together with 50 µL of protoplast suspension and 60 µL of PEG solution (0.1M $Ca(NO_3)_2$, 0.45M Mannitol, 25% PEG 6000) and incubated in the dark for 30 min. Then 140 µL of 0.275M $Ca(NO_3)_2$ solution was added to wash off PEG, wait for sedimentation of protoplasts and remove 240 µL of supernatant. The protoplast pellet was resuspended in 200 µL of B5 glucose-mannitol solution and incubated for 16 h in the dark at room temperature. Transfected protoplasts were mounted on the slides and viewed with Zeiss LSM 700 confocal scanning microscope.

Transient expression in Arabidopsis mesophyll protoplasts.

Mesophyll protoplasts were isolated from rosette leaves of 4-week-old Arabidopsis plants grown in soil under controlled environmental conditions in a 16:8 h light/dark cycle at 21 C.

Protoplasts were isolated and transient expression assays were carried out as described (Wu et al., 2009).

Coimmunoprecipitation (Co-IP) assays.

For the Co-IP assays, proteins were expressed in root suspension culture protoplasts (see above) and extracted from the cell pellet as described previously (Cruz-Ramírez et al., 2012); vectors containing ECH-HA and YIP4a-Myc were kindly provided by R.P. Bhalerao, Umea Plant Science Centre. 100 µg total protein extract was incubated in a total volume of 100 µL extraction buffer containing 150 mM NaCl and 1 µg anti-GFP (JL-8, Clontech) or 1.5 µg anti-cMyc (clone 9E10, Covance). After 2 h, 15 µL ProteinG-Magnetic Beads (BIO-RAD), which were previously equilibrated in TBS buffer we added and this mixture was further incubated for another 2 h on a rotating wheel at 4°C. The beads were then washed in 3x500 µL washing buffer (1xTBS, 5% glycerol, 0,1% Igepal CA-630) and eluted by boiling in 25 µL 1.5x Laemmli sample buffer. Proteins were then resolved with SDS-PAGE and blotted to PVDF transfer membrane (Millipore). The presence of the proteins of interest was tested by immunodetection using rat anti-HA-peroxidase (3F10, Roche).

Bimolecular fluorescence complementation (BiFC) assay.

To generate constructs for BiFC assay, the ORFs for SYAC1, YIP4a, YIP4b, YIP5b, ECH, KCR1 and PHB4 proteins were cloned into the pDONRZeo vector. Next, the ORFs were transferred from their respective entry clones to the gateway vector pSAT4-DEST-n(174)EYFP-C1 (ABRC stock number CD3-1089) or pSAT5-DEST-c(175-end)EYFP-C1 (ABRC stock number CD3-1097), which contained the N-terminal 174 amino acids of enhanced yellow fluorescent protein ($EYFP^N$) or the C-terminal 64 amino acids of EYFP ($EYFP^C$), respectively. The fusion constructs encoding cEYFP-SYAC1 and nEYFP-YIP4a, nEYFP-YIP4b, nEYFP-YIP5b, nEYFP-ECH, nEYFP-KCR1 or nEYFP-PHB4 proteins were mixed at a 1:1 ratio and transfection of root suspension culture protoplasts (see above) was performed. SYAC1 in P2YGW7 was used as a positive control.

Yeast two-hybrid assays.

Yeast two-hybrid assay was performed using the GAL4-based two-hybrid system (Clontech). Full-length SYAC1 and YIP4a, YIP4b, YIP5b, ECH, KCR1, DSK2, PHB4 ORFs were cloned into pGADT7 and pGBKT7 (Clontech) to generate the constructs AD-SYAC1 and BD-YIP4a (YIP4b, YIP5b, ECH, KCR1, DSK2, PHB4). The constructs were transformed into the yeast strain PJ69-4A with the lithium acetate method. The yeast cells were grown on

minimal medium (–Leu/–Trp), and transformants were plated (minimal medium, –Leu/–Trp/–His without or with increasing concentration of 3-Amino-1,2,4-trizol) to test the protein interactions.

α-Amylase enzymatic assay.

α-Amylase assays and calculations of the secretion index were performed as described (Früholz and Pimpl, 2017); α-Amylase expression constructs were kindly provided by P. Pimpl and transfections were performed in *Arabidopsis* mesophyll protoplasts (see above). α-Amylase activity was measured with a kit Ceralpha (Megazyme). The reaction was performed in a microtiter plate at 37 °C with 30 µL of extract and 30 µL of substrate. The reaction was stopped by the addition of 150 mL of stop buffer. The absorbance was measured at a wavelength of 405 nm. Experiment was performed three times with four replicates.

AFM measurements and Apparent Young's Modulus calculations.

The AFM data were collected and analyzed as described elsewhere with minor changes (Peaucelle et al., 2015). To examine extracellular matrix properties the turgor pressure was suppressed by seedlings immersion in a hypertonic solution (0.55 M mannitol). 4 days-old seedlings grown in darkness were placed on petri dishes filled with 1% Agarose and 10% Mannitol and immobilized by low melting agarose (0.7% with 10% Mannitol). The focus was set on the anticlinal (perpendicular to the organ surface) cell walls and its extracellular matrix. To ensure proper indentations (especially in the bottom of the doom shape between two adjacent cells regions), cantilevers with long pyramidal tip (14-16 µm of pyramidal height, AppNano ACST-10), with a spring constant of 7.8 N/m were used. The instrument used was a JPK Nano-Wizard 3.0 and indentations were kept to <10% of cell height. Three scan-maps per sample were taken over an intermediate region of the hypocotyls, using a square area of 25 x 25 µm, with 16 x 16 measurements, resulting in 1792 force-indentation experiments per sample. The lateral deflection of the cantilever was monitored and in case of any abnormal increase the entire data set was discarded. The apparent Young's modulus (EA) for each force-indentation experiment was calculated using the approach curve (to avoid any adhesion interference) with the JPK Data Processing software (JPK Instruments AG, Germany). To calculate the average EA for each anticlinal wall, the total length of the extracellular region was measured using masks with Gwyddion 2.45 software (at least 20 points were taken in account). The pixels corresponding to the extracellular matrix were chosen based on the topography map. For topographical reconstructions, the height of each

point was determined by the point-of-contact from the force-indentation curve. A total of 12-14 samples were analyzed. A standard t-test was applied to test for differences between genotypes.

Ruthenium red staining.

Mature seeds were incubated in 0.01% (w/v) aqueous solution of Ruthenium red. Seeds were mounted in water and viewed using a DIC Olympus BX53 microscope.

Tandem affinity purification.

Tandem affinity purification assay was performed in *Arabidopsis* cell suspension culture as described (Van Leene et al., 2014).

Fourier Transform Infrared Spectroscopy (FT-IR).

Spectra were recorded from the 4 days old dark grow hypocotyls sections in transmission mode on a Bruker Tensor 27 spectrometer equipped with a Hyperion 3000 microscopy accessory and a liquid N_2 cooled 64x64 mercury cadmium telluride (MCT) focal plane array (FPA) detector. 4 hypocotyls for each line were used and 5 spectra from each of 3 different regions were measured. The entire setup was placed on a vibration-proof table. Spectra were recorded in the region 900 – 3900 cm^{-1}, with 4 cm^{-1} spectral resolution and 32 scans co-added in double sided, forward-backward mode. FPA frame rate was 3773 Hz and integration time 0.104 ms, with offset and gain optimized for each sample between 180-230 and 0-1, respectively. A low pass filter and an aperture of 6mm were used. Background was recorded on a clean, empty spot on the CaF_2 carrier (Crystran Ltd, UK) and automatically subtracted. Fourier transformation was carried out using a zero filling factor of two, and Blackman-Harris 3-term apodization function. Phase correction was set to the built-in Power mode with no peak search and a phase resolution of 32. White light images were recorded with a Sony ExwaveHAD color digital video camera mounted on the top of the microscope and exported as jpg files. Spectra were recorded using OPUS (version 6.5 and 7, Bruker Optics GmbH, Ettlingen, Germany), cut to the fingerprint region of 900-1800 cm^{-1} and exported as .mat files for subsequent processing and analysis. The exported spectra were pre-processed by an open-source software developed at the Vibrational Spectroscopy Core Facility in Umeå (http://www.kbc.umu.se/english/visp/download-visp/), written in MATLAB (version 2014a, Mathworks, USA), using asymmetric least squares baseline correction (Eilers, 2004); (lambda: 100,000 and p=0.001), Savitzky-Golay smoothing (Savitzky and Golay, 1964); using a 1st order polynomial, with a frame number of 5; and total area normalization.

Multivariate Curve Resolution – Alternating Least Squares (MCR-ALS) analysis was performed on the spectra using 5 components based on singular value decomposition of the initial dataset. A maximum of 50 iterations and a convergence limit 0f 0.1 were used, with initial estimates in the spectrum direction and noise level of 10% given in the script. Only non-negativity constraints were used, both in the spectrum and concentration dimensions. For classification, k-means clustering was performed within the open-source software, using the resolved spectral profiles for each sample.

Accession numbers.

Sequence data from this article can be found in GenBank/EMBL data libraries under the following accession numbers: *SYAC1*, At1g15600; *YIP5b*, At3g05280; *YIP4a*, At2g18840; *YIP4b* At4g30260; *ECH*, At1g09330; *KCR1*, At1g67730; *DSK2*, At2g17200; *PHB4*, At3g27280.

