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Full Length Research Paper

Identification of differentially expressed genes from male and female flowers of kiwifruit

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Kiwifruit is one of the dioecious plant species, whereas most flowering plants are hermaphrodite. Sex chromosome-linked genes have been isolated mainly from the dioecious plant *Silene latifolia*. As an attempt to understand the molecular mechanisms of sex determination and differentiation in kiwifruit, we performed DEG (differentially expressed gene) screening using annealing control primer-based gene fishing method from male and female floral buds. As a result, 15 DEGs in total were isolated and then sequenced. RT-PCR analysis revealed that some of them were differentially expressed in either male or female floral buds. GenBank database searches revealed that 11 DEGs exhibited significant sequence similarity with known function of genes or ESTs of kiwifruit. Among them, kiwiDEG1 encodes partial open reading frame (ORF) for pectin methylesterase, which plays an important role in pollen development as a cell-wall modifying enzyme. It was abundantly expressed in male floral buds compared with those of female, suggesting its functional role in pollen development of kiwifruit. KiwiDEG9 encodes a full-length ORF for adenosine diphosphate (ADP)-ribosylation factor, which is a small guanosine-5'-triphosphate (GTP)-binding protein and plays an essential role in vesicle trafficking. Its expression level was higher in male floral buds than in female. The isolated kiwiDEGs will be useful resources to better understand the molecular events of sex differentiation in kiwifruit.

Key words: *Actinidia,* differentially expressed genes, dioecious plant, male flowers, female flowers, kiwifruit, pectin methylesterase, ADP-ribosylation factor.

INTRODUCTION

Most species of flowering plants are hermaphrodite and develop bisexual flowers. On the other hand, many dioecious species with separate male and female individuals are also found in approximately 5% of flowering plants. They are found in various taxa ranging from mosses and gymnosperms, to monocotyledonous and dicotyledonous angiosperms (Charlesworth, 2002; Vyskot and Hobza, 2004). The dioecy is one of the mechanisms to promote genetic variability and genetic exchange through cross-pollination (allogamy), the consequences of which are advantageous to the long-term survival and adaptation of a species (Dellaporta and Calderon-Urrea, 1993). As in animals, sex determination in most dioecious plants is generally based on X-to-autosome ratio (as in

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Drosophila and *Caenorhabditis elegans*), X/Y system witha dominant (active) Y chromosome (as in mammals), or rarely ZW system with heterogametic female (as in birds and butterflies). Heteromorphic or homomorphic sex chromosomes have been found in some dioecious plants (Dellaporta and Calderon-Urrea, 1993; Vyskot and Hobza, 2004). Genetic and molecular mechanisms controlling sex determination in animal system have been studied extensively, while those mechanisms in plants are largely unknown, although sex inheritance and sex chromosomes in plants are strikingly similar to those in animals. Sex determination in plants occurs at various developmental stages during flower organogenesis or reproductive organ differentiation depending on plant species. Previous studies show that plant sex determination genes act downstream or independently of the ABC homeotic functional network (Dellaporta and Calderon-Urrea, 1993; Negrutiu et al., 2001).

Since the first Y chromosome-linked gene, SlY1 encoding WD40 repeat protein, was isolated from a dioecious plant, *Silene latifolia* (Delichère et al., 1999), several sex chromosome-linked genes have been isolated through various molecular approaches. However, many of them were housekeeping genes such as protein kinase and fructose-2, 6-bisphosphatase, indicating no close relation between the gene products and sex determination. APETALA3-like MADS box gene, mapped on the Y chromosome of *S. latifolia*, showed the petal- and stamen-specific expression pattern (Matsunaga, 2006). Recently, Zluvova et al. (2006) suggested that *Arabidopsis thaliana* homologs of shootmeristemless (STM) and cup shaped cotyledon 1 (CUC1) and CUC2 genes in *S. latifolia* could be strong candidates in the involvement of sex determination.

All members of known species in the genus *Actinidia*, which encompass about 60 species, are functionally dioecious. Female plants possess pistillate flowers that are superficially hermaphroditic but produce only empty pollens due to degeneration after tetrad release, whereas male plants bear staminate flowers that are unisexual with numerous stamens surrounding a rudimentary pistil whose growth is arrested before style elongation or ovule initiation (Ferguson, 1984). In the dioecious genus, *Actinia*, sex-determining genes are postulated to be mapped on a pair of chromosomes which, although cytologically indistinguishable (homomorphic), function like an X/Y system with male heterogamety (Harvery et al., 1997; Testolin et al., 2004). In the X/Y system, there are at least two linked and dominant Y chromosome genes, an ovary suppressor (SuF) and an anther development promoting gene (M) (Charlesworth, 2002). The accumulating genetic evidence of sex determination in kiwifruit suggests that an active Y system functions in *Actinidia*, with maleness determined by two linked and dominant loci (Harvey et al., 1997; Testolin et al., 2004). Recently, using 644 microsatellite markers, Fraser et al. (2009) created gene-rich genetic linkage maps that define the 29 linkage groups of the haploid genome of *Actinidia chinensis* and revealed the position and recombination extent of the sex-determining locus of the kiwifruit species. According to the data, the flower-sex phenotype mapped to a single linkage group, in a sub-telomeric region of an autosome. The region contained markers of expressed genes, some of known and unknown function.

