

An auxin-repressed gene (*RpARP*) from black locust (*Robinia pseudoacacia*) is posttranscriptionally regulated and negatively associated with shoot elongation

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Summary The plant hormone auxin regulates various growth and developmental processes by controlling the expression of auxin-response genes. While many genes up-regulated by auxin have been characterized, less is known about the genes that are down-regulated by auxin. We isolated and characterized an auxin-repressed gene (*RpARP*) from the tree legume, *Robinia pseudoacacia* L. A sequence similarity search in public databases showed that the *RpARP* gene has homologs in various higher plants including monocots and dicots. The deduced amino acid sequences are highly conserved among these homologs (up to 85% identity). Northern blot analysis showed that auxin repressed *RpARP* gene expression and that repression was dependent on the presence of metabolizable sugar and on protein synthesis. In addition, cold treatment abolished the auxin-mediated repression of *RpARP* gene expression. Results from transgenic plant analyses suggest that *RpARP* gene expression is posttranscriptionally regulated by auxin and by the untranslated regions. Sequence analysis of the promoter region (–70 and –500 bp upstream of the putative transcription initiation site) of the *RpARP* gene identified four sucrose-repressible response elements (TATCCAT-motifs; Huang et al. 1990), suggesting that the *cis*-elements responsible for regulation by sucrose are located in the promoter region. In fact, the expression of the transgenic *RpARP* gene was unaffected by sucrose when driven by a CaMV 35S promoter. We present evidence that *RpARP* gene expression is negatively associated with hypocotyl elongation.

Keywords: auxin, auxin-repressed protein, posttranscriptional regulation.

Introduction

Auxin, a plant growth regulator produced mainly in apical buds, plays a key role in a wide variety of growth and developmental processes such as lateral root formation, apical dominance, tropism and differentiation of vascular tissue (Berleth et al. 2000, Hamann 2001, Muday 2001). Two major experimental approaches have been employed to investigate the mo-

lecular basis of auxin action on these developmental processes. One is to identify and analyze mutants that lack normal auxin responses. Two related families of proteins, the AUX/IAA proteins and auxin response factors (ARFs), were identified as key regulators of auxin-modulated gene expression (Ulmasov et al. 1997, Rouse et al. 1998). These protein families function as transcriptional regulators and are thought to interact with auxin-induced genes to activate or repress their expression. It was recently found that protein degradation by ubiquitin-mediated processes is important in the auxin response (Ruegger et al. 1998). The other approach involves the use of molecular biology tools to identify and characterize genes regulated by auxin signals. This approach has led to the identification of several classes of early auxin-response genes and auxin-responsive *cis*-elements within the promoters of these genes (Guilfoyle et al. 1998). The expression level of early (primary) auxin-response genes increases within minutes of auxin application, independent of *de novo* protein synthesis (Abel and Theologis 1996, Walker and Estelle 1998). Although many mutants and cDNA clones associated with auxin signaling have been isolated and characterized, our knowledge of the mechanisms by which auxin regulates various biological functions is still limited, partially because most molecular studies have focused on the primary “response genes” that are up-regulated by auxin signals (Hagen and Guilfoyle 2002).

Auxin-repressed protein (*ARP*) genes and their role in plant growth and development are relatively understudied. So far, three orthologs of *ARP* have been isolated and characterized. The *SAR5* gene was first isolated by differential screening of auxin-deprived strawberry receptacles (Reddy and Poovaiah 1990). Reddy and Poovaiah (1990) showed that the *SAR5* gene was repressed by auxin and that repression of the gene was positively correlated with strawberry fruit maturation. Another ortholog, *PsDRMI*, has been reported as a bud dormancy-related gene from pea. The abundance of *PsDRMI* transcripts in axillary buds declines within 6 h of apical bud removal. Transcripts re-accumulate when the axillary buds become latent again and the newly established apical bud

becomes active (Stafstrom et al. 1998). Therefore, the gene was named dormancy-related protein (DRP). However, it is unknown how the gene is regulated at the molecular level, or how the two different plant developmental processes are related to *ARP* expression. Steiner et al. (2003) reported a new member of the auxin-repressed protein family from tobacco, whose transcript levels were high during pollen maturation, but rapidly declined to minimal levels in germinating pollen.

Recently, we identified an *ARP* ortholog (named *RpARP*) from the expressed sequence tags (ESTs) derived from the trunk wood of 10-year-old black locust (*Robinia pseudo-acacia* L.) (Yang et al. 2003). The gene was expressed across the trunk wood (bark/cambium, sapwood and sapwood–heartwood transition zone). In this study, the auxin-repressed gene *RpARP* was isolated and characterized. A sequence similarity search in public databases was performed to identify any homologs. Northern blotting was carried out to determine the effects of auxin, sugar, protein synthesis, acidic pH, low temperature, and hormonal and abiotic conditions on auxin-induced repression of *RpARP* gene expression. Transgenic plants were produced to determine if the untranslated regions and sucrose regulatory elements play a role in the repression of the gene. The relationship between *RpARP* expression and hypocotyl elongation was also determined.

Materials and methods

Plant materials

Black locust seeds were germinated as described by Han et al. (1993). Seven-day-old hypocotyls (~2.5 cm long) from the seedlings were used. *Arabidopsis thaliana* (L.) Heynh. plants were grown under sterile conditions; ecotype Columbia seeds were surface-sterilized with 1.5% (v/v) sodium hypochlorite solution containing 0.02% (v/v) TritonX-100 for 5 min with vigorous shaking, and then washed several times with sterile water. Seeds were chilled in water at 4 °C for 2 to 4 days and then plated on MS medium (Murashige and Skoog 1962) containing 0.8% agar and 1% (w/v) sucrose (pH 5.7–5.8). The plants were grown at 25 °C in a 16-h daily photoperiod.

Isolation of the full-length *RpARP* gene and its promoter region

The *RpARP* gene (Genbank AY009094) was isolated through the black locust EST sequencing project (Yang et al., unpublished data). Total RNAs were purified from both the cambial and heartwood regions of 10-year-old black locust trees and used to construct directional cDNA libraries in the pTripleEx plasmid vector (Clontech, Palo Alto, CA). The resulting libraries were applied to EST sequencing. Based on a computational contig search, approximately 15 *RpARP* clones were detected out of the 4000 sequenced clones. The presumed whole transcript of *RpARP* was determined through multiple alignments. The *RpARP* gene promoter was cloned from 2.5 µg of genomic DNA isolated from a black locust seedling with the Genome Walker Kit (Clontech), according to the manufacturer's instructions.

