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ORIGINAL ARTICLE

Maria E. Eriksson · Thomas Moritz

Daylength and spatial expression of a gibberellin 20-oxidase isolated from hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.)

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Abstract Physiologically active gibberellins (GAs) are key regulators of shoot growth in trees. To investigate this mechanism of GA-controlled growth in hybrid aspen, we cloned cDNAs encoding gibberellin 20-oxidase (GA 20-oxidase), a key, highly regulated enzyme in the biosynthesis of GAs. Clones were isolated from leaf and cambium cDNA libraries using probes generated by polymerase chain reaction, based on conserved domains of GA 20-oxidases. Upon expression in *Escherichia coli*, the GST-fusion protein was shown to oxidise GA_{12} as well as oxidising the 13-hydroxylated substrate GA_{53}, successively to GA_9 and GA_{20}, respectively. The gene *PttGA20ox1* was expressed in meristematic cells and growing tissues such as expanding internodes, leaves and roots. The expression was negatively regulated by both GA_4 and overexpression of phytochrome A. RNA analysis also showed that the expression was down-regulated in late-expanding leaf tissue in response to short days (SDs). Actively growing tissues such as early elongating internodes, petioles and leaf blades had the highest levels of C_{19}-GAs. Upon transfer to SDs an accumulation of GA_{19} was observed in early elongating internodes and leaf blades. The levels of C_{19}-GAs were also to some extent changed upon transfer to SDs. The levels of GA_{20} were down-regulated in internodes, and those of GA_1 were significantly reduced in early expanding leaf blades. In roots the metabolites GA_{19} and GA_8 decreased upon shifts to SDs, while GA_{20} accumulated slightly. The down-regulation of GA 20-oxidase activity in response to SDs was further indicated by studies of [^{14}C]GA_{12} metabolism in shoots, demonstrating that the substrate for GA 20-oxidase, [^{14}C]GA_{53}, accumulates in SDs.

M.E. Eriksson · T. Moritz (✉)
Umeå Plant Science Centre,
Department of Forest Genetics and Plant Physiology,
Swedish University of Agricultural Sciences,
901 83 Umeå, Sweden
E-mail: thomas.moritz@genfys.slu.se
Fax: +46-90-7865901

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Abbreviations GA: gibberellin · GA 20-oxidase: gibberellin 20-oxidase · GST: glutathione *S*-transferase · IPTG: thiogalactopyranoside · LD: long day · phyA: phytochrome A · RACE: rapid amplification of cDNA ends · SD: short day

Introduction

Gibberellins (GAs) are a group of more than 100 naturally occurring plant and fungal products, a few of which are powerful growth promoters in plants. GAs have been implicated in a number of developmental processes such as seed germination, elongation growth, flowering and fruit development (Hooley 1994; Bethke et al. 1997). On the cellular level there is evidence that GAs affect both cell division and cell expansion (Daykin et al. 1997; Kende and Zeevaart 1997).

Studies of GA-biosynthesis mutants, and of cloned genes encoding enzymes involved in the biosynthesis of active GAs and their precursors, have elucidated many features of the GA-biosynthesis pathways, especially in *Arabidopsis*, pea, maize and pumpkin (for reviews, see Hedden and Kamiya 1997; Hedden and Philips 2000). The multifunctional 2-oxoglutarate-dependent dioxygenase, GA 20-oxidase is a key enzyme in controlling GA biosynthesis. It catalyses the stepwise conversion of the C-20 gibberellins, GA_{12}/GA_{53}, by three successive oxidations to GA_9/GA_{20}, which are the immediate precursors of the active gibberellins GA_4 and GA_1, respectively.

Genes encoding GA 20-oxidases have been cloned from various plant species, and have often been demonstrated to be part of a small multigene family. These enzymes have been suggested to be key regulators of GA biosynthesis. The expression of the genes are down-regulated by the action of $GA_{1/4}$ in several species,

suggesting that direct end-product repression is involved in regulation of the GA 20-oxidase gene (Phillips et al. 1995; Xu et al. 1999). They have also been suggested to be controlled by photoperiod (Xu et al. 1997) and light (Ait-Ali et al. 1999). The importance of the enzyme as a regulator of GA levels has been shown in transgenic plants constitutively overexpressing GA 20-oxidase. Increased GA levels have been found to be associated with accelerated growth in *Arabidopsis* (Huang et al. 1998; Coles et al. 1999), potato (Carrera et al. 2000) and hybrid aspen (Eriksson et al. 2000), indicating that GA 20-oxidase limits the production of active GAs in these plants.

In woody species the role of GAs in controlling growth and development has been less extensively studied than in many herbaceous species. However, it has been suggested that, amongst other things, GAs play an important role in the process of short day (SD)-induced growth cessation in such plants. Many woody species with indeterminate growth show complete cessation of elongation growth after only a few weeks in short photoperiods. Studies of these phenomena in the deciduous tree *Salix pentandra* have shown that the initiation of growth cessation by short photoperiods is preceded by a reduction of GA_1 in the subapical region (Olsen et al. 1995). After exposing seedlings to just five SDs, before visible reductions of growth had been observed, the levels of GA_1 had decreased by 50% within a specific region of the apex that normally contains the highest levels of this compound. Further evidence suggesting that GAs are involved in SD-induced growth cessation in trees has been provided by transgenic hybrid aspen overexpressing the oat phytochrome A (PHYA; Olsen et al. 1997). These transgenic trees do not sense short photoperiods, and are also apparently unable to down-regulate their GA levels in response to them.

In order to understand how GA metabolism is coupled to regulation of growth in trees, e.g. the photoperiodic control of growth, the cloning of genes involved in GA biosynthesis is essential. We hereby describe the cloning of a GA 20-oxidase cDNA from the model tree system, hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.). Following heterologous expression in *E. coli*, we showed that the cDNA product is a functional GA 20-oxidase. We have also investigated expression profiles of the corresponding gene, and its expression responses towards GA_4 and photoperiodic changes.

Materials and methods

Plant material

For preparation of genomic DNA, plants of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) clone T89 were grown in a greenhouse with day and night temperatures of 20 °C and 15 °C, respectively, and an 18-h photoperiod. For total extraction of RNA and quantification of GA, hybrid aspen was grown in a growth chamber as previously described (Eriksson et al. 2000). Plants were grown under long days (LDs) of 18 h: 10 h main photosynthetic period (300 μmol photons m^{-2} s^{-1} at 400–750 nm) and 8 h extended low-intensity light (20 μmol photons m^{-2} s^{-1}) (Osram Power Staw HQI-TS 400 W/D lamps). Samples in LDs were taken from 11-week-old plants. The remaining plants were transferred to SDs (10 h) and harvested after 4 days (except root samples for GA analysis, which were taken after five SDs). All samples were harvested in the middle of the photoperiod. Samples were collected from plants 1.7–1.9 m tall, from the apices and internodes 9, 27 and 68, together with adjacent leaf blades and petioles as well as actively growing roots. The sampling positions corresponded to tissues that were early expanding, late-expanding and mature non-expanding, respectively. Tissues from two plants were harvested and pooled for each sample. The 'first internode' was defined as the first internode below a leaf at least 1 cm long. Plants to be GA-treated were grown in LDs in a greenhouse, decapitated just above internode 7 and the excised stem and leaf tissue was placed in a solution containing 10^{-6} M GA_4 in half-strength MS medium (Sigma), pH 5.7, for 24 h. Plants overexpressing the oat *PHYA* (line 6) and the control (line 8) were grown in a growth chamber in LDs and samples of actively growing, expanding internodes with adjacent leaves were taken. GA_{12} metabolism was studied by growing plants in a growth chamber in LDs or SDs as described above, decapitating them above internode 7 and allowing the excised stem and leaf tissue to take up 3,333 Bq of [17-^{14}C]GA_{12} (1,936 GBq mol^{-1}; Prof. Lew Mander, Canberra, Australia) in 500 μl half-strength MS medium, (pH 5.7) by transpiration for 30 min. The stems were then left in half-strength MS medium for 24 h, after which the metabolites were extracted essentially as described by Peng et al. (1999). The metabolites were separated by HPLC, monitored by an on-line radioactivity detection system and identified by GC-MS following methylation and trimethylsilylation.

