Spatiotemporal gene expression profiles associated with male strobilus development in *Cryptomeria japonica* by suppression subtractive hybridization

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To identify the genes associated with the formation and development of the male strobilus of *Cryptomeria japonica* D. Don (Sugi), male strobilus-specific suppression subtractive hybridization (SSH) libraries were constructed from three different stages of male strobilus development. From these SSH libraries, 1,012 uni-gene sets including 314 contigs were assembled from 2,448 sequences. The profiles of the genes isolated from the SSH libraries differed strongly according to developmental stage: 49%, 13% and 29% of genes were unique to the early stage (stage E), tetrad stage (stage T), and mature stage (stage M) SSH libraries, respectively. To evaluate the reliability of the SSH libraries, we focused on the eight genes strongly concentrated in the three SSH libraries and performed RT-PCR analysis. All genes tested were expressed more strongly in the male strobilus than in the shoot. Our findings suggest that the gene profiles associated with male strobilus development differ in both number and kind of dominantly expressed genes.

Key Words: *Cryptomeria japonica*, male strobilus, gene profiling, suppression subtractive hybridization.

Introduction

Cryptomeria japonica D. Don (Sugi) is one of the most important commercial coniferous tree species in Japan. However, *C. japonica* pollen-induced pollinosis is the most widespread allergic disease in Japan, affecting over 15% of the population. Rapid improvement of this public health issue using a conventional breeding approach is not possible, because successful breeding of coniferous tree requires a very long time. Genetic modification might be a powerful tool to shorten the time needed to breed trees compared with traditional breeding methods because it is able to induce the favorable traits by introduction of specific genes in trees without unnecessary genetic transitions. A method for *Agrobacterium tumefaciens*-mediated transformation of embryogenic tissue was recently developed for *C. japonica* (Taniguchi *et al.* 2008). Introduction of a male sterile trait by genetic modification of *C. japonica* is a viable approach to address the medical and public health problems of *C. japonica* pollen-induced pollinosis. To use this approach for male sterilization, it is necessary to understand the molecular mechanism of the male strobilus and pollen formation of *C. japonica*.

Flower formation genes of *C. japonica*, such as the Class B MADS-box gene (Fukui *et al.* 2001) and functional analysis of *FLORICAULA/LEAFY* homolog genes (Shiokawa *et al.* 2008), have been studied. Global analyses of the expressed genes in mature pollen and the male strobilus of

C. japonica have been reported by Futamura *et al.* (2006, 2008). However, no profiling data are available of gene expression that is associated with different developmental stages of the male strobilus or pollen. We surmised that such spatiotemporal expression profiling would help us to understand male strobilus formation of *C. japonica* at the molecular level. Therefore, we created male strobilus-specific libraries associated with male strobilus development by a suppression subtractive hybridization (SSH) method.

SSH is an effective method of isolating genes that are expressed differentially between two tissues (Shukla *et al.* 2006, Sternberg *et al.* 2007). Using molecular hybridization and subtraction techniques, the SSH cDNA library approach reduces the cloning of abundantly expressed housekeeping genes or genes commonly expressed in both control and treated tissues, and thereby the SSH library comprises target tissue-specific genes. To isolate genes related to male strobilus formation and development, male strobilus-specific SSH libraries were derived from three different stages of male strobilus development. Herein, we describe the isolation of cDNAs that are expressed differentially in three different stages of *C. japonica* male strobilus development.

Materials and Methods

Plant materials

Male strobili and vegetative shoots were collected from adult *C. japonica* trees of Ohtawara 1, a plus tree clone of *C. japonica*, planted at a clone bank garden maintained at the Forest Tree Breeding Center (FTBC), Forestry and Forest Products Research Institute (FFPRI), Hitachi, Ibaraki,

Japan. Gibberellic acid (GA₃) (100 ppm) was sprayed on the trees on 7 July 2005 to induce flowering. Male strobili were sampled beginning on 18 August. Roots were also collected from cuttings of Ohtawara 1 grown in a tissue culture bottle at 25°C under a 16-h light/8-h dark cycle.

Scanning electron microscopy (SEM)

Male strobili were sampled to observe the stages of strobilus development by SEM. Strobili collected on 19 September (Sep), 20 Sep, 22 Sep, 25 Sep, 29 Sep, 2 October (Oct), 5 Oct, 9 Oct, 12 Oct, 17 Oct, 20 Oct, 26 Oct and 24 November (Nov) were dehydrated in a graduated series of ethanol to prevent dehydration-induced tissue shrinkage. Air-dried strobili were then cut along the axis using sharp razor blades to obtain smooth surfaces for SEM observation. The specimens were placed on brass specimen stubs with double-carbon-coated tape, and were coated with platinum by a magnetron-sputtering device (JUC-5000, JEOL, Tokyo, Japan). The specimens were observed with an SEM (JSM-5800LV, JEOL, Tokyo, Japan) with a 15-kV accelerating voltage. Based on our SEM observations of the male strobilus, we selected three different developmental stages of strobilus samples collected on 19 Sep, 9 Oct and 24 Nov. These strobilus pollen development stages were named the early stage (stage E), the tetrad stage (stage T), and the mature stage (stage M), respectively (Fig. 1).

