Analysis of differential gene expression during floral bud abortion in radish (*Raphanus sativus* L.)

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ABSTRACT. Radish floral bud abortion (FBA) is an adverse biological phenomenon that occurs during reproduction. Although FBA occurs frequently, its mechanism remains unknown. To elucidate the molecular mechanism underlying FBA, we detected gene expression differences between aborted and normal buds of radish using cDNA-amplified fragment length polymorphism (AFLP) and real-time polymerase chain reaction (real-time PCR). A total of 221 differentially expressed transcript-derived fragments (TDFs) were detected by 256 cDNA-AFLP primer combinations, of which 114 were upregulated and 107 were downregulated in the aborted buds. A total of 54 TDFs were cloned and sequenced. A BLAST search revealed that all TDFs have homologous sequences and 29 of these corresponded to known genes, whose functions were mainly related to metabolism, stimulus response, transcriptional regulation, and transportation. Expressions of 6 TDFs with different functions were further analyzed by real-time PCR yielding expression profiling results consistent with the cDNA-AFLP analysis. Our results indicated that radish FBA is related to abnormalities in

various physiological and biochemical plant processes.

Key words: Radish; Floral bud abortion; Gene expression; cDNA-AFLP; Real-time PCR

INTRODUCTION

Radish (*Raphanus sativus* L.) is a native Chinese vegetable belonging to the Cruciferae family. Its reproductive life cycle involves floral bud differentiation, blossoming, and silique bearing after vernalization. Radish is currently cultivated worldwide; small-rooted radishes are mainly cultivated in Europe and in the United States, while large-rooted radishes are cultivated in Asian countries, particularly in China, Japan, and South Korea. Radish can grow in all 4 seasons, but is most commonly cultivated in Autumn in most areas. Floral bud abortion (FBA), also known as “aborted bud”, is an adverse biological phenomenon for radish reproduction, which occurs frequently during radish breeding practices and in the creation of radish germplasm resources. During FBA, the floral bud first stops growing and then gradually turns from green to yellow, until it finally dries off completely. Plants exhibiting FBA are often eliminated during artificial breeding practices, even if they are otherwise productive, which is ultimately harmful for the selection of new radish cultivars.

FBA is a widespread phenomenon in plant reproduction and evolution, having been observed in *Cucurbita texana* (Krupnick et al., 2000), dwarf bean (Mouhouche et al., 1998), cowpea (Ahmed and Hall, 1993), rape (Bosac et al., 1994), Chinese cabbage (Zhang et al., 2010), radish (Liu et al., 2008; Jia and Zhang, 2008), and the model plant *Arabidopsis thaliana* (Warner and Erwin, 2005). FBA is most commonly thought to result from abiotic stress. Exogenous ethylene can induce *C. texana* to produce more female flowers and experience greater bud abortion in late developmental stages (Krupnick et al., 2000). Heat stress during vegetative and early reproductive stages induces FBA in the cowpea (Ahmed and Hall, 1993). Drought treatment increases the bud abortion rate of dwarf beans from 50 to 70% (Mouhouche et al., 1998). Finally, in *A. thaliana*, abortion of flower buds increased as temperature exposure increased between 200- and 300-degree hours (°C-h) above 33°C, and the entire primary inflorescence aborted when exposure exceeded 300°C-h above 33°C (Warner and Erwin, 2005).