Kiwifruit is an economically important crop plant grown in many different regions of the world. The underlying molecular and genetic mechanisms of sex determination and sex differentiation in kiwifruit are largely unknown despite its importance in agricultural application. In this study, as an attempt to understand molecular mechanisms of sex determination and sex differentiation in kiwifruit, we performed DEG (differentially expressed gene) screening for male and female floral buds, using annealing control primer-based gene fishing method (Kim et al., 2004). We identified 15 DEGs in total, and further characterized two DEGs: kiwiDEG1 encoding pectin methylesterase that is known to be involved in cell wall modification (Micheli, 2001; Pelloux et al., 2007) and kiwiDEG9 encoding ADP-ribosylation factor that plays an essential role in vesicle trafficking (Memon, 2004).

MATERIALS AND METHODS

Plant materials

Two kiwifruit cultivars, *A. chinensis* cv. 'Songongu' (as a male) and *A. chinensis* cv. 'Jecy Gold' (as a female) were used in this experiment. Floral buds and fully-opened flowers were harvested from male or female plants in the middle of May grown in the experimental kiwifruit plantation located at the Agricultural Research Center for Climate Change, Cheju, Korea. Harvested flowers were quickly frozen in liquid nitrogen for the RNA isolation and stored at -70℃ until use.

Total RNA isolation from male and female flowers of kiwifruit

Total RNA was isolated from the floral buds and fully-opened flowers using Plant RNeasy mini kit (Qiagen, Germany) according to the manufacturer's guide. The isolated total RNA was first treated with RNase-free DNase I (Promega, Madison, WI, USA) to remove any contaminated genomic DNA and further purified with an RNeasy mini kit (Qiagen). The purified total RNA was examined with UV/VIS spectrophotometer and agarose gel electrophoresis.

First-strand cDNA synthesis

The purified total RNAs were used for the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42℃ in a final reaction volume of 20 µl containing 3 µg of total RNA, 4 µl of 5× reaction buffer (Promega), 5 µl of dNTPs (each 2 mM), 2 µl of 10 µM dT-ACP1 (5'-CTGTGAATGCTGCGACTACGATIIIIIT(18)-3'), 0.5 µl of RNasin® RNase Inhibitor (40 U/µl; Promega), and 1 µl of Moloney-MurineLeukemia Virus (M-MLV) reverse transcriptase (200 U/µl; Promega). First-strand cDNAs were diluted by adding 80 µl of ultra-purified water for the GeneFishing™ PCR (Seegene, Seoul, Korea;

Table 1. Primer list used for RT-PCR in this experiment.

Gene	PCR primers (5' to 3')		Amplifying size (bp)	Cycle no.[a]
	Forward	Reverse		
KiwiDEG1	ACAAGAACGAGGTTCTCAAATAACT	ATTACAATTGATTGTTTCTCTCAGG	204	35
KiwiDEG2	TGAAGAAATAGAAGAAACACCAGAC	GTTTACAAAAATCCACTGTTTTACG	276	35
KiwiDEG3	GATAGGCTCGGTGACATTTTCTTTT	AGAAACCCACGAAAGTTGTAACATA	200	27
KiwiDEG4	GGATATGGAGTTCTAAACAAGGTCT	TTACATACAAAACCCAAAGAGGATA	363	35
KiwiDEG5	CTTGACTCTGCCTAGGGTTAATTG	AACAACTGCTTGAATCTTATTCACT	208	28
KiwiDEG6	AAGCCAAAGAAGAAGAGAAAGAAG	TCATGCTAGGTCGATTACATAAGAT	207	36
KiwiDEG7	ATACAAGTTCTCAAACCAAAAGCTA	AGACTTTTCTCTACCATCCATCTTT	213	35
KiwiDEG8	CTATTGATGGTGTGAAGAACACACT	CTAAATAAACAGCAACCTCACTAGG	218	40
KiwiDEG9	CCAATGCAATGAATGCTGCGGAAAT	TCTGTGTCTCCATGAATAGCCAAGT	258	35
KiwiDEG10	GTAGGTAAGCCTGTCGATCAAACC	CGCCATCTAAAAACGTAGTAATAAT	253	35
KiwiDEG11	CTCTTATTAATGGGCAACAAACTAC	CATCGAACTATTAACTAGCCTTCAA	266	37
KiwiDEG12	CAAGAACATTGAGAAAAGCCTAGTA	GACTTCTTCCTCATCTCATCAAGTA	229	37
KiwiDEG13	CTCTCTCTCATCTATCTATGGTTGG	CAATCTCTCTAAACTTCTGAGTTGC	390	37
KiwiDEG14	CGCTTCTCTTCTTCTTCTTCTCCGC	TATCAGGCCATTTCCCGGCTTCTCT	183	35
KiwiDEG15	CTCTACAGATTTGTTGCAGTATTGA	GAACCAGAGAGACAAATTACAGAGA	201	40
Kiwi Actin1	TCCATGAGACTACCTACAATTCTATC	AACTAAATAGCAAAGGAGAAACTCAC	354	35

[a]RT product (each 75 ng) synthesized using total RNA was used for PCR amplification.