Construction of binary vectors and plant transformation

The 700-bp fragment of *RpARP* cDNA including the 5'-UTR and 3'-UTR was prepared from pTripleARP by restriction digestion with *Sma*I and *Sac*I. The fragment was inserted into the corresponding sites of the binary vector pBI121 under the control of the CaMV 35S promoter to produce the recombinant vector, pBIARP (Figure 1A). To construct pBIARPGUS, a polymerase chain reaction (PCR) using primers containing linker sites, *Xba*I (5') and *Sma*I (3'), was performed on pTripleARP to yield 400 bp of *RpARP* coding region. The resulting PCR product was cloned into pGEM-T (Promega, Madison, WI) to produce the plasmid pGEMARP. The *Sma*I–*Xba*I fragment of pGEMARP was cloned into the corresponding sites of pBI101 (Genbank U12639.1) to yield pBIARPGUS (Figure 1A). *Arabidopsis* ecotype Columbia was transformed using recombinant *Agrobacterium tumefaciens* by the vacuum infiltration method (Bechhold et al. 1998). Transformants were selected on MS medium supplemented with 0.7% (w/v) agar, 1% (w/v) sucrose and 50 mg l⁻¹ kanamycin.

Elongation measurement

Black locust seedlings with uniform hypocotyl lengths (~25 mm) were selected and elongation was measured. Hypocotyls were cut into 5-mm segments and immersed in MS medium + 2% (w/v) sucrose (Figure 2), or in liquid MS medium with or without 2% sucrose, 10 µM naphthaleneacetic acid (NAA), 20 µM fusicoccin (FC), 2% mannose, or low temperature (4 °C). Segments were incubated at 25 °C in a 16-h daily

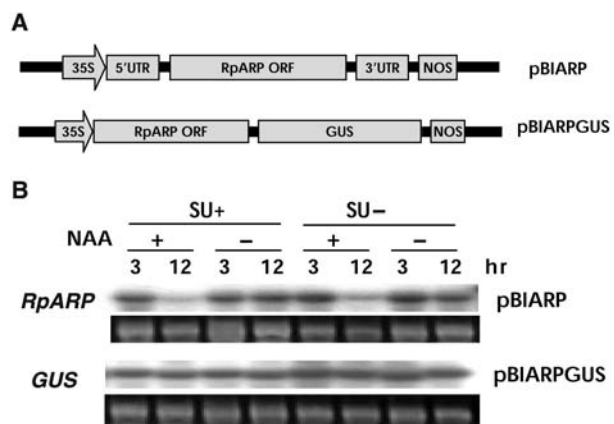


Figure 1. Posttranscriptional regulation of the *RpARP* gene. (A) Schematic representation of the plasmids used in the transformation of *Arabidopsis*. Abbreviations: 35S = CaMV 35S promoter; UTR = untranslated region; ORF = open reading frame; and NOS = *nos* terminator. (B) Northern blots (upper panels) of total RNA isolated from whole tissues of transgenic *Arabidopsis* overexpressing *RpARP* (pBIARP) or expressing *RpARP-GUS* (pBIARPGUS) after treatment with liquid MS medium in the absence (–) or presence (+) of 2% sucrose (SU) or 10 µM naphthaleneacetic acid (NAA) for 3 and 12 h. Each lane was loaded with 6 µg of total RNA. Agarose gels were stained with ethidium bromide (lower panels) to confirm equal loading of rRNA in each lane. *RpARP* and *GUS* were used as probes.

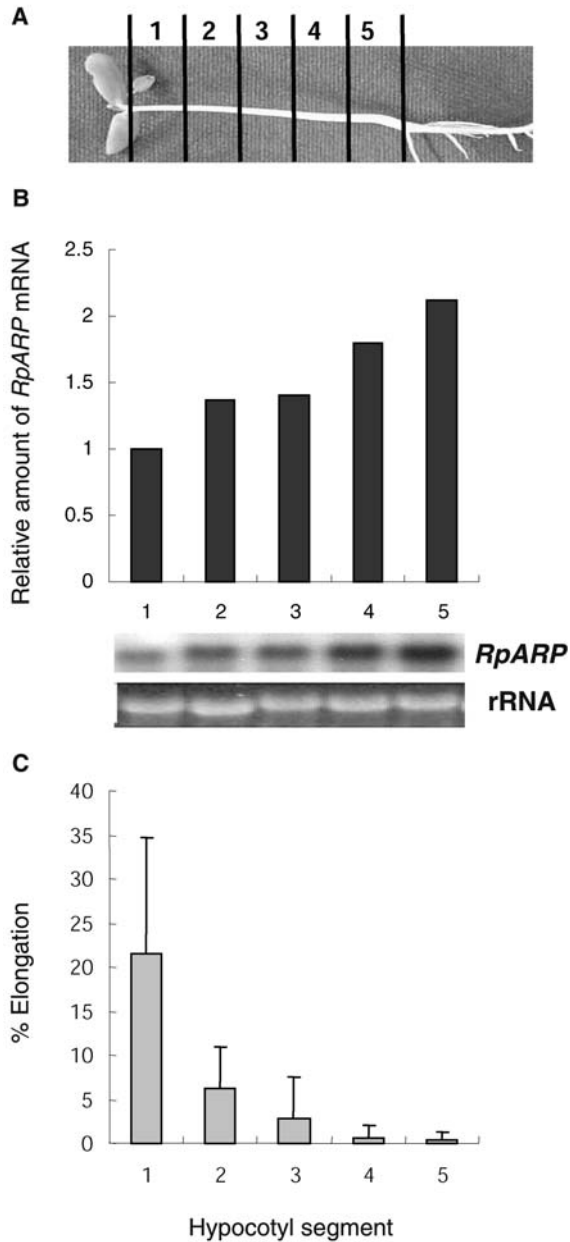


Figure 2. Expression pattern of *RpARP* mRNA is negatively correlated with hypocotyl elongation. (A) Hypocotyls (25 mm length) of black locust seedlings were excised into 0.5 mm segments from top to bottom (1, 2, 3, 4 and 5, respectively). (B) Expression of *RpARP* mRNA in each segment was determined by isolating 6 μ g of total RNA and quantifying the autoradiogram signal of the RNA gel blot using the ImageQuant program (Amersham) as shown in the upper panel. Agarose gels were stained with ethidium bromide (lower panel) to confirm equal loading of rRNA in each lane. Values are expressed relative to the *RpARP* mRNA level from the top segment (1), which is defined as 1. (C) Percent elongation was determined by measuring segments incubated in MS medium + 2% sucrose for 24 h. Data are means \pm SD.

photoperiod on a shaker at 30 cycles per min. Elongation ratios were determined by measuring the increase in segment length after 24 h of incubation.

