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Titel der Diplomarbeit

The role of strigolactones in the regulation of secondary growth in *Arabidopsis thaliana*

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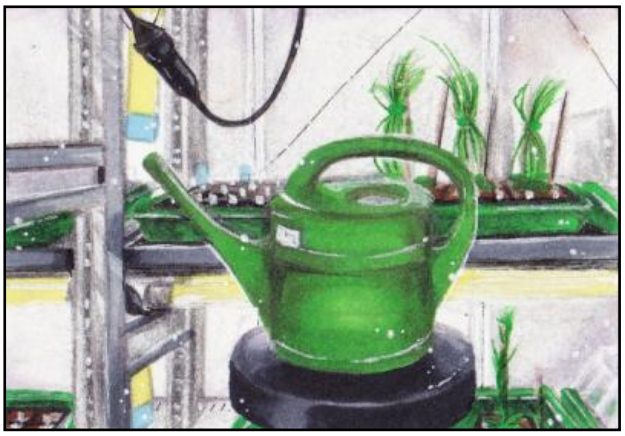

by Silvia Herold

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I. Abstract

Plant hormones control the life of a plant in many respects. They are crucial factors involved in numerous developmental and physiological processes. One of the best-studied and most comprehensive phytohormones is indole-3-acetic acid, also known as auxin. Besides cell division and elongation, auxin promotes the formation and differentiation of vascular tissues. In addition, it has been shown to be a key factor in the initiation and dynamics of secondary growth (Aloni et al., 1990; Ko and Han, 2004; Little et al., 2002) and in the maintenance of apical dominance (Cline, 1991; Tanaka et al., 2006). Recently, a long sought-after signalling molecule also involved in the maintenance of apical dominance has been identified, namely strigolactone (Gomez-Roldan et al., 2008; Umehara et al., 2008). Strigolactones are a group of carotenoid-derived plant hormones that have so far been known to stimulate the germination of parasitic weeds and symbiotic arbuscular fungi. Here we reveal a completely new function of strigolactones in the model plant *Arabidopsis thaliana*. We show that strigolactones, in combination with auxin, are involved in the promotion of secondary growth. Furthermore, the influence of strigolactones on auxin concentration and signalling was studied by the analysis of *DR5::GUS* and *DR5rev::GFP* reporter lines, as well as by direct auxin measurements. Strigolactone-deficient plants display severely increased levels of auxin and auxin signalling. *In vivo* treatments with the synthetic strigolactone analogue GR24 did not alter auxin transport in the stem, revealing that strigolactones do not affect the auxin flow directly. Moreover, we demonstrated that the expression levels of the auxin efflux transporters PIN1 and PIN3 are not elevated in the strigolactone-deficient mutant *max1-1* and that *in vivo* GR24 application does not increase the expression of these proteins. Taken together, our findings strongly support the hypothesis that strigolactones act downstream of auxin in the regulation of secondary growth

II. Zusammenfassung

Phytohormone regulieren und kontrollieren das Leben einer Pflanze in vielerlei Hinsicht. Sie sind wichtige Faktoren in unzähligen Entwicklungsprozessen, sowie bei physiologischen Vorgängen. Eines der am besten erforschten und funktionell vielseitigsten Phytohormone ist Indol-3-essigsäure, auch bekannt als Auxin. Neben Zellwachstum und –teilung, stimuliert Auxin auch die Entwicklung und Differenzierung des Vaskulärsystems der Pflanze. Desweiteren wurde gezeigt, dass das Hormon eine Schlüsselrolle bei Initiierung und Verlauf des sekundären Dickenwachstums (Aloni et al., 1990; Ko and Han, 2004; Little et al., 2002), sowie in der Vermittlung der Apikaldominanz spielt (Cline, 1991; Tanaka et al., 2006). Vor Kurzem konnte ein lange Zeit unbekanntes Signalmolekül, welches ebenfalls in die Erhaltung der Apikaldominanz involviert ist, als Strigolacton identifiziert werden (Gomez-Roldan et al., 2008; Umehara et al., 2008). Strigolactone sind eine Gruppe von Pflanzenhormonen, deren Synthese sich von Carotenoiden ableitet. Bisher waren Strigolactone bekannt dafür, die Keimung von parasitischen Kräutern und symbiotischen Pilzen zu stimulieren. In der vorliegenden Arbeit decken wir eine komplett neue Funktion der Strigolactone in der Modellpflanze *Arabidopsis thaliana* auf. Wir zeigen, dass die Hormone in Kombination mit Auxin die Entstehung von Sekundärem Dickenwachstum stimulieren. Allerdings sind sie nicht ausreichend, um Dickenwachstum in Abwesenheit von Auxin zu induzieren. Weiters wurde unter Verwendung der Reporterkonstrukte *DR5::GUS* und *DR5rev::GFP*, sowie direkten Auxin Konzentrationsmessungen, der Einfluss von Strigolactonen auf die Konzentration und die Signalwirkung von Auxin analysiert. *In vivo* Behandlungen mit dem synthetischen Strigolacton Analogon GR24 hatten keinen Einfluss auf den Auxin Transport im Stamm, was offenlegt, dass Strigolactone nicht direkt auf den Auxinfluss wirken. Außerdem demonstrieren wir, dass die Expressionslevel der Auxin Efflux Transporter PIN1 und PIN3 in der Strigolacton Biosynthese Mutante *max1-1* nicht erhöht sind. Auch *in vivo* Behandlungen mit GR24 konnten die Expression dieser Proteine nicht beeinflussen. Alles in allem stützen unsere Beobachtungen die Hypothese, dass Strigolactone innerhalb der Regulation des Sekundären Dickenwachstums downstream von Auxin agieren.

Contents

1. Introduction

Biomass is the major source of renewable energy worldwide. Besides crude oil, natural gas and coal, wood serves as the main donor of storable energy of high value. To enhance the efficiency and quality of wood production, understanding the molecular basis underlying the process of wood formation, i.e. secondary growth, is essential. A lot of new knowledge concerning the process of secondary growth has been gained over the past few years by research groups all over the world. Nevertheless, the detailed molecular mechanisms regulating secondary growth remain unknown.

Secondary growth occurs in dicotyledonous plants, especially woody species, whereas monocotyledonous plants lack cambium-derived secondary growth. The development of substantial stems found in some monocot species, for example palm trees, relies on the enlargement of parenchyma cells of the ground tissue (Esau, 1965).

1.1 *Arabidopsis thaliana* as a model to study secondary growth in higher plants

Due to the small genome (125 Mbp), the comfortable size and the short generation time, the herbaceous dicot *Arabidopsis thaliana* serves as an ideal model to study developmental processed in plants (Meinke et al., 1998). Its genome has been fully sequenced (AGI, 2000) and a remarkable number of reporter lines and molecular tools are available for this species. Another factor that makes *Arabidopsis thaliana* interesting especially as a model for secondary growth is that it undergoes the process of wood formation in the shoot, at least in the section from the base of the stem to around 1 cm above. The vascular tissues developed during this process (secondary phloem and secondary xylem) anatomically resemble those of angiosperm trees (Chaffey et al., 2002; Little et al., 2002).

1.2 The vascular system of plants

Higher plants (i.e. tracheophytes) as higher animals develop a vascular system throughout their bodies to guarantee that their organs are supplied fast and efficiently with nutrients and water. In addition, in plants the vasculature provides mechanical

support. The vascular system consists of two highly differentiated transport tissues, phloem and xylem, which are arranged together in strands (i.e. vascular bundles) that pervade the plant body. Phloem and xylem are composed of high specialised cells that originate from the procambium during primary growth. The procambium also gives rise to the cambium, which is located between the phloem and xylem (Fig. 1).

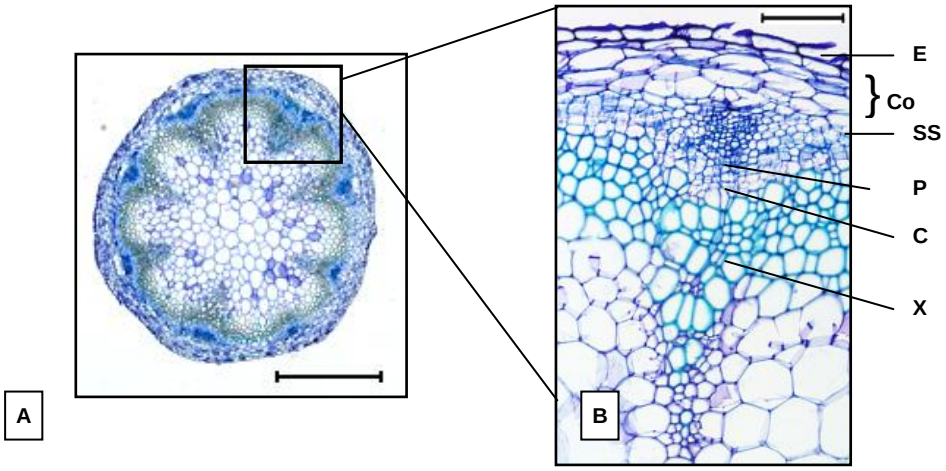


Fig. 1. Organisation of vascular bundles in the stem of *Arabidopsis thaliana*
(A) Transverse section of an *Arabidopsis* stem. The vascular bundles are organised in a radial manner. Scale bar = 0.5 mm. **(B)** A vascular bundle. Differentiated phloem cells [P] are represented by the dark blue stained compartments. The meristematic active cambium [C] is located between phloem and xylem [X]. [E] = Epidermis; [Co] = Cortex; [SS] = starch sheath, the innermost layer of the cortex; [P] = Phloem; [C] = Cambium; [X] = xylem; Scale bar = 0.1 mm

1.2.1 Vascular cambium

The vascular cambium is a tissue with meristematic activity. Its precursor, the procambium, originates from the apical meristem during primary growth of the plant. The procambium cells produce vascular cambium, primary phloem and xylem that together form primary vascular bundles. By the initiation of secondary growth, cambium is activated in the interfascicular regions and further develops secondary xylem (i.e. wood) and secondary phloem (i.e. bark) (Fig. 2).

1.2.2 Phloem

Phloem enables the distribution of soluble carbon metabolites from their sites of production (i.e. photosynthetically active regions) to sink tissues (Ward et al., 1998).

Furthermore, the phloem serves as a disseminator of RNA, proteins and amino acids (Citovsky and Zambryski, 2000; Fischer et al., 1998). Phloem consists of highly differentiated sieve elements and companion cells that build up the conducting system, as well as of parenchyma cells and fibres. The division of a phloem precursor cell generates a sieve element and a companion cell. The connection of several sieve elements via plasmodesmata, leads to the formation of sieve tubes, which are continuous compartments that load the nutrients and transport them to their target organs (Sjolund, 1997).

1.2.3 Xylem

The xylem transports water, nutrients and minerals from the root to the sites of transpiration. In addition, plant hormones (*e.g.* cytokinins) and amino acids are also transported and distributed through the xylem (Fischer et al., 1998; Haberer and Kieber, 2002). Xylem consists of conducting vessel elements, embedded in parenchyma and fibre cells. The differentiation of a cambium cell to a vessel cell starts with cell division, followed by growth in diameter and the formation of a lignified secondary cell wall. Finally, the cell undergoes apoptosis. The vessel elements are perforated at both ends and are connected to columns that build up a stable hollow transport pathway (Groover and Jones, 1999). The xylem of gymnosperms mainly consists of tracheids, which, like vessel elements, are dead cells with lignified cell walls. Tracheids are characterised by pointed ends that show an accumulation of pits. They guarantee wood of high stability, which is essential for trees carrying heavy crowns (Strasburger et al., 1991).

1.3 Secondary growth in *Arabidopsis thaliana*

During the initiation of lateral growth, stems undergo three stages of vascular tissue formation. In the earliest stage, the primary stage, primary vascular bundles, consisting of cambium, primary xylem and primary phloem are present. These tissues are formed from the procambium (Fig. 2A, D). This stage is followed by an intermediate stage in which interfascicular cambium, a secondary meristem, is initiated in the regions between the vascular bundles (Fig. 2B, E). During the secondary stage, the fascicular and the interfascicular cambium give rise to secondary xylem (wood) towards the proximal part of the stem, and secondary

phloem (bark) towards the outer side of the stem. The vascular tissues finally form a continuous ring throughout the stem (Fig. 2C, F).

In gymnosperms, wood deposition produces a supplementary ring of secondary xylem every year and, thus, the number of rings represents the age of a tree. The reasons for these visible concentric rings are the season-dependent changes of diameter of newly formed tracheary elements. In spring and summer, early wood, consisting of large tracheids, is produced. Towards the end of the year, late wood is developed, which is characterised by secondary xylem cells of small diameter. Early and late wood differ in their colour (light and dark brown) and together appear as one annual ring.

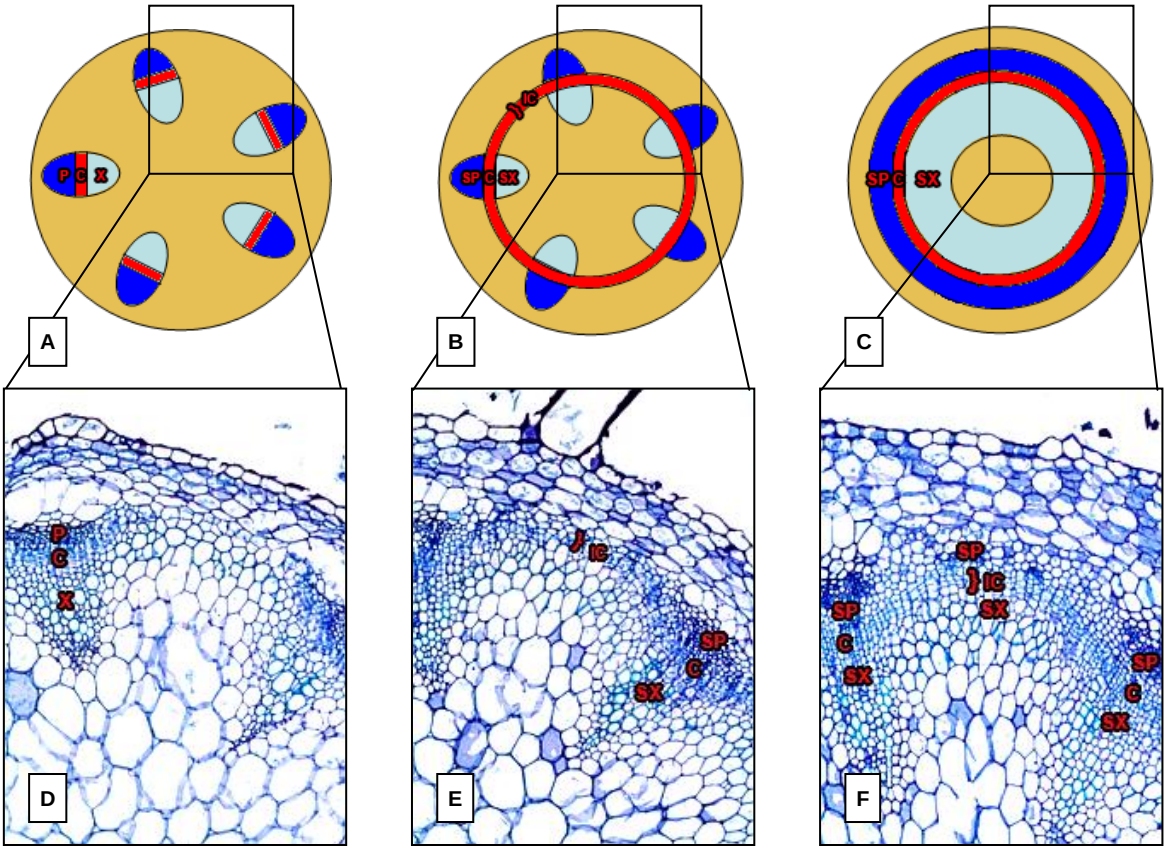


Fig. 2. Secondary growth in *Arabidopsis thaliana*

Images **A – F** represent cross sections of *Arabidopsis thaliana* stems at the three stages of secondary growth. Photos **D – F** show close-ups of the marked sectors in the schemes **A – C**. **(A, D)** Primary stage; Vascular bundles have been formed, consisting of cambium [C], phloem [P] and xylem [X]. **(B, E)** During the intermediate stage, interfascicular cambium [IC] is initiated in the regions between the vascular bundles. The fascicular cambium starts to develop secondary xylem [SX] and secondary phloem [SP]. **(C, F)** Finally, the stem reaches the secondary stage, characterised by the formation of a closed cylinder of cambium, secondary xylem and secondary phloem throughout the plant stem.

1.4 Hormonal control of secondary growth

1.4.1 Phytohormones

Hormones are small signalling molecules that act at very low concentrations and, partly, undergo long-distance transport from their site of synthesis to the target organ. Numerous hormones have been detected in plants that are involved in countless developmental processes. The five classical plant hormones and their main functions are listed in Table 1. There are other signalling molecules that have more recently been identified as being phytohormones. These are brassinosteroids, jasmonates, salicylic acid, oligosaccharins and strigolactones (Creelman and Mullet, 1997; Delaney et al., 1994; Umehara et al., 2008).

Trivial name	*Chemical nature*	*Main effects*
Auxin	Indole-3-acetic acid	◦ Cell division stimulation ◦ Apical dominance maintenance ◦ Vascular tissue differentiation
Cytokinin	Zeatin	◦ Cell division stimulation ◦ Morphogenesis ◦ Lateral bud outgrowth
Gibberellin	Gibberellin A_1, GA_1	◦ Stem elongation stimulation ◦ Fruit growth stimulation ◦ Induction of germination
Ethylene	Ethylene (gas)	◦ Stimulation of defence response ◦ Leaf and fruit abscission ◦ Fruit ripening
Abscisic acid	Abscisic acid	◦ Stomatal closure ◦ Shoot growth inhibition ◦ Wounding response

Table 1. Classical plant hormones

The five most important and best studied plant hormones are listed. Three of the main functions are shown for each hormone.

Phytohormones act in a complex network, either antagonistically or together, to direct a vast number of physiological and developmental processes in the plant. Some of

the hormones are involved in the process of secondary growth. In the course of this study I focused on auxin and strigolactone.

1.4.2 Auxin

Besides indole-3-ethanol and indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) (Fig. 3) is the most abundant naturally occurring auxin. In addition, a couple of phenolic acids (e.g. 2-phenylacetic acid) have been shown to feature auxin activity. In the cell, IAA occurs either in a free form or as conjugated to sugars, amino acids or proteins (Normanly et al., 2004). Most IAA is in the latter form.
There are a couple of industrially produced synthetic analogues of auxin. The most commonly used are α-Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D).

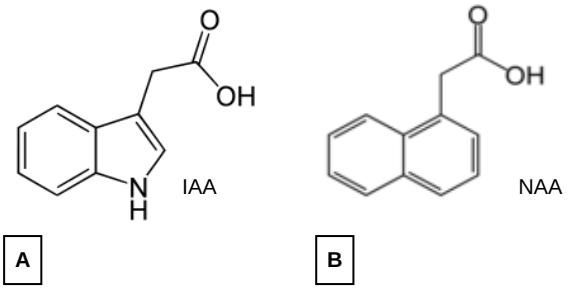


Fig. 3. Chemical structures of IAA and NAA
Auxins are chracterised by a heterocyclic indole ring. **(A)** IAA, a natural occurring auxin. **(B)** The synthetic auxin analogue NAA.

Auxin biosynthesis and action start during early embryogenesis. After the first asymmetric division of the zygote into a smaller apical and a bigger suspensor cell, an auxin gradient is formed along these cells establishing the apical-basal axis of the embryo (Friml et al., 2003). Furthermore, auxin maintains apical dominance (Cline, 1997) and induces cell elongation and division (Campanoni and Nick, 2005).