URL http://www.seegene.com), and stored at -20°C until use.

Reverse transcription-mediated polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized using 5 µg of total RNA with M-MLV reverse transcriptase (Invitrogen, Carlsbad, USA) and used as a template for PCR amplification. The conditions for PCR amplification were as follows: 96°C, 5 min for initial denaturation followed by 94°C for 15 s, 50°C for 30 s, and 72°C for 1 min (total 27 - 40 cycles) with 5 min of final extension at 72°C. PCR primer sets used in this experiment are listed in Table 1. The amplified PCR products were separated on a 1.3% agarose gel. Transcripts for actin gene of kiwifruit were amplified as a positive control for PCR.

ACP-based GeneFishing™ PCR

Differentially expressed genes were screened by ACP-based PCR method (Kim et al., 2004) using the GeneFishing™ DEG kits (Seegene). Briefly, second-strand cDNA synthesis was conducted at 50°C during one cycle of first-stage PCR in a final reaction volume of 20 µl containing 3 - 5 µl (about 50 ng) of diluted first-strand cDNA, 1 µl of dT-ACP2 (10 µM), 1 µl of 10 µM arbitrary ACP, and 10 µl of 2× Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After completion of the second-strand DNA synthesis, the second-stage PCR amplification was performed through 40 cycles of 94°C for 40 s, 65°C for 40 s and 72°C for 40 s, followed by 5 min of final extension at 72°C. The amplified PCR products were separated in a 2% agarose gel and stained with ethidium bromide.

Sequence analysis

The differentially expressed bands were re-amplified and extracted from the gel by using the GENCLEAN®II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly sequenced with ABI PRISM®3100-AvantGenetic Analyzer (Applied Biosystems, Foster City, CA, USA) using universal primer (5'-TCTACCAGGCATTCGCTTCAT-3'). Sequences were analyzed with the ExPASy Molecular Biology Server (URL http://kr.expasy.org), the BLAST program (Altschul et al., 1990), and BOXSHADE (version 3.2; URL http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis was performed with the Molecular Evolutionary Genetics Analysis (MEGA) software (version 4.0.; Tamura et al., 2007), and a phylogenetic tree was inferred by the Neighbor-joining tree method. The resulting tree topology was evaluated in bootstrap analyses based on 1,000 replicates.

RESULTS AND DISCUSSION

Screening of the differentially expressed genes from male and female flowers of kiwifruit

In order to identify genes that are specifically or abundantly expressed in male or female flowers of a dioecious plant kiwifruit, we performed DEG screening using annealing control primer (ACP)-based gene fishing method (Kim et al., 2004). Through the DEG screening, we isolated 15 putative DEGs in total (kiwiDEGs hereafter; Figure 1) and determined nucleotide sequences of the PCR products (data not shown).

Table 2. BlastX search results against NCBI nr database using kiwiDEGs from male and female flowers as queries. Threshold for bits score and E-value was 50 and e-5, respectively.

Query	Gene(s) producing significant alignment[a]	Score/E-value	Identities
KiwiDEG1 (527 bp)	Pollen development related protein [*Brassica rapa* subsp. chinensis] VANGUARD 1, pectin methylesterase [*Arabidopsis thaliana*]	67.0/3e-15 65.9/2e-13	60%(87%)[b] 60%(82%)
KiwiDEG2 (410 bp)	No significant similarity found		
KiwiDEG3 (251 bp)	Kelch domain-containing protein, putative [*Ricinus communis*]	54.3/ 4e-06	80%(83%)
KiwiDEG4 (412 bp)	Delta12-fatty acid desaturase [*Jatropha curcas*] Mocrosomal omega-6 fatty acid desaturase [*Glycine max*]	85.9/ 3e-33 84.0/ 4e-33	92%(97%) 90%(97%)
KiwiDEG5 (315 bp)	No significant similarity found		
KiwiDEG6 (492 bp)	Asr2, abscisic stress-ripening protein 2 [*Solanum lycopersicum*]	52.0/6e-07	80%(90%)
KiwiDEG7 (471 bp)	Cinnamoyl-CoA reductase [*Ricinus communis*]	77.8/5e-25	85%(90%)
KiwiDEG8 (508 bp)	UNE15 (unfertilized embryo sac 15) [*Arabidopsis thaliana*]	46.6/ 6e-11	77%(92%)
KiwiDEG9 (839 bp)	ADP-ribosylation factor [*Hyacinthus orientalis*]	94.7/1e-62	100%(100%)
KiwiDEG10 (405 bp)	Putative CONSTANS interacting protein 2b [*Capsicum chinense*]	67.4/5e-10	71%(84%)
KiwiDEG11 (457 bp)	No significant similarity found		
KiwiDEG12 (552 bp)	Chalcone synthase [*Vitis rotundifolia*]	85.1/3e-47	95%(95%)
KiwiDEG13 (640 bp)	No significant similarity found		
KiwiDEG14 (254 bp)	Hypothetical protein [*Vitis vinifera*] Monosaccharide transporter [*Populus tremula* x *Populus tremuloides*]	82.0/ 2e-14 71.6/2e-11	64%(76%) 74%(89%)
KiwiDEG15 (428 bp)	No significant similarity found		

[a]Representative gene(s) showing the highest sequence homology with each kiwiDEG.
[b]Values in parenthesis indicate % of similarity.