Hormone and chemical treatment

Hypocotyl segments (5 mm in length) were cut from below the apical meristem of 7-day-old seedlings and incubated in liquid MS medium supplemented with the indicated hormones or chemicals with gentle agitation on a shaker at 30 cycles per min at 25 °C. All hormones and chemicals were purchased from Sigma-Aldrich. Working and stock concentrations of hormones and chemicals were as follows unless otherwise noted: 10 μ M FC (diluted from 10 mM stock solution in dimethyl sulfoxide (DMSO)); 50 μ M cycloheximide (CHX, from 200 mM in DMSO); 20 μ M abscisic acid (ABA, from 10 mM in EtOH); 20 μ M 2-chloroethyl phosphonic acid (EP, from 100 mM in EtOH); 20 μ M gibberellic acid (GA, from 50 mM in H₂O); 20 μ M jasmonic acid (JA, from 200 mM in EtOH); and 10 μ M α -naphthaleneacetic acid (NAA, from 1 mM in H₂O). After the treatments, tissues were frozen in liquid nitrogen and stored at -80 °C for later RNA analysis.

Northern blot analysis

Total RNA was isolated from black locust hypocotyl segments or from whole tissues of transgenic *Arabidopsis* plants using the Trizol reagent (Invitrogen, San Diego, CA). Six micrograms of total RNA were separated on a formaldehyde-agarose gel and transferred to Hybond-N membranes. Hybridization of the northern blots was performed in UltraHyb solution (Amersham-Pharmacia, Piscataway, NJ) at 42 °C according to the manufacturer's instructions. The *RpARP* and *GUS* genes were amplified by PCR using gene-specific primers, labeled with ³²P-dCTP in a random primed reaction and used as probes. To quantify the northern signals, autoradiography films were scanned and analyzed with the ImageQuant program.

Results

Regulation of *RpARP* expression through mRNA stability

To investigate the regulation mechanism and biological function of *RpARP*, we generated transgenic *Arabidopsis* plants harboring T-DNA containing a full-length *RpARP* cDNA, with or without the untranslated regions (UTRs), driven by the CaMV 35S promoter (Figure 1A). The transgenic plants had a phenotype similar to that of the wild type. Expression of *RpARP* (with UTRs) was repressed in response to exogenous auxin in pBIARP transgenic plants, even though the gene is under the control of the constitutive CaMV 35S promoter (Figure 1B). On the other hand, the level of *RpARP* transcripts was unaffected by exogenous auxin in transgenic plants harboring the 35S::RpARPorf:GUS construct (pBIARPGUS, Figure 1A), which contains only 350 bp of the *RpARP* coding region and no UTRs. The expression of transgenic *RpARP* was unaffected by sucrose in transgenic plants transformed with either pBIARP or pBIARPGUS constructs. To determine if the *cis*-element(s) for sucrose signaling is located in the promoter region, we sequenced the 2 kb genomic region upstream of the *RpARP* coding region and searched for putative *cis*-elements. Four sucrose-repressible response elements (Huang et

al. 1990) were found between 70 and 500 bp upstream of the putative transcription start site (Figure 3).

RpARP gene expression is negatively correlated with hypocotyl elongation

We examined expression of the *RpARP* gene in segments of elongating hypocotyls with different elongation rates. Hypocotyls (25 mm in length) were cut into 5-mm segments and incubated on MS medium for 24 h. Then, percent elongation and *RpARP* transcript levels were measured at different regions. Hypocotyl segments showing higher percent elongation had a lower level of *RpARP* expression and vice versa (Figure 2). To further confirm that the expression of the *RpARP* gene was negatively associated with hypocotyl elongation, we examined the effects of various treatments that affect shoot elongation. In general, relatively lower levels of gene expression were observed in treatments that increased shoot elongation, with the exception of the SU+FC+ and Mn+N+ treatments (Figure 4).

The ARP gene family is specific to higher plants

The BLAST search against NCBI dbEST and The Institute For Genomic Research (TIGR) Plant Gene Indices (<http://www.tigr.org/tdb/tgi>) identified homologs of *RpARP*, all of which are from higher plant species (four monocots and eight dicots). No significant hits were obtained from other organisms such

as animal and fungal species using a $1.0E^{-5}$ E-value cutoff. Multiple alignments of the deduced amino acid sequences of the orthologs revealed the presence of highly conserved amino acid domains at both the N-terminal and C-terminal ends (Figure 5A). The deduced amino acid sequence of *RpARP* is highly conserved among plant genes (up to 85% identity at the amino acid level). To examine the evolutionary relatedness of the 12 putative ARP proteins, we generated an unrooted phylogenetic tree with the deduced amino acid sequences (Figure 5B). The tree showed that plant ARPs separated into two distinct clades of dicots and monocots. Sequences from all of the legume species (black locust, pea and soybean) were clustered together.

Suppression of RpARP gene expression by auxin

Expression of the *RpARP* gene in response to exogenous auxin treatment was analyzed by Northern blotting (Figure 6). Total RNA was extracted from black locust seedling hypocotyl fragments of black locust seedlings incubated in auxin-free MS medium after pretreatment with 10 μ M NAA for 24 h, or in MS medium + 10 μ M NAA after no pretreatment with auxin. *RpARP* transcript levels declined within 3 h of exogenous auxin application and were undetectable by Northern blot after 6 h. However, *RpARP* transcripts re-accumulated rapidly when tissues were transferred to auxin-free MS medium (Figure 6A). Auxin-mediated repression of *RpARP* was effectively

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1  tgggctgtgt  gggtgtatgg  acgaatgatc  cacttcggga  caggtccaga  tgacaaggta
61  agagggggta  tgatggtgca  catgaaatgg  gaagtggggg  tgtaaggaga  caagggataa
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1201  cttctcactc  tttccaccct  tcttttttcc  actatatcca  ctcactacca  cgtcaaaacg
1261  cagcgaata  agttacactt  ggaagcaaga  aaacactcca  tcccatttta  gttagagata

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Figure 3. Nucleotide sequence of the *RpARP* promoter region. The arrow indicates a putative transcriptional start site. The putative TATA box is underlined. The sucrose-repressible response element (TATCCAT-motif) is boxed (Huang et al. 1990). The analysis of *cis*-elements was performed with the PlantCARE database (<http://oberon.rug.ac.be/PlantCARE>).

