Breeding Science 58: 251–259 (2008)

Identification of genes specifically expressed in androgenesis-derived embryo in rapeseed (*Brassica napus* L.)

Ryo Tsuwamoto and Yoshihito Takahata*

Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan

Microspore culture is an efficient system to produce haploid and double-haploid plants, and is a useful model system in developmental research. Although many studies have been carried out to understand the mechanism of microspore embryogenesis, the details of molecular events occurring during its developmental process are unclear. During embryogenesis, a marked morphological change occurs just after globular stage embryos. To gain insight into the developmental process of androgenesis in rapeseed (*Brassica napus*), we isolated the genes expressed differentially during globular stage by suppression subtractive hybridization (SSH). Of 254 ESTs isolated and sequenced, 82 were singletons and the remaining 172 were assembled into 28 groups based on homology to the identical gene, resulting in the identification of 110 non-redundant EST groups, which are potentially up-regulated genes in androgenesis. Survey of the potential function of the genes showed that 96.4% of these genes were homologous to known genes with 42.7% of unclassified proteins and 13.6% of metabolism. To confirm the expression profiles of isolated genes during androgenesis and zygotic embryogenesis, six selected genes were examined by quantitative RT-PCR analysis. All genes were highly expressed in globular and other stages of androgenesis. The expressions of these genes were confirmed in zygotic embryogenesis. These results serve as a starting point for understanding the mechanism of androgenesis and zygotic embryogenesis.

Key Words: *Brassica napus*, microspore embryogenesis, suppression subtractive hybridization, ESTs, quantitative RT-PCR.

Introduction

Androgenesis, a type of embryogenesis from immature male gametes, was first reported in *Datura* (Guha and Maheshwari 1964). Since then, androgenesis has been reported in a large number of plant species, because of its potential to produce haploids and double-haploids for plant improvement (Khush and Virmani 1996). Many factors influencing androgenesis, such as genotypes, microspore developmental stage, culture medium and several stresses, have been evaluated. Although these optimized conditions could enhance the efficiency of androgenesis, recalcitrant species and cultivars are present in many important crops. In order to generalize androgenesis, it is essential to understand the induction mechanism of microspore embryogenesis and its developmental process.

Although anther culture is widely used as a method of androgenesis, isolated microspore culture, which has been developed in several crops, such as *Brassica* spp., tobacco and barley, has several advantages as a model in developmental research as well as in practical breeding because of its efficient embryogenesis from a single-cell. Morphological, genetic, biochemical and molecular studies using these materials, especially *Brassica* spp., have contributed to our understanding of androgenesis. Ultrastructural analysis using TEM and SEM clarified the early events of androgenesis and revealed that the symmetrical division of uni-nucleate microspores is a symptom of androgenic development (Zaki and Dickinson 1991, Telmer *et al.* 1993, Nitta *et al.* 1997, Ilic-Grubor *et al.* 1998). Genetic analysis of androgenesis in *Brassica* ssp. showed that both additive and dominant effects contribute to androgenesis with high heritability (Zhang and Takahata 2001). DNA markers linked to the ability of androgenesis were reported in *B. napus* (Cloutier *et al.* 1995, Zhang *et al.* 2003) and *B. rapa* (Ajisaka *et al.* 1999). A number of proteins and genes expressed in microspore embryogenesis have been isolated (Pechan *et al.* 1991, Boutilier *et al.* 2002).

Although these studies have elucidated part of the mechanism of androgenesis, most of its regulation mechanism is still unclear. EST analysis has been used extensively to discover novel genes. Recently, a large number of ESTs expressed in early microspore embryogenesis were identified in *B. napus* (Tsuwamoto *et al.* 2007, Malik *et al.* 2007); however, little has been reported in other developmental stages of embryogenesis. In the present study, we identified 110 non-redundant ESTs differentially expressed in microspore-derived globular embryos using suppression subtractive

Communicated by T. Sato
Received March 27, 2008. Accepted May 30, 2008.
*Corresponding author (e-mail: ytakahata@iwate-u.ac.jp)

hybridization (SSH), and confirmed the androgenesis-specific expression of isolated ESTs by quantitative RT-PCR.

Materials and Methods

Plant material and microspore culture

Rapeseed (*B. napus*) cv. Lisandra was used as a plant material. The plants were grown in a greenhouse and then transferred to an environmental chamber under a regime of 13/8°C (day/night) with a natural photoperiod at the beginning of bolting. Microspore culture was carried out as previously described by Takahata (1997). Sterilized flower buds, 3.0–4.0 mm in length, were homogenized in B5-13 medium (Takahata and Keller 1991). After washing the isolated microspores, they were resuspended in 1/2 NLN-13 medium (Takahata and Keller 1991) at a density of 1.0×10^5/ml, and then incubated at 32.5°C to induce androgenesis (embryogenic microspores), or at 20°C (non-embryonic microspores) for 4 days. For poly $(A)^+$ RNA isolation, embryonic and non-embryonic microspores, and fleshy isolated microspores were submerged in liquid nitrogen and then stored at −80°C.

After treatment at 32.5°C for 4 days, embryonic microspores were maintained at 25°C in darkness. After 2–3 weeks of incubation, microspore-derived embryos were divided based on size by sieving them through 1 mm nylon mesh followed by 200 μm mesh and 74 μm mesh, submerged in liquid nitrogen, and then stored at −80°C for poly $(A)^+$ RNA isolation (Takahata *et al.* 1991, Tsuwamoto *et al.* 2007). Embryos caught in the 1 mm, 200 μm and 74 μm nylon mesh consisted of cotyledonary, torpedo to heart and globular embryos, respectively.

