

Full Length Research Paper

Temporal expression of genes involved in the biosynthesis of gibberellins in birch (*Betula platyphylla*)

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Accepted 26 August, 2011

Gibberellins (GAs) are a large family of endogenous plant growth regulators. Bioactive GAs influence nearly all processes during plant growth and development. In the present study, we cloned and identified 10 unique genes that are potentially involved in the biosynthesis of GAs, including one *BpGGDP* gene, two *BpCPS* genes, one *BpKS* gene, two *BpKO* genes, three *BpGA20ox* genes and one *BpGA2ox* gene. The temporal expression of these 10 genes in birch was analyzed during a one-year growth cycle by real time reverse transcriptase-polymerase chain reaction (RT-PCR). The results show that their expression patterns were individually distinct throughout the one-year growth cycle. Three *BpGA20ox* genes, which are potential limiting factors of bioactive GA biosynthesis, displayed differential expression patterns during the course of the experiment. This study provides insights into the roles of genes involved in the biosynthesis of GAs during the early development of birch.

Key words: *Betula platyphylla* Suk, gene expression, gibberellins, real time reverse transcriptase-polymerase chain reaction (RT-PCR).

INTRODUCTION

Gibberellins (GAs) are a strikingly large family of endogenous growth regulators in higher plants (Sun, 2000; Swain and Singh, 2005; Yamamoto et al., 2010). Although, more than 100 GAs have been identified to date, only a few of these possess biologically prominent actions, including GA₁, GA₃, GA₄ and GA₇ (Bömke and Tudzynski, 2009; Huang et al., 2010). These bioactive GAs affect nearly all processes during plant development. Increasing evidence suggests that bioactive GAs can trigger cell division and can increase the hydrolysis of starch by breaking down fructose and sucrose into glucose, which are required for cell metabolic activity, seed germination and the mobilization of seed reserves (Joly et al., 2004; Swain and Singh, 2005). GAs also play certain various roles in many physiological phenomenon, including leaf expansion, auxiliary bud growth, root growth and internode length, because GAs can loosen primary cell walls and add new wall material to form the

architecture necessary for cellular elongation (Kawana et al., 2007; Deore and Johnson, 2008; Hirano et al., 2008). In addition, GAs can influence floral differentiation as they are most important factors controlling flowering in higher plants, and these can be employed effectively for artificially controlling flowering (Yoshino, 2000; Vieira et al., 2010; Yao et al., 2010). The process of fruit-set and growth also depend on GAs in tomato (Martí et al., 2010), and GA₃ affects the senescence of leaves in many species (Cheng et al., 2009). Furthermore, GAs are implicated in the transmission of light and temperature stimuli, which are mediated through their effects on GA biosynthesis and sensitivity (Phillips, 1998; Vieira et al., 2010).

GA biosynthesis is subject to feedback regulation that generally follows three steps (Bethke and Jones, 1996). First, geranylgeranyl diphosphate (GGDP) is converted to *ent*-kaurene by the enzymes *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) in the plastids of meristematic tissues (Martí et al., 2010). Second, a series of reactions are deduced to take place in the endoplasmic reticulum, whereby *ent*-kaurene oxidase (KO) catalyzes the conversion of *ent*-kaurene to *ent*-kaurenoic acid, followed by the conversion of *ent*-

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kaurenoic acid to GA₁₂ (catalyzed by *ent*-kaurenoic acid oxidase; KAO) (Bömke and Tudzynski, 2009; Dayan et al., 2010), then GA₁₂ is converted to GA₅₃. Finally, in the cytoplasm, GA₁₂ and GA₅₃ are converted to various GA intermediates and bioactive GAs, via a series of oxidation steps catalyzed by cytosolic dioxygenases (e.g., GA 20-oxidases [GA20ox] and GA 3-oxidases [GA3ox]). However, bioactive GAs and their precursors can be converted to inactive forms by GA 2-oxidases (GA2ox) and other catabolic enzymes (Žiauka and Kuusienė, 2010). Thus, the regulation of the expression of the GA20ox, GA3ox and GA2ox plays a critical role in GA homeostasis. GA-mediated responses can be controlled through the regulation of specific biosynthetic genes (Chen et al., 2007), including *GA20ox*, *GA3ox* and *GA2ox*. Transcript levels of many GA dioxygenases are subject to positive (*GA2ox*) and negative (*GA20ox* and *GA3ox*) feedback regulation by the GA signaling pathway (Hytönen et al., 2009). In addition, other hormones, mainly auxin and ethylene, also influence GA biosynthesis and catabolism (Martí et al., 2010).

