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MeJA-inducible expression of the heterologous *JAZ2* promoter from Arabidopsis in *Populus trichocarpa* protoplasts

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Abstract

Plant hormone jasmonates play an important role in the response of plants to environmental stress. Jasmonate ZIM-domain (JAZ) family proteins are key elements in the jasmonate signaling pathway. In *Arabidopsis thaliana* (Arabidopsis), the *JAZ2* (At1g74950) gene expresses at a low level in the entire plant, but the response can be strongly and quickly induced by MeJA. The promoter sequence (1389 bp) of *JAZ2* from Arabidopsis genome was cloned and linked to a GFP reporter gene, and we then investigated the transient expression of *GFP* in *Populus trichocarpa* protoplasts. After treating the transformed protoplasts with MeJA, we observed the GFP fluorescence and quantified its mRNA transcription level. The results showed that the Arabidopsis *JAZ2* promoter was able to drive *GFP* expression, and also responded to MeJA in *Populus trichocarpa* mesophyll protoplasts. The analysis of promoter sequences of *JAZ* gene family members showed that there were several conserved motifs, which were related to jasmonate signaling, such as G-box (CACGTG), CGTCA-motif (CGTCA) and TGACG-box (TGACG), and also some known cis-acting regulatory elements involved in other aspects, however the function of these motifs still needs further study.

Key words: GFP fluorescence, jasmonate, promoter

1 Introduction

As sessile organisms plants continuously need to adjust to environmental changes. Effective induction of the defense responses requires a network of signal transduction processes, resulting in the rapid activation of defense gene expression. A number of secondary signal molecules, such as salicylic acid, jasmonates and ethylene, act to amplify and regulate defense responses after initial activation (Farmer et al. 2003). Among these compounds, jasmonic acid and its derivatives, such as methyl jasmonate, collectively called jasmonates (JA), are lipid-derived signals. Recently, some studies have shown that the co-receptor complex of JA signaling perception in *Arabidopsis thaliana* (Arabidopsis) included the F-box protein coronatine insensitive 1 (COI1), transcription factor MYC2 and jasmonate ZIM domain (JAZ) proteins (Chini et al. 2007, Thines et al. 2007). In an unelicited state, JAZ proteins bind the transcription factor MYC2 and inactivate it. However in the presence of increased JA-Ile levels (the endogenous bioactive hormone), JAZ proteins interact with COI1 and are subsequently degraded by the 26S proteasome. The removal of JAZ proteins releases MYC2, enabling the transcriptional activator to regulate the expression of early jasmonate-responsive genes including *JAZ* itself. Apparently, there is a negative regulatory feedback loop, which mediated the response to JA signaling (Sheard et al. 2010, Chini et al. 2007, Thines et al. 2007).

The majority of JA-inducible promoters were cloned from the upstream region of the pathogen related genes (e.g. *PDF1.2*, *THI2.1* and *PR4*) (Sa et al. 2003, Creelman & Mullet 1997, Brown et al. 2003), however these genes were located downstream of the JA signaling pathway, so that they could not respond to the initial JA signaling. In contrast, *JAZs* and *MYC2* are primary response genes in the JA signaling pathway (Chung et al. 2008), their transcription levels peaked early (e.g., 0.5 h) after MeJA treatment, and the responses of *JAZs* were even earlier, within 5 min of wounding. It seems that the timing of JA-induced transcription in response to wounding is more rapid than previously thought, whereas the expression of other JA response genes, such as *VSP1* and *LOX2*, are delayed (Chung et al. 2008).

Populus trichocarpa, black cottonwood, is a deciduous broadleaf tree species native to western North America. It is used for timber, but diseases caused by pathogens and herbivores result in a reduction of quality and quantity. It is generally believed that homologous promoters should be avoided in transgenic projects, as they could lead to transgene and/or the resident genes silencing (Finnegan & McElroy 1994). So, it is becoming increasingly important to explore the potential of heterologous promoter activity for the purpose of producing transgenic *Populus trichocarpa* with specific induced expression of resistance genes against pests and diseases. Additionally, the effects of continuous expression of some defense genes are considered a waste of energy *in vivo* and harmful to plant growth and development. In the present study, the promoter sequence of Arabidopsis *JAZ2* gene was cloned and analysed, and we provide evidence that the MeJA-inducible promoter has potential applications for *Populus trichocarpa* transgenic projects.