DNA isolation and PCR with degenerate primers

Genomic DNA was extracted from leaf tissue of hybrid aspen using the hexadecyl-trimethylammonium bromide (CTAB) method (Doyle and Doyle 1990). Degenerate primers designed for amplifying GA 20-oxidases were obtained as a gift from Dr. P. Hedden (IARC LARS, Long Ashton, Bristol, UK). The primers encoded the conserved domains KLPWKET (sense) and HRAVVNS (antisense; Curtis et al. 2000). The primers incorporated a *Hind*III and an *Xba*I site at their 5′-ends, respectively, to facilitate cloning. DNA fragments were amplified by PCR using these primers and 250 ng hybrid-aspen genomic DNA as template under standard conditions, as described for *Taq* polymerase (Amersham Pharmacia Biotech), in a DNA Thermal Cycler (MJ Research, Watertown, Mass., USA). Samples were heated to 94 °C for 2 min then subjected to 39 cycles of 94 °C for 1 min, 50 °C for 2 min and 72 °C for 1 min, concluding with a further 5 min at 72 °C. The resulting PCR products were digested using *Hind*III and *Xba*I and ligated into the vector pUC 19, and subsequently transformed into XL1 Blue MRF′ cells (Stratagene) and sequenced.

Isolation of cDNA clones

Two different cDNA libraries from hybrid aspen were screened: one in λZap (Stratagene) constructed from mature leaves (Larsson et al. 1997) and one in λ gt11 (Gibco BRL Life Technologies) from the cambium region (Hertzberg and Olsson 1998). Positive plaques were identified using the PCR-derived genomic fragment as a probe. Membranes were pre-hybridised prior to addition of the probe for 1 h and hybridised overnight at 65 °C in Denhardt's solution [0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone and 0.1% (w/v) bovine serum albumin (BSA) in 5×SSPE with 0.5% (w/v) SDS]. They were then washed for 10 min, at 65 °C, in two changes of each of the following buffers: 2×SSPE (300 mM NaCl, 20 mM NaH_2PO_4, 4 mM EDTA, pH 7.4), 1×SSPE, 0.5×SSPE and 0.1×SSPE, all in 0.1% (w/v) SDS. Membranes were

autoradiographed for 1–4 days. Positive signals were re-screened as above until the plaques were pure. Positive clones from the leaf library were obtained by plasmid rescue as described in the manufacturer's (Stratagene) manual. Cambial clones were used for lambda DNA preparation as described in Current Protocols (Ausubel et al. 1995), digested with *Sal*I and *Not*I and subsequently ligated into the pOK12 vector (Vieira and Messing 1991). The ligation mixture was further used to transform XL1 blue MRF′ cells, and positive clones were selected and sequenced.

DNA gel blot analysis

Digested genomic DNA samples, 12 μg per sample, were separated by electrophoresis in a 0.7% (w/v) agarose gel, blotted onto nylon membranes (Hybond-N; Amersham) and cross-linked by UV-light (SpectroLinker XL-1000; Spectronics Corporation, Westbury, N.Y., USA). Pre-hybridisation and hybridisation steps were performed in Church buffer [Church and Gilbert 1984; 1% (w/v) BSA, 1 mM EDTA, 0.5 M Na_2HPO_4, 7% (w/v) SDS] overnight at 65 °C. The probe involved was the full-length *PttGA20ox1* cDNA, randomly labelled using the Ready-to-Go kit (Amersham Pharmacia Biotech) and purified by passage through NICK columns (Amersham Pharmacia Biotech). Membranes were washed at 65 °C in 2×SSPE, 1×SSPE, 0.5×SSPE, and 0.1×SSPE (all in 0.1% SDS; two 3-min washes for each solution), and were then exposed to Phosphoimager screens (Molecular Imager GS-525; Bio-Rad Laboratories). Hybridisation to the cDNA dot blot was done as described above, using an *Eco*RI/*Xba*I fragment specific for the 3′-end of *PttGA20ox1*. After the probe had been stripped from the membrane by boiling 0.1×SSPE, 0.1% SDS, it was re-probed with a conserved fragment of actin from *Populus trichocarpa* (a gift from Dr. Antje Rohde, University of Gent, Belgium).

Poly(A)$^+$ isolation and RNA gel blot analysis

Total RNA was extracted using a CTAB method (Chang et al. 1993), followed by poly(A)$^+$ purification on oligo (dT)-cellulose type 7 (Amersham Pharmacia Biotech) according to the manufacturer's recommendations, except that LiCl was substituted for NaCl. Poly(A)$^+$RNA (3–8 μg, see descriptions of specific blots) was loaded onto formaldehyde-agarose gels and separated according to Sambrook et al. (1989), followed by capillary blotting onto nylon membranes (Hybond-N; Amersham). The blots were probed with purified α-[^{32}P]dCTP (799 MBq nmol^{-1}; Amersham)-labelled DNA fragments, which were hybridised, washed and exposed to Phosphoimager screens as described for the DNA blots. Northern blots were probed with the *Eco*RI/*Xba*I fragment specific for the 3′-end of *PttGA20ox1*, stripped (as described above) and reprobed with the ubiquitin-like expressed sequence tags (ESTs), A046p07u (*PttUBQ1*) or A081p57u (*PttUBQ2*) (Sterky et al. 1998).

Rapid amplification of cDNA ends (RACE)

Adaptor-ligated cDNAs were obtained using a Marathon cDNA Amplification kit (Clontech Laboratories) and the poly(A)$^+$RNA extracted from LD-grown roots as a template. Subsequent PCR amplifications were prepared using an Advantage cDNA PCR kit (Clontech), following the manufacturer's protocol. The specific primers were the antisense primer complementary to nucleotide positions 842–871 and the sense primer at positions 679–706 for the 5′- and 3′- RACE of the *PttGA20ox1*, respectively. Either primer was used in conjunction with the adaptor primer 1 supplied by the manufacturer, and touch-down PCR was performed as described for a hot-lid thermal cycler. PCR fragments were isolated and purified prior to ligation in the TA-cloning vectors pGEM-T Easy (Promega, Madison, Wis., USA) or pDK 101 (Kovalic et al. 1991). The ligation mixture was used to transform XL1-blue MRF′ cells, and positive clones were isolated and sequenced.

DNA sequence analysis

Both strands were sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer) an automated sequencing system (ABI PRISM, model 377; Perkin Elmer) and M13/pUC forward and reverse sequencing primers. The results were analysed by the supplied program, AutoAssembler version 1.4 (Perkin Elmer). Derived protein sequences were analysed by the BLAST algorithm (Altschul et al. 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), and the highest scoring sequences (26) were fetched and protein comparisons processed by BEST FIT and PILEUP, Wisconsin Sequence Analysis Package version 8 (Genetics Computer Group, Madison, Wis., USA). Phylogenetic analysis was done according to the legend to Fig. 1.

Heterologous expression in *E. coli*

A full-length, cloned cDNA was expressed in *E. coli* as a fusion protein, after eliminating an upstream ATG and positioning the cDNA in-frame via a PCR approach. This strategy deployed a forward primer that has a *Sal*I and a *BamH*I restriction site inserted into its 5′-end and spanning over nucleotides 279–296, and a reverse primer complementary to the nucleotides 505–534, including an internal *Hind*III site. Both were used in a PCR reaction with a full-length *PttGA20ox1* cambial cDNA clone as template. The product was purified and subsequently filled in, digested with *Hind*III, purified and ligated between the *Hind*III and *Sma*I sites of pUC 19. The ligation products were trans-

