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The Role of Phytochrome A and Gibberellins in Growth under Long and Short Day Conditions

Studies in hybrid aspen

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Abstract

This thesis addresses questions concerning the regulation of growth and, specifically, the cessation of growth in response to short days in deciduous tree species. The model tree used in the studies was hybrid aspen (*Populus tremula* L. x *P. tremuloides* Michx.). We have exploited the possibility of transforming this species to modulate the level of expression of target genes using over-expression and antisense techniques.

The target genes in the studies were the photoreceptor phytochrome A (phyA) and gibberellin 20-oxidase (GA 20-oxidase), the latter being a highly regulated enzyme involved in the biosynthesis of gibberellins (GAs). The photoreceptor phyA has been implicated in photoperiodic regulation of growth, while GAs may regulate the physiological response further downstream. The endogenous expression of these genes has been investigated in parallel with studies of various plants with ectopic and reduced levels of expression. The main focus has been on the early stages of induction of growth cessation and its physiological and molecular mechanisms.

Studies of hybrid aspen plants with an increased or reduced expression of phyA, show this receptor to mediate the photoperiodic regulation of growth. Plants with ectopic expression could not stop growing despite drastically shortened photoperiods, while the antisense plants showed the reverse phenotype, with a higher sensitivity resulting in earlier cessation of growth. The role of GAs in growth inhibition was also addressed using plants with a reduction in GA levels. These plants showed early cessation of growth and dormancy, and thus an increased sensitivity toward daylength. Conversely, plants with increased rates of GA biosynthesis showed increased growth and stopped growing much later. Furthermore, increases in GA biosynthesis, resulting in high levels of GAs have a major impact on growth. Plants with high GA levels have increased elongation and diameter growth, due to higher rates of cell production in the apical meristem and cambium, respectively. Also, these plants have altered wood properties showing more numerous (71%) and longer (8%) fibres as compared to the control plants.

GA levels were modulated by altering the expression of the multifunctional enzyme GA 20-oxidase. This enzyme was shown by the over-expression studies to be a limiting factor in the biosynthesis of GAs. This enzyme was also shown to be regulated at the transcriptional level, both by photoperiod and active GA_4. Our studies indicate that GA 20-oxidase is very likely to be one of the most important factors in the GA-regulation of growth and growth cessation.

In conclusion, these studies have shed light on the early stages of growth cessation in deciduous trees, especially with respect to the role of phyA and GAs. It has also given new information on the importance of GAs in growth as such, with important implications for wood production.

Key words: gibberellin, GA 20-oxidase, phytochrome A, photoperiodism, growth cessation, growth, fibre, *Populus*, hybrid aspen, transgenic trees.

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"To see a world in a grain of sand
and a heaven in a wild flower
hold infinity in the palm of your hand
and eternity in an hour ..."

William Blake

Contents

Appendix

List of papers

The present thesis is based upon the following papers, which will be referred to by the corresponding Roman numerals.

I. Olsen, J.E., Junttila, O., Nilsen, J., Eriksson, M.E., Martinussen, I., Olsson, O., Sandberg, G., Moritz, T. (1997). Ectopic expression of oat phytochrome A in hybrid aspen changes critical daylength for growth and prevents cold acclimatization. *Plant J.* 12:1339-1350.

II. Eriksson, M.E. and Moritz, T. (1997). Isolation of a cDNA encoding a phytochrome A (Accession No. AJ00138) from *Populus tremula* x *tremuloides*. Plant Gene Register # PGR97-186, *Plant Physiol* 115:1731.

III. Eriksson, M.E., Mozley, D. and Moritz, T. (2000). Reduction in phytochrome A gene expression results in increased sensitivity to short day induction of growth cessation in hybrid aspen. (Submitted).

IV. Eriksson, M.E. and Moritz, T. (2000). A photoperiodically regulated gibberellin 20-oxidase isolated from hybrid aspen (*Populus tremula* L. *x P. tremuloides* Michx.). (Submitted).

V. 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. *Nat. Biotechnol.* 18: 784- 788.

VI. Eriksson, M.E., Mozley, D. and Moritz, T. (2000). The timing of shoot growth cessation and dormancy is dependent on proper regulation of GA biosynthesis. (Manuscript).

Introduction

Background

Plants, as stationary organisms, must be able to obtain sufficient information from the environment in order to adapt, in time, to new circumstances since they cannot physically move to avoid changes. This information could relate to drought, flooding, light, changes in daylength, cold or other important variables for which new strategies such as adaptation or escape (e.g. flowering and seed set, or growth cessation and dormancy if it is a perennial herb or a tree) would be appropriate responses from the plant. To obtain the information needed and respond to it, plants have developed a highly tuned system of "receptors", signal transduction pathways and growth regulators that can modify growth as required (Møller and Chua, 1999).

Light is the primary source of energy and the basis of photosynthesis. Light and its signalling pathways control a wide array of developmental and metabolic processes in plants, thereby optimising growth and photosynthesis. Light is perceived by the plant through receptors that monitor its direction, intensity, duration and wavelength. Such receptors have been studied intensively in the last few years. The most thoroughly characterised groups of photoreceptors are the phytochromes, which mainly monitor light in the red/far-red but also, to a lesser degree, in the blue spectra, and the cryptochromes as well as the phototropin, which mainly absorb blue/UVA. There are also likely to be other receptors, especially of light in the UVA and UVB region, but not much is known regarding their biochemical nature (Quail, 1994). These photoreceptors mediate information important for the regulation of many developmental processes, such as germination, de-etiolation of seedlings, shade avoidance and flowering. In addition they relay information to the endogenous time keeping machinery, the circadian clock, which coordinates physiological processes with the daily light and dark cycles. As an example, the movements of leaves are regulated by this mechanism to maximise light capture, and the activation of photosynthetic genes is timed to coincide with the light period, when photosynthesis is possible (Millar, 1999). In addition the circadian clock is also involved in regulating seasonal responses, described as photoperiodism. The photoreceptors not only pass the signals to the clock but have also been suggested to be a part of the actual clock itself (Bognar et al., 1999; Strayer and Kay, 1999). Downstream components of the light signalling pathways are being elucidated, but our knowledge of these components is still poor. However, it is clear that further downstream the information received by the plant modifies its growth patterns via the action of various growth regulators.

There are five classical groups of plant regulators or hormones in plants, the auxins, gibberellins, cytokinins, abscisic acid and ethylene (Davies, 1988; Kende and Zeevaart, 1997). The list has now been expanded to include several other com-

pounds with growth regulating effects, including brassinosteroids, jasmonates, salicylic acid and polyamines (Davies, 1995; Møller and Chua, 1999). Even though the substances are often referred to as hormones, these plant compounds do not meet the criteria of the classical hormone concept as defined in animals, stipulating that a hormone is synthesised in one organ and transported to the target organ where it exerts its effect. In contrast, plant hormones are often synthesised and active in the same organ. However, it must be emphasised that similar, local hormone-mediated signalling does occur in animals too, as shown, for instance, by the action of prostaglandins (Alberts et al., 1994).
Plant hormones can modulate growth either independently from or synergistically with each other. Since growth is a very complex process depending on both cell division and cell expansion, the importance of a specific hormone depends on the cell, organ and developmental phase being considered.

Light Perception and Regulation of Growth

Light receptors

Phytochromes

As mentioned above there is a wide range of photoreceptors in plants monitoring the properties of incoming light. The phytochromes, which are the most well described family of photoreceptors, absorb light in the red (λ_{max}~660 nm), far-red (λ_{max}~730 nm) and to a certain extent in blue wavelengths (Neff and Chory, 1998; Whitelam et al., 1998). They consist of an apoprotein with a linear tetrapyrrole pigment covalently attached. The chromophore is bound to the N-terminal while the C-terminal is implicated in dimerisation and signal output. Phytochromes have recently been acknowledged to carry PAS domains in their C-terminal regions, which have been shown to be essential for their interactions with several proteins (see Taylor and Zhulin, 1999). The phytochromes appear as dimers of indentical ~124 kDa polypeptide monomers, where each monomer has the ability to exist in either Pr or Pfr forms. The red (R) light absorbing form (Pr) and the far-red (FR) light absorbing form (Pfr), are interconvertible by light. The absorption spectra of Pr and Pfr overlap, so under most light conditions these species exist as a mixture of the two forms (Whitelam and Devlin, 1998). Phytochromes are thus chromoproteins that are reversibly photochromic and can exist in either of two stable forms. They are localised in the cytoplasm or nucleus, depending on the light conditions and type of phytochrome involved (Batschauer, 1998). Based on physiological and genetic studies the Pfr form is generally considered to be the active form (Whitelam et al., 1998). However, there is also evidence that the reverse may also be true in some cases, as shown for instance in *Arabidopsis* hypocotyl growth inhibition by high intensity FR light (Shinomura et al., 2000). These authors found the hypocotyl growth inhibition to be induced by FR and reversed by R, indicating that the Pr or an intermediary form can also act as the active species, in contrast to the classical concept.

There were also early suggestions that the phytochromes function as kinases, but until recently there has been little support for these controversial reports. However, it has now been shown that purified or recombinant oat phyA can both autophosphorylate and phosphorylate other substrates, preferably on serines or threonines, as reviewed in Yeh and Lagarias (1998). Two candidate genes, *PHYTOCHROME-INTERACTING FACTOR 3* (*PIF3*) (Ni et al., 1998) and *PHYTOCHROME KINASE SUBSTRATE 1* (*PKS1*) (Fankhauser et al., 1999), encoding substrates for phytochrome kinase activity have recently been isolated from two yeast hybrid screens. The corresponding proteins have been shown to interact with both phyA and phyB (Fankhauser et al., 1999; Ni et al., 1998).

In most plants the phytochromes are encoded by a gene family, for example there are five genes (*PHYA* to *PHYE*) encoding phytochromes in the genome of *Arabidopsis thaliana* (Clack et al., 1994; Sharrock and Quail, 1989). In flowering plants there appear to be three major types of phytochromes, phyA, phyB and phyC, which were phylogenetically separated in the earliest flowering plants. Together with *PHYB,* genes arising later, such as *PHYD* and *PHYE* in *Arabidopsis*, form a phylogenetic subgroup of the *PHY* family (Devlin et al., 1998). Among the phytochromes, phyA is the only light labile protein, being rapidly degraded in light, while the others are described as light stable. The conventional nomenclature for phytochromes and cryptochromes is such that the wild type gene, mutant gene, holoprotein, and apoprotein of phytochrome A, as an example, are designated *PHYA*, *phyA*, phyA and PHYA, respectively (Lin et al., 1998; Quail et al., 1994).

The analysis of mutants, especially of *Arabidopsis*, has been a fruitful approach to identify the receptors and to assign physiological functions to individual members of the photoreceptor families. At present, functions have been described for all members of the phytochrome family in *Arabidopsis*, but due to the lack of a *phyC* mutant its role has only been deduced from observations of over-expressing plants. The early downstream signalling events initiated by the different phytochromes, e.g. phyA and phyB, are believed to be distinct and there is a range of mutants that have been suggested to specifically affect one or the other signalling pathway. However, in addition to mutants impaired only in constitutive R or FR light, there are mutants defective in both R and FR, suggesting that the two pathways converge further downstream (Huq et al., 2000; Whitelam et al., 1998). This is also in accordance with microinjection and induction studies of different light regulated genes, as reviewed in Whitelam et al. (1998).

Cryptochromes and phototropin

The other photoreceptors that have been described are the blue (~450-480 nm) and UVA (~320-390 nm) light absorbing receptors, a group that has been recently identified and characterised. As yet, three blue light receptors have been identified in *Arabidopsis*, mainly through mutant screening: cryptochrome 1, cryptochrome 2 and phototropin. The cry1 photoreceptor consists of a chromophore binding domain with a flavin adenine dinucleotide (FAD) covalently attached to it, and in addition a

pterin has been found to bind to the N-terminal residues with photolyase homology *in vitro*. However, despite its structural homology, no photolyase activity has been detected (Lin, 2000). The structure of cry2 is similar to cry1, the N-terminal being identical, but structural differences appear in the C-terminal domain. There are also differences in stability, cry2 being less stable and rapidly degraded under light that activates the receptor (green, blue and UV). Functionally their domains seem to be interchangeable, as shown in studies of over-expressing plants carrying chimeric cry1 and cry2 proteins (Ahmad et al., 1998).

The phototropin gene (*NPH1*) encodes a ~120 kDa protein which is associated with the plasma membrane and undergoes phosphorylation in response to blue light. In studies of the recombinant protein it has been shown to bind flavin mononucleotide (FMN). Recently the FMN has been shown to attach to the two light oxygen and voltage (LOV) domains found in the N-terminal of the protein. Moreover, the apoprotein has a serine-threonine-kinase domain in its C-terminal. Thus, the nph1 protein is a kinase that catalyses autophosphorylation in response to blue light. In conclusion, there are two identified classes of blue light receptors in plants, the photolyase related cryptochromes, and the kinase and LOV domain containing photoreceptor, nph1 (Batschauer, 1998; Lin, 2000). Besides the already identified receptors, the results of physiological experiments on stomatal opening and phototropism suggest that there are additional receptors left to discover which absorb in the blue/UV spectra (Lin, 2000).