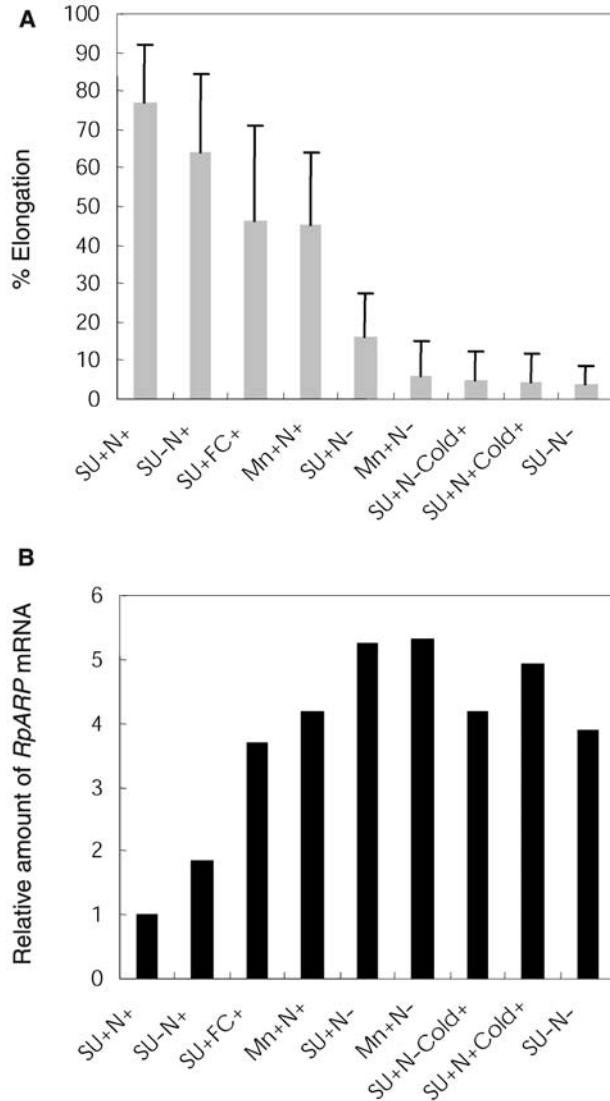


Figure 4. Repression of *RpARP* mRNA is correlated with elongation stimulation phenotype. Hypocotyls (25 mm length) of black locust seedlings were excised into 0.5 mm segments and were incubated in liquid MS medium with or without 2% sucrose (SU), 10 μ M naphthaleneacetic acid (N), 20 μ M fusicoccin (FC), 2% mannose (Mn), or low temperature (4 $^{\circ}$ C) (Cold) for 24 h. (A) Expression of *RpARP* mRNA was determined by isolating 6 μ g of total RNA from the segments after measuring their hypocotyl lengths, and the autoradiogram signal of the RNA gel blot was quantified using the ImageQuant program (Amersham). Values are expressed relative to the lowest amount of *RpARP* mRNA (value = 1).

achieved with NAA concentrations as low as 0.1 μ M NAA (data not shown). To determine if the auxin-mediated reduction of *RpARP* transcript was dependent on de novo protein synthesis, we carried out the same experiment with a protein synthesis inhibitor, cycloheximide. Cycloheximide treatment retarded the reduction of *RpARP* transcripts by auxin and blocked the re-accumulation of transcripts in the auxin-free medium (Figure 6B). The effects of various growth regulators, such as ABA, benzyladenine (BA), EP and GA, and

abiotic stresses, such as salt stress and cold treatment with or without exogenous auxin, on *RpARP* gene expression were determined (Figure 7). Cold treatment increased *RpARP* gene expression whether auxin was present or not, whereas salt slightly depressed the repression by auxin. Treatment with GA resulted in the loss of the *RpARP* transcript even in the absence of auxin. In the presence of sucrose, auxin effectively repressed *RpARP* gene expression in all treatments except cold stress.

RpARP gene expression is suppressed by fusicoccin and acidic pH

To determine the relationship between the physiological action of auxin and *RpARP* gene suppression, samples were treated with FC. Expression of *RpARP* was suppressed by both FC and acidic pH treatments (Figure 8). Under FC treatment, *RpARP* was suppressed in 1 h; however, FC and low pH treatments appear to be less effective than auxin in depressing the *RpARP* transcript. The *RpARP* transcript was undetectable after 2 h of auxin treatment (Figure 6), whereas it was clearly detectable even after 6 h of treatment with FC or low pH (Figure 8). In addition, the transcript level increased again after 9 h of FC treatment.

Effects of sucrose and low temperature on auxin suppression of *RpARP* mRNA expression

Our next question was whether factors associated with auxin-mediated growth can also affect expression of the *RpARP* gene. Sucrose-deprivation and low temperature conditions inhibited the auxin-modulated elongation of hypocotyl segments (Figure 4A). Sucrose deprivation increased *RpARP* expression, and in the low temperature treatment, *RpARP* gene expression was unaffected by exogenous auxin (Figure 9A).

In order to gain some insight into the physiological role of sucrose in auxin-mediated suppression of *RpARP*, we tested the effects of sugar analogs, mannose and mannitol, on the expression of the *RpARP* gene (Figure 9B). We analyzed the expression of the gene over time (0.5, 1, 3, 6, 9 and 24 h) in MS medium containing 2% sucrose, 2% mannose, or 2% mannitol. Mannitol had no effect on gene expression, whereas the effect of mannose was intermediate between that of the control and sucrose treatments.