Suppression subtractive hybridization (SSH) and sequence analysis

Poly $(A)^+$ RNA was isolated from collected materials using a Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen, San Diego, CA, USA). SSH was carried out according to Tsuwamoto *et al.* (2007) using the PCR-select subtractive hybridization kit (Clontech, Palo Alto, CA, USA). Two micrograms of poly $(A)^+$ RNA from globular embryos was employed to synthesize tester cDNA for forward subtraction, and a 2 μg mixture of poly $(A)^+$ RNA from non-embryonic microspores and fleshy isolated microspores was used to synthesize driver cDNA. The subtracted cDNAs (secondary PCR product), which were up-regulated in globular embryos, were cloned into a pCR2.1 cloning vector (Invitrogen). A total of 256 clones were randomly selected and were sequenced with a DNA sequencing kit (Applied Biosystems) using M13 universal primer on a model 310 DNA sequencer (PE Biosystems, Foster City, CA, USA). After elimination of SSH adaptor sequence, all ESTs were used for blast × homology search at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). For functional classification of ESTs, annotations of homologous genes in *Arabidopsis* to isolated ESTs referred to the MIPS website.

Quantitative RT-PCR

Isolation of poly $(A)^+$ RNA from microspores, microspore-derived embryos, developing ovules including zygotic embryos, siliques and leaves was carried out as mentioned above. Developing ovules excised from siliques at 16 days after pollination (DAP), 20 DAP, and 30 DAP and mature seeds were collected. After excision of ovules from siliques, the remaining empty siliques were collected as controls of non-embryogenic organ. Leaves were sampled from plants grown in a greenhouse at 2–3 weeks after germination.

cDNA synthesis from 150 ng of poly $(A)^+$ RNA was conducted with a First-strand cDNA synthesis kit (Amersham Bioscience UK Ltd, Buckinghamshire, UK), and quantitative RT-PCR reaction was performed on a LightCycler system (Roche, Mannheim, Germany) using LightCycler-FastStart DNA Master SYBR Green I (Roche). To equalize cDNA, the actin gene was used as a standardization control, and expression levels were normalized to globular stage embryos derived from microspores or ovules at 16 DAP. For all PCR reactions, no false amplicon was detected in meltcurve analysis (data not shown). The sequence of primers used in this analysis was as follows. BnGemb-4F, AGTGATCCTACTTCAGATCAT: BnGemb-4R, CCTCA GTGACTCATACGCATA: BnGemb-10F, ACTGAAGAG ACTGAATGAAGG, BnGemb-10R: TCAAGATTGAACC TTGGTGA: BnGemb-51F, TAGTTCGTAAATGCATTT GCA: BnGemb-51R, ATACCGTGGTCCTGGAGAGTT: BnGemb-53F, GCAGCCATGTAGGGATTCATA: BnGemb-53R, GAGATTAAGGAAGAAGCAATA: BnGemb-58F, CACAGCGGCTCTTTCCATGTAC: BnGemb-58R, GAGC TCGTAGTCATAGATCTCA: BnG-59 FW, ACGCTCCAG ATGGAAGTTTGA: BnG-59 RV, TGATGATATCAACAT GCTGCT.

Results

EST identification from the subtracted cDNA libary

To identify genes highly expressed in microspore-derived globular stage embryos, the cDNA library was prepared by subtracting non-embryonic and fleshy isolated microspore cDNA from microspore-derived globular stage embryos cDNA. A total of 254 clones were selected at random and sequenced. ESTs showing the highest homology to identical genes in the database were regarded as different parts of the same gene and grouped together as the same EST, although their sequences are not identical. Of 254 clones, 82 were singletons and the remaining 172 were assembled into 28 groups with occurrence of the common gene ranging from 2 to 76. Lipid transfer protein was the most frequently isolated gene, which is consistent with the result obtained in early embryogenesis (Tsuwamoto *et al.* 2007). In the results of this grouping, 110 non-redundant EST groups were identified.

The 110 genes identified were assigned to potential functional groups according to their sequence homology on

the MIPS website. Of these 110 EST groups, 106 (96.4%) showed high similarity to known genes registered in the database and 6 showed no matches to known genes. As shown in Fig. 1, a high proportion (42.7%) of the ESTs represented genes with unclassified proteins. Metabolism (13.6%), protein synthesis (9.1%), transcription (8.2%), and cellular communication/signal transduction mechanism (6.4%) were the highly represented classes of genes with known potential function. The number of clones in each EST group, putative function, e-value and accession number are shown in Table 1.

In a previous study, we isolated 136 EST groups differentially expressed in early stage of microspore embryogenesis (cultured at 32.5°C for 4 days) of rapeseed (Tsuwamoto *et al.* 2007). When the 110 EST groups, which were isolated from microspore-derived globular embryos in this study, were compared with early stage microspore embryogenesis-specific 136 EST groups, only 7 encoding lipid transfer protein (BnGemb-11, -12 and -14), glutathione *S*-transferase (BnGemb-64), GDSL-motif lipase/hydrolase (BnGemb-72), oleosin (BnGemb-34) and RNA helicase (BnGemb-18) were commonly found in both EST groups. As each library is almost entirely composed of different genes, this indicates the possibility that genes acting differentially in earlier stage embryos and globular embryos in androgenesis are considerably different.

Gene expression in microspore and zygotic embryogenesis

To evaluate the expression profile of genes isolated from globular embryos, six selected genes, which were functionally categorized into transcription (BnGemb-51, -53, -58), cellular communication/signal transduction (BnGemb-4, -10) and unclassified protein (BnGemb-59), were used for quantitative RT-PCR analysis. All genes showed a high expression in globular stage embryos, but less or no expression in freshly isolated microspores and vegetative leaves, except for BnGemb-53, which showed high expression in freshly isolated microspores (Fig. 2). Although BnGemb-53 exhibited an unexpected expression pattern, the other five genes (BnGemb-4, -10, -51, -58, -59) were highly expressed during androgenesis from an earlier embryogenesis stage (microspores cultured at 32°C for 4 days) to the torpedo stage. In particular, the highest expression of BnGemb-10 and -59 and BnGemb-51 and -58 was found in globular and torpedo stages, respectively, and BnGemb-4 showed the highest expression in an earlier embryogenesis stage. In contrast, the expression pattern of BnGemb-53 was different from those of other genes, and it was highly expressed in non-embryonic microspores (cultured at 20°C). Such discrepancies between SSH clones and their expression patterns by RT-PCR and/or Northern analysis have often been reported (Tsuwamoto *et al.* 2007, Xu *et al.* 2007).