Light regulated growth

Phytochrome functions

From studies of phytochrome mutants obtained thus far (again mainly in *Arabidopsis*) several functions have been attributed to individual phytochromes throughout the developmental cycle. Classically the actions of phytochromes are divided into three categories depending on the amount of light required to evoke a certain physiological response, i.e. the fluence of light, defined as the number of photons absorbed per unit surface area (mol m^{-2}). In addition, the irradiance of light may also be significant, measured as the number of photons received per square meter per unit time (mol m^{-2} s^{-1}). If the response, as in the germination of *Arabidopsis*, is induced by tiny amounts of virtually any light (Shinomura et al., 1996), the response is defined as a very low fluence response (VLFR). These responses cannot be reversed by giving FR light immediately after the first light flash. The phytochrome that mediates most VLFR responses is phyA. The second type of fluence response is the low fluence response (LFR), imposed by R and FR light of moderate illumination. These responses, generally activated by R and reversed or inhibited by FR, are generally mediated by phyB. The third and last class is called the high irradiance response (HIR). These responses require prolonged exposure to high irradiance, they may be photoreversible and they involve the action of phyA, phyB or cryptochrome, depending on the process (Smith, 1995).

Starting with germination, this early developmental stage can follow two markedly different patterns, depending on whether the seed is germinating in darkness or in the light. If germination takes place in the dark the seedling will develop according to the skotomorphogenic (etiolated) pattern, with undeveloped chloroplasts, closed and unexpanded cotyledons and an elongated hypocotyl. Such seedlings consume energy rapidly, in an adaptive response to reach the soil surface and light as fast as possible. When the seedling germinates in sunlight it will instead rapidly turn into a photosynthesising plant and most of its energy will be used to develop leaves, while elongation of the hypocotyl is minimised (von Arnim and Deng, 1996). Etiolated *phyA* seedlings of *Arabidopsis* are unable to inhibit hypocotyl elongation, cotyledon opening or activation of anthocyanin synthesis, and unable to regulate other light-responsive genes upon FR light exposure (Whitelam et al., 1998, and references therein). No other known phytochrome mutants are impaired in these responses to prolonged FR light (HIR), thus phyA is the only receptor to mediate these responses (Quail et al., 1995). Besides its role in prolonged FR exposure, phyA also seems to be crucial for germination under short exposures to R and FR (VLFR) (Shinomura et al., 1996), which may be important when it comes to establishing seedlings under very dense canopies. Under shading leaves the light reaching the soil is enriched in the FR spectra since the chlorophyll will absorb the R light. In light-grown seedlings the *phyA* mutant displays a wild type (WT) phenotype (Whitelam et al., 1998).

The *phyB* mutant is also, like *phyA*, deficient in the seedling de-etiolation response. It is generally unresponsive to R light of all intensities but it does respond to FR light by de-etiolating. In germinating *Arabidopsis*, phyB promotes germination in R light, which can be inhibited by a following pulse of FR light (LFR) (Shinomura et al., 1996). In *phyB* seeds this R-FR reversible response is almost absent (Whitelam et al., 1998, and references therein).

In later developmental stages, phyA is implicated in the photoperiodic control of flowering time: *Arabidopsis* deficient in phyA being late flowering, while plants overexpressing phyA are early flowering (Bagnall et al., 1995; Johnson et al., 1994). PhyB also affects flowering time and *phyB* mutants are early flowering under both short and long days. Thus in *Arabidopsis* phyA promote flowering while phyB has an inhibitory effect. The *phyB* mutant still, however, responds to the photoperiod since flowering is enhanced in response to long days (LD) (Whitelam et al., 1998). The opposite is true for *Sorghum bicolor*, where phyB seems to be very important for photoperiodic regulation of flowering. The ma_3^R mutant lacks phytochrome B and is insensitive to photoperiod, so it initiates flowers under any photoperiod (Childs et al., 1995; Childs et al., 1997). Potatoes with decreased phyB function, due to antisense expression, lose their requirement for SD, i.e. photoperiodic control, in tuberisation (Jackson and Prat, 1996). For tuberisation in potato, phyA has also been shown to be involved in daylength detection (Yanovsky et al., 2000). Further, in rice a mutant impaired in all phytochromes loses the photoperiodic control of flowering (Izawa et al., 2000). The last three examples are species of short day plants (SDPs) that are induced to flower or tuberise in response to SD. In these cases

phyB seems to play an important role, while in long day plants (LDPs) like *Arabidopsis* photoperiodic detection has been suggested to be mainly mediated through phyA (Whitelam et al., 1998). However, the role of phyB in detection of daylength in *Arabidopsis* seems to warrant more attention given very recent data on this topic, (see further discussion in Photoperiodic detection pp. 15-17).

Shade avoidance is the strategy used by shaded plants or plants having close neighbours, to outgrow low light conditions. The plant detects neighbours or low light conditions by the low R/FR ratio they cause, observed in the plant as a change in the Pr/Pfr ratio. The *phyB* mutants show a constitutive shade avoidance response, resulting in pale plants with increased elongation of internodes and petioles, reduced leaf area, increased apical dominance and accelerated flowering. In WT plants shade avoidance can be induced by an end of day FR (EOD FR) treatment, but the responsiveness to this treatment is greatly reduced in *phyB* plants. Recent data suggest that the phytochromes D and E are also involved in the shade avoidance response of *Arabidopsis*. Evidence for this assertion is most clearly visible in the *phyB* background or in *phyAphyBphyD* or *phyAphyBphyE* triple mutants. The non-responsiveness to EOD FR is especially pronounced in the *phyE* triple mutant (Devlin et al., 1998; Devlin et al., 1999; Whitelam et al., 1998). The role of phyC is at present not well known, but recent findings in *Arabidopsis* and tobacco over-expressing *Arabidopsis PHYC* indicate that it may have a role in leaf expansion and overall growth since over-expressing plants show increased expansion growth (Halliday et al., 1997; Qin et al., 1997). It may also be involved in the flowering response, as tobacco over-expressors tend to flower early (Halliday et al., 1997).

In summary, the phytochromes mediate light regulation of most developmental decisions in the plant life cycle, especially in germination, de-etiolation, flowering and shade avoidance. Also, both phyA and phyB are likely to be important parts of the global timing system, the circadian oscillator. For an overview of phyA and phyB mediated responses, see figure (Fig.) 1. Further, some phytochromes, e.g. phyA and phyB, are suggested to affect the regulation of several plant hormones e.g. auxins, brassinosteroids and gibberellins (see Chory and Li, 1997; Kamiya and Garcia-Martinez, 1999; Morelli and Ruberti, 2000). Phytochromes have been shown to influence both the biosynthesis of gibberellins (GAs), and plant sensitivity to them. Phytochrome B has been reported to affect responsiveness to gibberellins in *Arabidopsis* (Reed et al., 1996), for example, and in several species phyB and phyA may control GA biosynthesis (Chory and Li, 1997; Kamiya and Garcia-Martinez, 1999).

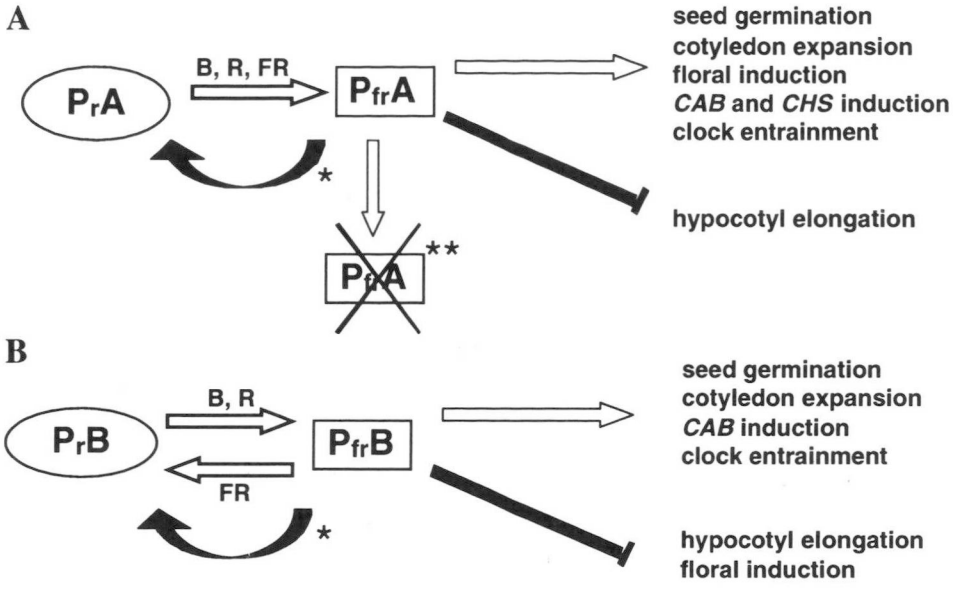

Fig 1. Phytochrome A and B mediated responses. The responses are shown in A) and B) respectively. Arrows indicate promotion and T-bars inhibition of the indicated process. (*), denotes dark reversion, and (**) light induced destruction of the protein. *CHS*, chalcone synthase gene, an enzyme in the anthocyanin biosynthesis pathway. *CAB*, chlorophyll a/b binding protein, a protein of the light harvesting complex. Active wavelengths mediated by the receptors are: R, red; FR, far-red and B, blue light. The figure is partly adapted from Chory (1997).

Cryptochrome and phototropin functions

In *Arabidopsis*, cry1, like phyA and phyB, has been found to be important for the de-etiolation of seedlings. The *hy4* mutant seedlings (deficient in cry1) are non-responsive to blue light and show an etiolated phenotype. *CRY1* overexpressing plants show reduced hypocotyl inhibition and increased anthocyanin production, confirming this function (Lin, 2000; Lin et al., 1996). Also, cry2 takes part in the de-etiolation process by inhibiting hypocotyl growth: a response that is especially clear in very low fluency blue light, and less apparent in high fluency blue light. Interestingly, like phyA, this receptor appears to be light labile and to mediate VLFR (Lin, 2000). Further, cry1 is implicated in the circadian regulation of gene expression (see below). Moreover, cry2 seems to be important for flowering, and it is allelic to a described late-flowering mutant, *fha* (Guo et al., 1998; Lin, 2000).

Phototropin (nph1) is the only characterised receptor that predominantly mediates blue light regulation of phototropism. The cryptochromes and phytochromes also contribute to the phototropic response, but so far no direct effect has been shown to be mediated by them (Lin, 2000).

To conclude, the cryptochromes mediate blue light regulation, especially in seed-

ling de-etiolation, in timing events such as flowering and, more generally, through the circadian oscillator (Fig. 2). Phototropin has only been found to mediate phototropic response (Fig. 2).

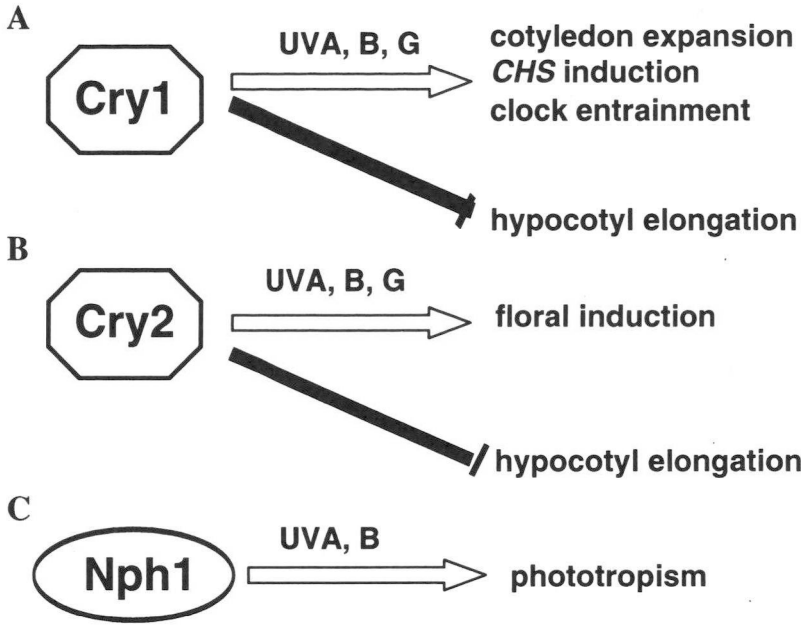

Fig. 2. Cryptochrome and phototropin mediated responses. A), Cryptochrome 1 (cry1) mediated responses. B), Cryptochrome 2 (cry2) mediated responses and C), phototropin (nph1) mediated response. *CHS*, chalcone syntase gene (see Fig. 2, p.). Active wavelengths mediated by the receptors are: UVA; B, blue and G, green light. The figure is partly adapted from Chory (1997).