Isolation of RNA and reverse transcription

Male strobili were sampled simultaneously for RNA extraction. Total RNA was isolated from the tissue of male strobili (stage E, stage T, stage M) and shoots (17 May, 19 Sep, 9 Oct, 24 Nov) of mature trees and roots of cuttings using the RNeasy Plant Mini kit (QIAGEN, Gaithersburg, MD) with minor modifications. The cDNA synthesis was performed using the Super SMART PCR cDNA Synthesis kit (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. Single-strand cDNAs were produced by reverse transcription from 1 µg total RNA of each sample.

SSH

We constructed three different subtraction libraries. The PCR-Select cDNA Subtraction kit (BD Biosciences) was used for the SSH to identify male strobilus-specific transcripts in *C. japonica* in a strategically designed experiment in which the male strobilus (stage E, stage T, stage M, respectively) and shoot (19 Sep, 9 Oct, 24 Nov, respectively) cDNA populations served as 'tester' and 'driver', respectively. Double-stranded cDNA was synthesized and digested with restriction enzyme *Rsa*I. Male strobilus cDNAs were divided into two groups and ligated to the specific adaptors 1 and 2R, respectively. Each denatured adaptor-ligated tester mix was separately hybridized with a single-stranded shoot cDNA driver for 8 h at 68°C, to enrich nonpairing sequences unique to the male strobilus cDNA pool. A second hybridization using single-strand driver cDNA as well as the prod-

Fig. 1. Micrographs showing male flower development. Parts A, D and G are by optical microscope. The other parts are by SEM. A–C, specimens were taken at stage E (19 Sep); D–F, J, stage T (9 Oct); G–I, K, stage M (24 Nov). J and K are enlargements of parts of F and I, respectively. Scale bars: 2 mm for A, D and G; 500 µm for B, E and H; 100 µm for C, F and I; 10 µm for J and K.

ucts of the first hybridization from both tester aliquots (adaptor 1- and adaptor 2R-linked molecules) was performed for 16 h at 68°C. Products from the second hybridization were diluted in 200 µl of dilution buffer of this kit, heated at 68°C for 7 min, and stored at –20°C.

PCR amplification of subtracted products

An ABI 9700 Thermal Cycler (Applied Biosystems, Foster City, CA) was used to perform PCR amplification of the subtracted tester products. Primary PCR amplifications were conducted for each tester using diluted subtracted products following the second hybridization. A 1-µl aliquot of sample was added to 24 µl of PCR master mix prepared using the reagents supplied in the kit, and cycling conditions were as follows: 75°C for 5 min to extend the adaptors; 94°C for 25 s; and 27 cycles at 94°C for 10 s, 66°C for 30 s and 72°C for 1.5 min. Amplified products were diluted 100-fold in sterile water, and 1 µl of diluted primary PCR products was added to 24 µl of secondary PCR master mix containing nested primers (1 and 2R) to ensure the specific amplification of double-stranded templates containing both adaptors. Secondary PCR was performed at 94°C for 15 s and 68°C for 3 min (cycle numbers were 15, 18 and 15 for stage E, stage T and stage M, respectively). PCR products were analyzed on 2% agarose gel.

Cloning of subtracted cDNA templates

Following secondary PCR amplification, subtracted products from each tester cDNA population were cloned into pCR 4-TOPO using a TA Cloning kit (Invitrogen, Carlsbad, CA) and transformed into competent DH5α *Escherichia coli* cells. Colonies were grown in a selective

Table 1. Primers used for the RT-PCR

Gene name	Forward primer	Reverse primer
<i>Cj_ssh_male_e_002</i>	5'-CTGTTAAGTATCTTGTGCTTCACCAC-3'	5'-CAACCACTGCCCCAACAATAGG-3'
<i>Cj_ssh_male_e_003</i>	5'-AAAGCAGAATGGTGGTGAGTG-3'	5'-ATGCTCCAGACAAAACCGTAGG-3'
<i>Cj_ssh_male_t_001</i>	5'-GCTGCACTGCCCTCAAATCG-3'	5'-CCTTGAGAGCTACTTTATAGCATAG-3'
<i>Cj_ssh_male_t_002</i>	5'-TGGCACAGGAAAAATCAGTCCAC-3'	5'-TCGCCCATCAAACATATGCATGATC-3'
<i>Cj_ssh_male_t_003</i>	5'-CATAACATTGCATTTCCTTGCAC-3'	5'-ACCCTCAAGAACATTGAGCAGG-3'
<i>Cj_ssh_male_m_001</i>	5'-CTGCCTGGTGTGTATTGTTGC-3'	5'-GAAGACACAACACAAGTTGGTGAG-3'
<i>Cj_ssh_male_m_002</i>	5'-ACAGACTGCCACAGATATACTTCTG-3'	5'-ACAGATTGGCTGCCTTATTTAGGC-3'
<i>Cj_ssh_male_m_003</i>	5'-TTCGAGAAGTGCAAAGTTGAGCG-3'	5'-AGTCACTTGAGAATCAGCAGAGC-3'
<i>Actin</i>	5'-ATGTTTGAGACCTTCAATACACCA-3'	5'-GAAGCTCATAACTCTTCTCAAGG-3'

Luria-Bertani agar medium containing X-gal and isopropyl β -D-1-thiogalactopyranoside for screening of blue/white colonies. For each tester, white colonies were randomly picked, placed on microtiter plates, and grown overnight in liquid Luria-Bertani medium.