Discussion

It has long been appreciated that auxin is an important regulator of developmental processes such as tropism, apical dominance and rhizoid initiation in higher plants, modifying the expression patterns of genes involved in these processes (Theologis 1989, Worley et al. 2000). This auxin-mediated regulation mechanism is often highly conserved. For instance, despite differences in the chemical composition of cell walls, the physiological response of all seed plants to auxin and acid is similar, and the key protein mediating the rapid elongation process, expansin, is highly conserved structurally and functionally among seed plants (Shcherban et al. 1995, Cooke et al.

A

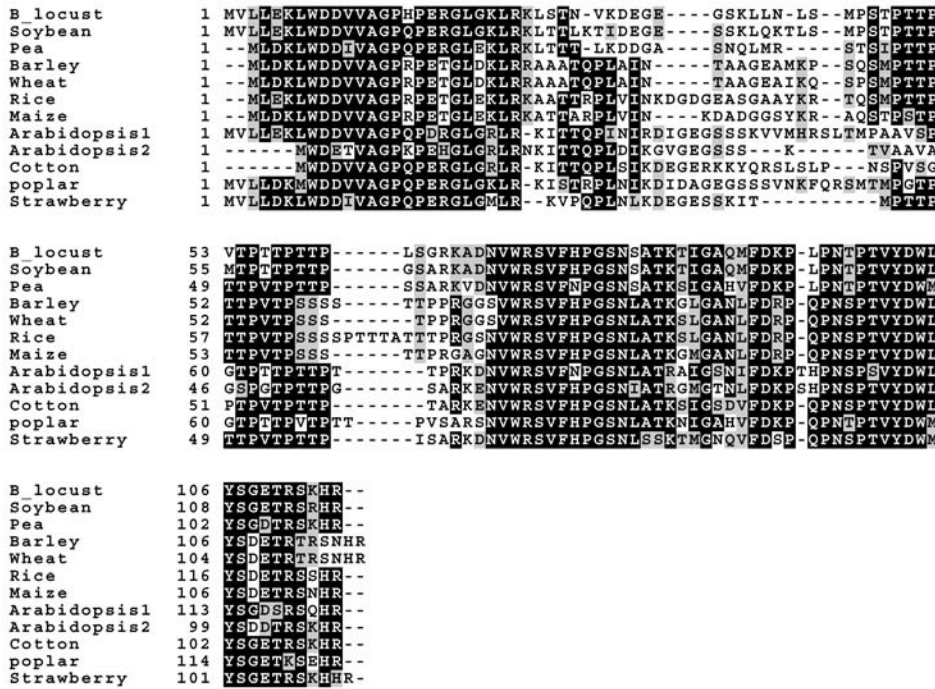
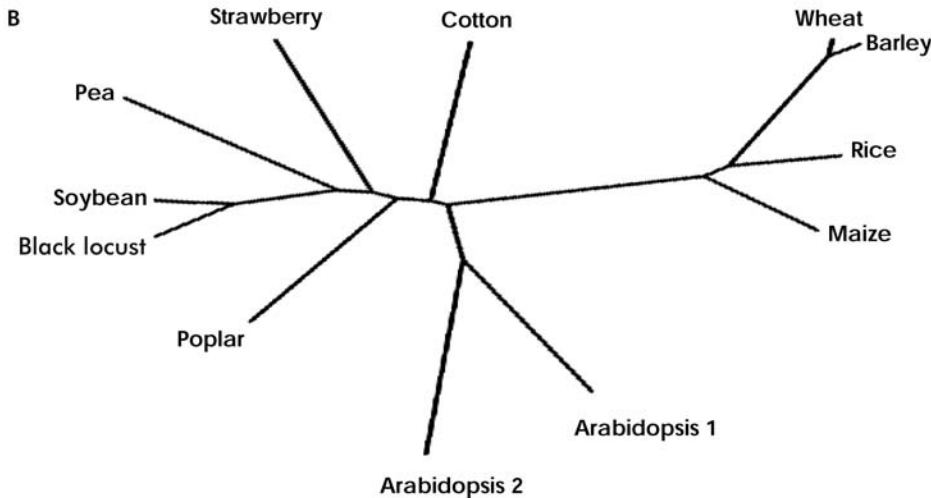


Figure 5. Multiple alignment and evolutionary tree of the predicted RpARP amino acid sequence with other plant ARPs. (A) Sequences of *Arabidopsis*1 (At1g28330)^a, *Arabidopsis*2 (At2g38330)^a, barley (TC26321)^b, cotton (TC9854)^b, maize (TC138763)^b, rice (TC82951)^b, soybean (TC132184)^b, wheat (TC40040)^b, pea (AF029242)^c, strawberry (L44142)^c and poplar (BI136544)^c are compared. Identical amino acid sequences are highlighted in black and sequence similarities in grey. Sequence alignment and sequence identity/similarity was displayed using Clustal W and BOXSHADE programs of the General Computer Group Package. (B) An unrooted phylogenetic tree of putative ARP proteins, shown in (A), was generated using the CTREE program based on the Neighbor-Joining algorithm from the San Diego supercomputer center (<http://biowb.sdsc.edu>). Symbols: ^a = AGI number; ^b = TIGR number; and ^c = Genbank number.



2002). The *ARP* gene is another example. We isolated an *ARP* ortholog (*RpARP*) from black locust, a tree legume. The *RpARP* gene was repressed rapidly by exogenous auxin, consistent with previous studies, supporting the idea that *ARP* genes might have common patterns of expression and hormonal regulation. A sequence homology search with dbESTs indicated that the *ARP* gene is present in many plant species and its amino acid sequence is highly conserved. The two clades shown in the phylogenetic tree of Figure 5B are consistent with the evolutionary distance between monocots and dicots (Soltis et al. 1999). Sequences from the legume species were clustered together, indicating that *RpARP* (from a legume tree) is structurally closer to *ARPs* of other legume species (pea and soybean) than to those of tree species such as

poplar. The high level of structural conservation between monocotyledonous and dicotyledonous plants suggests that (a) the *ARP* gene family might have formed before the evolutionary divergence of monocots and dicots; (b) the *ARP* protein has strict functional constraints that limit structural modification while maintaining function; and (c) the function of the protein is important to normal development or physiology of higher plants. Furthermore, *ARP* homologous sequences are found only in higher plants, suggesting that the gene plays a role in processes specific to higher plants. Although the *ARP* gene in black locust is repressed by exogenous auxin, as observed in previous studies with strawberry, it is still unknown if these genes are functionally conserved.

