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Understanding bamboo flowering based on large-scale analysis of expressed sequence tags

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ABSTRACT. Unlike other plants, bamboo (Bambusoideae) flowering is an elusive physiological phenomena, because it is unpredictable, long-periodic, gregarious, and uncontrollable; also, bamboo plants usually die after flowering. The flowering mechanism in *Arabidopsis thaliana*, a eudicot model species, is well established, but it remains unknown in bamboo species. We found 4470 and 3878 expressed sequence tags in the flower bud and vegetative shoot cDNA libraries, respectively, of the bamboo species, *Bambusa oldhamii*. Different genes were found expressed in bamboo flower buds compared to vegetative shoots, based on the Munich Information Center for Protein Sequences functional categorization; flowering-related genes were also identified in this species. We also identified *Arabidopsis* flowering-specific homologs

that are involved in its photoperiod in this bamboo species, along with autonomous, vernalization and gibberellin-dependent pathways, indicating that bamboos may have a similar mechanism to control floral transition. Some bamboo expressed sequence tags shared high similarity with those of rice, but others did not match any known sequences. Our data lead us to conclude that bamboo may have its own unique flowering genes. This information can help us understand bamboo flowering and provides useful experimental methods to study the mechanisms involved.

Key words: Bamboo; Expressed sequence tag; Flowering; Mechanism

INTRODUCTION

Bamboos (Bambusoideae) are arborescent and perennial plants, and are divided into approximately 1400 species growing mainly in Asia (Wu and Raven, 2006). Flowering is an important phase in a plant's life cycle. Molecular and genetic analyses in the eudicot model species, *Arabidopsis thaliana*, revealed that it was a complex network of four pathways that interact to regulate the floral transition (Sablowski, 2007). Flowering of bamboos depends on many factors including environment, nutrition, climate, and their physiological status. Several hypotheses have been proposed for their flowering mechanisms (Janzen, 1976; Gielis et al., 1999; Franklin, 2004). When two genes, *DlMADS8* and *DlMADS18*, cloned from young spikelets of a bamboo species (*Dendrocalamus latiflorus*) were overexpressed in *Arabidopsis*, curled leaves and early flowering was exhibited (Tian et al., 2005, 2006). Recently, a cDNA library from the *in vitro* grown shoots of a bamboo species (*Bambusa edulis*) was established and 987 ESTs (expressed sequence tags) were identified, but no flowering-related ESTs were specified (Liu et al., 2008).

The EST technique used to identify large numbers of expressed genes rapidly was developed by Adams et al. in 1991. The possible function of similar ESTs in one species could be determined, and more related or novel genes could be identified as compared with ESTs annotated in other species. Based on a recent database from NCBI, bamboos had only 2673 ESTs identified while other species such as *Pinus taeda, Populus* and *A. thaliana*, had 328,628, 418,090 and 1,526,133 ESTs, respectively. Therefore, it is necessary to identify more ESTs in bamboos.

In this study, we used the EST technique to determine ESTs in the bamboo species *Bambusa oldhamii*, and identified 4470 and 3878 ESTs from its flower bud and vegetative shoot cDNA libraries, respectively. All these ESTs were annotated, and some ESTs might act in bamboo flowering when these two cDNA libraries are compared and when compared to those in other species including *Arabidopsis* and rice. ESTs, which we have identified, provide important information for the study of the bamboo's flowering mechanism.

MATERIAL AND METHODS

Bamboo tissue culture

Bamboo tissue cultures were grown from 5-10-mm long shoot tips from newly emerging laterals from healthy, vigorously growing culms of *B. oldhamii*, as described in other study (Huang et al., 1989). Simply, the laterals were dislodged, washed with detergent solution, and

sterilized by immersion for 10 min in 0.5% (v/v) NaOCl solution containing two drops of Tween-20 emulsifier per 100 mL. The sterilized shoots were then rinsed several times with autoclaved distilled water and their tips were excised aseptically under a dissecting microscope. Initiated in 1991, shoot tips in each tube were inoculated in MS medium gelled with Gelrite (0.25%, w/v) and supplemented with 1 mg/L benzyladenine (Sigma, St. Louis, MO, USA) to develop individual plants. The plants were then maintained in the same medium that changed every 28 days. Some of the plants have been flowering since 2001. The flowering buds used in this study were derived from these flowered plants and were subcultured in the medium used for their ancestral plants. The vegetative shoots used in this study were derived from those plants that have never flowered since their initial culture in 1991. All plants were grown under a 16-h light/8-h dark photoperiod indoor at a temperature of 25-27°C.