The expression profile of the six genes was also investigated in various developmental stages of zygotic embryogenesis (Fig. 3). Because the isolation of zygotic embryos from ovules is difficult, developing ovules at 14 DAP, 16 DAP, 20 DAP, and 30 DAP, mainly including globular, heart, torpedo, and cotyledonary embryo respectively, were employed for mRNA isolation. All genes were highly expressed in developing ovules and/or mature seeds, while they were not or were less expressed in siliques used as a control of non-embryogenic tissues. BnGemb-10 and -58 showed a constant high expression level during zygotic embryogenesis, and BnGemb-58 was accumulated at an extremely high level in mature seeds. The expressions of other four genes peaked in mature seeds, and lower expressions were detected in developing ovules at each stage. Some genes showed a similar expression profile to those in microspore embryogenesis. BnGemb-53 and -59 showed higher expression in globular embryos and decreased as embryos developed in both microspore and zygotic embryogenesis. In contrast, an inconsistent expression pattern was also found, for example, BnGemb-10, which was expressed constantly during zygotic embryogenesis, decreased as embryo development progressed in androgenesis. Such inconsistence was observed in genes isolated from early embryogenesis derived from microspores (Tsuwamoto *et al.* 2007).

Discussion

In rapeseed, cultured microspores can alter their gametophytic development to embryogenic development with high efficiency, and this system has advantages not only to produce homozygous lines in plant breeding but also as a model in developmental research. In order to identify genes involved in the development of microspore-derived embryos, highly expressed genes in globular stage embryos were screened by SSH.

Many EST groups isolated in this study were homologous to the genes reported to be concerned with embryogenesis. Of these, several genes are related with epidermal development of embryos. BnGemb-8 is identical to *ARABIDOPSIS CRINKLY4* (*ACR4*), which encodes a receptor-like protein kinase. *ACR4* is expressed in the protoderm in the later stage of embryogenesis and is required for epidermal development (Gifford *et al.* 2003, Watanabe *et al.* 2004). BnGemb-56 showed high similarity to *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*), which is a HD-GL class transcription factor, an important gene for epidermal differentiation as well as *ACR4*, and expressed in the protoderm during embryogenesis (Lu *et al.* 1996). Double knockout of *ATML1* and *PROTODERMAL FACTOR2* (*PDF2*), another HD-GL class transcription factor expressed in the protoderm, exhibited severe defects in the epidermis of embryos (Abe *et al.* 2003). It has been reported that *ATML1* may regulate the expression of epidermis-specific genes via a *cis*-element named L1 box, and *PDF1* encoding a putative extracellular proline-rich protein is a target of *ATML1* (Abe *et al.* 2001). In our study, an EST similar to *PDF1* was also found as BnGemb-80. BnGemb-26 corresponded with an epidermis-specific gene *FIDDLEHEAD* (*FDH*), encoding a beta-ketoacyl-CoA synthase that mediates the addition of C2 unit to C16-C22 acyl-CoA to synthesize