GenBank nr database searches using BlastX algorithm were performed to deduce the potential functions of the kiwiDEGs (Table 2). Ten DEGs showed homology to sequences of known function. However, kiwiDEG3 and 14 were not long enough (approximately 250 bp) to be informative sequences, although they shared sequence homology with previously reported genes. Significant similarities were not found for the five remaining DEGs (kiwiDEG2, 5, 11, 13 and 15).

Crowhurst et al. (2008) produced a collection of 132,577 expressed sequence tags (ESTs) from various tissue types of mainly four *Actinidia* species, that is, *A. chinensis, Actinidia deliciosa, Actinidia arguta* and *Actinidia eriantha*. MegaBlast searches against the kiwifruit EST database in the GenBank revealed that 9 kiwiDEGs showed high sequence identity (more than 96%) with the ESTs of the database, indicating that each DEG could be the same gene product as the corresponding ESTs or simple sequence variation among kiwifruit cultivars. KiwiDEG4 and 8 shared 90 and 89% sequence identity with EST clones FG423355 (encoding putative fatty acid desaturase) and FG429611 (encoding unfertilized embryo sac 15 protein), respectively. Considering the homology value, kiwiDEG4 and 8 are more likely to belong to the same gene family as the corresponding ESTs rather than being either the same gene product or sequence variation. In contrast, the remaining 4 kiwiDEGs (kiwiDEG1, 2, 3 and 14) have no

Table 3. MegaBlast search results against kiwifruit EST database of NCBI using kiwiDEGs from male and female flowers as queries.

Query	GenBank acc. no.	Identity (%)	cDNA library	Species
KiwiDEG1	No significant similarity			
KiwiDEG2	No significant similarity			
KiwiDEG3	No significant similarity			
KiwiDEG4	FG423355	90	Young fruit	*A. eriantha*
KiwiDEG5	FG430475	97	Petal	*A. deliciosa*
KiwiDEG6	FG431070	98	Petal	*A. deliciosa*
KiwiDEG7	FG511455	98	Young leaf	*A. chinensis*
KiwiDEG8	FG429611	89	Petal	*A. deliciosa*
KiwiDEG9	FG508847	96	Petal	*A. eriantha*
KiwiDEG10	FG489478	97	Active meristems	*A. chinensis*
KiwiDEG11	FG427624	99	Petal	*A. deliciosa*
KiwiDEG12	FG528287	97	Young fruit	*A. chinensis*
KiwiDEG13	FG412031	97	Developing shoot buds	*A. deliciosa*
KiwiDEG14	No significant similarity			
KiwiDEG15	FG489974	96	Active meristems	*A. chinensis*

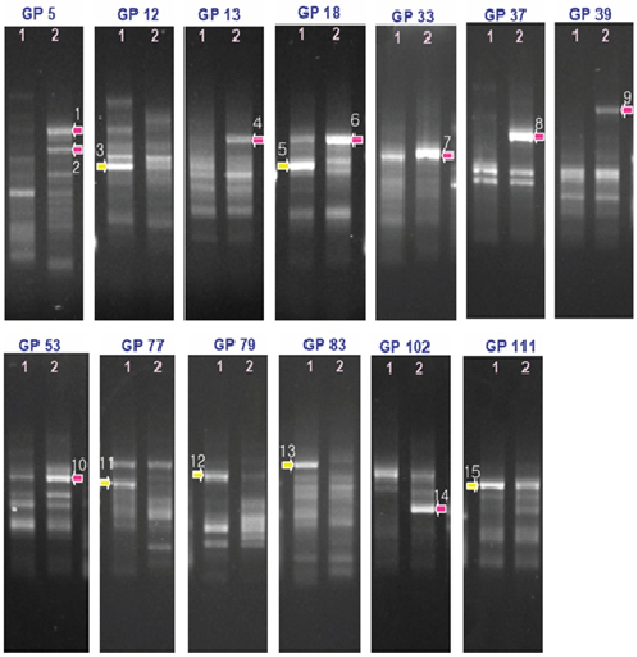

Figure 1. Gel images of DEG screening by ACP-based gene fishing system. Total 120 primer sets (GP numbers) were used for the screening of DEGs. Arrows with number indicate genes differentially expressed in female or male floral buds. Lane 1, female floral buds and lane 2, male floral buds.

significant similarity to the ESTs (Table 3).

In this study, we further characterize two kiwiDEGs; kiwiDEG1 showing sequence homology with pectin methylesterase genes and male flower-specific expression pattern, and kiwiDEG9 encoding a full-length ADP-ribosylation factor.

