Gibberellin Homeostasis and Biosynthesis in Relation to Shoot Growth in Hybrid Aspen

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Abstract

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The plant growth substance gibberellin (GA) is a key regulator of shoot growth in plants. In the work underlying this thesis I explored the biosynthesis and homeostasis of GA in the model tree hybrid aspen (*Populus tremula x tremuloides*). In addition, the effect of bioactive GA on growth and development was examined.

Two genes encoding key enzymes in GA biosynthesis, GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox), were individually over-expressed by standard transgenic techniques. The phenotype of GA20ox overexpressers (OEs) was associated with up to 20-fold increases in bioactive GA, leading to highly increased stem elongation and secondary growth. In contrast, ectopic expression of GA3ox did not dramatically alter growth and development. Thus it was concluded that the activity of GA20ox, relative to GA3ox, acts as a limiting step during GA biosynthesis in the control of shoot growth in hybrid aspen. Furthermore, several lines of evidence now suggest that GA_4 has a more pivotal role than GA_1 in controlling shoot elongation.

GA regulates wood formation as transgenic GA-overproducing plants developed 70% more and 8% longer xylem fibers as compared to WT. Curiously, no major alterations in phloem formation were observed. Based on the results of microarray analyses comparing the wood-forming tissues of GA20ox OE and WT, we suggest that GA mainly up-regulates the expression of genes implicated in cell wall formation and extension. Intriguingly, the microarray analysis helped uncover an interesting shift in the composition of lignin previously undetected in the transgenic trees.

The distribution of bioactive GA within the cambial region of *Populus* was mainly restricted to expanding xylem cells. We therefore suggest that the predominant effect of GA occurs during xylem expansion. Finally, the expression of two genes implicated in GA responsiveness correlated very well with the distribution of bioactive GAs in the cambial region.

Key words: *Populus tremula x tremuloides*, gibberellin, elongation, secondary growth, transgenic trees, GA20ox and GA3ox

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"Stig-Helmer, livet är fullt av möjligheter!"

Ole Bramserud i
Sällskapsresan 2 – Snowroller, 1985
Manus Lasse Åberg

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Appendix

List of papers

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Eriksson, M.E., Israelsson, M., Olsson, O. and Moritz, T. (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnology* 18: 784-788.

II. Israelsson, M., Mellerowicz, E., Chono, M., Gullberg, J. and Moritz, T. (2004) Cloning and overproduction of GA 3-oxidase in hybrid aspen. *Plant Physiology* In Press, to be published in Volume 135.

III. Israelsson, M., Eriksson, M.E., Hertzberg, M., Aspeborg, H., Nilsson, P. and Moritz, T. (2003) Changes in gene expression in the wood-forming tissue of transgenic hybrid aspen with increased secondary growth. *Plant Molecular Biology* 52: 893-903.

IV. Israelsson, M., Sundberg, B. and Moritz, T. Tissue-specific gibberellin biosynthesis and responsiveness during wood formation in aspen. Manuscript.

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Introduction

Plants cannot move. A consequence of this obvious statement is that plants must adapt their growth in accordance with external conditions in order to survive and successfully produce offspring. Although plants cannot physically move in the sense that animals can, they do posses a wide ability to modulate their growth and development. Environmental signals can be integrated into the developmental programs of a plant in order to maximize its competitiveness and survival. Light, for instance, may play several essential roles in the life cycle of plants (as well as providing an energy-source that drives photosynthesis), since both quantity and quality parameters may provide critical environmental cues. This enables the most favorable molecular and morphological responses available to the plant to be initiated under a given light regime.

Studying the action of plant hormones has helped to identify several pieces in the immensely complex and intricate puzzles that need to be solved to understand how plants live and grow. As regulators in various stages of plant development they have presented attractive fields to explore for many plant biologists, myself included. As a starting point for discussing hormones, the standard definition of a hormone is a substance produced in one tissue of an organism which can be transported to another where it exerts control over a specific physiological process (Lawrence, 1995). This definition does not fit the situation in plants, where a "hormone" may be produced at the site of its action, so the term plant growth regulator has been favored instead. However, for convenience I will sometimes refer to plant growth regulators as plant hormones in this text, although it may not be strictly correct biologically. In plants, the "big five" were the first characterized plant growth regulators, namely auxin, gibberellin, cytokinin, ethylene, and abscisic acid (Davies et al., 1995). These hormones have relatively well characterized functions, and they may act independently of each other, but are often found to have overlapping functions. Cross-talk between different growth-regulating substances increases the complexity and levels of control over growth and development. The number of identified plant growth regulators is growing, and jasmonic acid, the steroid- derived brassinosteroids and salicylic acid have all now been included in the group for a relatively long time. The ultimate task is, of course, to understand the individual roles all hormones may play in the life cycle of a plant and, more interestingly, their combined effects.

In this thesis I explore the regulation and biological functions of the group of plant growth regulators named gibberellins (GAs). The effects of GA were first described in the early 19th century when Asian farmers discovered a disease which caused the rice to grow very tall, pale and slender, named the foolish seedling disease, *bakanae*. In the 1930's the causative agent of the disease was identified as the fungus *Gibberella fujikoroi*, which was found to infect the rice and to cause the hyper-elongation by producing massive amounts of GA. Later, it was realized that GA is an essential endogenous regulator of plant growth. Nowadays, the plant growth processes known to be affected by GA have been expanded from shoot elongation to include seed germination, flowering induction, development of male floral organs and trichome development, to name but a few.

The main interest in GA-regulated development in my work has focused on different aspects of shoot growth. For this purpose I have used the tree model system hybrid aspen (*Populus tremula x tremuloides*) because it is convenient for studies of both shoot elongation and wood formation in conjunction with the regulation of GA biosynthesis.

Background

Shoot growth

In higher plants the shoot apical meristem (SAM) is the source of all aboveground organs, including leaves, stems and flowers. The SAM consists of undifferentiated, proliferating cells that have the capacity to differentiate into lateral organs or to keep dividing, thereby maintaining the stem cell pool (Medford, 1992). Although the leaf primordia and the tissues of the stem are initiated in the SAM, the expansion of leaves and internodes takes place in a distinct developmental phase, where cell division and (predominantly) cell elongation become evident (Steves and Sussex, 1989). The rate of cell divisions is specifically controlled by the cell cycle machinery, which limits the transitions between different checkpoints of the cycle (De Veylder et al., 2003). However, the greatest contribution to rapid elongation growth comes from cell expansion.

Cell expansion and the cell wall

Plant cell expansion *per se* is driven by internal turgor pressure, and is dependent on the flexibility of the cell wall – a mechanically strong structure enclosing the cell that is not present in animal cells (reviewed in Cosgrove, 1997). The increase in size is accomplished mainly by the uptake of water into the vacuole, and involves very little increase in the cytoplasm; hence it is a very cost-effective way to grow in terms of both energy and material. The primary cell wall consists of cellulose microfibrils (MF) that are synthesized by large complexes in the plasma membrane (Figure 1). Once secreted into the cell wall the unbranched MF associates with the polysaccharides hemicellulose and pectin, which are synthesized in the Golgi apparatus before being delivered to the wall by secretory vesicles. The hemicelluloses easily bind to and interconnect the cellulose MF, whereas pectins are generally acidic with a strong tendency to form ionic gels. Thus, hemicelluloses and pectins provide a hydrophilic matrix in which the crystalline cellulose becomes embedded. In dicotelydons, the primary cell wall consists of approximately 30% cellulose, 30% hemicellulose, 35 % pectin and 1-5% structural proteins, on a dry weight basis (Cosgrove, 1997). Plant cell walls typically expand faster at low pH in a process termed “acid growth”, which can be induced by auxin and is activated by the excretion of protons into the cell wall (Davies, 1995). Low pH in the apoplast can increase the activity of several cell wall associated enzymes. One such pH-dependent group of enzymes is the expansins, which may unlock the network of wall polysaccharides, thereby allowing turgor-driven cell enlargement (reviewed in Cosgrove, 2000). Because the extensibility of the cell wall limits plant growth it is

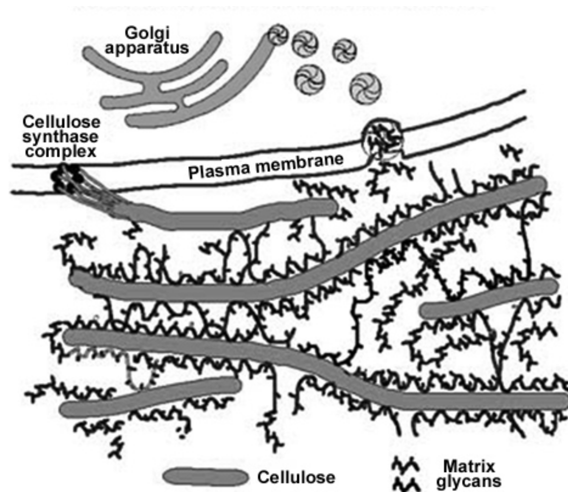

Fig. 1. A schematic model of cell-wall assembly. Large complexes in the plasma membrane synthesize cellulose microfibrils while hemicelluloses and pectins (matrix glycans) are synthesized in the Golgi apparatus and delivered to the cell wall by secretory vesicles. Reprinted by permission from Nature (Cosgrove) copyright (2000) MacMillan Publishers Ltd.

not surprising that the growth-promoting effect of many hormones ultimately affects a variety of different cell wall properties.

Internode formation and elongation

The plant shoot is made up of a repetitive structural unit, which consists of a leaf, a node, an internode and an axillary bud, often referred to as the phytomere. The number of phytomeres incorporated and the final length of the individual internodes therefore determine the total length of the stem. As the name implies, an internode is the part of a stem between two nodes (a node being defined as the joint in the stem where a leaf arises). During vegetative development, the SAM coordinately initiates the formation of leaves in an orderly manner referred to as phyllotaxis. Upon transition to flowering, a determinate floral meristem forms instead, from which the flower structures are initiated. A group of founder cells in the so-called peripheral zone at the flank of the vegetative meristem initiate leaf formation, and requirements for both auxin and auxin transport in this process have been established (Reinhardt et al., 2000; Reinhardt et al., 2003). Furthermore, local micro-induction of the cell wall-modifying enzyme expansin within the meristem is alone sufficient to initiate leaf development (Pien et al., 2001). GA has been shown to have a role in leaf formation by the observation that the expression of a gene encoding an enzyme catalyzing a key step in GA biosynthesis, GA 20-oxidase, is excluded from the apical meristem by the active repression of a knotted1-like homeobox (KNOX) protein that is preferentially accumulated in the indeterminate meristem cells (Sakamoto et al., 2001a). Hence, when *KNOX* genes are transgenically overexpressed they negatively affect the transcription of *GA 20-oxidase*, the GA levels decrease and aberrant leaf phenotypes are observed (Tanaka-Ueguchi et al., 1998; Kusuba et al., 1998; Rosin et al., 2003). These findings suggest that the role of KNOX in repressing the formation of GA is to maintain the indeterminate status of the SAM because GA is believed to be detrimental to meristem function, but required for normal leaf development. In fact, a complex interplay between KNOX and GA during leaf development has been

suggested to govern the generation of the dissected leaf morphologies observed in higher plants, for instance in tomato (Hay et al., 2002).