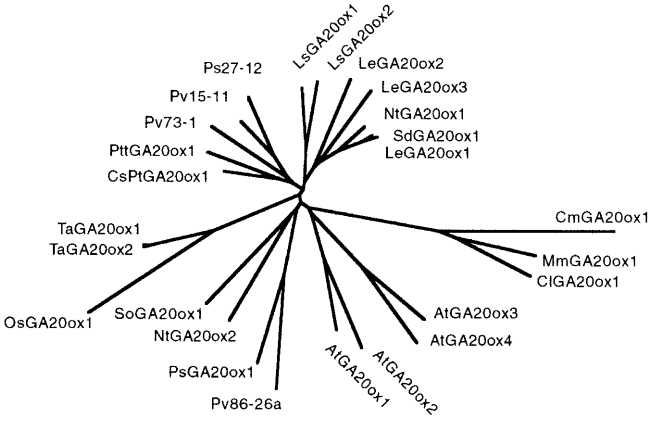

Fig. 1 Phylogenetic analysis using the deduced amino acid sequences of cloned GA 20-oxidases from diverse species. The tree was generated from PILEUP alignments using DISTANCES (Kimura protein distances matrix) and GROWTREE (the neighbour-joining method) in the GCG package (Genetics Computer Group, Madison, Wis., USA). The tree was plotted using TREEVIEW PPC. Shown are LsGA20ox1 and 2 from lettuce (*Lactuca sativa*); LeGA20ox1, 2 and 3 from tomato (*Lycopersicon esculentum*); NtGA20ox1 (accession number AB012856) and NtGA20ox2 (AB016084) from tobacco (*Nicotiana tabacum*); SdGA20ox1 from *Solanum dulcamara*; CmGA20ox1 from pumpkin (*Cucurbita maxima*); MmGA20ox1 from *Marah macrocarpus*; ClGA20ox1 from water melon (*Citrullus lananus*); and AtGA20ox1, 2, 3 and 4 (AC018908.8) from *Arabidopsis thaliana*. Pv15-11, Pv73-1 and Pv86-26A are GA 20-oxidases from French bean (*Phaseolus vulgaris*); PsGA20ox1 (U58830) and Ps27-12 are from pea (*Pisum sativum*); SoGA20ox1 is from spinach (*Spinacia oleracea*); OsGA20ox1 is from rice (*Oryza sativa*); TaGA20ox1 (AB05555) and TaGA20ox2 (Y14008) are from wheat (*Triticum aestivum*); CsPtGA20ox1 is from the *Citrus* hybrid *Citrus sinensis* × *Poncirus trifoliata*; and PttGA20ox1 is from hybrid aspen (*Populus tremula* L. × *P. tremuloides*)

formed into XL1 blue MRF′ cells and the target clones were identified. The selected clone was confirmed by sequencing, then digested with *BamH*I and *Hind*III and co-ligated with a *Hind*III and *Not*I fragment (representing the 3′-end of the cDNA) into the *BamH*I and *Not*I sites of the expression vector pGEX-4T-2 (Amersham Pharmacia Biotech). The expression vector thus derived, pGEX-GA20ox1, was introduced into *E.coli* host XL1 Blue MRF′ and incubated overnight. A single colony from this construct was inoculated into 1×LB medium with carbenicillin (50 μg ml^{-1}) and 2% (w/v) glucose, and grown overnight. From the overnight culture, 6-ml portions were inoculated into 250 ml LB (total volume) with carbenicillin, and grown at 30 °C, agitated by rotation at 180 rpm. When the OD at 600 nm reached 0.6–0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM, to induce expression in half of the cultures. After a further 3 h of growth, cells were harvested by centrifugation and protein lysate from induced and non-induced material was obtained and stored as previously described (Lange et al. 1994).

Enzyme activities

Cell lysates were incubated at 30 °C for 10 min with 4,167 Bq of [17-^{14}C]GA_{12}, [17-^{14}C]GA_{15}, [17-^{14}C]GA_{15}-open lactone, [17-^{14}C]GA_{24}, [17-^{14}C]GA_{44}, [17-^{14}C]GA_{44}-open lactone, [17-^{14}C]GA_{53}, or [17-^{14}C]GA_{19} (approx. specific radioactivity 1,900 GBq mol^{-1}; Prof. Lew Mander, Canberra, Australia). [17-^{14}C]GA_{15}-open lactone and [17-^{14}C]GA_{44}-open lactone were prepared by treating [17-^{14}C]GA_{15} and [17-^{14}C]GA_{44}, respectively, with 2 N NaOH at 21 °C for 30 min. In additional assays, [17-^{14}C]GA_{15}, [17-^{14}C]GA_{44} and the corresponding open lactones were also incubated with cell lysate at 30 °C for 22 h. Enzyme activities were assayed in 100 mM Hepes buffer (pH 7.6) and a cofactor solution mixture (final concentration 40 mM ascorbate, 40 mM α-ketoglutarate, 2 mM $FeSO_4$ and 2 mg ml^{-1} catalase). Lowering the pH to 3 with acetic acid stopped the enzyme reaction. The products were applied to a 100 mg C_{18}-column (Isolute; Sorbent AB, Västra Frölunda, Sweden), pre-washed with methanol and equilibrated with 1% (v/v) aqueous acetic acid, and the sample was eluted with 2 ml of methanol. For metabolic profiles, metabolites were separated by HPLC, monitored by an online radioactivity detection system and identified by GC/MS following methylation and trimethylsilylation.

Quantification of GAs

GA levels in samples of 200–300 mg fresh weight were analysed as described earlier (Peng et al. 1999) by GC/MS-selected reaction monitoring (SRM) using a JEOL SX/SX102A four-sector mass spectrometer (JEOL, Tokyo, Japan). [$^2H_2$]GAs (purchased from Prof. Lew Mander) were added as internal standards.

Results

Isolation of cDNA clones and Southern blot analysis

In order to clone GA 20-oxidase-like genes, degenerate primers based upon conserved domains were used to generate a PCR fragment from genomic DNA of hybrid aspen. The PCR fragment contained two introns of 112 and 173 bp, which included the exact splicing sites predicted for the *Arabidopsis GA5* gene (Xu et al. 1995). This genomic fragment was used to screen two cDNA libraries, one from mature leaves and the other from the cambium region. Screening 100,000 plaque-forming units (pfu) from the leaf library resulted in the recovery of one partial cDNA clone. From the cambium library, four full-length and one partial cDNA clones encoding putative GA 20-oxidase were recovered from a screen of 200,000 pfu. All clones were sequenced and were found to be identical, except that most cambium-derived sequences lacked one triplet in a region conserved in most GA 20-oxidases. This deletion was not found in the genomic probe. The longest isolated *PttGA20ox1* cDNA clone had 1,787 nucleotides, and encoded a putative protein of 385 amino acids (accession number AJ001326). 3′-RACE analysis of several products detected two poly(A) addition sites, one transcript being 315 bp shorter than expected according to the cDNA sequence. In contrast, there were also transcripts that had longer untranslated 5′-ends, since we isolated multiple 5′-RACE products with additional bases upstream of the start of the cDNA. The GA 20-oxidase from hybrid aspen shows the highest similarity and phylogenetic homology to the deduced protein sequence of the *Citrus sinensis* × *Poncirus trifoliata* cross (accession number AJ250187). The *Citrus* sequence shares about 80% identity and 85% similarity with PttGA20ox1, based upon their deduced amino acid sequences (BEST FIT, GCG). Phylogenetically, PttGA20ox1 groups with the mentioned *Citrus* clone (Fig. 1). In the next group the French bean clone Pv 73-1 (accession number U70531) is placed the closest and thereafter the French bean clone Pv 15-11 (accession number U70530) and pea clone Ps 27-12 (accession number U70471) (García-Martínez et al. 1997) are found.

To investigate the copy number of *PttGA20ox1* genes, Southern blot analysis was performed in which a full-length *PttGA20ox1* cDNA probe was hybridised at high stringency using genomic DNA from *P. tremula* L. × *P. tremuloides* (Fig. 2). The band patterns obtained were consistent with those expected for a single gene locus for *PttGA20ox1*. However, hybridisation at low stringency indicated that related genes might be present in the genome (data not shown).

Heterologous expression of the PttGA20ox1 cDNA

To confirm that the *PttGA20ox1* clone encodes an active GA 20-oxidase, the full-length coding sequence was expressed as a GST-fusion protein in *E. coli*. The protein lysates from IPTG-induced and non-induced cells were incubated with ^{14}C-labelled substrates involved in both the non-hydroxylated and the early 13-hydroxylated pathways. The reaction products were separated by HPLC and detected by an on-line radioactivity monitoring system. All products were identified by GC/MS (data not shown). The expressed protein converted both 13-hydroxylated C_{20}-GAs and non-13-hydroxylated C_{20}-GAs (Table 11). When [^{14}C]GA_{15} and [^{14}C]GA_{44} were incubated for 10 to 60 min with the lysate, conversions occurred only when they were in the open-lactone forms. However, when the duration of the enzyme assay was extended to 22 h, about 25% of the lactone

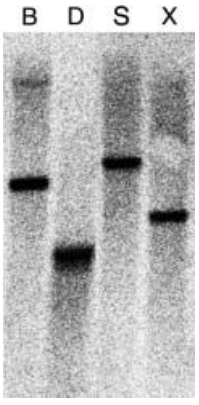

Fig. 2 Southern blot showing the hybridisation pattern of *PttGA20ox1* hybrid aspen clone T89 (*P. tremula* × *P. tremuloides*). The DNA was cut with *Bgl*II (*lane B*), *Dra*I (*lane D*), *Sph*I (*lane S*) and *Xba*I (*lane X*)

form of [^{14}C]GA_{15} was also converted to GA_9. No conversion of [^{14}C]GA_{44} was observed. The heterologous expression shows that this gene encodes a true GA 20-oxidase which is also capable of using GA_{15} in its lactone form, not previously shown for any other recombinant GA 20-oxidases.