Sucrose and auxin regulate *RpARP* gene expression by dif-

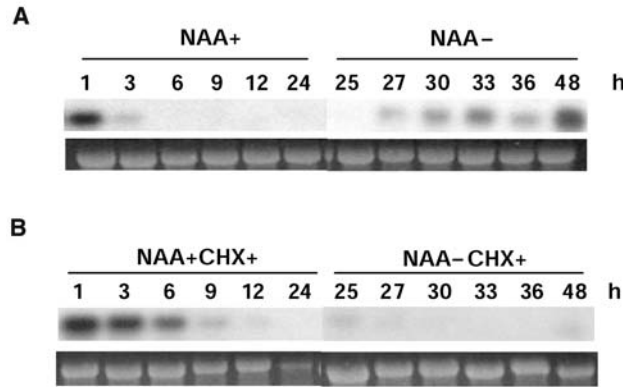


Figure 6. Expression of *RpARP* mRNA in response to auxin and cycloheximide treatments. (A) Hypocotyl segments of a black locust seedling were treated with MS + 2% (w/v) sucrose medium containing 10 μ M naphthaleneacetic acid (NAA+) for the indicated time, or without NAA (NAA-) after 24 h pretreatment with 10 μ M NAA. (B) Hypocotyl segments were treated as in (A), with the addition of 40 μ M cycloheximide (CHX+). Total RNA was extracted at the indicated times, and 6 μ g of total RNA was loaded per lane. Agarose gels were stained with ethidium bromide (lower panels) to confirm equal loading of rRNA in each lane. Blots were probed with a random-primer-labeled *RpARP* cDNA.

ferent mechanisms. For example, the transcript level of *RpARP* was effectively reduced by exogenous auxin in transgenic plants transformed with the 35S::*RpARP* construct containing the *RpARP* structural gene with UTRs, but not in transgenics carrying 35S::*RpARP*orf:GUS, a construct that has the coding region but no UTRs. On the other hand, sucrose had no effect on either construct (Figure 1B). These results suggest that auxin regulates the *ARP* gene posttranscriptionally, and that the responsible *cis*-elements are likely located in the UTR regions. A number of mRNA instability sequences have been identified in the UTR regions. For example, AU-rich elements (AREs), known as the archetypal mRNA instability determinant, were found in the 3'-UTR of mammalian genes such as lymphokine and cytokine (Chen

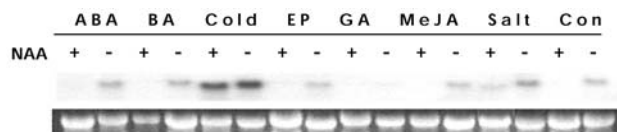


Figure 7. Comparison of *RpARP* transcript levels under various hormonal and abiotic conditions. Total RNA was extracted from black locust hypocotyl segments immersed for 24 h in liquid MS + 2% (w/v) sucrose medium containing 20 μ M of the hormones abscisic acid (ABA), benzyladenine (BA), ethephon (EP), gibberellin (GA) and methyl jasmonate (MeJA), or treated at 4 $^{\circ}$ C (Cold) or with 300 mM NaCl (Salt) in the absence (-) or presence (+) of 10 μ M naphthaleneacetic acid (NAA) as indicated. For the control (Con), samples were incubated only in MS medium + sucrose. Six micrograms of RNA was loaded per lane and hybridized with *RpARP* cDNA. Agarose gels were stained with ethidium bromide (bottom) to confirm equal loading of rRNA in each lane.

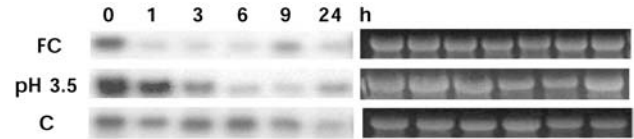


Figure 8. Fusicoccin and acidic pH can repress *RpARP* mRNA expression. Excised hypocotyl segments of black locust seedlings (7 days old) were treated with MS + 2% (w/v) sucrose medium buffered with 5 mM MES (pH 3.5), with or without 10 μ M fusicoccin (FC and C (control), respectively). Total RNA (6 μ g) was isolated after treatment at the indicated times (0.5, 1, 3, 6, 9, 12 and 24 h) and subjected to RNA blot hybridization using probes specific for *RpARP*. The 25S rRNA bands of the corresponding ethidium bromide-stained gels are shown on the right.

and Shyu 1995). The downstream element, which is responsible for mRNA instability in plants, was found in the 3'-UTR of the small auxin-up RNA (SAUR) transcripts (Gil and Green 1996). However, although UTRs are involved in auxin-mediated regulation of the *RpARP* gene, no known regulatory element was found in the UTRs. Therefore, auxin-mediated regulation of *RpARP* transcript stability might represent a novel mechanism for posttranscriptional regulation of gene expression.

Treatment of hypocotyl fragments with the protein synthesis inhibitor, cycloheximide, slowed the repression of *RpARP* transcription and blocked the re-accumulation of transcripts in

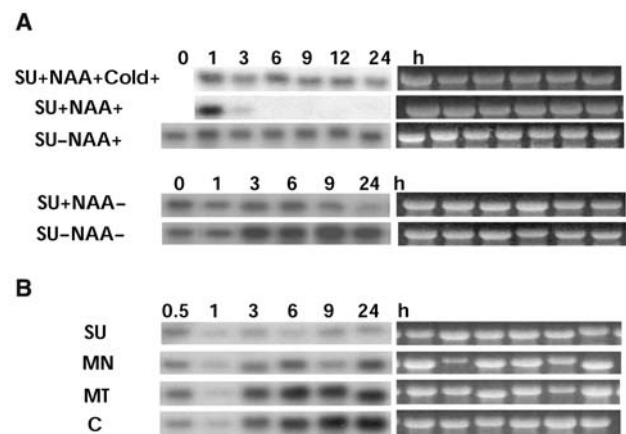


Figure 9. Effects of sucrose and low temperature on auxin suppression of *RpARP* mRNA expression. (A) Hypocotyl segments were treated with liquid MS medium containing 10 μ M naphthaleneacetic acid (NAA) and 2% (w/v) sucrose (SU+NAA+), 10 μ M NAA only (SU-NAA+), 2% sucrose only (SU+NAA-), MS medium only (SU-NAA-), or 10 μ M NAA and 2% sucrose with cold treatment, 4 $^{\circ}$ C (SU+NAA+Cold+). (B) Analysis of *RpARP* mRNA expression under various sucrose isomers. Hypocotyl segments were incubated for 0.5, 1, 3, 6, 9 and 24 h in MS medium containing 2% sucrose (SU), 2% mannose (MN), or 2% mannitol (MT). For the control (C), only MS medium was used. Total RNA (6 μ g) was isolated after treatment at the indicated times and subjected to RNA blot hybridization with probes specific for *RpARP*. Agarose gels were stained with ethidium bromide to confirm equal loading of rRNA in each lane. (SU+NAA+ is a duplication of the left half of Figure 6a.)

auxin-free medium (Figure 6B), indicating that protein synthesis is required for auxin-mediated suppression of *ARP* gene expression.