In the past, numerous studies proposed that auxin is a key factor in the regulation of vascular tissue development. The development of vascular tissue starts during primary growth with the formation of procambium cells out of precursors derived from the apical meristem. A ‘canalisation hypothesis’ was set up which proposed that auxin induces the formation of vascular tissues along its basipetal transport route. Indeed, it has been shown that auxin flow plays an important role in determining the

sites of procambium formation and that it promotes the process by stimulation of cell division and differentiation (Berleth et al., 2006; Mattsson et al., 2003; Przemeck et al., 1996; Sachs, 1981; Scarpella et al., 2006). Furthermore, auxin together with gibberellins and cytokinins trigger the development of the transport tissues phloem and xylem out of the vascular cambium. For a long time, auxin accumulation at the base of the stem was linked to the initiation of secondary growth. Little et al., for example, showed, that the inhibition of auxin transport by NPA, and the consequential accumulation of the hormone above the NPA treated zone, induced secondary growth (Aloni et al., 1990; Ko et al., 2004; Little et al., 2002). It has also been proposed that the vascular tissues themselves are sites of auxin biosynthesis. In addition, Schrader et al. revealed that the initiation of the cambium in the stem is correlated with the upregulation of auxin transport factors in this meristematic tissue, which implies that more auxin is present for further transport (Cheng et al., 2006; Schrader et al., 2003; Sheldrake, 1971).

1.4.3 Polar auxin transport

Auxin is transported in a polar manner from the main sites of biosynthesis (i.e. meristematic tissues such as young leaf primordia or shoot apical meristem) towards the root (Cheng et al., 2006; Muday and DeLong, 2001). Three systems of transmembrane proteins enable active auxin transport, establishing a continuous flow of the hormone through the plant body. These are PINs, PGPs and members of the AUX1 family (Fig. 4). PGPs are located in the plasma membrane of auxin transporting cells in a non-polar manner and facilitate auxin export by stabilisation of auxin efflux complexes (Geisler et al., 2005). AUX1 family members serve as auxin influx carriers (Yang et al., 2006), while the PIN proteins are responsible for auxin efflux (Petrasek et al., 2006). In the course of this stud,y the focus was on the PIN proteins, especially PIN1 and PIN3.

Eight members of the PIN-FORMED protein family, PIN1-PIN8, have been characterised so far. They are expressed in different, but partly overlapping regions throughout the whole plant. Moreover, some PIN proteins show similarities in structure and partly redundant functions. The structure of PIN1 is shown in Fig. 4 (B). It is characterised by ten transmembrane helices and a hydrophilic loop in the middle.

PIN1 is localised in a polar manner in the membrane of cells related to vascular tissues (i.e. vascular bundles, interfascicular cambium). PIN3 is expressed at the basal site of cells in the starch sheath, as well as in pericycle cells of the root elongation zone. PIN4 accumulation is characteristic of cells within and around the quiescent centre and as PIN7 and PIN3, it was also detected in procambial cells. In addition, PIN7 as PIN3 is located in columella cells. PIN5, PIN6 and PIN8 have been proposed to form their own evolutionary group among the PINs. They have recently been shown to be localised at the ER and to be involved in auxin homeostasis (Blilou et al., 2005; Friml et al., 2002; Gälweiler et al., 1998; Mravec et al., 2009; Paponov et al., 2005).

(Sieberer and Leyser, 2006) (Mravec et al., 2009)

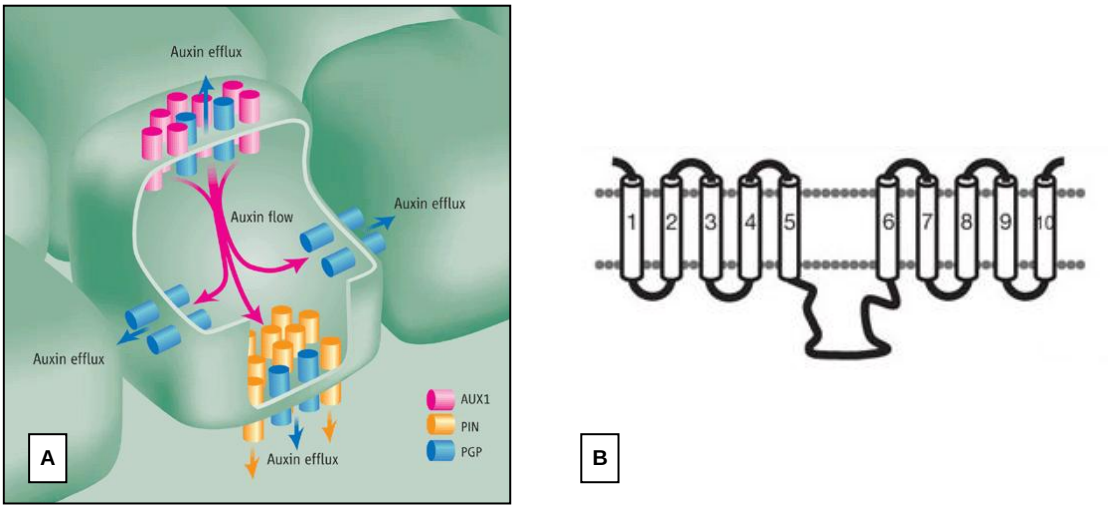


Fig. 4. Auxin transport sytems

Three classes of transmembrane auxin transporting proteins are known: AUX1 family members, PINs and PGPs. **(A)** A scheme that illustrates the subcellular localisation of auxin transporters and their direction of transport relative to the cell. **(B)** Simplified scheme illustrating the structure and localisation of the transmembrane protein PIN1.

1.4.4 Strigolactones

Strigolactones are a group of carotenoid-derived signalling molecules that, among others, include strigol (Fig. 5A), sorgolactone and orobanchol. There are some synthetic strigolactone analogues available (Binne et al., 2009). The highly active GR24 was utilised in this study (Fig. 5B).

Strigolactones have been isolated and identified in connection with the induction of germination of parasitic plants. Strigolactones are released from the host plant's

roots, and can be sensed by seeds of symbiotic mycorrhizal fungi (glomeromycota) and parasitic weeds (*e.g. Orobanche, Striga*), thus stimulating their germination (Akiyama and Hayashi, 2006). In 2008, strigolactones were found to be crucial factors in the regulation of shoot branching and it was proposed that they are phytohormones (Gomez-Roldan et al., 2008; Umehara et al., 2008).

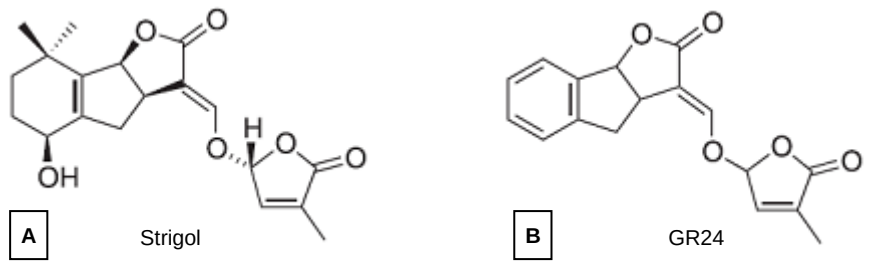


Fig. 5. Chemical structures of strigol and GR24

Strigolactones are sesquiterpene lactones. **(A)** Structure of the naturally occurring strigolactone strigol. **(B)** Structure of the synthetic strigolactone analogue GR24.

1.4.5 The *MAX* pathway of strigolactone biosynthesis

In 2005, Booker et al. proposed the role of the MORE AXILLARY BRANCHING (MAX) proteins in the biosynthesis pathway of an, at that time, unidentified signalling molecule. MAX1, MAX3 and MAX4 were suggested to act together in a pathway to produce this substance out of a carotenoid precursor (Booker et al., 2005). In 2008, the substance has been revealed as strigolactone (Gomez-Roldan et al., 2008; Umehara et al., 2008).

MAX3 and MAX4 were identified as carotenoid cleavage deoxygenases (CCD) that catalyse the cleavage of β-carotene in order to generate a secondary intermediate, which is further processed by MAX1, a member of the cytochrome P450 monooxygenase family (Booker et al., 2004; Booker et al., 2005; Sorefan et al., 2003). A strigol biosynthesis pathway had been proposed by Matusova et al. (Fig. 6). However, the definite substrates and enzymatic reactions, especially the function of MAX1 within the pathway remain to be elucidated (Matusova et al., 2005).

Unlike the other MAX proteins, MAX2 is not involved in the biosynthesis of strigolactones, but as been proposed to be their receptor. MAX2 is an F-Box protein, that participates in the SCF^{MAX2} complex, which is involved in protein ubiquitination

(Stirnberg et al., 2007). SCF also degrade transcription factors required for hormone dependent signalling (McSteen and Zhao, 2008). However, the targets of SCF^{MAX2} have not been identified so far.

(Pichersky, 2008)

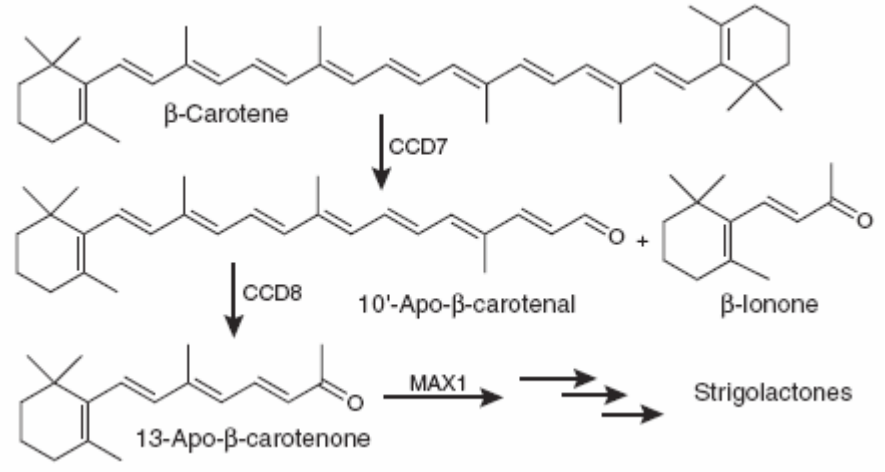


Fig. 6. Proposed strigolactone biosynthesis pathway

The scheme illustrates the proposed strigolactone biosynthesis pathway. MAX3 [CCD7] and MAX4 [CCD8] catalyse cleavage of the carotenoid precursor, whose secondary intermediate product is further processed to strigolactone by MAX1.

Homologues of the *Arabidopsis MAX* genes have been identified in several other model plants. These are *RAMOSUS* (*RMS*) in pea, the *DWARF* (*D*) family in rice and *DECREASED APICAL DOMINANCE* (*DAD*) in petunia. Plants mutated in these genes show a similar phenotype, namely an increase in the number of lateral branches (Schachtschabel and Boland, 2009).

1.4.6 Strigolactones act as inhibitors of lateral branching

In 2008 strigolactones were identified as the long sought-after substances, that are synthesised by the MAX pathway and are involved in the regulation of lateral shoot branching. They act as inhibitors of lateral bud outgrowth. This is the reason why strigolactone biosynthesis mutants (i.e. *max* mutants) show an increased number of side branches (Fig. 7) (Gomez-Roldan et al., 2008; Umehara et al., 2008).

(Stirnberg et al., 2002) (Bennett et al., 2006)

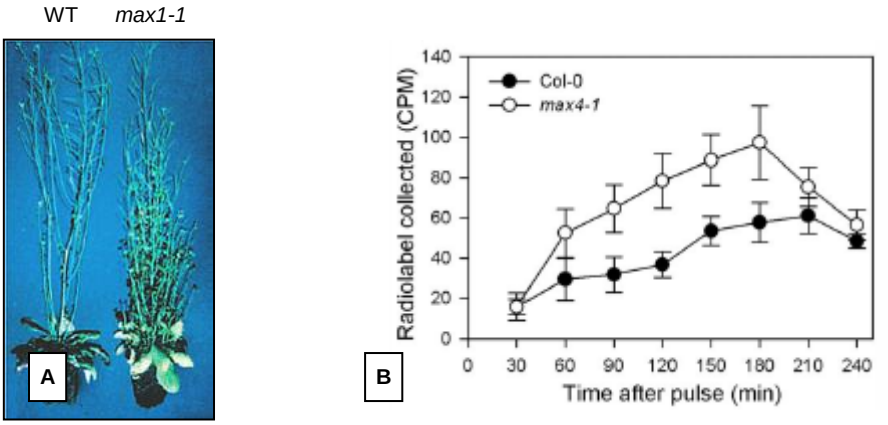


Fig. 7. *max* mutants show increased lateral branching and elevated auxin transport capacity
(A) The *max* mutants show increased lateral branching in comparison to WT plants. **(B)** By measuring the transport rate of radiolabelled auxin through stem segments, Bennett et al. showed that *max* mutants display increased auxin transport capacity.

The detailed mechanism of branching inhibition by strigolactones is not yet known. However, there are two different models suggesting that strigolactones act either upstream or downstream of auxin. Auxin itself has been shown to indirectly inhibit lateral branching. Bennett et al. proposed that strigolactones lower auxin transport in order to repress the outgrowth of axillary buds. This model was supported by their finding that auxin transport capacity is increased in strigolactone-deficient *Arabidopsis* plants, an observation that was confirmed by other groups (Brewer et al., 2009; Lazar and Goodman, 2006) (Fig. 7). Furthermore, when auxin levels are decreased by *e.g.* the auxin transport inhibitor NPA, the wild type branching phenotype is restored (Bennett et al., 2006). On the contrary, Christine Beveridge's group showed that lateral branching could be repressed by strigolactones in the absence of auxin, hinting that strigolactones may act downstream of auxin (Brewer et al., 2009).

However, no increase in the auxin transport capacity was found in *rms* mutant pea plants. This may indicate that strigolactone regulated processes differ among different plant species (Brewer et al., 2009).

1.5 Aim of the thesis

Bennett et al. proposed that auxin transport capacity is increased in *max* mutants (Bennett et al., 2006) (Fig. 7) and since auxin had been shown to be a key factor in the regulation of secondary growth, we suspected that strigolactone-deficient plants would show an altered secondary growth-related phenotype compared to wild type. In order to address this issue, we analysed *max1-1* plants histologically and observed a reduction of secondary growth in the mutant (Fig. 8). This finding suggests that strigolactones may play a role in the regulation of secondary growth.

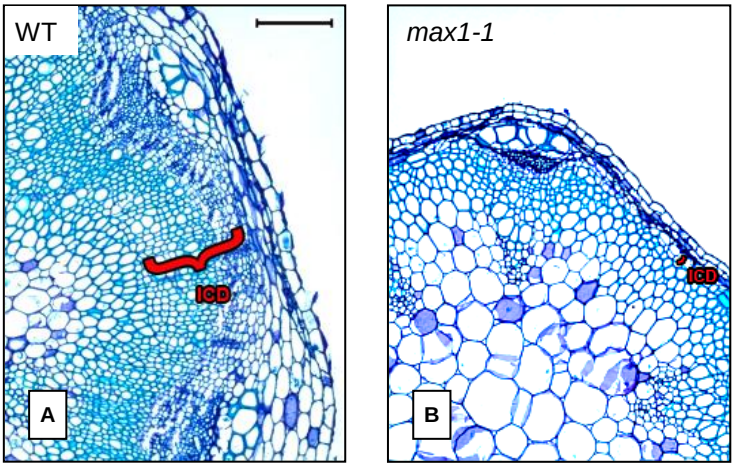


Fig. 8. Extension of secondary growth in *max1-1* and WT

Transverse stem sections of a WT **(A)** and a *max1-1* mutant **(B)** plant. The extension of the interfascicular cambium derived tissues [ICD] is reduced in the strigolactone biosynthesis mutant. Scale bar = 0.1 mm

The aim of the thesis was to further investigate the role of strigolactones in the process of secondary growth. The main goal was to elucidate the influence of strigolactones on auxin levels, signalling and transport in order to possibly identify a new powerful mediator of the plant hormone auxin in the initiation and activity of secondary growth. This was achieved by using a broad range of different approaches, including *in vivo* hormone treatments, histological and confocal analysis of reporter lines and the generation of expression constructs.

2. Material and methods

2.1 Material

2.1.1 Plant material

All the plants used were *Arabidopsis thaliana* (L.) Heynh. of the Columbia ecotype (Col). Seeds were sown on special soil for young plants and incubated at 4°C in the dark for 3-4 days to synchronize their germination. The seedlings were raised in a growth chamber under short day conditions (8 h light/16 h dark; 22°C). After 3 weeks, the plants were shifted to long day conditions (16 h light/8 h dark; 22°C) to induce flowering and stem elongation.

2.1.1.1 Plant lines

Name	mutated gene	locus	mutation type	Construct	References/Supplier
max1-1	***MAX1***	**at2g26170**	**point mutation; conversion of amino acid 117 from Pro to Leu**	***DR5::GUS***	**(Booker et al., 2005) (Ulmasov et al., 1997) (Bennett et al., 2006) from Ottoline Leyser**
max1-1	***MAX1***	**at2g26170**	**point mutation; conversion of amino acid 117 from Pro to Leu**	***DR5::GFP***	**(Booker et al., 2005) (Ulmasov et al., 1997) (Friml et al., 2003) from Tobias Sieberer**
max1-1	***MAX1***	**at2g26170**	**point mutation; conversion of amino acid 117 from Pro to Leu**	***PIN1:: PIN1-GFP***	**(Booker et al., 2005) (Friml et al., 2003) (Benkova et al., 2003) from Ottoline Leyser**
max1-1	***MAX1***	**at2g26170**	**point mutation; conversion of amino acid 117 from Pro to Leu**	***PIN3:: PIN3-GFP***	**(Booker et al., 2005) (Friml et al., 2003) from Eva Sehr**
max2-1	***MAX2***	**at2g42620**	**point mutation; conversion of amino acid 581 from Asp to Asn**	***DR5::GUS***	**(Stirnberg et al., 2002) (Ulmasov et al., 1997) (Bennett et al., 2006) from Ottoline Leyser**
max3-9	***MAX3***	**at2g44990**	**exchange of 16 nucleotides in exon 2 with 42 nucleotides of unknown origin; truncated protein**	***DR5::GUS***	**(Booker et al., 2004) (Ulmasov et al., 1997) (Bennett et al., 2006) from Ottoline Leyser**
max4-1	***MAX4***	**at4g32810**	**transposon insertion in intron 2**	***DR5::GUS***	**(Sorefan et al., 2003) (Ulmasov et al., 1997) (Bennett et al., 2006) from Ottoline Leyser**

las-4 max1-1	***LAS MAX1***	**at2g26170**	**las-4: deletion of 20 nucleotides at position 484 max1-1: point mutation; conversion of amino acid 581 from Asp to Asn**	**-**	**(Booker et al., 2005) (Greb et al., 2003) from Klaus Theres**
WT	**-**	**-**	**-**	***DR5::GUS***	**(Ulmasov et al., 1997) (Bennett et al., 2006) from Ottoline Leyser**
WT	**-**	**-**	**-**	***DR5::GFP***	**(Ulmasov et al., 1997) (Friml et al., 2003) from NASC**
WT	**-**	**-**	**-**	***PIN1:: PIN1-GFP***	**(Friml et al., 2003) (Benkova et al., 2003) from Jiří Friml**
WT	**-**	**-**	**-**	***PIN3:: PIN3-GFP***	**(Friml et al., 2003) from Jiří Friml**
WT	**-**	**-**	**-**	**-**	

Table 2. *Arabidopsis thaliana* lines used in this study.