Despite the widespread occurrence of FBA, there is limited knowledge about its molecular mechanism, with only a few preliminary studies published to date. Zhang et al. (2010) analyzed gene expression during 10 continuous developmental periods of FBA in Chinese cabbage using cDNA amplified fragment length polymorphism (cDNA-AFLP), and obtained 72 novel expressed sequence tags related to the aborted buds. The results of this study suggested that FBA in Chinese cabbage resulted from the regulation of a series of genes closely related to metabolism. Liu et al. (2008) demonstrated that the aborted buds in radish display characteristics of programmed cell death at the DNA level. Jia and Zhang (2008) analyzed the aborted and normal buds of radish and obtained 107 differentially expressed fragments related to FBA. Their results also indicated that PCD may be a physiological and biochemical phenomenon that occurs in FBA, plant aging, and in plants under stress. However, the underlying mechanism of radish FBA remains unknown. To demonstrate the sequence of molecular events occurring during FBA, this study analyzed gene expression differences between aborted and normal buds of radish using cDNA-AFLP, which is more suitable than differential display reverse transcription-polymerase chain reaction (PCR) for differentially expressed gene analyses (Simões-Araújo et al., 2002). Our results will provide base-

line data for relevant gene cloning and further molecular studies of radish FBA.

MATERIAL AND METHODS

Plant materials

Radish (*R. sativus* L.) FBA material 09L9 was provided by the Chinese Cabbage Laboratory of the Northwest A&F Agriculture and Forestry University. The radish was planted in the greenhouse in spring. When the plants began to bolt and bud, 3 kinds of floral bud samples, including 1) aborted buds, 2) buds transitional between aborted and normal buds, and 3) normal buds, were collected and labeled accordingly based on their phenotype (Figure 1). The bud samples were stored at -70°C for future use.

Figure 1. Floral bud abortion of radish. 1 = Aborted buds; 2 = transitional buds; 3 = normal buds.

RNA extraction and cDNA synthesis

Total RNA was isolated from the floral bud samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. Recombinant DNase (Takara, Japan) was used to remove the trace DNA in the total RNA. The quality of total RNA was determined by agarose gel electrophoresis and ultraviolet spectrophotometry (NanoDrop 2000C, Thermo Scientific, Wilmington, DE, USA).

First-strand cDNA was obtained using a PrimeScript™ First-Strand cDNA Synthesis Kit (Takara, Dalian, China), according to manufacturer instructions. Double-stranded cDNA synthesis was carried out using DNA Polymerase I, *Escherichia coli* DNA Ligase, RNaseH, and T4 DNA polymerase (Takara), in accordance with the instruction manual. Phenol:chloroform:isoamyl alcohol (25:24:1) was used to purify the double-stranded cDNA. Purified cDNA was dissolved in ddH₂O and stored at -20°C for future use. The concentration of double-stranded cDNA was measured by ultraviolet spectrophotometry.

cDNA-AFLP analysis

The cDNA-AFLP reaction was performed according to the protocol described in Bachem et al. (1998). Double-stranded cDNA was digested using *TaqI* and *AseI* (Takara). The digested products were ligated to adapters with the following sequences: *TaqI* adapter, 5'-GACGATGAGTCCTGAC-3' and 5'-CGGTCAGGACTCAT-3'; *AseI* adapter, 5'-GCGTAGACTGCGTACC-3' and 5'-TAGGTACGCAGTC-3'. The ligated products were pre-amplified with their corresponding primers (*TaqI*: 5'-GACGATGAGTCCTGACCGA-3'; *AseI*: 5'-CTCGTAGACTGCGTACCTAAT-3'). The pre-amplified products were diluted 20-fold with ddH₂O and used for selective amplification with their corresponding primers (*TaqI*: 5'-GATGAGTCCTGACCGANN-3'; *AseI*: 5'-GACTGCGTACCTAATNN-3', where N refers to either A, T, C, or G). Selected amplification products were separated with 6% polyacrylamide gel and silver stained.

Isolating and sequencing the transcript-derived fragments (TDFs)

TDFs that showed differential expressions were excised from the gel, soaked in 50 μ L sterile ddH₂O overnight at -20°C, and boiled for 10 min. A 5- μ L aliquot was used as a re-amplified template in a total volume of a 20- μ L PCR mixture, using the same set of corresponding selective primers. The PCR products were checked on 1.5% agarose gel, and each unique single band was isolated and purified using an agarose gel extraction kit (OMEGA, USA). The purified TDFs were subcloned individually into the pGM-T vector (TIANGEN, China) and transformed into *E. coli* (Top10) following standard procedures. Positive clones were screened with ampicillin selection and sent for sequencing (Sangon, China).