Fusicoccin, which is known to induce rapid cell elongation through the activation of the transport protein H^+ -ATPase in the plasma membrane (Blatt 1988), causing strong acidification of the cell walls (Marre 1979, Kutschera and Schopfer 1985), also repressed *RpARP* gene expression (Figure 8). The increase in *RpARP* transcript levels after 9 h of FC treatment (Figure 8) suggests that the FC effect might be transient. Whether H^+ -ATPase activation is involved in the auxin-mediated repression of *RpARP* is not known.

Sucrose-deprivation and low temperature conditions inhibited auxin-modulated elongation of hypocotyl segments (Figure 4A). Sucrose has been known to affect auxin-mediated elongation and growth (Stevenson and Cleland 1981, Seyedin et al. 1982, Gray et al. 1998), and low temperature is known to be a general inhibitor of auxin transport and metabolism (Keitt et al. 1967).

Cis-elements responsible for the sucrose signal might be located at genomic regions upstream of the *RpARP* gene. Four copies of conserved TATCCA *cis*-elements were present in the promoter region of *RpARP*. This sucrose-repressible response element was reported to be responsible for sugar repression of the α -amylase gene in rice (Lu et al. 1998, Toyofuku et al. 1998). Lu et al. (1998) identified nuclear proteins binding to TATCCA in a sequence-specific and sugar-dependent manner.

To further understand the physiological role of sucrose in auxin-mediated repression of *ARP* genes, we tested various sugar analogs for their effects on *RpARP* expression. Mannitol (non-permeable through plasma membranes) mimicked the effect of the control treatment lacking sucrose, whereas mannose (a permeable but non-metabolizable sugar; Gibson 2000) showed an intermediate effect between the control and the sucrose treatments (Figure 9B). These results indicate that sucrose is needed for both intracellular osmotic regulation and metabolic energy. Sugars are important regulators of various processes associated with plant growth and development and function as metabolic resources and as structural constituents of cells. In recent studies, sugar was found to favor the expression of growth-related genes while repressing the expression of the stress-related gene (Ho et al. 2001).

Both auxin and sucrose are required to completely repress the expression of *RpARP* gene. This is consistent with the auxin-induced elongation process. According to the acid growth hypothesis (Kutschera 1994), auxin stimulates proton pumping, which results in hyperpolarization of the plasma membrane, thereby inducing cell elongation through acid-dependent cell wall loosening (Senn and Goldsmith 1988, Rayle and Cleland 1992). But after 4 to 6 h, the growth rate begins to decrease without a supply of exogenous sucrose, which is needed for osmotic regulation and the formation of new cell wall compounds (Cheung and Cleland 1991, Inouhe and Yamamoto 1991). Previous studies have indicated that *ARP* gene expression is low in actively growing tissues (Reddy and Poovaiah 1990, Stafstrom et al. 1998). These results led us to

hypothesize that the *RpARP* gene must be down-regulated for auxin-mediated elongation to occur.

Here, we present two pieces of evidence in support of this hypothesis. First, *RpARP* mRNA expression was lower at the upper positions of the hypocotyl, which have more enhanced elongation ability than the lower positions (Figure 2). Second, physiological conditions that stimulate elongation effectively repressed *RpARP* mRNA expression, except in the SU+FC+ and Mn+N+ treatments (Figure 4). This may be due to the transient nature of the FC and mannose effects on gene expression. The *RpARP* gene is expressed at various developmental stages (e.g., from seedling to mature wood) and in various tissues (i.e., leaves, roots, hypocotyl and stem), and most abundantly in non-growing tissues. It is feasible that the *RpARP* gene needs to be repressed for cell growth, probably through cell wall loosening, structural modification to the cell wall, or an increase in elasticity of the cytoskeleton. This seems to corroborate previous results with *ARP* genes isolated and characterized from pea (*PsDRMI*; Stafstrom et al. 1998) and strawberry (*SAR5*; Reddy and Poovaiah 1990). Repression of *SAR5* was associated with strawberry fruit maturation, whereas the expression level of *PsDRMI* was high in dormant axillary buds. These different plant developmental processes are related to cell elongation or expansion processes regulated by auxin.

In conclusion, the *RpARP* gene likely plays a role in biological processes that are characteristic under non-growing (i.e., dormant) or stress conditions such as cold and sucrose-starvation. Subcellular localization of the *RpARP* protein, its interaction with other proteins, and analysis of transgenic plants that over-express or suppress the *RpARP* gene will help determine the biological function of this gene. We are in the process of generating transgenic plants for such studies.

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References

- Abel, S. and A. Theologis. 1996. Early genes and auxin action. *Plant Physiol.* 111:9–17.
- Berleth, T., J. Mattsson and C.S. Hardtke. 2000. Vascular continuity and auxin signals. *Trends Plant Sci.* 5:387–393.
- Blatt, M.R. 1988. Mechanisms of fusicoccin action—a dominant role for secondary transport in a higher-plant cell. *Planta* 174:187–200.
- Chen, C.Y. and A.B. Shyu. 1995. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci.* 20: 465–470.
- Cheung, S.P. and R.E. Cleland. 1991. Galactose inhibits auxin-induced growth of *Avena* coleoptiles by 2 mechanisms. *Plant Cell Physiol.* 32:1015–1019.
- Cooke, T.J., D. Poli, A.E. Sztein and J.D. Cohen. 2002. Evolutionary patterns in auxin action. *Plant Mol. Biol.* 49:319–338.
- Gibson, S.I. 2000. Plant sugar-response pathways. Part of a complex regulatory web. *Plant Physiol.* 124:1532–1539.