2.1.2 Bacterial strains

For cloning, competent cells of the *Escherichia coli* strain DH5α were used. Competent *Agrobacterium tumefaciens* cells of the strain C58C1 were used to transform *Arabidopsis* plants.

2.1.3 Buffers and Solutions

50x TAE (1 L)

242 g TRIS

100 ml EDTA (0.5 M)

57.1 ml acetic acid

pH7.6

Loading buffer

0.25% xylene cyanole

0.25% bromphenol blue

50% glycerol

10 mM TRIS, pH8

1 mM EDTA, pH8

LB medium

1% peptone

1% NaCl

0.5% yeast extract

For plates: 1.5% bacteria agar

For Kan selection medium: 0.005% Kanamycin

pH7

YEB medium

0.5% Meat extract

0.1% yeast extract

0.5% sucrose

0.5% peptone

0.5% NaCl

2 mM $MgSO_4$

For plates: 1% bacteria agar

Antibiotics:

50 µg/ml Rifampicin

5 µg/ml Tetracyclin

50 µg/ml Kanamycin

Toluidine Blue staining solution (50 ml)

25 mg of Toluidine Blue (AppliChem) were dissolved in 50 ml ddH_2O

***GUS* staining buffer (1 L)**

100 mM NaH2PO4

10 mM EDTA (pH8)

0.1 % Triton X-100

pH7

X-Gluc stock solution

10.4 mg/ml X-Gluc was dissolved in DMF

FAA fixation solution

50% 100%-EtOH

10% glacial acetic acid

5% Formaldehyde

35% ddH_2O

2.1.4 Primers

All Primers were designed by using INVITROGEN™ Vector NTI software and purchased from SIGMA-ALDRICH®

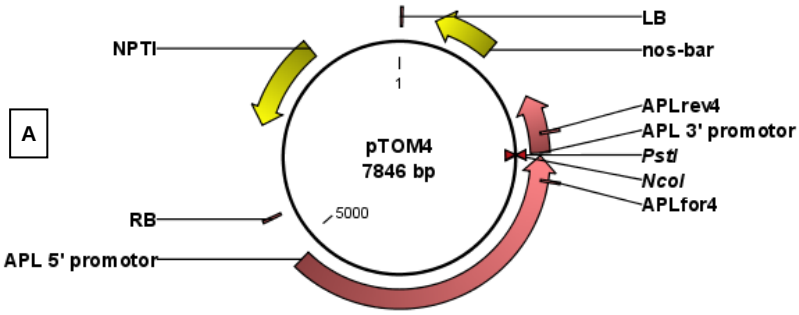
Primer name	Sequence	Application
SCR_Prom_R	ACTAGGATCCAGATGCATGGAGATTGAAGGGTTGTTGGTC	Ampl of 5'-*pSCR* and cloning into *pGreen0229*; contains BamHI and NsiI rs
SCRprom3	ACTAGCGGCCGCCGACCACCACCGTCAACAATTTTG	Ampl of 5'-*pSCR* and cloning into *pGreen0229*; contains NotI rs
SCR_Prom3´_F	ACTACCCGGGCAGCTTGGACGCCTCGTTCTTAG	Ampl of 3'-*pSCR* and cloning into *pGreen0229*; contains XmaI rs
SCR_Prom3´_R	AGAAATGAATTCAGAGCTCCACGGTGGTGG	Ampl of 3'-*pSCR* and cloning into *pGreen0229*; contains EcoRI rs
MAX2for3	ACTAATGCATGCTTCCACTACTCTCTCCGACC	Ampl of *MAX2*, cloning into *pSH4*; contains NsiI rs
MAX2rev3	ACTAGGATCCTCAGTCAATGATGTTGCGGCTGTTC	Ampl of *MAX2*, cloning into *pSH4*, *pTOM33* and *pTOM49*; contains BamHI rs
MAX2for1	ACTAACATGTTGGCTTCCACTACTCTCTCCGACC	Ampl of *MAX2*, cloning into *pTOM4*, *pTOM33* and *pTOM49*; contains PciI rs
MAX2rev1	ACTACTGCAGTCAGTCAATGATGTTGCGGCTGTTC	Ampl of *MAX2,* cloning into *pTOM4*; contains PstI rs
APLfor4	TGCGTTCAAGGCGATTCAG	Sequencing of *pSH1*
APLrev4	AAACACGAGCAAAACTAATCCG	Sequencing of *pSH1*
MAX2for2	CCTTGAGTCTTTAGATCTCTCCAACTTC	Sequencing of *pSH1* Sequencing of *pSH2* Sequencing of *pSH3* Sequencing of *pSH5*
MAX2rev2	CTGTCATAGGTAATGGCGTCAAAA	Sequencing of *pSH1* Sequencing of *pSH2* Sequencing of *pSH3* Sequencing of *pSH5*

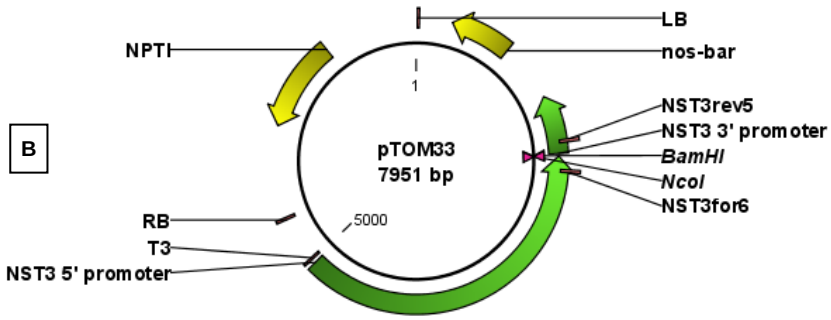
NST3for6	CTCGTCGAGTCCTACCACCATTAT	Sequencing of *pSH2*
NST3rev5	CCCTCCACCATTAACAAATGTTAAA	Sequencing of *pSH2*
T7	GTAATACGACTCACTATAGGGC	Sequencing of *pSH3*
SCRfor3	AACACACAACGACGAACATTTTCCG	Sequencing of *pSH3*
WOX4for10	CCGACGTCCCACTTTTGTAAAA	Sequencing of *pSH5*
WOX4rev5	TTGATTCCTCTGAACCTAAACCAGAT	Sequencing of *pSH5*

Table 3. Primers used in the course of molecular cloning
[Ampl] = Amplification; [rs] = restriction site(s)

2.1.5 Cloning vectors

The vector *pGreen0229* (Hellens et al., 2000) served as the backbone for all of the constructs used and generated in this study. *pSoup* (Hellens et al., 2000) was used as helper plasmid, enabling replication of *pGreen0229* in the transformed plant cells. The promoter constructs *pTOM4* (carrying *pAPL*), *pTOM33* (carrying *pNST3*) and *pTOM49* (carrying p*WOX4*) were generated by Thomas Greb (unpublished).





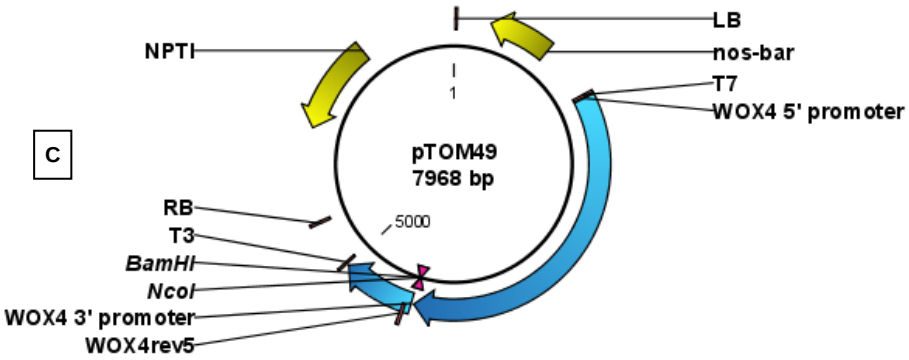


Fig. 9. Expression vectors used for cloning of *MAX2*

(A) *pTOM4*: vector carrying *pAPL*. **(B)** *pTOM33*: vector containing *pNST3*. **(C)** *pTOM49*: vector containing *pWOX4*. All the vectors contain the NPTI gene that mediates resistance to kanamycin, as well as nos-bar that confers resistance against the herbizide BASTA®. The vector *pGreen0229* served as the basic backbone for the constructs shown.

2.1.6 Reporter elements

2.1.6.1 *DR5*

DR5 is a synthetic auxin response element consisting of 11 bp tandem direct repeats that include the auxin response motif *TGTCTC*. Fused to marker genes such as *GUS* or *GFP*, the constructs serve as reporters for auxin-responsive transcription (Ulmasov et al., 1997).

2.1.6.2 *GUS*

The *E. coli* protein β-Glucuronidase (GUS) is used as a stable expression marker in higher plants (Jefferson et al., 1987). *GUS* expression is detected by treating transgenic plant tissue with a solution containing β-glucuronides (X-Gluc), which serve as cleavage substrates for GUS. Further progression of the cleavage product XH results in the formation of diXH-indigo that appears as blue staining.

2.1.6.3 *GFP*

GFP was originally isolated from jellyfish and is used as a reporter protein in most model organisms. After stimulation by UV light, *GFP* emits bright green light at a

wavelength of around 500 nm, which can be detected by fluorescence microscopy (Kain et al., 1995).

2.1.7 Enzymes

The enzymes and their corresponding reaction buffers used in this study were purchased either from NEW ENGLAND BIOLABS® or Fermentas®.

2.1.8 Hormones and related substances

The synthetic strigolactone analogue GR24 was purchased from Prof. Dr. Binne Zwanenburg, University Nijmegen (The Netherlands). NPA was purchased from DUCHEFA, lanolin from SIGMA-ALDRICH®.

2.2 Methods

2.2.1 DNA and RNA work

2.2.1.1 Standard PCR

The following protocol was used for the standard PCR approach. The reaction was performed by either a BIORAD® iCycler or Biometra® T3000 thermocycler. Amplification primers were designed using INVITROGEN™ Vector NTI software. A general annealing temperature of 55°C was used for all primer combinations. Elongation time was set to 2 min, 40 cycles were performed.

Standard PCR approach:

2 µl DNA template
2 µl 10x *Taq* buffer
1.2 µl 25mM $MgCl_2$
0.4 µl 10 mM dNTP Mix
1 µl 10 mM Primer forward
1 µl 10 mM Primer reverse
0.2 µl *Taq* polymerase
12.2 µl ddH_2O
---
20 µl

2.2.1.2 Agarose gel electrophoresis

In general, 1% agarose gels were used. For this, a flask of 1x TAE buffer mixed with 1% agarose powder was boiled in a microwave. The flask was taken out of the microwave from time to time and agitated until it turned into a clear solution without visible agarose fibres. The solution was cooled down to approximately 50°C by treating the flask with cold water. Subsequently the liquid gel was poured into a gel tray carrying a suitable comb, and was allowed to set until the gel turned solid. The DNA sample to be loaded on the gel was mixed with 0.8% loading buffer. In parallel 7.5 µl of size markers *Fermentas GeneRuler™ Mix* or *Fermentas GeneRuler™ 1kb* were loaded. The run was performed with 90–120 V in a gel electrophoresis chamber

2.2.1.3 Ethidium bromide staining and analysis of the gel

The gel was incubated in an ethidium bromide staining bath (50 µl ethidium bromide in 500 ml 1x TAE buffer) for 30-60 min to visualise DNA or RNA under UV-light. The gel was, subsequently, analysed and photographed using the KODAK Gel Logic 200 Imaging System.

2.2.1.4 Rough DNA extraction

Plant material (~two small leaves) was collected in a 1.5 ml plastic tube containing 200 µl of extraction buffer at room temperature. The tissue was ground with a micropestle to break up the cells. An additional 200 µl of extraction buffer were added and the suspension was mixed by inverting the tube. After 5 min centrifugation at 13.2 krpm 350 µl of the supernatant were transferred to a new tube and 350 µl of isopropanol were added. The tubes were agitated and left at room temperature for 10 min. The DNA was collected in a pellet by 5 min centrifugation at 13.2 krpm. The supernatant was removed and 300 µl of 70%-ethanol were added to wash the pellet. After another round of centrifugation (1 min, 13.2 krpm) the supernatant was discarded and the pellet dried and dissolved in 50 µl of ddH_2O by incubation at 65°C for 10 min.

2.2.1.5 RNA extraction

Fresh plant material was collected in a plastic tube, shock frozen in liquid nitrogen and stored at -80°C. 400 µl of INVITROGEN TRIzol® reagent were added and ground with an iced plastic pestle. An additional 600 µl of TRIzol® were added and the tube inverted to mix the suspension. After 5 min of incubation at room temperature the tube was centrifuged at 4°C for 5 min at 14 krpm. The supernatant was transferred to another plastic tube containing 500 µl of chlorophorm and was kept at room temperature for 5 min. Subsequently, the suspension was centrifuged at 4°C for 5 minutes at 14 krpm. The aqueous layer was removed and transferred to another plastic tube containing 500 µl of isopropanol, followed by an incubation step for at least 20 min at -20°C. The tube was centrifuged again at 4°C for 10 min at 14 krpm. After removal of the supernatant, 1 ml of 70%-ethanol was added to wash the pellet and it was subsequently centrifuged for 5 min at 1 krpm. The supernatant was removed and the pellet dried in a vacuum centrifuge. Finally the pellet was dissolved in 50 µl ddH_2O and kept at -80°C.

2.2.1.6 cDNA generation

3 µg of RNA was mixed with 1 µl of oligo dT primer (0.5 µg/µl) and ddH_2O to make up a total volume of 12 µl. The mix was incubated at 70°C for 5 min and subsequently extended by the addition of 4 µl of 5x reaction buffer, 2 µl dNTPs (10 mM) and 0.5 µl of RiboLock^{TM} RNAse inhibitor. After another 5 minutes incubation period at 37°C, 1 µl of reverse transcriptase was added to the mix. A reverse transcriptase reaction was performed using the following settings:

42°C for 60 min
70°C for 10 min

The reaction was, subsequently, chilled on ice and stored for further use at -20°C.

2.2.2 Histology

2.2.2.1 Fixation

Freshly collected plant material was fixed in 100% ethanol:acetic acid (3:1). The tissue was infiltrated under a vacuum for 10 min and then incubated at 4°C O/N.

2.2.2.2 Washing

The fixed plant material was transferred to SANOWA Biopsie embedding cassettes and washed for at least 1 h in 70% ethanol to get rid of residual acetic acid.

2.2.2.3 Paraffin embedding

Embedding of plant material in wax was performed using a THERMO Shandon Tissue Excelsior following a standard embedding protocol. Afterwards the plant material was taken out of the embedding cassettes and embedded in liquid paraffin manually at the THERMO Shandon Embedding Center using metal moulds. After at least 30 min on the cooling platform, the moulds could be peeled off the wax blocs that enclosed the plant material. The blocs were stored at 4°C to keep them solid.

2.2.2.4 Cross sectioning

The wax-embedded plant material was cut into 10 µm thick cross sections utilizing a MICROM microtome. The sections were placed on a glass slide covered with water and then dried at 42°C on the heating bank O/N.

2.2.2.5 Toluidine Blue staining

The slides were de-waxed and stained by incubating them as follows:

Histoclear	10 min
Histoclear	10 min
100% ethanol	1 min
100% ethanol	1 min

96% ethanol	1 min
85% ethanol	1 min
50% ethanol	1 min
30% ethanol	1 min
ddH_2O	1 min
ddH_2O	1 min
Toluidine Blue	4 min
ddH_2O	1 min
ddH_2O	1 min
ddH_2O	30 s
96% ethanol	30 s
100% ethanol	30 s
100% ethanol	30 s

The slides were air dried and cover glasses attached using Entellan (MERCK). After O/N incubation at room temperature the slides were ready for analysis.

2.2.3 *GUS* staining

2.2.3.1 *GUS* staining reaction

20 mM X-Gluc (Duchefa), 200 mM Ferricyanide and 200 mM Ferrocyanide were added to the *GUS* staining buffer. The plant material was transferred to a plastic tube containing the staining mix. Vacuum infiltration was performed. Subsequently the samples were incubated O/N at 37°C.

2.2.3.2 Fixation and de-staining

The *GUS* staining solution was replaced by FAA and the samples incubated O/N at 4°C. On the following day, the FAA solution was removed and 70% ethanol was added to de-stain the samples by elimination of chlorophyll. After 30 min the 70% ethanol was exchanged with 100%-ethanol. The samples were stored at 4°C.

2.2.4 *In vivo* hormone treatment

2.2.4.1 Preparation of hormone-containing lanolin paste

Lanolin paste was melted in a microwave and then cooled down to 50°C in a water bath. The required volume of lanolin was pipetted onto a plastic weighing pan and mixed well with the hormone in order to reach a concentration of 10 mg/g hormone in lanolin.

2.2.4.2 Application of lanolin paste

Plants between 10 and 20 cm in height were used for the treatment. The lanolin-hormone mix was applied to the stem at a height of 1 cm above the base utilizing a small scoop. These plants were further grown under long day growth conditions for 16 days. After half of this period (8 days), the hormone paste was renewed completely.

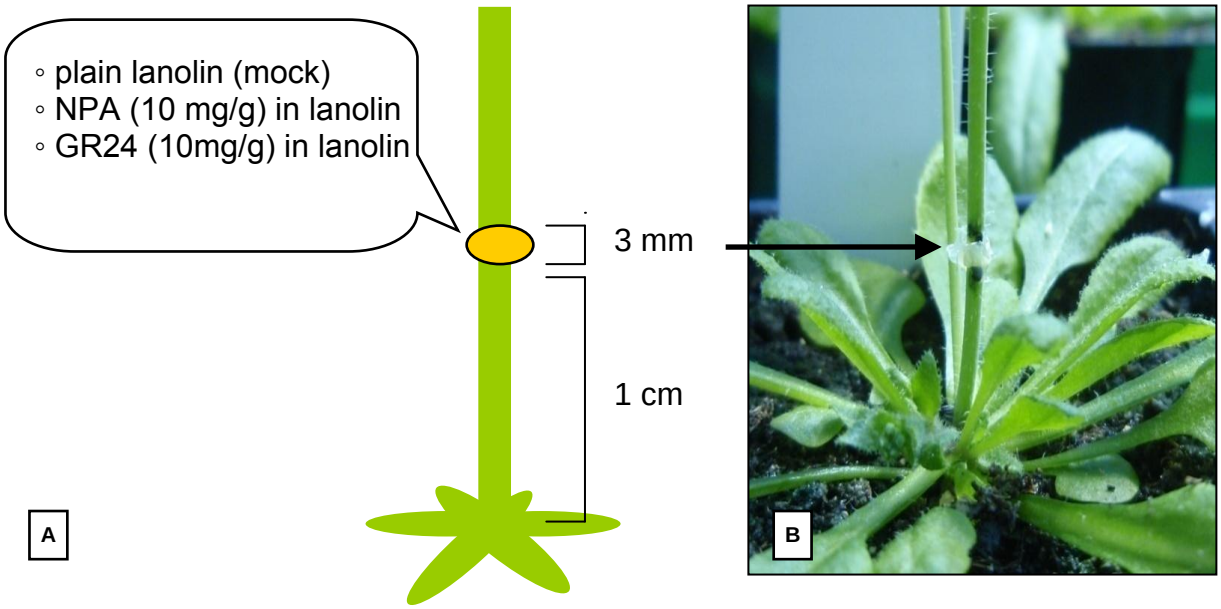


Fig. 10. *In vivo* hormone treatment utilising lanolin

(A) Schematic view of an *Arabidopsis* stem ringed with lanolin paste containing either NPA or GR24 with a concentration of 10 mg/g. Plain lanolin treatment was used as a control. **(B)** Photograph of an *Arabidopsis* stem ringed with lanolin.

