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## Changes in growth conditions alter the male strobilus gene expression pattern in *Cryptomeria japonica*

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**Abstract** Two-year old saplings grown from cuttings of *Cryptomeria japonica* D. Don initiate strobilus development following treatment with gibberellic acid under long-day photoperiods. At 25 °C with a 14-h photoperiod in a phytotron, male strobili initiated normally; however, they remained green and fell from the saplings prematurely. To examine the change in male strobilus development at the molecular level, three genes expressed specifically in male strobili were analyzed. Two were MADS box genes homologous to the B-function genes in angiosperms, *CjMADS1* and *CjMADS2*, and the third was *Cry j I*, which encodes an allergen protein, and this gene is expressed mainly in microspores. Under phytotron growing conditions, the homeotic genes were expressed constantly, which reflected the extended early developmental stage of male strobili. On the other hand, *Cry j I* expression was detected after a long delay just before strobilus development ceased. These results indicate that the expression of the genes related to male reproductive development in *C. japonica* is regulated by a factor(s) that is sensitive to environmental signals.

**Keywords** B-function gene · *Cryptomeria* · Gymnosperm · MADS box gene

**Abbreviation** GA<sub>3</sub>: gibberellic acid

### Introduction

*Cryptomeria japonica* (Taxodiaceae) is widely distributed in Japan. The male strobilus primordia form from late June to late August in the axils of small branches.

Meiosis of the microsporocytes occurs in October and mature microspores are formed from late October to late November. In December, the generative cell and the tube cell are formed. The staminate strobili then remain in an arrested state of development until the following March when pollen grains are disseminated (Hashizume 1962; Nagao et al. 1989).

*Cryptomeria japonica* was the first coniferous species found to initiate strobilus development by treatment with gibberellic acid (GA<sub>3</sub>; Kato et al. 1958). Generally, *C. japonica* tends to initiate more male strobili when treated with a low concentration (at least 28 μM) of GA<sub>3</sub> together with a long daylength and at a high temperature (25–30 °C; Nagao et al. 1989). These conditions reflect the natural conditions in late June to late August in Japan when male strobili are initiated. It is important to note that even 1-year old saplings could be induced to initiate male strobili and produce normal pollen under natural conditions (Hashizume 1959).

Since the original report by Kato et al. (1958), *C. japonica* has served as the model system for the study of strobilus development of conifers, both in relation to the use of plant growth substances and other treatments (Nagao et al. 1989). Besides growth substances, it has also been shown that environmental factors such as temperature, light, water stress, and nutrient conditions exert a strong influence on strobilus initiation and development of *Cryptomeria* (Nagao et al. 1989). When 2-year old saplings were grown in a phytotron at a lower temperature, i.e. 25 °C with a 14-h photoperiod, male strobili were initiated normally; however, they remained green and fell from the saplings prematurely.

Using *Arabidopsis* and *Antirrhinum* as model plant systems, endogenous and environmental influences on flowering have been studied at the molecular level (e.g., Theissen 2002; Zhao et al. 2002). It is generally accepted that floral initiation and development are controlled by genetic interactions of many genes including MADS box genes (Theissen 2002; Zhao et al. 2002).

Homologs of angiosperm floral homeotic genes have also been isolated from different gymnosperm

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species (Tandre et al. 1995; Liu and Podila 1997; Mellerowicz et al. 1998; Rutledge et al. 1998; Mouradov et al. 1998a, 1998b, 1999; Shindo et al. 1999; Sundström et al. 1999; Winter et al. 1999; Fukui et al. 2001). Two MADS box genes, *CjMADS1* and *CjMADS2*, expressed specifically in male strobili were isolated (Fukui et al. 2001). These genes are homologous to the B-function genes in angiosperms and are considered to be involved in the formation of the male reproductive organs. *CjMADS1* was expressed in male strobili throughout its development, while *CjMADS2* was strongly expressed 10–13 weeks after GA<sub>3</sub> treatment. In addition to these MADS box genes, another gene, *Cry j I* encoding an allergen protein in the pollen with pectate lyase activity, was also identified (Fukui et al. 2001). The expression of *Cry j I* was first detected at the mature microspore stage and then it was expressed constantly through pollen maturation, suggesting its role in pollen maturation (Fukui et al. 2001).