DNA sequencing and analysis

SSH clone inserts were sequenced using a cloning vector primer (M13R) and an Applied Biosystems 3100 sequencer with the BigDye Terminator v3.1 cycle Sequencing kit (Applied Biosystems). The sequences were edited using Sequencer Software (Gene Codes Corporation, Ann Arbor, MI). Sequence analysis was performed using the BLASTX programs of the National Center for Biotechnology Information (NCBI) and The Arabidopsis Information Resource (TAIR).

RT-PCR

RT-PCR amplifications were performed with 1 μ g total RNA from the male strobilus (stage E, stage T, stage M), shoots (17 May, 19 Sep, 9 Oct, 24 Nov), and roots. First-strand cDNA synthesis was performed using Superscript and oligo-dT primers (Invitrogen). RT-PCR primer sets are listed in Table 1. The reaction mixture for RT-PCR contained 0.5 μ l of the solution of the first-strand cDNA added to 49.5 μ l of KOD plus Master Mix (TOYOBO, Osaka, Japan) and 0.4 μ M of gene-specific primers (Table 1), for a total volume of 50 μ l. The program used for RT-PCR was 94°C for 5 min followed by 25 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and finally 72°C for 1 min. The PCR product was separated on 2% agarose gel and stained with SYBR SAFE (Invitrogen).

Results

Gene expression profile in the three developmental stages of male strobilus

A total of 2,448 clones was isolated and sequenced. The results of sequence analysis by BLASTX were filtered using an E-value of $1e-4$ (Table 2; full data are shown in Supplemental Table 1). As a result of the BLASTX search, 458 unigene sets including 123 contigs were derived from 873 ESTs isolated from stage E. The most frequently isolated genes in

this stage had similarity with a bark protein-like protein and a pollen Ole e 1 allergen and extensin family protein (Table 2). Similarly, 727 ESTs isolated from the stage T library formed 175 unigene sets including 68 contigs. In the stage T library, there was a strong concentration of three genes. The most frequently isolated gene had similarity with Male cone protein 1 (PrMC1), the second most frequently isolated gene was a lipase, and the third was a PrMALE1 (Table 2). In stage M, 848 clones consisting of 379 unigene sets including 123 contigs were isolated. The most frequently isolated genes obtained from this stage were similar to SF18 from sunflower, CjMP1 of *C. japonica*, and peroxidase, respectively (Table 2). The profiles of the genes isolated from the SSH libraries differed strongly depending on the developmental stage of the strobilus: 49%, 13% and 29% of genes were isolated only from stage E, T and M SSH libraries, respectively (Fig. 2). The genes isolated from SSH libraries of all stages constituted only 1% of all genes (Fig. 2). For the purpose of the analysis of the trait of each developmental stage, each library-specific gene was analyzed by TAIR GO annotation search (Fig. 3) (<http://www.arabidopsis.org/index.jsp>). In stage E, many genes were categorized into cell organization and biogenesis compared with other libraries; in stage T, many genes were categorized into metabolic processes and unknown biological processes compared with other libraries. Many genes categorized into response to stress compared with other libraries were included in stage M (Fig. 3).

Expression analysis of the genes isolated from SSH libraries

Although SSH can be used to isolate spatiotemporal-specific genes, the reliability of the SSH libraries must be evaluated. Therefore, we evaluated their reliability by RT-PCR analysis. The eight genes that were main components of each SSH library were used for RT-PCR (from stage E: *Cj_ssh_male_e_002* (3.4%) and *Cj_ssh_male_e_003* (2.1%); from stage T: *Cj_ssh_male_t_001* (24%), *Cj_ssh_male_t_002* (22%) and *Cj_ssh_male_t_003* (14%); from stage M: *Cj_ssh_male_m_001* (12%), *Cj_ssh_male_m_002* (5.1%) and *Cj_ssh_male_m_003* (2.8%) (Table 2)).

We designed gene-specific primers for each gene (Table 1), and investigated the patterns of expression

Table 2. The twenty most highly expressed unigenes in the stage E, T and M SSH libraries