2.2.5 Microscopy

2.2.5.1 Differential interference contrast microscopy (DIC)

The toluidine-stained cross sections were analysed using a Zeiss Axio Imager.M1 microscope. Photographs were taken with a Visitron Systems camera and analysed using SPOT™ Imaging software.

2.2.5.1.1 Quantification of secondary growth

To quantify the extent of secondary growth, on stem cross sections, the lateral extension of the ICD was measured utilising the measurement tool of SPOT™ Advanced Imaging software (Fig. 11). The measurements were performed in mm. The length of the ICD was determined for every interfascicular region of a stem cross section. The individual average extension of ICD was calculated from these values.

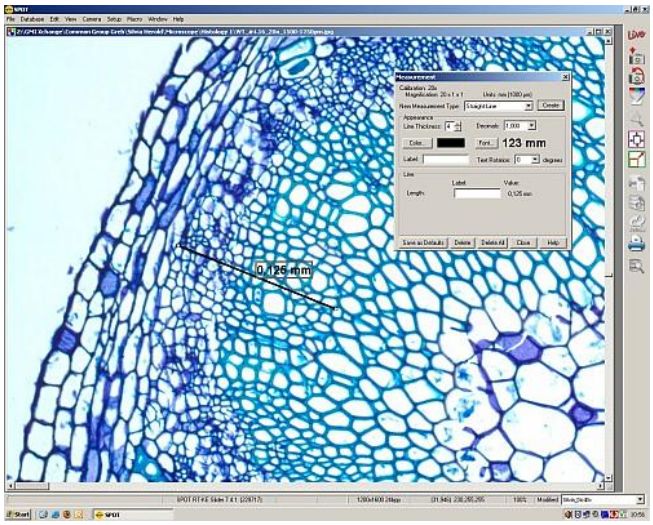


Fig. 11. Quantification of secondary growth with SPOT™ Advanced Imaging software
The length of one single row of dividing cambium cells was measured, representing the lateral extension of the ICD.

2.2.5.2 Laser scanning confocal microscopy

A Leica TCS SP confocal microscope was used to analyse GFP signalling in cross sections of stems. Photographs were analysed using Leica light Imaging software. Cross sections were prepared for analysis as follows: Fresh plant samples were fixed between two plates of soft plastic and cut into thin slices by hand. These were

incubated in propidiumiodol (0.25 ml in 1 ml) for a few minutes to stain cell walls, then put onto a wetted slide and covered by a cover glass.

2.2.5.3 Stereomicroscopy

GUS-stained samples were photographed using the Leica MZ 16 FA fluorescence stereomicroscope. The pictures were analysed with Leica Application software.

2.2.6 Molecular cloning

2.2.6.1 Amplification of a DNA sequence

To amplify the desired DNA sequence (*MAX2*, *3'-pSCR*, *5'-pSCR*) for cloning into a vector, primers, containing unique restriction sites resulting in sticky ends after digestion, were used. A PCR was performed on wild type Col genomic DNA (in case of the amplification of *3'-pSCR* and *5'-pSCR*) or cDNA (in case of the amplification of *MAX2,* to get rid of introns) using NEW ENGLAND BIOLABS® Phusion™ High-Fidelity DNA Polymerase with proofreading activity following the manufacturer's protocol. The PCR product was purified by a QUIAGEN QIAquick PCR purification kit following the manufacturer's instructions.

2.2.6.2 Restriction enzyme digestion

The PCR product was double digested by the enzymes cutting within the primer. In parallel, the binary plasmid vector *pgreen0229* (Hellens et al., 2000) was digested with the same enzymes as the insert. Enzymes and related buffers were purchased from Fermentas®. The digestion mix was set up according to the instructions on the Fermentas® webpage (http://www.fermentas.com). The mix was incubated at 37°C for 3-16 h.

2.2.6.3 Ligation

DNA concentration was measured with the NanoDrop® ND-1000 spectrophotometer and a ligation of digested *pgreen0229* and PCR product was set up.

Ligation mix

10 µg/µl digested PCR product

10 µg/µl digested *pgreen0229*

1 µl T4 DNA ligase

x µl 10x T4 DNA ligase buffer

x µl ddH_2O, to reach a total volume of 20 µl

The mix was incubated 1 h at 20°C. It was then used for transformation

2.2.6.4 Heat shock transformation of *E. coli*

An aliquot of competent DH5α *E.coli* cells was taken from -80°C and kept on ice until the cell suspension started to melt. 10 µl of the ligation mix were then added slowly to the tube. To physically stabilize the bacteria the suspension was kept on ice for 20 min and subsequently put on 42°C for 1.5 min. Afterwards the tube was put on ice again for 5 min. The heat shock and subsequent coldness cause uptake of the foreign plasmid. To enforce cell proliferation the suspension was incubated at 37°C on a shaker for 1 h. The bacteria were pelletized by a 3 min centrifugation at 3600 rpm and then plated onto LB + Kanamycin plates. These were incubated O/N at 37°C.

2.2.6.5 Plasmid preparation

A colony was inoculated in 3 ml LB medium containing 3 µl Kanamycin (50 mg/ml) and grown O/N on a shaker at 37°C. A QUIAGEN QIAprep Spin Miniprep kit was used, following the manufacturer's protocol, to isolate the plasmids out of the bacteria suspension.

2.2.6.6 Sequencing

A BigDye® reaction was performed according to the manufacturer's protocol including between 250–300 ng of DNA. Afterwards, an equivalent amount of ddH_2O was added to the reaction to get it ready for sequencing. The sequencing was performed by the VBC Biotech sequencing service. Subsequently the sequence was checked with INVITROGEN^{TM} Vector NTI software.

2.2.6.7 Heat shock transformation of *Agrobacterium tumefaciens*

500 ng of the plasmid to be transformed into the bacteria was added to an aliquot of 200 µl of competent *Agrobacterium* cells. The mix was put on ice for 5 min, followed by 5 min incubation in liquid nitrogen. The tube was then transferred again to a 37°C hot heating block for 5 min. After this heat shock step, 800 µl of LB medium was added to the bacteria suspension and it was grown for 2 h at 28°C on a shaker. Subsequently, 100 µl of the suspension was plated onto YEB agar and the residue centrifuged for 3 min at 3.6 krpm. The supernatant was partly removed and the pellet resuspended in the remaining liquid, which was then plated on YEB agar. The plates were incubated at 28°C for 3 days.

2.2.6.8 Culture of transformed *Agrobacterium tumefaciens* cells

One big *Agrobacterium* colony was chosen and inoculated into 3 ml of liquid YEB medium containing 50 µg/ml Rifampicin, 5 µg/ml Tetramycin and 50 µg/ml Kanamycin. The culture was incubated O/N at 28°C on a shaker. The following day the 3 ml cell suspension was added to a 1 L Erlenmeyer flask containing 400 ml YEB medium, 5 µg/ml Tetramycin and 50 µg/ml Kanamycin. The bacteria were grown O/N at 28°C on a shaker. The cell culture was then transferred to 500 ml plastic centrifugation tubes and centrifuged for 15 minutes at 5000 rpm. The supernatant was removed and the pellet resuspended in 500 ml of a 5% sucrose solution containing 200 µl/L silwet.

2.2.6.9 Transformation of *Arabidopsis thaliana*

80 plants were used for transformation with each one of the constructs. Siliques were removed from the *Arabidopsis* plants to be transformed to decrease the amount of untransformed seeds. The plants were inverted and the flowers dipped into the *Agrobacteria* suspension for approximately 5 min. Subsequently, the plant was transferred to a tray with a closed, airproof lid. The lid was removed the following day. Seeds were collected from these T1 generation plants for further analysis.

2.2.6.10 Selection of transformed plants

Seedlings of the T1 generation were sprayed with the herbicide BASTA® (40 mg/L) every third day, three times in total. Successfully transformed plants were resistant to the reagent and transferred to new pots.

3. Results

3.1 Histological characterisation of the *max* mutants

Auxin has been reported to be an essential factor for the initiation and activity of secondary growth (Little et al., 2002; Snow, 1935; Uggla et al., 1998). Strigolactones have been suggested to be connected to auxin signalling (Brewer et al., 2009; Ferguson and Beveridge, 2009) and to decrease auxin transport capacity (Bennett et al., 2006). Analysis of *max1-1* showed that secondary growth is decreased in the strigolactone biosynthesis mutant.

To determine whether secondary growth is also affected in the other *max* mutants (i.e. *max2, max3, max4*), I histologically analysed them and quantified secondary growth by measuring the lateral extension of the ICD (interfascicular cambium-derived tissues). Stems of 20-25 cm tall plants were harvested, fixed and embedded in wax (see Chapter 2.2.2). Cross sections were taken at an interval of 1 mm, from the base of the stem to 7 mm above the base. The cross sections were stained with toluidine blue and analysed by DIC microscopy. For each plant line, 5 individual plants were examined. Secondary growth was quantified as explained in Chapter 2.2.5.1.1.

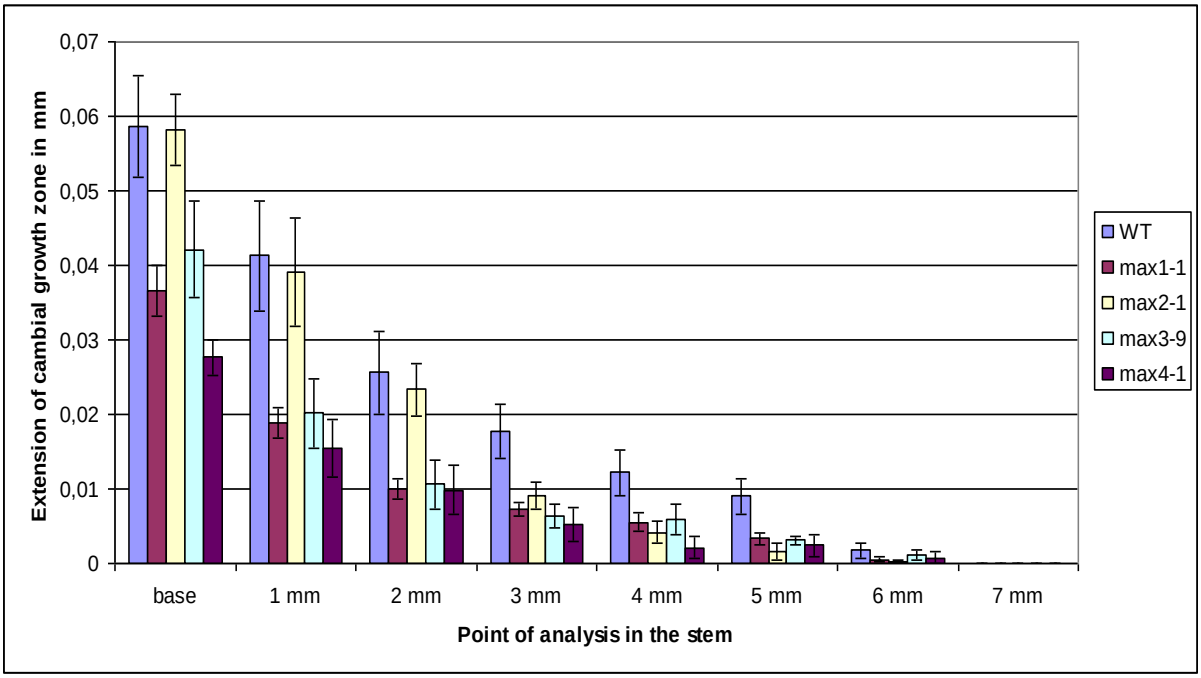


Fig. 12. Quantification of secondary growth in WT, *max1-1, max2-1, max3-9 and max4-1*

Each bar represents the average lateral extension of the ICD of 5 individual plants at a defined point of analysis. Error bars represent SEM.

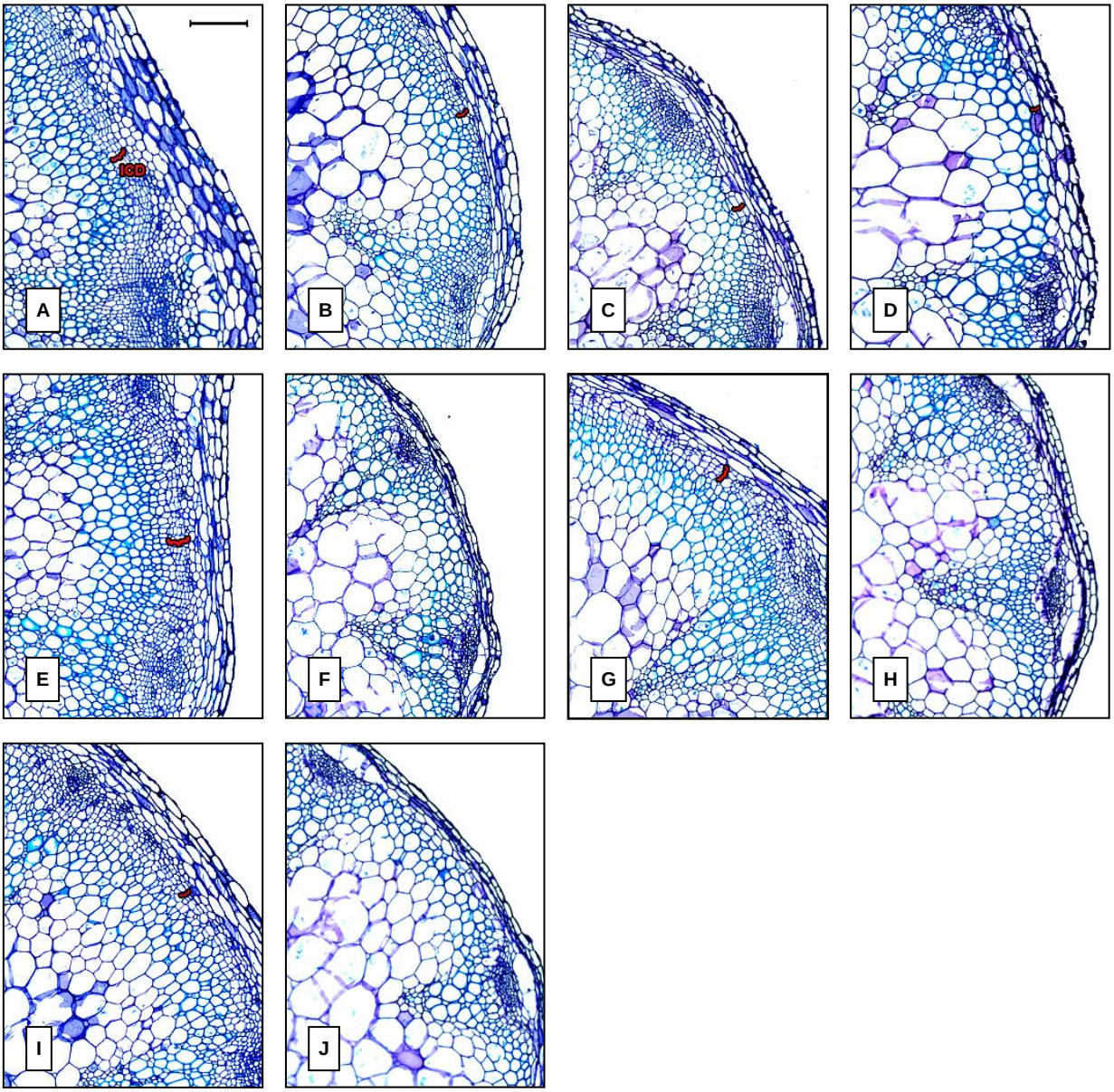


Fig. 13. Transverse stem sections of WT and the *max* mutants

Transverse sections through the base of a stem of WT **(A)**, *max1*-1 **(C)**, *max2-1* **(E)**, *max3-9* **(G)** and *max4-1* **(I)**; transverse stem sections at a height of 3 mm above the base of WT **(B)**, *max1*-1 **(D)**, *max2-1* **(F)**, *max3-9* **(H)** and *max4-1* **(J)**; The red clamp marks the lateral extension of the ICD. Scale bar = 0.1 mm.

In the strigolactone biosynthesis mutants, *max1-1, max3-9* and *max4-1*, secondary growth was reduced. Considering all the measurements shown in Fig. 12, in *max1-1* the lateral extension of the ICD was reduced to an average of 66% compared to wild type. In *max3-9,* we detected an average reduction of 60% and in *max4-1* the extension of the interfascicular cambium was reduced by 71% (all percentages were rounded to the nearest integer). However, in *max2-1* from the base of the stem to 2 mm above the base, secondary growth was present to a similar extent as in wild

type. However, *max2-1* showed an average reduction in secondary growth of 71% in upper regions (from 3 mm to 6 mm above the stem) compared to wild type. At a height of 7 mm in the stem, no secondary growth was detectable at all in neither of the plant lines analysed (wild type*, max1-1, max2-1, max3-9, max4-1*). In summary, all *max* mutants were defective in secondary growth, which implies that strigolactones play a role in this process.

3.2 Analysis of auxin signalling in *max1-1* and wild type

To further examine the causes for the reduction of secondary growth in strigolactone-deficient plants, the levels of auxin signalling in *max1-1* and wild type were determined.

3.2.1 Analysis of *DR5::GUS* activity in *max1-1* and wild type

To investigate auxin signalling, we used the auxin-responsive reporter constructs *DR5::GUS* and *DR5rev::GFP*. An analysis of *DR5* driven *GUS* signalling in the *max* mutants was already performed by Bennett et al. in 2006. However, we repeated the experiment as in Bennett's study the exact location of analysis in the stem was not specified.

For this experiment, stems 15-20 cm in height from *DR5::GUS* plants were collected and stained for 48 h following the *GUS* staining protocol as explained in Chapter 2.2.3 of material and methods section.

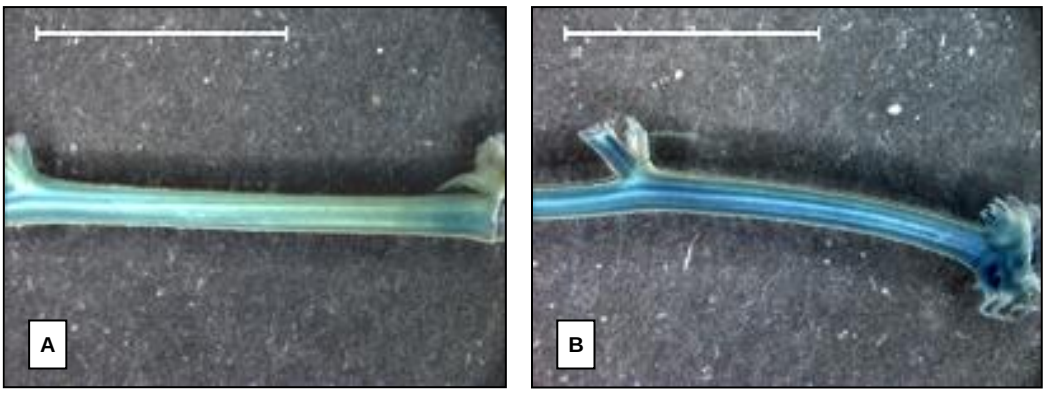


Fig. 14. *DR5::GUS* signalling in WT and *max1-1*

First internodes of *GUS*-stained WT **(A)** and *max1-1* **(B)** stems. The base of the stem is shown at the right side of the images. Scale bar = 1 cm.

As shown in Fig. 14, in *max1-1* we detected *GUS* signals of increased intensity relative to wild type, representing an elevation in auxin signalling in the strigolactone biosynthesis mutant. This observation agrees with the findings described by Bennett et al. They also showed that this is true for the other strigolactone-lacking plants, *max3-9* and *max4-1*, and *max2-1* mutants, lacking the putative strigolactone receptor (Bennett et al., 2006). Furthermore, we observed a slight accumulation of auxin at the base of the stem, especially in the wild type. The *max1-1* mutant showed a more steady level of *DR5*-driven *GUS* expression along the analysed stem regions.