2 Materials and methods

2.1 Plant materials and growth conditions

Seeds of Arabidopsis (Columbia ecotype) were grown on a medium with half strength Murashige and Skoog salts (Sigma-Aldrich, USA), 10 g l^{-1} sucrose. pH is adjusted to 5.8 with 1M KOH, and 8 g l^{-1} agar is added. Tissue culture plantlets of *Populus trichocarpa* (about 3 cm height) were grown on the same medium as Arabidopsis. The two plant species were cultivated in an environmentally controlled chamber for 3 weeks, with photosynthetic flux of 150 µmol m^{-2} s^{-1}, a photoperiod of 16 h light at 22°C and 8 h dark at 18°C, and a relative humidity of approximately 60%.

2.2 MeJA treatment

Arabidopsis plants were sprayed with MeJA (final concentration 50 µM, in 0.001% ethanol) and control plants were

treated with 0.001% ethanol. *Populus trichocarpa* protoplasts were treated with the same concentration of MeJA as the Arabidopsis plants.

2.3 Promoter cloning and promoter::GFP fusion vector construction

The promoter sequence of *JAZ2* gene was PCR amplified with specific primers 5′(*Hin*dIII)-CCCAAGCTTGTGACCACCCC-ACTTGCCTTCTT-3′ and 5′(*Bam*hI)-CGCGGATCCCGTTGA-AACCGAAATTGAAATCG-3′ (restriction endonuclease sites are shown with underlined letters), using the *Arabidopsis thaliana* genomic DNA as a template. Next, the amplified fragment (1389 bp) was digested with *Hin*d III and *Bam*h I endonuclease. The binary vector pBIG (pBI121 with *GFP-S65T* instead of *GUS*, stored in our laboratory) was digested by *Hin*d III and *Bam*h I to delete the CaMV 35S promoter. Then the digested fragment and pBIG were ligated by the T4 ligation kit (Takara, Japan), the constructed vector with the *JAZ2* promoter upstream of *GFP* was named pJAZ2G and verified by sequencing.

2.4 Preparation of protoplasts, transformation and visualisation of GFP fluorescence

Young leaves of *Populus trichocarpa* was used as starting material. Mesophyll protoplasts were isolated from the leaves and transformed by PEG 4000, according to Kang et al. (1997) and Nowak et al. (2004). One µg of pBIG (*CaMV 35S::GFP*) or pJAZ2G (*pJAZ2::GFP*) plasmids were suspended in liquid cultivation medium (Nowak et al. 2004), transferred into cell culture plates and maintained on an orbital shaker with 50 rpm min^{-1} under weak light. After 23 h of cultivation, the protoplasts transformed with pJAZ2G plasmid were divided into two groups. One group served as a control; the other group was treated with 50 µM MeJA. All samples were incubated for another 1 h and then protoplasts were harvested.

The transformed protoplasts were visualised with a confocal laser scanning microscope ZeissLSM700 (Carl Zeiss, Göttingen, Germany), set at 485/20 nm excitation and 530/25 nm emission. All pictures were taken using the same settings, such as exposure time.