Table 1. Specification of identified ESTs and their putative function

No. of clone	Clone ID[a]	Functional categories/Homology	E-value
BIOGENESIS OF CELLULAR COMPONENTS			
1	BnGemb-1	similar to histone H4.1, partial [*Bos taurus*]	7.00E-36
CELL CYCLE AND DNA PROCESSING			
1	BnGemb-2	splicing factor Prp8, putative [*Arabidopsis thaliana*]	5.00E-55
1	BnGemb-3	DNA polymerase epsilon subunit B-like protein [*Arabidopsis thaliana*]	5.00E-49
CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM			
2	BnGemb-4	leucine-rich repeat transmembrane protein kinase, putative [*Arabidopsis thaliana*]	9.00E-39
2	BnGemb-5	leucine-rich repeat family protein/protein kinase family protein [*Arabidopsis thaliana*]	4.00E-17
2	BnGemb-6	leucine-rich repeat protein kinase, putative [*Arabidopsis thaliana*]	5.00E-30
1	BnGemb-7	calcium/calmodulin-dependent protein kinase CaMK3 [*Arabidopsis thaliana*]	5.00E-55
3	BnGemb-8	receptor protein kinase, putative (ACR4) [*Arabidopsis thaliana*]	1.00E-57
1	BnGemb-9	leucine rich repeat protein (LRP), putative [*Arabidopsis thaliana*]	5.00E-42
1	BnGemb-10	leucine-rich repeat transmembrane protein kinase, putative [*Arabidopsis thaliana*]	3.00E-51
CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES			
76	BnGemb-11	non-specific lipid transfer protein [*Brassica napus*]	2.00E-23
13	BnGemb-12	lipid transfer protein [*Brassica oleracea*]	4.00E-23
1	BnGemb-13	plasma membrane aquaporin (PAQ1) [*Raphanus sativus*]	1.00E-30
3	BnGemb-14	nonspecific lipid-transfer protein precursor-like [*Arabidopsis thaliana*]	6.00E-29
4	BnGemb-15	putative nonspecific lipid-transfer precursor [*Arabidopsis thaliana*]	1.00E-22
CLASSIFICATION NOT YET CLEAR-CUT			
1	BnGemb-16	putative calmodulin-binding protein [*Arabidopsis thaliana*]	2.00E-54
1	BnGemb-17	putative epoxide hydrolase [*Arabidopsis thaliana*]	1.00E-20
1	BnGemb-18	putative RNA helicase [*Arabidopsis thaliana*]	1.00E-38
ENERGY			
2	BnGemb-19	phosphoenolpyruvate carboxykinase (ATP)-like protein [*Arabidopsis thaliana*]	7.00E-62
INTERACTION WITH THE CELLULAR ENVIRONMENT			
1	BnGemb-20	ferritin [*Brassica napus*]	1.00E-45
METABOLISM			
3	BnGemb-21	ascorbate peroxidase [*Brassica oleracea*]	6.00E-74
1	BnGemb-22	putative invertase [*Arabidopsis thaliana*]	5.00E-23
1	BnGemb-23	isocitrate dehydrogenase, putative [*Arabidopsis thaliana*]	3.00E-14
1	BnGemb-24	endo-1,4-beta-glucanase (EGASE)/cellulase [*Arabidopsis thaliana*]	3.00E-68
1	BnGemb-25	ribonucleotide reductase [*Nicotiana tabacum*]	2.00E-60
2	BnGemb-26	beta-ketoacyl-CoA synthase (FIDDLEHEAD) [*Arabidopsis thaliana*]	7.00E-60
1	BnGemb-27	glycosyl transferase family 48 protein [*Arabidopsis thaliana*]	4.00E-47
1	BnGemb-28	6-phosphogluconate dehydrogenase family protein [*Arabidopsis thaliana*]	4.00E-42
2	BnGemb-29	putative chloroplast-targeted beta-amylase [*Brassica napus*]	2.00E-63
1	BnGemb-30	anthranilate phosphoribosyltransferase-like protein [*Arabidopsis thaliana*]	6.00E-18
1	BnGemb-31	transketolase-like protein [*Arabidopsis thaliana*]	2.00E-42
1	BnGemb-32	S-adenosyl-L-homocystein hydrolase [*Arabidopsis thaliana*]	1.00E-17
1	BnGemb-33	YUC4 (YUCCA4); monooxygenase [*Arabidopsis thaliana*]	6.00E-58
1	BnGemb-34	oleosin, isoform 21K [*Arabidopsis thaliana*]	2.00E-41
1	BnGemb-35	putative pyruvate decarboxylase [*Arabidopsis thaliana*]	1.00E-59
PROTEIN SYNTHESIS			
3	BnGemb-36	putative Argonaute (AGO1) protein [*Arabidopsis thaliana*]	1.00E-68
1	BnGemb-37	cytoplasmic ribosomal protein S14 [*Brassica napus*]	2.00E-55
1	BnGemb-38	40S ribosomal protein S2 homolog [*Arabidopsis thaliana*]	6.00E-61
1	BnGemb-39	60S ribosomal protein L37a (RPL37aC) [*Arabidopsis thaliana*]	1.00E-19
1	BnGemb-40	ribosomal protein L11-like [*Arabidopsis thaliana*]	4.00E-53
1	BnGemb-41	26S proteasome, non-ATPase regulatory subunit [*Arabidopsis thaliana*]	9.00E-70

Table 1. (continued)

No. of clone	Clone ID[a]	Functional categories/Homology	E-value
1	BnGemb-42	40S ribosomal protein S3 (RPS3C) [*Arabidopsis thaliana*]	2.00E-63
1	BnGemb-43	60S ribosomal protein L5 [*Arabidopsis thaliana*]	6.00E-55
1	BnGemb-44	elongation factor 1-alpha [*Arabidopsis thaliana*]	9.00E-62
1	BnGemb-45	40S ribosomal protein S28 [*Arabidopsis thaliana*]	2.00E-20
PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT			
1	BnGemb-46	tubulin beta-7 chain (TUB7) [*Arabidopsis thaliana*]	3.00E-58
1	BnGemb-47	serine/threonine kinase [*Brassica oleracea*]	2.00E-41
1	BnGemb-48	putative receptor protein kinase [*Arabidopsis thaliana*]	2.00E-62
SUBCELLULAR LOCALIZATION			
1	BnGemb-49	putative nucleolar protein AtNAP57 [*Arabidopsis thaliana*]	5.00E-12
1	BnGemb-50	H+-transporting ATP synthase beta chain (mitochondrial)-like protein [*Arabidopsis thaliana*]	6.00E-71
TRANSCRIPTION			
1	BnGemb-51	PHD finger family protein/GYF domain-containing protein [*Arabidopsis thaliana*]	1.00E-15
1	BnGemb-52	ethylene-induced calmodulin-binding protein 1 [*Arabidopsis thaliana*]	8.00E-55
1	BnGemb-53	putative bZIP transcription factor [*Arabidopsis thaliana*]	7.00E-09
1	BnGemb-54	ABI3 protein [*Arabidopsis thaliana*]	1.00E-26
3	BnGemb-55	SCARECROW1 [*Arabidopsis thaliana*]	2.00E-70
1	BnGemb-56	L1 specific homeobox gene ATML1/ovule-specific homeobox protein A20 [*Arabidopsis thaliana*]	2.00E-50
1	BnGemb-57	transcription activator (GRL2) [*Arabidopsis thaliana*]	6.00E-22
1	BnGemb-58	AP2/EREBP transcription factor [*Arabidopsis thaliana*]	4.00E-56
2	BnGemb-59	unknown protein [*Arabidopsis thaliana*]	9.00E-33
UNCLASSIFIED PROTEINS			
1	BnGemb-60	unknown protein [*Arabidopsis thaliana*]	4.00E-18
1	BnGemb-61	expressed protein [*Arabidopsis thaliana*]	4.00E-36
3	BnGemb-62	selenoprotein family protein [*Arabidopsis thaliana*]	2.00E-57
2	BnGemb-63	delta 9 desaturase (ADS1) [*Arabidopsis thaliana*]	6.00E-64
5	BnGemb-64	glutathione S-transferase, putative (ERD9) [*Arabidopsis thaliana*]	2.00E-62
1	BnGemb-65	ferredoxin--NADP(+) reductase, putative/adrenodoxin reductase, putative [*Arabidopsis thaliana*]	3.00E-67
1	BnGemb-66	heavy-metal-associated domain-containing protein/unknown protein [*Arabidopsis thaliana*]	5.00E-13
1	BnGemb-67	pollen Ole e 1 allergen and extensin family protein [*Arabidopsis thaliana*]	1.00E-39
5	BnGemb-68	GDSL-motif lipase/hydrolase family protein [*Arabidopsis thaliana*]	3.00E-60
1	BnGemb-69	DNA polymerase A family protein, putative [*Arabidopsis thaliana*]	3.00E-59
1	BnGemb-70	expressed protein [*Arabidopsis thaliana*]	8.00E-47
1	BnGemb-71	putative 3-ketoacyl-CoA reductase 2 [*Brassica napus*]	7.00E-44
9	BnGemb-72	GDSL-motif lipase/hydrolase family protein [*Arabidopsis thaliana*]	5.00E-63
1	BnGemb-73	thaumatin-like protein, putative/pathogenesis-related protein, putative [*Arabidopsis thaliana*]	9.00E-70
1	BnGemb-74	putative amino acid or GABA permease [*Arabidopsis thaliana*]	5.00E-42
1	BnGemb-75	expressed protein [*Arabidopsis thaliana*]	5.00E-09
1	BnGemb-76	expressed protein [*Arabidopsis thaliana*]	1.00E-40
1	BnGemb-77	unknown protein [*Arabidopsis thaliana*]	1.00E-29
1	BnGemb-78	hypothetical protein [*Arabidopsis thaliana*]	2.00E-40
2	BnGemb-79	translin family protein [*Arabidopsis thaliana*]	9.00E-31
1	BnGemb-80	protodermal factor 1 [*Arabidopsis thaliana*]	0.24
1	BnGemb-81	late embryogenesis abundant family protein/LEA family protein [*Arabidopsis thaliana*]	5.00E-63
3	BnGemb-82	expressed protein [*Arabidopsis thaliana*]	2.00E-36
1	BnGemb-83	universal stress protein (USP) family protein [*Arabidopsis thaliana*]	1.00E-09
1	BnGemb-84	putative epoxide hydrolase [*Arabidopsis thaliana*]	1.00E-20
1	BnGemb-85	kinesin (centromere protein) like heavy chain-like protein [*Arabidopsis thaliana*]	2.00E-31
1	BnGemb-86	short-chain dehydrogenase/reductase (SDR) family protein [*Arabidopsis thaliana*]	4.00E-36
6	BnGemb-87	disease resistance-responsive protein-related/dirigent protein-related [*Arabidopsis thaliana*]	4.00E-13
1	BnGemb-88	GDSL-motif lipase/hydrolase family protein [*Arabidopsis thaliana*]	2.00E-13
1	BnGemb-89	60S ribosomal protein L144 [*Brassica rapa* subsp. pekinensis]	2.00E-39
1	BnGemb-90	glycine-rich protein [*Arabidopsis thaliana*]	1.00E-06
2	BnGemb-91	synaptic glycoprotein SC2-like protein [*Arabidopsis thaliana*]	4.00E-31