Although genes related to male strobilus development have been identified in gymnosperms, no information is available on the environmental effects on the expression of these genes. In this study, *C. japonica* is used as an experimental system to study whether environmental effects could influence the gene expression pattern during male strobilus development.

## Materials and methods

### Plant material

Two-year old saplings from cuttings of Boka-sugi, *Cryptomeria japonica* D. Don (Nagao et al. 1989), a local cultivar of Japanese cedar, were grown in a phytotron at 25 °C with a 14-h photoperiod. Control saplings were grown in the field. Saplings were sprayed with GA<sub>3</sub> (140 µM) three times per week at the beginning of July to induce flowering. Male strobili were periodically collected from saplings grown under artificial or natural conditions for the extraction of RNA. Total RNA was extracted using standard procedures as previously described (Fukui et al. 2001) and stored at –80 °C until use.

### RNA gel blot analysis

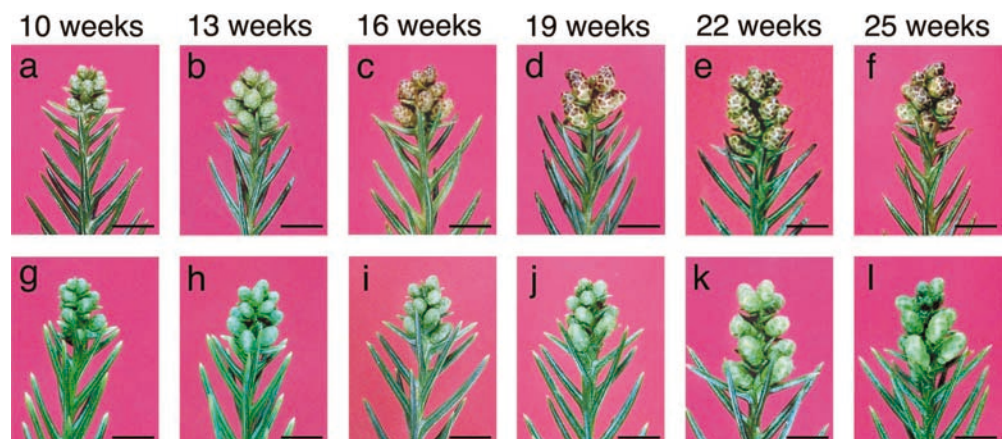
The analysis was conducted using methods similar to those employed in our earlier studies (Fukui et al. 2001). Total RNA samples (10 µg each) were separated by electrophoresis on a 1% agarose gel containing 5% formaldehyde and 1×MOPS solution [20 mM 3-(N-morpholino)propanesulfonic acid (Mops), 5 mM sodium acetate, 1 mM EDTA], and transferred onto Hybond-N+ membranes (Amersham) using standard blotting techniques (Sambrook et al. 1989). RNA gel blots were hybridized with <sup>32</sup>P-labeled cDNA fragments of *CjMADS1* and *CjMADS2*, corresponding to nucleotides 691–929 and 557–727, respectively. The full-length cDNA of *Cry j I*, encoding an allergen protein with a pectate lyase activity included in the pollen of sugi (Miki-Hirosige et al. 1994; Sone et al. 1994; Taniguchi et al. 1995), was also used as a probe. A gene encoding light-harvesting chlorophyll *a/b*-binding protein (*cab*) isolated from Japanese cedar (Mukai et al. 1998) was used as an internal control. Hybridization and washing were performed as described previously (Fukui et al. 2001).