3.5 Figures and Tables

Figure 1

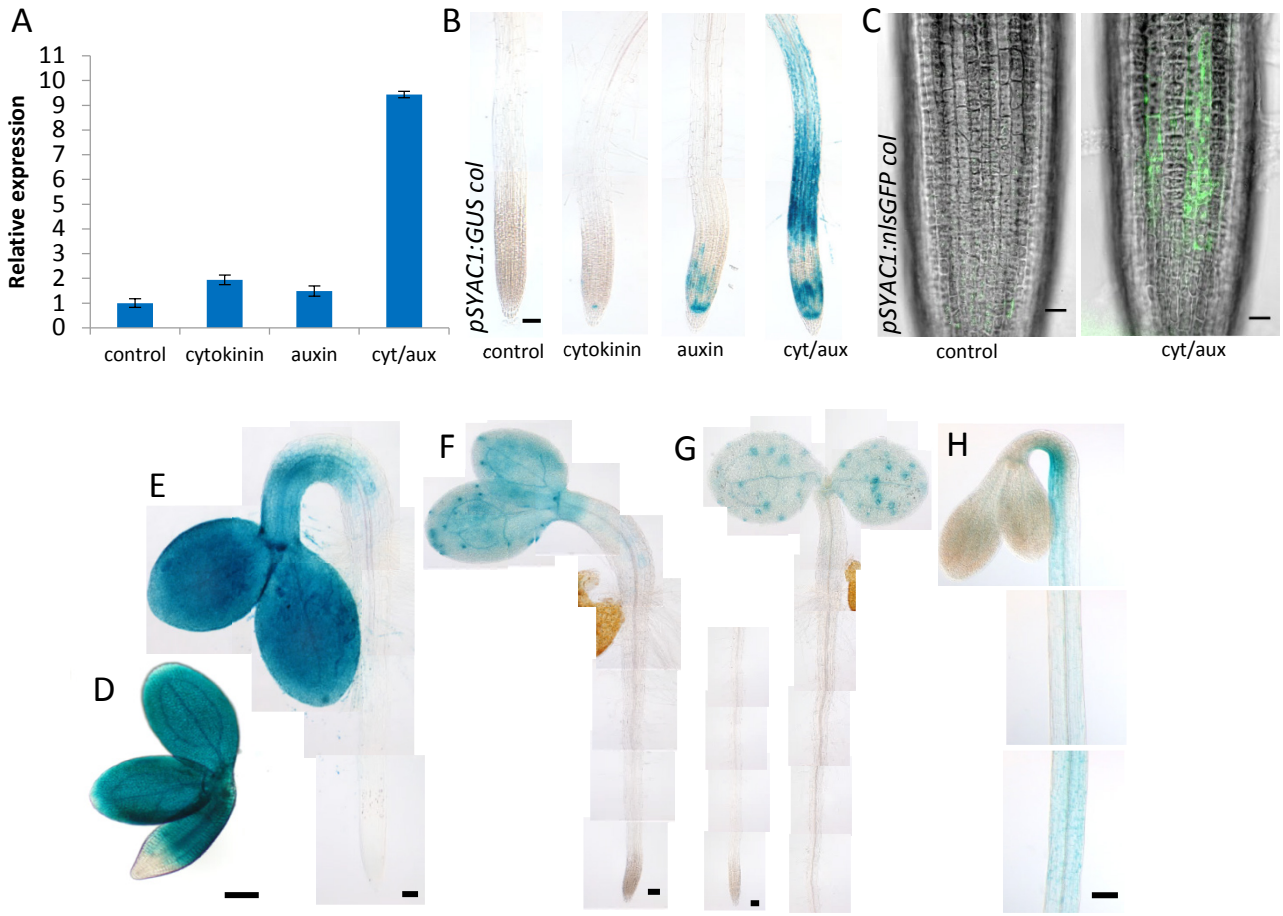


Figure 1. Developmentally specific expression of *SYAC1* and in response to hormonal treatment
(A-C) Expression of *SYAC1* in 5 days old roots is synergistically upregulated after 6 hours treatment with 1 µM auxin and 10 µM cytokinin. *SYAC1* expression in *Columbia* roots monitored by qRT–PCR(A), expression of GUS. (B) Expression of *nlsGFP* (C). (D-H) Expression pattern of *SYAC1*in mature embryo and seedlings. Mature embryo (D), 3, 4 and 5 days old seedling, respectively (E-G). (H) Hypocotyl and apical hook of 3 days old etiolated seedling. Error bars represent standard error. Scale bar 50 µm (B) 20 µm (C), 200 µm (D), 50 µm (E-G), 100 µm (H).

Figure 2

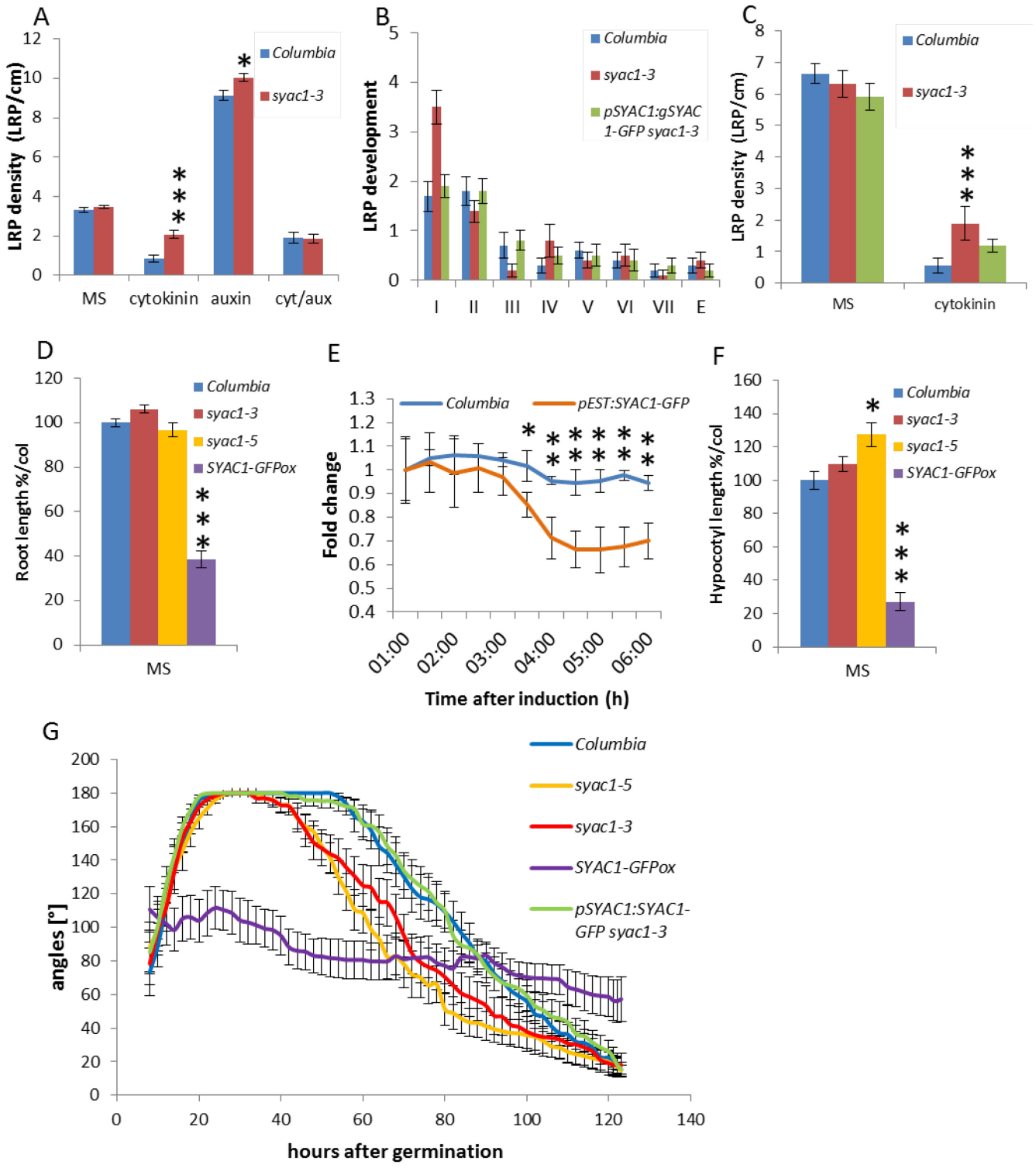


Figure 2. SYAC1 regultes plant growth by modification of cell elongation and by fine tuning LRP response to auxin and cytokinin inputs. (A,C) The inhibitory effect of cytokinin on LRP is reduced in *syac1* mutant, *pSYAC1:gSYAC1-GFP* complements *syac1* defect. (B) *syac1* exhibits delay in LR development, and this phenotype defect is recovered by *pSYAC1:gSYAC1-GFP*. (D,F) *syac1* exhibits longer etiolated hypocotyls, whereas overexpression of *SYAC1-GFPox* causes a strong reduction in root and etiolated hypocotyl growth. (E) Significant reduction of root growth was observed within 4 hours after induction of *SYAC1* expression by

estradiol. (G) Apical hook in *syac1* opens 20 hours earlier when compared to Columbia and *pSYAC1:gSYAC1-GFP*, *syac1* complementation line. *SYAC1-GFPox* overexpressor fails to form apical hook. For each experiment n = 15, average ± SE. Significant differences are indicated as *P < 0.05, **P < 0.01, and ***P < 0.001 (t test). Each graph shows one experiment, representative 2 additional repeats.