Following the induction of leaves by the SAM, which indirectly governs the formation of internodes, the stem tissue between two nodes may divide and expand in the subapical meristem and the subapical region to its final length (Steves and Sussex, 1989). Work from GA-deficient mutants has established that GA has a role in promoting shoot growth and stem elongation, as GA mutants are associated with severe dwarfism (reviewed in Ross, 1997). Conversely, WT plants treated with GA elongate rapidly, becoming more slender than normal. GA has also been shown to promote shoot elongation in gymnosperms such as Scots pine and white spruce (Little and MacDonald, 2003). In addition to GA, the plant growth substances auxin, brassinosteroids and ethylene have been suggested to affect stem elongation (Davies, 1995). The cell wall-associated expansins are clearly involved in leaf formation, as described earlier, but investigations of deep water rice have suggested that they may have functions in internodal growth, and that some are GA-inducible (Cho and Kende, 1997; Lee and Kende, 2001). Recent data imply that different expansins have different, specific roles during internode elongation in tomato (Vogler et al., 2003). The mRNA encoding one GA-induced expansin was detected specifically in the submeristematic zone where cell divisions cease, while mRNA for another was found in young stem tissues where cell elongation is predominant. These findings imply that the role of GA in regulating shoot growth, at least through the activity of expansins, may be restricted to the actively elongating subapical region and not to the meristem. Studies in deepwater rice have shown that GA-induced internodal growth is due to increased cell division and cell elongation (Sauter and Kende, 1992; Daykin et al., 1997). However, GA's effect on cell division may be indirect, as increased cell elongation has been detected before the onset of increased cell division (Sauter and Kende, 1992). These findings suggest that GA first promotes cell elongation, and that cell division could be stimulated as a result of cell growth. Finally, it is not fully understood how the rate of leaf formation is coordinated with the extent of internode elongation, but together they determine the number and length of internodes.

Wood formation

An economically important aspect of shoot growth is the circumferential growth of the stem, since the fifth most important commodity in world trade is wood (Plomion et al., 2001). The formation of wood is initiated in the SAM where a procambium is initiated at an early stage in the plant's development. In species undergoing wood formation the procambium then progressively develops into a lateral meristem called the vascular cambium, which is ultimately responsible for the formation of secondary xylem - wood (Larson, 1994). The vascular cambium is generally believed to be composed of initials with stem cell characteristics, and the relatively more differentiated phloem and xylem mother cells originating from and flanking each side of the initials (Fig. 2, reviewed in Mellerowicz et al., 2001). The xylem transports water along with mineral nutrients and provides mechanical support for the plant, while the main function of phloem is transport of sugars and nutrients (Esau, 1960). Xylem mother cells divide more than phloem mother cells, explaining the considerable disproportion in volume between the phloem and xylem

tissues (Fig. 2). There are two types of initials. Radial initials give rise to rays, which are essential for the translocation of nutrients between phloem and xylem and the storage of nutrients. In contrast, fusiform initials produce (in angiosperms) either xylem cells (fibers for support and vessels for water conductivity) or phloem cells (sieve tubes for transport along with companion cells, fibers and parenchyma) (Larson, 1994). The fusiform initials may divide anticlinally thereby enabling a harmonious increase in width of the cambium in the growing stem.

The formation of wood by xylem differentiation (xylogenesis) is an ordered process with distinct developmental stages (reviewed in Mellerowicz et al. 2001; Plomion et al. 2001). Following cell divisions in the cambial region, the newly produced xylem daughter cells embark on a process that will end in programmed cell death: only the ray cells will remain alive. Initially the cells expand and elongate longitudinally and radially to their final size. Once the expansion of the primary cell wall is complete, deposition of a thick secondary cell wall is initiated. The secondary cell wall is composed of three layers (S1, S2 and S3) that differ in the ordered orientation of their cellulose microfibrils (MF). The non-random orientation

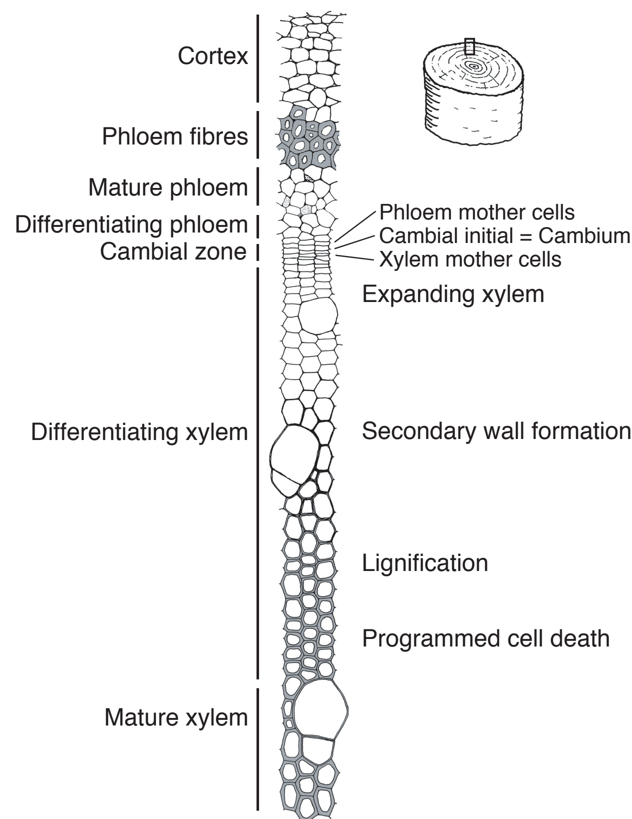

Fig. 2. Terminology of wood-forming tissues in *Populus* (Schrader, 2003). (Figure reproduced with the kind permisson of J. Schrader).

of MF in the S-layers helps terminate the extensibility of the cell wall, which is further cemented by the deposition of lignin. Once the lignification is complete, first the vessels and later the fibers undergo programmed cell death. During this process the vacuoles collapse with the release of hydrolases that degrade the cellular content while leaving the cell wall intact (Jones, 2001).

Lignin formation (from the Latin *lignum*: wood)

Functionally, lignin in plants is associated with mechanical support, defense mechanisms and sap conductance through the lignified vascular elements (Larson, 1994). However, the lignin in wood is an undesirable component for the pulp and paper industry, as it must be removed from the cellulose by expensive and polluting chemical processes. Furthermore, any remaining lignin in paper reduces the paper quality. But how is lignin formed?

Lignins are complex phenolic polymers that are formed by the dehydrogenative polymerization of three different hydroxycinnamyl alcohols (reviewed in Boudet et al. 1995; Boudet 2000). These hydoxycinnamyl alcohols (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) are often referred to as monolignols and differ only in their methylation degree. Upon cross-linking into lignin they give rise to the hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of the lignin polymer, respectively. In angiosperm trees the polymerization of S and G monolignols gives rise to an S/G-ratio of ≈2-2.5 and alterations in this ratio strongly affect the properties of the wood. For instance, every unit increase in the lignin S/G-ratio doubles the amount of lignin that must be removed by the pulp production industry (Chang and Sarkanen, 1973). Because of the strong interest in understanding and altering lignin content and composition, transgenic approaches have been employed to modify different steps of the monolignol pathway (thoroughly reviewed in Anterola and Lewis, 2002). Recently, by the simultaneous modification of two monolignol biosynthesis genes, Vincent Chiang's group produced transgenic aspen trees with 50% less lignin, a 60% higher S/G-ratio and 30% more cellulose than WT (Li et al., 2003).

Regulation of wood formation

Xylogenesis is, at least partly, controlled by the levels of and responsiveness to different plant growth regulators. Considerable amounts of data support the hypothesis that the hormone auxin (indole-3-acetic acid, IAA) plays a key role in this process (reviewed in Sundberg et al., 2000). Convincingly, auxin alone can induce differentiation of vascular strands in callus and explants (Jacobs, 1952; Mattsson et al., 1999). Moreover, IAA is present in a steep concentration gradient across the cambial region, peaking in the cambium and its most recent derivatives, as demonstrated in both Scots pine and hybrid aspen (Uggla et al., 1996; Tuominen et al., 1997). Collectively, these results imply a role for auxin as a positional signal in patterning cambial development.

The application of IAA and/or GA has previously demonstrated a synergistic effect on xylem development and a role for GA in stimulating meristematic activity and xylem fiber elongation (Wareing, 1958; Digby and Wareing, 1966). High ratios of IAA/GA led to more xylem differentiation, whereas low ratios induced phloem production, thereby suggesting a role for GA during phloem development (Digby

and Wareing, 1966). However, a GA-induced increase in both the number and length of xylem fibers in transgenic GA-overproducing trees suggests that GA is mainly active during xylem fiber development (Eriksson et al., 2000).

Gibberellins

GAs are a large family of tetracyclic diterpenoids, some of which are involved in the control of various stages in the life cycle of a plant, including seed germination, stem elongation, root growth, leaf expansion, trichome development, flowering and fruit development (Davies, 1995; Hooley, 1994; Ross, 1997). To date, an astounding 126 different GAs have been identified in bacteria, fungi and vascular plants (MacMillan, 2001). However, only a few of these compounds, including GA_4 and GA_1, have been shown to elicit biological responses in plants (Hedden and Phillips, 2000). Mutants with impaired GA biosynthesis ability are generally dwarfed, due to shortened internodes, in addition to having small leaves, delayed flowering and varying degrees of male sterility, but a supplement of bioactive GA can restore their growth (Ross, 1997). The availability of many mutants affected either in the biosynthesis of, or the response to, GA has helped researchers to build an almost complete picture of GA biosynthesis and an emerging understanding of GA signaling processes.

GA Biosynthesis

The formation of GAs, together with numerous other plant compounds, such as steroids, chlorophylls, carotenoids and the plant hormone abscisic acid, stems from the complex isoprenoid pathway (McGarvey and Croteau, 1995). The starting blocks for these widely different pathways are obtained by variations in the repetitive joining of the five-carbon (C_5) precursor isopentenyl diphosphate (IPP). The precursor for GA formation is the C_{20} unit geranylgeranyl diphosphate (GGPP). GGPP can be produced via a mevalonate-dependent pathway in the cytosol or a mevalonate-independent pathway in the plastids (Fig. 3), but the latter is the main contributor to the GGPP required for diterpenoid biosynthesis (Schwender, 1996; 1997; reviewed in Sponsel, 2002). Accordingly, the mevalonate-independent pathway was recently demonstrated to be the predominant supplier of GGPP destined for GA formation in *Arabidopsis* seedlings (Kasahara et al., 2002).

The biosynthesis of GAs can be divided into three main stages: (i) the formation of *ent*-kaurene in plastids, (ii) conversion of *ent*-kaurene to GA_{12} by membrane-bound cytochrome P450 monooxygenases and (iii) formation and deactivation of bioactive C_{19}-GA in the cytoplasm (Figs. 3 and 4; reviewed in Hedden and Phillips, 2000; Yamaguchi and Kamiya 2000; Olszewski et al., 2002). So what enzymes are responsible for catalyzing each step and where in the plant are they localized? Unless otherwise stated, the abovementioned reviews form the basis for the following section on GA biosynthesis.

1. The conversion of GGPP to *ent*-kaurene is catalyzed by the enzymes *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). In *Arabidopsis*, high activity of the *AtCPS* promoter has been detected in rapidly growing tissues, including shoot apices, root tips, developing anthers and seeds (Silverstone et al.,

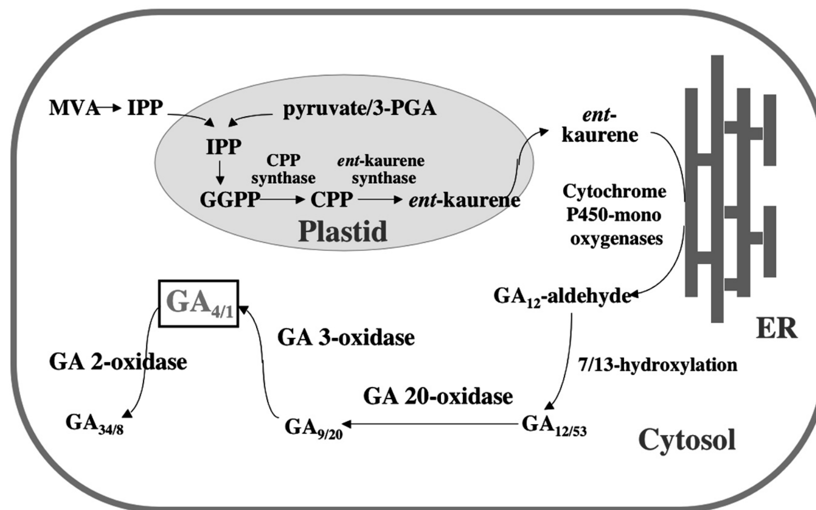

Fig. 3. Schematic representation of the cellular localization of different steps in the gibberellin biosynthesis pathway.