Gene ID	Accession No.	# ESTs (% in the library)	highest homology (NCBI) highest homology (TAIR)	Homology accession locus name	E-value
Stage E					
<i>Cj_ssh_male_e_001</i>	FS942126	30 (3.4)	bark protein-like protein catalytic	AAV65286 AT4G28940.1	2e-73 1e-59
<i>Cj_ssh_male_e_002</i>	FS942127	30 (3.4)	unknown pollen Ole e 1 allergen and extensin family protein	ABK26139 AT5G15780.1	9e-18 9e-10
<i>Cj_ssh_male_e_003</i>	FS942128	18 (2.1)	unknown unknown protein	ABK22759 AT5G48480.1	9e-63 6e-21
<i>Cj_ssh_male_e_004</i>	FS942129	17 (1.9)	unknown MT3 (METALLOTHIONEIN 3)	ABK21034 AT3G15353.1	4e-10 1e-04
<i>Cj_ssh_male_e_005</i>	FS942130	15 (1.7)	conserved hypothetical protein	YP_001680918	8e-28
<i>Cj_ssh_male_e_006</i>	FS942131	14 (1.6)	hypothetical protein	CAN70790	9e-36
<i>Cj_ssh_male_e_007</i>	FS942132	13 (1.5)	hypothetical protein PhapfoPp090	YP_358636	6e-45
<i>Cj_ssh_male_e_008</i>	FS942133	11 (1.3)	putative cyclosporin A-binding protein ROC1 (ROTAMASE CYP 1); peptidyl-prolyl cis-trans isomerase	CAC81066 AT4G38740.1	1e-56 2e-53
<i>Cj_ssh_male_e_009</i>	FS942134	11 (1.3)	limonene/borneol synthase terpene synthase/cyclase family protein	BAC92722 AT3G29410.1	7e-44 2e-17
<i>Cj_ssh_male_e_010</i>	FS942135	10 (1.1)	hypothetical protein	CAN65763	5e-25
<i>Cj_ssh_male_e_011</i>	FS942136	9 (1.0)	translation elongation factor-1 alpha elongation factor 1-alpha	AAV92325 AT5G60390.3	2e-123 e-117
<i>Cj_ssh_male_e_012</i>	FS942137	8 (0.9)	unknown xyloglucan:xyloglucosyl transferase, putative	ABK22211 AT3G23730.1	3e-97 1e-77
<i>Cj_ssh_male_e_013</i>	FS942138	8 (0.9)	unnamed protein product	CAO18260	2e-24
<i>Cj_ssh_male_e_014</i>	FS942139	8 (0.9)	unknown LTP; lipid binding	ABK21262 AT2G15050.3	2e-18 6e-08
<i>Cj_ssh_male_e_015</i>	FS942140	8 (0.9)	hypothetical protein Bm1_17870	XP_001895031	4e-18
<i>Cj_ssh_male_e_016</i>	FS942141	7 (0.8)	unknown pollen Ole e 1 allergen and extensin family protein	ABK24101 AT5G15780.1	7e-20 1e-11
<i>Cj_ssh_male_e_017</i>	FS942142	6 (0.7)	Os01g0317800 esterase/lipase/thioesterase family protein	NP_001042883 AT5G19290.1	4e-62 1e-63
<i>Cj_ssh_male_e_018</i>	FS942143	5 (0.6)	flavonol synthase FLS (FLAVONOL SYNTHASE)	AAS21058 AT5G08640.1	1e-80 1e-67
<i>Cj_ssh_male_e_019</i>	FS942144	5 (0.6)	unknown glycosyl hydrolase family 17 protein	ABK92452 AT2G05790.1	2e-62 3e-62
<i>Cj_ssh_male_e_020</i>	FS942145	5 (0.6)	delta-selinene synthase GA1 (GA REQUIRING 1); ent-copalyl diphosphate synthase	AAK83561 AT4G02780.1	9e-41 3e-16
Stage T					
<i>Cj_ssh_male_t_001</i>	FS942584	175 (24)	Male-cone protein 1 precursor (PRMC1) protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	O24493 AT5G62080.1	1e-15 1e-13
<i>Cj_ssh_male_t_002</i>	FS942585	161 (22)	similar to Bile-salt-activated lipase precursor (BAL) protein kinase family protein	XP_859406 AT1G49270.1	2e-08 1e-05
<i>Cj_ssh_male_t_003</i>	FS942586	104 (14)	PrMALE1 protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	AAD02170 AT5G62080.1	6e-18 4e-08
<i>Cj_ssh_male_t_004</i>	FS942587	10 (1.3)	unknown MT3 (METALLOTHIONEIN 3)	ABK21034 AT3G15353.1	1e-10 6e-05
<i>Cj_ssh_male_t_005</i>	FS942588	6 (0.8)	defensin PDF2.1; peptidase inhibitor	ABO61349 AT2G02120.1	3e-23 1e-10

Table 2. (continued)