Spatial expression of the GA 20-oxidase gene

The expression pattern of the *PttGA20ox1* gene in different tissues of hybrid aspen was investigated. Plants were grown under a long photoperiod, and poly $(A)^+$ RNA was extracted from young roots, the apex, early expanding, late-expanding and mature non-expanding leaf blades, and their respective petioles as well as internodes. Northern blot analysis using the *PttGA20ox1* 3′-end *EcoR*I/*Xba*I fragment as a probe, showed that the highest levels of expression of the transcript occur in late-expanding tissues of internodes and leaf blades (Fig. 3A, B). However, strong expression was also found in root tissue. Surprisingly, the expression of the gene was found to be low in the apex. As the apex sample contained not only meristematic tissue but also primordia and young leaves, the gene expression in the apical meristem was further investigated by means of a cDNA dot blot, as was specific expression in different cell layers of the stem (Fig. 4). The blot carried cDNA from PCR-amplified transcriptomes of differentiating phloem, cambial zone, xylem expansion zone and the xylem maturation zone, as well as the apical meristem of hybrid aspen (for details see Hertzberg and Olsson 1998). The blot demonstrated that there is strong expression of *PttGA20ox1* transcription in cambial meristematic tissues. This was confirmed by conventional northern hybridisation (data not shown) and further supported by the high number of cDNA clones found in the cDNA library from cambium as compared with the leaf library. The highest signal in the cDNA blot was found in the apical meristem (Fig. 4). The relatively strong expression found in the apical meristem contrasts with the weak signal obtained from the apex by the northern analysis. The measurement of the isolated apical meristem was, however, far more specific, and it avoided dilution of the signal from *PttGA20ox1*-expressing cells with non-expressing cells from young leaves and primordia.

Table 1 Conversion of $^{14}C_1$-GAs by lysate of *Escherichia coli* expressing the PttGA20ox1 fusion protein. [$^{14}C_1$]GA_{15} and [$^{14}C_1$]GA_{44} were incubated for 22 h, all other substrates for 10 min. Asterisks (*) indicate that incubation of [$^{14}C_1$]GA_{15} and [$^{14}C_1$]GA_{44} for 10 min did not result in any conversion (even after 22 h there was no conversion of [$^{14}C_1$]GA_{44})

Substrate	Products identified by GC/MS
[$^{14}C_1$]GA_{12}	[$^{14}C_1$]GA_{15}, [$^{14}C_1$]GA_{24}, [$^{14}C_1$]GA_9
[$^{14}C_1$]GA_{15}-open lactone	[$^{14}C_1$]GA_{24}, [$^{14}C_1$]GA_9
[$^{14}C_1$]GA_{15}	[$^{14}C_1$]GA_9*
[$^{14}C_1$]GA_{24}	[$^{14}C_1$]GA_9
[$^{14}C_1$]GA_{53}	[$^{14}C_1$]GA_{44}, [$^{14}C_1$]GA_{19}, [$^{14}C_1$]GA_{20}, [$^{14}C_1$]GA_{17}
[$^{14}C_1$]GA_{44}-open lactone	[$^{14}C_1$]GA_{19}, [$^{14}C_1$]GA_{20}
[$^{14}C_1$]GA_{44}	–*
[$^{14}C_1$]GA_{19}	[$^{14}C_1$]GA_{20}, [$^{14}C_1$]GA_{17}

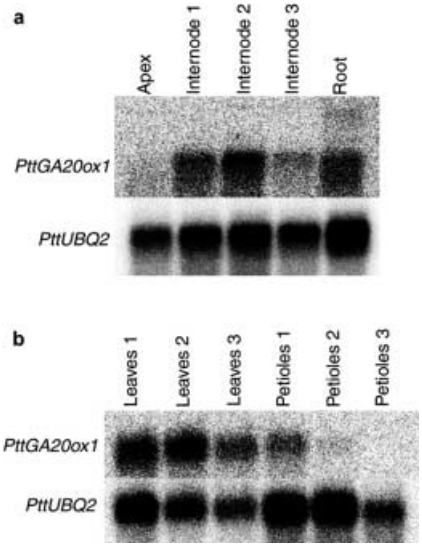

Fig. 3a, b Northern analysis showing the expression of *PttGA20ox1* in hybrid aspen in LDs. **a** Eight micrograms of poly$(A)^+$ RNA was loaded in each lane from: the apex, an early expanding internode (*1*), a late-expanding internode (*2*), a mature, fully expanded internode (*3*) and young roots. **b** Expression pattern in the leaves and petioles from: an early expanding internode (*1*), from a late-expanding internode (*2*) and a mature non-expanding internode (*3*). Three micrograms of poly$(A)^+$ was loaded per lane. The blots were probed with a 3′-end-specific probe for *PttGA20ox1* and re-probed with an endogenous ubiquitin, *PttUBQ2*

Feedback inhibition of the GA 20-oxidase gene

It has already been shown that transcription of GA 20-oxidase genes is down-regulated, in several species, by

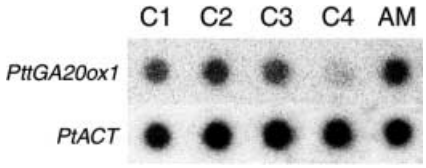

Fig. 4 Expression of *PttGA20ox1* in specific cell layers of hybrid aspen stem and the apical meristem, as shown by a cDNA dot blot from PCR-amplified transcriptome. Transverse sections (2.5×15 mm, 50 μm thick) were pooled into groups according to their developmental status. *C1*, *C2*, *C3* and *C4* represent differentiating phloem, cambial cells (mainly), enlarging xylem and maturing xylem, respectively. As a control, a conserved actin fragment (*PtACT*) from *Populus trichocarpa* was used

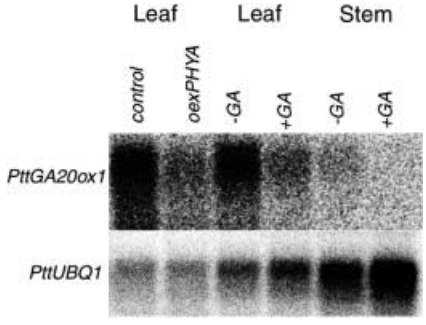

Fig. 5 Feedback inhibition of *PttGA20ox1* transcription in hybrid aspen treated with 10 μm GA_4, and expression of *PttGA20ox1* in transgenic hybrid aspen expressing the oat phytochrome A gene (*oexPHYA*) compared with expression in a transformed control (*control*). Poly(A)$^+$RNA (3 μg) was loaded into each lane. Ubiquitin (*PttUBQ1*) was used as a reference

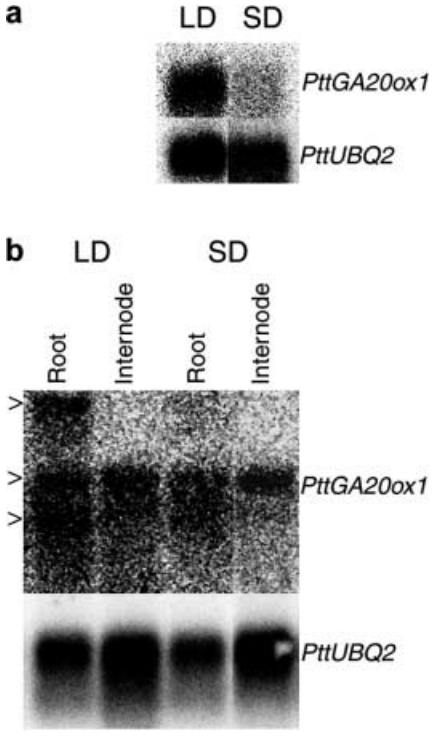

Fig. 6a, b Daylength-regulated expression of *PttGA20ox1* in hybrid aspen. Sampling was done during LDs and after four SDs. **a** Expression in late-expanding leaf blades; 3 μg of poly(A)$^+$RNA was loaded in each lane. **b** Young roots and late expanding internodes; 8 μg of poly(A)$^+$RNA was loaded in each lane. Endogenous ubiquitin, *PttUBQ2*, was used as a reference in both **a** and **b**

the action of active GAs. To investigate if the *PttGA20ox1* gene is feedback-regulated, shoots of hybrid aspen were fed with 10 μM GA_4 for 24 h. Subsequent northern analysis confirmed that the transcript level of the *PttGA20ox1* gene is down-regulated in both leaf and stem tissue by the GA_4 treatment (Fig. 5).