1997). Vascular tissue of some non-growing organs like expanded leaves also exhibit *AtCPS* promoter activity, suggesting that CPS present in leaves may initiate GA biosynthesis destined for transport to other organs. In contrast to the tissue- and developmental stage- specific expression pattern of *CPS*, *KS* transcript has been found in all organs analyzed (Smith et al., 1998; Yamaguchi et al., 1998), and at least in *Arabidopsis* is present at much higher levels than that of *CPS* (Yamaguchi et al., 1998). Thus, CPS may be the main regulatory enzyme in the formation of *ent*-kaurene.

2. The oxidation steps from the non-polar *ent*-kaurene to the soluble GA_{12} are catalyzed by the generally membrane-bound *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO) (reviewed in Helliwell et al., 2002). Interestingly, in tobacco leaves, the N-terminal transit peptides of CPS and KS direct green fluorescent protein (GFP) to chloroplasts (Helliwell et al., 2001a). Moreover, similar fusions using N-terminal regions of KO and KAO show that KO is targeted to the outer envelope of the chloroplast, while KAO is targeted to the endoplasmic reticulum (ER). These findings suggest that KO may link the two spatially separated parts of the GA biosynthesis pathway, but the mechanism whereby the non-soluble *ent*-kaurene is transported to the ER-bound enzymes remains to be elucidated. At a tissue specificity level, at least the two KAO present in *Arabidopsis* are expressed in all tissues thus far examined (Helliwell et al., 2001b).

3. Once GA_{12} has been formed it can be 13-hydroxylated into GA_{53}, marking the starting points of two parallel pathways in the last stage of GA formation: the early non-hydroxylation pathway and the early 13-hydroxylation pathway (Fig 4). The relative predominance of the two pathways is species-dependent, for instance in pea, rice and lettuce more GA_1 is formed, whereas higher levels of GA_4 are formed in *Arabidopsis* and cucumber (fig 1 in Kamiya and Garcia-Martinez, 1999). The

penultimate step in both pathways is carried out by the multifunctional enzyme GA 20-oxidase (GA20ox) and involves a stepwise oxidation of the C-20 in GA_{12} and GA_{53}, giving rise to the C_{19}-compounds GA_9 and GA_{20}, respectively. All bioactive GAs are C_{19}-metabolites, but to obtain bioactivity GA 3-oxidase (GA3ox) must convert the inactive GA_9 and GA_{20} into GA_4 and GA_1, respectively. The level of bioactive GA is thus governed by the rate of biosynthesis, but the rate of turnover or deactivation catalyzed by the enzyme GA 2-oxidase (GA2ox) also plays a role. In this process, GA_4 and GA_1 are catabolized into the inactive forms GA_{34} and GA_8, respectively. In addition, the products of the enzyme GA20ox may be diverted away from the route towards bioactive GA by 2-hydroxylative degradation. A novel class of GA2ox has recently been identified in *Arabidopsis* that possesses the ability to 2-hydroxylate C_{20}-GAs and not C_{19}-GAs, thereby further diversifying GA catabolism (Schomburg et al., 2003). Moreover, the GA levels may be affected by conjugation, as both bioactive GAs and precursors can exist in conjugated, inactive forms (Moritz and Monteiro, 1994; reviewed in Schneider and Schliemann, 1994). GA20ox, GA3ox and GA2ox are soluble dioxygenases that use 2-oxoglutarate as a co-substrate and are dependent on Fe^{2+} and ascorbate for high activity. Unlike CPS, KS and KO (which are encoded by single-copy genes in most plant species examined), the GA dioxygenases belong to small multigene families: in *Arabidopsis* potentially between four and eight genes each (Hedden et al., 2001; Schomburg

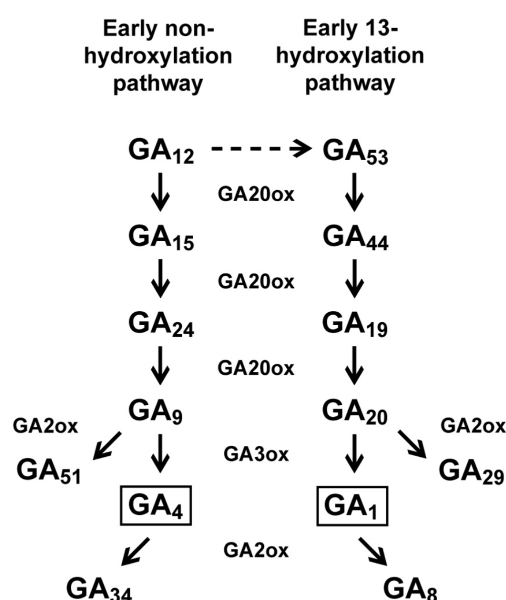

Fig. 4. Two parallel pathways at the last stage of gibberellin biosynthesis, the early non-hydroxylation pathway and early 13-hydroxylation pathway. The activity of three dioxygenase enzymes is shown, bioactive GAs are depicted in boxes.

et al., 2003). No general pattern of expression can be discerned for the different dioxygenase genes in any of these families, instead their expression patterns may differ both spatially and temporally.

Aspects of the regulation of GA biosynthesis

The amount of GA present in any tissue is controlled by its rate of biosynthesis and deactivation, but this in turn is governed by factors like transport, tissue type, developmental stage and environmental cues. Below, I summarize key findings related to GA regulation.

Transport or biosynthesis at the site of action?

Bioactive GAs are mainly present in actively growing and elongating tissues, such as shoot apices, young leaves and flowers, prompting the suggestion that they are synthesized at their site of action. Accordingly, during the vegetative and floral stages in rice, the expression of several GA biosynthesis and signaling genes has been found to overlap, although the patterns during seed germination are different (Kaneko et al., 2003). Seed germination is an excellent system in which to explore the GA formation and response systems, but the cited authors emphasize the unusual feature that the seed tissue where the GA signal is perceived lacks the capacity to synthesize GA. Therefore, general assumptions on the lack of a spatial correlation between GA biosynthesis and signaling are best avoided. Studies of different GA biosynthesis transcripts during seed germination have suggested subcellular compartmentation of the expression of different GA genes in pumpkin and *Arabidopsis* (Frisse et al., 2003; Yamaguchi et al., 2001). This implies that the synthesis of GA, at least in germinating seeds, requires intercellular transport of intermediate compound(s). Other evidence for GA transport, albeit over long distances, has been provided by the presence of some GAs in xylem and phloem exudates (reviewed in Hoad, 1995; Lang, 1970). In line with these findings, grafting experiments in pea between WT rootstocks and GA mutant scions have established that the precursor GA_{20} is predominantly transported rather than the bioactive GA_1 (Proebsting et al., 1992). More recent studies in pea shoots have demonstrated that mature tissues possess a capacity for *de novo* GA_{19} production in addition to the ability to convert GA_{19} to GA_{20} and GA_{20} to GA_1 (Ross et al., 2003). The low actual levels of GA_1 in mature tissues were explained in the cited study by an increased rate of deactivation catalyzed by abundant amounts of GA2ox. However, it must be emphasized that in plants the main bulk of GA required for shoot growth probably generally originates from the young expanding tissues themselves, although compartmentation of the biosynthesis within these tissues could occur.

Hormonal regulation

The endogenous levels of GA have been shown to affect the transcript levels of the GA dioxygenase genes, but not the expression of genes acting earlier in the pathway (reviewed in Hedden and Phillips, 2000). Plants with reduced bioactive GA contents, due either to mutation in the GA biosynthesis enzymes or treatment with chemical inhibitors, display elevated levels of *GA20ox* and *GA3ox* transcripts, while the addition of GA may down-regulate the levels of these transcripts (Phillips

et al., 1995; Carrera et al., 1999; Xu et al., 1995; Martin et al., 1996; Xu et al., 1999; Ross et al., 1999; Sakamoto et al., 2003; Vidal et al., 2003; Thomas et al., 1999; Yamaguchi et al., 1998; Cowling et al., 1998; Eriksson and Moritz, 2002). Mutants with impaired GA responses also have altered expression of these genes (reviewed in Bethke and Jones, 1998; Hedden and Phillips, 2000). There are exceptions to the rule of dioxygenase feed-back regulation, as only one of the two *GA3ox* genes expressed during *Arabidopsis* germination is subject to feed-back regulation (Yamaguchi et al., 1998). Taken together, however, the results provide strong evidence that lowered levels of bioactive GA trigger an up-regulation of genes in the GA biosynthesis pathway, whereas highly increased levels initiate a negative feed-back response. Less is known about possible effects that altered GA levels may have on the expression of the deactivating enzyme GA2ox. However, results from *Arabidopsis*, pea and rice suggest a feed-forward mechanism is induced in the presence of high GA levels, and down-regulation occurs when GA levels are lowered or not properly perceived (Thomas et al., 1999; Elliot et al., 2001; Sakamoto et al., 2003). The action of endogenous GA levels on both biosynthetic and catabolic genes enables the plant to maintain its concentration of bioactive GA within appropriate limits.

The plant growth regulator auxin has been implicated in regulating the expression of *GA20ox* and *GA3ox* in a species-dependent manner, thus controlling the rate of GA formation (reviewed in Ross et al., 2001). The removal of the auxin-producing apical bud has been shown to negatively affect the expression of *GA3ox* in decapitated pea and barley plants, while *GA20ox* becomes down-regulated following decapitation in tobacco (Ross et al., 2000; Wolbang and Ross, 2001; Wolbang et al., 2004). Expression of the GA biosynthetic genes was restored by IAA application in these studies and consequently the GA levels recovered, at least partially, towards those of intact plants. Auxin may be able to exert differential control over *GA20ox* in tobacco and *GA3ox* in pea because these steps could be rate limiting in the formation of GA in the respective species (Ross et al., 2001). The mechanism whereby auxin restores GA biosynthesis in decapitated pea shoots is rapid (taking 2-4 hrs) and, at least for *PsGA3ox1*, requires *de novo* protein synthesis (O'Neill et al., 2002).

Light regulation

As well as providing energy and essential environmental cues, light is also known to regulate the formation of GA in a number of developmental processes (reviewed in Kamiya and Garcia-Martinez, 1999; Garcia-Martinez and Gil, 2001; Olszewski et al., 2002). Red light, for instance, promotes seed germination in a number of plant species by increasing GA biosynthesis (reviewed in Yamaguchi and Kamiya, 2002). The expression of *AtGA3ox1* and *AtGA3ox2* in soaked *Arabidopsis* seeds was induced within 1 hr after a brief red light treatment (Yamaguchi et al., 1998), and similar results have been obtained for *Ls3h1* in germinating lettuce seeds (Toyomasu et al., 1998). Light-induced changes in expression in *Arabidopsis* were found to be reversible by a far-red pulse, indicating that the genes are controlled via the red and far-red sensing phytochromes. Interestingly, only the *AtGA3ox1* gene was subject to feed-back inhibition by the GA levels produced *de novo*. The cited authors suggested that by alleviating the germinating-seed-specific *AtGA3ox2* from down-regulation, high levels of GA could be maintained, thus allowing germination

to be completed.

Tuberisation in potato is a photoperiod-dependent process that is promoted (or strictly required, at least in some potato species) by short day (SD) conditions, and inhibited by GA (reviewed in Martinez-Garcia et al., 2002). Consequently, over-expression of *StGA20ox1* in transgenic potato led to increased growth with taller stems and other typical GA-associated traits, in addition to delayed tuberisation (Carrera et al., 2000). Conversely, the inhibition of *GA20ox* in transgenic potato plants resulted in shorter plants with premature tuberisation. Another level of photoperiodic control of GA formation is observed during growth cessation in woody perennial trees that alternate between active shoot growth and vegetative dormancy. In *Salix pendantra*, SD are required for growth cessation and cold hardening (Junttila and Kaurin, 1990). Furthermore, SD conditions have been shown to induce rapid reductions in GA levels in the subapical region (Olsen et al., 1995). The reduction of GA levels is correlated with a lower mitotic index in these tissues, which can be reversed and even increased by the application of GA (Hansen et al., 1999). Hence, it has been suggested that the lowered GA levels are causally associated with reductions in mitotic activity, causing the apical bud to stop growing. In hybrid aspen, SD-promoted reductions in bioactive GA were paralleled by decreased *PttGA20ox1* transcript levels in a study by Eriksson and Moritz (2002).