Gene ID	Accession No.	# ESTs (% in the library)	highest homology (NCBI) highest homology (TAIR)	Homology accession locus name	E-value
<i>Cj_ssh_male_t_006</i>	FS942589	6 (0.8)	predicted protein kinesin light chain-related	XP_001774076 AT3G27960.1	9e-30 2e-30
<i>Cj_ssh_male_t_007</i>	FS942590	5 (0.7)	unknown LTP; lipid binding	ABK21262 AT2G15050.3	2e-18 6e-08
<i>Cj_ssh_male_t_008</i>	FS942591	5 (0.7)	unnamed protein product LPD1 (LIPOAMIDE DEHYDROGENASE 1)	CAO44892 AT3G16950.2	5e-50 5e-51
<i>Cj_ssh_male_t_009</i>	FS942592	4 (0.6)	unknown protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	ABK22309 AT5G64080.2	1e-19 7e-14
<i>Cj_ssh_male_t_010</i>	FS942593	4 (0.6)	unknown AWPM-19-like membrane family protein	ABK23412 AT1G29520.1	2e-12 6e-08
<i>Cj_ssh_male_t_011</i>	FS942594	4 (0.6)	unknown ADF6 (ACTIN DEPOLYMERIZING FACTOR 6)	ABK23967 AT2G31200.1	2e-63 1e-55
<i>Cj_ssh_male_t_012</i>	FS942595	4 (0.6)	Os01g0151700 short-chain dehydrogenase/reductase (SDR) family protein	NP_001042042 AT5G02540.1	7e-93 1e-93
<i>Cj_ssh_male_t_013</i>	FS942596	4 (0.6)	esterase/lipase/thioesterase family protein esterase/lipase/thioesterase family protein	NP_197430 AT5G19290.1	4e-62 5e-64
<i>Cj_ssh_male_t_014</i>	FS942597	3 (0.4)	Peptidase aspartic, active site	ABD33091	1e-08
<i>Cj_ssh_male_t_015</i>	FS942598	3 (0.4)	unknown	ABK22573	3e-15
<i>Cj_ssh_male_t_016</i>	FS942599	3 (0.4)	unknown calreticulin 2 (CRT2)	ABK24327 AT1G09210.1	5e-59 1e-58
<i>Cj_ssh_male_t_017</i>	FS942600	3 (0.4)	male sterility-related protein MS2 (MALE STERILITY 2)	ACA28679 AT3G11980.1	5e-39 6e-40
<i>Cj_ssh_male_t_018</i>	FS942601	3 (0.4)	hypothetical protein BEST A. thaliana protein match is: unknown protein (TAIR:AT3G49590.2)	BAF00799 AT3G18770.1	7e-08 3e-11
<i>Cj_ssh_male_t_019</i>	FS942602	3 (0.4)	hypothetical protein GDLS-motif lipase/hydrolase family protein	CAN75831 AT2G23540.1	8e-42 5e-41
<i>Cj_ssh_male_t_020</i>	FS942603	3 (0.4)	unnamed protein product ADL6 (DYNAMIN-LIKE PROTEIN 6); GTPase	CAO46903 AT1G10290.1	1e-32 1e-30
Stage M					
<i>Cj_ssh_male_m_001</i>	FS942760	101 (12)	Anther-specific protein SF18 PDF1.3 (plant defensin 1.3)	P22357 AT2G26010.1	2e-05 3e-05
<i>Cj_ssh_male_m_002</i>	FS942761	43 (5.1)	CjMPI1	BAE93119	1e-28
<i>Cj_ssh_male_m_003</i>	FS942762	24 (2.8)	peroxidase peroxidase, putative	CAH10840 AT5G05340.1	1e-59 1e-46
<i>Cj_ssh_male_m_004</i>	FS942763	20 (2.4)	unknown MT3 (METALLOTHIONEIN 3)	ABK21034 AT3G15353.1	3e-10 1e-04
<i>Cj_ssh_male_m_005</i>	FS942764	19 (2.2)	hypothetical protein	CAN78649	5e-08
<i>Cj_ssh_male_m_006</i>	FS942765	16 (1.9)	peroxidase peroxidase, putative	CAH10839 AT5G05340.1	2e-48 1e-39
<i>Cj_ssh_male_m_007</i>	FS942766	12 (1.4)	unknown unknown protein	ACG33260 AT5G55620.1	7e-16 5e-15
<i>Cj_ssh_male_m_008</i>	FS942767	12 (1.4)	RNase H family protein	ABD63162	2e-34
<i>Cj_ssh_male_m_009</i>	FS942768	7 (0.8)	unknown unknown protein	ACG36115 AT5G55620.1	5e-16 5e-15
<i>Cj_ssh_male_m_010</i>	FS942769	7 (0.8)	unknown MT2A (METALLOTHIONEIN 2A)	ABK20934 AT3G09390.1	1e-11 2e-06
<i>Cj_ssh_male_m_011</i>	FS942770	7 (0.8)	pollen allergen	BAF32143	2e-06

Table 2. (continued)

Gene ID	Accession No.	# ESTs (% in the library)	highest homology (NCBI) highest homology (TAIR)	Homology accession locus name	E-value
<i>Cj_ssh_male_m_012</i>	FS942771	7 (0.8)	Gag/pol polyprotein, 3'-partial, putative	ABI34371	2e-10
<i>Cj_ssh_male_m_013</i>	FS942772	7 (0.8)	Peptidase aspartic, active site	ABD33091	2e-09
<i>Cj_ssh_male_m_014</i>	FS942773	6 (0.7)	translation elongation factor-1 alpha elongation factor 1-alpha	AAV92325 AT5G60390.3	2e-123 e-118
<i>Cj_ssh_male_m_015</i>	FS942774	6 (0.7)	hypothetical protein RNA binding / nucleic acid binding / nucleotide binding	CAN70556 AT5G66010.1	4e-72 6e-63
<i>Cj_ssh_male_m_016</i>	FS942775	6 (0.7)	unnamed protein product	BAB08213	1e-37
<i>Cj_ssh_male_m_017</i>	FS942776	5 (0.6)	unknown GPT2; antiporter/ glucose-6-phosphate transmembrane transporter	ABK24716 AT1G61800.1	4e-148 e-130
<i>Cj_ssh_male_m_018</i>	FS942777	5 (0.6)	unknown AILP1	ABK21853 AT5G19140.1	9e-93 4e-77
<i>Cj_ssh_male_m_019</i>	FS942778	5 (0.6)	hypothetical protein OsJ_034246 HSP93-III	EAZ20037 AT3G48870.1	3e-60 4e-61
<i>Cj_ssh_male_m_020</i>	FS942779	5 (0.6)	ribosomal protein L18A 60S ribosomal protein L18A (RPL18aB)	AAT08714 AT2G34480.1	3e-16 9e-18