RNA isolation and cDNA library construction

Vegetative shoots and flower buds collected from the cultured plants as described above were ground in liquid nitrogen using a mortar and pestle. Total RNA was isolated with RNAgent (BIOMAN). cDNA libraries were constructed from corresponding vegetative shoots and flower buds using a SMART cDNA Library Construction Kit (BD Biosciences) and cDNA clones were converted to plasmid pDNR-LIB vector according to manufacturer instructions.

EST sequencing, assembly and sequence analysis

Plasmid DNAs were used as sequence templates. Sequence reaction was performed using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (version 3.0) with a primer (5'-ATTATACGAAGTTATCAGTCGAC-3') to generate 5'-end sequences of the cDNA clones. Sequencing was done with an ABI PRISM 3700 DNA Sequencer (PE Applied Biosystems).

Sequence data were analyzed by the Phred program (Ewing et al., 1998; Ewing and Green, 1998), and base calls with Phred quality values under 16 were considered to be ambiguous. Sequences were trimmed when five ambiguous bases were observed within 15 continuous bases. The vector-derived sequence was also removed. The clustering of ESTs was performed at the criterion of 95% identity for 50 bases. ESTs were similarity searched against public protein databases by the BLASTX program (Altschul et al., 1997). Functional categorization of ESTs was performed by aligning translated amino acid sequences with the Munich Information Center for Protein Sequences (MIPS) database (Frishman et al., 2001).

Real-time polymerase chain reaction

Total RNA of flower buds and vegetative shoots of *B. oldhamii* was extracted with Trizol reagent (Dingguo). First-strand cDNA was synthesized with SuperScript II (Invitrogen) using oligo $(dT)_{20}$ and treated with RNase H. The resulting cDNA was analyzed by real-time polymerase chain reaction (PCR) with an SYBR green kit (Takara) and detected in a Lightcycler according to manufacturer instructions. At least three PCR using the same templates were performed to get average values of expression levels. The PCR conditions were 10 s at 95°C, followed by 40 cycles of 5 s at 95°C, and 20 s at 60°C. The homolog of the *Actin* gene of *B. oldhamii* was used as a control. The primer pairs were actin-F (5'-TGGAGACCGCAAAGACGA-3') and actin-R

(5'-GAAGGATGGCTGGAAGAGGA-3'). The gene specific primer sets were listed as follows: *Bo004*-F (5'-CGCTTCATGAGGGAGGAAA-3') and *Bo004*-R (5'-CCTCGTTGCTGTTGGAAG TAGA-3'), *Bo009*-F (5'-CGCGCTCATCATCTTCTCC-3') and *Bo009*-R (5'-GTAGTTGCAGGT GCGGTATCTCT-3'), *Bo012*-F (5'-GCTGAGAAGATGGGGAGGG-3') and *Bo012*-R (5'-GCTG GAGAAGACGATGAGGG-3'), *Bo024*-F (5'-CAGCGAACGCAAAGGAAT-3') and *Bo024*-R (5'-TGATGTGCTTGAAGGACGAA-3'), *Bo032*-F (5'-CTTGACAATGGGCTGACGAA-3') and *Bo032*-R (5'-GCGACATCTTGCTGGTGAA-3').

RESULTS

Bamboo plants growing on culture medium flowered

Bamboo plants derived from tissue cultures initiated in 1991 and growing on the culture medium, as shown in Figure 1A, began to flower after a 10-year subculture. Inflorescences (spikelets) grew out from their vegetative shoots (Figure 1B). Florets from bamboo plants outdoor and on culture medium possessed all lemma, palea, lodicules, androecium, and gynoecium. The spikelets and florets from plants growing on culture medium were similar to those from outdoor plants but their floral parts were smaller (data not shown). Flower buds derived from bamboo plants growing on culture medium proliferated in the same medium, and vegetative shoots regenerated from the inflorescences (Figure 1C). Flowering of bamboo growing on culture medium provides a useful means to study its flowering mechanism.

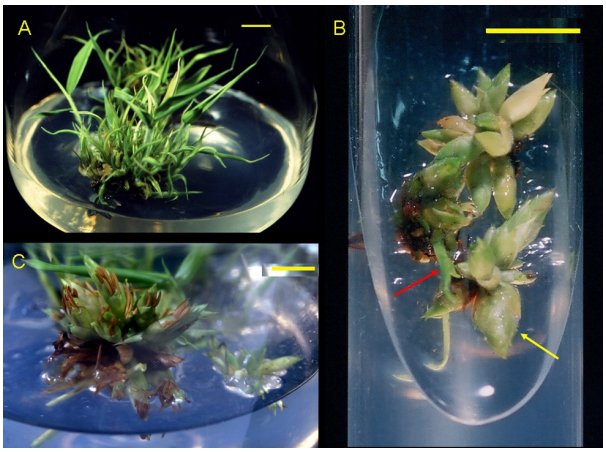

Figure 1. Tissue culture of bamboo (*Bambusa oldhamii*). **A.** Vegetative shoot growing on culture medium. **B.** Bamboo plants flowered *in vitro*. **C.** Flower buds proliferated, as indicated with a yellow arrow, and vegetative shoots, as indicated with a red arrow, regenerated from flower buds (scale bar = 1 cm).