Photoperiodic detection

The predictable daily cycles of light and dark provide eukaryotic organisms and some prokaryotes signals to synchronise growth processes to conditions that are most favourable. These organisms not only respond to light signals, but can also anticipate the onset of light, using a biological clock that is synchronised to the day/night cycle. This endogenous, daily (or "circadian") oscillator keeps the plant in time with its environment, mediating the rhythmic regulation of many processes. Rhythms of leaf movement and stomatal opening, for example, are regulated to place the leaves in a horizontal position during the day, to maximise light capture and to optimise gas exchange, respectively. In addition to the daily regulation this oscillator also participates in the detection of photoperiod and the timing of seasonally regulated events (reviewed by McClung, 2000). A principal model of the circadian clock is shown in Fig. 3.

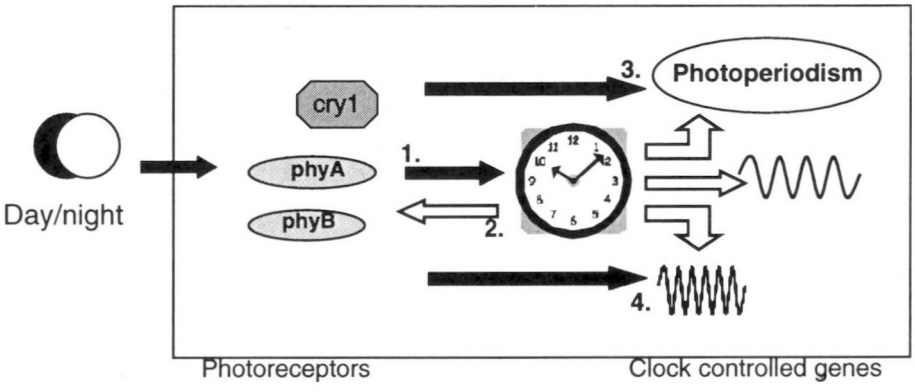

Fig.3. A conceptual model of a circadian system in a plant cell. The circadian oscillator is depicted as a clock **1.** Light mediated entrainment (or "resetting") of the clock, mainly mediated through the photoreceptors phyA, phyB and cry1. **2.** Clock gating (or regulation) of circadian sensitivity of the input pathway. **3.** Photoperiodic time measurement e.g. circadian regulation of the sensitivity to inductive light. **4.** Dual control, there is both a circadian and light regulation of the indicated gene's expression. The model is partly adapted from Kondo and Ishiura (1999).

Circadian rhythms share similar properties in all organisms: they persist under constant environmental conditions with a period close to 24 hours, and are reset (or "entrained") by light-dark transitions. Our understanding of the circadian clock mechanism at the molecular level is rapidly evolving and our knowledge of its biochemical properties has been greatly assisted by the recent identification and cloning of mutants aberrant in clock-related functions of the fly *Drosophila melanogaster*, the fungus *Neurospora crassa* and the plant *Arabidopsis thaliana*, amongst other organisms (Millar, 1999; Strayer and Kay, 1999). In animals it has been shown that there are many peripheral clocks capable of entraining their own rhythms, as shown for dissected organs, but they too are influenced and entrained through coupling to central clocks, such as the mammalian suprachiasmatic nucleus (SCN) in the hypothalamus (Green, 1998; Yamazaki et al., 2000). No main clock setting of gene

expression has been shown in plants, in fact parts of the same organ can be entrained *in planta* to different times (Thain et al., 2000), and several photoreceptors have been shown to mediate the light entrainment of the clock.

In order to study regulation of circadian gene expression a chlorophyll a/b binding protein promoter fused to the reporter gene luciferase (*CAB2::LUC*) has been the main tool. The function of individual receptors is investigated through monitoring the expression of the reporter gene in response to different light qualities and in different mutant backgrounds. Using this strategy, phyA has been demonstrated to mediate low intensity FR light and low intensity B together with cry1, while phyB is relaying high intensity R light (Somers et al., 1998). These authors also suggest that the cry2 protein mediates signals from very low blue light intensities. Typically, light information delivered to the clock alters the level of critical clock components such as *PERIOD* (*PER*) in *Drosophila*, and the chemical basis of the clock is thought to depend on the rhythmic accumulation of RNA transcript and protein of central clock components including the PER and TIMELESS (TIM) proteins (Green, 1998). Recent findings in mouse, where the clock function appears to be dependent on Cry1 and Cry2, which is homologues to the plant cryptochromes, suggest an additional role for the photoreceptors in the circadian mechanism itself (van der Horst et al., 1999). Structural similarities between photoreceptors and putative clock genes have also been described recently. These genes typically contain a PAS domain, as found in phytochromes or a degenerate variant of this kind of motif, like the LOV domains of nph1 (Taylor and Zhulin, 1999). The PAS domain has also been shown to be essential *in vitro* for interaction between phytochrome and a potential signalling partner, PIF3 (Ni et al., 1998). Further, both phyA and phyB have been shown to be translocated to the nucleus in response to inductive light qualities, as reviewed in Reed (1999). Both findings argue for phytoreceptors having a potential function in the circadian clock. To find out if individual photoreceptors are implicated in the actual mechanism a first step could be to investigate whether the expression of the receptor is regulated in a circadian fashion, as would be predicted for a putative clock gene or a downstream signalling molecule. Indeed, the expression of *PHYB* has recently been found to be regulated in such a manner, both at the RNA and the protein levels (Bognar et al., 1999). This shows that besides providing input signals to the clock, phyB has a function in the circadian output pathway. In general, no such circadian expression has been detected for other phytochromes despite the possibility being explored in several investigations (Bognar et al., 1999, and references therein).

In the photoperiodic regulation of flowering a new link to phyB has recently been discovered. A mutant impaired in R light de-etiolation was cloned and found to be identical to a previously described mutant named *gigantea* (*gi*) (Fowler et al., 1999; Huq et al., 2000; Park et al., 1999; and references therein). This mutant shows a significant delay in flowering under LD but not under SD, and its gene product is thought to be important in the photoperiodic regulation of flowering in *Arabidopsis*. The expression of the *GI* gene has been shown to have circadian regulation: its

expression changes in response to daylength, being higher late in LD photoperiods than in SD (Fowler et al., 1999; Park et al., 1999). In the cell the protein has been found to be associated with the nucleoplasm (Huq et al., 2000). Further, the mutation reduces the expression of several genes linked to the clock: *CIRCADIAN CLOCK ASSOCIATED 1*, *CCA1* and *LATE ELONGATED HYPOCOTYL*, *LHY* (Fowler et al., 1999). Also, Fowler et al found that *GI* shows arrhythmic expression in an *early flowering 3* (*elf3*) background (Zagotta et al., 1996). The *elf3* itself is impaired in the input or regulation of circadian signalling, showing arrhythmic *CAB2::LUC* expression, so *GI* expression seems to be regulated via ELF3 (Fowler et al., 1999). A range of other flowering time genes have been isolated and described in *Arabidopsis,* some of which are reviewed by Piñeiro and Coupland (1998). However, their individual roles in this process and their relation, if any, to the actual clock mechanism remain to be determined.

Shade avoidance

As mentioned earlier (p. 12), the shade avoidance response is a strategy whereby plants extend towards better light conditions in shaded or competitive environments. Hence, this response is most strongly developed in angiosperms adapted to open habitats (Smith, 1995). *Arabidopsis* is typical of plants with this shade avoidance ability, and at present three phytochromes seems to mediate the response in this species, namely phyB, phyD and phyE (Devlin et al., 1998; Devlin et al., 1999; Whitelam et al., 1998). Recent progress in linking phytochrome regulation to the actual growth response includes the isolation and characterisation of some homeodomain-leucine zipper (HD-zip) transcription factors (Ruberti et al., 1991; Schena and Davis, 1992). One of the first genes to be isolated encoding HD-zip proteins was *Athb-2* (also known as *HAT4*) (Ruberti et al., 1991; Schena and Davis, 1992). Its expression was found to be highly up-regulated in leaf tissue, together with that of a closely related gene, *Athb-4*, in response to FR light treatment (Carabelli et al., 1993). Further studies of *Athb-2* expression have clearly shown that its expression is induced in response to FR light and that this is mediated through a phytochrome other than phyA or phyB. EOD FR also strongly induces *Athb-2* and the expression is maintained, albeit at a lower level in the *phyB* background, in line with the role of phyB in shade avoidance. Both experiments show that phyB and other phytochromes, possibly including phyD and/or phyE, mediate the increase in *Athb-2* expression in parallel to inducing shade avoidance, thus coupling the expression with the response (Carabelli et al., 1996). In contrast, a brief exposure of etiolated seedlings to R or FR light will inhibit elongation growth and promote de-etiolation. Consequently, the cited authors found that *Athb-2* expression was repressed under these light conditions. It was observed that the repression was mediated through phytochromes but not through phyA or phyB.

The role of *Athb-2* in *Arabidopsis* growth responses has been studied in antisense and over-expressing transformants (Steindler et al., 1999). These studies confirm that this transcription factor is involved in shade avoidance, and define a role for it in hypocotyl elongation and the secondary growth of stem and root tissues. Over-

expressing plants showed decreased expansion of cotyledons, both in length and width, their hypocotyls were elongated (since their cells were longer) and their root masses were reduced. The reduction in root mass was due to both reductions in the number of lateral roots and to the main root being shorter and thinner. Conversely, all of these phenotypic features were reversed in the antisense *Athb-2* plants. The growth effects found were suggested to result from an *Athb-2* promoted change in auxin response, since many of the features altered by *Athb-2* expression could be mimicked by applying auxin inhibitors (Steindler et al., 1999). The facts that several of the features affected by *Athb-2* expression, and that the shade avoidance phenotype of the *Arabidopsis phyB* mutant can be phenocopied by gibberellin treatments (Reed et al., 1996), was unfortunately not discussed or addressed by the cited authors. Light regulated growth has previously been coupled to both brassinosteroids and gibberellins (Chory and Li, 1997; Kamiya and Garcia-Martinez, 1999) and more recently to auxins (see references in Morelli and Ruberti (2000).

Gibberellins and their Regulation of Growth

Biosynthesis

Gibberellins (GAs) are a large group of terpenoid compounds found in both plants and fungi. Today more than 120 GAs have been identified, of which only a few are considered to be biologically active. The active GAs have 19-carbon atoms (C_{19}-GAs), with a hydroxyl group at carbon-3β (GA_1, GA_3, GA_4, GA_7 and GA_{32}) or an unsaturated carbon-3 atom (GA_5). Their activities have been defined in bioassays and they are described as plant hormones (Lange, 1998).
The first GAs were discovered in the 1930s and isolated from the fungus *Gibberella fujikoroi*. This fungus infects rice, causing it to hyper-elongate. The resulting state is called bakanae disease and the elongation is an effect of massive GA production by the fungus (Hedden and Proebsting, 1999).

The importance of GAs in regulation of growth and development has been nicely shown by GA-deficient mutants with very low levels of bioactive GAs due to mutations in biosynthetic genes. These mutants from species such as pea, *Arabidopsis* and maize are dwarfs with short internodes, reduced leaf size and delayed flowering time. They are often male sterile but, if seeds are produced, they are usually found to be impaired in germination (Hedden and Proebsting, 1999). All these defects can be reversed by application of a bioactive GA species. At the cellular level GAs have been shown to induce cell elongation as well as cell division (Daykin et al., 1997; Kende and Zeevaart, 1997). In trees this is well illustrated since GAs increase cell division in both the apical meristem and cambium, resulting in cell production from these meristems, and to increase the length of the xylem fibres (Digby and Wareing, 1966; Eriksson et al., 2000; Hansen et al., 1999; Ridoutt et al., 1996; Wareing, 1958).

Gibberellins are synthesised in fungi and plants via the isoprenoid pathway, from which several groups of compounds, such as steroids and carotenoids are derived.

Geranyl geranyl disphosphate (GGPP) is a branching point compound, common to the biosynthesis of both carotenoids and gibberellins since it is a substrate for both phytoene synthase in the carotene pathway and copalyl diphosphate sythase (CPS) in the GA pathway (Hedden and Proebsting, 1999; Mende et al., 1997). The cyclisation of GGPP takes place in proplastids and requires the CPS to form the intermediate copalyl diphosphate and *ent*-kaurene synthase (KS) to form *ent*-kaurene (Aach et al., 1997). *ent*-kaurene is converted to the bioactive GAs by a series of oxidative reactions (Hedden, 1997; Lange, 1998). The first part of the pathway from *ent*-Kaurene takes place at the endoplasmatic reticulum, where it is oxidised in six steps by cytochrome P450-dependent monooxygenases giving rise to GA_{12}, which is 13-hydroxylated to GA_{53}. The conversion of GA_{12}-aldehyde to GA_{12} and the 13-hydroxylation giving GA_{53} have also been reported to be catalysed by soluble 2-oxoglutarate dependent dioxygenases (2-ODDs). The subsequent steps involved in GA biosynthesis and deactivation are also carried out by 2-ODDs. The dioxygenases of GA biosynthesis are often found to be multifunctional, having broad substrate specificity resulting in the numerous GAs found in higher plants. The majority of 2-ODDs, including the ones involved in GA biosynthesis, use 2-oxoglutarate as a co-substrate, while Fe^{2+} and ascorbate increase their activity. GA_{12} and GA_{53} are the first compounds in the parallel non-hydroxylation and early 13-hydroxylation pathways (Fig. 4), respectively, and they are oxidised by two dioxygenases to their respective bioactive GAs: GA_4 and GA_1. In these paths, GA 20-oxidase converts the methyl group of C-20 first to an alcohol, then an aldehyde, and then removes the C-20 altogether to form a γ-lactone in the remaining C_{19}-gibberellin. Next the GA 3β-hydroxylase introduces a hydroxyl group at the 3β position of the compound, giving it its activity. Deactivation of active GAs is carried out by a third group of 2-ODDs, the GA 2β-hydroxylases, by hydroxylation at the 2β position (Hedden, 1997; Hedden and Kamiya, 1997; Lange, 1998).