KiwiDEG1 encodes partial pectin methylesterase

Blast search against GenBank nr database using kiwi DEG1 as a query revealed that kiwiDEG1 shared sequence homology with members of plant pectin methylesterase (PME) gene family (Table 2). Up to now, genes encoding PME have not been isolated from kiwifruits yet. Pectins are major constituent of the primary cell wall in higher plants and primarily composed of polygalacturonic acid (PGA). Pectins are highly methylesterified in the golgi body, and are secreted into the cell wall and subsequently modified by PME (EC3.1.1.11), which catalyzes the demethylesterification of homogalacturonan to release acidic pectins and methanol. PMEs have been known to be implicated in various biological processes through modification of the cell wall, such as plant reproduction, vegetative growth, cellular differentiation, and plant-pathogen interactions (Micheli, 2001; Pelloux et al., 2007).

Figure 2 illustrates multiple sequence alignment of kiwiDEG1 with previously isolated plant PMEs. The alignment revealed that kiwiDEG1 encodes partial PME. According to the comprehensive analysis using genome sequence information, PMEs can be classified into two groups. PMEs belonging to group I contain signal peptide (SP), transmembrane domain (TM) and PME domain. In contrast, group II PMEs have additional domains between SP/TM and PME domain, that is, PME inhibitor (PMEI) domain and a processing motif (PM). PMEI domain is processed at the PM site during the protein maturation (Micheli, 2001; Pelloux et al., 2007). PME domain contains five highly conserved motifs (44_GxYxE, 113_QAVAL, 135_QDTL, 157_DFIFG and 223_LGRPW; based on the carrot PME numbering). A highly conserved and catalytically important arginine

Figure 2. Multiple sequence alignment of kiwiDEG1 and previously reported plant pectin methylesterases. The sequence alignment and conserved residues were displayed using the CLUSTALW and the BOXSHADE programs. Identical amino acid sequences are highlighted in black, and similar sequences in grey. Asterisk indicates catalytically important arginine (R) residue. The pectin methylesterase sequences used in this alignment are as follows: kiwiDEG1, kiwifruit (in this study, GenBank Acc. No. GU327819); BP19, *Brassica napus* (X56195); B8 and B16, tomato (X74638 and X74639); PPE1, *Petunia inflata* (L27101); ZmC5*, Zea mays* (Y13285); P65, alfalfa (U28148); VGD1, *Arabidopsis thaliana* (At2g47040).

(R) residue was found in the LGRPW motif from the sequence alignment of kiwiDEG1 with plant PMEs (Figure 2).

KiwiDEG9 encodes ADP-ribosylation factor

Blast search against GenBank nr database using kiwiDEG9 as a query revealed that kiwiDEG9 shared strong sequence identity with ADP-ribosylation factors (ARFs), small GTP-binding proteins that are members of Ras-like GTPase superfamily. An EST sharing 96% sequence identity with kiwiDEG9 was identified from the petal cDNA library of *A. eriantha* (Table 3). Figure 3 illustrates multiple sequence alignment of kiwiDEG9 with ARFs isolated from plants, human and yeast. The alignment revealed that kiwiDEG9 encodes a full-length ARF protein composed of 181 amino acid residues and also showed that ARF proteins consist of a protein family highly conserved across biological kingdoms.

The ARF protein was first identified in mammalian cells as a cofactor required for cholera toxin-mediated ADP-ribosylation of adenylate cyclase $G\alpha_s$ subunit (Kahn and Gilman, 1984). ARF-type GTP-binding proteins play an essential role during intracellular membrane trafficking of cytosolic coat proteins (ex, COPI and clathrin coats) through the secretory pathway in eukaryotic cells. They operate as molecular switches that cycle between active GTP-bound and inactive GDP-bound states (Vernoud et al., 2003; Memon, 2004). The cycling between active and inactive conformational states occurs through association of the GTP-binding protein with accessory proteins, guanine nucleotide exchange factors (GEFs) that convert inactive/ cytosolic GDP-ARF to active/ membrane-associated GTP-ARF and GTPase-activating proteins (GAPs) hydrolyzing bound GTP to reform GDP-ARF (Memon, 2004). A putative ARF protein encoded by kiwiDEG9 shares many molecular features with other typical ARFs (Figure 3). These include four conserved GTP-binding motifs (denoted with I - IV), GAP-binding region (Leu-37 to Ile-49), two regions binding conserved Sec7 domain of GEFs (Gly-40 to Thr-55, Gly-70 to His-80), a potential myristoylation site at Gly-2 that is likely involved in the protein binding to the membrane, and domain that activates phospholipase D (Tyr-35 to Ser-94; Figure 3).