2.5 Isolation of RNA and real-time PCR

The expression profiles of the *JAZ2* gene in Arabidopsis and the *GFP* gene in *Populus trichocarpa* protoplasts were analysed by real-time PCR. The GFP transcription level in protoplasts was checked with samples taken immediately after transformation (without subsequent cultivation) as a negative control, to verify that the results were not affected by the vector DNA or protoplasts themselves. Total RNA was extracted and purified using the Qiagen Plant RNAeasy kit (Qiagen Inc., USA). RNA integrity was checked on denaturing 37% formaldehyde agarose gel and RNA quality was measured, using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). First-strand cDNA was synthesised, using the Transcriptor First Strand cDNA synthesis kit (TaKaRa Inc., Japan). The absence of genomic contamination was confirmed by using a primer pair designed on the basis of the intron-exon over-spanning region. Quantitative real-time PCR performed on the Opticon Monitor 2 (MJ Research Inc., USA), using SYBR Green I reagents (TaKaRa Inc., Japan), and PCR reacting condition were performed, according to the manufacturer's instructions. Information on the primers is shown in Table 1; Sequence information came from the TAIR (http://www.arabidopsis.org) and NCBI (http://www.ncbi.nlm.nih.gov/) databases. At the end of each PCR program, a melting curve was generated and analysed with Dissociation Curves Software (MJ Research Inc., USA). PCR products were verified by sequencing. The mean normalised expression was calculated using Q-Gene software (http://www.biotechniques.com/softlib/qgene.html) based on the formula of Müller et al. (2002). The reference gene for *JAZ2* was Arabidopsis *Actin-2* (At3g18780), and for *GFP* the *Populus*18S rRNA (Xu et al. 2011). The results included three independent biological replicates.

Table 1: Primers used for Real-time PCR.

Gene	Primer	Sequences(5'-3')
JAZ2	RTJAZ2-S	CAGGAGTAGTAAGAGGGTGAAATGG
	RTJAZ2-A	TTTTGTCACGAGGAAGAAATGGA
ACTIN2	ACT-S	TCCAGGAATCGTTCACAGAA
	ACT-A	GCTACAAAACAATGGGACTAA
GFP	*GFP*-S	ATTAGCAGTGGTATCAACGCAG
	GFP-A	ATACAACATTGGGAAACAACAC
18S rRNA	18S-S	GTATGGTCGCAAGGTGAAAC
	18S-A	TTAGCAGGCTGAGGTCTCGT

3 Results

3.1 Expression profiles of *JAZ2* in response to MeJA

The *JAZ2* gene was expressed in the aerial and root parts of the Arabidopsis plant. Compared with the reference gene *Actin2*, the expression level of *JAZ2* was lower (Fig. 1). One hour after spraying the plants with 50 µM MeJA, the expression level of *JAZ2* significantly increased in both aerial and root parts. In general, the transcription level of *JAZ2* in Arabidopsis was low but could be strongly upregulated by MeJA.

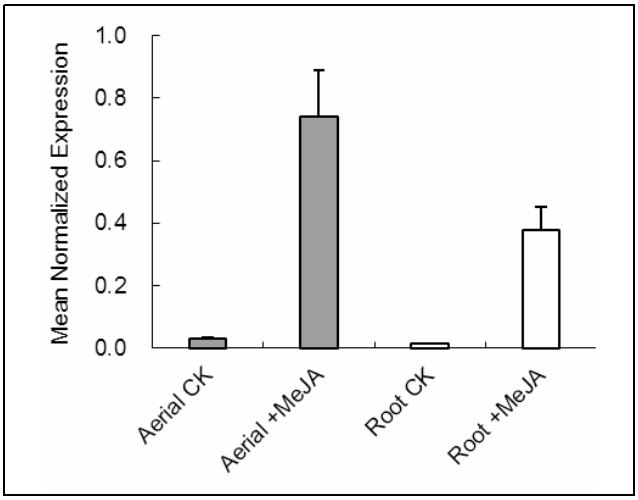

Fig. 1: Real-time PCR analysis of the expression level of *JAZ2* response to MeJA in the aerial and root part of Arabidopsis plants. The results included three independent biological replicates.