- Gil, P. and P.J. Green. 1996. Multiple regions of the *Arabidopsis* SAUR-AC1 gene control transcript abundance: the 3' untranslated region functions as an mRNA instability determinant. *EMBO J.* 15: 1678–1686.
- Gray, W.M., A. Ostin, G. Sandberg, C.P. Romano and M. Estelle. 1998. High temperature promotes auxin-mediated hypocotyl elongation in *Arabidopsis*. *Proc. Natl. Acad. Sci.* 95:7197–7202.
- Guilfoyle, T., G. Hagen, T. Ulmasov and J. Murfett. 1998. How does auxin turn on genes? *Plant Physiol.* 118:341–347.
- Hagen, G. and T. Guilfoyle. 2002. Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Mol. Biol.* 49: 373–385.
- Hamann, T. 2001. The role of auxin in apical-basal pattern formation during *Arabidopsis* embryogenesis. *J. Plant Growth Reg.* 20: 292–299.
- Han, K.H., D.E. Keathley, J.M. Davis and M.P. Gordon. 1993. Regeneration of a transgenic woody legume (*Robinia pseudoacacia* L., black locust) and morphological alterations induced by *Agrobacterium rhizogenes*-mediated transformation. *Plant Sci.* 88: 149–157.
- Ho, S., Y. Chao, W. Tong and S. Yu. 2001. Sugar coordinately and differentially regulates growth- and stress-related gene expression via a complex signal transduction network and multiple control mechanisms. *Plant Physiol.* 125:877–890.
- Huang, N., T.D. Sutliff, J.C. Litts and R.L. Rodriguez. 1990. Classification and characterization of the rice alpha-amylase multigene family. *Plant Mol. Biol.* 14:655–668.
- Inouhe, M. and R. Yamamoto. 1991. Effects of 2-deoxygalactose on auxin-induced growth and levels of UDP-sugars in higher plants. *Plant Cell Physiol.* 32:433–438.
- Keitt, G.W., Jr. and R.A. Baker. 1967. Acropetal movement of auxin: dependence on temperature. *Science* 156:1380–1381.
- Kutschera, U. 1994. The current status of the acid growth hypothesis. *New Phytol.* 126:549–569.
- Kutschera, U. and P. Schopfer. 1985. Evidence for the acid growth theory of fusicoccin action. *Planta* 163:494–499.
- Lu, C.A., E.K. Lim and S.M. Yu. 1998. Sugar response sequence in the promoter of a rice α -amylase gene serves as a transcriptional enhancer. *J. Biol. Chem.* 273:10,120–10,131.
- Marre, E. 1979. Fusicoccin: a tool in plant physiology. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 30:273–288.
- Muday, G.K. 2001. Auxins and tropisms. *J. Plant Growth Reg.* 20: 226–243.
- Rayle, D.L. and R.E. Cleland. 1992. The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiol.* 99: 1271–1274.
- Reddy, A.S.N. and B.W. Poovaiah. 1990. Molecular cloning and sequencing of a cDNA for an auxin-repressed messenger-RNA—correlation between fruit growth and repression of the auxin-regulated gene. *Plant Mol. Biol.* 14:127–136.
- Rouse, D., P. Mackay, P. Stirnberg, M. Estelle and O. Leyser. 1998. Changes in auxin response from mutations in an AUX/IAA gene. *Science* 279:1371–1373.
- Ruegger, M., E. Dewey, W.M. Gray, L. Hobbie, J. Turner and M. Estelle. 1998. The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes Develop.* 12:198–207.
- Senn, A.P. and M.H.M. Goldsmith. 1988. Regulation of electrogenic proton pumping by auxin and fusicoccin as related to the growth of *Avena* coleoptiles. *Plant Physiol.* 88:131–138.
- Seyedin, N., J.S. Burris, C.E. Lamotte and I.C. Anderson. 1982. Temperature-dependent inhibition of hypocotyl elongation in some soybean cultivars. 1. Localization of ethylene evolution and role of cotyledons. *Plant Cell Physiol.* 23:427–431.
- Shcherban, T.Y., J. Shi, D.M. Durachko, M.J. Guiltinan, S.J. McQueen-Mason, M. Shieh and D.J. Cosgrove. 1995. Molecular cloning and sequence analysis of expansins—a highly conserved, multigene family of proteins that mediate cell wall extension in plants. *Proc. Natl. Acad. Sci.* 92:9245–9249.
- Soltis, P.S., D.E. Soltis and M.W. Chase. 1999. Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature* 402:402–404.
- Stafstrom, J.P., B.D. Ripley, M.L. Devitt and B. Drake. 1998. Dormancy-associated gene expression in pea axillary buds. *Planta* 205:547–552.
- Steiner, C., J. Bauer, N. Amrhein and M. Bucher. 2003. Two novel genes are differentially expressed during early germination of the male gametophyte of *Nicotiana tabacum*. *Biochim. Biophys. Acta* 1625:123–133.
- Stevenson, T.T. and R.E. Cleland. 1981. Osmoregulation in the *Avena* coleoptile in relation to auxin and growth. *Plant Physiol.* 67:749–753.
- Theologis, A. 1989. Auxin-regulated gene expression in plants. *Biotechnology* 12:229–243.
- Toyofuku, K., T. Umemura and J. Yamaguchi. 1998. Promoter elements required for sugar-repression of the *RAmy3D* gene for α -amylase in rice. *FEBS Lett.* 428:275–280.
- Ulmasov, T., G. Hagen and T.J. Guilfoyle. 1997. ARF1, a transcription factor that binds to auxin response elements. *Science* 276: 1865–1868.
- Walker, L. and M. Estelle. 1998. Molecular mechanisms of auxin action. *Curr. Opin. Plant Biol.* 1:434–439.
- Worley, C.K., N. Zenser, J. Ramos, D. Rouse, O. Leyser, A. Theologis and J. Callis. 2000. Degradation of Aux/IAA proteins is essential for normal auxin signalling. *Plant J.* 21:553–562.
- Yang, J., S. Park, D.P. Kamdem, D.E. Keathley, E. Retzel, C. Paule, V. Kapur and K.-H. Han. 2003. Novel gene expression profiles define the metabolic and physiological processes characteristic of wood and its extractive formation in a hardwood tree species, *Robinia pseudoacacia*. *Plant Mol. Biol.* In press.