Phase transition from vegetative to reproductive growth

Gibberellins are capable of inducing flowering in the facultative long-day (LD) plant *Arabidopsis* in a daylength-dependent fashion. Mutants with severely reduced GA levels, such as *ga1-3*, are unable to flower in SD (Wilson et al., 1992), whereas LD conditions only slightly delay the onset of flowering (Wilson et al. 1992; Silverstone et al., 1997). The induction of flowering by GA in *Arabidopsis* has been associated with activation of the promoter of the flower meristem gene *LEAFY (LFY)* (Blazquez et al., 1998). More recently, a *cis*-element shown to confer GA responsiveness in the *LFY* promoter (GOF9) was identified (Blazquez and Weigel, 2000). A GA-inducible MYB transcription factor, GAMYB33, can bind to GOF9, suggesting that this transcription factor may mediate the influence of the GA signal over *LFY* expression, and thus control flowering induction (Gocal et al., 2001). Further evidence for the regulation of GA and flowering comes from *in situ* hybridization analysis in rice, which has revealed a ring-shaped expression pattern of *OsGA2ox1* around the shoot apex (Sakamoto et al., 2001b). Following the transition from vegetative to reproductive growth, the expression of this GA-deactivating enzyme was greatly reduced, only to become absent in the floral meristem. The cited authors suggest GA2ox may have a role in preventing bioactive GA accumulating in the shoot apical meristem, and thus promoting precocious flowering.

The GA response pathway

No GA receptor has been cloned yet, but there is evidence to suggest that the GA signal is perceived at the plasma membrane, based on results from aleurone cells (Gilroy and Jones, 1994; Hooley et al., 1991). Studies in cereal aleurone have further shown that Ca^{2+}, calmodulin, cyclic GMP, heterotrimeric G proteins, GAMYB and

protein kinases may all be involved in early GA signaling events (reviewed in Bethke and Jones, 1998; Lovegrove and Hooley, 2000). During the later stages of the GA response pathway, evidence suggests that *PHOTOPERIOD RESPONSIVE 1*, *SLEEPY* and *PICKLE* may act as positive components, while *SPINDLY* may act in a negative manner (Amador et al., 2001; Steber et al., 1998; Ogas et al., 1999; Jacobsen et al., 1996). However, the most central players in GA signaling are the growth repressor proteins of the DELLA family (reviewed in Olszewski et al., 2002). The members of this family for which most progress towards understanding has been made are *RGA* ("*REPRESSOR OF ga1-3*") and *GAI* ("*GIBBERELLIN INSENSITIVE*").

The *gai-1* allele was initially detected in a GA-insensitive mutant with a GA-deficient phenotype (Korneef et al., 1985). RGA, however, was isolated as a repressor of GA signaling, as *rga* partially rescued the stem growth defect of the GA-deficient *ga1-3* mutant (Silverstone et al., 1997). The RGA/GAI family is classified as a subgroup of the GRAS protein superfamily, and is comprised of putative transcription factors (Pysh et al., 1999). What discriminates the RGA/GAI family from other GRAS proteins is the presence of a DELLA domain in the N-termini of the proteins (Peng et al., 1997; 1999; Silverstone et al., 1998). Mutations affecting genes coding for RGA/GAI have been identified in a variety of species; *Arabidopsis* (*rga* and *gai*), barley (*sln1*), maize (*d8*), rice (*slr1*) and wheat (*rht*) (Peng et al., 1997; Silverstone et al., 1998; Ogawa et al., 2000; Ikeda et al., 2001; Chandler et al., 2002; Gubler et al., 2002). The type of mutation involved can differ: in *Arabidopsis*, maize, wheat, and barley semi-dominant gain-of-function mutations cause dwarfism, while recessive loss-of-function mutations can cause increased growth, as observed in *Arabidopsis*, barley and rice. Based on the recessive loss-of-function mutants, the DELLA proteins are believed to act as repressors of GA-activated processes.

Many of the dwarfed, high-yielding varieties introduced during the "Green Revolution" during the 1960's and 1970's have now been ascribed to defects in the GA biosynthesis or signaling systems, gain-of-function RGA/GAI mutations (Peng et al., 1999; reviewed in Hedden, 2003). An impaired GA response may, thus, lead to valuable dwarfed plants with increased grain yield that are better capable of supporting heavy grain and withstanding wind. Interestingly, by transgenically expressing mutated *RGA* and *SLR1* carrying deletions in the DELLA domain, semi-dwarfism can be obtained in *Arabidopsis* and rice (Dill et al., 2001; Ikeda et al., 2001). Thus, the DELLA domain is believed to be essential for the repressor effect exerted by bioactive GA on RGA/GAI during GA signaling.

In *Arabidopsis*, GAI and RGA act as negative regulators of GA-mediated growth and have largely overlapping roles in the regulation of stem elongation (King et al., 2001). Apart from *GAI/RGA* there are three RGA-like (RGL) proteins: *RGL1, RGL2* and *RGL3* (Dill and Sun, 2001; Lee et al., 2002; Wen and Chiang, 2002). RGL2 has been suggested to repress seed germination (Lee et al., 2002), but other evidence suggests RGL1 plays a role in this process (Wen and Chang, 2002). Although the function of all five *Arabidopsis* DELLA proteins remains to be characterized, they may clearly differ functionally, with GAI/RGA being responsible for GA-mediated stem elongation and RGL1 and/or RGL2 involved in the regulation of

seed germination. Recently, it was shown that GA promotes *Arabidopsis* petal, stamen and anther development by opposing the function of RGA, RGL1 and RGL2 (Cheng et al., 2004).

Nuclear localization has been established for RGA, GAI and RGL2 in *Arabidopsis*, SLR1 in barley and SLN1 in rice by using DELLA fusion proteins carrying a GFP-tag (Silverstone et al., 2001; Fleck and Harberd, 2002; Wen and Chang, 2002; Gubler et al., 2002; Itoh et al., 2002). Treatment with GA induces a rapid (30 min-2 hr) disappearance of signal from the nucleus for RGA, SLR1 and SLN1, suggesting that GA negatively affects the DELLA proteins, thus alleviating growth repression (Silverstone et al., 2001; Wen and Chang, 2002; Gubler et al., 2002; Itoh et al., 2002). The GA-induced disappearance of RGA from nuclei in *Arabidopsis* roots is suggested to be dependent on shoot-derived auxin (Fu and Harberd, 2002). Interestingly, the role of auxin in controlling root growth could therefore be, at least partly, to modify the responsiveness of root cells to GA. The integrative role of DELLA proteins in hormone signaling is further corroborated by findings from the triple response of *Arabidopsis* seedlings, where gibberellin, auxin and ethylene were all found to modify DELLA function (Achard et al., 2003). Unlike RGA, the nuclear signal of GAI-GFP and RGL2-GFP fusion proteins is unaltered following GA applications (Fleck and Harberd, 2002). The cited authors suggest a role for GAI and RGL2 in providing a background level of growth repression that is not GA responsive. The existence of such non-responsive DELLA proteins could partly explain the differences in GA responsiveness between species.

Ubiquitin-mediated proteolysis is known to play an important role in hormone signaling in general (reviewed in Frugis and Chua, 2002), e.g. in the targeted destruction of AUX/IAA proteins in auxin signaling. A major type of E3 ubiquitin ligase complex involved in the ubiquitin-proteasome pathway is SCF, which is composed of four subunits: SKP1, Cullin, RBX1 and F-box protein (Patton et al., 1998). The F-box protein in the SCF complex confers target specificity. Recent results indicate that the route whereby GA stimulates DELLA protein destabilization involves phosphorylation, polyubiqutination via a specific SCF E3 ubiquitin ligase complex, and destruction in the 26S proteasome (Fu et al., 2002;

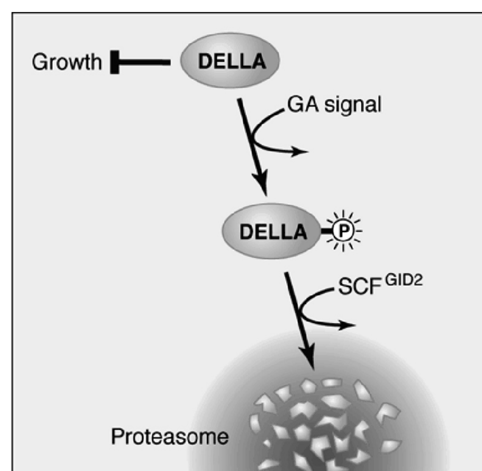

Fig. 5. A model for GA signaling in rice. The function of the DELLA proteins is to repress growth. In response to a GA signal, the DELLA proteins are first phosphorylated, then targeted for destruction in the proteasome by polyubiquitination. The addition of a polyubiquitin chain is catalyzed by the SCF^{GID2} E3 ligase. Degradation of the DELLA proteins releases the repression of plant growth. (Figure reproduced from Harberd, 2003 with kind permission from the publisher).

McGinnis et al., 2003; Sasaki et al., 2003). A key break-through came with the characterization of a putative F-box mutant in rice, *gid2*, which is believed to confer specificity of the SCF E3 ubiquitin ligase complex to the DELLA protein SLR1, following GA-signaling induced phosphorylation of SLR1 (Figure 5; Sasaki et al., 2003). In *Arabidopsis,* a similar role was ascribed to the putative F-box protein SLEEPY1, which presumably targets RGA for destruction (McGinnis et al., 2003). An inventory of the *Arabidopsis thaliana* genome revealed nearly 600 putative F-box protein genes, indicating that the involvement of proteasome degradation will be discovered in a multitude of plant processes, including hormone signaling (Kuroda et al., 2002).

Objectives

The broad aim of this project was to gain a better understanding of how GA biosynthesis is controlled and how GA regulates different aspects of shoot growth and development. In order to facilitate detailed studies of internode elongation and wood formation I employed the tree model system hybrid aspen (*Populus tremula x tremuloides*). Most importantly, I focused on the following questions:

- What is the effect of transgenically modified GA levels on shoot growth in trees?

- What is the relative importance of GA20ox and GA3ox in regulating GA homeostasis?

- What genes may contribute to an increased wood formation?

- Are GA biosynthesis and GA responsiveness specifically regulated in the wood-forming tissues of hybrid aspen?

Methodological overview

Hybrid aspen as a model tree system

When choosing a model system that is capable of wood formation the choice readily falls on the woody, perennial trees in the genus *Populus* (Klopfenstein et al., 1997). Species of *Populus* are fast growing, easy to propagate, have a relatively small genome of approximately 500 Mb (5x the size of *Arabidopsis*) and are transformable by *Agrobacterium*-mediated transformation. Because the regeneration time of *Populus* is several years, the transgenic plants we have worked with have been primary transformants. Consequently, one of the drawbacks of using *Populus* is that it is not possible to perform controlled crosses with interesting genotypes. The work presented in this thesis is based on a male hybrid between *Populus tremula* (L.) and *Populus tremuloides* (Michx.), also denoted hybrid aspen. Although secondary growth can be induced in *Arabidopsis thaliana* by prolonged

SD conditions (Chaffey et al., 2002), discovering the true nature of different aspects of xylogenesis requires a model tree system. Furthermore, due to the small size of *Arabidopsis* it is relatively difficult to sample different types of tissues. In contrast, in hybrid aspen, high-resolution tangential cryo-sectioning of the wood-forming tissues allows nearly cell-specific resolution and the scope to study gene expression and metabolite profiles in distinct developmental gradients (see, for instance Uggla et al., 1996; Hertzberg et al., 2001).