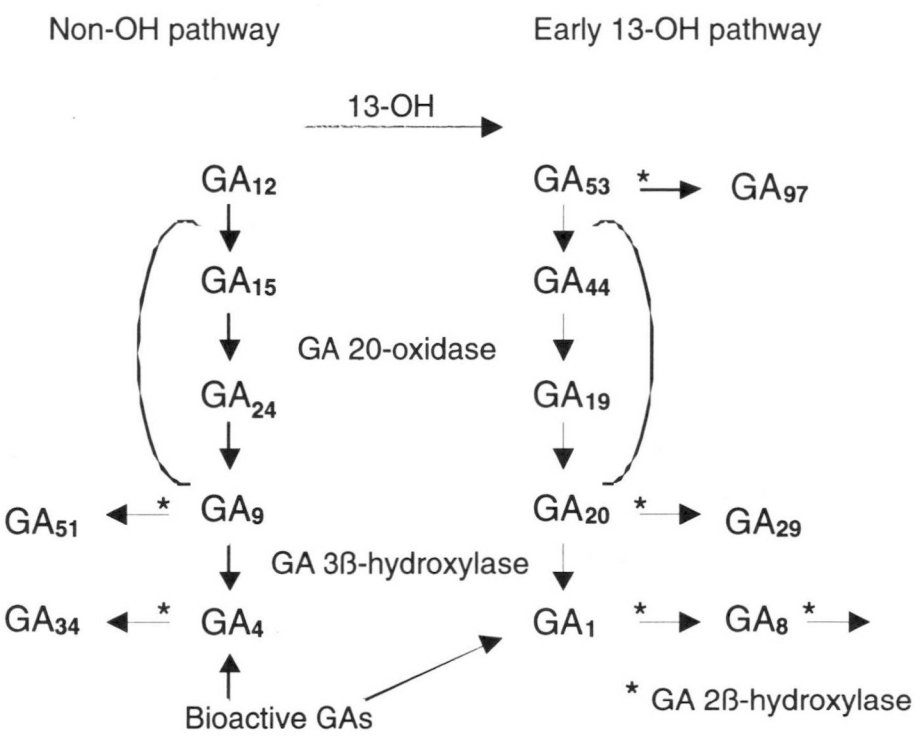

Fig. 4. The non-hydroxylated and early 13-hydroxylated GA biosynthetic pathways. The three oxidations carried out by GA 20-oxidase are in brackets, the last step giving active GAs is mediated through GA 3 ß-hydroxylase. The steps which are catalysed by 2 ß-hydroxylase are indicated (*).

Transcriptional control of biosynthetic genes

Feedback regulation

Being such powerful growth promoters the regulation of GAs is under strict control. The transcription of the central enzymes GA 20-oxidase and GA 3β-hydroxylase have both been shown to be regulated by bioactive GAs via negative feedback (Bethke and Jones, 1998; Hedden and Proebsting, 1999), by photoperiod and by certain light qualities (Kamiya and Garcia-Martinez, 1999). In addition, their expression is also developmentally regulated (Garcia-Martinez et al., 1997; Itoh et al., 1999; Kang et al., 1999; Phillips et al., 1995; Rebers et al., 1999). Uncoupling the transcriptional control of GA 20-oxidase by over-expression has been shown to greatly increase the biosynthesis of active GAs and thereby to increase growth (Carrera et al., 2000; Coles et al., 1999; Eriksson et al., 2000; Huang et al., 1998).

Genes encoding GA 20-oxidases and GA 3β-hydroxylases have been cloned from various species (Carrera et al., 1999; Chiang et al., 1995; Garcia-Martinez et al., 1997; Itoh et al., 1999; Kang et al., 1999; Lange et al., 1994; Lester et al., 1997; Martin et al., 1997; Phillips et al., 1995; Proebsting et al., 1996; Rebers et al., 1999; Toyomasu et al., 1998; Toyomasu et al., 1997; Wu et al., 1996; Xu et al., 1995). In many of these plants, negative regulation by bioactive GAs such as GA_3 and GA_4 at the level of transcription has been described. For example, the transcription of the *GA4* and *GA5* genes of *Arabidopsis,* encoding GA 3β-hydroxylase and GA 20-oxidase, respectively, is inhibited by bioactive GAs (Chiang et al., 1995; Cowling et al., 1998; Phillips et al., 1995; Xu et al., 1999; Xu et al., 1995). The expression patterns of these genes following the spraying of 100 µM GA_4 was studied in stems of the Landsberg *erecta* (L*er*) ecotype (WT), and the mutants *ga4* and *ga5* (Xu et al., 1999). The expression of *GA5* was higher in the mutants compared to WT, and highest in the *ga4* mutant. Upon GA_4 application the transcript level of *GA5* was reduced in all three genotypes. Further, application of the inactive 20-carboxylic acid, GA_{17}, did not down-regulate *GA5* expression in the *ga4* mutant. The cited authors also over-expressed the pumpkin gene *P16* (*CmGA20ox1*) in *Arabidopsis,* resulting in a diversion of GA biosynthesis from the bioactive GAs to the inactive 20-carboxylic acids GA_{25} and GA_{17}. The *GA4* and *GA5* expression patterns in these plants proved to be greatly up-regulated, but despite the reduced levels of active GAs found in them, their growth was almost like WT. The over-expression of the same pumpkin gene in the plant *Solanum dulcamara* reduced the active GA_1 in favour of the inactive GA_{17}, but it did not reduce the level of GA_4 (Curtis et al., 2000) The lower GA_1 levels, however, increased the transcription from the endogenous GA 20-oxidase gene *SdGA20ox1* in a similar fashion to the results in *Arabidopsis.* However, these plants were semi dwarfed, and apparently unable to compensate for the growth inhibition by increasing GA 20-oxidase/ GA 3β-hydroxylase activity. The feedback effects of *GA4* were also investigated in the *ga1-3* mutant of *Arabidopsis* (Sun and Kamiya, 1994), which is deficient in copalyl diphosphate synthase, resulting in a severely dwarfed and male sterile plant. The effects of the

immediate precursor to GA_4, GA_9, and those of active GAs other than GA_4 were investigated too (Cowling et al., 1998). Again the feedback was shown to be due to active GAs, and was correlated with hypocotyl growth. These studies of the feedback control of the expression of *GA4* and *GA5* show it to be evoked only by physiologically active GAs, although not necessarily by a 3ß-hydroxylated GA.

Information on the feedback regulation of the GA biosynthetic genes has also been obtained from two different GA signalling mutants. The first are the GA insensitive (*gai*) *Arabidopsis* mutant: a semi-dwarf in which length is not restored by GA application (Koornneef et al., 1985; Peng et al., 1997). The second is *spindly* (*spy*) which mimics a plant fed with high concentrations of GAs, being pale and elongated, and is also insensitive to GA inhibitors (Jacobsen and Olszewski, 1993). Both *gai* and, surprisingly, *spy* show elevated transcript levels of *GA4* and no feedback regulation (Cowling et al., 1998). In contrast, the slender *spy*-like pea mutant *la cry*^{s}, does show lower transcript levels of GA 20-oxidase. This is the opposite to what was found for the *spy* mutant, which did not show any evidence of feedback on *GA4* expression. It is suggested therefore that there may be two subclasses of mutants; one mimicking WT receiving a non-saturating GA dose and the other like plants receiving a saturating dose. If so, *spy* would belong to the first category since it shows no feedback and *la cry*^{s} to the second, showing down-regulation of GA 20-oxidase transcript levels (Cowling et al., 1998). In GA perception the SPY protein has been suggested to be an activator of GAI, which acts as an inhibitor of GA-induced responses e.g. elongation. The action of SPY is inhibited by GAs. GAI also has a homologue, called RGA/GRS, acting at the same level as GAI (Bethke and Jones, 1998; Ogas, 1998). In conclusion, the transcript abundance of GA 20-oxidase and GA 3β-hydroxylase genes is inversely related to the GA responses, and their regulation is only susceptible to physiologically active GAs (Bethke and Jones, 1998; Cowling et al., 1998; Curtis et al., 2000; Xu et al., 1999).

Photoperiodic and light regulation

Long day plants (LDPs) that have a rosette growth habit under short days (SD) can be induced to elongate and flower by long day (LD) exposure. All LDPs will respond by increasing stem growth and flower formation after a SD to LD transfer, but the effect of a short LD exposure varies. Brief LD exposure will definitely transform *Silene armeria* from vegetative to flowering stages, but not necessarily spinach (Zeevaart et al.1990). However, transferring spinach plants from SD to LD will result in stem and petiole elongation. The same phenotype can also be obtained by applying GA_3 in SD conditions. Conversely, the same responses can be suppressed in LD by feeding the plant GA biosynthesis inhibitors, and released by simultaneously feeding inhibitor plus GA (Zeevaart et al.1990). The results of several studies imply that the C20-oxidation is the main step in the GA biosynthesis that is controlled by photoperiod (Gilmour et al., 1986; Wu et al., 1996; Xu et al., 1997; Xu et al., 1995). In spinach the GA 20-oxidase transcript levels are reduced in SD as compared to LD when trancript levels of GA 20-oxidase start to accumulate and elongation is induced (Wu et al., 1996). Similar results have been obtained in investiga-

tions of the regulation of GA 20-oxidase (*GA5*) transcript levels by photoperiod in elongating *Arabidopsis* stems (Xu et al., 1997; Xu et al., 1995), but no such regulation of the GA 3ß-hydroxylase (*GA4*) was found.

The light regulation of these enzymes has also been carefully studied in germinating *Arabidopsis* and lettuce, and also in pea seedlings (Ait-Ali et al., 1999; Toyomasu et al., 1998; Yamaguchi et al., 1998). In *Arabidopsis*, as in lettuce, germination is under the control of phytochromes, and R light will induce germination while FR light will inhibit the response if given directly after the R light pulse (Borthwick et al., 1952; Shinomura et al., 1996). Furthermore, germination can also be obtained by application of GAs, circumventing the requirement for R light (Ikuma and Thimann, 1960; Kanhn and Goss, 1957). In *Arabidopsis* the GA 3ß-hydroxylase gene, *GA4*, and its homologue, *GA4H*, are both activated by R light in germinating seeds. The *GA4H* expression was shown to be dependent on functional phyB, since it could not be induced by R light in a phyB-deficient background. The *GA4* was, however, still activated by R light in the mutant, indicating that its regulation is mediated through another phytochrome (Yamaguchi et al., 1998). Similar results were obtained in lettuce, where the expression of two GA 20-oxidase (*Ls20ox1* and *2*) and two GA 3ß-hydroxylase (*Ls3h1* and *2*) genes were studied in response to R and/or FR light (Toyomasu et al., 1998). The *Ls3h1* transcript was found to be under phytochrome regulation since it was up-regulated by R and down-regulated by FR light. The *Ls20ox1* and *Ls20ox2* genes were both induced by imbibition of the seed, but only *Ls20ox2* seemed to be affected by the light quality, since its expression was down-regulated by R light. This down-regulation of *Ls20ox2* by R light could either result from direct inhibition by phytochrome or be due to negative feedback by GA_1, which started to accumulate at the same time.

In de-etiolation of pea seedlings both phyA and phyB appear to regulate GA 20-oxidase transcription, according to studies in pea mutants deficient in these phytochromes. There was also a large increase of transcript corresponding to both GA 20-oxidase and GA 3ß-hydroxylase upon transfer of etiolated seedlings to the light (Ait-Ali et al., 1999; Gil and Garcia-Martinez, 2000). At the same time, the GA_1 levels dropped drastically, while those of the inactivated GA_8 species rose. The increased transcription of these genes is likely to be due to the decrease in GA_1, and subsequent release of the feedback inhibition (Ait-Ali et al., 1999; Gil and Garcia-Martinez, 2000; Kamiya and Garcia-Martinez, 1999). The observations also indicate that deactivation by GA 2ß-hydroxylase may be light regulated. From the case studies presented for the LDPs above, it is clear that phytochromes do regulate the expression of GA 20-oxidase and/or GA 3ß-hydroxylase in these species. It is also clear that the control depends on what developmental process is being considered. In germination, GA 3ß-hydroxylase has been shown to be regulated via phytochrome in both *Arabidopsis* and lettuce (Toyomasu et al., 1998; Yamaguchi et al., 1998). On the other hand, in de-etiolation only the GA 20-oxidase transcription seems to be regulated through phytochrome (Ait-Ali et al., 1999; Gil and Garcia-Martinez, 2000).