3.2.2 Analysis of *DR5rev::GFP* activity in *max1-1* and wild type

To be sure about this issue we additionally had a look at the *DR5rev::GFP* line, as *GFP* is a more sensitive reporter than *GUS*. The construct allows auxin-induced *GFP* expression to be followed in specific tissues by analysing stem cross sections. In comparison to *GUS*, the risk of diffusion to other tissues is reduced, because the GFP protein is not as stable as *GUS*.

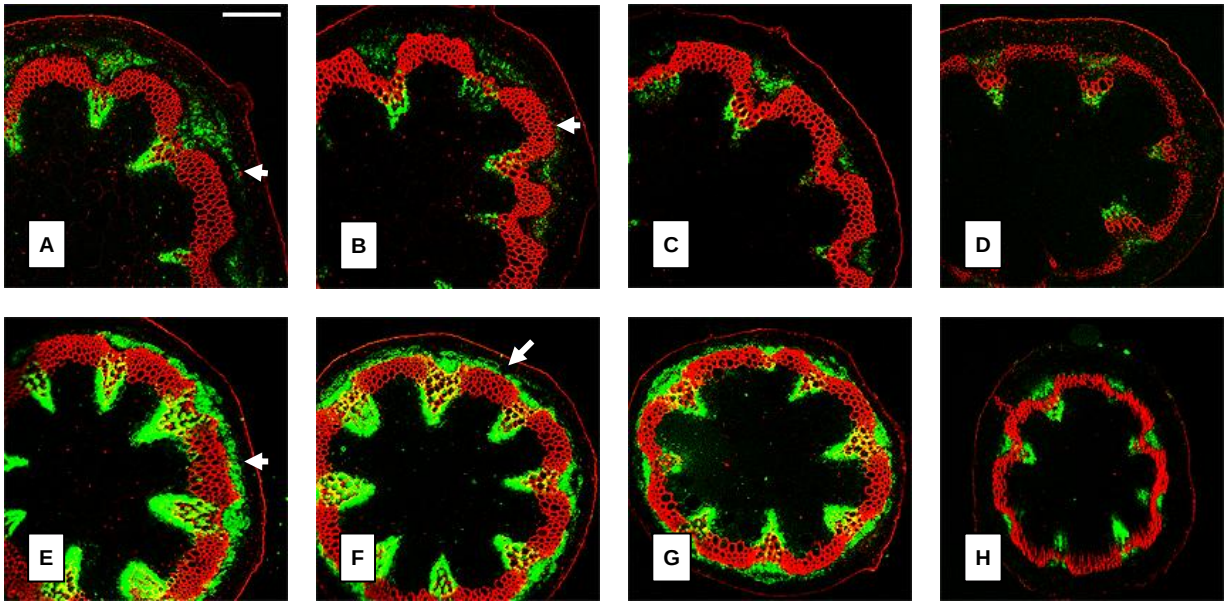


Fig. 15. *DR5rev::GFP* signalling in WT and *max1-1*

Transvers stem sections of *DR5rev::GFP* carrying WT **(A – D)** and *max1-1* **(E – H)** plants were analysed at the base **(A, E)**, at 5 mm above the base **(B, F)**, 1 cm above the base **(C, G)** and 10 cm above the base **(D, H)**. Arrowheads point to interfascicular regions. Green = GFP expression; Red = propidiumiodol staining. The plants analysed were between 18 and 25 cm in height. Scale bar = 200 µm.

Transverse cross sections were taken by hand at four different positions (i.e. base, 5 mm, 1 cm and 10 cm above the base) along the stems of wild type and *max1-1* plants. These sections were analysed by laser scanning confocal microscopy (see Section 2.2.5.2). The relative expression levels of the *GFP* reporter resembled those of the *DR5::GUS* lines. Auxin signalling was increased in the *max1-1* mutant compared to wild type at all analysed stem positions (Fig. 15). Very strong auxin signalling was detectable, particularly in tissues associated with secondary growth. For example, the *max1-1* mutant displayed an increased *GFP* signal in the interfascicular regions, especially at the base and at 5 mm above the base, but hardly develops any secondary growth (Fig. 15, arrowheads). In the wild type, *GFP* activity already decreases at a height of 5 mm, while secondary growth was shown to be still present at this stem height. These observations led us to the conclusion that auxin signalling is increased in strigolactone-deficient plants and that this increase is not positively correlated with the extent of secondary growth.

3.3 Direct auxin concentration measurements in *max1-1* and wild type

DR5 promoter-reporter constructs were proposed to indirectly represent auxin concentrations. To address the question of whether increased *DR5*-driven *GUS* and *GFP* expression in the *max1-1* mutant were due to elevated auxin concentrations or because of higher sensitivity to auxin, auxin concentration measurements were performed in *max1-1* and wild type.

To compare auxin concentrations in several stem sections of *max1-1* and wild type plants, direct auxin concentration measurements were performed. We collected 3 mm long samples at four positions of the stem (base, within first internode, within second internode, within third internode). For both, *max1-1* and wild type, we had 5 replicates of material. Each replicate contained samples of the same position in the stem of 10 individual plants. The auxin measurements themselves were performed by our collaborator Karin Ljung (Plant Science Centre, Umeå/Sweden) using a mass spectrometry-based approach (Edlund et al., 1995). Overall, we detected an increase in auxin concentrations in the *max1-1* mutant compared to wild type (Fig. 16). At the base of the stem, the level of free auxin was found to be 5.5 times higher in *max1-1* than in wild type. Within the first internode we detected 3.8 times more IAA in *max1-1* and within the second intenode 2.2 times more. Within the third internode the

concentration of auxin in *max1-1* was still 1.8 times the concentration in wild type. These results confirmed what had already been indicated by *DR5::GUS* and *DR5rev::GFP* activity in the *max1-1* mutant: auxin concentrations are severely increased throughout the stems of strigolactone biosynthesis mutants.

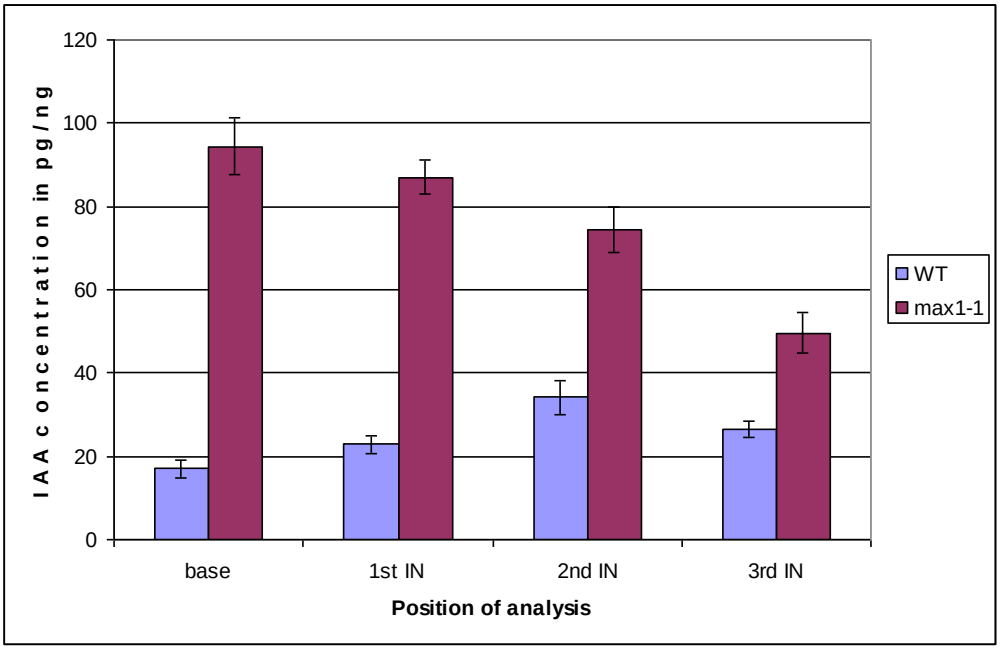


Fig. 16. Auxin concentrations along the stem in wild type and *max1-1*

The bars represent the concentrations of free auxin [IAA] (pg/mg) in four positions along WT and *max1-1* stems. Measurements were performed at the base, within the 1st internode [IN], the 2nd internode and the 3rd internode. Shown is the average concentration of free IAA detected in 5 replicates per line (WT, *max1-1*). Error bars represent SEM.

3.4 Do strigolactones promote secondary growth? – *In vivo* hormone treatment

To investigate one of the very basic questions of this study, namely whether strigolactones have the potential to initiate secondary growth, an *in vivo* hormone treatment was performed. Lanolin (i.e. wool wax) has been established as a suitable carrier for hormones in the course of *in vivo* treatments (Laibach, 1933; Little et al., 2002; Redemann et al., 1950), for which reason we used it in our experiments. All the treatments were performed with the synthetic strigolactone analogue GR24 and the auxin transport inhibitor NPA, as well as with plain lanolin as a mock control. The treatment started when the plants reached a height of 10–15 cm. The lanolin-hormone mixture was prepared and applied to the plant stems as described in Chapter 2.2.4 of the material and methods section. After 16 days of incubation, the

treated plants were histologically characterized within and 3 mm above the treatment zone. Ten individual plants were analysed per treatment.

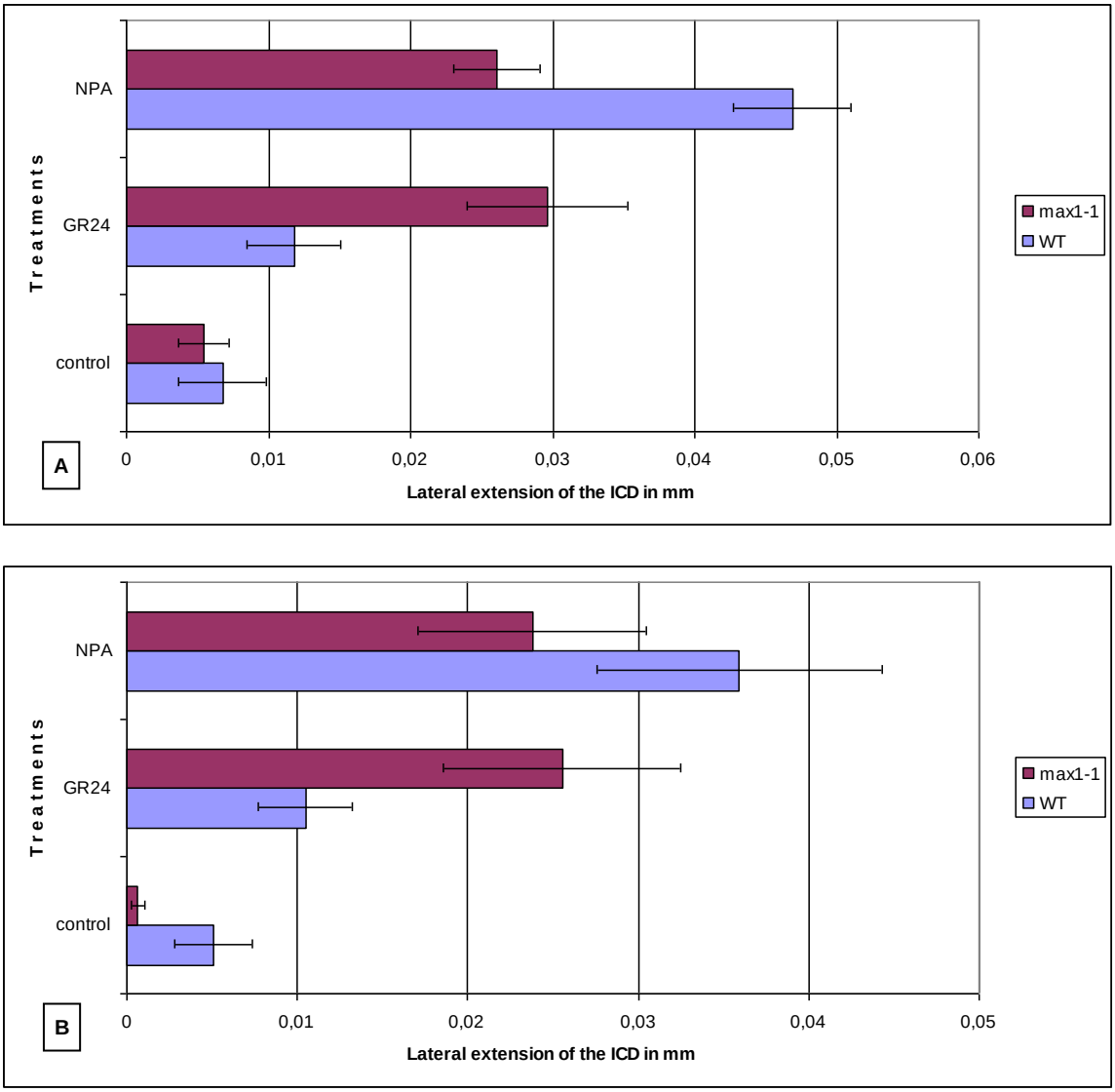


Fig. 17. Quantification of secondary growth induced by GR24 and NPA treatments

The lateral extension of the ICD was measured within the GR24, NPA and mock-treated zones **(A)** and 3 mm above this region **(B)**. Data of *max1-1* (magenta bars) and WT (lilac bars) are shown. Error bars represent SEM.

In 2002, Little et al. showed that ringing an *Arabidopsis* stem with a mixture of NPA and lanolin induced secondary growth in the treated regions and above. This finding was confirmed by our experiment. However, in the *max1-1* mutant, the effect of NPA, regarding the induced extension of the ICD, was weaker than in wild type. In *max1-1,* compared to the wild type, the NPA-promoted lateral extension of the ICD was reduced by 44.3% on average within the treatment zone and by 33.8% in the stem regions above the treatment zone. Treatments with GR24 also promoted secondary

growth in *max1-1* and wild type stems. Strigolactone occurred to stronger effect *max1-1* mutants than wild type. The extension of the ICD was increased by an average of 60.2% in the *max1-1* mutant, relative to wild type, within the GR24-treated regions. The upper parts of the *max1-1* stems still showed an increase of 59% on average in the formation of interfascicular cambium. The NPA and GR24-induced secondary growth was still present 3 mm above the treatment zone, even though the intensity was decreased. The results of this experiment suggest that strigolactone is directly involved in the promotion of secondary growth.

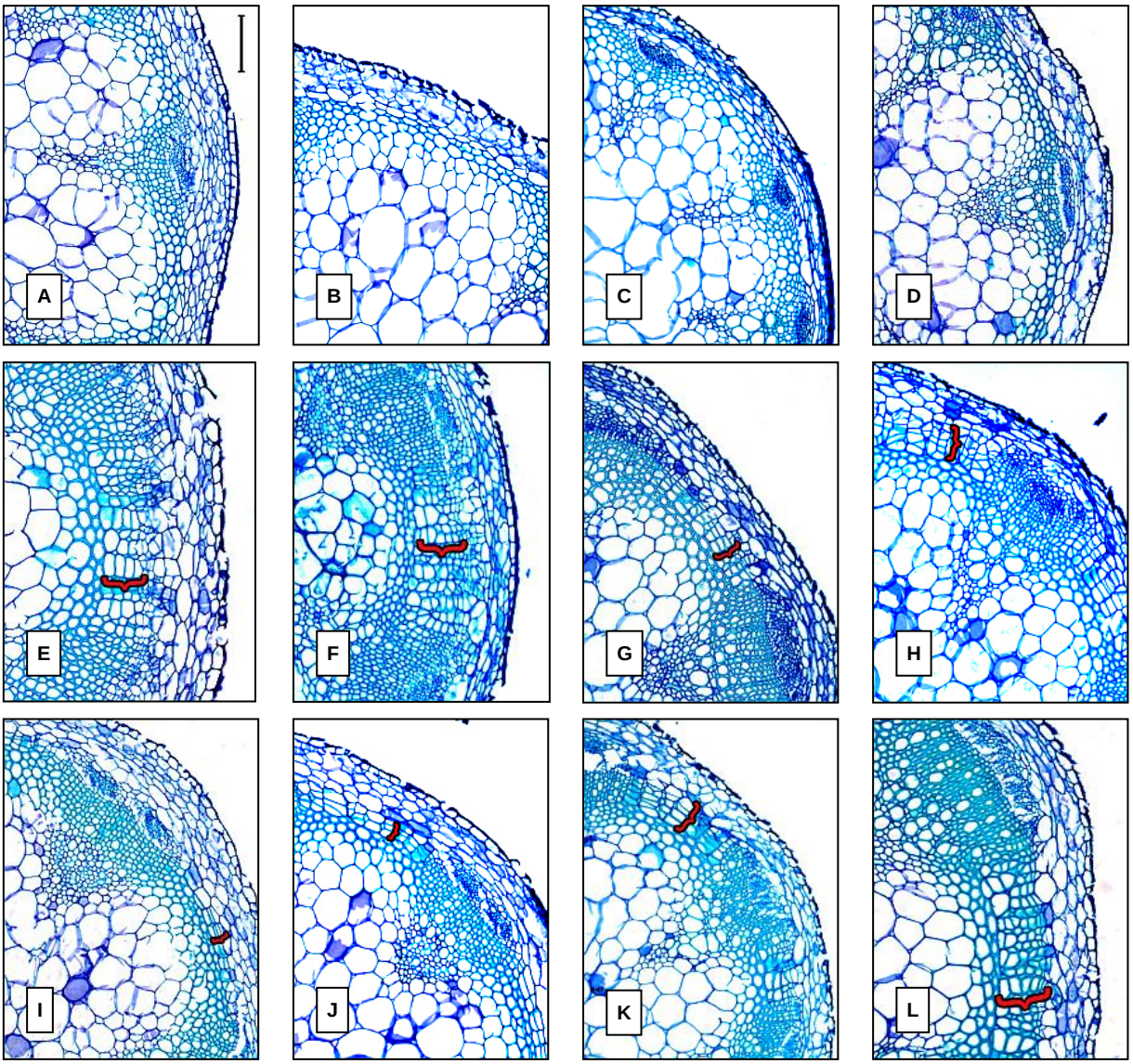


Fig. 18. *In vivo* GR24 and NPA treatment induced secondary growth in WT and *max1-1*
Transverse stem sections of mock **(A – D)**, NPA **(E – H)** and GR24 **(I – L)** treated *max1-1* **(C, D, G, H, K, L)** and WT **(A, B, E, F, I, J)** plants are shown. The stems were analysed at the region of the hormone-lanolin application [trz] **(A, C, E, G, I, K)** and 3 mm above the treated zone [atrz] **(B, D, F, H, J, L)**. The red clamp marks the extension of the ICD. Scale bar = 0.1 mm.

The measured extension of the ICD shown in Fig. 17 differs from that of the images in Fig. 18. In fact, NPA and GR24 treatments did not induce secondary growth in each interfascicular region to the same extent. Fig. 18 represents examples of regions with highly NPA or GR24-stimulated secondary growth formation, while the average values shown in Fig. 17 also consider regions displaying low levels of secondary growth.

3.5 The effect of *in vivo* strigolactone treatment on auxin signalling

3.5.1 Analysis of *DR5::GUS* activity in *max1-1* and wild type

In both NPA and GR24-treated plants, the outcomes looked similar: interfascicular cambium initiation and activity had been stimulated.