3.2 Cloning and analysis of the *JAZ2* promoter sequence

A 1389 bp region at the upstream of the *JAZ2* gene transcription start site (ATG) was cloned from the *Arabidopsis thaliana* genome by PCR, according to the sequence information from the TAIR database. The interaction of transcription factors with cis-acting elements is a key step involved in the regulation of plant gene expression response to hormone and environmental stimuli (Li et al. 2008), so the potential cis-acting elements of *JAZ2* promoter sequence were predicted and analysed with the plant promoter database, plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). In the *JAZ2* promoter, there are several known cis-elements, which are involved in the response to MeJA, including G-box (CACGTG), CGTCA-motif (CGTCA) and TGACG-motif (TGACG). Among these elements, the G-box could be recognised by the transcription factor MYC2, which has an important role in the activation of early jasmonate-responsive genes (Boter et al. 2004). However, GCC-box (AGCCGCC), which is known to respond to, was not found. There were two common core promoter elements, TATA-box and CAAT-box; several light responsive elements, and elements (e.g. HSE, LTR, MBS, and TC-rich repeats) which are known to be formed in respond to other stresses.

3.3 Visualisation of the GFP expression in transformed *Populus trichocarpa* protoplasts

The treatments, the control (protoplasts with pJAZ2G not treated with MeJA) and the protoplasts with pBIG were observed under the laser confocal scanning microscope. In the transformed protoplasts, the fluorescence of GFP could be detected (Fig. 2). It revealed that Arabidopsis *JAZ2* promoter could drive the expression of *GFP* with or without MeJA treatment in *Populus trichocarpa* protoplasts.

3.4 The activity of *JAZ2* promoter could be induced by MeJA in *Populus trichocarpa* protoplasts

After 23 h of cultivation and 1 h of MeJA treatment, the *GFP* transcription level was significantly higher in the pJAZ2G transformed protoplasts that were treated with MeJA than in the protoplasts that were not treated with MeJA, whereas the expression level was lower in the protoplasts that were treated with MeJA than in the pBIG protoplasts not treated with MeJA (Fig. 3). In *Populus trichocarpa* protoplasts the non-activated *JAZ2* promoter (immediately after transformation) had a low level of transcription activity, while it could quickly and strongly respond to MeJA.

4 Discussion

4.1 Is there a connection between the co-expression of *JAZ* gene family and their promoter?

According to information from the ATTED-II database (Obayashi et al. 2007; http://www.atted.bio.titech.ac.jp), there is a co-expression network of *JAZ* gene family in Arabidopsis (Fig. 4). Most of the *JAZ* family members are upregulated by wounding or MeJA treatment (Chung et al. 2008). It suggests that the expression of the *JAZ* gene family might be regulated by some common factors, which are induced by JA signaling. We, therefore, downloaded the gene promoter sequences (up to 1500 bp upstream of the start codon) of 12 *JAZ* family genes and predicted the cis-acting elements in the promoters, using