## Results and discussion

To examine the effect of the environmental conditions on the development of the male strobilus, saplings were grown in a phytotron and strobilus initiation was artificially induced by GA<sub>3</sub> (Fig. 1). For control plants growing under natural conditions, male strobili were detected ca. 5 weeks after GA<sub>3</sub> treatment and developed normally. At the 10th week, archesporial cells were present within the microsporangium. The strobili began to turn brown 13 weeks after GA<sub>3</sub> treatment when the meiosis of pollen mother cells occurred. Pollen was fully mature 31 weeks later and began to shed from the microsporangia. The mature strobili in the field were dark brown in color and remained attached to the branches even after pollen had been released.

Under the artificial condition, male strobili were also detected ca. 5 weeks after GA<sub>3</sub> treatment. The male strobili at the 10th week after GA<sub>3</sub> treatment showed sporophyll development, but the microsporangia had just been initiated. At the 13th week, sporogenous cells were observed within microsporangia; however, distinct archesporial or pollen mother cells were absent. Furthermore, these developing strobili remained green.

**Fig. 1a–l** Male strobili of *Cryptomeria japonica*. **a–f** Strobili grown in the field. **g–l** Strobili grown in a phytotron at 25 °C in a cycle of 14 h light/10 h darkness. Numbers above strobili refer to the weeks after GA<sub>3</sub> treatment. Bars = 5 mm



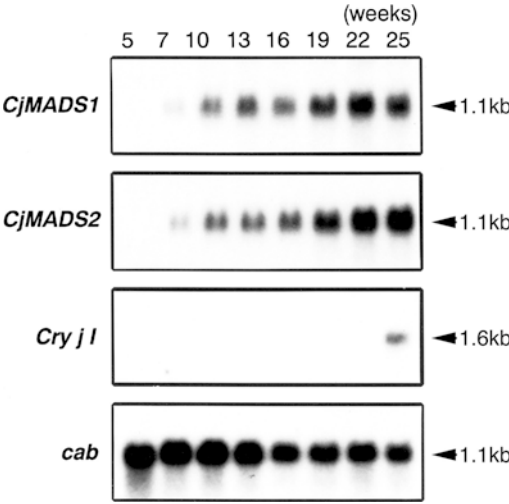


**Fig. 2** Northern blot analysis of *CjMADS1* and *CjMADS2* expression during development of *C. japonica* strobili. Total RNAs were isolated from male strobili grown in a phytotron at 25 °C in a cycle of 14 h light/10 h darkness. Numbers above lanes refer to the weeks after GA<sub>3</sub> treatment. The blots contain 10 µg of total RNA loaded in each lane and were hybridized with 3'-specific probes of *CjMADS1* and *CjMADS2* and with full-length cDNA probes of *Cry j I* and *cab*

After 25 weeks, most of the green strobili dropped off the saplings in the phytotron.

The temporal gene expression pattern of strobili from phytotron-grown plants was examined by Northern blot analysis with total RNAs isolated from male strobili after the artificial induction of reproductive development by GA<sub>3</sub> treatment (Fig. 2). An RNA gel blot was probed with four probes: 3'-specific probes of *CjMADS1* and *CjMADS2*, *Cry j I* and *cab*. *CjMADS1* and *CjMADS2* expression was first detected at the 7th week after GA<sub>3</sub> treatment and then gradually increased till the 25th week. In contrast, *Cry j I* was moderately expressed at the 25th week just prior to the premature dropping of strobili. The *cab* gene, which served as an internal control, was expressed almost constantly throughout the treatment period.