Phytochrome and photoperiodic regulation of GA 20-oxidase expression

It has previously been shown that overexpression of the oat *PHYA* gene in hybrid aspen and tobacco results in shortened internodes and decreased GA levels (Jordan et al. 1995; Olsen et al. 1997). Hybrid aspen overexpressing oat *PHYA* resume a normal stem growth upon feeding with GA_4 (data not shown); therefore, we decided to investigate *PttGA20ox1* expression in stem tissue of oat *PHYA* overexpressors (Fig. 5). The northern analysis showed that the transcript is down-regulated in the overexpressor compared with the transformed control plant. The lower levels of GAs (Olsen et al. 1997) and the associated dwarf phenotype found in these plants are therefore, at least partly, likely to be due to a decrease in the transcription of the GA 20-oxidase gene.

GAs have been suggested to be involved in the photoperiodic cessation of shoot growth in deciduous tree species. To investigate the relationship between photoperiodically controlled growth and GAs, *PttGA20ox1* transcript levels in late-expanding tissues and young roots were analysed under different photoperiods. Plants grown under long photoperiods were transferred to a short photoperiod. Plants were harvested both before the transfer and after four SDs. Results of the subsequent northern analysis show that there was a clear down-regulation of *PttGA20ox1* transcripts in response to SDs in late-expanding leaf blades (Fig. 6A) and a slight reduction in young root tissue (Fig. 6B). However, in contrast, there was a slight upregulation of *PttGA20ox1* transcript in response to SDs in late-expanding internodes (Fig. 6B). On a well-separated gel blot, two bands of about 1.8 kb clearly hybridising to the specific 3′-end probe can be distinguished in most tissues, and in addition one or possibly two bands of 2.5–2.6 kb in the root sample(s) (Fig. 6B). By 3′-RACE we determined that the 1.8-kb transcript could potentially show two alternative sites of polyadenylation, one transcript being 315 bp shorter than the other, which might explain the lower band. The 2.5- to 2.6-kb band is also likely to represent a transcript of the *PttGA20ox1* gene. This is supported by the single band found in Southern analysis and after hybridisation at high stringency (Fig. 2).

GA levels in different tissues and photoperiods

To compare the *PttGA20ox1* transcript levels with GA levels in hybrid aspen we quantified GAs of the early 13-hydroxylation pathway in different tissues, as well as under different photoperiods (Fig. 7). Samples of the same tissues were analysed as for the northern analysis, making it possible to compare the abundance of the transcript with GA levels. The data show that actively growing tissues, such as early elongating internodes, petioles and leaf blades had the highest levels of C_{19}-GAs. Mature and fully elongated tissues had lower levels of all GAs analysed, with the exception of fully elongated internodes that had high levels of GA_{19}. This might be due to reduced GA 20-oxidase activity. Young expanding internodes contained the highest levels of GA_1, as well as GA_8. The levels of GA_1 in the apex were moderate but, in contrast, the level of GA_{20} was high, indicating that 3β-hydroxylation was limiting in the apex. Transfer to short photoperiods affected only actively growing tissues. An accumulation of GA_{19} was observed in early elongating internodes and leaf blades, indicating that short photoperiods are associated with a reduction in GA 20-oxidase activity. The levels of C_{19}-GAs were also to some extent changed upon transfer to SDs. The levels of GA_{20} were decreased in internodes, and those of GA_1 were reduced by 50% in early expanding leaf blades, but less reduced in internode tissue.

The levels of GA metabolites were, overall, lower in roots than in internodes and leaf blades, with the active GA_1 content being approximately 1/10th of that in the actively growing internodes under LDs. The photoperiodic regulation of GA levels in roots was found to be complex, with a high accumulation of GA_8 and GA_{19} in the long photoperiod and a decrease in SDs of all metabolites except GA_{20}, which accumulated slightly.

GA_{12} metabolism in different photoperiods

Metabolism of GA_{12} in different photoperiods was studied by decapitating shoots of hybrid aspen. The apical shoot was thereafter infiltrated via the transpiration stream with [17-^{14}C]GA_{12}, and harvested after 24 h. Metabolites were detected by on-line HPLC radioactivity detection, and finally identified after methylation and

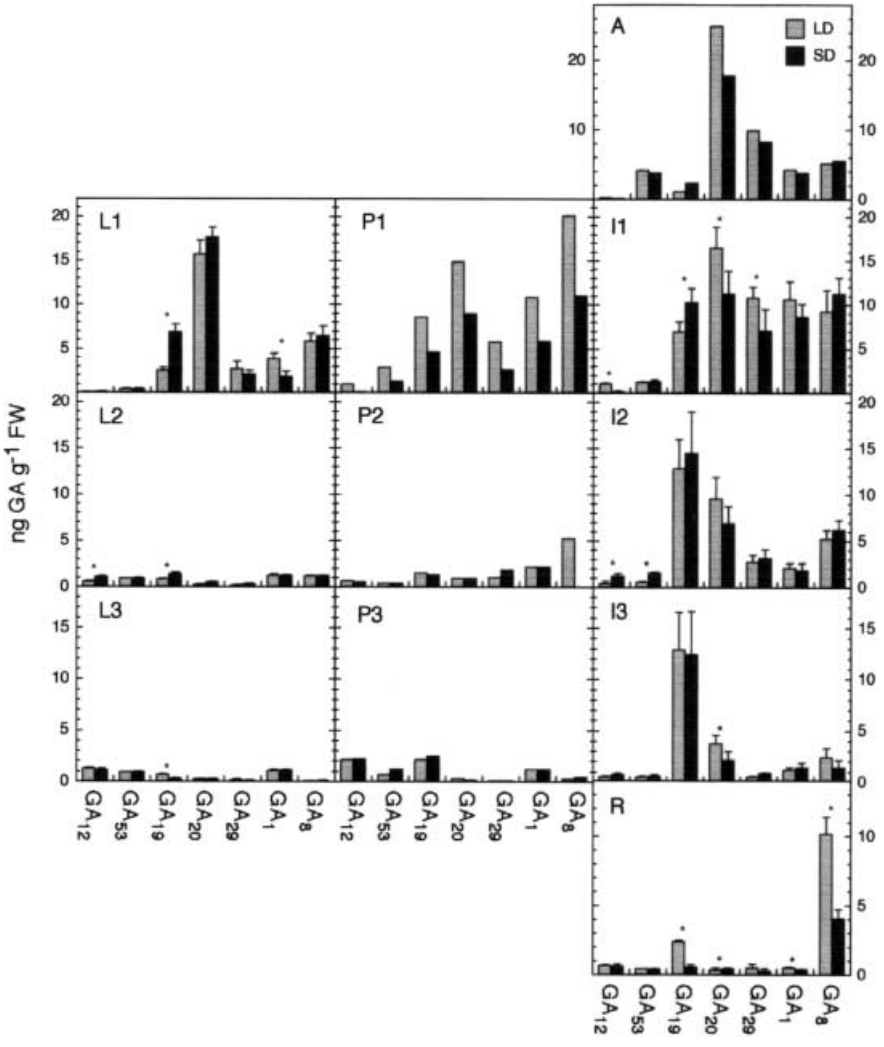

Fig. 7 Levels of GAs in different tissues of hybrid aspen under LDs or SDs. The tissues are: the apex (*A*), early expanding internode (*I1*), a late-expanding internode (*I2*), a mature, fully expanded internode (*I3*), corresponding petioles (*P*) and leaf blades (*L*), and young roots (*R*). The sample numbers are three for leaf, internode and root tissue (bars ± SD), and one for apex and petiole tissue as the amount of tissue was limited. Statistically significant differences between LD and SD samples are indicated at the 5% level (*), student's *t*-test

trimethylsilylation by GC/MS (data not shown). In LDs, all fed [17-^{14}C]GA_{12} was metabolised to two major metabolites at a retention time of 35 min (Fig. 8), identified tentatively as hexose conjugates of [17-^{14}C]GA_{12} (data not shown). Another prominent metabolite was [17-^{14}C]GA_{53} at 30 min. Besides several polyhydroxylated C_{20}-GAs, such as 12β-OH-GA_{12} and 16β,17-H_2-17-OH-GA_{12}, only metabolites of the early 13-hydroxylation pathway were identified, including GA_{44} and GA_{19} but not GA_{20} and GA_1. [17-^{14}C]GA_{53} accumulated after only 2 days in SDs (Fig. 8). Also, a polar metabolite at retention time of 12 min accumulated in SDs. This metabolite was identified as GA_{97} (2β-OH-GA_{53}), based on published mass spectrum and GC retention index (Mander et al. 1996). The metabolism data suggest that GA 20-oxidase is photoperiodically regulated in hybrid aspen.