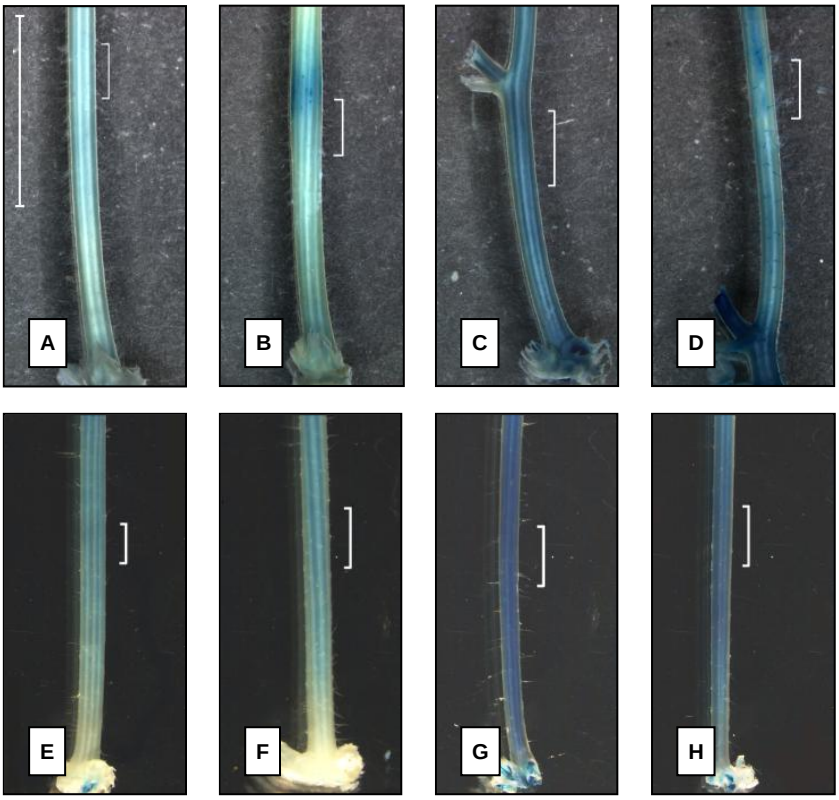


Fig. 19. The effect of NPA and GR24 on *DR5::GUS* activity in WT and *max1-1* stems
GUS stained stems of WT **(A, B, E, F)** and *max1-1* **(C, D, G, H)** plants carrying the reporter construct *DR5::GUS*. The stems had been ringed for 3 days with lanolin paste containing NPA **(B, D)** or GR24 **(F, H)**. Mock treated controls are shown in images **A, C, E, G**. The clamps represent the areas of treatment. Scale bar = 1 cm.

To further examine whether strigolactones as NPA induce secondary growth by auxin transport inhibition, *DR5::GUS* transgenic *max1-1* and wild type plants were treated *in vivo* with GR24 or NPA. Ten individual plants of each line (wild type and *max1-1*) were treated with plain lanolin, NPA or GR24, (10 mg/g in lanolin), as described in Chapter 2.2.4 of the materials and methods section. After 3 days of incubation, the plants were analysed using stereomicroscopy. A strong effect was detected in wild type stems after NPA treatment. NPA-induced auxin accumulation was represented by a maximum of *GUS* activity above the stem section treated with the auxin transport inhibitor. A slight increase of *DR5::GUS* activity also occurred in the *max1-1* mutant, though it was not as significant as in wild type. GR24 treatments did not affect auxin signalling, neither in the wild type nor in the *max1-1* mutant.

3.5.2 Analysis of *DR5rev::GFP* activity in *max1-1* and wild type

In vivo treatments with NPA and GR24 were also performed on the *DR5rev::GFP* reporter lines in wild type and *max1-1* backgrounds. Again, ten individual 15–20 cm tall plants of each genotype were treated with NPA or GR24, (10 mg/g in lanolin), as described in Chapter 2.2.4 of the materials and methods section. The hormone paste remained on the stems for 3 days of incubation. Hand sections of the stems were produced as described in Chapter 2.2.5.2 of the material and methods section. The plants were analysed at 3 positions along the stem (5 mm under the hormone treated zone, within the treatment zone and 5 mm above the treated zone), using confocal microscopy. The outcome of this experiment supports the results shown in Chapter 3.5.1. As expected, a light increase in *GFP* activity was observed in wild type and *max1-1* stems treated by the auxin transport inhibitor NPA (Fig. 20). In wild type, especially the interfascicular regions displayed an increase in signal above the treated zone (Fig. 20 arrowheads), representing the initiation of secondary growth. However, there was no significant difference in the *GFP* activity between mock treated plants and those covered with strigolactone. All in all, these results suggest that there is no direct influence of strigolactones on auxin signalling. Furthermore, GR24 was shown not to inhibit auxin transport as NPA does, so we suggest that the induction of secondary growth by these two substances follows different mechanisms. Additionally, the finding that the auxin flow cannot be affected by strigolactone supply hints that strigolactones may act downstream of auxin.

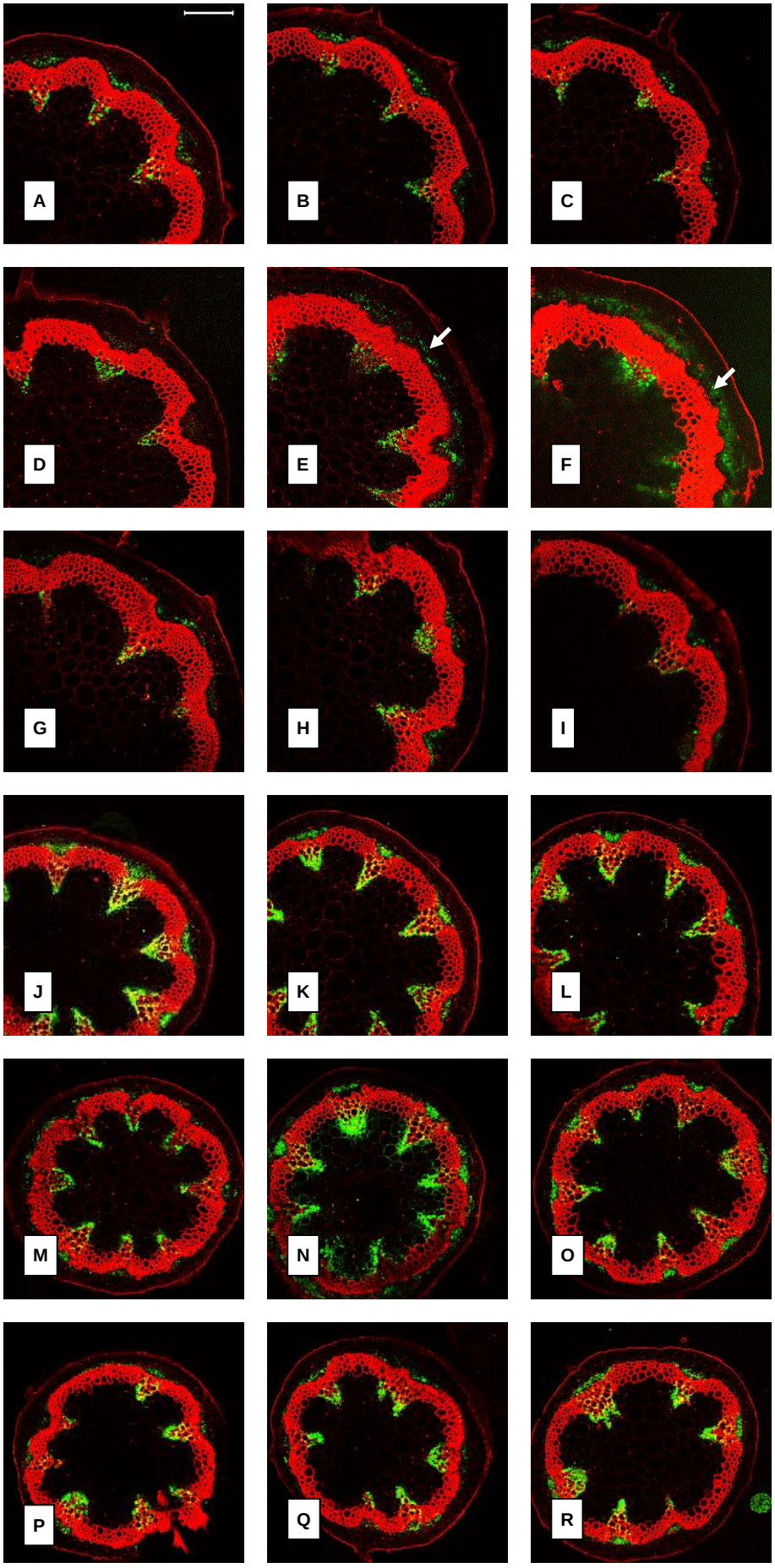

A
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Fig. 20. The effect of NPA and GR24 on *DR5rev::GFP* activity in WT and *max1-1* stems

Transverse stem sections of WT **(A – I)** and *max1-1* **(J – R)** plants carrying the reporter construct *DR5rev::GFP*. The stems had been ringed for 3 days with lanolin paste containing GR24 **(G – I, P – R)** or NPA **(D – F, M – O)**. Mock-treated plants are shown in images **A – C, J – L**. The stems had been analysed 5 mm below the treatment zone **(A, D, G, J, M, P)**, within the hormone-treated zone **(B, E, H, K, N, Q)** and 5 mm above the treated zone **(C, F, I, L, O, R)**. Arrowheads point to interfascicular regions. Scale bar = 200 µm.

3.6 Expression patterns of the auxin transporters PIN1 and PIN3 in *max1-1*

3.6.1 Analysis of PIN3 expression in *max1-1* and wild type

In 2006, Bennett and others proposed that, based on RT-PCR data, the expression of the auxin transporters PIN1 and PIN3 is upregulated in the *max* mutants. In addition, they analysed the PIN1 expression in *max1-1 PIN1::PIN1-GFP* reporter lines and showed an enhanced expression level (Bennett et al., 2006).

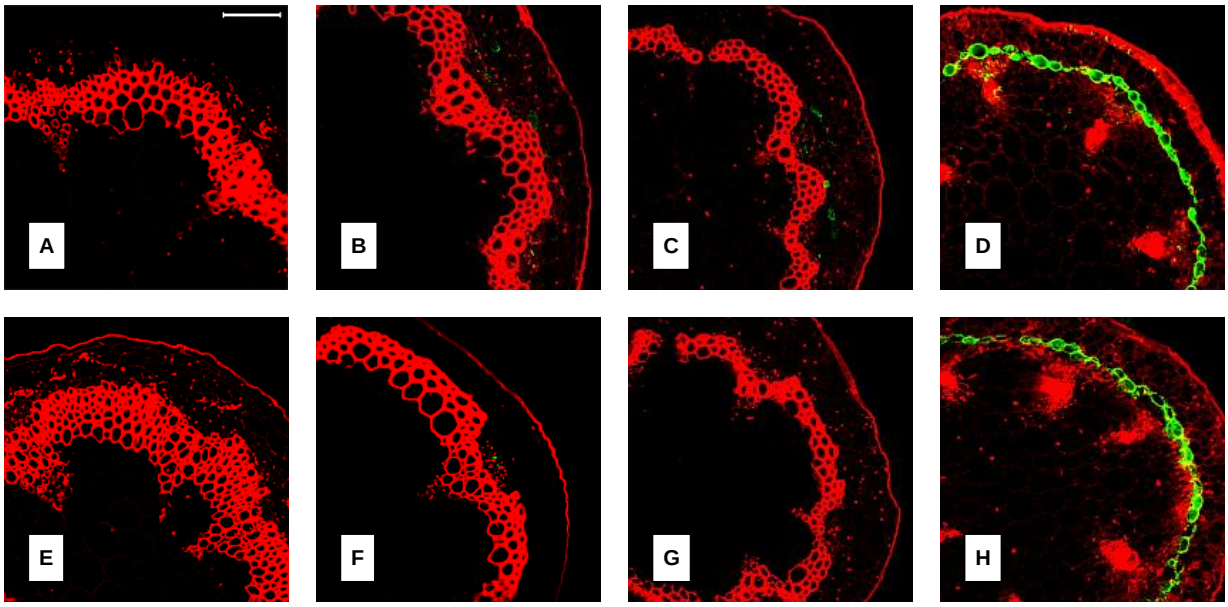

Fig. 21. PIN3 expression in *max1-1* and WT stems

Transverse stems sections of WT (**A – D**) and *max1-1* (**E – H**) plants that carry the reporter construct *PIN3::PIN3-GFP* are shown. The stems were analysed at the base (**A, E**), at a height of 5 cm above the base (**B, F**), 10 cm above the base (**C, G**) and 15 cm above the base (**D, H**) using confocal microscopy. Images shown are representative of 19 cm tall plants. Scale bar = 200 µm

To further investigate the expression of PIN3 in *max1-1*, we analysed the *max1-1 PIN3::PIN3-GFP* lines. Plants transgenic for the construct express PIN3 fused to the

GFP reporter protein under the control of the PIN3 promoter. Ten plants of a height of 18–20 cm were examined per line (wild type and *max1-1*). The analysis was performed as explained in Chapter 2.2.5.2 of the material and methods section.

As shown in Fig. 21, no PIN3 expression was detected at the base or 5 cm above the base of the stem, neither in wild type nor in the *max1-1* mutant. At a height of 10 cm above the base, there was a weak *GFP* signal in the starch sheath of both lines. We detected stronger PIN3 expression at a height of 15 cm above the base in *max1-1* and wild type, although *GFP* activity was not higher in the *max1-1* mutant relative to wild type. In contrast to RT-PCR data, we could not show an elevated PIN3 expression level in *max1-1* (Bennett et al., 2006). We therefore decided to examine *PIN1::PIN1-GFP* activity in the *max1-1* background as well.

3.6.2 Analysis of PIN1 expression in *max1-1* and wild type

Bennett et al. already analysed PIN1 expression in the *max1-1* mutant using *PIN1::PIN1-GFP*. In the course of their studies in 2006, they detected an increase in the expression of PIN1 in *max1-1* (Bennett et al., 2006). For the repetition of the experiment, we analysed ten plants per line (*max1-1* and wild type) at a height of 16–20 cm. Confocal analysis was performed as explained in chapter 2.2.5.2 of material and methods.

PIN1 is expressed in auxin transporting vascular tissues (Gälweiler et al., 1998). Thus, in wild type, strong PIN1 expression in the interfascicular regions was detected at the base of the stem, while in *max1-1,* we found a decrease in GFP signal in these regions (Fig. 22, arrows). At a height of 5 cm above the base, PIN1 expression was restricted to the vascular bundles in both wild type and *max1-1*. Furthermore, no visible difference in the expression pattern of PIN1 was detectable at this point of the analysis in *max1-1* compared to wild type. This was also true at a height of 10 cm above the stem. Thus, in the course of our experiment we did not observe an upregulation of PIN1 expression in strigolactone-deficient plants, a finding that is contrary to that of Bennett et al.

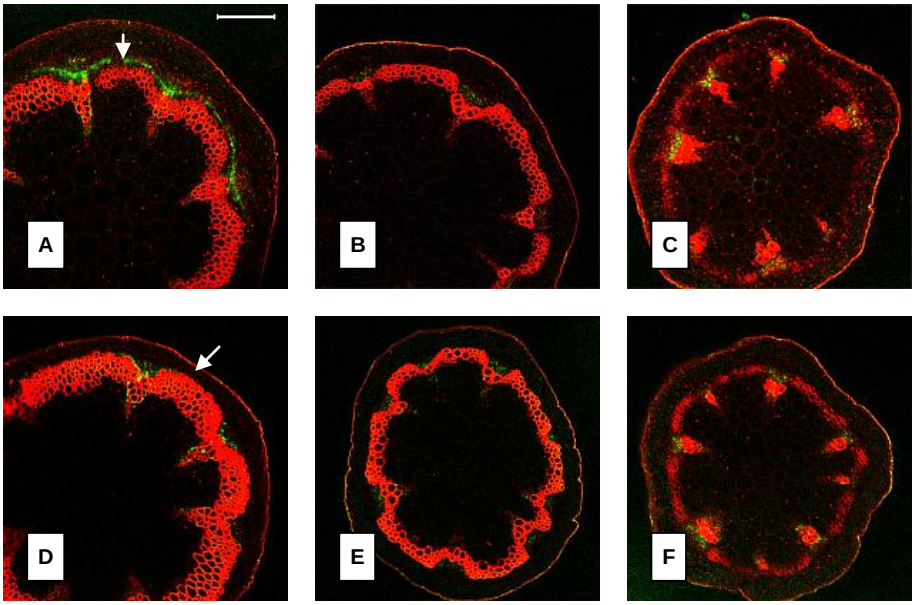

Fig. 22. PIN1 expression in *max1-1* and WT stems

Transverse stems sections of WT **(A – C)** and *max1-1* **(D – F)** plants that carry the reporter construct *PIN1::PIN1-GFP*, are shown. The stems were analysed at the base (**A, D**), at a height of 5 cm above the base **(B, E)** and 10 cm above the base **(C, F)**. Images shown are representative of 19 cm tall plants. The arrowhead points to the interfascicular region. Scale bar = 200 µm

3.7 Effect of *in vivo* strigolactone treatments on PIN1 and PIN3 expression

3.7.1 Analysis of the effect of strigolactones on PIN1 expression

To examine whether direct application of strigolactone has an effect on PIN1 expression in the stem, wild type and *max1-1 PIN1::PIN1-GFP* plants were treated *in vivo* with GR24 as described in Chapter 2.2.4 of the material and methods section. Wild type and *max1-1* mutant plants 15–20 cm in height were treated either with plain lanolin (mock) or GR24 (10 mg/g). Ten individual plants were used per line and treatment. The hormone paste was left on the stems for an incubation period of 3 days. The stems were analysed 5 mm below, within and 5 mm above the hormone-treated zone (Fig. 23).

No significant difference in the expression level and pattern of PIN1 was detected after the treatment with GR24, neither in the wild type nor in the *max1-1* mutant (Fig. 23). These results led us to propose that there is no direct influence of strigolactone signalling on the expression of the auxin transporter PIN1.