Figure 3

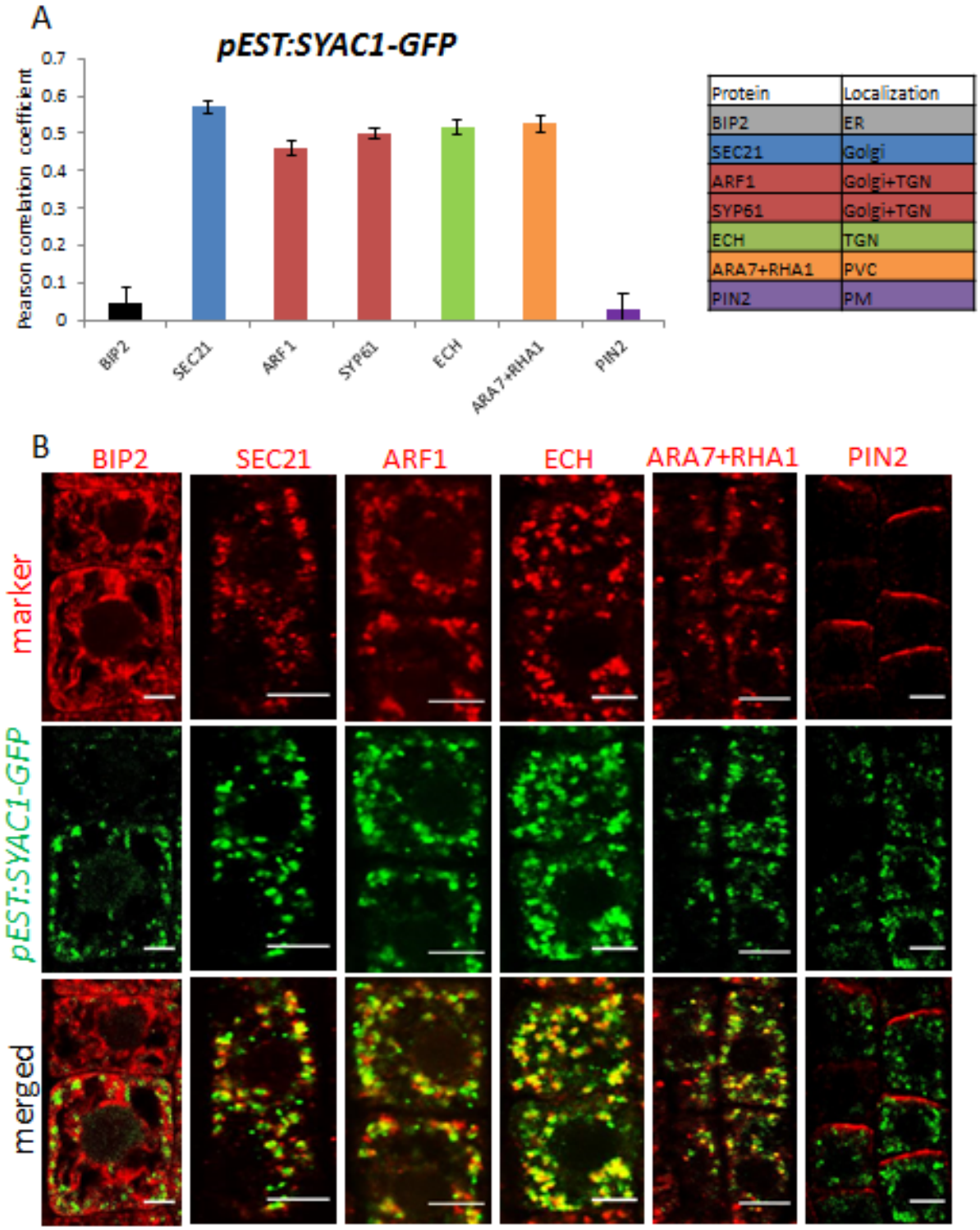


Figure 3. SYAC1 colocalizes with markers for Golgi/TGN/Endosomal/PVC compartments. (A) Quantitative measurement of colocalization of SYAC1 with various markers and their localization by evaluation of Pearson correlation . (B) Colocalization between SYAC1 and various markers for intracellular compartments detected by immunolocalisation. *pEST:SYAC1-GFP* line was grown 5 days on MS media then transferred for 6 hours on plates with 5 µM estradiol. (n = 10 roots with 5 cells each; average ± SE). Scale bars = 5 µm.

Figure 4

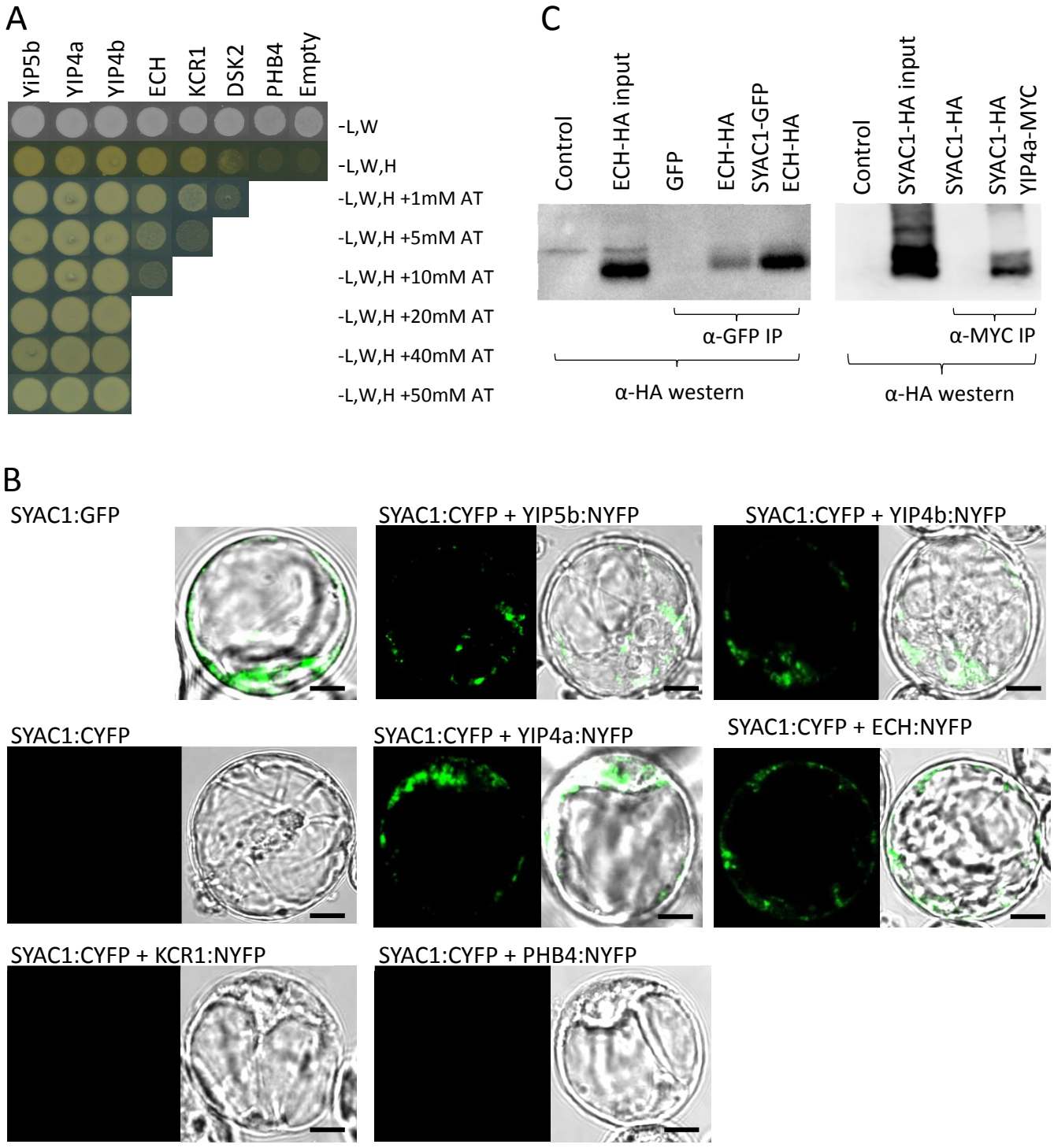
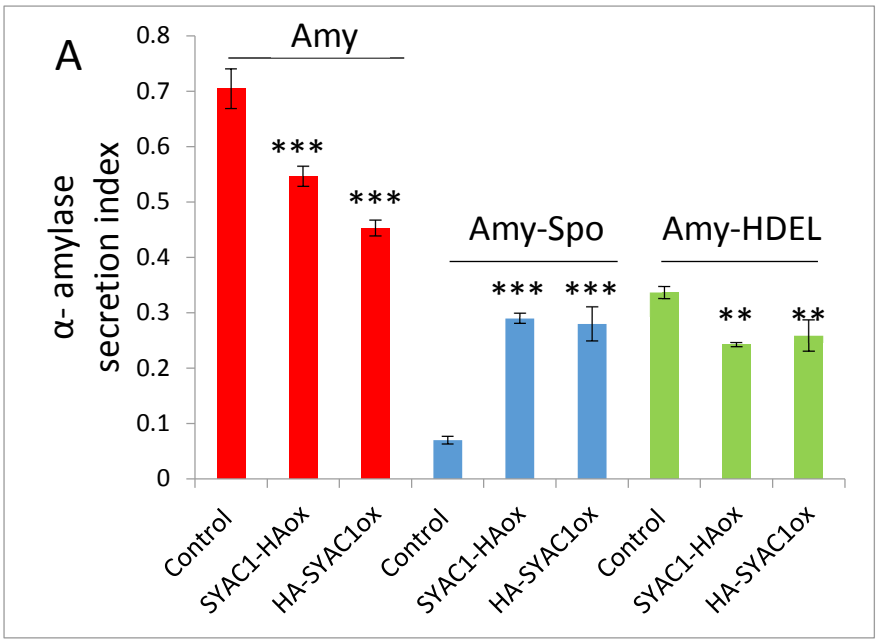
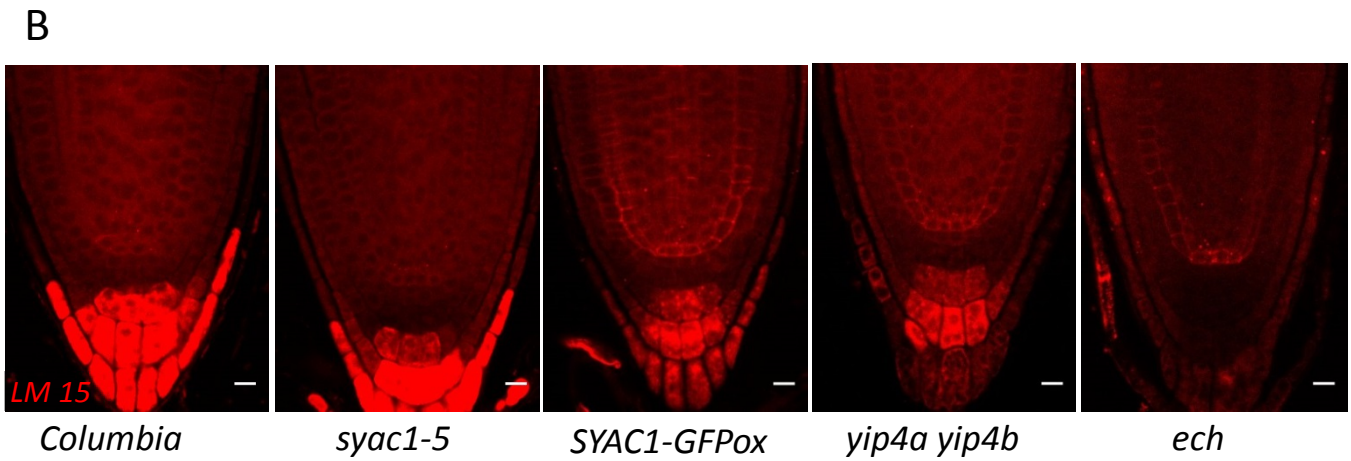


Figure 4. SYAC1 interacts with YIP4a, YIP4b, YIP5b and ECH protein. (A) Y2H assay confirms SYAC1 interaction with YIP4a, YIP4b YIP5b, ECH, KCR1. Weak or no interaction recovered for DSK2 and PHB4 protein. Yeast cells were grown on SD-LWH minimal media without histidin (H), leucin (L) and tryptophan (W), supplemented with 3-amino-1,2,4-triazole (3AT). Empty vectors were used as a negative control. (B) BiFC assay between SYAC1 with YIP4a, YIP4b YIP5b, ECH, KCR1, DSK2 and PHB4 protein performed in *Arabidopsis* root cell culture protoplasts. *SYAC1:GFP* and *SYAC1:CYFP*(C-terminal part of YFP) were used as a positive and negative control, respectively. Scale bar 5 µm. (C) Co-immunoprecipitation (CoIP) assay of SYAC1-GFP with ECH-HA and SYAC1-HA with YIP4a-MYC transiently expressed in *Arabidopsis* root cell

culture protoplasts. Plots show the GFP or MYC immunoprecipitation (IP), and the protein gel blot was performed using an anti-HA antibody.