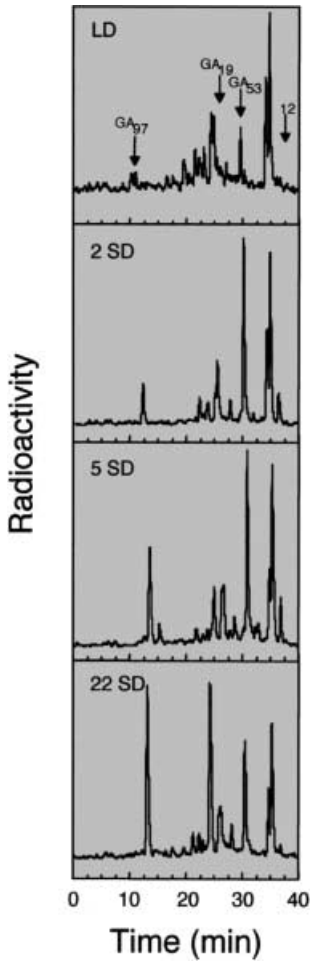

Fig. 8 Metabolism of [17-^{14}C]GA_{12} in the apical shoot of hybrid aspen grown in LDs and the indicated numbers of SDs. The substrate was infiltrated via the transpiration stream, and metabolised for 24 h. Metabolites were detected by on-line HPLC radioactivity detection

Discussion

Genes encoding GA 20-oxidase have previously been cloned and analysed from a wide range of plant species (Phillips et al. 1995; Xu et al. 1995; Martin et al. 1996; García-Martínez et al. 1997; Toyomasu et al. 1997; Carrera et al. 1999; Kang et al. 1999). In order to probe some of the mechanisms involved in the control of elongation growth in deciduous trees we have now identified a gene encoding GA 20-oxidase from hybrid aspen. The isolated *PttGA20ox1* exhibits features that indicate it plays an important role in regulating GA-dependent growth in aspen.

The *PttGA20ox1* sequence contains several components characteristic of 2-oxoglutarate-dependent dioxygenases, including an Fe^{2+}-binding motif containing the conserved residues H-253, D-255, and H-309, and the suggested 2-oxoglutarate (co-substrate)-binding residues R and S comprising residues 319 and 321, respectively (Valegard et al. 1998). The *PttGA20ox1* is an active GA 20-oxidase, as shown by expression and characterisation of the full-length cDNA as a fusion protein in *E. coli* (Table 1). The recombinant protein oxidised all C_{20}-GAs available, both 13-hydroxylated and non-13-hydroxylated. Differences in affinity to the substrates GA_{12} and GA_{53} have been observed in GA 20-oxidases derived from *Arabidopsis* (Phillips et al. 1995) and pumpkin (Lange et al. 1997) but, in general, previously studied GA 20-oxidases in plants can use both types of GA as substrates. In hybrid aspen, other evidence suggests that the early 13-hydroxylated pathway is the most important metabolic route, since little GA_9, GA_4 and GA_{34} can be detected in these trees (Eriksson et al. 2000). Furthermore, upon feeding radioactively labelled GA_{12} to such plants, rapid conversion to GA_{53} has been detected, but not to non-13-hydroxylated GAs such as GA_{15}, GA_{24} and GA_9 (Fig. 8, and unpublished data). The *PttGA20ox1* preferentially oxidised GA_{15} in the open-lactone form, as do other recombinant GA 20-oxidases. However, when incubated for longer times, the GA_{15}-lactone was converted to GA_9 by the expressed GA 20-oxidase. To our knowledge, recombinant GA 20-oxidases generally convert the open-lactone form of GA_{15} (Ward et al. 1997), as do cell-free systems from pumpkin (Lange and Graebe 1989) and pea (Kamiya and Graebe 1983). However, there are examples of other cell-free systems that can convert the closed-lactone form of $GA_{15/44}$, and a GA_{44} 20-oxidase, which converts the GA_{44}-lactone, has been partly purified from spinach (Gilmour et al. 1987). The slow conversion of the GA_{15}-lactone to GA_{24} and GA_9 suggests that the GA_{15}-open lactone is the natural substrate for the GA 20-oxidase from hybrid aspen, and that long incubation times may result in a slow chemical equilibration towards the open-lactone form of GA_{15}. However, it is also possible that the enzyme can use both forms of GA_{15}, while having a very low affinity for the GA_{15}-lactone, resulting in a slow conversion to GA_9. No conversion of GA_{44} in its lactone form was detected.

Northern analysis showed that the *PttGA20ox1* transcript is expressed in a wide range of tissues, but at very low levels. The multiple transcripts seen in the

analyses (Fig. 6B) suggest alternative polyadenylation of this transcript, which has not been described amongst GA 20-oxidase transcripts in previous studies of other species. However, it is commonly encountered in eu#132;caryote mRNA, and may be important in translational control (Gibbs et al. 1998), for the stability of the transcripts, or for other regulatory functions.

GA 20-oxidase has been suggested to be a key regulator in the biosynthesis of active GAs (see Hedden and Kamiya 1997). This implies that it may be possible to examine the site(s) of C_{19}-GA biosynthesis by mapping GA 20-oxidase expression. However, it must be emphasised that the level of transcription activity is not the sole determinant of enzyme activity. Other important factors include transcript stability, post-translational modifications and the availability of substrates. In the present study the expression of the gene was low in most of the tissues analysed, except for late-expanding tissues of internodes and leaf blades (Fig. 3A, B). This was surprising since the highest levels of C_{19}-GAs were detected by in the early expanding tissues and apical region (Fig. 7). This shows the importance of GA analysis, and how difficult it is to conclude where the highest levels of GAs are actually found, based on transcript levels. The discrepancy in our study could be explained by the active transport of C_{19}-GAs (such as $GA_{20,}$ which has been suggested to be the predominant GA transport form in pea; Proebsting et al. 1992) from potential sources like leaves to actively growing tissues. This hypothesis may appear inconsistent with the high levels of *PttGA20ox1* transcript detected in meristematic tissues like the apical meristem and the cambium in the blot carrying cDNA from dissected tissues (Fig. 4). However, the low amount of meristematic to non-meristematic tissues in apex or stem will surely dilute this transcript. Alternatively, the expression of *PttGA20ox1* may be inhibited by negative feedback imposed by the high GA_1 levels in younger tissues such as the apex and early expanding internodes (Fig. 7). By infiltrating aspen shoots with or without GA_4 we confirmed that *PttGA20ox1* gene expression is indeed feedback-regulated, both in the stem and leaves (Fig. 5). Moreover, the low transcript levels found in mature tissues are likely to be due to a general reduction of *PttGA20ox1* transcription as the tissues age, which is also reflected in a decline in the measured GA levels in mature leaf or stem tissues (Fig. 7).