Fig. 2. Venn diagram showing the overlaps between SSH libraries. The genes that have sequence similarity with NCBI database (E-value < 1e-4) were used. Stage E: early stage of pollen development, stage T: tetrad stage, stage M: mature pollen stage. The genes used for this analysis are shown in the Supplemental Table 1.

associated with the eight genes in each tissue of *C. japonica* using semi-quantitative RT-PCR (Fig. 4). In stage E, *Cj_ssh_male_e_002* showed stronger expression in the male strobilus than in the shoot. *Cj_ssh_male_e_003* showed the strongest expression in stage E male strobilus (Fig. 4). The other genes isolated from stage T and M SSH libraries were expressed only in male strobilus tissue. *Cj_ssh_male_t_001*,

Cj_ssh_male_t_002 and *Cj_ssh_male_t_003* were strongly detected in stage T male strobilus tissue, and *Cj_ssh_male_t_003* expression was also detected in stage E male strobilus tissue (Fig. 4). Similarly, *Cj_ssh_male_m_001*, *Cj_ssh_male_m_002* and *Cj_ssh_male_m_003* were detected only in stage M male strobilus tissue (Fig. 4). None of the eight genes were expressed in root tissue (Fig. 4).

Discussion

SSH is an effective method for isolating genes that are differentially expressed between two tissues (Shukla *et al.* 2006, Sternberg *et al.* 2007). We focused on isolating genes from the three different stages of male strobilus development, early, tetrad and mature pollen, of *C. japonica* (Fig. 1), using the SSH method. Ma *et al.* (2008) reported that approximately 80% of 15,950 transcripts shared across all anther stages are also expressed in leaves. Becker *et al.* (2003) analyzed transcriptional profiling of *Arabidopsis* pollen. Of the 1,584 genes detected in pollen, 1,422 (90%) showed an overlap with the genes detected in vegetative tissues. These observations showed that there were very few tissue-specific transcripts. Although Futamura *et al.* (2008) reported the construction of a full-length cDNA library of the male strobilus of *C. japonica* and globally characterized expressed genes, many of the genes they isolated may not show male strobilus-specific expression. Moreover, the gene profiling associated with each developmental stage of the male strobilus of *C. japonica* has not been reported. We subtracted the housekeeping genes using the shoot as a driver, thereby effectively isolating male strobilus-specific genes.

Fig. 3. Overview of the functional classification of stage-unique genes listed in Table 2 and Supplemental Table 1. Sequence homology search was analyzed by BLASTX program of TAIR (E-value < 1e-4). The highest hits locus names were used for functional classification analysis by TAIR GO annotation search. Stage E: early stage of pollen development, stage T: tetrad stage, stage M: mature pollen stage.

We evaluated the SSH libraries by RT-PCR (Fig. 4), and believe that the subtraction was successful. *Cj_ssh_male_e_002* and *Cj_ssh_male_e_003* were also expressed in shoot. In *C. japonica*, male strobilus primordium is formed in the axil of the shoot (Fukui *et al.* 2001, Hashizume *et al.* 1973, Nagao *et al.* 1989). Stage E is an early stage of differentiation, so it has been assumed that the qualitative border of male reproductive tissue and vegetative tissue was not yet clear. Nevertheless, the genes that were expressed more strongly in the male strobilus than in shoot, such as *Cj_ssh_male_e_002* and *Cj_ssh_male_e_003*, were isolated from our library. This result shows that SSH is an effective method for isolating genes that also differ quantitatively in expression. The other genes analyzed by RT-PCR showed not only tissue-specific expression but also stage-specific expression (Fig. 4).

In stage E, the genes that have sequence similarity with the genes expressed in flower tissue were isolated (e.g., *Cj_ssh_male_e_002*, *Cj_ssh_male_e_003* and *Cj_ssh_male_e_016*). The genes expressed in meristematic cells, which actively produced protein, were also isolated (e.g., *Cj_ssh_male_e_011* and *Cj_ssh_male_e_020*). *Cj_ssh_male_e_002* and *Cj_ssh_male_e_016* had sequence similarity with Pollen Ole e 1 family protein (Table 2). *Pollen Ole e 1* gene was expressed in pollen (Alche *et al.* 1999). *Cj_ssh_male_e_003* is similar to early tobacco anther 1 (Accession No. AAO43000) (data not shown). *Cj_ssh_male_e_011* and *Cj_ssh_male_e_020* had sequence similarity with translation elongation factor (*EF-1a* and *GA requiring 1* (*GAI*) genes, respectively. A high level of *EF-1a* expression was detected in the regions of high protein synthesis, including meristems, rapidly growing tissues, and de-