Different approaches to investigate gene expression

Pros and cons of studying gene expression

One way to analyze the biological relevance of a gene of interest is to determine the spatial and temporal distribution of its transcription. However, it must be emphasized that expression data only offer indirect evidence about the actual distribution of a translated protein, and even less direct evidence about its activity distribution. With the advent of the post-genomic era, microarray techniques have enabled large-scale analysis of transcript changes, while proteomics can offer large-scale investigations at the protein level. Researchers in the GA field have seized the opportunities offered by proteomics (see, for instance Gallardo et al., 2003; Tanaka et al., 2004) and microarray analysis (see, for instance Ogawa et al., 2003) to better understand GA-regulated responses. Importantly, metabolomics will soon offer perhaps the most direct link to processes that occur in plants (Fiehn, 2002). This prompts the question, why study gene expression? There are two good reasons. First, large-scale microarray technology still offers higher resolution data, with more accurately annotated results, than proteomics can (currently, at least). Secondly, gene-by-gene analysis through RT-PCR is a more cost-effective and possibly more specific and easier process than immuno-blot analysis. Although there are many technical advantages of expression analysis it must still be remembered that it does not necessarily reflect the true abundance and activity of the protein(s) observed.

GUS histochemical localization

Reporter genes are routinely used as tools in molecular biology to investigate the expression pattern of a given promoter, and the *uid* gene, referred to as GUS, is one of the most widely used systems (Jefferson, 1987). By linking the promoter of a gene to GUS and introducing it into a plant, the expression of the gene can be mapped by monitoring GUS activity, detected through its conversion of the substrate X-gluc, which yields a blue precipitate. The tissues where the reporter gene activity is detected then presumably reflect the expression pattern of the endogenous gene. This is not always the case, as regulatory *cis*-elements may or may not have been included in the promoter construct. Notably, *cis*-elements crucial to a correct expression pattern may be present in the intragenic regions, which are often ignored in the making of GUS constructs (see, for instance, Sieburth and Meyerowitz, 1997). In my work I used GUS to analyze the expression profile of *PttGA20ox1* in hybrid aspen and in the heterologous system *Arabidopsis thaliana*. However, GUS studies are generally only a preliminary way to analyze expression: to confirm the findings *in situ* hybridization or even Northern blot analysis or reverse transcriptase

polymerase chain reaction (RT-PCR) analyses must be performed.

Semi-quantitative and Real-Time RT-PCR

When dealing with very weakly expressed genes, such as most genes coding for enzymes in the GA biosynthesis pathway, Northern blot analysis may not be sensitive enough for detection. However, RT-PCR can offer the ability to detect transcripts present in scarce amounts. I have used a two-step RT-PCR approach to analyze GA dioxygenase transcripts, where isolated RNA is first reverse transcribed into cDNA and then used as template in gene-specific PCR amplifications. Semi-quantitative RT-PCR was performed in the studies reported in Paper II, with *18S* RNA amplified as an internal standard in the same reaction tube as the gene under investigation. In order to compensate for the higher abundance of rRNA, competitive primers of *18S* were included so that the amplification efficiencies of the control and gene of interest were roughly in the same linear range. In this approach, after the optimization steps for the semi-quantitative RT-PCR, the products are analyzed on agarose gels, or further processed and hybridized, so that the calculated ratio of intensity between the two gene products can give a relative expression value. Thus, the relative value of different samples can be compared, provided that the expression level of the control gene remains constant.

An even more sensitive approach for detection is Real-Time PCR, which enables the starting amount of nucleic acid to be quantitatively determined, or a relative expression ratio with much higher resolution than described above. Here, the amplification of the targeted gene product is monitored cycle by cycle and the increase in product level is measured in terms of the increase in incorporated fluorescent DNA dye. A Real-Time PCR machine can simultaneously detect increases in fluorescence in an entire 96 or 384 well plate, providing a very fast detection system for analyzing large numbers of samples. I used Real-Time PCR in the studies described in Paper IV when I investigated the expression of GA biosynthesis and GA response genes in distinct parts of the wood-forming tissues of hybrid aspen. Because the starting material was very restricted, approximately 1-2 mg per tissue type, it is highly unlikely that I could have monitored the expression of eight genes per sample using any other method without further amplification of the cDNA pool.

Microarray analysis

Microarray technology (originally developed by Schena et al., 1995) has rapidly emerged as the method of choice for analyzing differences in transcript levels of a large number of genes between two samples. The key components are the microarray slides onto which nucleic acids representing thousands of genes are first immobilized. RNA is subsequently isolated from the samples under investigation, reverse transcribed, labeled with separate fluorescent dyes (routinely, the cyanine dyes Cy3 and Cy5) and hybridized to the DNA on the microarray. The differentially labeled fragments in the hybridization will then form duplexes with any complementary DNA on the microarray. By measuring the strength of the fluorescent signals associated with each spot, the relative abundance of transcript in the two samples can then be determined. A laser scanner coupled to a confocal microscope offers the required high-resolution detection of the hybridization signals.

The data analysis that follows is a time-consuming process involving image analysis and the manual flagging of bad spots in addition to appropriate normalization.

In my work, microarray analysis was employed to detect genes that were differentially expressed in the wood-forming tissues of WT and a fast-growing GA-overproducing transgenic line, see Paper III. This microarray analysis was performed on the first generation spotted cDNA microarray slides, containing 2995 EST clones obtained from cambial zone cDNA libraries (Sterky et al., 1998).

GA Analysis

Crucial elements of any investigation of plant growth substances are, of course, accurate analyses that can specifically detect and distinguish between similar compounds. In the case of GA, which is present in very scarce amounts, as well as in several structurally related forms, a sensitive and specific method of detection is required. In our laboratory, detection of GA in plant samples usually involves purification by HPLC coupled to GC/MS for final analysis. The extensive purification by HPLC separates the many GA metabolites as well as other structurally related compounds. Finally, accurate and precise results are obtained by GC/MS analysis. In Paper IV, GA analysis was performed with approximately just 1 mg of starting plant material. Due to the very low amounts of plant material and the type of tissues that were analyzed, the samples were not purified by HPLC after the solid phase extraction column purification. However, due to the specific GC/MS analysis that was performed, the distribution of various GA metabolites in developmentally distinct samples in the cambial region of aspen trees could be determined.

Results and discussion

The aim of my work was to elucidate the regulation of GA biosynthesis genes and to improve our understanding of the role of GA during different stages of shoot growth. In the first part of this section of the thesis I discuss aspects of the characterization of *PttGA20ox1* and *PttGA3ox1* and the effects on shoot growth and GA homeostasis when two orthologous *Arabidopsis* genes were individually over-expressed in hybrid aspen. In the second part I analyze differentially expressed genes in the wood-forming tissues of fast-growing GA-over-producing trees. Finally, I discuss the regulation of GA formation and responsiveness during distinct phases of wood formation.

GUS histochemical analysis of *PttGA20ox1* promoter activity (-)

The spatial and temporal transcript distribution of *PttGA20ox1* was investigated by promoter-GUS histochemical analysis. Prior to my work a cDNA clone encoding PttGA20ox1 was isolated, and the enzymatic activity of its product was subsequently confirmed (Eriksson and Moritz, 2002). Based on information from the cDNA clone a genomic *PttGA20ox1* clone was identified, in a genomic hybrid aspen library screen, which was further subcloned and sequenced (data not

shown). The obtained clone contained a 1.6 kb promoter sequence upstream of the start ATG codon, a coding region encompassing two introns and a 0.65 kb 3'-untranslated region (UTR). Four different promoter-GUS constructs were produced from the genomic clone: two translational (pA and pB) and two transcriptional (pC and pD) fusions, see Fig. 6. The different constructs were made so that positional information concerning *cis*-elements could be obtained by comparing differences in promoter activity. Importantly, translational fusions were made to include regulatory intragenic *cis*-elements, which are often essential for correct expression patterns, as shown for the GA biosynthetic genes *CPS* (Chang and Sun, 2002), *GA3ox* (Itoh et al., 1999) and *GA20ox* according to unpublished results in tobacco (referred to in Sakamoto et al., 2001). The constructs were introduced into hybrid aspen by *Agrobacterium*-mediated transformation and individual transgenic lines were obtained for each construct (data not shown). Hybrid aspen has a regeneration time of several years. So, since we were interested in *GA20ox* expression during flowering we used *Arabidopsis thaliana*, ecotype *Landsberg erecta*, as a heterologous system for our GUS studies. Although the activity of a given promoter might differ, both temporally and spatially, when under the control of *trans*-acting factors in another species, *Arabidopsis* has often been used as a heterologous host (Avila et al., 2001; Baumlein et al., 1991, Hofig et al., 2003).

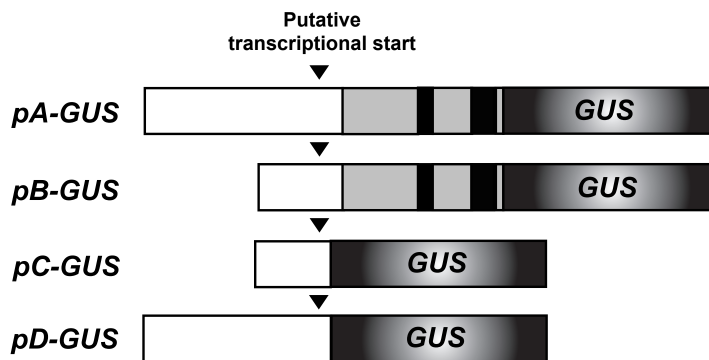

Fig 6. Different promoter-GUS constructs based on a genomic clone of *PttGA20ox1*. The constructs named *pA* and *pB* are translational fusions, while *pC* and *pD* are transcriptional fusions. White coloring marks the promoter region, grey exons and black introns.

Translational versus transcriptional fusions

GUS histochemical analysis in transgenic lines of both hybrid aspen and *Arabidopsis* generally showed the activities of the translational fusions pA and pB to be much weaker than those of pC and pD (data not shown). In the case of the pB construct, GUS activity was never detected visually. However, a quantitative MUG assay of *Arabidopsis* pB-GUS seedlings did reveal weak, but detectable, activity levels (data not shown). The low activity of the translational fusions can be explained in at least two ways. First, negatively acting *cis*-elements may be present in the intragenic regions that would be included in the pA and pB constructs, but lacking

in pC and pD. Secondly, the structure of the chimeric PttGA20ox-GUS enzyme might interfere with the GUS activity, thus explaining the low activity levels. By measuring the levels of the different GUS transcripts it would be possible to disprove or confirm the latter hypothesis. The pD construct conferred stronger GUS activity than pC, which is consistent with the stronger activity of pA over pB. This could be explained by the presence of negatively acting *cis*-elements in the 0.7 kb 5' UTR, or alternatively by the inclusion of expression-enhancing elements in the 0.7-1.6 kb 5' UTR.

Young vegetative shoots

PttGA20ox1 is expressed in young leaves, according to the patterns of pA, pC and pD GUS activity observed in hybrid aspen and *Arabidopsis* (Fig. 7 A-C; data not shown). Notably, the overall activity of pA-GUS is very low, and the only visible histochemical localization occurs in leaf tissue. Previous results from hybrid aspen confirm the presence of *PttGA20ox1* transcript in young leaves and show weaker expression in young expanding internodes (Eriksson and Moritz, 2002). The internode expression data correlate well with the pith- (data not shown) and cortex-specific GUS activity of pC and pD (Fig. 7A). The largest constraint on internode elongation has been suggested to reside in the epidermal cell wall layers (see, for instance, Kutschera et al, 1987). Interestingly, immunolocalization studies have revealed a cortex-specific distribution for a protein with a proposed role in elongation growth, yieldin (Okamoto-Nakazato et al., 2001). The cited authors suggest that yieldin may be involved in regulation of the turgidity of cortical cells, which forces the epidermal cell wall layers to expand. It is interesting to speculate that GA20ox could enable the formation of GA in the cortex, which in turn could promote cortical cell elongation and thus influence the extension of the thick epidermal cell wall layers. Similarly, promoter-GUS studies of a *GA3ox* in tobacco have revealed GUS activity in the cortex and pith, but not in the epidermis or vascular tissues (Itoh et al., 1999).