The relatively strong expression of the *PttGA20ox1* gene in root tissues was unexpected, as the previously studied GA 20-oxidases have very little or no expression in roots, except for the French bean clone Pv15-11 (accession number U70530; García-Martínez et al. 1997). However, there are studies on the expression of other GA-biosynthesis and GA-response genes suggesting that roots are sites of both GA biosynthesis and action. Promoter-GUS and northern analysis of *ent*-copalyl diphosphate synthase (CPS) from *Arabidopsis* (Silverstone et al. 1997), GA 2-oxidase from pea (Martin et al. 1999) and GA 3β-hydroxylase from tobacco (Itoh et al. 1999) have shown that these GA biosynthetic genes are expressed in the roots. Studies of the GA-induced gene *GASA4* from *Arabidopsis* have found that it, too, is strongly expressed in these meristematic tissues (Aubert et al. 1998). The strong expression of the *PttGA20ox1* gene in root tissues was associated with moderate levels of GA metabolites. The GA levels in root tissues of different plants have been little described, but recently the importance of GAs for normal root growth has been shown in GA-deficient mutants of pea (Yaxley et al. 2001). The importance of GAs in roots is also demonstrated by the GA-deficient tomato mutant, *gib-1*, which exhibits altered cell division patterns in the root meristem due to its GA deficiency (Barlow et al. 1991).

Phytochrome has been shown to regulate the transcript levels of GA biosynthetic genes in several species. It affects the transcription of both GA 20-oxidase and/or GA 3β-hydroxylases in germinating lettuce (Toyomasu et al. 1998), in *Arabidopsis* seeds (Yamaguchi et al. 1998) and in pea seedlings during the process of de-etiolation (Ait-Ali et al. 1999; Gil and García-Martínez 2000). In the present study we analysed transcript levels of *PttGA20ox1* in hybrid aspen overexpressing the oat *PHYA* cDNA. These transgenic plants exhibit a dwarf phenotype, and it has already been shown that GA_1 levels are lower in oat *PHYA* overexpressors than in control plants, both in tobacco and hybrid aspen (Jordan et al. 1995; Olsen et al. 1997). Northern analysis showed that the *PttGA20ox1* transcript levels are lower in the transgenic aspen plants than in the transformed control lines (Fig. 5). This indicates that strong expression of phyA results in either a direct down-regulation of *PttGA20ox1* transcription, or enhances the negative feedback regulation of the transcript. In pea it has been shown that both phyA and phyB regulate accumulation of GA 20-oxidase transcripts, and that negative feedback regulation is important in controlling levels of GA 20-oxidase transcripts during de-etiolation (Ait-Ali et al. 1999). Negative regulation of the levels of GA_1 and GA 20-oxidase transcripts has also been reported when the rice homeobox gene *OSH1* is overexpressed in tobacco (Kusaba et al. 1998).

Photoperiodic regulation of GA 20-oxidase transcription has been shown in spinach (Wu et al. 1996), potato (Carrera et al. 1999) and *Arabidopsis* (Xu et al. 1997). In spinach, for instance, the up-regulation of GA 20-oxidase transcripts observed following transfer of plants to LD conditions correlates with an increase in GA levels. In potato there is a diurnal variation in the GA 20-oxidase levels, and non-inductive conditions for tuber formation promote the accumulation of a specific GA 20-oxidase transcript. In the present study, the GA 20-oxidase transcript levels were also down-regulated in late-expanding leaf tissue after only 4 days under short photoperiods (Fig. 6A). The down-regulation of GA 20-oxidase transcript was followed by an accumulation of GA_{19} in early elongating internodes and leaf blades, and reduced GA_1 levels in young leaf blades (Fig. 7), indicating that short photoperiods are

associated with a reduction in GA 20-oxidase activity. These results support the hypothesis that reduction in GA biosynthesis upon transfer to SDs is at least partly responsible for the cessation of shoot elongation growth in temperate woody species (Olsen et al. 1995). Surprisingly, however, the GA 20-oxidase transcript levels were slightly up-regulated in the internodes upon transfer to SDs (Fig. 6B). It must also be emphasised that the levels of GA_1 did not change significantly in internode tissues in response to SDs. This is surprising, as Olsen et al. (1997) reported that the GA_1 levels were reduced in internodes of hybrid aspen after 1 week in SDs.

Photoperiodic changes in GA 20-oxidase activity in shoot tissue was further investigated by studies of [17-^{14}C]GA_{12} metabolism (Fig. 8). The accumulation of [^{14}C]GA_{53} after only 2 days in SDs is consistent with reduced activity of GA 20-oxidase, as GA_{53} is the first 13-hydroxlyated substrate for the GA 20-oxidase. The observed accumulation of [^{14}C]GA_{53} could be a result of higher rates of 13-hydroxylation in SDs and lower production of GA_{12}-conjugates. However, the accumulation of [^{14}C]GA_{97} (2β-OH-GA_{53}) under SDs suggests that accumulation of [^{14}C]GA_{53} results in increased 2β-hydroxylation, probably due to increased concentration of substrate for the GA 2-oxidase. This is interesting as the levels of GA_{97} in spinach are higher in SDs than in LDs (Mander et al. 1996). It is possible a similar accumulation of GA_{97} is occurring in hybrid aspen in SDs, but as no internal standard was available for GA_{97} we could not quantify that metabolite.

In the present study we also show that the biosynthesis of GA_1 decreases in root tissue upon transfer to SDs. The decrease in GA_1 levels was correlated with a slightly decreased level of *PttGA20ox1* expression in root tissue (Fig. 6). A change also occurs in other GA levels following a transition to short photoperiods, e.g. GA_{19} is reduced by a factor of approximately 4 in such conditions, and it can not be ruled out that roots play an important role as a source of GAs for the shoots. Indications that communications between root and shoot influence the biosynthesis of GAs were reported in some early papers (for references, see Graebe and Ropers 1978). Grafting experiments using northern and southern ecotypes of *Salix pentandra* have also shown that daylength regulates communication between shoot and root (Junttila 1988). Although the *Salix pentandra* study did not involve any quantitative analysis of GA levels, it is tempting to speculate that the delay imposed by a southern rootstock on SD-induced cessation of apical growth may be mediated by changes in GA metabolism. The hypothesis that roots provide an important source of GAs must now be further studied.

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References

Ait-Ali T, Frances S, Weller JL, Reid JB, Kendrick RE, Kamiya Y (1999) Regulation of gibberellin 20-oxidase and gibberellin 3β-hydroxylase transcript accumulation during de-etiolation of pea seedlings. Plant Physiol 121:783–791

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Aubert D, Chevillard M, Dorne AM, Arlaud G, Herzog M (1998) Expression patterns of *GASA* genes in *Arabidopsis thaliana*: the *GASA4* gene is up-regulated by gibberellins in meristematic regions. Plant Mol Biol 36:871–883

Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K (1995) Current protocols in molecular biology. Wiley, New York

Barlow PW, Brain P, Parker JS (1991) Cellular growth in roots of a gibberellin-deficient mutant of tomato (*Lycopersicon esculentum* Mill) and its wild type. J Exp Bot 42:339–351

Bethke PC, Schuurink R, Jones RL (1997) Hormonal signalling in cereal aleurone. J Exp Bot 48:1337–1356

Carrera E, Jackson SD, Prat S (1999) Feedback control and diurnal regulation of gibberellin 20-oxidase transcript levels in potato. Plant Physiol 119:765–773

Carrera E, Bou J, García-Martínez J, Prat S (2000) Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. Plant J 22:247–256

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Coles JP, Phillips AL, Croker SJ, García-Lepe R, Lewis MJ, Hedden P (1999) Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. Plant J 17:547–556

Curtis IS, Ward DA, Thomas SG, Phillips AL, Davey MR, Power B, Lowe KC, Croker SJ, Lewis MJ, Magness SL et al (2000) Induction of dwarfism in transgenic *Solanum dulcamara* by over-expression of a gibberellin 20-oxidase cDNA from pumpkin. Plant J 23:329–338

Daykin A, Scott IM, Francis D, Causton DR (1997) Effects of gibberellin on the cellular dynamics of dwarf pea internode development. Planta 203:526–535

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Eriksson ME, Israelsson M, Olsson O, Moritz T (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. Nat Biotechnol 18:784–788

García-Martínez JL, Lopez-Díaz I, Sanchez-Beltran MJ, Phillips AL, Ward DA, Gaskin P, Hedden P (1997) Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. Plant Mol Biol 33:1073–1084

Gibbs L, Willis D, Morgan MJ (1998) The identification and expression of heme oxygenase-2 alternative transcripts in the mouse. Gene 221:171–177