Table 1. (continued)

No. of clone	Clone ID[a]	Functional categories/Homology	E-value
1	BnGemb-92	F-box family protein (FBL22) [*Arabidopsis thaliana*]	2.00E-19
2	BnGemb-93	SEC14 cytosolic factor family protein/phosphoglyceride transfer family protein [*Arabidopsis thaliana*]	1.00E-67
1	BnGemb-94	COBRA-like protein 7 precursor/phytochelatin synthetase-related [*Arabidopsis thaliana*]	5.00E-25
1	BnGemb-95	putative protein [*Arabidopsis thaliana*]	2.00E-43
1	BnGemb-96	seed specific protein Bn15D18B [*Brassica napus*]	1.00E-45
3	BnGemb-97	unknown protein [*Arabidopsis thaliana*]	2.00E-40
1	BnGemb-98	GDSL-motif lipase/acylhydrolase-like protein [*Arabidopsis thaliana*]	1.00E-40
1	BnGemb-99	GDSL-motif lipase/hydrolase family protein [*Arabidopsis thaliana*]	7.00E-60
2	BnGemb-100	putative acyltransferase [*Arabidopsis thaliana*]	1.00E-40
1	BnGemb-101	tryptophan synthase beta chain [*Arabidopsis thaliana*]	4.00E-38
6	BnGemb-102	disease resistance-responsive protein-related [*Arabidopsis thaliana*]	2.00E-20
1	BnGemb-103	thaizole biosynthetic enzmye [*Alnus glutinosa*]	3.00E-19
1	BnGemb-104	RNA-binding protein 45 (RBP45), putative [*Arabidopsis thaliana*]	1.00E-20
1	BnGemb-105	microtubule associated protein (MAP65/ASE1) family protein [*Arabidopsis thaliana*]	9.00E-46
1	BnGemb-106	expressed protein [*Arabidopsis thaliana*]	9.00E-44
NOT FOUND			
1	BnGemb-107	Not found	—
1	BnGemb-108	Not found	—
1	BnGemb-109	Not found	—
1	BnGemb-110	Not found	—

[a] Accession number in DDBJ genebank: BnGemb-1 (DB999559) to BnGemb-110 (DB999668)