Different genes present in bamboo flower buds and its vegetative shoots

To determine the genes responsible for bamboo flowering, we utilized the EST technique to construct cDNA libraries for the flower buds and vegetative shoots of bamboo plants growing on the culture medium. We identified 4470 and 3878 ESTs from flower bud and vegetative shoot

cDNA libraries, respectively, using DNA sequencing (Table 1). All ESTs from the flower bud cDNA library were grouped into 757 contigs and 2308 singletons that consist of 3065 unigenes, while ESTs from the vegetative shoot cDNA library were grouped into 483 contigs and 1767 singletons that consist of 2250 unigenes. As shown in Table 1, gene redundancy of ESTs in flower bud and vegetative shoot cDNA libraries was 31 and 42%, respectively. Moreover, the percentage of unigenes fell but gene redundant level rose when sequence numbers increased. Compared to the corresponding sequences in rice in the GenBank nr database (TBLASTX, E ≤ 10-5), 47% ESTs in flower buds and 55% ESTs in vegetative shoots were found to be similar, respectively.

Table 1. Redundancy of sequences of *Bambusa oldhamii* flower bud and vegetative shoot cDNA libraries.

Flower bud cDNA library				Vegetative shoot cDNA library			
Sequence	Unigene	Percentage	Redundancy	Sequence	Unigene	Percentage	Redundancy
96	90	94%	6%	2246	1401	62%	38%
480	394	82%	18%	3878	2250	58%	42%
1011	750	74%	26%				
4470	3065	69%	31%				

All ESTs in bamboo flower buds and vegetative shoots were annotated to determine functions of their corresponding unigenes. Putative functions of the bamboo unigenes were classified into 19 categories using the MIPS functional categorization system by aligning them with *A. thaliana*, as shown in Table 2. Among 19 functional categories, the most abundant category was unknown proteins, representing approximately 25.8% in flowering buds and 24.0% in vegetative shoots, respectively. The other proteins were involved in specific functions including metabolism, subcellular localization, transcription, and cellular communication/signal transduction mechanism. The composition of flower bud and vegetative shoot unigene-related proteins was similar, but there were more proteins responsible for cell cycle and DNA processing, protein synthesis, etc., in the flower buds than in the vegetative shoots. Taken together, these data suggest that genes in the flowering buds are different from those in the vegetative shoots in bamboos.

Table 2. Function annotation and classification of unigenes created from flower bud and vegetative shoot of *Bambusa oldhamii.*

Function classification	Flower	%	Shoot	%
01 Metabolism	360	11.7%	297	13.2%
02 Energy	31	1.0%	38	1.7%
03 Cell cycle and DNA processing	105	3.4%	73	3.2%
04 Transcription	182	5.9%	136	6.0%
05 Protein synthesis	154	5.0%	111	4.9%
06 Protein fate (folding, modification, destination)	171	5.6%	123	5.5%
08 Cellular transport and transport mechanisms	82	2.7%	67	3.0%
10 Cellular communication/signal transduction mechanisms	170	5.5%	136	6.0%
11 Cell rescue, defense and virulence	101	3.3%	86	3.8%
13 Regulation of/interaction with cellular environment	24	0.8%	21	0.9%
25 Development (systemic)	26	0.8%	24	1.1%
29 Transposable elements, viral and plasmid proteins	28	0.9%	18	0.8%
30 Control of cellular organization	35	1.1%	29	1.3%
40 Subcellular localization	229	7.5%	185	8.2%
63 Protein with binding function or co-factor requirement (structural or catalytic)	120	3.9%	85	3.8%
65 Storage protein	0	0.0%	1	0.0%
98 Classification not yet clear-cut	29	0.9%	33	1.5%
99 Unclassified proteins	170	5.5%	106	4.7%
The number of "unknown protein"	792	25.8%	539	24.0%
The number of "No hits found"	256	8.4%	142	6.3%
Total	3065	100.0%	2250	100.0%

Unigenes responsible for flowering were observed in bamboo flower buds

To determine flowering-related genes in bamboos, we analyzed all the unigenes in bamboo flower buds and in their vegetative shoots using TBLASTX. We found "no hits found" for unigenes present in both flower bud and vegetative shoot cDNA libraries, as shown in Table 3. When the factor e is less than 1, 41 "no hits found" unigenes present in the flower buds, while only 33 "no hits found" unigenes were observed in its vegetative shoots. Among the 41 "no hits found" unigenes that are specific for bamboo flower buds, 18 unigenes were specific for bamboos compared to the other plants (data not shown). Thus, it is likely that genes specific for flowering are present in the flower buds, and some genes specific for flowering are present only in bamboos rather than in the other plants.