Figure 5





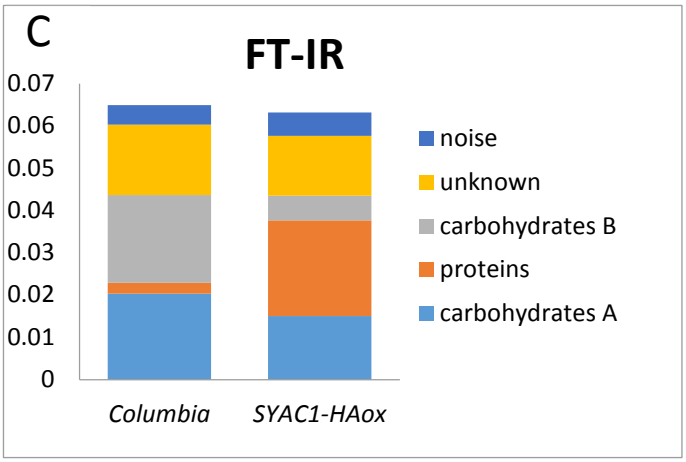


Figure 5. SYAC1 regulates secretion of cell wall components. (A) *SYAC1* affects α-amylase secretion index. Transient co-expression of *SYAC1* with *α-amylase* (Amy) and its derivatives carrying different C-terminal

sorting motifs, including ER retention (Amy-HDEL) and vacuolar sorting motif (Amy-spo). Error bars indicate standard error calculated from 4 independent measurements. Significant differences are indicated as **P < 0.01, and ***P < 0.001 (t test). (B) Immunolacalization of xyloglucan with LM 15 antibody shows increase and change in xyloglucan localization in *SYAC1* overexpressor line, *yip4a yip4b* and *ech* mutant.. Scale bar 10 μm. (C) FT-IR measurements in 4 days old etiolated hypocotyls show alternations in cell wall composition in *SYAC1-HAox* lines.

Figure 6

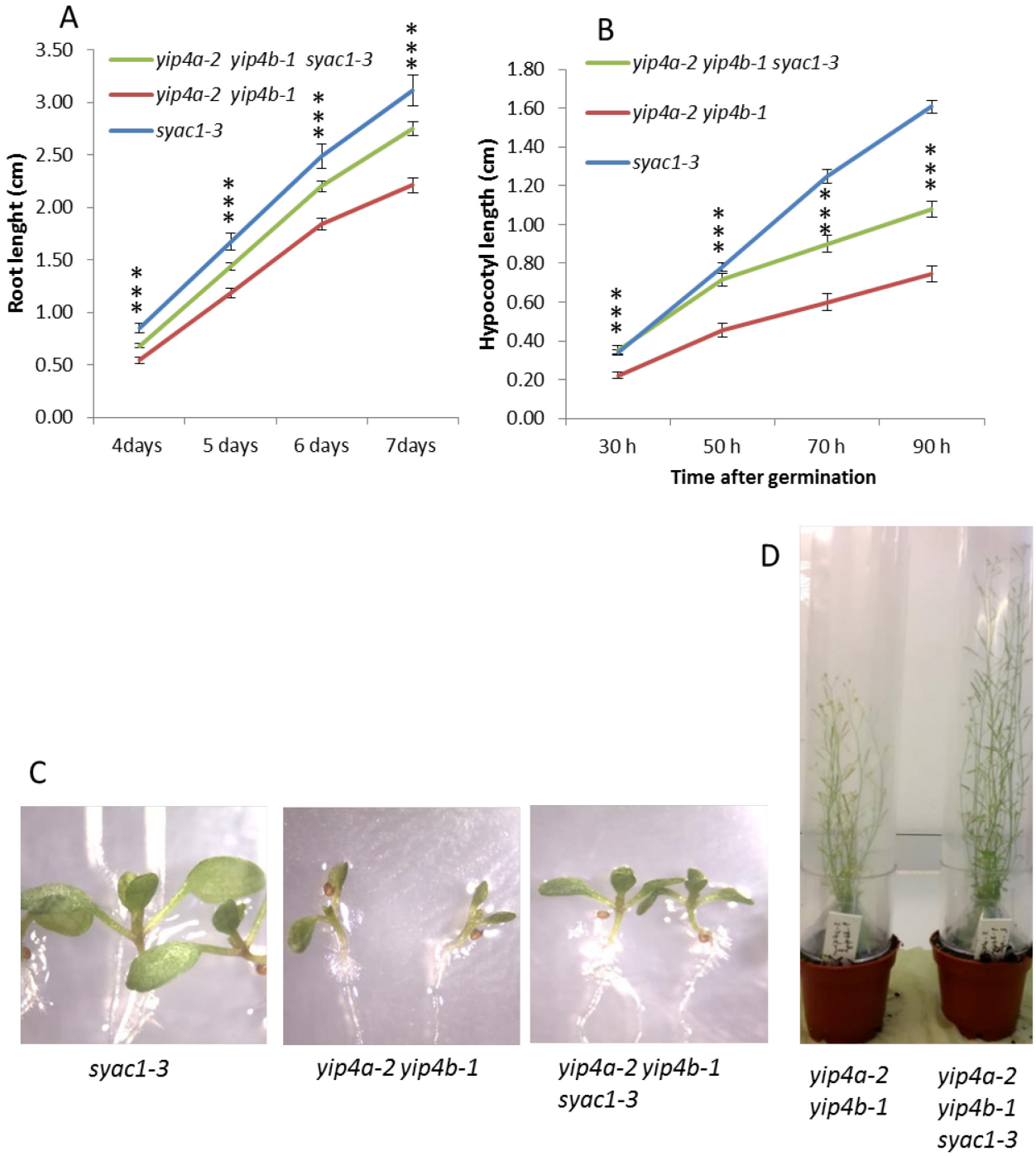
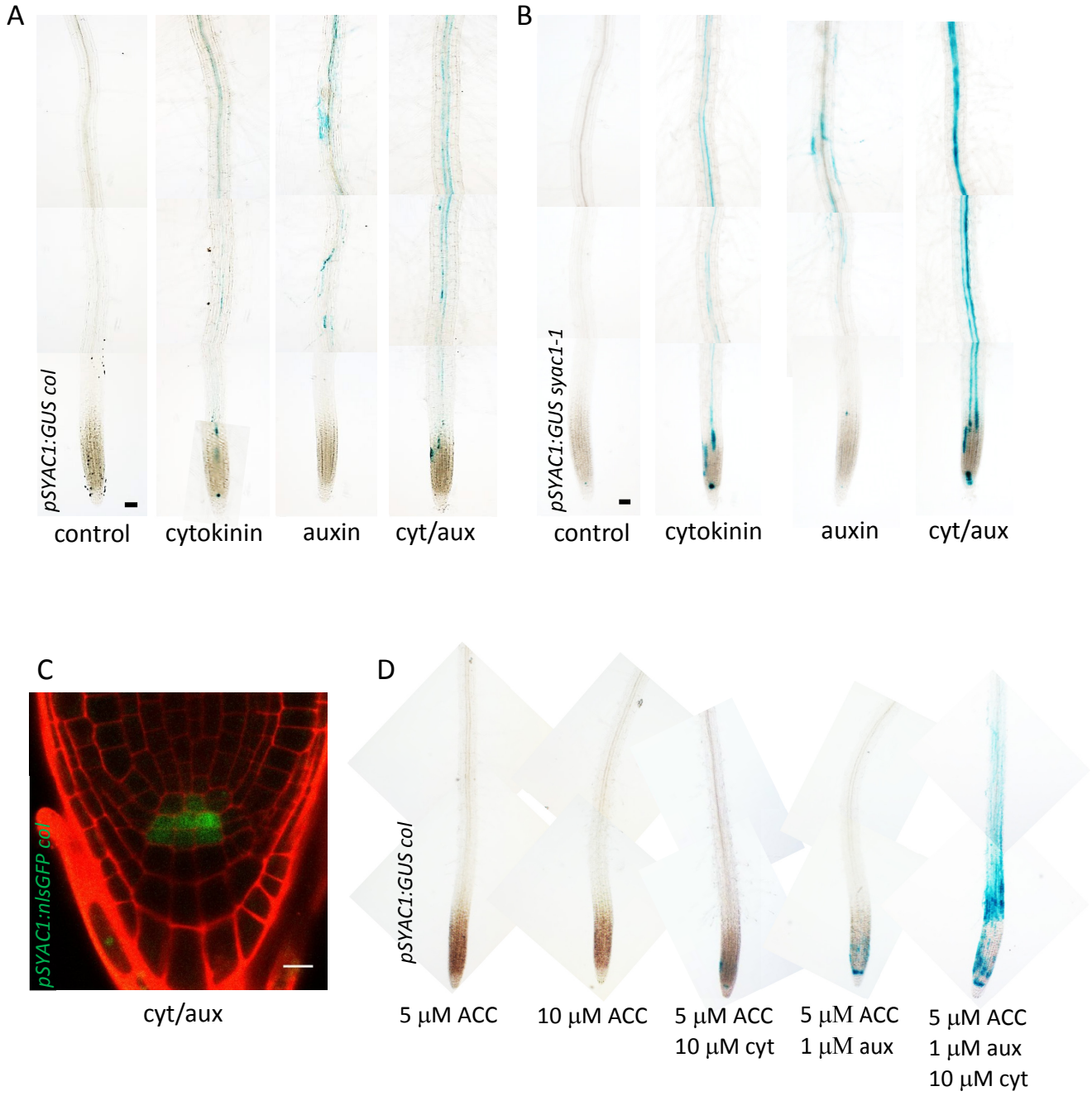