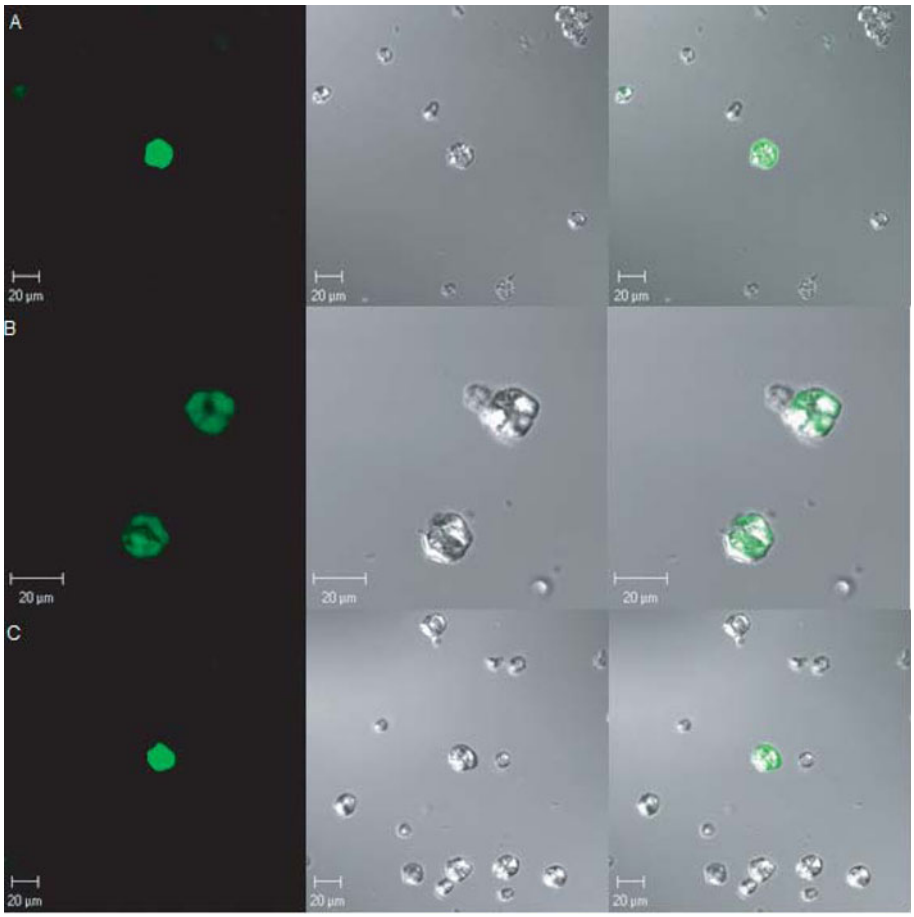

Fig. 2: Visualisation of GFP in the transformed *Populus trichocarpa* protoplasts. A) Expression of GFP in pBIG (*CaMV 35S::GFP*) transformed protoplasts; B) Expression of GFP in pJAZ2G (*pJAZ2::GFP*) transformed protoplasts without MeJA treatment; C) Expression of GFP in pJAZ2G transformed protoplasts with MeJA treatment. The graphic panel from left to right: Image in fluorescence channel, bright fields and the merge of fluorescence and bright field.

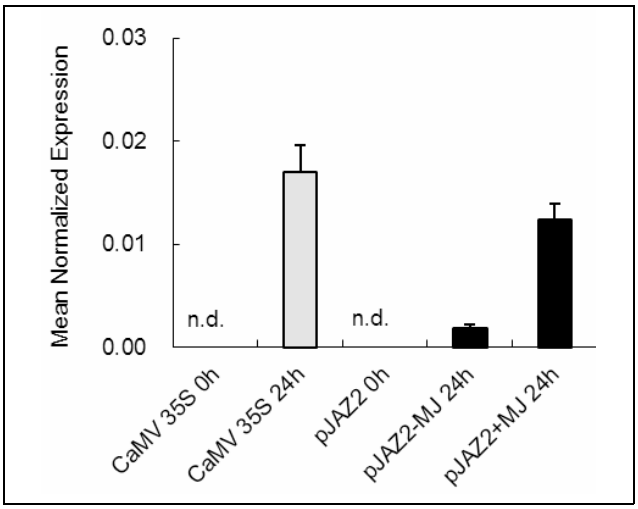

Fig. 3: Real-time PCR analysis of the expression level of *GFP* in the transformed protoplasts.
'CaMV 35S 0 h' represents the protoplasts transformed with pBIG vector without cultivation, 'CaMV 35S 24 h' represents the sample after 24 h of cultivation; 'pJAZ2 0 h' represents the protoplasts transformed with pJAZ2G vector without cultivation; 'pJAZ2-MJ 24 h' represents the sample without MeJA treatment; 'pJAZ2+MJ 24 h' represents the sample with MeJA treatment. The results included three independent biological replicates.

plantCARE database, and discovered some conservative elements (Table 2), including three known MeJA responsive elements (CGTCA-motif, G-box and TGACG-motif), several light responsive elements, and some elements (e.g. HSE, LTR, MBS and TC-rich repeats) which responded to other stresses. It suggests that there might be a connection between the co-expression of *JAZ* gene family and their promoters, and further studies will be required to confirm and elucidate this finding.