Photoperiodic control of GA 20-oxidase expression has also been reported in the short day plant (SDP) potato, *Solanum tuberosum* subsp. *andigena*. This potato is strictly dependent on SD for tuber formation, and SD treatment will also reduce GA activity in its leaves. Tuberisation is delayed by feeding bioactive GAs. Furthermore, a GA deficient mutant (with a suggested block at the hydroxylation of GA_{12} to GA_{53}) emphasises the importance of GAs in controlling tuberisation, since it can develop tubers even in LD (Carrera et al., 1999).

The SD dependence of tuberisation is lost in potatoes expressing phytochrome B (*PHYB*) in antisense orientation (Jackson et al., 1996), and in grafting experiments a transmissible inhibitor of tuber formation (from leaves) was shown to be missing in these transgenic potatoes (Jackson et al., 1998). Recently, antisense *PHYA* (phytochrome A) potato plants were shown to have earlier tuber formation in SD extended by FR (Yanovsky et al., 2000) and taken together these experiments indicate that both phyA and phyB control tuber formation. Tuber formation seems to be controlled through diurnal regulation of GA biosynthesis, and one specific GA 20-oxidase gene (*StGA20ox1*) seems to be a possible key regulator. Its expression increases in SD with night breaks (non-inductive for tuberisation) but not in SD (inductive) (Carrera et al., 1999), thus it shows a daylength dependent regulation of transcription. Recent experiments with sense and antisense *StGA20ox1* plants have corroborated its importance in GA homeostasis and for tuber formation (Carrera et al., 2000). Over-expressors were shown to have increased levels of active GAs, which delayed tuberisation, while under-expressors were shown to have lower levels of active GAs and earlier tuberisation. In potato, therefore, as in *Arabidopsis* and spinach, phyA and phyB seem to regulate GA 20-oxidase expression by means of photoperiodic regulation. The photoperiodic mechanism may differ between LDPs and SDPs, for instance LD induces flowering in *Arabidopsis* and pea (LDPs) and this process is delayed in mutant phyA plants (Reed et al., 1994; Weller et al., 1997; Whitelam et al., 1998), while the opposite is true for tuberisation in potato (SDP), which is promoted by SD and phyB deficiency (Jackson et al., 1996).

Growth Cessation in Deciduous Trees

Trees of boreal, temperate and subtropical regions are subjected to large seasonal variations in temperature and daylength. Hence, woody perennial plants have adapted to these conditions by evolving an annual growth cycle, alternating between active shoot growth and vegetative dormancy (endodormancy) that is closely synchronised with the seasonal changes of the local climate (Howe et al., 1999). In temperate and boreal trees the time of productive growth occurs between spring bud flush and bud set in the autumn. The timing of these processes is of the utmost importance for the survival of the plant, and for breeders the goal is to maintain an adequate level of dormancy to maximise wood quality and productivity without compromising survival (Rhode et al., 2000).

Populus spp. grow continuously in long days and the induction of endodormancy is directly coordinated with the end of the growth season by short days (Howe et al., 1999). Growth cessation, and the following bud formation, is a prerequisite for leaf abscission and the development of cold resistance, the last two phases also requiring a drop in temperature.

Although the process of growth cessation and bud formation is crucial for survival of deciduous trees in temperate and boreal conditions, little is known about the molecular mechanisms involved. The availability of photoperiodic ecotypes in *Salix pentandra* has allowed the physiological process to be studied, and it has been shown to be dependent on SD for cessation of growth and cold hardening (Junttila and Kaurin, 1990). In this species SD leads to a rapid reduction in GAs in the subapical region prior to any visible signs of growth cessation and bud set (Olsen et al., 1995). Growth cessation has earlier been shown to be suppressed by feeding bioactive GAs in SD (Junttila and Jensen, 1988). The SD induced reduction in GAs is paralleled by a reduction in mitotic activity in the subapical region, and if GAs are supplied to SD induced buds the activity increases several-fold (Hansen et al., 1999). These results imply that gibberellins are involved in the process of apical growth cessation, possibly by affecting the mitotic activity in the meristem

Daylength regulated traits have been shown to be mediated through the action of phytochromes, a finding that prompted us to study the effect of *PHYA* over-expression in hybrid aspen (Olsen et al., 1997, Paper I). These studies showed that *PHYA* overexpression affects daylength regulated growth in this species. The over-expressing plants were unable to sense and/or respond to daylength shortening and consequently to set buds or develop cold resistance. In these plants the biosynthesis of auxin and gibberellins was affected so that the level of the active species was reduced. The down-regulation in gibberellins that normally occurs when plants are exposed to SD did not take place, indicating a phytochrome effect on biosynthesis. These interesting findings were the first of their kind in trees, and set the scene for the subsequent studies that we conducted and which, together with Paper I form the basis of this thesis.

Aims of the Project

The aims of the work described in this thesis were to increase our knowledge about the growth and the photoperiodic regulation of growth in deciduous trees. The focus has been on both physiological and molecular mechanisms of the early induction of growth cessation. The main model system used in these studies was the hybrid aspen, *Populus tremula* L. x *P. tremuloides* Michx., and the following questions have been addressed:

- What phytochrome(s) mediate growth cessation?
- Is daylength detection changed when the expression of phytochrome is altered?
- Do changes in phytochrome expression alter the biosynthesis of GAs?
- Do increased or decreased levels of GAs affect the response to daylength?
- How is growth affected by increased or decreased GA levels in hybrid aspen?

Methodological overview

Techniques used

General technologies and plant transformation

The studies described in this thesis relied on standard molecular biology techniques, such as gene cloning, gene expression studies, PCR based methods, and heterologous protein expression in combination with fundamental biochemical methods. Further, analytical techniques such as HPLC and GC-MS have been used to measure GA metabolites and GC-MS to quantify IAA.

For transformation of hybrid aspen, sterile, *in vitro* cultured cuttings of clone T89 were used. Internodes from such plants were cut into pieces about 1 cm long and infected with *Agrobacterium tumefaciens* strains carrying the gene construct of interest according to a protocol developed by Nilsson et al. (1992). After the infection, cells that incorporated the new gene also carried resistance to certain antibiotics. A complete plant medium (Murashige and Skoog, MS) with plant hormones, sucrose and appropriate antibiotics was then used to select for the transformed cells, since only these cells would have the competence to develop into plants in the presence of the antibiotic. Selected, antibiotic resistant plants were further grown on an elongation-inducing medium without antibiotics. Cuttings from these elongated plantlets were then taken for rooting on a half-strength MS medium without plant hormones and sucrose. Cuttings of transformed plants were regularly transferred to this hormone-free medium, rooted and maintained *in vitro* or transferred to soil. Since the regeneration time of this species is at least ca. 10 years, we only worked with primary transformants. For details of these and other techniques used, see Papers I-VI.

Over-expression and antisense techniques

In our studies we used the promoter from Cauliflower Mosaic Virus (CaMV) 35S, giving a strong constitutive expression in most tissues. The expression pattern of this promoter has been carefully investigated in hybrid aspen and it has been shown to be fairly ubiquitous (Nilsson et al., 1992). The promoter was used to obtain both the over-expressing (sense) and antisense plants in the presented experiments.

Down-regulation of transcription and thus, hopefully, of the corresponding protein can be obtained by the use of antisense techniques. The antisense effect can be obtained by transcribing the target gene in the reverse of its wildtype direction (i.e. from its end to its start). The gene fragment in antisense transformations is inserted behind a strong promoter such as CaMV 35S and followed by a poly adenylation site. The gene product obtained from such a construction is believed to hybridise with the endogenous copy and affect RNA stability, transcription and/or translation directly, or to generate a signal for gene silencing and pathogen attack (Fagard and Vaucheret, 2000). This will result, normally, in a reduction or lack of transcript of

the target gene. This is a commonly used way to eliminate the function of genes in plants where screening for mutants is not possible. To be successful with this technique it is necessary to use a gene or a piece of a gene that has very high homology to the corresponding endogenous gene.

To express a gene that is not normally expressed, or to increase the level of expression of a gene, the whole coding region is usually fused behind a strong promoter. In this case a gene from any source can be used, and it may in fact be an advantage if the gene of interest has a low homology to the endogenous gene. If the gene is nearly identical to the normal gene it could cause down-regulation of the gene instead of over-expression, a phenomenon termed co-suppression (Fagard and Vaucheret, 2000), which is another technique, like antisense transformation, that can sometimes be used to obtain plants with a loss of function phenotype. Recently it has also been acknowledged that there are naturally occuring antisense transcripts in plants (Terryn and Rouze, 2000).

Results and Discussion

Phytochrome A Regulation of Growth

Endogenous expression and function

In order to study the endogenous *PHYA* in hybrid aspen, PCR with degenerate primers was used on genomic DNA to obtain a probe for screening. A genomic PCR fragment encoding phyA was obtained and cDNA clones corresponding to the *PHYA* were successfully obtained and characterised in a preliminary fashion (II). The corresponding gene was shown to be single copy, in agreement with what has been found in *Populus tricocarpa* (Howe et al., 1998). In *P. trichocarpa*, where the phytochrome family has been thoroughly characterised, there was found to be one *PHYA* and two *PHYB* loci.

The expression of phytochrome A in hybrid aspen (*PttPHYA*) is low but detectable throughout the plant (III). Its level of expression seems to be correlated with the level of light to which the various tissues were exposed, as shown in Paper III (Fig. 4A). The highest levels of the photoreceptor transcript are found in lower parts of the stem and in root tissue under long days. This is not a unique trait, however, since high levels of both the transcript and the protein have been found in the roots of light-grown oat, *Avena fatua*, seedlings (Wang et al., 1993). Investigations of Phytochrome A expression in various species, for example *Arabidopsis* (Sharrock and Quail, 1989), tobacco (Adam et al., 1994) and *Pharbitis nil* (Carter et al., 2000), have all shown transcription to take place in most tissues and to be down-regulated by light.

In the study of *PttPHYA* expression, the effect of photoperiod length upon its transcription was investigated by exposing plants to long (18 h) or short (10 h) days. In LD the main photoperiod of 10 h (Photosynthetically Active Radiation) was supplemented with low intensity light, roughly equivalent to 4 h of dawn and 4 h of dusk before and after the main light period. In the SD treatment no such extensions were given, but just the main photoperiod of 10 h. Plant tissues were sampled in the middle of the main 10 h photoperiods in both cases. These Northern studies (III, Fig. 4B) show that the expression differed substantially between treatments: the SD plants showing, in comparison to LD plants, a shift in expression from the lower parts of the plants to the apical part and to younger internode tissues. However, expression was very low in leaves in either case (data not shown). There are many possible reasons for this shift, one being that that it could be a direct effect of the duration of light exposure experienced by the tissues: a high level of apical expression persisting in SD, but not LD, since the tissue was exposed for a shorter time. A second possibility is that the expression could be subject to a circadian rhythm, and the difference in the timing of the lights-on signal could alter the circadian period, such that expression peaks at another time, which is observed as a change in the level of expression. As a comparison, the expression of *PHYB* in tobacco, which is known to have circadian regulation, peaks 4-6 hours after lights-on (Bognar et al., 1999), which is approximately when we find the high expression under short photoperiods for *PttPHYA*. The second hypothesis could be tested by following the expression of *PttPHYA* over 24 hours. However, circadian regulation of *PHYA* expression has not been reported in any species so far despite the possibility being investigated at both the RNA level (Adam et al., 1994; Kolar et al., 1995) and in *PHYA* promoter::luciferase studies (Kolar et al., 1998). A third hypothesis is that the spatial expression of *PttPHYA* could be regulated by daylength, and besides playing a role in daylength detection, the redistribution of transcripts could reflect some as yet unknown physiological function of phyA. It has been speculated that phyA could have a function in phloem loading (Adam et al., 1994) since it is reportedly associated with these elements in mature tobacco plants, but so far there are no firm data supporting this idea. From the LDPs *Arabidopsis* and pea it can be learned that phyA in the green plant affects their photoperiodically regulated flowering (see also Introduction, p. 12). Flowering in both species is dependent upon functional phyA which, in pea, has been postulated to counteract an inhibitor of flowering in response to LD (Reed et al., 1994; Weller et al., 1997; Whitelam et al., 1998). In the SDP potato, phyA inhibits the formation of tubers in long days (Yanovsky et al., 2000).