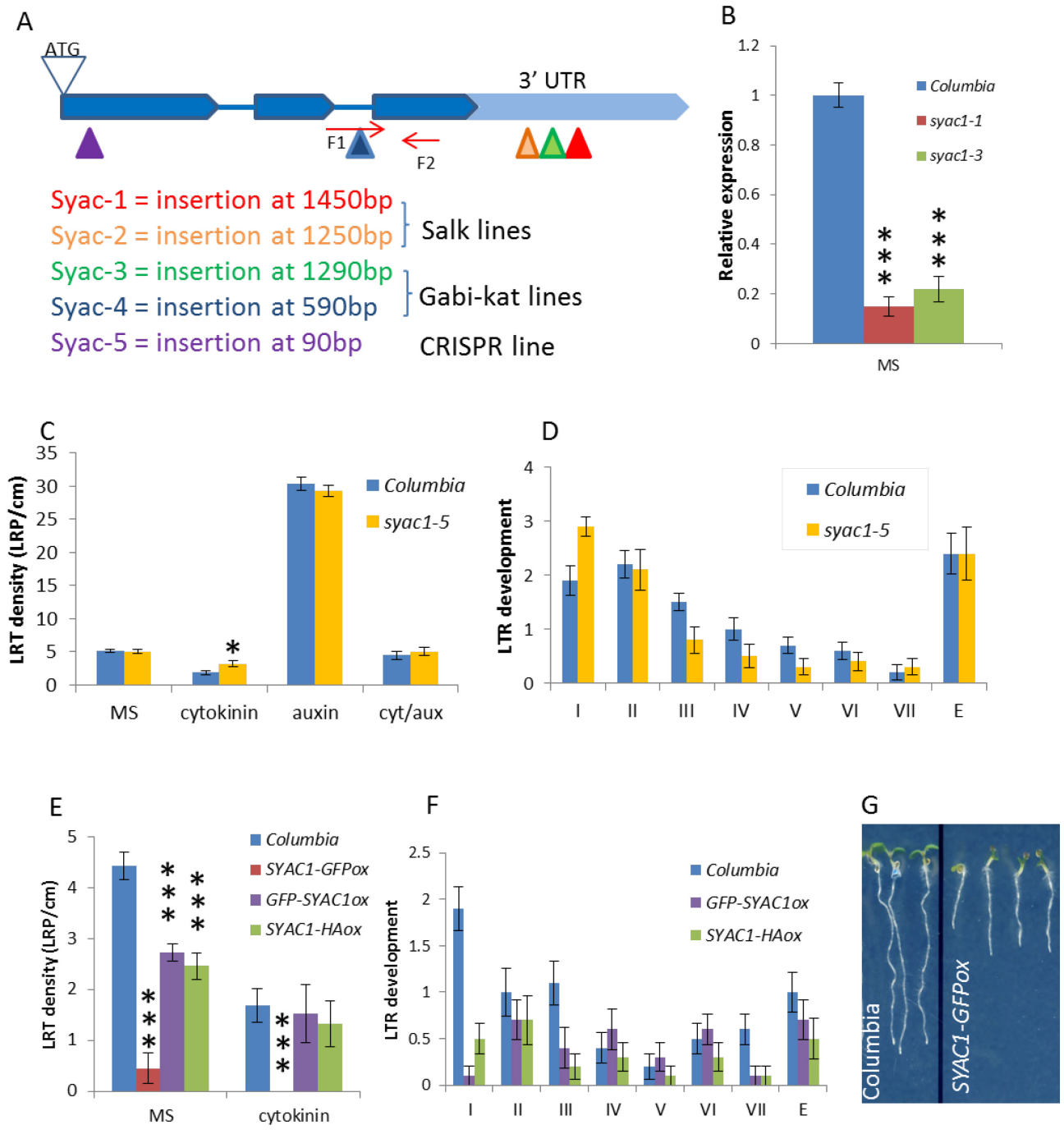
Figure 6. *syac1* partially rescues the *yip4a yip4b* elongation deficit. (A) Root growth kinetics of the *yip4a yip4b* double and *yip4a yip4b syac1* triple mutant (n = 30, average ± SE). (B) Etiolated hypocotyl growth kinetics of the *yip4a yip4b* double and *yip4a yip4b syac1* triple mutant (n = 15, average ± SE). Significant differences calculated for double and triple mutant are indicated as ***P < 0.001 (t test). Each graph shows one experiment, representative 2 additional repeats. (C) Representative images of 7-day-old *syac1*, *yip4a yip4b* and *yip4a yip4b syac1* seedlings grown *in vitro*. (D) Representative images of 2-months-old, *yip4a yip4b* and *yip4a yip4b syac1* plants.

Supplementary figure 1



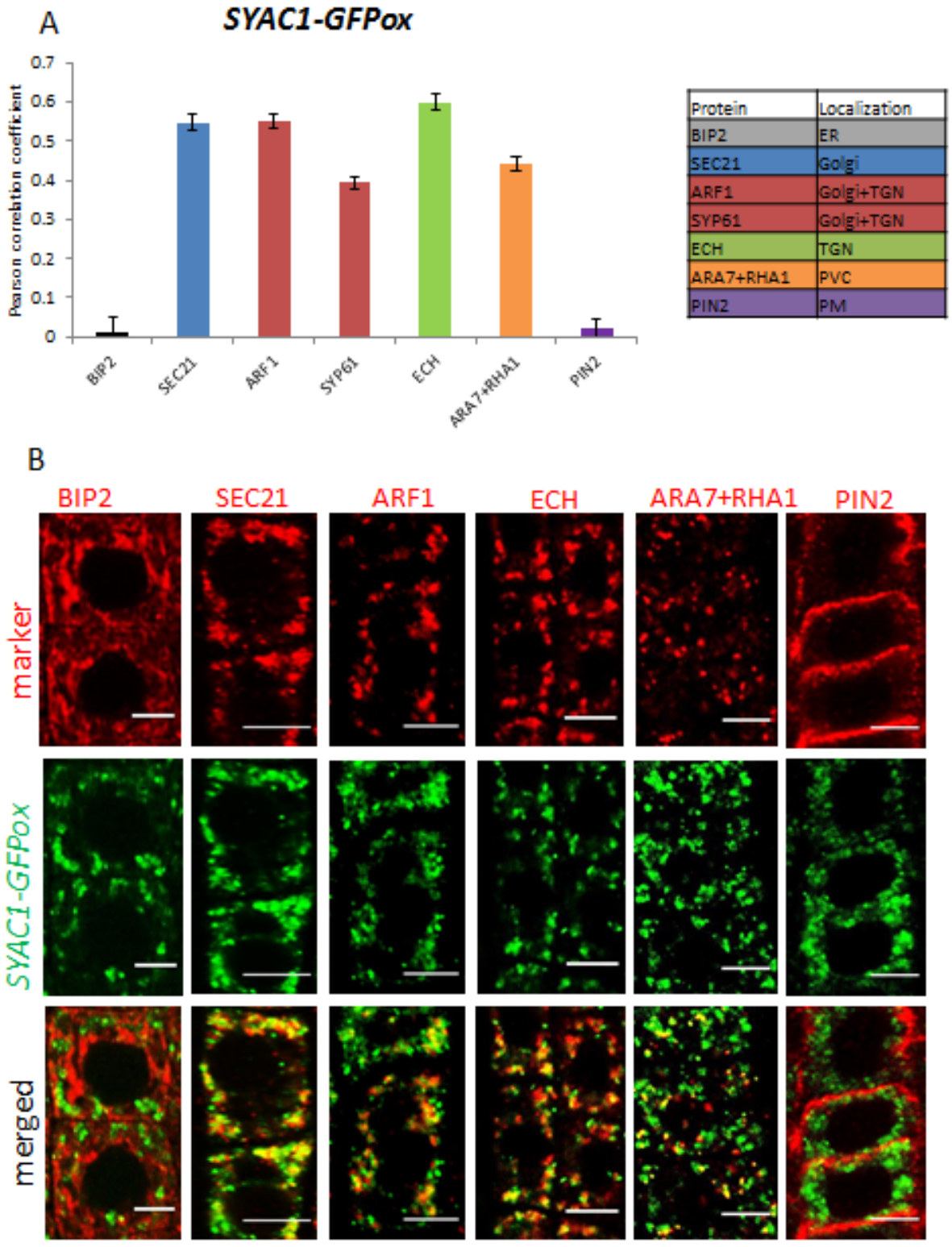
Supplementary Figure 1. Expression pattern of *SYAC1* in response to hormonal treatments in root. (A-B) Expression of *SYAC1* in roots of 5 days old plants grown on media supplemented 100 nM cytokinin and 100 nM auxin when treated separately and 50 nM when auxin and cytokinin added together is synergistically upregulated. Scale bar 50 µm. (C) Expression of *SYAC1:nlsGFP* in quiescent centre and collumela initials of 5 days old seedlings treated with auxin and cytokinin for 6 hours. Scale bar 10 µm. (D) Treatment for 6 hours with ACC (ethylene precursor) or ACC in combination with either cytokinin or auxin does not trigger *SYAC1:GUS* transcription. No ACC interference with auxin and cytokinin triggered *SYAC1:GUS* expression observed as well.