Table 3. Comparison of unigenes in *Bambusa oldhamii* in flower bud and vegetative shoot cDNA libraries.

	e < 1	e < 0.1	e < 0.001
Flower specific unigenes	41	805	1437
Shoot specific unigenes	33	531	968

Flowering-specific homologs of *Arabidopsis* present in bamboos

As described earlier, four major signaling pathways of *Arabidopsis* flowering were suggested (Sablowski, 2007). We compared all ESTs derived from bamboo flowering buds and vegetative shoots with those in *Arabidopsis*, and found that bamboos had many homologs including *CO* (*CONSTANS*), *FLD* (*Flowering locus D*), *VRN1* (*Vernalization1*), *SPY* (*Spindly*), and *PIF3* (*Phytochrome interacting factor 3*), as shown in Table 4. It is likely that these homologs may be related to bamboo flowering.

Table 4. *Bambusa oldhamii* homologs of *Arabidopsis* flowering-related genes.

Function of bamboo ESTs	Flower	Shoot	*Arabidopsis* homolog	Pathway/Function
Zinc finger protein	56	51	CO (CONSTANS)	Long day pathway
MADS protein	16	3	MADS box	Floral promoter
RING zinc finger protein	9	2	HOS1 (high expression of osmotically responsive genes1)	Cold signaling
MYB transcription factor	8	4	CCA1 (Circadian Clock Associated 1)	Circadian clock
F-box protein (kelch repeat-containing)	8	8	UFO (unusual floral organ)	Floral promoter
WD40-repeat protein	8	10	FVE	Autonomous pathway/ Flower repressor
Chromatin remodeling factor	3	1	PIE 1 (phytochrome independent early flowering 1)	Floral repressor
Histone deacetylase	3	1	FLD (Flowering locus D)	Autonomous pathway/ Flower repressor
Phytochrome	3	0	PHY (Phytochrome)	Light perception
Casein kinase	2	0	CK2 (Casein kinase 2)	Circadian clock
Gigantea-like protein	2	2	GI (Gigantea)	Long day pathway
Cryptochrome	2	0	CRY (Cryptochrome)	Light perception
Drooping leaf protein	2	0	DL (Drooping leaf)	Carpel development
Polyadenylation factor	2	0	FY	Autonomous pathway
YABBY protein	2	0	CRC (Crabs claw)	Floral promoter
N-acetylglucosaminyl transferase	1	0	SPY (Spindly)	GA pathway
Vernalization independence 4	1	1	VIP 4	Flower repressors
AP2 transcription factor	0	1	TOE1-2 (target of eat 1 and 2)	Flower repressors
Polycomb group protein	0	1	VRN1 (Vernalization1)	Vernalization pathway
Helix-loop-helix transcription factor	0	1	PIF3 (Phytochrome interacting factor 3)	Light signaling

ESTs = expressed sequence tags; GA pathway = gibberellin-dependent pathway.

Flowering-related genes significantly expressed in bamboo flower buds

To examine flowering-related gene expression levels in bamboo flower buds and vegetative shoots, five genes (*Bo004*, the homolog of *CRABS CLAW*; *Bo009*, the homolog of *SEPALLATA1*; *Bo012*, the homolog of *AGAMOUS*; *Bo024*, the homolog of *SEPALLATA3*; *Bo032*, the homolog of *PISTILLATA*) that are randomly selected based on the above sequence analysis were determined by real-time PCR. As shown in Figure 2, their expression levels were all significantly higher in the flower buds than in the vegetative shoots, indicating that these five genes are more active in the flower buds than in the vegetative shoots. These data suggest that these five genes may be involved in bamboo flowering.