Thus it seems phyA has opposite roles in LDPs and SDPs. The induction of growth cessation in *Populus* spp. is dependent upon SD (as reviewed by Howe et al., 1999). The growth cessation response has been investigated in hybrid aspen by giving EOD FR treatments. These studies show the hybrid aspen responds to SD plus EOD FR by ceasing growth, and dormancy (I). Thus, growth cessation in this species seems to be a LDP response, i.e. LD inhibits growth cessation in it.

The absolute levels of *PHYA* expression affect the plant's ability to sense a decrease in daylength. This is shown by physiological data on growth of hybrid aspen in both Paper I and Paper III. The levels of *PttPHYA* detected in antisense lines are decreased in leaves of LD grown plants (III, Fig. 2). The general down-regulation of *PttPHYA* in the antisense plants was found to make plants more sensitive to short days, resulting in earlier growth cessation and bud set (III, Fig. 3, Table 1). Conversely, it was shown by ectopic expression of oat *PHYA* that high levels of expression (I, Fig 1C) of this gene make the aspen trees insensitive to daylength shortening (I, Fig 5). In conclusion we have shown by altering the levels of *PHYA* expression that the photoreceptor phytochrome A mediates the photoperiodic regulation of growth in hybrid aspen (I, III). The spatial pattern of *PttPHYA* expression, in addition to the level of expression, changes in response to the light conditions. Both the amount of light and the length of the photoperiod are likely to regulate the spatial distribution of its transcription. This conclusion is supported both by the light exposed tissues having lower expression of *PttPHYA* in LD (III, Fig. 4A) and by the up-regulation of expression seen in apices and stems in response to SD (III, Fig. 4B). The up-regulation in response to SD in these organs does not seem to have a counterpart in leaf tissues. Leaves are suggested to be the major receptor organ of the photoperiodic signal in trees and other plants (Thomas and Vince Prue, 1997). From this perspective it may only be the leaves that are of interest for the photoperiodic control, while the redistribution of transcript levels along the vertical plant axis could have other functions, which remain to be determined.

Ectopic expression

The oat *PHYA* was coupled to the strong constitutive promoter CaMV 35S, giving a strong constitutive expression in most tissues. Ectopic oat *PHYA* expression was shown to affect the whole appearance and growth pattern of the plant. The most apparent changes were the decrease in internode length and increase in anthocyanin levels that were found in all over-expressing lines. Dramatic effects on the growth of plants over-expressing the oat *PHYA* gene grown in white fluorescent light had already been reported, for example in *Arabidopsis* and tobacco (Boylan and Quail, 1991; Cherry et al., 1991). It has been suggested that this effect could be due to an accumulation of the more stable monocotolydenous oat phyA, which may not have been recognised by the endogenous deactivation machinery (Cherry et al., 1991). However, potato over-expression studies carried out using the endogenous *PHYA* have demonstrated that these plants also show reduced height and increased anthocyanin synthesis proportional to the amount of phyA expressed in the plant (Yanovsky et al., 1998). The short internodes found in the oat *PHYA* over-expressing aspen are the result of fewer cells, not of a reduction in cell length (I, Fig. 2). The short internodes are likely to result mainly from a decrease in gibberellin biosynthesis, since the levels of GA_{20} and GA_1 in the severe over-expressors (lines 6 and 22) in apical stem and leaf tissues are just 20-40% of the levels in control plants in LD. As shown in Paper IV (Fig. 5), the expression of the GA 20-oxidase (*PttGA20ox1*) is reduced in the strong oat *PHYA* over-expressor (line 6) as compared to trans-

formed controls. It has earlier been suggested that phyA controls the biosynthesis of GA 20-oxidase, based mainly on the results of over-expression (Chory and Li, 1997; Kamiya and Garcia-Martinez, 1999). The down-regulation of GA 20-oxidase transcripts found in our study, together with the fact that the short internodes can be restored by application of GA_4 (Eriksson and Moritz, unpublished), also support this notion.

Another interesting feature is that the levels of the auxin, indole acetic acid (IAA) are reduced in the over-expressors, most markedly in the stem of lines 6 and 22, which contain 30-50% of control plant levels. There is also a decrease of auxin in leaves, but generally the difference is much smaller. Why the IAA levels are down-regulated is not clear, but it may be a phyA-mediated effect. Auxin distribution has been suggested to be regulated in a light dependent manner in the shade avoidance response, and to be regulated through phytochromes other than phyA and phyB via *Athb-2* (Morelli and Ruberti, 2000) (see Introduction pp. 17-18). The ectopic expression of *PHYA* could potentially interfere with this regulation. Also, the cromophore mutant *pew1*, which is reduced in all phytocromes, and *pew2*, which is reduced in a photoreversible phytochrome in dark grown seedlings of tobacco, show increased levels (three- and two- fold, respectively) of free IAA (Kraepiel et al., 1995). Moreover, the efficiency of the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) has been shown to be light dependent and specifically mediated through cry1, phyA and phyB (Jensen et al., 1998).

In addition, there is cross talk between auxin and GAs. In pea, the biosynthesis of GA_1 from the substrate GA_{20} is dependent on the presence of auxins, at least in stem tissues (Ross et al., 2000). Conversely, increases in the levels of IAA can be promoted by increased levels of GAs (Law and Davies, 1990; and references therein). The reduction of IAA levels detected in the oat over-expressors could also be an indirect effect resulting from the reduction in growth that is typical for these plants.

Ectopic expression makes the plant insensitive to daylength changes, and not even a photoperiod of 6 h can induce cessation of growth in the over-expressors (I, Fig. 5). The WT and transformed controls stop growing at about 16 h, the cessation being very clear under a 14 h photoperiod (I, Fig. 5). In woody species the cessation of growth and bud set is a prerequisite for the ability to develop cold resistance (Junttila and Kaurin, 1990; Nitsch, 1957), hence the over-expressors are also aberrant in this respect (I, Table 1). As mentioned above (Introduction p. 25) growth cessation has been postulated to depend on the down-regulation of bioactive GAs. Therefore, the inability of *PHYA* over-expressors to stop growing under SD may be due to interference with the GA biosynthesis. It was shown that no down-regulation of auxin or GA biosynthesis takes place in response to SD in these plants (I, Fig. 6). The down-regulation of GA 20-oxidase (IV, Fig. 5), together with the data showing the endogenous GA 20-oxidase to be photoperiodically regulated in hybrid aspen (IV, Fig. 6A, B, Fig. 7), suggests that the ectopic expression of *PHYA* specifically interacts with the regulation of this biosynthetic enzyme.

In summary, the ectopic expression of *PHYA* and thus, possibly, increased phyA levels, interferes with the biosynthesis of auxin and GAs. The decrease in GA (or both GA and IAA) results in stunted growth via a shortening of the internodes, which can be reversed by GA application. Further, the transgenic plants cannot down-regulate GAs in response to a decreasing daylength. Thus, the plants maintain vegetative growth rates, and they are prevented from entering the growth cessation phase and developing cold resistance in response to SD. The effect of increased phyA levels on GA biosynthesis is here suggested to involve the regulation of GA 20-oxidase, possibly by both direct and photoperiodic mechanisms.

Gibberellin 20-oxidase and its Effect of GAs upon Growth

Endogenous expression and function

The possible role of GA 20-oxidase in SD regulation of growth suggested by Talon and Zeevaart (1991), the GA profiles of *S. pentandra* in response to SD (Olsen et al., 1995) and the cloning of GA 20-oxidases in *Cucurbita* (Lange et al., 1994) and *Arabidopsis* (Phillips et al., 1995; Xu et al., 1995) prompted us to clone GA 20-oxidase from hybrid aspen. First we obtained a genomic fragment of a putative GA 20-oxidase by PCR with degenerate primers. The genomic fragment was also used to screen cDNA libraries from leaf and cambium, and several cDNAs were isolated (IV). All clones were found to be identical apart from a three basepair (bp) deletion found in some of them. The deletion was not, however, found in the corresponding genomic DNA and may have resulted from an error in the cDNA synthesis at the library construction stage. The putative GA 20-oxidase was shown to hybridise to a single locus (*PttGA20ox1*) in the genome by Southern analysis (IV, Fig. 2). Southern hybridisation at low stringency also indicates that there may be additional GA 20-oxidase like genes in the genome (data not shown). The presence of a multigene family would be expected since this is the rule rather than the exception for this enzyme. As an example, at least three differentially expressed genes have been described in each case for *Arabidopsis*, bean, and potato (Carrera et al., 1999; Garcia-Martinez et al., 1997; Phillips et al., 1995; Xu et al., 1995). It is possible that we would have found more members of this family by screening for cDNAs in a more diverse set of tissues.

To probe the possible role of PttGA20ox1 in growth regulation we began by characterising the expression pattern of the corresponding gene by means of Northern analyses of actively growing plants under LD. All the GA 20-oxidases genes isolated so far have been shown to be expressed in many different tissues. In *Arabidopsis*, there are at least four gene members (Philips *et al.*, Poster abstract S35-31, 6th ISPMB Congress, 18-24 June 2000, Québec, Canada). Each of the three genes described thus far has a specific pattern of expression. *AtGA20ox1* is mostly expressed in the stem and inflorescence, *AtGA20ox2* is expressed in the inflorescence and developing silique, while *AtGA20ox3* is expressed only in the silique (Coles et al., 1999; Phillips et al., 1995). Hence there seems to be a developmentally regulated speciali-

sation within this gene family. We investigated *PttGA20ox1* expression only in vegetative tissues, and found it to be strongest in young and late expanding tissues such as stem and leaves, and in young roots (IV, Fig. 3 A, B). In contrast, the expression was very low in apices, petioles and mature fully expanded tissues. Overall, the level of expression generally is very low, but highest in leaves. The large number of clones found in the cambium library, together with Northern analyses from this area (data not shown), indicates that localised tissues can have higher expression levels. We carefully investigated the more specific expression in layers of a stem exhibiting secondary growth and in the apical meristem by means of a cDNA dot blot. The blot carried cDNA from a PCR-amplified transcriptome of differentiating phloem, cambium, enlarging xylem, maturing xylem and apical meristem respectively (IV, Fig. 4). The results show that there is indeed a high specific level of expression of *PttGA20ox1* in the primary and secondary meristems, since there were strong signals in the apical meristem and cambial region samples. We did not detect expression in the apical meristem with our Northern analysis, probably because the signal from the expressing cells was diluted by the non-expressing tissues. The indication that expression is strong in the apical meristem has recently been supported independently by analysis of *PttGA20ox1* promoter-GUS expression in hybrid aspen (Israelsson, Eriksson and Moritz, unpublished).

We also wanted to characterise the expression in response to SD by comparing RNA expression in plants grown under LD (18 h) and SD (10 h). In several species it has been shown that photoperiod regulates the level of GA 20-oxidase gene expression (Carrera et al., 1999; Wu et al., 1996; Xu et al., 1997). In aspen the expression was compared for late expanding leaves and internodes, as well as for young roots. We found that there was a clear down-regulation in leaves and a slight reduction in roots, while almost no detectable down-regulation was found in internodes (IV, Fig. 4 A, B). The expression of GA 20-oxidases in roots has only previously been reported for the bean clone Pv 15-11 (accession number U70531) (Garcia-Martinez et al., 1997), which is quite phylogenetically close to *PttGA20ox1* (IV). Recent proposals that roots have active GA biosynthesis and are the site of GA action have been supported by promoter-GUS and northern analysis of both *ent*-copalyl diphosphate synthase (CPS) from *Arabidopsis* (Silverstone et al., 1997) and GA 3ß-hydroxylase from tobacco (Itoh et al., 1999). These GA biosynthetic genes, which catalyse very early and late stages of synthesis, respectively, are both expressed in the tips of roots (including lateral roots). The GA-induced gene *GASA4* from *Arabidopsis* is also strongly expressed in these meristematic tissues, indicating that GAs are active in roots (Aubert et al., 1998). GA levels have been measured in roots of *S. pentandra* (Olsen et al., 1994) and hybrid aspen (IV, Fig. 7), and in both cases levels of GA_1 were found to be lower in roots than in shoot tissue by a factor of about ten. The amount of active GA needed for normal root elongation is much lower than for shoot elongation, as shown in pea (Tanimoto, 1994). There is also a mutant in tomato, *gib-1*, which is deficient in GAs due to an early block in the biosynthesis pathway. The mutant is impaired in root cell division, showing the physiological importance of GAs in the root (Barlow et al., 1991).

Moreover, on a well separated blot (IV, Fig. 6B) we detected two bands of about the same size as *PttGA20ox1* in all tissues, and a third larger band seen only in roots. The possible origins of the smaller bands and the larger band was investigated by means of 3'- and 5'-RACE. For this analysis the mRNA from the roots of LD grown plants that were used in the Northern experiment was used as the cDNA template. The results from 3'-RACE indicated that there may be an alternative poly adenylation site about 315 bp upstream from the cloned cDNA site, since we recovered many independent RACE clones with this shorter length. The origin of the larger 2.5-2.6 kbp band found only in roots has not yet been determined, we found many clones with extensions at their 5'-ends compared to the cDNA, but not as extensive as would be required for such a large band. The putative upstream poly adenylation site and the extra root band do not have any counterpart in any of the described GA 20-oxidase genes. Whether this has any physiological relevance is difficult to decide at present, but alternative transcripts are common in eukaryote mRNA, and may be important in translational control (Gibbs et al., 1998), in the stability of the transcripts, or for other regulatory functions.