Supplementary figure 2



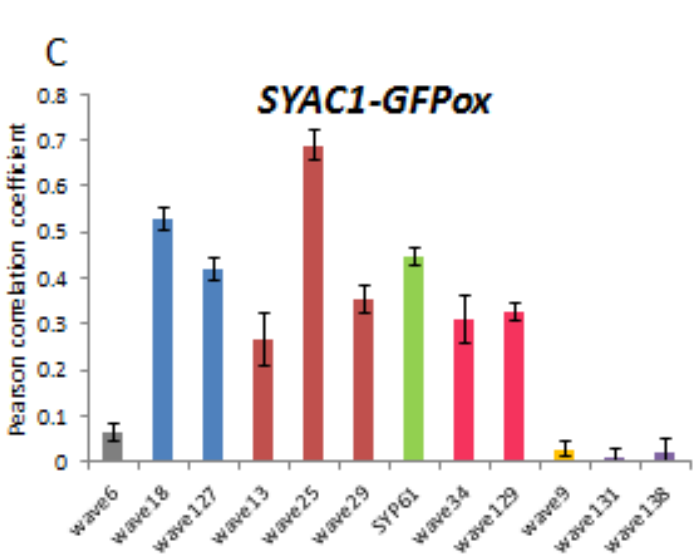
Supplementary figure 2. SYAC1 regulates LR organogenesis.(A) Exon/intron maps of the *SYAC1* genes (from TAIR) and T-DNA and CRISPR insertion positions. F1 and F2 arrows indicate the position of primers used for qRT-PCR. (B) *SYAC1* expression in Columbia, *syac1-1* and *syac1-3* monitored by qRT–PCR. Error bars represent standard error. (C) *syac1* mutant is resistant to cytokinin inhibitory effect on LR organogenesis. (D) *syac1* shows a delay in lateral root development. (E,F) Overexpression of SYAC1 is causing a significant decrease in LRT density and interfere with LRP development. (n = 10, average ± SE). (G) Representative images of 5 days old Columbia and *SYAC1-GFPox* lines. Significant differences are indicated as *P < 0.05 and ***P < 0.001 (t test). Each graph shows one experiment, representative 2 additional repeats.

Supplementary figure 3 A,B

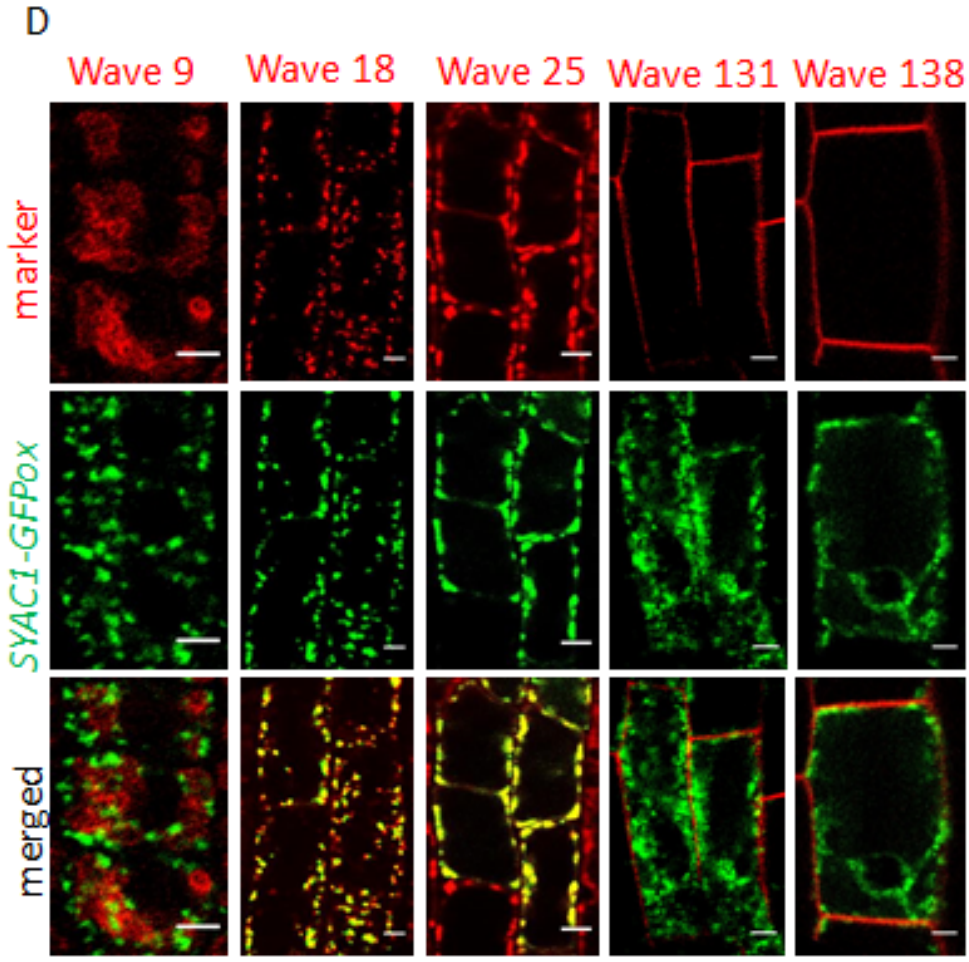


Supplementary figure 3A,B. SYAC1 colocalizes with markers for Golgi/TGN/Endosomal/PVC compartments. (A,C) Quantitative measurement of colocalization of SYAC1 with various markers and their localization using Pearson correlation coefficient (n = 10 roots with 5 cells each; average ± SE). Scale bars 10 µm.

Supplementary figure 3C,D

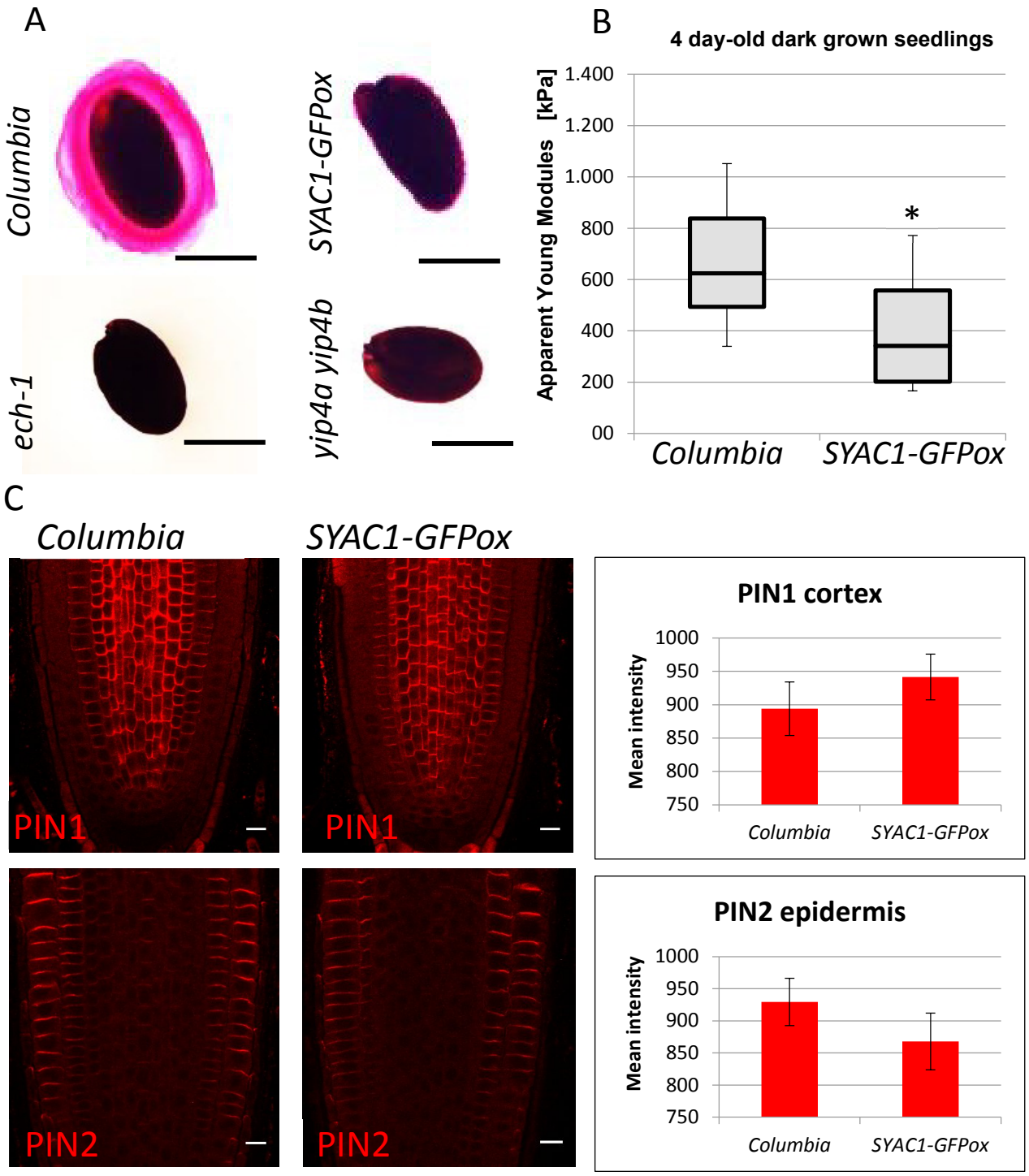


	Protein	Localization
wave6	NIP1;1	ER+PM
wave18	Got1	Golgi
wave127	MEMB12	Golgi
wave13	VTI12	TGN+EE
wave25	Rab D1	Post -golgi +endosomal
wave29	Rab D2a	Golgi+endosomal
SYP61	SYP61	TGN
wave34	Rab A1e	Endosomal+RE
wave129	RabA1g	Endosomal+RE
wave9	VAMP711	Vacuole
wave131	NPSN12	PM
wave138	PIP1;4	PM



Supplementary figure 3C,D. SYAC1 colocalizes with markers for Golgi/TGN/Endosomal/PVC compartments. Colocalization between SYAC1 and various markers for intracellular compartments detected by immunolocalisation (B), by monitoring *SYAC1:GFP* and RFP fused to respective subcellular marker (D). (n 10 roots with 5 cells each; average ± SE). Scale bars 5 µm.