Birch (*Betula platyphylla*) is distributed widely in temperate and sub-tropical areas of Asia (Zhang et al., 2011). It is a deciduous broad-leaved tree with high photosynthetic capacity and is able to adapt to soils that are low in water content or nutritionally poor (Kitao et al., 2001). Birch is used as a building material and it can also be pulped for use by the paper industry. Moreover, birch sap can be drunk as a tonic and its bark is used in folk medicine for the treatment of inflammatory diseases (Kim et al., 2010). Therefore, it is important to study its development and physiological processes.

In the present study, ten unique genes potentially involved in GAs biosynthesis were cloned from young birch leaves. The temporal expression of these genes was analyzed by real time reverse transcriptase-polymerase chain reaction (RT-PCR) during a one-year growth cycle. The results showed that the expression patterns and abundances of these genes altered significantly during the experimental period. This study provides useful insight into the roles of the genes involved in GA biosynthesis and the roles of GAs in birch development.

MATERIALS AND METHODS

Four-year-old birch trees (*B. platyphylla* Suk) were grown in a field under natural conditions in Harbin (China) located at 45° 44' N and 126° 36' E. Young leaves from these trees (sprouting leaves near the shoot tips) were harvested at 13:00 h at different stages of growth on certain days during the 2009 growth season (May 1st, May 15th, June 1st, June 15th, July 1st, July 15th, August 1st and August 15th). All leaf samples were frozen immediately in liquid nitrogen, and were used for real time RT-PCR.

Temporal expression analysis of genes potentially involved in GAs biosynthesis

A transcriptome from the shoots of birch was constructed using the

solexa method, and this was used to identify 10 unique genes potentially involved in the biosynthesis of GAs, including one gene for *BpGGDP*, two genes for *BpCPS*, one gene for *BpKS*, two genes for *BpKO*, three genes for *BpGA20ox* and one gene for *BpGA2ox*. Total RNA was extracted from each sample using a CTAB method and digested with DNase I (Promega) to remove any DNA contamination. One microgram of total RNA from each sample was reverse transcribed into cDNA using oligo-deoxythymidine as primer in a final volume of 10 μ L. The synthesized cDNAs were diluted to 100 μ L with water and used as the template for the RT-PCR performed using an MJ Opticon^{TM2} machine (Bio-Rad, Hercules, CA). The genes for actin, β -tubulin and ubiquitin were selected to act as internal controls to enable normalization of the amount of total RNA present in each reaction. The primers used for real time RT-PCR are shown in Table 1.

The reaction mixture (final volume of 20 μ L) contained 10 μ L of SYBR Green Real Time PCR Master Mix (Toyobo), 0.5 μ M each of forward and reverse primers and 2 μ L of cDNA template (equivalent to 100 ng of total RNA). The amplification was performed using the following cycling parameters: 94°C for 30 s followed by 45 cycles at 94°C for 12 s, 58°C for 30 s, 72°C for 45 s and 79°C for 1 s. A melting curve was generated for each sample at the end of each run to assess the purity of the amplified products. All experiments were conducted with three biological repeats, and each biological repeat was conducted with three technical repeats. The expression levels were calculated from the threshold cycle according to the delta-delta CT (cycle threshold) method. The relative expression ratios were calculated as the transcription level at different times divided by the transcription level for the first sample collected (May 1st). All relative expression level values were log₂ transformed.

Statistical analyses

Excel 2007 software (Microsoft Company, USA) was used for data analyses and the preparation of histograms. In each case, total RNA was extracted for three parallel samples (biological repeats). Data presented in figures are means \pm standard deviation (SD) of these replicates.