In conclusion we found the expression of *PttGA20ox1* to be linked to meristems and late expanding tissues. The strong expression in young roots we hypothesise may be due to the high meristematic activity found in these tissues. The high level of expression could also result from possible derepression of the negative feedback on this gene by the lower GA levels found in tissues such as late expanding leaves and young roots compared to the very high levels found in the young expanding shoot tissues. We have shown that *PttGA20ox1* does indeed show negative feedback in response to applied GA_4 (10 µM), most clearly in expanding leaves and internodes (IV, Fig. 5). The negative feedback by active GAs is reviewed in the Introduction, pp. 22-23, and has been shown to be a general trait for most GA 20-oxidases (Carrera et al., 1999; Curtis et al., 2000; Kang et al., 1999; Martin et al., 1996; Phillips et al., 1995; Toyomasu et al., 1997; Xu et al., 1995).

To investigate the function of the putative GA 20-oxidase a GST fusion protein was expressed in *E. coli* and the purified lysate was tested with all possible substrates of the non-hydroxylated and early 13-hydroxylated pathways *in vitro* (IV). At the first attempt a clone with the three basepair deletion (which removes a centrally placed threonine) was unknowingly used, giving only a slow conversion of GA_{12} to GA_{15}, and virtually abolishing all GA 20-oxidase activity (Eriksson and Moritz, unpublished). When using the intact sequence, however, the approach was very successful. The results from these feedings showed the recombinant protein to have GA 20-oxidase activity on GA_{12}, GA_{15}-open lactone, and GA_{24} of the non-hydroxylated pathway as well as GA_{53}, GA_{44}-open lactone, and GA_{19} of the early 13-hydroxylated pathway (for a review of the pathways, see Introduction Fig. 4 p. 20). We also observed conversion of the GA_{15}-lactone, with long incubation times (IV, Table 1). The GA_{15}-lactone has not previously been shown to be a substrate of this enzyme, in contrast to the GA_{44}-lactone (Gilmour et al., 1987). Hence, PttGA20ox1 was shown to be a true GA 20-oxidase.

We next addressed the activity of the enzyme *in planta*, as measured by quantitative analysis of GAs. One could be led to think that the expression of the protein should be tightly correlated to the expression pattern at the RNA level, but this could be deceptive. Various factors important in the regulation of proteins are not detected by Northern analysis, such as transcript stability, post-translational modifications and substrate availability. In the case of the GA 20-oxidase, the negative feedback of the active species might also complicate the interpretation of expression analyses. We consequently investigated the levels of key metabolites in the same tissues sampled for the RNA analysis, in LD as well as in SD. In hybrid aspen GA_1 is the predominant, bioactive GA. We have shown this indirectly by demonstrating the early 13-hydroxylation pathway to be the dominant biosynthetic route in tracer experiments (IV, Fig. 8) and, further, by analysis of GA_9 (the precursor of GA_4), which is not detectable in WT controls (V, Table 1). The levels of the final GA 20-oxidase product, GA_{20}, were shown in LD to be present at high levels in all tissues but mature expanding, mature non-expanding leaves and petioles, and young roots. The highest GA_1 levels were also found in the younger tissues (IV, Fig. 7). Roots show, overall, low levels of GAs with about 10% of the GA_1 found in actively growing internodes. A short photoperiod seems to affect the GA 20-oxidase activity since we can detect a build up of GA_{19} after four short photoperiods. This is true for all tissues except the petioles, mature fully expanded tissues and young roots (IV, Fig. 7). SD also induces lower levels of GA_{20} in all tissues but roots, indicating that there is still a turnover of this species by GA 2β-hydroxylase and/or 3β-hydroxylase under SD. The metabolite pattern in the roots is distinct from the patterns in the other tissues, since there is a build up of GA_{19} in LD. The GA_{19} accumulation is not reflected in any downstream increase of GA_{20}, and is accompanied by only a slight increase in the deactivation of GA_{20} to GA_{29}. Where the unaccounted surplus of GA_{19} ends up is impossible to conclude, but there may be a net export from young roots to other tissues. In pea, Proebsting et al. (1992) have shown by grafting studies with the GA deficient mutant *le* and WT, that GA_{20} can be easily transported. No transport of GA_{19} was noted in this study but it is not unlikely to occur, since it is a quite polar species. Also, GA_{19} has been found to be easily transported when fed to aspen plants (Moritz, unpublished).

We have shown *PttGA20ox1* to encode a functional GA 20-oxidase with all of the earlier reported characteristics such as negative feedback by active GAs and photoperiodic regulation. It also displays some additional characters, having multiple transcripts, high expression in young roots and a potential to metabolise GA_{15} in its lactone form *in vitro*. When we compare the location of GA metabolites with the expression of the gene, the patterns do not correlate entirely. This shows that there may be differences between detected transcript levels and protein activity at the same location, as exemplified by the high levels of GA 20-oxidase expression, but very low levels of metabolites, in late expanding leaves and young roots (IV, Fig 3A) (IV, Fig. 7). This discrepancy may be the result of a net export of metabolites from these tissues, or their low levels may be due to fast turnover of the protein. Alternatively, they may result from a low availability of substrates and/or enzyme

activity. Whatever the cause, the highest expression of *PttGA20ox1,* which is found in mature expanding leaves, is not correlated with high levels of GA metabolites.

Ectopic expression

In order to find out more about the role of GAs and, specifically, the constraints of GA biosynthesis, we expressed a GA 20-oxidase gene from *Arabidopsis*, *AtGA20ox1*, in hybrid aspen. The full *AtGA20ox1* cDNA (At2301; Phillips et al., 1995) was inserted behind the CaMV 35S promoter and was used to transform hybrid aspen. Out of 14 independently obtained transformants, 10 were selected for further characterisation and growth analysis. All showed stable integration of the *AtGA20ox1* by Southern blotting (data not shown), and a very high level of expression at the RNA level in Northern analysis (V, Fig. 2). Even *in vitro* these plants showed features typical of GA over-expressing plants, with a pale, slender phenotype characterised by elongated internodes and petioles as well as smaller, narrower than WT leaves. The rooting capability was highly reduced, which became obvious when the plantlets were transferred to soil, since their poor rooting was the main obstacle to establishing them in soil. The decreased initial rooting capacity resulted in a much lower survival rate (32%) for transgenes compared to WT control plants (100%).

The levels of the active GAs, both GA_1 and GA_4, were strongly increased in all these transgenic lines (V, Table 1). As stated above, the early 13-hydroxylated pathway is the major route of GA biosynthesis in aspen. The non-hydroxylated GA pathway resulting in GA_4 is the most important in *Arabidopsis* and GA_{12} has been reported to be the preferred substrate for AtGA20ox1 (Phillips et al., 1995). Hence it seemed likely that the protein resulting from the expression of *AtGA20ox1* would have a high affinity for GA_{12}, and also divert some of the GA produced along this pathway when expressed in aspen. The strong expression of *AtGA20ox1* does, indeed, give an increased flux through both of the pathways, resulting in high levels of both GA_4 and GA_1 (V, Table 1). GA_4 has activity when applied to hybrid aspen since internode length is regained in oat *PHYA* transgenes following this treatment (Eriksson and Moritz, unpublished) and it induces feedback inhibition of *PttGA20ox1* (IV, Fig. 5). However, it is not known if there are any qualitative differences between GA_1 and GA_4 with respect to growth.

When established, all the transgenic lines showed dramatic increases in elongation (resulting from increased internode elongation) and in overall growth compared to WT (V, Fig. 3A). The number of nodes was the same in transgenes and WT, but the number of cells per internode was about 55% higher for both epidermal and pith cells in the former (V, Fig. 4 B). This increase in cell number indicates that division in the apical meristem increases in response to the higher levels of GAs found in these plants. It also agrees with the higher cell division rates found when GAs are applied to SD induced buds of *S. pentandra* (Hansen et al., 1999), and the reduced rates found in oat *PHYA* over-expressors, which have decreased GA levels (I, Figs. 2, 3). The effect of GAs on mitotic activity has been thoroughly demonstrated, especially for cell division in rice intercalary meristem (Kende and Zeevaart, 1997;

Lorbiecke and Sauter, 1998). No effect on the length of epidermis and pith cells was detected in our study of the effects of the *Arabidopsis AtGA20ox* gene in aspen (V, Fig. 4 A), our oat *PHYA* over-expressor study (I, Fig. 2) or in the *S. pentandra* experiment (Hansen et al., 1999). The increases in bioactive GAs also give increased rates of cell division at the secondary meristem, the cambium, leading to increased diameter growth.

The *AtGA20ox1* over-expressing plants investigated show a dramatic rise in xylem fibre number: a 71% increase compared with the numbers in WT (V, Fig. 4 C). Interestingly, the fibres are not only more numerous, they are also about 8% longer than in WT (V, Fig. 4 D). The effect of GAs on fibre differentiation and elongation was reported very early by Digby and Wareing (1966) and Wareing (1958), who concluded that IAA and GA have a synergistic action on fibre differentiation and elongation from application studies using *Populus robusta*. Digby and Wareing (1966) also found that no fibre elongation took place in the absence of IAA and that fibre length increased with increased GA application only in the presence of IAA. The optimal IAA concentration was, however, reached at 100 ppm, higher concentrations being shown to be inhibitory, while GA was shown to still increase fibre length at 500 ppm. The effect of GAs on fibre length has also been investigated by Ridoutt et al. (1996). These authors showed (by applying GA biosynthesis inhibitor to growing *Eucalyptus globulus*) that fibre length was positively correlated with GA levels.

The role of GAs on fibre development has not been as intensively studied as the effect of IAA. However, none of the IAA experiments, to our knowledge, have shown such a clear effect on both fibre numbers and length. Our study is the first in which the levels of GAs have been modified with transgenic technology, thereby increasing the endogenous levels of bioactive GAs and avoiding the problems of concentration control and localisation that might arise upon exogenous application of hormones or inhibitors. In the strongest GA 20-oxidase over-expressors there is an increase of about 20-fold increase for both GA_1 and GA_4 in stem tissue, while the increase in IAA levels is about two-fold (Eriksson and Moritz, unpublished). It is likely that this promotes the formation of fibres, since it has been shown that both auxin and GAs are required for the process (Digby and Wareing, 1966). If GAs play only a minor role compared to IAA *in planta* it is difficult to explain why effects on fibre production as strong as those seen in the GA over-producers have not been observed earlier e.g. in IAA-overproducing aspen (Tuominen et al., 1995).

The stimulatory effect found in the GA over-producers on both elongation and diameter growth, is also reflected in an increase in weight both on a fresh and dry matter basis (V, Table 2). The overall increase in dry weight for shoot tissues of transgenic plants was 64%, but if only the stems are compared it was even higher, at 126%. Furthermore, even though root growth was initially weak in the over-expressors, there was no statistical difference in root mass of established WT and over-expressors on a dry matter basis.

Interestingly enough we also noted a difference in leaf development between the genotypes (V, Table 2). We could show by careful measurements of two independent transgenic lines and WT plants, that the leaf elongation was increased in transgenes in the early developmental stages compared to WT. In later stages the leaf to width ratio was similar to WT, except that transgenic plants had both longer and broader leaves. The expansion of leaves has been shown to be regulated by two independent and polarised processes. These have been coupled to three main genes in *Arabidopsis*, affecting leaf length *ROTUNDIFOLIA* (*ROT1*, leaf expansion; *ROT3*, short leaf blade) and leaf width *ANGUSTIFOLIA* (*AN*; narrow leaf) (Tsuge et al., 1996). The relation between the products of these genes and GAs is not known. Our plants, however, show an interesting phenotype, which indicates that GAs can affect the elongation of leaves both length- and width-wise at different developmental stages.

In summary, we have shown that by expression of GA 20-oxidase under a constitutive promoter, and thus uncoupling it from endogenous regulation, GA biosynthesis is increased. The constraints on the over-expression, set by the capacity of the deactivating enzymes, GA 2β-hydroxylase and GA 3ß-hydroxylase, which catalyse the conversion of GA_9 or GA_{20} to GA_4 and GA_1, respectively, seem to be minor. Possibly the most limiting factor in our experiment was the trade-off between high GA levels and survival of the transgenic lines, which inevitably selected against the most extreme phenotypes *in vitro*. The take home message from our ectopic expression study in aspen, as well as the studies in *Arabidopsis* and potato, is that GA 20-oxidase is really a key, regulatory enzyme in the biosynthesis of GAs. It is likely to be the most important when trying to increase GA levels, since the effects of overproduction of other GA biosynthetic enzymes, like the downstream GA 3β-hydroxylase, would still be limited by the negative feedback of active GA on the GA 20-oxidase. The GA 20-oxidase also catalyses as many as three steps in GA biosynthesis, which comprise a major part of the committed GA pathways. Further, the increased levels of GAs resulting from over-expression, besides confirming the role of GAs in processes like elongation growth, give new information on the GA effect in secondary growth and fibre development, as well as its intriguing effect on leaf development.