Fig. 4. RT-PCR analysis of genes isolated from subtracted libraries. The template cDNAs were synthesized from shoot (S), male strobilus (M), young shoot (L), and root (R). The samples of shoot and male strobilus were collected on 19 Sep (stage E), 9 Oct (stage T) and 24 Nov (stage M).

veloping gametophytes (Ursin *et al.* 1991). *GAI* promoter activity is highest in rapidly growing tissue; for example, shoot apices, root tips, developing flowers, and seeds (Silverstone *et al.* 1997). Stage E is the time when a male strobilus is enlarging. Therefore, we consider that these results show that cell division or protein synthesis was active in stage E.

In stage T, a number of genes have sequence similarity with tapetum-specific genes of other species. One of the characteristics of this library is that three kinds of genes of

Cj_ssh_male_t_001-3 occupied 60% of all sequences. Two of these genes, *Cj_ssh_male_t_001* and *Cj_ssh_male_t_003*, have peptide sequence similarity with PrMC1 (Walden *et al.* 1999) and PrMALE1 (Höfig *et al.* 2006), respectively, which were isolated from *Pinus radiata* (Table 2). These genes share similarity with several anther-expressed cDNAs, of which the most well characterized is *A9* from *Brassica napus* and *Arabidopsis* (Walden *et al.* 1999). *B. napus A9* and *Arabidopsis A9* expression is restricted to the tapetum (Paul *et al.* 1992). *Cj_ssh_male_t_017*, which has sequence similarity with the *Arabidopsis thaliana MS2* gene, was also isolated from this SSH library. The expression of the *MS2* gene was observed in the tapetum of wild-type flowers at, and shortly after, the release of microspores from tetrads (Aarts *et al.* 1997). The tapetal cells have a major role in pollen development by contributing to microspore release, nutrition, pollen wall synthesis, and pollen-coat deposition (Bedinger 1992, Goldberg *et al.* 1993). In the present study, the genes that have sequence similarity with tapetum-specific genes were highly concentrated in the stage T SSH library, which might reflect an increase in tapetum activity in stage T.

A number of genes have sequence similarity with the expressed genes of pollen in stage M. In a study of ESTs from *C. japonica* pollen (Futamura *et al.* 2006), the existence of the *SF18*-like gene and *CjMPI*-related genes in a pollen EST library was reported. Our stage M SSH library also contained these two genes, which occupied 12% and 5.1% of all the sequences in this library, respectively (Table 2). Moreover, many sequences had similarity with peroxidase. Flower-specific peroxidase gene, *Ghpod*, was isolated from cotton (*Gossypium hirsutum*) (Chen *et al.* 2009). *Ghpod* is a member of class III peroxidase genes and is predominantly expressed during pollen development. Chen *et al.* (2009) presumed that *Ghpod* might play a role in stress response. The data in Fig. 3 show that the stage M SSH library included many genes categorized into response to stress compared with other libraries.

The number of unigenes isolated from each library differed. Genes isolated from the E, T and M SSH libraries numbered 458, 176 and 379, respectively. Because not only the genes for morphogenesis of the male strobilus (e.g., *Cj_ssh_male_e_196* (Supplemental Table 1)) but also the genes that contributed to active cell proliferation (e.g., *Cj_ssh_male_e_020* (Table 2 and Supplemental Table 1)) were included, many kinds of genes might have been isolated from the stage E library. In the stage T library, the variety of genes was greatly decreased. The phenomenon of the decrease in number of expressed genes in the tetrad stage was also observed in other species (Ma *et al.* 2008). This result might show that pollen development nursed by the tapetum was active in this stage. However, it might also show that other processes cease, such as anther development. In stage M, pollen-specific genes were isolated and the variety of isolated genes increased. In maize, the number of expressed genes increased after meiosis (Ma *et al.* 2008). These authors

considered the anther to be an integrated system, in which activation of anther maturation in the somatic cell layers is contingent on the successful completion of meiosis. Because not only pollen specific-genes but also the anther maturation-related genes were expressed, the kinds of expressed genes of stage M might have increased in *C. japonica*.

This is the first report on spatiotemporal gene profiling of the developmental stage of the male strobilus of *C. japonica*. Through the analysis of gene expression profiling, we showed that the expressed genes different significantly among developmental stages of the male strobilus. One of our goals is to clarify the broad cascade of gene expression associated with male strobilus development at various developmental stages and in various cell types. Using the results of this study and knowledge of the full-length cDNA library, we will attempt to develop male sterile technology to resolve *C. japonica* pollen-induced pollinosis, which is a serious allergic disease.