Gil J, García-Martínez JL (2000) Light regulation of gibberellin A(1) content and expression of genes coding for GA 20-oxidase

and GA 3β-hydroxylase in etiolated pea seedlings. Physiol Plant 108:223–229

Gilmour SJ, Bleecker AB, Zeevaart JAD (1987) Partial-purification of gibberellin oxidases from spinach leaves. Plant Physiol 85:87–90

Graebe JE, Ropers H (1978) Hormone levels in xylem sap and roots in relation to shoot development and physiology. In: Letham DS, Goodwin PB, Higgins TJV (eds) Phytohormones and related compounds: a comprehensive treatise, vol 2. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 226–228

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol 48:431–460

Hedden P, Philips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci 5:1323–1330

Hertzberg M, Olsson O (1998) Molecular characterisation of a novel plant homeobox gene expressed in the maturing xylem zone of *Populus tremula* × *tremuloides*. Plant J 16:285–295

Hooley R (1994) Gibberellins – perception, transduction and responses. Plant Mol Biol 26:1529–1555

Huang SS, Raman AS, Ream JE, Fujiwara H, Cerny RE, Brown SM (1998) Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in *Arabidopsis*. Plant Physiol 118:773–781

Itoh H, Tanaka-Ueguchi M, Kawaide H, Chen XB, Kamiya Y, Matsuoka M (1999) The gene encoding tobacco gibberellin 3β-hydroxylase is expressed at the site of GA action during stem elongation and flower organ development. Plant J 20:15–24

Jordan ET, Hatfield PM, Hondred D, Talon M, Zeevaart JAD, Vierstra RD (1995) Phytochrome A overexpression in transgenic tobacco – Correlation of dwarf phenotype with high-concentrations of phytochrome in vascular tissue and attenuated gibberellin levels. Plant Physiol 107:797–805

Junttila O (1988) Effect of rootstock on photoperiodic control of elongation growth in grafted ecotypes of *Salix*. Physiol Plant 74:39–44

Kamiya Y, Graebe JE (1983) The biosynthesis of all major pea gibberellins in a cell-free system from *Pisum sativum*. Phytochemistry 22:681–689

Kang HG, Jun SH, Kim J, Kawaide H, Kamiya Y, An C (1999) Cloning and molecular analyses of a gibberellin 20-oxidase gene expressed specifically in developing seeds of watermelon. Plant Physiol 121:373–382

Kende H, Zeevaart JAD (1997) The five ''classical'' plant hormones. Plant Cell 9:1197–1210

Kovalic D, Kwak J-H, Weisbaum B (1991) General method for direct cloning of DNA fragments generated by primed enzymatic amplification. Nucleic Acids Res 19:4560–4563

Kusaba S, Fukumoto M, Honda C, Yamaguchi I, Sakamoto T, Kano-Murakami Y (1998) Decreased GA_1 content caused by the overexpression of *OSH1* is accompanied by suppression of GA 20-oxidase gene expression. Plant Physiol 117:1179–1184

Lange T, Graebe JE (1989) The partial-purification and characterization of a gibberellin C-20 hydroxylase from immature *Pisum sativum* L seeds. Planta 179:211–221

Lange T, Hedden P, Graebe JE (1994) Expression cloning of a gibberellin 20-oxidase, a multifunctional enzyme involved in gibberellin biosynthesis. Proc Natl Acad Sci USA 91:8552–8556

Lange T, Kegler C, Hedden P, Phillips AL, Graebe JE (1997) Molecular characterisation of gibberellin 20-oxidases. Structure-function studies on recombinant enzymes and chimaeric proteins. Physiol Plant 100:543–549

Larsson S, Bjorkbacka H, Forsman C, Samuelsson G, Olsson O (1997) Molecular cloning and biochemical characterization of carbonic anhydrase from *Populus tremula* × *tremuloides*. Plant Mol Biol 34:583–592

Mander LN, Owen DJ, Croker SJ, Gaskin P, Hedden P, Lewis MJ, Talon M, Gage DA, Zeevaart JAD, Brenner ML, Sheng CX (1996) Identification of three C-20-gibberellins: GA_{97} (2 beta-hydroxy-GA_{53}), GA_{98} (2 beta-hydroxy-GA_{44}) and GA_{99} (2 beta-hydroxy-GA_{19}). Phytochemistry 43:23–28

Martin DN, Proebsting WM, Parks TD, Dougherty WG, Lange T, Lewis MJ, Gaskin P, Hedden P (1996) Feed-back regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. Planta 200:159–166

Martin DN, Proebsting WM, Hedden P (1999) The *SLENDER* gene of pea encodes a gibberellin 2-oxidase. Plant Physiol 121:775–781

Olsen JE, Junttila O, Moritz T (1995) A localised decrease of GA_1 in shoot tips of *Salix pentandra* seedlings precedes cessation of shoot elongation under short photoperiod. Physiol Plant 95:627–632

Olsen JE, Junttila O, Nilsen J, Eriksson ME, 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

Peng JR, Richards DE, Moritz T, CanoDelgado A, Harberd NP (1999) Extragenic suppressors of the *Arabidopsis gai* mutation alter the dose-response relationship of diverse gibberellin responses. Plant Physiol 119:1199–1207

Phillips AL, Ward DA, Uknes S, Appleford NEJ, Lange T, Huttly AK, Gaskin P, Graebe JE, Hedden P (1995) Isolation and expression of three gibberellin 20-oxidase cDNA clones from *Arabidopsis*. Plant Physiol 108:1049–1057

Proebsting WM, Hedden P, Lewis MJ, Croker SJ, Proebsting LN (1992) Gibberellin concentration and transport in genetic lines of pea – effects of grafting. Plant Physiol 100:1354–1360

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Silverstone AL, Chang CW, Krol E, Sun TP (1997) Developmental regulation of the gibberellin biosynthetic gene *GA1* in *Arabidopsis thaliana*. Plant J 12:9–19

Sterky F, Regan S, Karlsson J et al (1998) Gene discovery in the wood-forming tissues of poplar: analysis of 5,692 expressed sequence tags. Proc Natl Acad Sci USA 95:13330–13335

Toyomasu T, Kawaide H, Sekimoto H, von Numers C, Phillips AL, Hedden P, Kamiya Y (1997) Cloning and characterization of a cDNA encoding gibberellin 20-oxidase from rice (*Oryza sativa*) seedlings. Physiol Plant 99:111–118

Toyomasu T, Kawaide H, Mitsuhashi W, Inoue Y, Kamiya Y (1998) Phytochrome regulates gibberellin biosynthesis during germination of photoblastic lettuce seeds. Plant Physiol 118:1517–1523

Valegard K, van Scheltinga ACT, Lloyd MD, Hara T, Ramaswamy S, Perrakis A, Thompson A, Lee HJ, Baldwin JE, Schofield CJ, Hajdu J, Andersson I (1998) Structure of a cephalosporin synthase. Nature 394:805–809

Vieira J, Messing J (1991) New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene 100:189–194

Ward JL, Jackson GJ, Beale MH et al (1997) Stereochemistry of the oxidation of gibberellin 20-alcohols, GA_{15} and GA_{44}, to 20-aldehydes by gibberellin 20-oxidases. Chem Commun 1:13–14

Wu K, Li L, Gage DA, Zeevaart JAD (1996) Molecular cloning and photoperiod-regulated expression of gibberellin 20-oxidase from the long-day plant spinach. Plant Physiol 110:547–554

Xu YL, Li L, Wu KQ, Peeters AJM, Gage DA, Zeevaart JAD (1995) The *GA5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: molecular cloning and functional expression. Proc Natl Acad Sci USA 92:6640–6644

Xu YL, Gage DA, Zeevaart JAD (1997) Gibberellins and stem growth in *Arabidopsis thaliana* – effects of photoperiod on expression of the *GA4* and *GA5* loci. Plant Physiol 114:1471–1476

Xu YL, Li L, Gage DA, Zeevaart JAD (1999) Feedback regulation of *GA5* expression and metabolic engineering of gibberellin levels in *Arabidopsis*. Plant Cell 11:927–935

Yamaguchi S, Smith MW, Brown, RGS, Kamiya Y, Sun TP (1998) Phytochrome regulation and differential expression of gibberellin 3β-hydroxylase genes in germinating *Arabidopsis* seeds. Plant Cell 10:2115–2126

Yaxley JR, Ross JJ, Sherriff LJ, Reid JB (2001) Gibberellin biosynthesis mutations and root development in pea. Plant Physiol 125:627–633