The change in the gene expression pattern under the different growth conditions is shown in Table 1. The expression data for male strobili grown in the field is based on previous work (Fukui et al. 2001). The

expression of *CjMADS1* was strong; it was first detected at the 5th week and remained strongly expressed through the maturation stages. *CjMADS2* was strongly expressed 10–13 weeks after GA<sub>3</sub> treatment and this time corresponded to the period from the end of September to the middle of October, when meiosis of pollen mother cells occurs. *Cry j I* was expressed from the 16th week to the 25th week in the male strobili at the time of microspore formation and pollen maturation. The most notable difference in the gene expression pattern between the phytotron-grown and field-grown plants is that there is a delay in *CjMADS1*, *CjMADS2* and *Cry j I* expression. Weak *CjMADS1* and *CjMADS2* expression is seen starting at week 7. The stage-specific expression of *CjMADS2* is absent from phytotron-grown plants and *Cry j I* was just weakly detected at the 25th week just prior to strobilus abortion (Table 1). The continuous expression of *CjMADS2* may have resulted in the altered *Cry j I* expression. These changes in the gene expression pattern mostly result in the delay of development and premature dropping of male strobili in the phytotron-grown plants.

From the results, judging from the successful initiation of strobili and the gene expression pattern, the constant environment of the phytotron provides appropriate signals that are essential to the early stages of strobilus development in *C. japonica*. However, this constant environment also prevents the normal progression of strobilus maturation and maintained the early status of strobilus development. These results clearly indicate that the genes related to male reproductive development are strongly influenced by environmental signals.

In *Arabidopsis*, genetic interactions among genes that regulate flower development have been studied (Zhao et al. 2002). One of the signaling pathways is based on the photoperiod and its central effector gene is *CONSTANS* (*CO*; Putterill et al. 1995). The *CO* gene was suggested to act upstream of *LEAFY* (*LFY*; Weigel et al. 1992; Simon et al. 1996), which is one of the meristem-identity genes. The *Arabidopsis* B-function genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), were suggested to be downstream of *LFY* (Weigel and Meyerowitz 1993; Jack et al. 1994; Goto and Meyerowitz 1994). And for the up-regulation of *AP3* and *PI*, other genes, *UNUSUAL FLORAL ORGANS* and *Arabidopsis*

**Table 1** Comparison of expression patterns of *CjMADS1*, *CjMADS2* and *Cry j I* in male strobili of saplings of *Cryptomeria japonica* grown under different conditions. Weeks represent the period after GA<sub>3</sub> treatment. S Strong, W weak, VW very weak, – not detected

| Weeks | Field-grown saplings <sup>a</sup> |                |                | Phytotron-grown saplings |                |                |
|-------|-----------------------------------|----------------|----------------|--------------------------|----------------|----------------|
|       | <i>CjMADS1</i>                    | <i>CjMADS2</i> | <i>Cry j I</i> | <i>CjMADS1</i>           | <i>CjMADS2</i> | <i>Cry j I</i> |
| 5     | S                                 | VW             | –              | –                        | –              | –              |
| 7     | S                                 | VW             | –              | W                        | W              | –              |
| 10    | S                                 | S              | –              | S                        | S              | –              |
| 13    | S                                 | S              | –              | S                        | S              | –              |
| 16    | S                                 | VW             | S              | S                        | S              | –              |
| 19    | S                                 | VW             | S              | S                        | S              | –              |
| 22    | S                                 | VW             | S              | S                        | S              | –              |
| 25    | S                                 | VW             | S              | S                        | S              | W              |

*SKPI-LIKE1* genes, were suggested to be required as well (Lee et al. 1997; Hill et al. 1998; Tilly et al. 1998; Samach et al. 1999; Zhao et al. 1999; Honma and Goto 2000). Hence, the photoperiod could influence the expression of B-function genes through a complex sequence of gene interactions in *Arabidopsis*.

This study provides the first evidence that the expression of B-function genes in a conifer (*C. japonica*) is regulated by factors sensitive to environmental stimuli. However, the genetic mechanism involved in the environmental regulation of male strobilus formation is unknown. I propose that the expression of B-function genes in conifers is under the control of environment-sensitive signaling pathway(s).

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