ARF-type GTP-binding proteins can be classified into two groups, ARFs and ARLs (ARF-like proteins), depending on sequence relatedness and their functional activity. ARLs could not act as cofactors to activate cholera toxin A or could not rescue the $arf1^{-}$ $arf2^{-}$ double mutant of yeast (Clark et al., 1993; Vernoud et al., 2003; Memon, 2004). A phylogenetic tree was generated using homologues from Arabidopsis (total 19 genes; Vernoud et al., 2003; Gebbie et al., 2005), human and yeast (Figure 4). KiwiDEG9 was closely clustered with ARF group including Arabidopsis ARF1 (At2g47170), which was known to play a role in the intracellular trafficking of cargo

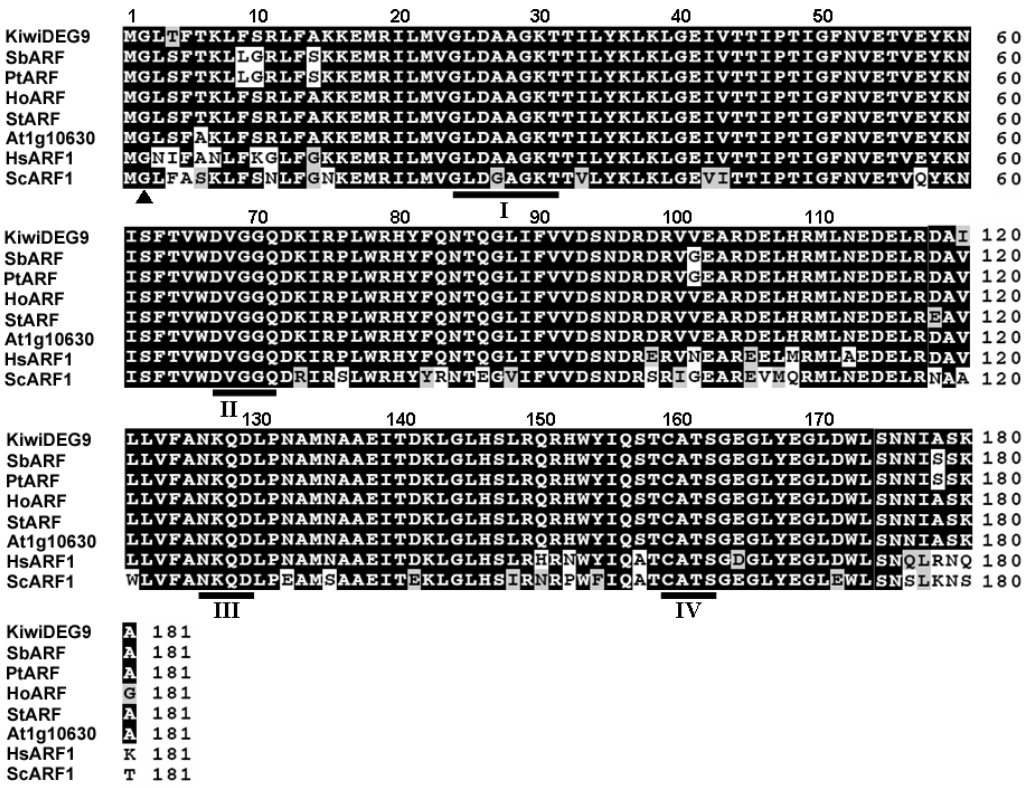

Figure 3. Multiple sequence alignment of KiwiDEG9 and previously reported ADP-ribosylation factors. The sequence alignment and conserved residues were displayed using the CLUSTALW and the BOXSHADE programs. Identical amino acid sequences are highlighted in black and similar sequences in grey. Arrow head indicates Gly-2 myristoylation site. Four conserved motifs (I - IV) responsible for GTP binding were underlined. The ADP-ribosylation factor sequences used in this alignment are as follows: KiwiDEG9, kiwifruit (in this study, GenBank Acc. No. GU327818); SbARF, *Salix bakko* (AB003377); PtARF, *Populus trichocarpa* (EEF01158); HoARF, *Hyacinthus orientalis* (AAT08648); StARF, *Solanum tuberosum* (ABB16972); At1g10630, *Arabidopsis thaliana*; HsARF1, human (P32889); ScARF1, yeast (P11076).

proteins (Lee et al., 2002).

Expression pattern of kiwiDEGs in male and female flowers of kiwifruit

RT-PCR was performed to compare expression patterns of kiwiDEGs in floral buds from male and female plants (Figure 5A). Several DEGs showed enhanced expression pattern in either male (kiwiDEG1, 4, 7, 8, 9 and 14) or female (kiwiDEG3, 11, 12, 13 and 15) floral buds. However, the rest (kiwiDEG2, 5, 6 and 10) had no difference in the expression level, even though they showed differential expression pattern from the DEG screening (Figure 1).

KiwiDEG1 encoding partial PME was specifically expressed in the floral buds from male plant (Figure 5A). However, kiwiDEG1 transcripts were also weakly detected in the floral buds from female plant, but not in the opened flowers of the plant, when the PCR cycle numbers were increased (Figure 5B). In case of male plant, kiwiDEG1 was almost equally expressed in the floral buds and the opened flowers (Figure 5B). A PME gene showing male flower-specific expression was isolated from a dioecious willow (*Salix gilgiana* Seemen). However, the gene was detected in the genomes of both male and female plant (Futamara et al., 2000).