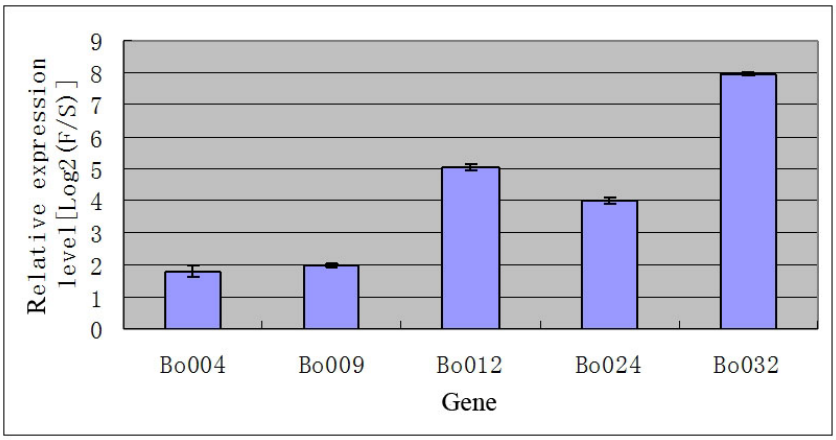

Figure 2. Expression profile of flower specific genes of *Bambusa oldhamii* by real-time PCR. The relative levels of gene expression are represented in base 2 logarithm. F/S represents the ratio of the gene expression in flower buds versus vegetative shoots.

DISCUSSION

In vitro bamboo flowering was reported in *Bambusa arundinacea*, *Dendrocalamus brandisii*, and *D. strictus* as well as other species but in most of these reports the juvenile shoots that precociously flowered were derived from seedlings (Nadgauda et al., 1990; Ramanayake et al., 2001; Lin et al., 2003). However, we observed *in vitro* bamboo flowering that occurred in bamboo plants developed from bamboo tissue culture initiated in 1991 and thereafter subcultured continuously on culture medium. It is difficult to study bamboo flowering because in the wild it is unpredictable and factors affecting bamboo flowering are uncontrollable. Also, morphology of spikelets and florets from plants in our experiments was similar to those from outdoor bamboo plants, indicating that bamboo flowering in our culture system is comparable to that in outdoor environments. Furthermore, when the full-length cDNA of *Bo009* cloned from *B. oldhamii* was over expressed in *Arabidopsis* and rice, the transgenic *Arabidopsis* and rice exhibited early flowering (data not shown). Taken together, our data thus suggest that our tissue culture system provides a useful means to study the flowering mechanism of bamboo plants.

We constructed the flower bud and vegetative shoot cDNA libraries of *B. oldhamii* using the EST technique and identified many flower-specific unigenes. Some of those unigenes did not match any known sequences, indicating that bamboo may have its own unique flowering genes (Table 2). Because many identified cDNA clones from bamboo flower buds encoded proteins whose functions are not known, further study is thus required to determine those protein functions. Bamboo ESTs shared high similarity with those of rice, and the relationship between bamboo and rice was closer than that between rice and wheat or barley since the grass family had high colinearity (Moore et al., 1995; Feuillet and Keller, 2002). Therefore, rice genomic information is useful to study flower-related genes or flowering mechanisms in bamboos.

Arabidopsis is a plant whose flowering mechanism is well known and possesses the clearest genetic network of flowering among higher plants. Flowers of grasses and *Arabidopsis* differ in perianth structure and arrangement. However, grasses have many *Arabidopsis* flowering-related homologs including *LFY* and *SOC1*, and they also have the corresponding ABCDE model of eudicots with lodicules as modified petals, and palea and lemma as modified sepals, indicating that their flowers probably develop through similar genetic control mechanisms (Ambrose et al., 2000; Tadege et al., 2003; Kater et al., 2006; Alexandre and Hennig, 2008). A flowering model of rice is proposed based on the *Arabidopsis* flowering model and rice genomic sequences (Goff et al., 2002; Izawa et al., 2003). The flowering models of rice and *Arabidopsis* present differences, but their frameworks are similar (Izawa et al., 2003; Andersen et al., 2004; Laurie et al., 2004; Winichayakul et al., 2005; Cockram et al., 2007; Izawa, 2007).

Although many bamboo homologs of *Arabidopsis* flower-related genes were identified by our group and others, bamboo flowering mechanisms remain elusive. To understand molecular mechanisms of bamboo flowering, we identified *LFY*, *FT*, *AP1*, *EMF*, *SOC1*, and *FCA* homologs of *Aradidopsis* from *Bamboo* (data not shown). The presence of *LFY*, *FT*, *AP1*, *EMF*, and *FCA* in *B. oldhamii* as well as its homologs of *Arabidopsis* flower-specific genes (Table 4) showed that bamboo had many proteins similar to those that are involved in *Arabidopsis* flowering mechanisms including photoperiod, autonomous, vernalization, and gibberellin-dependent pathways. Therefore, it is likely that bamboo also have similar mechanisms to control floral transition.

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