RESULTS AND DISCUSSION

Cloning of birch genes involved in the biosynthesis of GAs

A transcriptome from birch leaves was constructed and used to identify 10 unique genes that are potentially involved in the biosynthesis of GAs, including a *GGDP* gene (*BpGGDP*), two *CPS* genes (*BpCPS*), a *KS* gene (*BpKS*), two *KO* genes (*BpKO*), three *GA20ox* genes (*BpGA20ox*) and a *GA2ox* gene (*BpGA2ox*). These gene sequences have been submitted to GenBank with the accession numbers from HO112169 to HO112178 (Table 1).

Relative abundance of the 10 GA biosynthesis-related genes

The relative abundances of the ten genes potentially involved in GA biosynthesis were determined for young leaves from birch trees in the early stages of development (May 1st). The gene with the lowest expression

Table 1. The primers used in real time RT-PCR.

Gene	GenBank number	Forward primer (5'- 3')	Reverse primer (5' -3')
<i>BpDDGP</i>	HO112169	GCCAATTACATTGCTCACAG	ACGCTAGTCAAGATTCAACC
<i>BpCPS1</i>	HO112170	CTGCGAATGAACCTCTTTGAC	GCTTGGCAATTGTTGTAGTC
<i>BpCPS2</i>	HO112171	GAGCTGCTGTCCCATCCTC	CAGAATCAATATCATCTGG
<i>BpKS</i>	HO112172	GGTTATTGGATAATCAACTC	AGTTGGTTCTCATCGGTAGC
<i>BpKO1</i>	HO112173	CTGGTTACAACAGAATGG	TCCAGCAGGAACATAGTACC
<i>BpKO2</i>	HO112174	AGAAGAGCTTGTGCAGGTG	AGGTTTCAGTAGTTACTAG
<i>BpGA2ox</i>	HO112175	GAAACCTAGCCTGTTATCAC	CTTCAGGTAATGAGCAATGG
<i>BpGA2ox1</i>	HO112176	GTGATAATGGAGGTGTTGGC	GTGTCACCAATGTTGATGAC
<i>BpGA2ox2</i>	HO112177	GCAACAAGTTCTTCTCACTC	GATTTCCATCGTATGTCTCC
<i>BpGA2ox3</i>	HO112178	GATCAAAAGGCTTAAAGAGC	ACTTTCATTGTCTATGATTGG
<i>Actin</i>	HO112155	GCATTGCTGATAGGATGAGC	CCACTTGATTAGAAGCCTCTC
<i>β-tubulin</i>	HO112154	ACGCCAAACCCTAAATCTGG	GGATCGGATGCTGTCCATG
<i>Ubiquitin</i>	HO112156	CCATCTGGTGCTAAGACTGAG	AGGACCAGATGGAGAGTGC

abundance (the highest delta Ct value) was used as the calibrator (designated to be a magnitude of expression of 1) to determine the relative expression levels of the other nine genes (Figure 1). The results show that these ten genes displayed marked differences in expression. *BpCPS1*, *BpCPS2*, *BpKS* and *BpGA2ox3* were expressed in low abundance on May 1st, with *BpCPS2* having the lowest expression among these genes. Conversely, *BpGGDP* had the greatest expression level at this time point and its abundance was far greater than that of the other nine genes.

Temporal expression of GA biosynthesis-related genes

Temporal expression levels of the ten genes were assessed during a one-year growth cycle. The gene expression patterns divided the genes into five groups (Figure 2). The first group contained *BpCPS2* and *BpGA2ox2*, whose expression increased from May 1st to June 1st. Subsequently, the expression of *BpCPS2* and *BpGA2ox2* reduced, but peaked on July 15th before reducing again. The second group included the *BpKS*, *BpKO1*, *BpKO2*, *BpGA2ox* and *BpGA2ox3* genes, whose expression increased on May 15th, but then reduced from May 15th to June 15th. The expression of these genes was initially up-regulated, but then down-regulated from July 1st to August 15th. The third group contained only *BpDDGP* and its expression was down-regulated from May 1st to June 1st, but this recovered to the May 1st level from June 15th to July 15th. *BpDDGP* expression decreased at August 1st, but it recovered again to approximately the May 1st level. Only *BpCPS1* was in the fourth group, whose expression was up-regulated before June 15th, but then down-regulated from June 1st to August 15th. The fifth group contained the gene for *BpGA2ox1*; its expression was similar to the

second group, excluding the down-regulation of expression at May 15th.