The increasing amount of data shows that PMEs likely play an essential role in the sexual reproduction of plants. Pollen grains- or pollen tube-specific PME genes have been isolated from a variety of monoecious and dioecious plant species (Albani et al., 1991; Mu et al., 1994; Wakeley et al., 1998; Futamura et al., 2000; Bosch et al., 2005; Tian et al., 2006), indicating that plant PMEs may be involved in the processes of pollen formation and/or pollen tube development. Recent molecular genetic approaches using Arabidopsis mutant resources produced more direct evidences for the functional roles of PMEs in the sexual reproduction of plants. Arabidopsis loss-of-function mutants for the PME genes that were specifically or highly expressed in pollen grain and/or pollen tube displayed abnormality

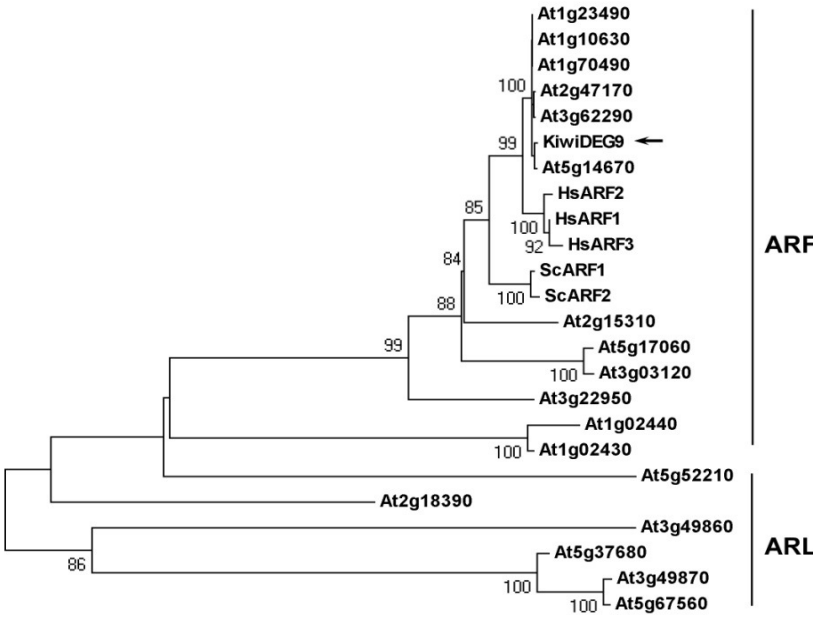

Figure 4. A phylogenetic tree of kiwiDEG9 and ARF-type GTP-binding proteins from Arabidopsis, human and yeast. KiwiDEG9 was marked with an arrow. A Neighbor-joining tree was generated using MEGA4 software. This bootstrap consensus tree was based on 1,000 replicates. Bootstrap values were shown on the nodes. The values of less than 80% (of total 1,000 replicates) were omitted to simplify the tree.

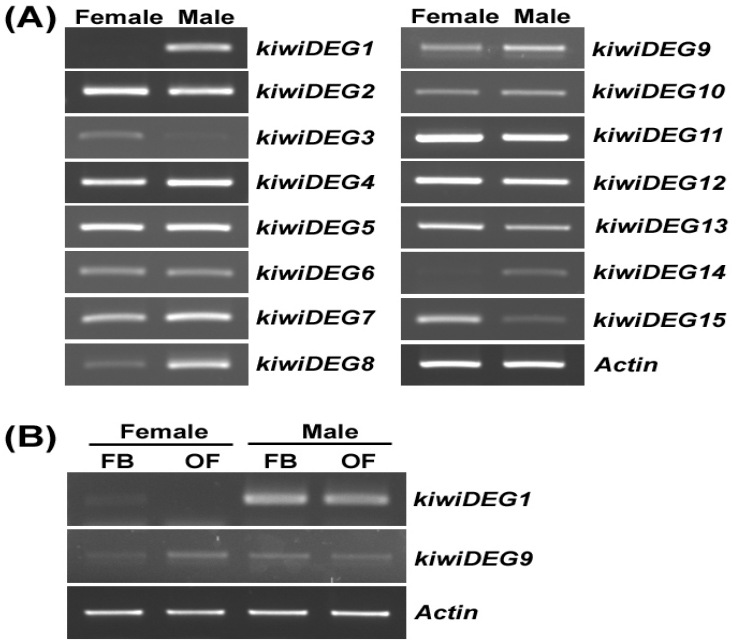

Figure 5. Expression patterns of kiwiDEGs in male and female flowers of kiwifruit. Total RNA was isolated from floral buds and fully opened flowers and used for RT-PCR analysis. Actin (GenBank acc. no. EF063572; *actin1* gene from *Actinidia deliciosa*) was used as an internal control for PCR. (A) Expression patterns of kiwiDEGs in male and female floral buds; (B) Expression pattern of kiwiDEG1 and kiwiDEG9 in floral buds (FB) and fully opened flowers (OF) of male and female plants. PCR cycle number was increased to detect kiwiDEG1 expression in the female floral buds as compared to (A).

in separation of pollen tetrads (Francis et al., 2006) or unstable and retarded pollen tube growth (Jiang et al., 2005; Tian et al., 2006). KiwiDEG1 expression disappeared in the opened flowers after weak detection in the floral buds of female plant (Figure 5B). This expression pattern of kiwiDEG1 is likely related to the programmed cell death occurring in microspores after the release from pollen tetrads in the anthers of *A. deliciosa* female flowers (Coimbra et al., 2004). Taken altogether, kiwiDEG1 encoding PME might be implicated in pollen development, as studied in pollen-specific PME genes from diverse plant species (Jiang et al., 2005; Francis et al., 2006; Tian et al., 2006), although it is not linked to sex-determining chromosomes in a dioecious plant kiwifruit (Fraser et al., 2009). This was further supported by the genomic PCR data that kiwiDEG1 was detected in both male and female plants (data not shown).