GUS-activity of the transcriptional fusions pC and pD was observed in the meristem of hybrid aspen and *Arabidopsis* (Fig. 7A, C). In tobacco, there is an active repression of *GA20ox* in the SAM by a KNOX homeodomain protein preferentially expressed in indeterminate cells (Tanaka-Ueguchi et al.; 1998; Sakamoto et al., 2001). The repression is mediated via two eight bp *cis*-elements, one in the 5' UTR and one in the first intron of *Ntc12* (Sakamato et al., 2001), both of which are present at equivalent locations in *PttGA20ox1* (data not shown). GUS-activity of pC and pD in the meristem could therefore be explained by the fact that these promoter constructs did not encompass the first intron, and therefore the second *cis*-element required for repression was missing.

Wood-forming tissues

In stems with secondary growth the activity of pC was detected in expanding xylem (Fig. 7D), unlike the situation in pD transgenic lines where activity was mainly specific to cortex and phloem fibers (data not shown). According to Real-Time PCR data of WT hybrid aspen, the expression of *PttGA20ox1* is measurable in xylem-enriched tissues, but not detectable under the same reaction conditions in phloem scrapings (data not shown). This pattern is also supported by Real-Time PCR

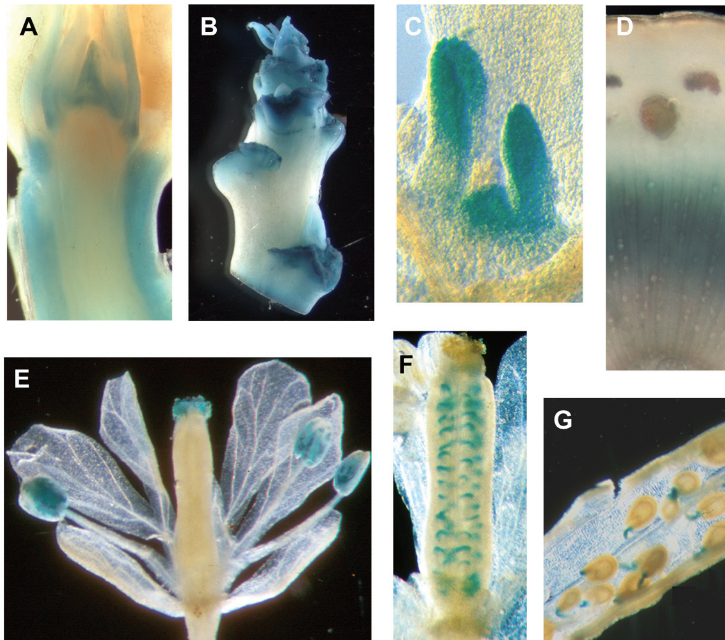

Fig. 7. Histochemical localization of *PttGA20ox1* promoter-GUS activity. A-B + D depicts GUS activity in *Populus tremula x tremuloides* and C + E-G represents GUS activity patterns detected in *Arabidopsis thaliana*. All images show transgenic lines carrying the *pD-GUS* construct except D, which shows a line carrying *pC-GUS*.

analysis of distinct zones of the developing xylem, since the highest expression is observed in expanding xylem cells (Paper IV, Fig. 3). The significance of enriched *PttGA20ox1* transcript levels in developing xylem is further discussed in the "GA formation and responsiveness during wood formation" section below. However, the activity of pD in the phloem and pC in the xylem during wood formation suggests that *cis*-elements involved in conferring tissue specificity are differently represented in the two promoter constructs. During development in the young expanding shoot, however, no differences in tissue specificity were observed between pC and pD (data not shown).

Flower and seed development

In the heterologous host *Arabidopsis,* pD GUS activity was detected in pollen and in funiculi (Fig 7E-G). The presence of GUS-stained pollen was also observed on the stigmatic surface of the carpel and occasionally in elongating pollen tubes (data not shown). A role for GA during stamen development and pollen formation is well established as GA-deficient mutants in *Arabidopsis* and tomato are male sterile (Nester and Zeevaart, 1998; Goto and Pharis, 1999). A more recent study claims that GA is required not only for seed development, as previously established, but also during pollen tube growth (Singh et al., 2002), which is in agreement with our data.

Feed-back regulation

To assess the regulation of the promoter constructs, we treated transgenic *Arabidopsis* GUS seedlings with GA_4 and the GA biosynthesis inhibitor paclobutrazol (pac) to induce the well-characterized feed-back regulation of *GA20ox* (reviewed in Hedden and Phillips, 2001). Although *PttGA20ox1* has also been shown to be feed-back regulated by bioactive GA in previous studies (Eriksson and Moritz, 2002), we were unable to detect any significant differences in quantitative MUG assays (data not shown). Similarly, the pac treatments did not cause any detectable differences in activity. In contrast, *Arabidopsis* reporter gene studies of *AtGA20ox1* using luciferase (luc) revealed a 50% reduction following GA_3 treatment, and a two-fold induction following GA biosynthetic inhibitor treatment as measured by luc activity (Meier et al., 2001). The reasons for the lack of similar results in our GUS lines remain to be resolved.

Characterization of a *Populus* GA 3-oxidase (II)

Cloning and functional characterization

The role of GA3ox in catalyzing the conversion of inactive GA species into GAs with biological activity is well established and the gene encoding it has been cloned in a variety of species (Chiang et al., 1995; Lester et al., 1997; Williams et al., 1998; Martin et al., 1997; Itoh et al., 1999; Rebers et al., 1999; Lee and Zeevart, 2002; Kang et al., 2002). Paper II describes the isolation of a full-length *GA3ox* in hybrid aspen named *PttGA3ox1*, which contained an open reading frame (ORF) of 1,122 bp encoding a putative protein of 374 amino acids, Genbank accession number AY433958.

To test whether *PttGA3ox1* encoded a functional GA3ox, the coding region was sub-cloned in an expression vector to produce an in-frame fusion protein that was expressed simultaneously with an empty vector construct serving as control. Lysates of bacteria in which recombinant GA3ox protein production had been induced, and controls, were used for functional assays. Initially, ^{14}C-labeled GA_{20} and GA_9 were mixed with the recombinant PttGA3ox1 protein and found to be converted to GA_1 and GA_4 respectively, while no conversion took place in the controls (data not shown). Once activity had been confirmed, a competitive assay using unlabeled substrates at various concentrations was performed. Interestingly, the recombinant PttGA3ox1 protein converted GA_9 to GA_4 more efficiently than GA_{20} to GA_1, suggesting that it may make a more important contribution to the early non-hydroxylated pathway than the early 13-hydroxylation pathway (Fig. 8).

According to BLAST searches in the *P. trichocarpa* genome-sequencing program (http://genome.jgi-psf.org/poplar0/poplar0.home.html) there are four *GA3ox* genes in *Populus* (data not shown). Similarly, in *Arabidopsis* GA3ox is encoded by four genes, and the function of all four gene products has been confirmed by heterologous expression in *E. coli* (Williams et al., 1998; Yamaguchi et al., 1998; Hedden et al., 2001).

Regulation of PttGA3ox1 expression

GA-induced feed-back regulation of *GA3ox* transcription has been demonstrated

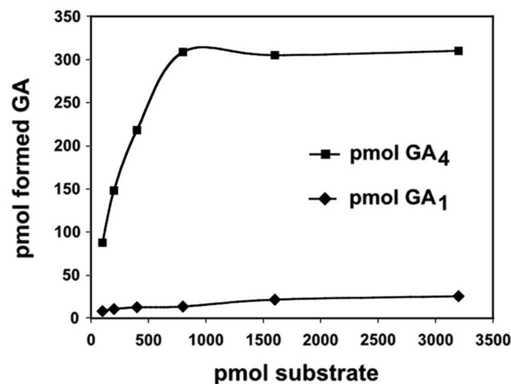

Fig. 8. Competitive assay for GA 3β-hydroxylation with cell lysates from recombinant *E. coli* expressing *PttGA3ox1*. Cell lysates were incubated with cofactors and a mixture of equal amounts of the substrates GA_9 and GA_{20} at varying concentrations for 1 hr at 20ºC and the resulting formation of GA_4 and GA_1 was monitored by GC/MS using deuterated GAs as internal standards.

in several species (Martin et al., 1996; Cowling et al., 1998; Itoh et al., 2001, Lee and Zeevart, 2002). Accordingly, our isolated *PttGA3ox1* exhibits a 40% reduction in expression upon GA treatment in *Populus tremula* seedlings as judged by semi-quantitative RT-PCR analysis (M. Chono, in preparation). Evidence has now been gathered showing that *PttGA3ox1* is expressed not only during early seedling development, but also in apical tissue and young expanding internodes (Paper II, Fig. 5). In addition, low levels of transcript were detected in young leaves. Similarly, in tobacco and pea the expression of *Nty* and *PsGA3ox1* is higher in internode tissue as compared to leaves (Itoh et al., 1999; Martin et al., 1997; Ross et al., 2003).

A reverse genetics approach to study GA mediated shoot growth and GA homeostasis in hybrid aspen (I, II)

Comparison of GA20ox and GA3ox OEs

In order to assess the effect of altered GA levels in trees and to compare the relative contribution of two key enzymes to GA biosynthesis, transgenic plants over-expressing *AtGA20ox1* or *AtGA3o1x* were produced and analyzed (described in detail in Papers I and II; see Figs. 9 and 10 for a selected summary).

Over-expression of *GA20ox* in *Arabidopsis* (Huang et al., 1998; Coles et al., 1999), potato (Carrera et al., 2000) and tobacco (Vidal et al., 2001) has been demonstrated to raise bioactive GA levels and to accelerate growth. In hybrid aspen, ectopic *GA20ox* expression led to up to 20-fold higher GA_1 and GA_4 levels and trees with a dramatically faster growth rates, in both height and girth (Fig. 9A-B; 10). The levels of deactivated GA, both GA_{34} and GA_8, increased approximately 4-5 fold, but the raised GA 2β-hydroxylation activity was apparently not sufficient to maintain GA homeostasis. The increased growth rate was ascribed to longer internodes (Fig. 9C) while the total number of internodes was not affected. At a cellular level, the length of epidermal and pith cells did not increase. Instead, the longer internodes were due to an increased number of cells. The elevated growth rate in *GA20ox* over-expressers (OEs) resulted in 126% higher stem dry weight, on average, than in the control. Initially the OE lines had poor rooting capacities, but the root dry weight of mature trees compared to WT was not significantly different. The marked increase in shoot biomass subsequently led to a shift in shoot to root ratios from 2:1 in WT to 4:1 in the transgenics.

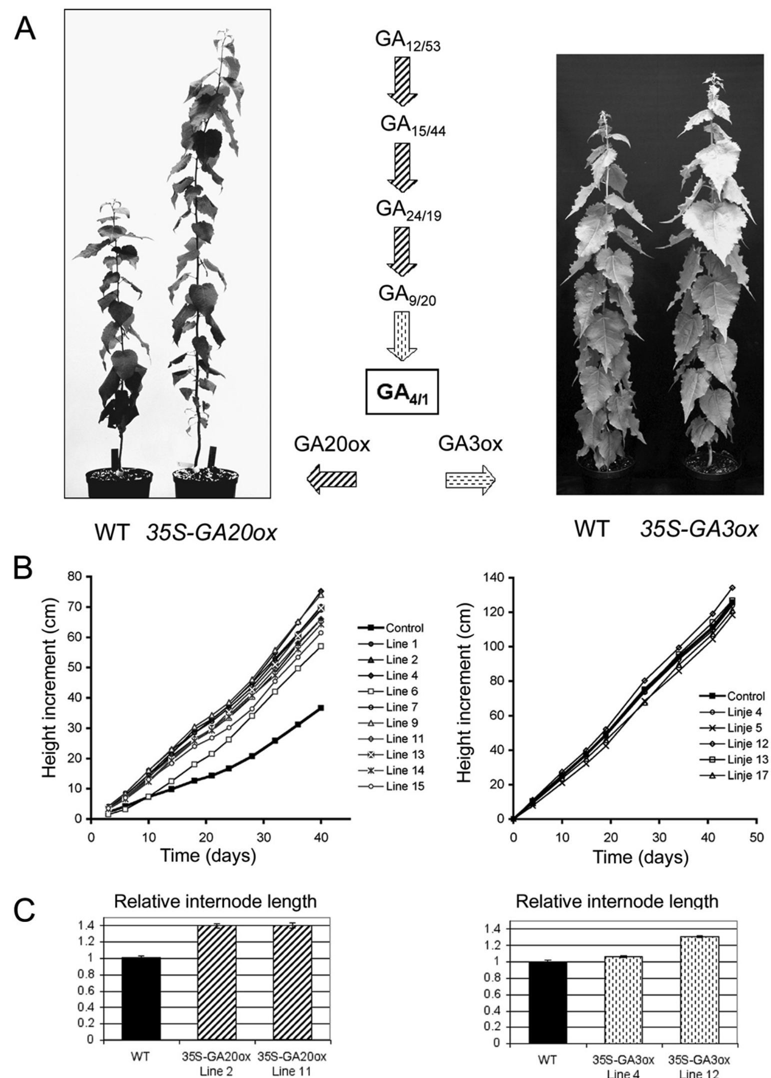

Fig. 9. Summary of data from transgenic hybrid aspen trees over-expressing *AtGA20ox1* or *AtGA3ox1* under the consitutive *CaMV35S* promoter. (A) Shoot phenotypes of transgenic plants and WT. (B) Height increment of different lines as compared to WT (C) Differences in internode length between control and two selected transgenic lines of each construct. For a more detailed description see Papers I and II.