4.2 JA signaling pathway was conserved in Arabidopsis and *Populus trichocarpa*

Wound-induced accumulation of JA and the expression of related genes (such as *JAZ*, *MYC2* and *PRs*) had been observed in several plant species (Major & Constabel 2006, Leron et al. 2008). In Arabidopsis, COI1, MYC2 and JAZs complexes form the critical components of JA signaling (Chini et al. 2007, Thines et al. 2007). We found several proteins in the *Populus trichocarpa* Non-RefSeq protein database with high homology to Arabidopsis COI1, JAZ2 and MYC2 (Table 3). The phylogenetic tree based on the deduced amino acid sequences of AtCOI1, AtJAZ2 and AtMYC2 (generated by NCBI Blast Tree View program, data not shown) as well as previous reports (Major & Constabel 2006, Leron et al. 2008) and our results (Fig. 2) support that the COI1-MYC2-JAZ2 complex is conserved within different plant species and may play a key role in the activation of JA-induced defense gene.

4.3 *JAZ2* promoter is a MeJA-inducible promoter, which could be applied in *Populus trichocarpa* transgenic projects

Populus trichocarpa is used as a model organism in plant biology, because its entire genome has been sequenced (http://genome.jgi-psf.org).

Plant defense responses against herbivores and pathogens are supposed to be activated upon wound-induced accumulation of endogenous JA (Creelman & Mullet 1997, Farmer et al. 2003). The expression of several *JAZs* in Arabidopsis and hybrid poplar were upregulated within 2 h by both mechanical wounding and herbivory, and both the local and the systemic responses were rapid (Yan et al. 2009, Major & Constabel 2006).

It is believed that homologous promoters could lead to transgene and/or resident gene silencing (Finnegan & McElroy 1994). Therefore, in plant transgenic projects the use of heterologous promoters is preferred to drive the target gene expression. The constitutive promoter, such as CaMV 35S, could keep the target gene expressing continuously and at a high level. However, the continuous expression of some defense genes was considered a waste of energy *in vivo* and could even be harmful to plant growth and development. Consequently, turning the pathway of JA-dependent transcriptional activation on and off should be tightly regulated to avoid harmful responses. In Arabidopsis plants, *JAZ2* is a low-expressed gene, but its expression quickly and strongly responds to MeJA (Fig. 1). Furthermore, the transient expression of *GFP* driven by *pJAZ2* in *P. trichocarpa* protoplasts was induced by MeJA (Fig. 2; Fig. 3). It suggests that the *JAZ2* promoter from Arabidopsis could serve as a potential MeJA-inducible heterologous promoter for the application in *P. trichocarpa* transgenic projects. However, this conclusion needs to be further verified by the results involving stable transformed *P. trichocarpa*.