KiwiDEG4 shared high sequence identity with fatty acid desaturases (FADs) (Table 2). Fatty acids are the primary components of membrane phospholipids and storage triacylglycerols in all organisms including plants. C16:0- and C18:1-fatty acids that are synthesized in the stroma of plastids are further desaturated through the action of FADs (designated from FAD2 to FAD8) localized in either chloroplast or endoplasmic reticulum membranes (Somerville et al., 2000). Linolenic acid, a trienoic fatty acid, also serves as the precursor for the biosynthesis of jasmonic acid, which plays essential roles in defense responses and diverse developmental processes including pollen maturation (Park et al., 2002; Cheong and Choi, 2007). Among five different genes encoding FAD2 isolated from cotton, *FAD2-1* gene was specifically expressed in both developing flower buds and seeds (Zhang et al., 2009). Expression level of kiwiDEG4 encoding a putative FAD was higher in the male floral buds than in the female (Figure 5A). The increased expression of kiwiDEG4 in the male floral buds might be related to demands for considerable lipid biosynthesis to support male reproductive development in kiwifruit.

KiwiDEG8 showed much higher expression level in the male floral buds than in the female (Figure 5A) and shared the highest sequence similarity to *UNE15* (*unfertilized embryo sac 15*) gene from Arabidopsis (Table 2), which encodes a LEA (late embryogenesis abundant) domain-containing protein (At4g13560). *UNE15* gene was highly expressed in floral buds and flowers of Arabidopsis compared to vegetative tissues such as root, stem and silique (Hundertmark and Hincha, 2008) and its protein product was also identified from mature pollen proteome analysis of Arabidopsis (Noir et al., 2005). A mutation in Arabidopsis *UNE15* gene resulted in the fertilization failure probably due to abnormal pollen tube growth (Pagnussat et al., 2005). Based on these previous reports, kiwiDEG8 is likely to play a role during sex development or differentiation in kiwifruit.

Expression level of kiwiDEG9 was higher in the male floral buds than in the female counterparts (Figure 5A). In the female plant, it was highly expressed in the opened flowers as compared with the floral buds, whereas its expression level in the male plant was similar between the floral buds and the opened flowers (Figure 5B). An EST clone corresponding to kiwiDEG9 was isolated only from the petal cDNA library of *A. deliciosa* (Table 3). A cDNA clone encoding ARF was isolated from a dioecious woody plant, Japanese willow (*Salix bakko* Kimura). Its expression was higher in male flowers than in female flowers and in the vegetative tissues of both sexes (Futamura et al., 1998). A DEG encoding ARF-type GTPase was also abundantly expressed in the flower buds of a terrestrial orchid species, *Calanthe discolor* (Park et al., 2010). Mem-brane trafficking is fundamental to tip growing cells such as pollen tubes and root hairs, to supply lipid and cell wall materials to the new plasma membrane and cell wall that extends the apex of the cell (Campanoni and Blatt, 2007). Arabidopsis *titan* mutant defective in an ARL gene (At2g18390) exhibited dramatic alterations in both embryo and endosperm development, which were caused by abnormality in mitosis and cell cycle control during seed development (McElver et al., 2000). Therefore, kiwiDEG9 encoding ARF-type GTPase might play a functional role during flower development of kiwifruit.

In summary, we isolated several genes highly expressed in either male or female flowers of kiwifruit by the DEG screening using ACP-based PCR method. Among them, kiwiDEG1 was a partial clone encoding PME, a cell wall-modifying enzyme, and highly expressed in the male floral buds, indicating that it might play a role in pollen development. KiwiDEG9 was a full-length clone encoding ARF-type small GTP-binding protein, playing an essential role in vesicle trafficking, and also showed higher expression in the male flowers than in the female flowers. The isolated kiwiDEGs will be useful resources to better understand molecular events of sex differentiation in kiwifruit.

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Abbreviations

DEG, Differentially expressed gene; **ORF,** open reading frame; **STM,** shootmeristemless; **CUC1,** cup shaped cotyledon 1; **RT-PCR,** reverse transcription-mediated polymerase chain reaction; **ESTs,** expressed sequence tags; **PME,** pectin methylesterase; **PGA,** polygalacturonic acid; **SP,** signal peptide; **TM,** transmembrane domain; **PMEI,** pectin methylesterase inhibitor; **PM,** processing motif; **ARFs,** adenosine diphosphate-ribosylation factors; **ARL,** ARF-like proteins; **GEFs,** guanine nucleotide exchange factors; **FADs,** fatty acid desaturases; **LEA,** late embryogenesis abundant.

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