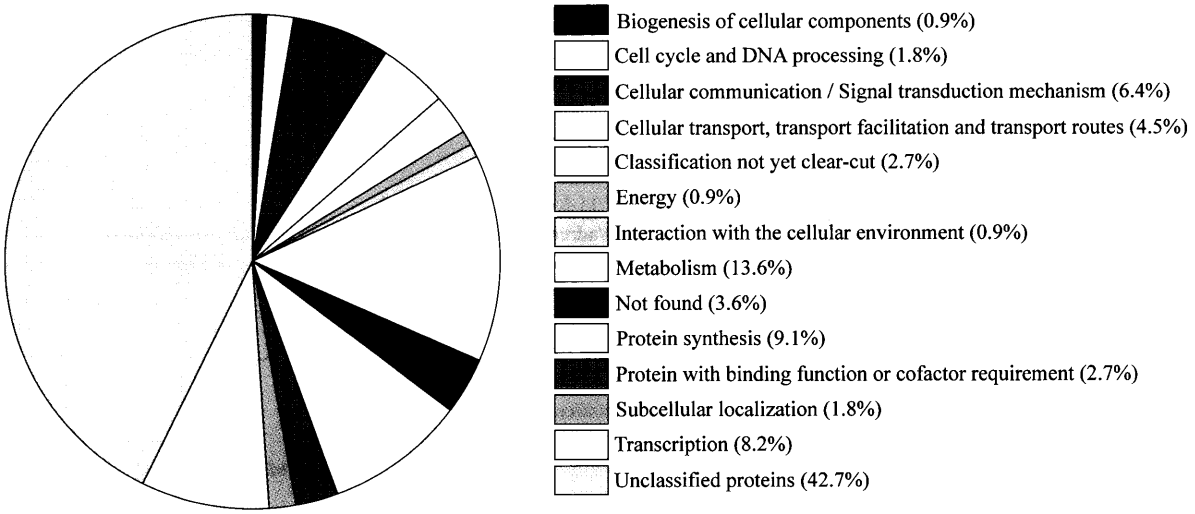

Fig. 1. Percentage distribution of the 110 isolated EST groups in functional categories. The putative functions of identified ESTs were determined by sequence comparison with the GenBank database (http://www.ncbi.nlm.nih.gov) using a blast × homology search program. All ESTs were functionally classified based on the MIPS website (http://mips.gsf.de/proj/thal/db/search/blast_arabi.html).

very long chain fatty acid (Yephremov *et al.* 1999, Pruitt *et al.* 2000). It is known that *FDH* is expressed in the protoderm in embryogenesis, ovule primodia and epidermis, and is essential for cuticle generation (Lolle *et al.* 1992, Lolle and Cheung 1993, Tanaka *et al.* 2007), and *FDH* is also expressed in the somatic embryo of white spruce (Stasolla *et al.* 2003). Identification of such epidermis-specific genes suggests that epidermis differentiation is ongoing in globular embryos derived from micropores as well as zygotic embryogenesis.

Goldberg *et al.* (1994) suggested that a prepattern of the different transcriptional regulatory domain has been established in the globular embryo before the morphogenetic events that lead to differentiation of cotyledon and axis regions at the heart stage. After the globular stage, remarkable morphological changes occur: initiation of cotyledons from two lateral domains at the apical parts, start of hypocotyl elongation, and differentiation of root meristem at the basal part. Isolated ESTs contained genes related with the formation of shoot or root meristem. BnGemb-36 showed high similarity to the *ARGONAUTO1* (*AGO1*) gene, which has an overlapping function with *ZWILLE/PINHEAD* (*ZLL/PNH*)

Microspores	Cultured microspores				Microspore-derived embryos			Leaf	Clone ID	Putative function
	20 °C 1day	20 °C 4days	32 °C 1day	32 °C 4days	Globular	Torpedo	Cotyledonary			
0.08	0.12	0.32	0.28	4.00	1.00	1.20	0.28	0.00	BnGemb-4	leucine-rich repeat transmembrane protein kinase
0.00	0.01	0.06	0.01	0.24	1.00	0.44	0.24	0.01	BnGemb-10	leucine-rich repeat transmembrane protein kinase
0.14	0.59	0.56	0.44	0.57	1.00	1.32	0.32	0.06	BnGemb-51	PHD finger family protein / GYF domain-containing protein
1.75	5.39	34.00	1.82	3.57	1.00	0.50	0.29	0.04	BnGemb-53	putative bZIP transcription factor
0.00	0.00	0.03	0.00	0.41	1.00	2.15	0.52	0.00	BnGemb-58	AP2/EREBP transcription factor
0.07	0.19	0.20	0.10	0.43	1.00	0.54	0.04	0.00	BnGemb-59	unknown protein

0.00-0.10
0.11-0.50
0.51-0.99
1.00
1.01-2.00
2.01-

Fig. 2. Expression profiles of selected ESTs during embryogenesis from microspores. Expression levels of six selected genes were investigated by quantitative RT-PCR, and the results were denoted by relative values based on expression intensity in microspore-derived globular embryos.

Silique	Ovule stage				Seed	Clone ID	Putative function
	14 DAP	16 DAP	20 DAP	30 DAP			
0.22	1.00	0.54	0.49	0.31	7.32	BnGemb-4	leucine-rich repeat transmembrane protein kinase
0.11	1.00	1.08	1.57	1.15	1.02	BnGemb-10	leucine-rich repeat transmembrane protein kinase
0.11	1.00	0.50	0.31	0.27	3.95	BnGemb-51	PHD finger family protein / GYF domain-containing protein
0.11	1.00	1.03	0.78	0.60	3.54	BnGemb-53	putative bZIP transcription factor
0.00	1.00	2.06	1.80	2.66	88.0	BnGemb-58	AP2/EREBP transcription factor
0.00	1.00	0.55	0.28	0.07	1.23	BnGemb-59	unknown protein

0.00-0.10
0.11-0.50
0.51-0.99
1.00
1.01-2.00
2.01-

Fig. 3. Expression profiles of selected ESTs in zygotic embryogenesis. Expression levels of six selected genes were investigated by quantitative RT-PCR, and the results were denoted by relative values based on expression intensity at 14 DAP.

for the formation of shoot apical meristem through regulation of the expression of *SHOOT MERISTMLESS* (*STM*) (Lynn *et al.* 1999, Fagard *et al.* 2000, Kidner and Martienssen 2005). BnGemb-55 is homologous to the *SCARECROW* (*SCR*) gene, which is known to be expressed in the quiescent center (QC) during embryogenesis and contributes to the maintenance of root apical meristem and radial pattern formation by modulating asymmetrical cell division to generate cortex and endodermis (Di Laurenzio *et al.* 1996, Sabatini *et al.* 2003). Isolation of ESTs corresponding to these genes indicates that androgenic embryos undergo similar controls to zygotic embryos to establish a fundamental body plan.