Supplementary figure 4



Supplementary figure 4. *SYAC1* overexpression inhibits seed coat mucilage secretion, reduces stiffnes of the cell wall but doesn't interfere with plasma membrane protein accumulation. (A) Ruthenium red–stained seed coat mucilage after imbibition of *Columbia*, *SYAC1-GFPox, ech-1* and *yip4a yip4b* seeds. Scale bar 200 µm. (B) The apparent Young modules measured by AFM in 4 day-old etiolated hypocotyls. (n = 10-14, average ± SE). Significant differences are indicated as *P < 0.05 (t test). (C) PIN1 and PIN3 intensity at the plasma membrane of *Columbia* and *SYAC1-GFPox*. Scale bar 10 µm. Average of intensity measured in 10 roots (10 cells for each) ± SE. No significant difference (t-test).

Supplementary Table 1: Sequences of primers

Gene and purpose	Name	Sequence (5'-3')
Genotyping *syac1-1* SALK_151420C	Syac1-1_LP	GTGTTCTCCTTG CTGTGGAAG
	Syac1-1_RP	ACATAAAGCCCTCTCCGATTC
Genotyping *syac1-2* SALK_151662B	Syac1-2_LP	TGCGTATCAAAC GAAATTTCC
	Syac1-2_RP	ACATAAAGCCCTCTCCGATTC
Genotyping *syac1-3* GABI-KAT 760F05	Syac1-3_LP	TGACCTTTCTTA TACTCATGCCTT
	Syac1-3_RP	TTCCACTTGTAAATGGACAACTCC
Genotyping *syac1-4* GABI-KAT 961C03	Syac1-4_LP	GGGTGATCCAT ACACAAATGAAAG
	Syac1-4_RP	CCAATTATTAGTCAATTGTAGCCCG
Genotyping *ech-1* SAIL 163E09	Ech-1_LP	AAACGGAAAGGGAAACACAAC
	Ech-1_RP	AGAGAAGAGTTATCGGGCTCG
Genotyping *yip4a-2* SALK_021897	Yip4a-2_LP	GTTCTTGTGGCATTGCTTCTC
	Yip4a-2_RP	TGATCTGGTTTCCACATTTCC
Genotyping *yip4b-1* SALK_129888	Yip4b-1_LP	TGTTACTTCCGC ATAAGTCGG
	Yip4b-1_RP	GCGGCTGGAGAATTCTCTATC
Genotyping T-DNA SALK	LBb1.3	ATTTTGCCGATTTCGGAAC
Genotyping T-DNA SAIL	LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
Genotyping T-DNA GABI-KAT	RBGK.08474	ATAATAACGCTGCGGACATCTACATTTT
SYAC1 promoter cloning	pSYAC1_attB4	GGGGACAACTTTGTATAGAAAAGTTGGGGAAGACCTAGCCGTAGTT
	pSYAC1_attB1r	GGGGACTGCTTTTTTGTACAAACTTGTGATCACTTTTGGTTTTTCC
SYAC1 ORF cloning	SYAC1_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAGGGCCCTTTGTTGAG
	SYAC1_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAGCAGATGCATGATACAA
SYAC1 ORF fusion with GFP tag cloning	SYAC1_attB1_Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAGGGCCCTTTGTTGA

	GFP_attB2_Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTA CTTGTACAGCTCGTCCA
	SYAC1linGFP _Fw	ATTGTATCATGCATCTGCGGAGGTGGAGGTGGA GCTATGGTGAGCAAGGGCGAG
	GFPlinSYAC1 _Rv	CTCGCCCTTGCTCACCATAGCTCCACCTCCACCT CCGCAGATGCATGATACAAT
SYAC1 ORF fusion with 3xHA tag cloning	SYAC1_attB1 _Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAT GGAGGGCCCTTTGTTGA
	3xHAattB2_R v	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAT GCATAGTCCGGGACG
	SYAC1linHA_ Fw	ATTGTATCATGCATCTGCGGAGGTGGAGGTGGA GCTTTCCCATATGACGTTCCA
	HAlinSYAC1_ Rv	TGGAACGTCATATGGGAAAGCTCCACCTCCACCT CCGCAGATGCATGATACAAT
3xHA tag fusion with *SYAC1* ORF cloning	3xHA_attB1_F w	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAT GTTCCCATATGACGTT
	SYAC1_attB2 _Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCA GCAGATGCATGATAC
	HAlinSYAC1_ Fw	GACGTCCCGGACTATGCAGGAGGTGGAGGTGGA GCTATGGAGGGCCCTTTGTTG
	SYAC1linHA_ Rv	CAACAAAGGGCCCTCCATAGCTCCACCTCCACCT CCTGCATAGTCCGGGACGTC
YIP4a ORF cloning	YIP4a_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAT GTCACAAGGCGATACAGT
	YIP4a_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCA ATTGATGGCTATGATGA
YIP4b ORF cloning	YIP4b_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAT GTCGCACAACGATACGAT
	YIP4b_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCA ATTAATGGCAATGATTA
YIP5b ORF cloning	YIP5b_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAT GATGTCCGGCGGGAACTA
	YIP5b_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCA

		TACTTTGACATTGAAGA
ECH ORF cloning	ECH_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGACCCTAATAATCAGAT
	ECH_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAGACAAGGGTGAAGGCAG
KCR1 ORF cloning	KCR1_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAGATCTGCACTTACTT
	KCR1_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCATTCTTTCTTCATGGAGT
DSK2 ORF cloning	DSK2_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGGTGGAGAGGGAGATTC
	DSK2_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTACTGTCCGATACTCCCCA
PHB4 ORF cloning	PHB4_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGGAAGTCAACAAGTAGC
	PHB4_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAACGACCAGGGTTCAGAT

3.6 References

Bassham, D.C., Brandizzi, F., Otegui, M.S., and Sanderfoot, A.A. (2008). The Secretory System of Arabidopsis. Arab. Book *6*, e0116.

Beemster, G.T.S., and Baskin, T.I. (2000). STUNTED PLANT 1 Mediates Effects of Cytokinin, But Not of Auxin, on Cell Division and Expansion in the Root of Arabidopsis. Plant Physiol. *124*, 1718–1727.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell *115*, 591–602.

Bielach, A., Podlesakova, K., Marhavy, P., Duclercq, J., Cuesta, C., Muller, B., Grunewald, W., Tarkowski, P., and Benkova, E. (2012). Spatiotemporal Regulation of Lateral Root Organogenesis in Arabidopsis by Cytokinin. Plant Cell *24*, 3967–3981.

Bishopp, A., Help, H., El-Showk, S., Weijers, D., Scheres, B., Friml, J., Benková, E., Mähönen, A.P., and Helariutta, Y. (2011). A Mutually Inhibitory Interaction between Auxin and Cytokinin Specifies Vascular Pattern in Roots. Curr. Biol. *21*, 917–926.

Chae, H.S., Faure, F., and Kieber, J.J. (2003). The eto1, eto2, and eto3 Mutations and Cytokinin Treatment Increase Ethylene Biosynthesis in Arabidopsis by Increasing the Stability of ACS Protein. Plant Cell *15*, 545–559.

Chen, C.Z., Calero, M., DeRegis, C.J., Heidtman, M., Barlowe, C., and Collins, R.N. (2004). Genetic Analysis of Yeast Yip1p Function Reveals a Requirement for Golgi-Localized Rab Proteins and Rab-Guanine Nucleotide Dissociation Inhibitor. Genetics *168*, 1827–1841.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. Cell Mol. Biol. *16*, 735–743.

Cruz-Ramírez, A., Díaz-Triviño, S., Blilou, I., Grieneisen, V.A., Sozzani, R., Zamioudis, C., Miskolczi, P., Nieuwland, J., Benjamins, R., Dhonukshe, P., et al. (2012). A bistable circuit involving SCARECROW-RETINOBLASTOMA integrates cues to inform asymmetric stem cell division. Cell *150*, 1002–1015.

Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and 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, M.T., Aoyama, T., Costantino, P., and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. Science *322*, 1380–1384.

Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Ehrismann, J.S., Jürgens, G., and Estelle, M. (2005). Plant Development Is Regulated by a Family of Auxin Receptor F Box Proteins. Dev. Cell *9*, 109–119.

Drakakaki, G., van de Ven, W., Pan, S., Miao, Y., Wang, J., Keinath, N.F., Weatherly, B., Jiang, L., Schumacher, K., Hicks, G., et al. (2012). Isolation and proteomic analysis of the SYP61 compartment reveal its role in exocytic trafficking in Arabidopsis. Cell Res. *22*, 413–424.

Driouich, A., Follet-Gueye, M.-L., Bernard, S., Kousar, S., Chevalier, L., Vicré-Gibouin, M., and Lerouxel, O. (2012). Golgi-Mediated Synthesis and Secretion of Matrix Polysaccharides of the Primary Cell Wall of Higher Plants. Front. Plant Sci. *3*.

Eilers, P.H.C. (2004). Parametric time warping. Anal. Chem. *76*, 404–411.

Foresti, O., and Denecke, J. (2008). Intermediate Organelles of the Plant Secretory Pathway: Identity and Function. Traffic *9*, 1599–1612.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003). Efflux-dependent auxin gradients establish the apical|[ndash]|basal axis of Arabidopsis. Nature *426*, 147–153.

Früholz, S., and Pimpl, P. (2017). Analysis of Nanobody–Epitope Interactions in Living Cells via Quantitative Protein Transport Assays. In Plant Protein Secretion, (Humana Press, New York, NY), pp. 171–182.

Geldner, N., Dénervaud-Tendon, V., Hyman, D.L., Mayer, U., Stierhof, Y.-D., and Chory, J. (2009). Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set. Plant J. *59*, 169–178.

Gendre, D., Oh, J., Boutte, Y., Best, J.G., Samuels, L., Nilsson, R., Uemura, T., Marchant, A., Bennett, M.J., Grebe, M., et al. (2011). Conserved Arabidopsis ECHIDNA protein mediates trans-Golgi-network trafficking and cell elongation. Proc. Natl. Acad. Sci. *108*, 8048–8053.

Gendre, D., McFarlane, H.E., Johnson, E., Mouille, G., Sjodin, A., Oh, J., Levesque-Tremblay, G., Watanabe, Y., Samuels, L., and Bhalerao, R.P. (2013). Trans-Golgi Network Localized ECHIDNA/Ypt Interacting Protein Complex Is Required for the Secretion of Cell Wall Polysaccharides in Arabidopsis. Plant Cell *25*, 2633–2646.