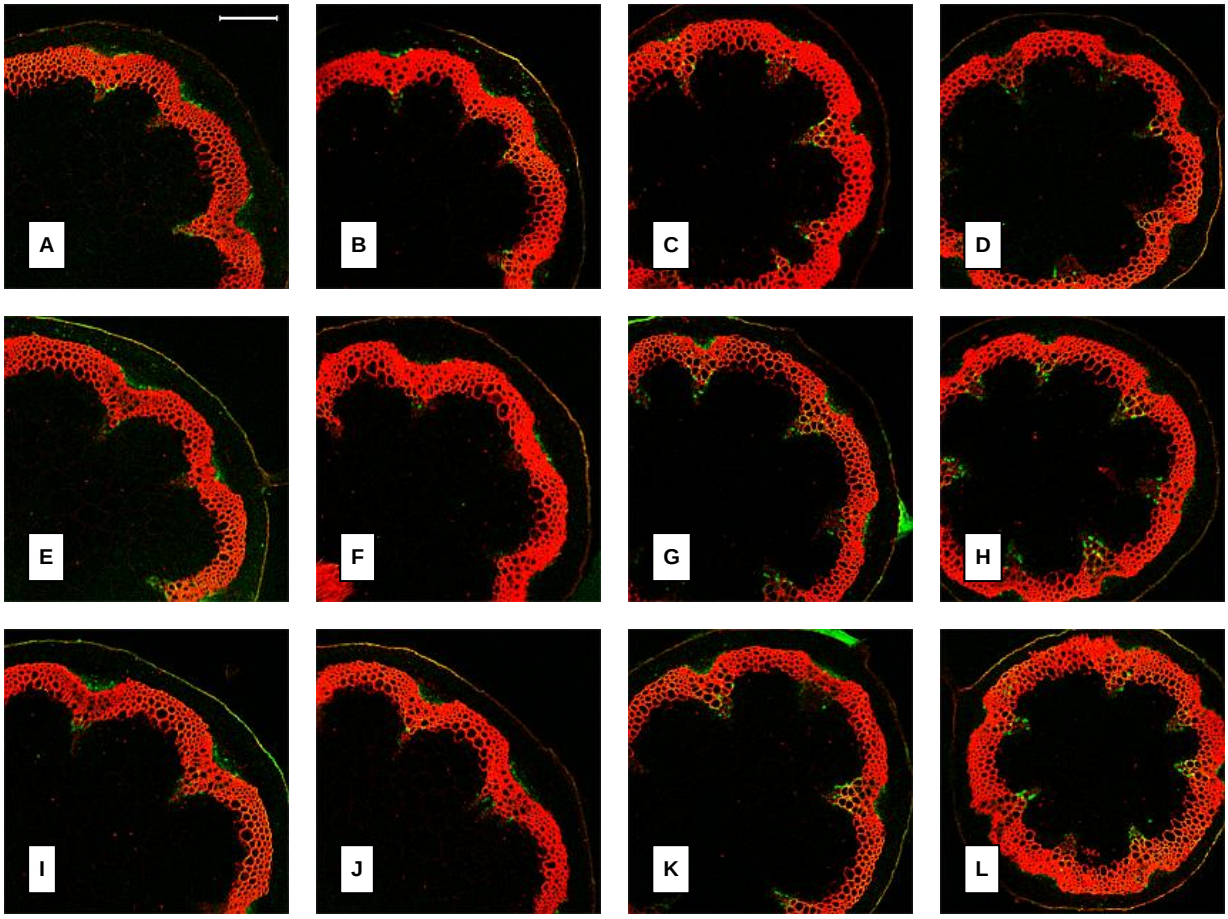

Fig. 23. Effect of GR24 treatment on the expression level of PIN1 in *max1-1* and WT stems
Transverse sections of WT **(A, B, E, F, I, J)** and *max1-1* **(C, D, G, H, K, L)** stems treated with plain lanolin **(A, E, I, C, G, K)** or GR24 **(B, F, J, D, H, L)** for 3 days. PIN1 expression (using *PIN1::PIN1-GFP*) was analysed in the stems by confocal microscopy at 5 mm below the treatment zone **(A – D)**, within the treatment zone **(E – H)** and 5 mm above the treatment zone **(I – L)**. *GFP* signalling is represented by green staining. Scale bar = 200 µm.

3.7.2 Analysis of the effect of strigolactones on PIN3 expression

We wanted to investigate whether *in vivo* strigolactone treatment has an effect on PIN3 expression in the stem of wild type and *max1-1*. This was done by analysis of the activity of *PIN3::PIN3-GFP* in the stem after GR24 treatment. The experimental procedure was the same as described in Chapter 3.7.2. As expected, in mock-treated wild type plants, no PIN3 expression was detectable at all, at least within the analysed region from 5 mm to 15 mm above the base. In this respect, *max1-1* behaved like the wild type, namely that it showed no PIN3 activity within lower stem segments. *In vivo* treatments with strigolactones did not induce PIN3 expression in the analysed stem regions, neither in the wild type nor in the *max1-1* mutant.

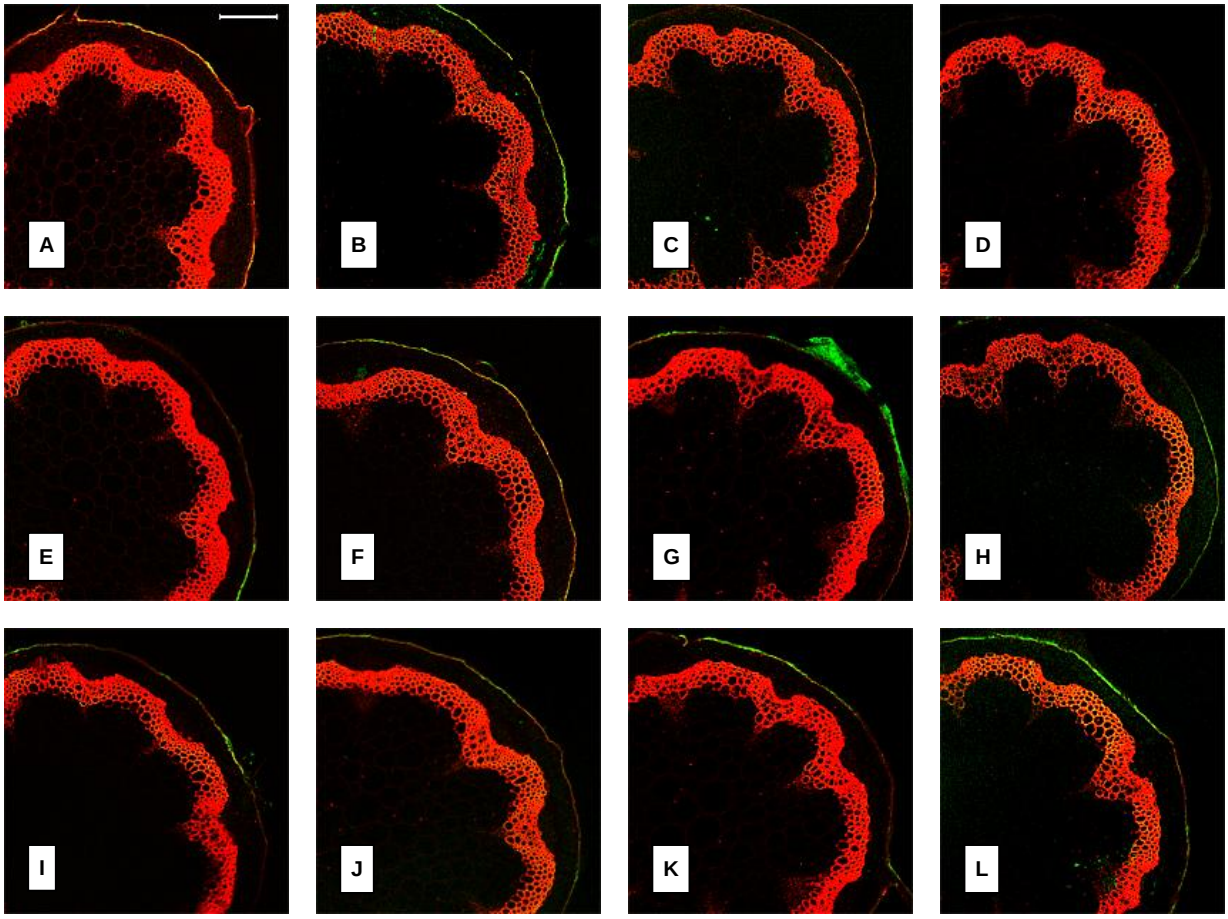

Fig. 24. Effect of GR24 treatment on the expression of PIN3 in *max1-1* and WT stems
Transverse sections of WT **(A, B, E, F, I, J)** and *max1-1* **(C, D, G, H, K, L)** stems treated with plain lanolin **(A, E, I, C, G, K)** or GR24 **(B, F, J, D, H, L)** for 3 days. PIN3 expression was analysed using the *PIN3::PIN3-GFP* reporter construct. The stems were analysed by confocal microscopy at 5 mm below the treatment zone **(A – D)**, within the treatment zone at 1 cm above the base of the stem **(E – H)** and 5 mm above the treatment zone **(I – L)**. Green staining represents autofluorescence of the epidermis. Scale bar = 200 µm.

We conclude that strigolactones do not elevate the expression of PIN3 directly, though PIN3 is regularly not expressed in lower stem sectors (Fig. 21). We cannot, therefore, exclude a possible downregulation of the protein by strigolactones. In order to address this issue, GR24 treatments have to be performed in stem regions where PIN3 is present.

3.8 Analysis of the effect of strigolactones on auxin-depleted stems

To investigate whether strigolactone is sufficient to induce secondary growth, auxin-depleted *max1-1* and wild type plants were treated *in vivo* with NPA and GR24. As mutations in auxin biosynthesis genes are lethal, another way had to be chosen to

remove the auxin from the plants. A well established method for depleting plant stems of auxin is decapitation (Brewer et al., 2009; Thimann and Skoog, 1933). The shoot apical meristem and young leaf primordia are the major sources of auxin biosynthesis (Cheng et al., 2006; Sieburth, 1999) in the stem. Decapitation, i.e. removal of the apex, leads to the loss of these auxin suppliers. Fifteen individual plants each of *max1-1* and wild type backgrounds, transgenic for the reporter *DR5::GUS*, were decapitated when they had reached a height of 15–20 cm. The cut was performed within the first internode to prevent auxin feed-in by axillary leaves. The cut surface of the stem was sealed with lanolin and the plants were left for 3 days to guarantee total depletion of auxin out of the decapitated stem. *In vitro* treatments with plain lanolin, NPA and GR24 were performed as described in Chapter 2.2.4 of the material and methods section. Five plants of each background (wild type and *max1-1*) were used per treatment. The hormone mix was left on the stem for an incubation period of 14 days. Then, samples of the treatment zone were taken, embedded in wax (see Section 2.2.2) and analysed histologically.

To examine whether the stem was totally depleted of auxin, transversal stem sections were checked on *GUS* expression. No *GUS* activity was found in any of the sections, thus showing the absence of auxin in all of the samples. As controls, non-decapitated stems were investigated on *DR5*-driven *GUS* expression (Fig. 25A – D). These were found to show *GUS* activity within the vascular tissues. Additional cross sections were stained by toluidine blue and analysed for secondary growth activity. In the case of NPA treatment, no induction of secondary growth was expected, because NPA inhibits auxin transport, which leads to accumulation of auxin and, hence, to the initiation of secondary growth. This is not possible if there is no auxin in the stem. Our observations agreed with these expectations as we could not detect secondary growth in NPA-treated, auxin-depleted stems. However, treatments with GR24 also did not stimulate the initiation of secondary growth, which may demonstrate that strigolactones are not sufficient to induce secondary growth and auxin is required in addition. However, the outcome of the experiment has to be evaluated critically, as no positive controls were run in parallel. This issue is discussed further in Chapter 4.7.

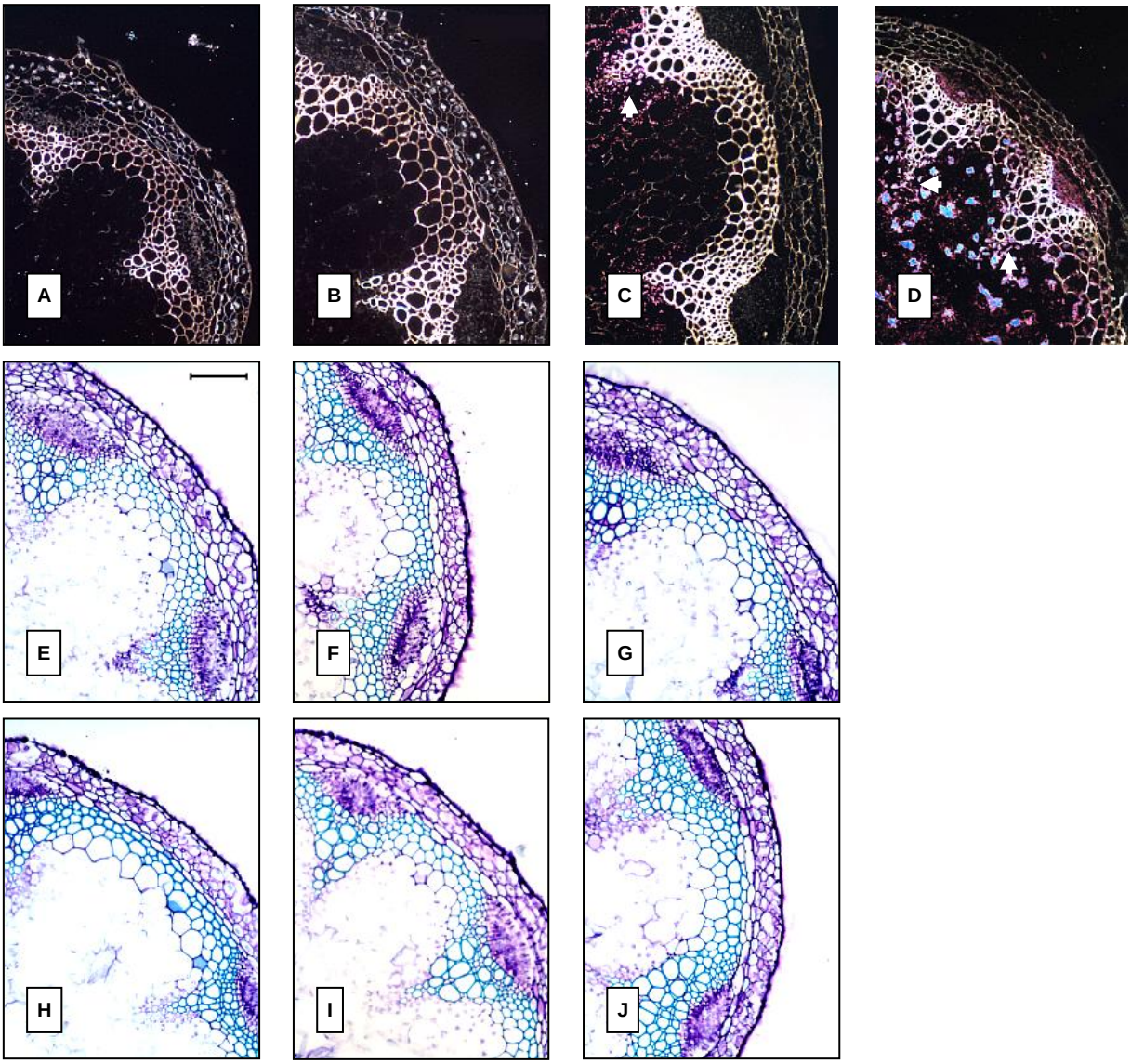

Fig. 25. Effect of NPA and GR24 treatements on auxin-depleted stems

Transverse stem sections of *DR5::GUS*-carrying, auxin-depleted WT **(E – G)** and *max1-1* **(H – J)** plants, that have been treated for 14 days with plain lanolin **(E, H)**, NPA **(F, I)** or GR24 **(G, J)**. **(A)** Dark field caption of a transverse section through a *GUS*-stained, auxin-depleted WT stem. **(B)** Dark field caption of a transverse section through a *GUS*-stained, auxin-depleted *max1-1* stem. No *GUS* activity was detectable in the decapitated stems. **(C)** Dark field caption of a transverse section through a *GUS*-stained, non-decapitated WT stem. *GUS* expression is represented by lilac staining (arrowhead). **(D)** Dark field caption of a transverse section through a *GUS*-stained, non-decapitated *max1-1* stem. *GUS* expression is represented by lilac staining (arrowheads). Scale bar = 0.1 mm.

3.9 Generation of vascular tissue-specific MAX2 expression vectors

In previous studies *MAX2* has been suggested to be a putative receptor for strigolactones (Bennett et al., 2006; Booker et al., 2005). Ectopic expression of *MAX2* in the *max2-1* background would restrict the perception and, hence, the scope

of strigolactone signalling to a specific tissue. Thus, to investigate the effect of strigolactone signalling on diverse types of vascular tissues, constructs expressing *MAX2* under tissue-specific promoters were generated. Vectors containing the promoters *pAPL*, *pNST3* or *pWOX4* were produced by Thomas Greb before I started my work in his group (unpublished). *APL* initiates and maintains phloem identity (Bonke et al., 2003), *NST3* serves as marker for the xylem and fibers (Mitsuda et al., 2007) and *WOX4* was found to be expressed in the cambium (unpublished). In addition, I generated a construct containing *pSCR*, a promoter that drives the expression of the starch sheath-maintaining transcription factor *SCARECROW* (Wysocka-Diller et al., 2000).

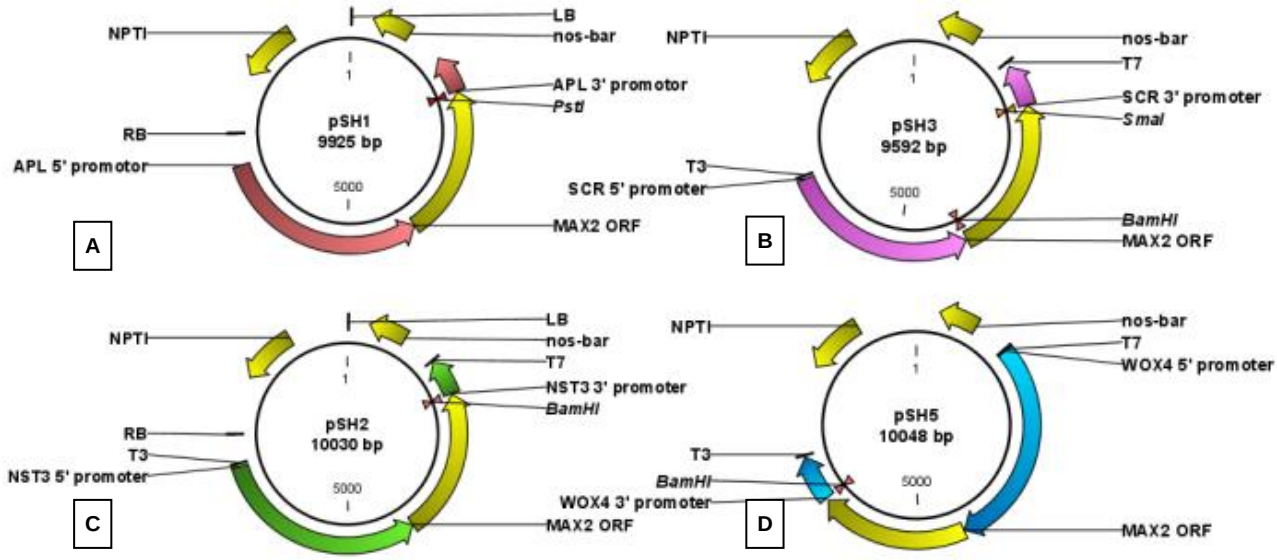

Fig. 26. Constructs for the ectopic expression of *MAX2* in four types of vascular tissue

Diagrams of the generated constructs for the ectopic expression of *MAX2* under the phloem-specific promoter *pAPL* [*pSH1*] **(A)**, *pSCR,* a marker for the starch sheath [*pSH3*] **(B)**, the xylem-specific promoter *pNST3* [*pSH2*] **(C)**, and the cambium-specific promoter *pWOX4* [*pSH5*] **(D)**. [LB] = left border, [RB] = right border, [nos-bar] = BASTA® resistance maintenance gene, [NPTI] = Kan resistance maintenance gene, [T3] = binding site of T3 primer, [T7] = binding site of T7 primer.

The *SCR* promoter consists of a 2517 bp long 5' and a 554 bp long 3' part, which were cloned into *pgreen0229*. Subsequently, the *MAX2* ORF was cloned between the promoter fragments as explained in Chapter 2.2.6 of the material and methods section.

15 cm high *max2-1 Arabidopsis* plants were transformed with the constructs as described in Section 2.2.6.9. Positive transformants of the T1 generation have already been identified. These lines have to be checked for T-DNA copy numbers

and, furthermore, the T-DNA insertions have to be stabilized in the genome. After the establishment of homozygous transgene lines, it is planned to histologically analyse them to see, in which tissues strigolactone perception is sufficient to establish a wild type-like behaviour concerning secondary growth.