Growth Cessation and GAs

Growth cessation in plants with altered GA levels

In our studies of GA biosynthesis, we have focussed on the GA 20-oxidase since it is likely to be the key enzyme regulating biosynthesis in LD (V). We have also shown it to be photoperiodically regulated (IV). Previously, the role of GAs in cessation of growth in deciduous trees has mainly been studied by observing the effects of exogenous applications. However, by constructing plants over-expressing GA 20-oxidase, and thus GAs, we have obtained the means to study the effect of high levels of GAs *in planta* (V). With the cloning of the endogenous GA 20-oxidase gene (IV) we also provided the possibility of creating GA 20-oxidase antisense plants with reduced levels of GAs. For the *PttGA20ox1* antisense construct a 1252 bp

fragment of the cDNA clone was introduced in a reverse direction behind the 35S CaMV promoter of the vector pPCV702.kana. The antisense construct was further used to transform hybrid aspen (VI). Sixteen independent transformant lines were obtained, all of which showed slow growth and short internodes in tissue culture compared to WT controls. We characterised six of the lines and found that they had all incorporated the antisense construct, having one to three insertions per line (VI, Fig. 1). With Northern analysis no significant down-regulation was detected (data not shown). The low expression of the endogenous gene makes it difficult to detect minor changes, so this experiment needs to be repeated. The transformants we studied probably have only a subtle down-regulation of the gene since we can only detect small differences in GA synthesis under LD (VI, Table 1), which do not cause any obvious growth retardation in these conditions (data not shown). We are probably selecting against severe phenotypes, since even the obtained lines are significantly more slow-growing than WT under *in vitro* conditions.

When studying this gene we are also working against the transcriptional feed-back control systems that will try to compensate for any decrease in bioactive GAs by increasing transcription (see Introduction p. 21-22). Indeed, it has been shown in *Arabidopsis* by Coles et al. (1999) that it is difficult to down-regulate GA 20-oxidase by antisense techniques. In potato, Carrera et al. (2000) were very successful in obtaining antisense *StGA20ox1* plants, but the low percentage (20%) of recovered plants they obtained with reduced expression indicate that it was problematic.

Under SD conditions we could detect clear phenotypic differences in the antisense lines compared to WT. All transgenic lines were more sensitive towards SD, resulting in an earlier cessation of growth and bud set (VI, Table 2). The response is significant since in experiment 2, for example, 43% of WT and 100% of antisense line 5 plants had formed an apical bud after 27 days in SD (VI, Table 2; Exp. 2). The difference was also confirmed by anatomical inspection of stem sections, which showed antisense line 5 to have dormant cambium prior to WT plants (VI, Fig. 4). We investigated the response of GA over-producing plants in the same experiment, but none of these plants showed any growth cessation or bud set at this point (VI, Table 2). The levels of GA_{19}, GA_{20}, GA_1, and GA_8 were measured in the three genotypes (antisense, over-expressors and WT) in LD and in response to two and six SD photoperiods. These measurements showed that there was a substantial reduction in GA biosynthesis in antisense and WT plants, possibly mediated through the decreased GA 20-oxidase activity that we also have demonstrated (IV, fig 7). Moreover, the antisense line analysed in this way (line 2) has lower than WT levels of all of the measured GA species. The plants over-expressing GA 20-oxidase show about 80 times higher than WT levels of bioactive GA_1 after two SD, and only a moderate down-regulation of GA_1 after six SD (VI, Fig. 2A).

In parallel to the SD treatment we grew a set of the same genotypes in SD plus end-of-day FR (EOD FR). This treatment changes the photoequilibrium of phytochrome from the FR light absorbing (Pfr) toward the R light absorbing (Pr) form, which is

supposed to be the inactive form, at least for light stable phytochromes such as phyB. The EOD FR treatment would therefore be expected to mimic the loss of function of these phytochromes (Reed et al., 1994). This treatment has earlier shown the photoperiodic regulation of growth cessation not to be mediated by phyB in hybrid aspen (Olsen et al., 1997a), and this was confirmed in these experiments since the plants stop growing and set bud at the same time in SD, irrespective of the FR treatment (VI, Table 2). What could be detected, however, were dramatic increases in GA contents and elongation rate following EOD FR treatment as compared to only SD (VI, Fig. 2A). The strongest response was shown by the antisense plants, while the over-expressors did not respond at all. The overall increases in length in SD plus EOD FR relative to SD were 79% and 41% for antisense line 2 and WT, respectively. The reduction of GA_{19} and increased GA_{20} and GA_1 levels of the antisense line 2 and WT suggest that GA 20-oxidase activity increased in response to EOD FR. Since the over-expressor already has a constitutive expression of this enzyme it is logical that it gives no response to this treatment and further supports the conclusion.

We speculate that the apparently conflicting data from SD and SD plus EOD FR can be explained by two independent regulatory mechanisms. First, the down-regulation in response to SD could result purely from photoperiodic regulation of GA 20-oxidase, possibly mediated through phyA. The EOD FR effect, on the other hand, may result from down-regulation of GA 20-oxidase transcription through a stable phytochrome (possibly phyB). The FR treatment will inhibit the repression of GA transcription by phyB, giving increases in GA biosynthesis and subsequent elongation. A suppression of transcription of phyB would also explain the data from aspen over-expressing oat *PHYA*. These plants are dwarfed and non-responsive towards photoperiod, but when subjected to SD with EOD FR they elongate in a WT fashion, without affecting their impaired daylength response (I, Fig. 4). The transcript level of endogenous GA 20-oxidase has been investigated in LD in these over-expressors and was found to be down-regulated (IV, Fig. 5). Following the same reasoning, this repression is likely to have resulted from the fact that the more stable oat phyA is able to substitute for photostable phytochrome (Cherry et al., 1991). The repression of GA 20-oxidase transcription would consequently be released by EOD FR, resulting in GA biosynthesis and elongation. The photoperiodism would not be affected, however, since its regulation likely lies elsewhere. The increased levels of GA_1 of antisense plants in comparison to WT remains to be explained, but presumably the transcript inhibition and the potential of feedback by active GAs are lower in the antisense plants. To probe these questions it would be interesting to investigate the transcript levels in two SD photoperiods as compared to two SD plus EOD FR and to investigate the feedback response in the antisense plants.

In short, by investigating the response to SD of plants with increased or reduced levels of active GAs we can show that cessation of growth is dependent on proper regulation of GA biosynthesis. We also confirm the importance of the role of GA 20-oxidase. We have earlier shown phyA to mediate the cessation of growth in as-

pen (I, III), and the response of antisense plants, with increased daylength sensitivity, is the same for both phytochrome A and GA 20-oxidase. Further, we have shown that it is likely that phyB or some other photostable phytochrome represses GA 20-oxidase transcription, but it seems not to affect the photoperiodic regulation of growth cessation in this species.

Conclusions

• Phytochrome A mediates daylength control of growth cessation - as shown in the results of daylength experiments using WT, *PHYA* over-expressors and antisense plants (Papers I and III).

• GAs are part of the growth regulation machinery under both LD and SD and the ability to regulate GA levels is crucial, especially for the timing of growth cessation (Papers I, V and VI).

• GAs seem to act on mainly meristems (apical meristem and cambium), specifically affecting cell production, and thus controlling growth in hybrid aspen (Papers I and V).

• The enzyme GA 20-oxidase is a major factor in the control of GA biosynthesis in aspen, regulated at the transcriptional level through a photoperiodic mechanism, and probably light qualities as well as by the active $GA_{1/4}$ (Papers I, IV, V and VI).
For an overview, see Fig. 5, p.42.

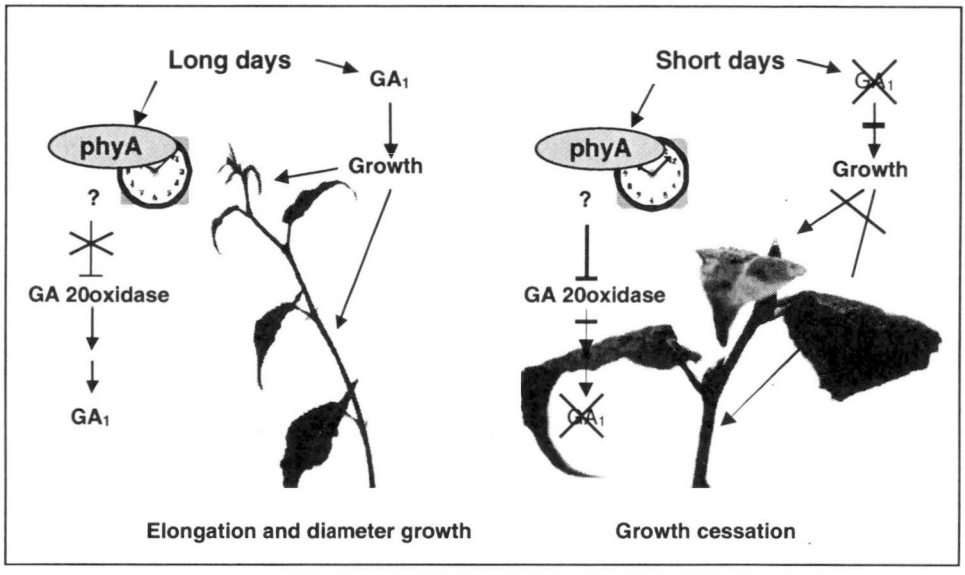

Fig. 5. Tentative model of daylength dependent regulation of growth through GAs. The growth response exerted through the action of GA(s) in response to photoperiod is shown to the right in A) and B), respectively. A possible model of regulation by daylength through phyA and/or the circadian clock on GA biosynthesis is shown to the left in both A) and B) respectively. Arrows indicate a positive regulation, while T-bars show repressive effects. Crosses (X) show loss of that specific regulation or product. Blocks in the signalling are indicated by (-).

Sammanfattning

Den här avhandlingen behandlar tillväxtreglering i lövträd, speciellt tillväxtavslutning som induceras av korta dagar. Det modelsystem som vi har använt oss av är hybridasp (*Populus tremula* L. x *P. tremuloides* Michx.). Hybridasp kan lätt modifieras m h a transgen teknik, något som vi har utnyttjat genom att öka eller minska uttrycket av vissa "nyckelgener".

De gener som vi har studerat är ljusreceptorn fytokrom A (phyA) och gibberellin 20-oxidase (GA 20-oxidase), den senare är ett enzym med stor betydelse för syntesen av växthormonet gibberellin (GA). Ljusreceptorn phyA medierar troligtvis information med betydelse för daglängdsreglerad tillväxt, emedan GA är involverad i den fysiologiska responsen lite längre ned i signalkedjan.

Vi har undersökt växtens normala uttryck av de här generna liksom växter som har ökat eller minskat uttryck. Målet har varit att studera de tidiga tillväxtavslutnings responserna, fysiologiska såväl som molekylära.

Studier av hybridasp med ökad eller minskad mängd phyA visar att just den här ljusreceptorn har betydelse för kortdagsreglerad tillväxtavslutning. Växter med ökat uttryck kan ej förmås att sluta växa ens vid mycket korta dagar, emedan de med lägre uttryck slutar att växa tidigare än normala, oförändrade plantor. GAs betydelse för tillväxtavslutning har också undersökts. Aspar med lägre halter av GAs slutar att växa tidigare i korta dagar, det omvända gäller för de med förhöjda halter som slutar att växa senare än normalt.

Vidare resulterar förhöjd GA produktion i en kraftigt ökad tillväxt. De plantor som har förhöjda halter har snabbare längd och diameter tillväxt än oförändrade aspar. Den ökade tillväxten beror av ökad cell produktion i det apikala meristemet och i kambium. De har även förändrade vedegenskaper såsom fler (71%) och längre (8%) fibrer jämfört med kontroll plantor.

Modifieringen av GA mängder uppnåddes genom att specifikt förändra uttrycket av gener som kodar för enzymet GA 20-oxidase. Våra studier visar att enzymet är ett begränsande steg i syntesen av GA i asp. Enzymet är också reglerat på transkriptionell nivå av både dagslängd och mängd aktivt GA_4. Därför drar vi slutsatsen att GA 20-oxidase troligen är ett av de viktigaste stegen för GA reglerad tillväxt och tillväxtavslutning.

Våra studier har kastat nytt ljus över det tidiga skedet av tillväxtavslutning i lövträd, speciellt m a p phyA och GAs roll. De har också gett ny kunskap om GAs betydelse för tillväxt som sådan, med viktiga tillämpningar speciellt för vedbildning.

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