Previous studies have shown that GAs modulate gene expression and protein synthesis, and ultimately affect the physiological and biochemical processes of plants (Wu et al., 2008). The biosynthesis and metabolism of GAs involves complex pathways, and many enzymes are involved in its signaling pathways (Huang et al., 2010). In the present study, 10 unique genes that are potentially involved in GA biosynthesis were cloned and identified. The present study show that the expression of the *BpGGDP* gene had greatest abundance during the early stages of development (Figure 1). Relative expression of *BpGGDP* remained stable from May 1st to August 15th (Figure 2). This may correlate with GGPP synthase activity that catalyzes the consecutive condensation of IPP to FPP then to GGPP. GGPP is the precursor for diterpenoids and can be regarded as a branching point to many important isoprenoid compounds (Lin et al., 2010; Singkaravanit et al., 2010). In addition, in our results, *BpCPS1*, *BpCPS2* and *BpKO1* displayed a relatively steady pattern of expression (Figure 2). This phenomenon concurred with the results of Chen et al. (2007) in that gene expression and enzyme activities of *CPS*, *KS* and *KAO* are relatively consistent, and the over expression of *CPS* or *KS* in *Arabidopsis* only increased levels of upstream precursors but not bioactive GAs (Chen et al., 2007).

The regulation of *GA2ox* expression plays a pivotal role in GA homeostasis and overexpression of *GA2ox* in transgenic *Arabidopsis* results in longer hypocotyls, early flowering, increased stem elongation and reduced seed dormancy, which are associated with an increase in the main bioactive GA (Hedden and Phillips, 2000; Hytönen et al., 2009; Martí et al., 2010). These results suggest that *GA2ox* activity is a limiting factor in GA biosynthesis. In *Arabidopsis*, *GA2ox* is encoded by at least three different genes that show differential expression

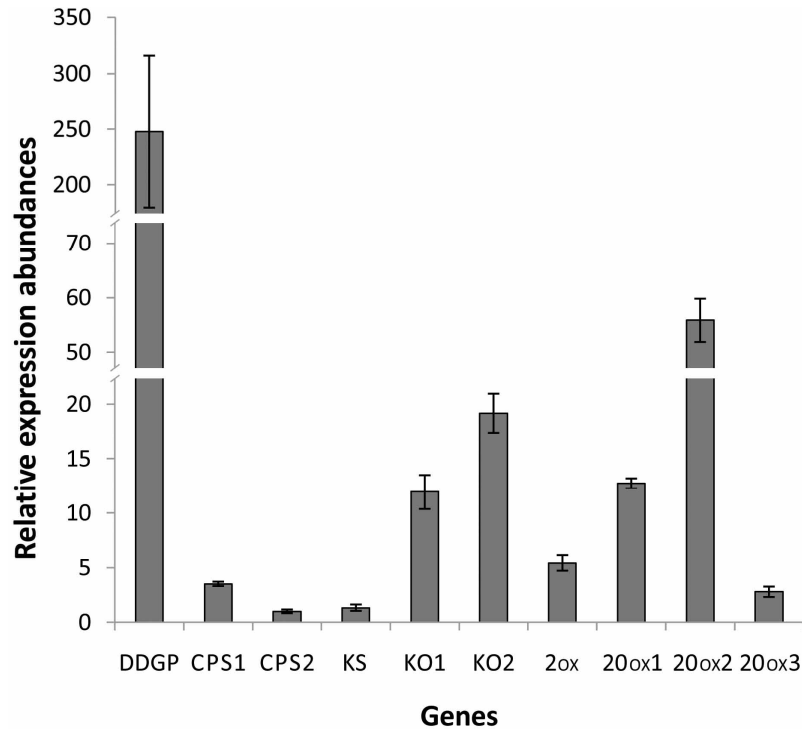


Figure 1. Relative expression of the ten genes involved in GA biosynthesis on May 1st (early stage of development).