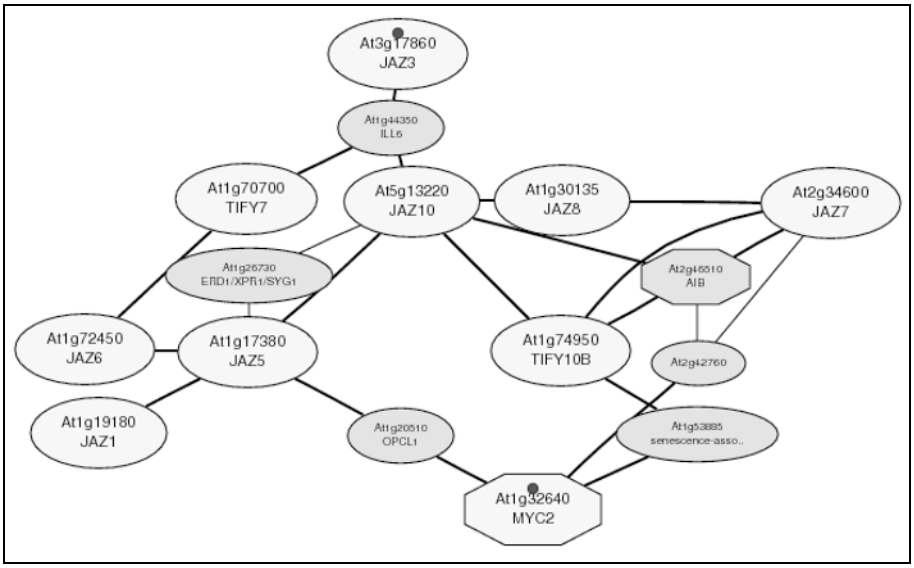

Fig. 4: The co-expression of *JAZ* gene family analysed by using the information of ATTED-II database. *TIFY10B* is another name of *JAZ2*.

Table 2: The predicted cis-action elements in promoter sequences of JAZ gene family.
The numbers 1~12 represented the members of *JAZ* gene family, *JAZ 1~12*.

Element	Sequences	Members of JAZ gene family												Functional annotation
		1	2	3	4	5	6	7	8	9	10	11	12	
ABRE	CACGTG	•	•	•			•	•	•	•	•		•	abscisic acid responsiveness
ACE	ACGTGGA	•		•	•	•	•	•	•			•	•	light responsiveness
AE-box	AGAAACAA	•	•			•	•				•		•	light responsiveness
ARE	TGGTTT	•	•	•	•	•	•	•		•	•	•		anaerobic induction
Box 4	ATTAAT	•	•	•	•		•	•	•	•	•	•	•	light responsiveness
Box I	TTTCAAA	•				•		•	•	•	•	•		light responsiveness
CAAT-box	CCAAT	•	•	•	•	•	•	•	•	•	•	•	•	common promoter element
CGTCA-motif	CGTCA	•	•	•			•	•			•	•		MeJA responsiveness
G-Box	CACGTG/T	•	•	•	•	•	•	•	•	•	•	•	•	MeJA and light responsiveness
GT1-motif	GGTTAA		•	•	•		•	•		•			•	light responsiveness
HSE	AGAAAATTCG	•	•		•	•	•	•		•	•	•		heat stress responsiveness
I-box	TATTATCTAGA	•	•	•	•			•	•	•	•	•		light responsiveness
LTR	CCGAAA		•		•	•			•		•	•		low-temperature responsiveness
MBS	TAACTG		•				•	•		•	•			drought-inducibe MYB binding site
Skn-1_motif	GTCAT	•	•	•	•		•	•	•	•		•	•	required for endosperm expression
TATA-box	TATAA	•	•	•	•	•	•	•	•	•	•	•	•	core promoter element
TC-rich repeats	ATTTTCTCCA		•	•		•	•		•	•				defense and stress responsiveness
TCA-element	TCAGAAGAGG		•		•		•		•	•	•		•	salicylic acid responsiveness
TCT-motif	TCTTAC	•	•		•	•		•	•		•			light responsive element
TGACG-motif	TGACG		•	•			•	•			•	•		MeJA-responsiveness
Unnamed 1	CGTGG	•	•	•	•	•			•					unknown
Unnamed 4	CTCC	•	•	•	•	•	•	•	•	•	•	•	•	unknown
Circadian	CAANNNNATC	•	•				•	•	•			•		circadian control

Table 3: The BLASTX of Arabidopsis *COI1*, *JAZ2* and *MYC2* in *Populus trichocarpa* Non-RefSeq protein database.

Arabidopsis	*Populus trichocarpa*	Score	Identities	Positives
COI1 (At2g39940)	gb|EEE89507.1|	777	70%	82%
	gb|EEF01349.1|	770	70%	82%
JAZ2 (At1g74950)	gb|EEE71804.1|	181	46%	60%
MYC2 (At1g32640)	gb|EEE72523.1|	558	54%	69%
	gb|EEF07352.1|	555	55%	69%

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