In contrast, over-expression of *GA3ox* in hybrid aspen did not lead to pronounced changes in shoot growth, the lines grew only slightly faster or slower than WT (Fig. 9A-B). In contrast to the unaltered number of nodes in *GA20ox* OEs, the *GA3ox* OEs generally formed fewer nodes than WT. Notably, the most divergent line, with the fewest internodes (line 12), concomitantly developed 30% longer internodes, thereby making its total growth increment faster than WT (Fig. 9A-C).

The substrates of GA3ox, GA_{20} and GA_9, were dramatically reduced, in accordance with ectopic GA3ox expression (Paper II; Fig. 4). However, the bioactive GA levels did not increase accordingly. In fact, in apical tissue and young expanding internodes the GA_1 levels generally decreased or remained unchanged, while the amount of GA_4 significantly increased in line 12. The absence of highly elevated GA levels in *GA3ox* OEs correlates well with their less affected phenotype compared to *GA20ox* transgenics. In order to establish whether the transcription of GA dioxygenase genes had been altered in the *GA3ox* OEs, thus explaining their lack of increased GA levels, several dioxygenase genes were assayed by semi-quantitative RT-PCR. We did not detect any down-regulation of either *PttGA20ox1* or the endogenous *PttGA3ox1* (Paper II, Fig. 5). Similarly, there was no evidence indicating up-regulation of the expression of either of two recently isolated putative GA 2-oxidases: *PttGA2ox1* and *PttGA2ox2*. Thus, the lack of increased bioactive GA levels in the transgenics was not apparently due to any homeostasis-maintaining mechanism acting via GA dioxygenase transcription.

Since the GA homeostasis could be largely maintained in *GA3ox* transgenic lines in contrast to *GA20ox* OEs, we propose that GA 20-oxidation is a more rate-limiting step in GA biosynthesis than GA 3β-hydroxylation in the control of shoot elongation and wood formation. Further support for the presence of a rate-limiting step in the later stage of GA biosynthesis comes from a recent study where over-expression of *CPS* and *KS* increased the levels of *ent*-kaurene without affecting the levels of bioactive GA (Fleet et al., 2003).

Which GA controls shoot elongation in hybrid aspen?

In order to study if GA, and if so which one, determines final internode length in hybrid aspen, the strongest GA3ox OE line (line 12, which has 30% longer internodes than WT) was grown together with WT in an additional experiment described in Paper II. The GA_4 levels of line 12 were consistently higher in internodes representing three stages of elongation than their WT counterparts (Paper II, Table 2). Interestingly, the GA_4 (but not GA_1) levels of both genotypes were correlated with the developmental stage of internode elongation (Paper II, Fig 6B). It is possible that GA_4 is the main bioactive GA involved in *Populus* shoot growth, explaining how the consistently higher GA_4 concentrations in elongating internodes of line 12 can induce 30% longer internodes.

To further investigate the role of GA in *Populus* shoot elongation, the relative sensitivity of *Poplus tremula* seedlings to GA_4 and GA_1 was determined. Both GAs induced increased hypocotyl elongation compared to the control, and the responsiveness was slightly higher towards GA_4 (Paper II, Fig. 7). In *Arabidopsis*, it is widely accepted that GA_4 is the main bioactive GA since the species is more sensitive to GA_4, and has higher concentrations of this compound than GA_1 (Cowling et al., 1998; Xu et al., 1997). We have now established that there are: considerably higher levels of GA_4 than GA_1 in hybrid aspen (Paper II, table 2); correlations between GA_4 levels and final internode length (Paper II; Fig 6B); a slightly higher sensitivity to GA_4 than to GA_1 in *P. tremula* seedlings (Paper II, Fig. 7); and higher formation rates of GA_4 than GA_1 by the PttGA3ox enzyme (Fig. 8). Collectively, these data indicate that GA_4 is the main bioactive GA in hybrid aspen.

Effects on wood formation of increased GA levels

GA positively influences the formation of xylem fibers, as judged by effects of GA over-production on xylogenesis in the *GA20ox* OEs (Figure 10A-C). The transgenic lines had 70% more xylem fiber cells as compared to WT, and the xylem fibers were approximately 8% longer. These findings are consistent with the results of application studies with both GA (Digby and Wareing, 1966) and inhibitors of GA biosynthesis (Ridoutt et al., 1996). In contrast to findings that low ratios of IAA/GA lead to increased phloem production (Digby and Wareing, 1966), the extent of phloem formation was not greatly altered in our GA over-producing plants (Fig. 10C). This difference can be ascribed to the fact that we altered GA levels *in planta* rather than by GA application involving detrimental plant decapitation. The cambial zone apparently widened due to ectopic *GA20ox* expression (Fig. 10C) and it is possible that the zones of cell expansion and secondary cell wall formation were also increased. In deepwater rice GAs have been shown to increase internode elongation by increasing the zone of cell expansion (Sauter et al., 1993). Since the time allowed for the expansion zones of a cell to mature will ultimately determine its size, the possibility cannot be excluded that the action of elevated levels of bioactive GA extends this transition time, thereby increasing the fiber length. Finally, the effect of GA on cell division may be an indirect result of increased cell elongation, as studies in deepwater rice have detected a temporal separation between GA-induced cell elongation and cell division (Sauter and Kende, 1992).

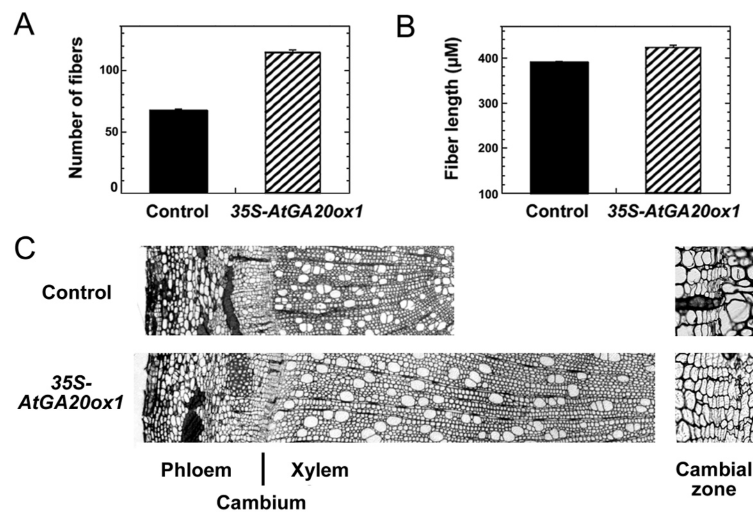

Fig. 10. Wood formation in fast-growing *35S-GA20ox* hybrid aspen trees. (A) Number of xylem fibers and (B) xylem fiber length in transgenic lines as compared to WT (C) Anatomy of the wood-forming tissues of control and a representative *35S-GA20ox* line.

Genes implicated in GA-induced increased wood formation (III)

From the studies reported in Paper I it was evident that ectopic *GA20ox* expression led to elevated bioactive GA levels and, subsequently, highly increased shoot growth. The pronounced effect on xylogenesis presented a unique opportunity to use

transgenic trees to identify important genes implicated in wood formation. Paper III describes a microarray experiment in which genes that are differentially expressed in the expanding xylem of a *GA20ox* OE line and WT were identified.

Cell wall formation and extension

Among the up-regulated genes in the GA over-producing trees with increased secondary growth, many appear to encode proteins with a role in cell wall formation and cell wall extension (Paper III, Table 1). Notable examples are genes encoding pectin methyl esterase (PME) and pectate lyase, suggesting that GA production may induce pectin modifications. Pectin is abundant in the primary cell wall, where its degree of methylation influences the cell wall extensibility (Micheli et al., 2001; Ridley et al., 2001). PME acts to demethylate the pectin while pectate lyase further degrades it, hence these enzymes may modify the plant's pectin status and thus allow cell wall extensibility to increase. To further investigate the role of pectate lyase we have cloned a full-length hybrid aspen pectate lyase (data not shown) and transformed it into hybrid aspen under the control of the *35S* promoter. The analysis of these transgenic trees and a functional characterization of the gene product are in progress. The transcription of pectate lyase is auxin-inducible (Domingo et al., 1998) and, interestingly, the transgenic *GA20ox* OEs possessed twice as high levels of IAA as WT plants (Paper III, p 897). Together with the up-regulation of an IAA-induced gene (Paper III, Table 1) this implies that the induced secondary growth is caused by a combined effect of auxin and GA. However, the relative amount of GA compared to IAA is still higher in the *GA20ox* OEs (Paper III, p 897), suggesting a pivotal role of GA in the increased secondary growth.

Other differentially expressed genes encoded the following enzymes with functions ascribed to cell wall loosening and expansion: basic cellulase and putative endoglucanases, xylosidase, expansin, sucrose synthase, GDP-mannose pyrophosphorylase, aquaporin and a K^+ channel protein (Paper III, Table 1). Furthermore, a recent up-date of cDNA clones on the array, using the improved PopulusDB with 120 000 ESTs (R. R. Bhalerao, 2003), improved the annotation of five *major latex allergen* ESTs (Paper III, Table 2) into another *expansin* gene (data not shown).

Uncovering changes in lignin composition through microarray analysis

The results from the microarray analysis suggested an alteration in lignin formation as genes coding for caffeoyl-coenzyme A *O*-methyltransferase (CCoAOMT) and a dirigent protein were induced (Paper III, table 1). However, only a sub-set of genes involved in lignin formation was differentially expressed and it is not clear whether the total lignin content of the transgenic plants was altered.

The enzyme cinnamyl alcohol dehydrogenase (CAD) is associated with G-lignin formation. Interestingly, two *CAD* ESTs (AI162401, AI161452) showed unchanged ratios on the microarray, whilst those of another *CAD* (AI165107) increased more than 2-fold. Subsequent full-length sequencing of the latter clone revealed a putative *sinapyl alcohol dehydrogenase* (*SAD*) gene similar to the *Populus tremula SAD* (*PtSAD*), which has been suggested to be essential for the biosynthesis of S-lignin in angiosperms (Li et al., 2001). Spurred by this finding, we found increased transcript

ratios for two other genes implicated in S-lignin formation: ferulate 5-hydroxylase (F5H) (1.5) and caffeate *O*-methyltransferase (COMT) (1.9). The transcript ratios for both of these genes were less than two, but all measurements on these genes were consistent. G-lignin is deposited developmentally earlier than S-lignin in xylem fibers and spatio-temporal links between CAD and SAD protein to G and S lignin biosynthesis, respectively, have been described in aspen (Li et al., 2001). Similarly, the expression of *PttCAD* in hybrid aspen exhibited an earlier transcript profile peak compared to our putative *PttSAD* in distinct zones of xylem development (Hertzberg et al., 2001). Finally, in order to confirm any alterations of the S-lignin composition, the S/G ratios of WT and transgenic plants were determined. In agreement with the microarray data, the S/G ratio of transgenic plants was shown to be increased by up to 7% (Paper III, Table 2).