Several other ESTs similar to lipid transfer protein (BnGemb-11, -12, -14, -15), oleosin (BnGemb-34), *ABSCISIC ACID–INSENSITIVE3* (*ABI3*) (BnGemb-54), delta 9 desaturase (BnGemb-63), glutathione *S*-transferase (BnGemb-64) and glycine-rich protein (BnGemb-90) were also identified. All of these genes have been reported to be expressed in zygotic and/or somatic embryogenesis (Fernandez *et al.* 1991, Giraudat *et al.* 1992, Zou *et al.* 1995, Sterk *et al.* 1991, Vrinten *et al.* 1999, Slocombe *et al.* 1992, Galland *et al.* 2001, Magioli *et al.* 2001). These results indicate that the subtraction was performed successfully and our subtracted library is likely to contain unidentified genes acting in embryogenesis.

Quantitative RT-PCR analysis for several selected genes, which are related to the transcription factor and/or signal transduction, demonstrated that they are highly expressed in not only androgenesis but also zygotic embryogenesis; however, in some genes, their expression profiles between both types of embryogenesis were quantitatively inconsistent. We therefore cannot describe accurate causes of such phenomena; however, it is likely that this discrepancy is due to mRNAs used as zygotic embryos, which were isolated from ovules instead of embryos, and due to a partly different regulation mechanism between androgenesis and zygotic embryogenesis. In particular, different features between both types of embryogenesis are well known, for example, androgenic embryos have no desiccation tolerance and dormancy in comparison with zygotic embryos.

Although our results show that isolated genes are involved in androgenesis, isolation of ESTs is an key to

understanding the mechanism of androgenesis, and further analysis of individual genes is needed. We are carrying out functional analysis of isolated genes using homologous genes in *Arabidopsis*, which will provide more information to elucidate androgenesis and zygotic embryogenesis.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Science, Culture, Sports and Technology, Japan. R.T. is a recipient of the student support program of Grants-in-Aid for the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Literature Cited

Abe, M., T. Takahashi and Y. Komeda (2001) Identification of a cis-regulatory element for L1 layer-specific gene expression, which is targeted by an L1-specific homeodomain protein. Plant J. 26: 487–494.

Abe, M., H. Katsumata, Y. Komeda and T. Takahashi (2003) Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in *Arabidopsis*. Development 130: 635–643.

Ajisaka, H., Y. Kuginuki, M. Shiratori, K. Ishiguro, S. Enomoto and M. Hirai (1999) Mapping of loci affecting the cultural efficiency of microspore culture of *Brassica rapa* L. syn. *campestris* L. using DNA polymorphism. Breed. Sci. 49: 187–192.

Boutilier, K., R. Offringa, V.K. Sharma, H. Kieft, T. Ouellet, L. Zhang, J. Hattori, C. Liu and A.A.M. Lammeren (2002) Ectopic expression of *BABY BOOM* triggers a conversion from vegetative to embryonic growth. Plant Cell 14: 1737–1749.

Cloutier, S., M. Cappadocia and B.S. Landry (1995) Study of microspore culture responsiveness in oilseed rape (*Brassica napus* L.) by comparative mapping of a F_2 population and two microspore-derived populations. Theor. Appl. Genet. 91: 841–847.

DiLaurenzio, L., J. Wysocka-Diller, J.E. Malamy, L. Pysh, Y. Helariutta, G. Freshour, M.G. Hahn, K.A. Feldmann and P.N. Benfey (1996) The *SCARECROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsis* root. Cell 86: 423–433.

Fagard, M., S. Boutet, J.B. Morel, C. Bellini and H. Vaucheret (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc. Natl. Acad. Sci. 97: 11650–11654.

Fernandez, D.E., F.R. Turner and M.L. Crouch (1991) *In situ* localization of storage protein mRNAs in developing meristems of *Brassica napus* embryos. Development 111: 299–313.

Galland, R., B. Randoux, J. Vasseur and J.L. Hilbert (2001) A glutathione S-transferase cDNA identified by mRNA differential display is upregulated during somatic embryogenesis in *Cichorium*. Biochim. Biophys. Acta. 1522: 212–216.

Gifford, M.L., S. Dean and G.C. Ingram (2003) The Arabidopsis *ACR4* gene plays a role in cell layer organisation during ovule integument and sepal margin development. Development 130: 4249–4258.

Giraudat, J., B.M. Hauge, C. Valon, J. Smalle, F. Parcy and H.M. Goodman (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. Plant Cell 4: 1251–1261.

Goldberg, R.B., G. de Paiva and R. Yadegari (1994) Plant embryogenesis: zygote to seed. Science 266: 605–614.

Guha, S. and S.C. Maheshawari (1964) In vitro production of embryos from anthers in *Datura*. Nature 204: 497.

Ilic-Grubor, K., S.M. Attree and L.C. Fowke (1998) Comparative morphological study of zygotic and microspore-derived embryos of *Brassica napus* L. as revealed scanning electric microscopy. Ann. Bot. 82: 157–165.

Kidner, C.A. and R.A. Martienssen (2005) The role of *ARGONAUTE1* (*AGO1*) in meristem formation and identity. Dev. Biol. 280: 504–517.

Khush, G.S. and S.S. Virmani (1996) Haploids in plant breeding. *In*: Hain, S.M., S.K. Sopory and R.E. Veilleux (eds.) In Vitro Haploid Production in Higher Plants Vol. 1, Kluwer Academic Publishers, Dordrecht, pp. 11–33.

Lolle, S.J., A.Y. Cheung and I.M. Sussex (1992) *Fiddlehead*: an *Arabidopsis* mutant constitutively expressing an organ fusion program that involves interactions between epidermal cells. Dev. Biol. 152: 383–392.