4. Discussion

4.1 Strigolactones are involved in the promotion of secondary growth

A histological characterisation of wild type and *max1-1* stems led to the finding that secondary growth is reduced in this strigolactone-deficient mutant. We were curious to know whether this phenotype is shown by all of the *max* mutants. Thus, another histological characterisation was performed, this time also including *max2-1*, *max3-9* and *max4-1* (3.1). The mutants defective in strigolactone biosynthesis, *max1-1*, *max3-9* and *max4-1*, all show a similar phenotype, namely a reduction in secondary growth compared to the wild type. These findings are consistent with a role of strigolactones in the maintenance of proper secondary growth in *Arabidopsis thaliana*. However, other possible reasons for the reduction of secondary growth in *max* mutants also have to be considered. Compared to wild type, *max* mutants show an increased number of branches and develop a thinner main stem, which could also be a reason for the decreased secondary growth.

The next step in dealing with these questions was a direct treatment of strigolactone deficient stems with strigolactones. We chose to perform the hormone applications *in vivo*, which has the advantage of having intact, non-wounded plant tissue responding to the substance. Lanolin was chosen as the medium for the substances to be applied to the plant. Over the years, it had been established as a suitable carrier for hormones (mainly auxin and NPA) in the course of *in vitro* systems (Laibach, 1933; Little et al., 2002; Redemann et al., 1950). In 2002, Little et al. performed a series of *in vivo* NPA and NAA treatments on *Arabidopsis* stems, using lanolin as a carrier (Little et al., 2002). We adopted the concentration of hormones they used to our system, namely 10 mg of the hormone in 1 g of lanolin. Due to limited time, a dose-response experiment for strigolactone was not performed before in order to find out the ideal concentration for the *in vivo* treatment. Therefore, the proposed concentration for NPA, 10 mg/g, was also tried for GR24, the synthetic strigolactone analogue. In our experiment, NPA served as a positive control. In the case of NPA secondary growth is triggered by an accumulation of auxin in the stem, caused by auxin transport inhibition. As expected, we could detect secondary growth in NPA treated wild type and *max1-1* stems. Secondary growth was also induced in the

plants ringed with the GR24-lanolin mixture (Section 3.4). This observation supports our suggestion that strigolactones are involved in the promotion of secondary growth in *Arabidopsis thaliana*. Moreover, we show that it is not the weak constitution of *max* stems that impedes the formation of proper secondary growth. Quite well developed secondary growth can be detected in the mutant after hormone treatment, while the branching phenotype is still present. This implies that the process of secondary growth formation is independent of axillary branching. *max1-1* plants displayed an even stronger response to GR24 treatment than wild type. Thus, we assume that strigolactone biosynthesis mutants are more sensitive to the hormone, because these plants may try to compensate their strigolactone deficiency by increased uptake and/or perception capacities for GR24.

Auxin has been shown to be a key factor for the initiation and activity of the cambium (Section 1.4.2). Thus, in order to reveal the particular role of strigolactones in the regulation of secondary growth, we focused on the elucidation of the interplay between strigolactones and auxin. We chose *max1-1* for the experiments in this study because homozygous transgene lines of interest were available for this mutant and it is expected to represent the situation of the other strigolactone biosynthesis mutants, *max3* and *max4*.

4.2 Auxin levels and signalling are increased in *max1-1*

By the analysis of the *DR5::GUS* reporter line, Bennet et al. showed that auxin signalling is increased in the *max* mutants, compared to wild type (Bennett et al., 2006). Our repetition of their experiment, as well as the analysis of *DR5rev::GFP* expression in wild type and *max1-1,* confirmed that auxin signalling may be increased in strigolactone biosynthesis mutants (Section 3.2). Although *DR5* is a well-established marker for auxin signalling and concentration, one has to consider that *DR5* is still an artificial construct, whose activity may be altered by factors other than auxin. For example, it has also been shown to respond to brassinolides (Nakamura et al., 2003). To confirm whether auxin concentration is increased in plants lacking strigolactone or whether they are just more sensitive to auxin signalling, direct auxin measurements along wild type and *max1-1* stems have been performed (Section 3.3). The results of the measurements went along with the *DR5* analysis, as auxin levels were severely increased in *max1-1* mutants compared to

wild type. This observation is supported by the recent finding that *max1-1* is involved in the biosynthesis of flavonoids that are inhibitors of auxin transport (Brown et al., 2001; Lazar and Goodman, 2006). Therefore, the increased auxin levels and signalling in the *max* mutants can be interpreted to be a result of decreased flavonoid concentrations in the stem.

It was shown that accumulation of auxin stimulates secondary growth induction (Little et al., 2002). Furthermore, we detected an increase in auxin signalling at the base of the stem using *DR5rev::GFP* (Section 3.2.2). However, in the course of the auxin measurements, in wild type we detected no increase in auxin levels at the base in comparison to upper regions of the stem. The auxin measurements represent global auxin concentrations in particular stem sectors, but accumulation of auxin may be restricted to certain tissues. Furthermore, to show whether the small differences in auxin concentrations between the base and internodes are statistically significant, many more replicates have to be analysed.

4.3 Strigolactones do not block auxin transport directly

In vivo treatments of *Arabidopsis* stems with NPA and strigolactones showed a similar result, namely the induction of secondary growth. To further investigate whether the action of both is based on the same basic mechanism, the inhibition of auxin transport, *DR5::GUS* and *DR5rev::GFP* activities in wild type and *max1-1* stems were checked after *in vivo* treatments by those substances (Section 3.5). NPA application on wild type stems resulted in a strong increase in *GUS* activity above the treatment zone, while in the *max1-1* mutant just a slight effect was detectable. However, we suspect that the *max1-1* stems are already saturated with auxin. After staining, *DR5::GUS* transgenic *max1-1* plants displayed a dark-blue colouration, representing the high level of auxin signalling, which made it difficult to distinguish between regular stain and increased *DR5::GUS* signalling, which would be expected, for example, after NPA treatment. In this aspect, *DR5rev::GFP* appeared to be a more sensitive reporter line for auxin signalling, as the stems can be analysed in detail by confocal microscopy. However, NPA treatments on *DR5rev::GFP* transgenic wild type and *max1-1* plants resulted in a slight increase in auxin signalling within and above the treated stem segments, while an effect of GR24 on *DR5rev::GFP* activity was not detectable at all.

In comparison to NPA, GR24 application did not influence the activity of *DR5*, neither in the wild type nor in *max1-1*. This leads to the conclusions that strigolactones do not influence auxin signalling directly, and that they, unlike NPA, do not promote secondary growth by the inhibition of auxin transport. In this case, it is important to compare individuals of the same treatment. *GFP* expression differs from one individual to another, also depending on the size of the plant. In comparison to the experiment explained in Section 3.2.2, where the situation in plants of exactly the same height is shown, in the experiment in Section 3.5.2, plants of 15–20 cm height are shown.

To further unravel the molecular mechanisms and differences underlying the induction of secondary growth by NPA and strigolactone treatments, it is important to identify the genes that specifically respond to those substances. A microarray-based transcriptional profiling is currently ongoing in the Greb lab. A comparison of the gene expression pattern in NPA, GR24 and non-treated plant stems would presumably allow the identification of new factors involved in strigolactone signalling and secondary growth initiation.

4.4 Levels of the auxin transporters PIN1 and PIN3 are not increased in *max1-1*

Supported by RT PCR data, Bennett et al. proposed PIN1 and PIN3 expression to be upregulated in *max* backgrounds. An analysis of PIN1 expression levels using *PIN1::PIN1-GFP* confirmed this result (Bennett et al., 2006). As we were interested in the expression pattern of PIN3, we analysed *PIN3::PIN3-GFP* activity in *max1-1* and wild type. Unlike suspected, no increase in PIN3 expression was detectable in the mutant (Section 3.6.1). Furthermore, a repetition of the PIN1 analysis using *PIN1::PIN1-GFP* was performed in order to see whether we could confirm at least the proposed increase in PIN1 levels in *max1-1*. However, again we could not detect an increased amount of the auxin transporter in the strigolactone-deficient mutant (Section 3.6.2). The interpretation of these results is based on the visible GFP signal. No detailed quantification of GFP fluorescence intensity was performed. We conclude that, at least under the conditions of our experiments, the expression of PIN1 and PIN3 is not upregulated in *max1-1*. To investigate this issue further, a detailed analysis of PIN1 and PIN3 protein concentrations along the stem of wild type and *max* mutants is planned using western blotting.

4.5 Directly applied strigolactones do not alter PIN1 and PIN3 expression

To investigate whether a locally applied high concentration of strigolactones alters the expression of PIN1 and/or PIN3, the activities of *PIN1::PIN1-GFP* and *PIN3::PIN3-GFP* were analysed after *in vivo* treatments with GR24 (Section 3.7). We could not detect an increase in PIN1 expression after strigolactone treatment, neither in the wild type, nor in *max1-1*. Similarly, *PIN3::PIN3-GFP* could not be induced by GR24. Together with the finding that PIN1 and PIN3 are not significantly upregulated in strigolactone-deficient plants, it occurs that strigolactones are not directly involved in the regulation of auxin transport via the alteration of PIN1 and PIN3 expression. Nevertheless it is possible, that strigolactones affect polar PIN localisation in the cells. This will be examined in the future in the Greb lab by analysis of the subcellular localisation of PIN1 and PIN3 in plants lacking strigolactones. This will be done by analysing longitudinal cuts of *max1-1* and wild type stems, utilizing *PIN1::PIN1-GFP* and *PIN3::PIN3-GFP*. In addition, other PIN proteins could be checked for their response to strigolactones.

4.6 Strigolactones act downstream of auxin

The finding that auxin transport as well as PIN1 and PIN3 expression are upregulated in *max* mutants led to a model suggesting that auxin acts downstream of strigolactones (Bennett et al., 2006). Recently, an alternative model was proposed, suggesting that strigolactones act downstream of auxin. This model is supported by the findings that strigolactone completely inhibits bud outgrowth in decapitated plants and that strigolactone biosynthesis genes are stimulated by auxin (Bainbridge et al., 2005; Brewer et al., 2009). The results presented in this study rather support the hypothesis of auxins being upstream of strigolactones. However, in contrast to shoot branching inhibition, secondary growth could not be stimulated by strigolactones independently of auxin. Several studies have suggested flavonoids as regulators of auxin transport (Brown et al., 2001). Recently, it has been shown that MAX1 is involved in the biosynthesis of these polyphenolic compounds (Lazar and Goodman, 2006). We did not further focus on flavonoids in the course of this study but it might be important to consider them by setting up new models describing the interaction between auxin and strigolactones. We did not find that strigolactones influence auxin transport directly, a fact that highlights, that there is something between

strigolactones and the increased levels of auxin detected in the *max* mutants. Flavonoid seems to be a potential candidate linking MAX genes with the modulation of auxin transport and signalling (Fig. 27).

4.7 Strigolactone and auxin are both required for proper secondary growth

Strigolactones were shown to promote secondary growth (Section 3.4). The next question to answer was whether they are sufficient to induce secondary growth in an auxin free environment. For this, *in vivo* strigolactone treatments were performed on decapitated *DR5::GUS* transgenic wild type and *max1-1* plants (Section 3.8). Treatments with NPA, as expected, could not induce secondary growth. In addition GR24 application was not detected to stimulate cambial activity in either wild type or *max1-1* stems. This finding indicates that strigolactones alone may not be able to initiate secondary growth. However, the result has to be critically examined, as no positive controls could be used. It may happen that decapitated stems desiccate, leading to stressed tissues that would not respond to hormone treatments. We tried to overcome this issue by placing the cut 3–4 cm above the zone of lanolin application and sealed the lesion with lanolin. The next step would be a repetition of the experiment including non-decapitated controls and decapitated plants that are offered an auxin source by application of a lanolin-IAA mixture to the cut, to show that decapitated stems are able to initiate secondary growth under suitable conditions.

The proposal that strigolactones need auxins in order to promote secondary growth is based on very preliminary results. Cambial activity in strigolactone-deficient plants is reduced, but not completely absent (Section 3.1). In addition, *in vivo* treatments with NPA on the *max1-1* mutant induced secondary growth, even though not to the same extent as in wild type (Section 3.4). Furthermore, we showed that increased levels of auxin in *max1-1* do not automatically lead to an increase in secondary growth. Taken together, these results suggest that strigolactones combined with auxin are required for proper secondary growth. Auxin is essential for the initiation of the process and strigolactones may serve as enhancing factors.

Auxin present Strigolactone present	normal secondary growth
Auxin present Strigolactone absent	reduced secondary growth
Auxin absent Strigolactone present	no secondary growth
Auxin absent Strigolactone absent	no secondary growth

Table 4. Proper secondary growth requires both auxin and strigolactones

4.8 The role of MAX2 in secondary growth

The F-Box protein MAX2 is supposed to be a receptor for strigolactone (Stirnberg et al., 2007; Stirnberg et al., 2002). Histological analysis of the *max2-1* mutant revealed that it displays secondary growth to a similar extent as wild type, at least from the base of the stem to a height of around 3 mm above the stem (Section 3.1). This finding strongly suggests that MAX2 might not be required for successful strigolactone signal perception in order to promote secondary growth at the very base of the stem. On the other hand, it is possible that there exists another strigolactone receptor involved in secondary growth establishment in this part of the stem. However, in upper stem sectors of *max2-1* plants, secondary growth rapidly reduced to the level of the other *max* mutants (Section 3.1). Thus, the question arises as to whether the reduction in secondary growth in this particular part of the stem is due to the position of the tissue (i.e. from 3 mm above the base on) or the developmental stage. If it is the position of the tissue, the proposed second strigolactone receptor may just be present or active at the base of the stem, but not in the more apical part. Another possible explanation is that the expression of this proposed receptor might be dependent on the developmental stage of the tissues. The base represents tissues older in age than the regions above. In order to address this issue, *max2-1* plants of different developmental stages have to be histologically analysed. It might be that the assumed second strigolactone receptor is essential in this process in the region from 3 mm above the base onwards. In order to test this, *in*

vivo strigolactone treatments will also be performed on *max2-1* mutants. In addition, a series of *MAX2* expression constructs have been generated to ectopically express the protein in different types of vascular tissue in the *max2-1* background (i.e. cambium, phloem, xylem, starch sheath) (Section 3.9). This allows the restriction of strigolactone perception to defined areas. In the future, transformed plants will be histologically analysed in more apical parts of the stem, to investigate whether strigolactone signalling has a direct effect on the structure of vascular tissues and where it is essential to possibly induce secondary growth. We conclude that MAX2 is involved in the regulation of cambial activity, as *max2-1* is defective in secondary growth as the other *max* mutants in particular sectors of the stem.

4.9 Summary

On the basis of all the data produced in the course of this study, a model was created, describing the relationship between auxin and strigolactones regarding secondary growth. Auxin acts upstream of strigolactones, promoting the biosynthesis of strigolactones by the stimulation of the carotenoid cleavage deoxygenase MAX3 (Bainbridge et al., 2005; Hayward et al., 2009). At the base of the stem, auxin together with strigolactones induce secondary growth, while auxin is the essential factor and strigolactones act as kind of enhancers in order to establish proper secondary growth. In the *max* mutants (excluding *max2*), strigolactones are lacking. Auxin concentration increases, possibly because of the lack of flavonoids, which have been proposed to be inhibitors of auxin transport (Brown et al., 2001). In the mutant, secondary growth is initiated, but cambial activity is reduced in comparison to wild type. This defect cannot be compensated for by higher auxin levels in the mutant.

One has to be aware that the model does not involve additional factors that could possibly interact with strigolactones and auxin. One of these could be the comprehensive phytohormone cytokinin. Cytokinin has been shown to be involved in the differentiation of vascular tissues and was recently assumed to interact together with strigolactones and auxin in the regulation of axillary bud outgrowth, possibly as an antagonist of strigolactone (Aloni et al., 2006; Ferguson and Beveridge, 2009; Sachs, 2000).

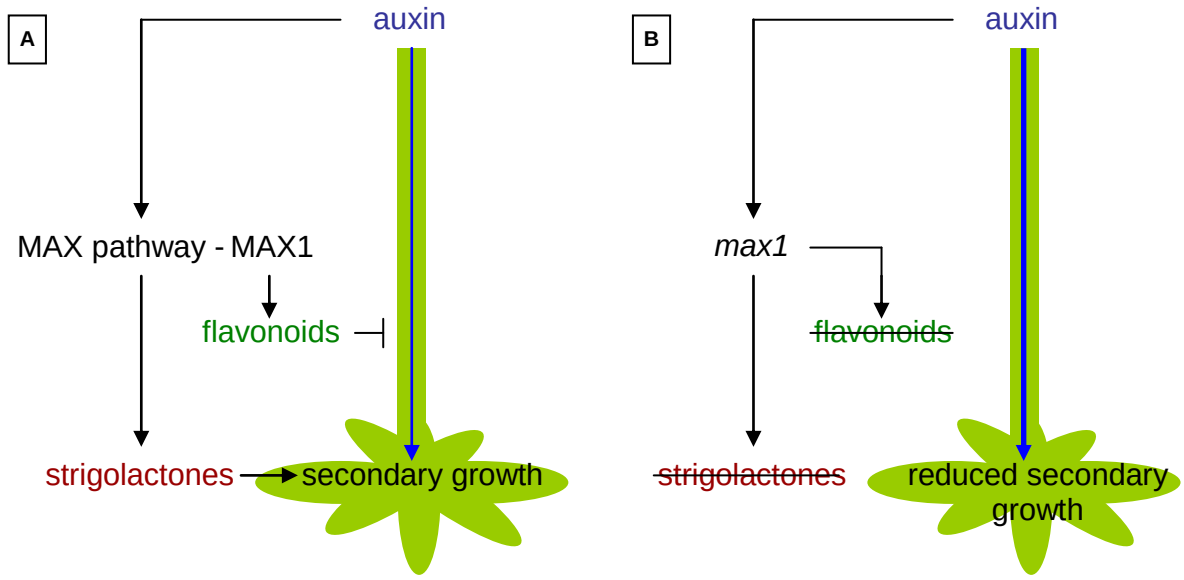

Fig. 27. Model describing the interaction between auxin and strigolactones

The simplified diagrams illustrate the interaction between auxin and strigolactones regarding secondary growth in WT **(A)** and in the strigolactone biosynthesis mutant *max1* **(B)**.

5. Index of tables

6. Index of figures

7. Abbreviations

Units were used and abbreviated according to the International System of Units (SI).

A	Adenine
AGI	*Arabidopsis* Genome Initiative
APL	*ALTERED PHLOEM DEVELOPMENT*
bp	base pairs
C	Cytosine
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
ddH_2O	Double distilled water

diXH-indigo	5,5'-Dibromo-4,4'-dichloro-indigo
dNTP	Deoxyribonucleotide triphosphate
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FAA	Formalin/acetic acid/alcohol
Fig	Figure
G	Guanine
GFP	Green Fluorescent Protein
GUS	β-Glucuronidase
IAA	Indole-3-acetic acid
ICD	Interfascicular cambium derived tissues
i.e.	id est
LB	Lysogeny Broth medium
MAX	*MORE AXILLARY BRANCHING*
NPA	1-Naphthylphthalamic acid
NST3	*NAC SECONDARY WALL THICKENING PROMOTING FACTOR 3*
O/N	Over night
ORF	Open reading frame
p	Promoter
PCR	Polymerase Chain Reaction
PGP	P-Glycoprotein
PIN	*PIN-FORMED*
RNA	Ribonucleic acid
rpm	Rounds per minute
RT-PCR	Real Time Polymerase Chain Reaction
SCR	*SCARECROW*
SEM	Standard error of the mean
T	Thymine
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TRIS	Tris(hydroxymethyl)aminomethane
WOX4	*WUSCHEL RELATED HOMEOBOX 4*
WT	Wild type
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronide
XH	5-Bromo-4-chloro-3-indoxyl
YEB	Yeast and beef extract medium

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