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Literature Cited

- Aarts, M.G., R. Hodge, K. Kalantidis, D. Florack, Z.A. Wilson, B.J. Mulligan, W.J. Stiekema, R. Scott and A. Pereira (1997) The *Arabidopsis MALE STERILITY 2* protein shares similarity with reductases in elongation/condensation complexes. *Plant J.* 12: 615–623.
- Alche, J. D., A.J. Castro, A. Olmedilla, M.C. Fernandez, R. Rodriguez, M. Villalba and M.I. Rodriguez-Garcia (1999) The major olive pollen allergen (*Ole e 1*) shows both gametophytic and sporophytic expression during anther development, and its synthesis and storage takes place in the RER. *J Cell Sci.* 112: 2501–2509.
- Becker, J.D., L.C. Boavida, J. Carneiro, M. Haury and J.A. Feijó (2003) Transcriptional profiling of *Arabidopsis* tissues reveals the unique characteristics of the pollen transcriptome. *Plant Physiol.* 133: 713–725.
- Bedinger, P. (1992) The remarkable biology of pollen. *Plant Cell.* 4: 879–887.
- Chen, D., Y. Ding, W. Guo and T. Zhang (2009) Molecular cloning and characterization of a flower-specific class III peroxidase gene in *G. Hirsutum*. *Mol. Biol. Rep.* 36: 461–469.
- Fukui, M., N. Futamura, Y. Mukai, Y. Wang, A. Nagao and K. Shinohara (2001) Ancestral MADS box genes in Sugi, *Cryptomeria japonica* D. Don (Taxodiaceae), homologous to the B function genes in angiosperms. *Plant Cell Physiol.* 42: 566–575.
- Futamura, N., T. Ujino-Ihara, M. Nishiguchi, H. Kanamori, K. Yoshimura, M. Sakaguchi and K. Shinohara (2006) Analysis of expressed sequence tags from *Cryptomeria japonica* pollen reveals

- novel pollen-specific transcripts. *Tree Physiol.* 26: 1517–1528.
- Futamura, N., Y. Totoki, A. Toyoda, T. Igasaki, T. Nanjo, M. Seki, Y. Sakaki, A. Mari, K. Shinozaki and K. Shinohara (2008) Characterization of expressed sequence tags from a full-length enriched cDNA library of *Cryptomeria japonica* male strobili. *BMC Genomics* 9: 383.
- Goldberg, R.B., T.P. Beals and P.M. Sanders (1993) Anther development: Basic principles and practical applications. *Plant Cell.* 5: 1217–1229.
- Hashizume, H. (1973) Studies on flower bud formation, flower sex differentiation and their control in conifers. *Res. Bull. Tottori Univ. For.* 7: 1–139.
- Höfig, K.P., R. Möller, L. Donaldson, J. Putterill and C. Walter (2006) Towards male sterility in *Pinus radiata*—a stilbene synthase approach to genetically engineer nuclear male sterility. *Plant Biotechnol. J.* 4: 333–343.
- Ma, J., D.S. Skibbe, J. Fernandes and V. Walbot (2008) Male reproductive development: gene expression profiling of maize anther and pollen ontogeny. *Genome Biol.* 9: R181.
- Nagao, A., S. Sasaki and R.P. Pharis (1989) *Cryptomeria japonica*. In: Halevy, A.H. (ed.) *CRC Handbook of Flowering*, vol. VI, CRC Press; Boca Raton, FL, pp. 247–269.
- Paul, W., R. Hodge, S. Smartt, J. Draper and R. Scott (1992) The isolation and characterisation of the tapetum-specific *Arabidopsis thaliana* A9 gene. *Plant Mol. Biol.* 19: 611–622.
- Shiokawa, T., S. Yamada, N. Futamura, K. Osanai, D. Murasugi, K. Shinohara, S. Kawai, N. Morohoshi, Y. Katayama and S. Kajita (2008) Isolation and functional analysis of the *CjNdly* gene, a homolog in *Cryptomeria japonica* of *FLORICAULA/LEAFY* genes. *Tree Physiol.* 28: 21–28.
- Shukla, A.K., A.K. Shasany, M.M. Gupta and S.P. Khanuja (2006) Transcriptome analysis in *Catharanthus roseus* leaves and roots for comparative terpenoid indole alkaloid profiles. *J. Exp. Bot.* 57: 3921–3932.
- Silverstone, A.L., C. Chang, E. Krol and T. Sun (1997) Developmental regulation of the gibberellin biosynthetic gene *GA 1* in *Arabidopsis thaliana*. *Plant J.* 12: 9–19.
- Sternberg, M.B. and S. Gepstein (2007) Subtractive hybridization techniques to study cellular senescence. *Methods Mol. Biol.* 371: 289–305.
- Taniguchi, T., Y. Ohmiya, M. Kurita, M. Tsubomura and T. Kondo (2008) Regeneration of transgenic *Cryptomeria japonica* D. Don after *Agrobacterium tumefaciens*-mediated transformation of embryogenic tissue. *Plant Cell Rep.* 27: 1461–1466.
- Ursin, V.M., J.M. Irvine, W.R. Hiatt and C.K. Shewmaker (1991) Developmental analysis of elongation factor-1 α expression in transgenic tobacco. *Plant Cell* 3: 583–591.
- Walden, A.R., C. Walter and R.C. Gardner (1999) Genes expressed in *Pinus radiata* male cones include homologs to anther-specific and pathogenesis response genes. *Plant Physiol.* 121: 1103–1116.