Guo, Y., Yang, F., and Tang, X. (2017). An Overview of Protein Secretion in Yeast and Animal Cells. In Plant Protein Secretion, (Humana Press, New York, NY), pp. 1–17.

Haas, T.J., Sliwinski, M.K., Martínez, D.E., Preuss, M., Ebine, K., Ueda, T., Nielsen, E., Odorizzi, G., and Otegui, M.S. (2007). The Arabidopsis AAA ATPase SKD1 Is Involved in Multivesicular Endosome Function and Interacts with Its Positive Regulator LYST-INTERACTING PROTEIN5. Plant Cell *19*, 1295–1312.

Hayashi, T., and Kaida, R. (2011). Functions of Xyloglucan in Plant Cells. Mol. Plant *4*, 17–24.

Heidtman, M., Chen, C.Z., Collins, R.N., and Barlowe, C. (2003). A role for Yip1p in COPII vesicle biogenesis. J. Cell Biol. *163*, 57–69.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature *413*, 383–389.

Inadome, H., Noda, Y., Kamimura, Y., Adachi, H., and Yoda, K. (2007). Tvp38, Tvp23, Tvp18 and Tvp15: Novel membrane proteins in the Tlg2-containing Golgi/endosome compartments of Saccharomyces cerevisiae. Exp. Cell Res. *313*, 688–697.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. Nature *409*, 35059117.

Kano, F., Yamauchi, S., Yoshida, Y., Watanabe-Takahashi, M., Nishikawa, K., Nakamura, N., and Murata, M. (2009). Yip1A regulates the COPI-independent retrograde transport from the Golgi complex to the ER. J. Cell Sci. *122*, 2218–2227.

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

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M.B., et al. (2007). Cytokinins Act Directly on Lateral Root Founder Cells to Inhibit Root Initiation. PLANT CELL ONLINE *19*, 3889–3900.

Lewis, D.R., Negi, S., Sukumar, P., and Muday, G.K. (2011). Ethylene inhibits lateral root development, increases IAA transport and expression of PIN3 and PIN7 auxin efflux carriers. Dev. Camb. Engl. *138*, 3485–3495.

Leyser, O. (2009). The control of shoot branching: an example of plant information processing. Plant Cell Environ. *32*, 694–703.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development *124*, 33–44.

Müller, B., and Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature *453*, 1094–1097.

Paciorek, T., Zazímalová, E., Ruthardt, N., Petrásek, J., Stierhof, Y.-D., Kleine-Vehn, J., Morris, D.A., Emans, N., Jürgens, G., Geldner, N., et al. (2005). Auxin inhibits endocytosis and promotes its own efflux from cells. Nature *435*, 1251–1256.

P**eaucelle, A., Wightman, R., and Höfte, H.** (2015). The Control of Growth Symmetry Breaking in the Arabidopsis Hypocotyl. Curr. Biol. CB *25*, 1746–1752.

Pernisová, M., Klíma, P., Horák, J., Válková, M., Malbeck, J., Souček, P., Reichman, P., Hoyerová, K., Dubová, J., Friml, J., et al. (2009). Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. Proc. Natl. Acad. Sci. U. S. A. *106*, 3609–3614.

Raz, V., and Ecker, J.R. (1999). Regulation of differential growth in the apical hook of Arabidopsis. Development *126*, 3661–3668.

Raz, V., and Koornneef, M. (2001). Cell Division Activity during Apical Hook Development. Plant Physiol. *125*, 219–226.

Reinhardt, D., Pesce, E.-R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. Nature *426*, 255–260.

Ruzicka, K., Ljung, K., Vanneste, S., Podhorská, R., Beeckman, T., Friml, J., and Benková, E. (2007). Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell Online *19*, 2197–2212.

Růžička, K., Šimášková, M., Duclercq, J., Petrášek, J., Zažímalová, E., Simon, S., Friml, J., Van Montagu, M.C.E., and Benková, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. Proc. Natl. Acad. Sci. U. S. A. *106*, 4284–4289.

Sanderfoot, A.A., Kovaleva, V., Bassham, D.C., and Raikhel, N.V. (2001). Interactions between syntaxins identify at least five SNARE complexes within the Golgi/prevacuolar system of the Arabidopsis cell. Mol. Biol. Cell *12*, 3733–3743.

Sauer, M., Paciorek, T., Benkova, E., and Friml, J. (2006). Immunocytochemical techniques for whole-mount in situ protein localization in plants : Article : Nature Protocols. Nat Protoc. *1*, 98–103.

Savitzky, A., and Golay, M.J.E. (1964). Smoothing and Differentiation of Data by Simplified Least Squares Procedures. Anal. Chem. *36*, 1627–1639.

Schaller, G.E., Bishopp, A., and Kieber, J.J. (2015). The Yin-Yang of Hormones: Cytokinin and Auxin Interactions in Plant Development. Plant Cell Online *27*, 44–63.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji - an Open Source platform for biological image analysis. Nat. Methods *9*.

Shakoori, A., Fujii, G., Yoshimura, S., Kitamura, M., Nakayama, K., Ito, T., Ohno, H., and Nakamura, N. (2003). Identification of a five-pass transmembrane protein family localizing in the Golgi apparatus and the ER. Biochem. Biophys. Res. Commun. *312*, 850–857.

Siemens, J., Keller, I., Sarx, J., Kunz, S., Schuller, A., Nagel, W., Schmülling, T., Parniske, M., and Ludwig-Müller, J. (2006). Transcriptome Analysis of Arabidopsis Clubroots Indicate a Key Role for Cytokinins in Disease Development. Mol. Plant. Microbe Interact. *19*, 480–494.

Sivars, U., Aivazian, D., and Pfeffer, S.R. (2003). Yip3 catalyses the dissociation of endosomal Rab–GDI complexes. Nature *425*, nature02057.

Soonthornsit, J., Sakai, N., Sasaki, Y., Watanabe, R., Osako, S., and Nakamura, N. (2017). YIPF1, YIPF2, and YIPF6 are medial-/trans-Golgi and trans-Golgi network-localized Yip domain family proteins, which play a role in the Golgi reassembly and glycan synthesis. Exp. Cell Res. *353*, 100–108.

Stepanova, A.N., Yun, J., Likhacheva, A.V., and Alonso, J.M. (2007). Multilevel Interactions between Ethylene and Auxin in Arabidopsis Roots. Plant Cell *19*, 2169–2185.

Street, I.H., Mathews, D.E., Yamburkenko, M.V., Sorooshzadeh, A., John, R.T., Swarup, R., Bennett, M.J., Kieber, J.J., and Schaller, G.E. (2016). Cytokinin acts through the auxin influx carrier AUX1 to regulate cell elongation in the root. Dev. Camb. Engl. *143*, 3982–3993.

Swarup, R., Perry, P., Hagenbeek, D., Van Der Straeten, D., Beemster, G.T.S., Sandberg, G., Bhalerao, R., Ljung, K., and Bennett, M.J. (2007). Ethylene Upregulates Auxin Biosynthesis in Arabidopsis Seedlings to Enhance Inhibition of Root Cell Elongation. Plant Cell *19*, 2186–2196.

Tanimoto, K., Suzuki, K., Jokitalo, E., Sakai, N., Sakaguchi, T., Tamura, D., Fujii, G., Aoki, K., Takada, S., Ishida, R., et al. (2011). Characterization of YIPF3 and YIPF4, cis-Golgi Localizing Yip domain family proteins. Cell Struct. Funct. *36*, 171–185.

Van Leene, J., Eeckhout, D., Cannoot, B., De Winne, N., Persiau, G., Van De Slijke, E., Vercruysse, L., Dedecker, M., Verkest, A., Vandepoele, K., et al. (2014). An improved toolbox to unravel the plant cellular machinery by tandem affinity purification of Arabidopsis protein complexes. Nat. Protoc. *10*, 169–187.

von Wangenheim, D., Hauschild, R., Fendrych, M., Barone, V., Benková, E., and Friml, J. (2017). Live tracking of moving samples in confocal microscopy for vertically grown roots. ELife *6*.

Wolf, S., and Greiner, S. (2012). Growth control by cell wall pectins. Protoplasma *249*, 169–175.

Wu, F.H., Shen, S.C., Lee, L.Y., Lee, S.H., Chan, M.T., and Lin, C.S. (2009). Tape-Arabidopsis Sandwich-a simpler Arabidopsis protoplast isolation method. Plant Methods *5*, 16.

Yang, X., Matern, H.T., and Gallwitz, D. (1998). Specific binding to a novel and essential Golgi membrane protein (Yip1p) functionally links the transport GTPases Ypt1p and Ypt31p. EMBO J. *17*, 4954–4963.

Yang, Z., Liu, G., Liu, J., Zhang, B., Meng, W., M?ller, B., Hayashi, K., Zhang, X., Zhao, Z., De Smet, I., et al. (2017). Synergistic action of auxin and cytokinin mediates aluminum?induced root growth inhibition in *Arabidopsis*. EMBO Rep. e201643806.

Yoshida, Y., Suzuki, K., Yamamoto, A., Sakai, N., Bando, M., Tanimoto, K., Yamaguchi, Y., Sakaguchi, T., Akhter, H., Fujii, G., et al. (2008). YIPF5 and YIF1A recycle between the ER and the Golgi apparatus and are involved in the maintenance of the Golgi structure. Exp. Cell Res. *314*, 3427–3443.

Žádníková, P., Petrášek, J., Marhavý, P., Raz, V., Vandenbussche, F., Ding, Z., Schwarzerová, K., Morita, M.T., Tasaka, M., Hejátko, J., et al. (2010). Role of PIN-mediated auxin efflux in apical hook development of Arabidopsis thaliana. Development *137*, 607–617.

Zhao, Z., Andersen, S.U., Ljung, K., Dolezal, K., Miotk, A., Schultheiss, S.J., and Lohmann, J.U. (2010). Hormonal control of the shoot stem-cell niche. Nature *465*, 1089–1092.

Zhu, Q., Žádníková, P., Smet, D., Van Der Straeten, D., and Benková, E. (2017). Real-Time Analysis of the Apical Hook Development. In Plant Hormones, J. Kleine-Vehn, and M. Sauer, eds. (New York, NY: Springer New York), pp. 1–8.