Lolle, S.J. and A.Y. Cheung (1993) Promiscuous germination and growth of wildtype pollen from *Arabidopsis* and related species on the shoot of the *Arabidopsis* mutant, *fiddlehead*. Dev. Biol. 155: 250–258.

Lu, P., R. Porat, J.A. Nadeau and S.D. O'Neill (1996) Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes. Plant Cell 8: 2155–2168.

Lynn, K., A. Fernandez, M. Aida, J. Sedbrook, M. Tasaka, P. Masson and M.K. Barton (1999) The *PINHEAD/ZWILLE* gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the *ARGONAUTE1* gene, Development 126: 469–481.

Magioli, C., R.M. Barroco, C.A.B. Rocha, L.D.S. Fernandes, E. Mansur, G. Engler, M. Margis-Pinheiro and G.S. Martins (2001) Somatic embryo formation in *Arabidopsis* and eggplant is associated with expression of a glycine-rich protein gene (*Atgrp-5*). Plant Sci. 161: 559–567.

Malik, M.R., F. Wang, J.M. Dirpaul, N. Zhou, P.L. Polowick, A.M.R. Ferrie and J.E. Krochko (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol. 144: 134–154.

Nitta, T., Y. Takahata and N. Kaizuma (1997) Scanning electron microscopy of microspore embryogenesis in *Brassica* spp. Plant Cell Rep. 16: 406–410.

Pechan, P.M., D. Bartels, D.C.W. Brown and J. Schell (1991) Messenger-RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. Planta 184: 161–165.

Pruitt, R.E., J.P. Vielle-Calzada, S.E. Ploense, U. Grossniklaus and S.J. Lolle (2000) *FIDDLEHEAD*, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. Proc. Natl. Acad. Sci. USA 97: 1311–1316.

Sabatini, S., R. Heidstra, M. Wildwater and B. Scheres (2003) SCARECROW is involved in positioning the stem cell niche in the *Arabidopsis* root meristem. Genes Dev. 17: 354–358.

Slocombe, S.P., I. Cummins, R.P. Jarvis and D.J. Murphy (1992) Nucleotide sequence and temporal regulation of a seed-specific *Brassica napus* cDNA encoding a stearoyl-acyl carrier protein (ACP) desaturase. Plant Mol. Biol. 20: 151–155.

Stasolla, C., L. van Zyl, U. Egertsdotter, D. Craig, W. Liu and R.R. Sederoff (2003) The effects of polyethylene glycol on gene expression of developing white spruce somatic embryos. Plant Physiol. 131: 49–60.

Sterk, P., H. Booij, G.A. Schellekens, A. Van Kammen and S.C. De Vries (1991) Cell-specific expression of the carrot *EP2* lipid transfer protein gene. Plant Cell 3: 907–921.

Takahata, Y. and W.A. Keller (1991) High frequency embryogenesis and plant regeneration in isolated microspore culture of *Brassica oleracea* L. Plant Sci. 74: 235–242.

Takahata, Y., D.C.W. Brown and W.A. Keller (1991) Effect of donor plant age and inflorescence age on microspore culture of *Brassica napus* L. Euphytica 58: 51–55.

Takahata, Y. (1997) Microspore culture. *In*: Kalia, H.R. and S.K. Gupta (eds.) Recent Advances in Oilseed Brassicas, Kalyani Publishers, Ludhiana, pp. 162–181.

Tanaka, H., M. Watanabe, M. Sasabe, T. Hiroe, T. Tanaka, H. Tsukaya, M. Ikezaki, C. Machida and Y. Machida (2007) Novel receptor-like kinase ALE2 controls shoot development by specifying epidermis in *Arabidopsis*. Development 134: 1643–1652.

Telmer, C.A., W. Newcomb and D.H. Simmonds (1993) Microspore development in *Brassica napus* and the effect of high temperature on division *in vivo* and *in vitro*. Protoplasma 172: 154–165.

Tsuwamoto, R., H. Fukuoka and Y. Takahata (2007) Identification and characterization of genes expressed in early embryogenesis from microspores of *Brassica napus*. Planta 225: 641–652.

Vrinten, P.L., T. Nakamura and K.J. Kasha (1999) Characterization of cDNAs expressed in the early stages of microspore embryogenesis in barley (*Hordeum vulgare* L.) Plant Mol. Biol. 41: 455–463.

Watanabe, M., H. Tanaka, D. Watanabe, C. Machida and Y. Machida (2004) The ACR4 receptor-like kinase is required for surface formation of epidermis-related tissues in *Arabidopsis*. Plant J. 39: 298–308.

Xu, B.Y., W. Su, J.H. Liu, J.B. Wang and Z.Q. Jin (2007) Differentially expressed cDNAs at the early stage of banana ripening identified by suppression subtractive hybridization and cDNA microarray. Planta 226: 529–539.

Yephremov, A., E. Wisman, P. Huijser., K. Welleson and H. Saedler (1999) Characterization of the *FIDDLEHEAD* gene of *Arabidopsis* reveals a link between adhesion response and cell differentiation in the epidermis. Plant Cell 11: 2187–2201.

Zaki, M.A.M. and H.G. Dickinson (1991) Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex. Plant Reprod. 4: 48–55.

Zhang, F.L. and Y. Takahata (2001) Inheritance of microspore embryogenic ability in *Brassica* crops. Theor. Appl. Genet. 103: 254–258.

Zhang, F., S. Aoki and Y. Takahata (2003) RAPD markers linked to microspore embryogenic ability in *Brassica* crops. Euphytica 131: 207–213.

Zou, J., G.D. Abrams, D.L. Barton, D.C. Taylor, M.K. Pomeroy and S.R. Abrams (1995) Induction of lipid and oleosin biosynthesis by (+)-abscisic acid and its metabolites in microspore-derived embryos of *Brassica napus* L. cv Reston (biological responses in the presence of 8[prime]-hydroxyabscisic acid). Plant Physiol. 108: 563–571.