The confirmation of increased S-lignin biosynthesis in the fast-growing transgenic trees demonstrates the usefulness of studying large-scale transcript changes. Without the microarray analysis, this highly interesting phenotype (see section on Lignin formation in the Background section above) would most probably have been missed. In contrast to the descriptive microarray results previously obtained from hybrid aspen (eg. Hertzberg et al., 2001; J. Schrader, 2003), we have now been able to link a microarray-derived transcript profile to a biologically relevant phenotype that would otherwise have been overlooked.

Reduced stress responses in GA over-producing trees?

Among the genes with repressed transcription there were many that coded for stress-related proteins (Paper III, p 900), e.g. glutathione *S*-transferase, a multi-functional enzyme that is known to play a critical role in several stress responses (Dixon et al., 1998), and a down-regulated wounding induced protein that is obviously stress-related. The transcriptional down-regulation of a phosphate-induced protein (phi) is noteworthy as studies in tobacco imply that *PHI* is induced in response to changes in cytoplasmic pH, and is thus a stress indicator, rather than being involved in phosphate-induced processes as previously believed (Sano and Nagata, 2002). A recent up-date of the annotation of down-regulated cDNA clones with unknown functions, using the improved PopulusDB with 120 000 ESTs (R. R. Bhalerao, 2003), revealed genes with implied involvement in cold acclimation and disease resistance (data not shown). Curiously, several of the repressed genes can be induced by the plant growth substance abscisic acid (ABA) (Machuka et al., 1999; Moons, 2003; Sano and Nagata, 2002; data not shown). Similarly, during GA-induced *Arabidopsis ga1-3* seed germination, several of the down-regulated genes identified by microarray analysis contained an ABA-responsive element for ABA-induced gene expression in their promoters (Ogawa et al., 2003). In the abovementioned *Arabidopsis* study, the negative effect on ABA-induced expression was not ascribed to lowered ABA levels, but rather to effects on the signal transduction pathway. Similarly, increased GA levels could negatively affect the induction of ABA-induced genes in our GA over-producing transgenic trees. For now we can only speculate that if the induction of stress-induced genes is impaired in the *GA20ox* OEs, they may consequently be more susceptible to stress.

GA formation and responsiveness during wood formation (IV)

From the studies reported in Paper I it was evident that increased GA levels lead to increases in both the length and number of xylem fibers (Fig. 10). Because we wanted to gain a better understanding of the role of GA during xylogenesis, we accurately measured GAs, in conjunction with the transcription of several GA-related genes, in minute samples representing distinct stages during wood formation (Paper IV).

Differences in the distribution of auxin and GA

In order to confirm that our approach for measuring hormones in tangential cryo-sections was valid we initially determined the distribution of auxin along the developmental gradient (Paper IV, Fig. 2A, B). In accordance with previous results from hybrid aspen and pine, the IAA levels exhibit a characteristic peak around the secondary meristem, the cambium (Uggla et al., 1996; Tuominen et al., 1997). Because of this concentration gradient, auxin has been suggested to act as a morphogen in patterning cambial development and vascular differentiation. By using a very sensitive analysis we were also able to detect GAs, of both the early non-hydroxylation and early 13-hydroxylation pathway, in individual tangential cryo-sections. In contrast to IAA, the concentration of bioactive GA_1 and GA_4 exhibited a clear peak outside the cambium in the expanding xylem. The distinct distribution pattern of bioactive GA suggests it has a role primarily during xylem expansion and elongation.

How is bioactive GA formed in the expanding xylem?

The immediate precursors of bioactive GA, GA_{20} and GA_9, peak in the phloem, but there is also a second peak in the zone of expanding xylem (Paper IV, Fig. 2CD). Interestingly, the expression of *PttGA20ox1,* as measured by Real-Time PCR, is considerably higher in the developing xylem as compared to the phloem (Paper IV, Fig. 3D-F). This prompts questions about how the pools of GA_{20} and GA_9 in the phloem and the xylem arise – from transport via the phloem and/or xylem stream, and/or by *de novo* production? In our cutting series, the expression of the first committed GA biosynthesis enzyme, CPS, was considerably higher in the phloem than in the xylem (Paper IV, Fig 3A-F). Non-overlapping expression patterns of *CPS* and GA dioxygenase genes have previously been observed during *Arabidopsis* seed germination (Yamaguchi et al., 2001). The cited authors suggested that the highly hydrophobic GA metabolite *ent*-kaurene could be transported between tissues, possibly by association with a carrier protein. One hypothesis to explain our results is that any *ent*-kaurene, or other precursor, formed in the phloem of aspen could be transported, possibly by carrier proteins, into the xylem. This transport could take place through the ray cells, which readily transport phloem assimilates between phloem and xylem. Once present in the differentiating xylem cells, *ent*-kaurene could be further converted into GA_{12}, thus providing substrate for the presumably highly abundant GA20ox enzyme. Finally, conversion of GA_{20} and GA_9 by GA3ox would give rise, or at least contribute, to the high concentration of bioactive GA detected in expanding and elongating xylem. Real-Time PCR data for one *GA3ox* gene did not reveal any conclusive tissue-specificity (Paper IV, Fig. 3G-I). However, we have previously shown that GA 3β-hydroxylation is not a rate-limiting step as

compared to GA 20-oxidation in GA biosynthesis in the control of shoot elongation and secondary growth in hybrid aspen (Papers I and II). Therefore, expression data for all of the *GA3ox* genes would be superfluous to explain the final bioactive GA levels, since the GA20ox activity would act as the limiting step and *PttGA20ox1* expression in expanding xylem was high.

Several studies support evidence that GA is present in xylem and phloem, suggesting that GA is transported in the vascular tissues of the plant (reviewed in Hoad, 1995; Lang 1970). Furthermore, grafting studies in pea have revealed that GA_{20} can be transported within the plant, and the transport efficiency of GA_{20} is higher than that of GA_1 (Proebsting et al., 1992). Given that GA_{20} and GA_9 are part of the phloem stream in hybrid aspen, they could be transported through the ray cells into the developing xylem, offering an alternative explanation for the second peak of C_{19}-GA. The possibility of GA transport between phloem and xylem, in both directions, has previously been demonstrated in *Salix* (Bowen and Wareing, 1969). The proportions of GA_{20} and GA_9 in the xylem that originate from the root system remains to be established, but it is likely that at least some is translocated from the phloem via the ray cells. However, because there is a high level of limiting *GA20ox* expression, correlated with high bioactive GA levels in the developing secondary xylem, we also suggest that *de novo* GA biosynthesis occurs in the expanding xylem.

Correlation of bioactive GA with expression of GA response genes

In order to assess responsiveness to bioactive GA, the expression of two GA response-related genes (*DELLA-like1* and *GIP-like1*) were assayed. To start with the first of these, RGA/GAI proteins of the DELLA family are known to act as repressors of GA-mediated processes (reviewed in Olszewski et al., 2002). Following a GA signal, some of them become degraded through targeted polyubiquitination and subsequent degradation by the 26S proteasome (Fu et al., 2002; McGinnis et al., 2003; Sasaki et al., 2003). In *Arabidopsis,* treatment of bioactive GA increases the transcription of *RGA* (Silverstone et al., 1998), possibly as a compensatory mechanism for the increased turn-over rate at the protein level (Silverstone et al., 2001). In line with this hypothesis, the transcript profile of a *DELLA-like* gene in aspen was found to be relatively high in expanding and elongating xylem tissues, where the bioactive GA levels are high (Paper IV, Fig. 4A-C). Secondly, the expression of a GA-induced gene, *GIP-like1*, was more than 100-fold higher in the developing xylem as compared to sample A (phloem) in all trees analyzed (Paper IV, Fig. 4D-F). In summary, the expression of genes encoding proteins acting downstream of the site of GA perception correlates well with the observed distribution of bioactive GA.

Conclusions

The work presented in my thesis was performed to increase our understanding of the plant growth substance GA and its effect on shoot growth in the model tree hybrid aspen. In the young vegetative shoot, reporter gene analysis of a gene encoding a key GA biosynthesis enzyme, *PttGA20ox1*, revealed expression in expanding leaves and elongating internodes. PttGA20ox1 was also implicated in the development of the male floral organ, pollen development and seed development, based on results from the heterologous host *Arabidopsis thaliana*. An enzyme catalyzing the conversion of inactive GA into GA with biological activity, PttGA3ox1, was cloned and functionally characterized. In line with the role of GA during shoot elongation, expression of this gene was detected in young elongating internodes.

Over-expression of GA20ox increased bioactive GA levels up to 20-fold, leading to fast-growing trees with highly increased elongation and secondary growth. In contrast, ectopic expression of GA3ox did not dramatically alter shoot growth and development. Thus, it was concluded that GA20ox acts more as a limiting step than GA3ox in GA biosynthesis, controlling shoot elongation and secondary growth in hybrid aspen. Furthermore, several lines of evidence now suggest that GA_4, rather than GA_1, has a pivotal role in controlling shoot elongation.

The effect of GA on wood formation was ascribed to increased xylem fiber formation and extension since 70% more fibers were formed, which were 8% longer than in WT. However, no major effect on phloem development was observed. Based on microarray results, comparing the wood-forming tissues of WT and one transgenic line, we suggest that GA mainly up-regulates the expression of genes implicated in cell wall formation and extension. In addition, the microarray analysis helped uncover an interesting shift in S-lignin composition previously undetected in the transgenic trees.

A role of GA during xylem differentiation was further corroborated by the specific distribution of GA_4 and GA_1 in expanding xylem cells. We hypothesize that the effect on cell division exerted by GA in *Populus* could be an indirect consequence of its primary effect on cell elongation. Furthermore, the absence of high amounts of bioactive GA in phloem tissue indicates that any role GA may have during phloem development is less important than its function during xylem expansion.

The biosynthesis of GA in expanding xylem was suggested to be associated with intercellular transport of GA intermediate/s. It is interesting to speculate that *ent*-kaurene could be produced in the phloem and then transported by the phloem assimilate stream via the ray cells into the xylem. The strongest evidence for *de novo* production of bioactive GA in expanding xylem was the xylem-enriched expression of *PttGA20ox1.* This is based on the OE studies showing GA 20-oxidation to be a limiting step in GA biosynthesis in the control of secondary growth. Finally, the expression of two genes implicated in GA responsiveness correlated very well with the distribution of bioactive GAs.

Future Plans

The increased understanding related to GA and shoot growth provided by the work presented in this thesis has prompted new questions that need to be addressed, some of which are outlined below:

- How is the deactivation of bioactive GAs regulated during plant growth and development? Functional characterization and transcript distribution analysis of two putative GA 2-oxidases is underway.
- What is the role of some of the proteins that presumably limit secondary growth? Over-expression of an arabinogalactan peptide and a pectate lyase in hybrid aspen, which were up-regulated in GA20ox OEs, in addition to their functional analysis, may help resolve this question. Furthermore, it would be interesting to analyze their temporal expression patterns after the addition of GA.
- Is the effect of GA on xylem fiber length due to increased fiber tip growth or are the initial cells already longer?
- How is the formation of bioactive GAs in the wood-forming tissues regulated? We need to determine whether GA biosynthesis in expanding xylem requires transport of the intermediate *ent*-kaurene from the phloem. This could be done by using an inducible system, for instance RNAi, to repress the amount of *CPS* mRNA in the phloem.
- Does GA play a role during phloem development? If possible, it would be interesting to specifically block the GA response pathway in phloem cells.
- Similarly, what is the role of GA during xylem development? It would be informative, if possible, to specifically block the perception of GA during xylem differentiation.
- Once the *Populus trichocarpa* genome sequences have been properly assembled and annotated it will enable analysis of all known genes that encode GA biosynthesis enzymes. Real-Time PCR analysis of the expression of these genes in hybrid aspen, including entire gene families, would increase our understanding of GA biosynthesis and deactivation in the model tree.

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