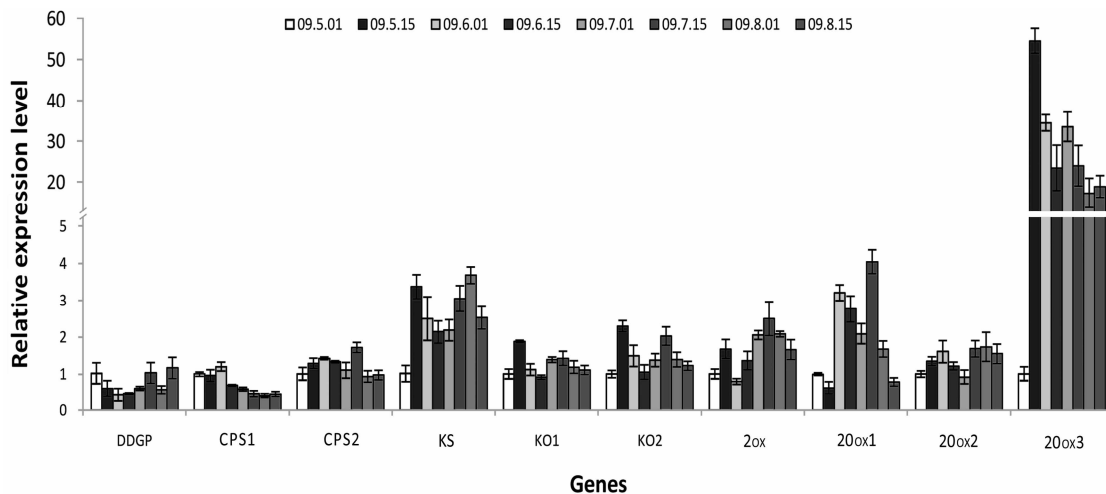


Figure 2. Temporal expression of the ten genes involved in GA biosynthesis during a one-year growth cycle.

patterns, and specific reductions in mRNA levels for each individual gene may identify the developmental roles of each enzyme (Hedden and Phillips, 2000). In the present study, three unique genes of *BpGA20ox* were cloned. *BpGA20ox3* expression was the lowest of the *BpGA20ox* genes and showed similar expression levels to *BpCPS1* (Figure 1). The relative expression of *BpGA20ox2* was greater than the other two *BpGA20ox* genes. Furthermore, these three *BpGA20ox* genes had distinct

expression patterns throughout the life cycle of birch (Figure 2), which suggested that they may have different functions in GA biosynthesis. The function of these *BpGA20ox* genes will be investigated further in a future study.

GA2ox are encoded by a small gene family in pea (*Pisum sativum*) and these negatively regulate the levels of bioactive GAs (Swain and Singh, 2005). The expression of *AtGA2ox1* and *AtGA2ox2* can be up-

regulated by the application of GA₃ to maintain GA homeostasis during stem elongation (Huang et al., 2010). Schomburg et al. showed that overexpression of GA2ox decreased GA levels and created a dwarf phenotype in transgenic Arabidopsis, and reduced GA levels in seeds promoted dormancy in tobacco plants (Huang et al., 2010). The results of the present study showed that only a single gene of *BpGA2ox* was cloned from young leaves of birch and its transcript abundance was only half of that of *BpGA20ox1* (Figure 1). The *GA20ox* gene positively regulates the biosynthesis of GA; however, the *GA2ox* plays an opposing role in GA biosynthesis, since its expression can be induced by GA treatment (Bömke and Tudzynski, 2009). The expression pattern of *BpGA2ox* was similar to that of *Bp20ox3*, indicating that *BpGA2ox* may be involved in maintaining bioactive GA biosynthesis homeostasis.

In conclusion, ten genes with potential involvement in GA biosynthesis were cloned. The temporal expression of these genes was studied during a one-year growth cycle and their roles in birch development were discussed. This study may provide useful insight into the roles of these genes in GA biosynthesis and in birch development.

ACKNOWLEDGEMENTS

This work was supported by National High Technology Research and Development Program of China (863 Program) (2009AA10Z107), Special Research Funds for Public Welfare Forestry (No. 200904039) and Heilongjiang Postdoctoral Financial Assistance (LBH-Z09287).

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