Sequence analysis

A BLAST search of TDF sequencing data was conducted in the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/BLAST>), using the BLASTN algorithm (Altschul et al., 1997) against publicly available non-redundant genes. The genes were classified according to their putative functions using Gene Ontology.

Real-time PCR

Real-time PCR was performed to confirm the validity of the cDNA-AFLP analysis. Six differentially expressed TDFs were selected as samples. Total RNA was extracted from the normal and aborted buds of sterile and fertile plants, respectively. First-strand cDNA was obtained using the PrimeScriptTM RT reagent Kit (Takara). Primers were designed according to the TDF sequences using the Primer 5.0 software (Table 1). Real-time PCRs were performed in triplicate in a total volume of 20 μ L, containing 2X 10 μ L SYBR[®] Premix Ex TaqTM, 0.5 μ L 10 μ M forward primer, 0.5 μ L 10 μ M reverse primer, 2 μ L diluted cDNA, and sterile ddH₂O, under the following program: 95°C for 3 min; 95°C for 20 s, 58°C for 30 s, and 72°C for 30 s, for 40 cycles. Melting curve analysis was performed to check the occurrence of primer dimers and non-specific PCR products. The relative mRNA levels of each TDF were normalized to EF-1- α (GO479260) using the 2^{- $\Delta\Delta$ C_t} method (Livak and Schmittgen, 2001) in the Bio-Rad iQ5 System software (Foster City, CA, USA).

Table 1. Sequences of the primers used for real-time qRT-PCR.

TDF clone	Forward sequence (5'-3')	Reverse primer (5'-3')
17	CTAATGGCGGAAGCGGTGGAACTA	ATGAGTCCTGACCGATTCCCTGTGG
22	GACTGTTGCTGTAAACACGGACT	GGCTCAGAGAGGTCTACTCCATTC
78	ATGTTGGATGGGTTGACGCTTTTAA	AGCACATTGGAGGTCTAACC GAAGG
85-1	CCGAAAGATGTAAAGATGGGAGG	CTTCCACTACCAAACGGCTACAAT
86	CGGGTTGGCGAGTGTGTA	ACACCAATGCCAGAACCATACCA
124	TCCTGTAACCCATAACATCCACGG	ACAACGGGACGATAGGCGAAAAAGC

TDF = transcript-derived fragment.

RESULTS

cDNA-AFLP analysis

A total of 256 primer combinations were used to perform cDNA-AFLP in order to examine gene expression differences between aborted and normal radish buds. A total of 221 differentially expressed TDFs were obtained, ranging in size from 100 to 800 bp. According to the expression patterns, TDFs of “aborted buds”, “transitional buds”, and “normal buds” could be divided into the following 4 types (Figure 2): type I: presence → presence → absence, which had 82 bands (37.1%); type II: presence → absence → absence, which had 32 bands (14.5%); type III: absence → presence → presence, which had 71 bands (32.1%); type IV: absence → absence → presence, which had 36 bands (16.3%). Interestingly, the numbers of upregulated and downregulated TDFs in the aborted buds were almost equal. Among these differentially expressed TDFs, 84 were cloned and sequenced. The sequencing data showed that the sequence lengths of these TDFs were consistent with their band sizes, and the sequences at both ends of the TDFs were identical to the primers used, which indicated that the sequencing data were reliable and could be used for further analysis. The redundancy of the obtained nucleotide sequence was analyzed using the DNASTAR software, and a final total of 54 TDFs, with sizes greater than 100 bp, were obtained.

Figure 2. Types of transcript-derived fragments displayed by cDNA-AFLP. Lane 1 = aborted buds; lane 2 = transitional buds; lane 3 = normal buds. I = presence → presence → absence; II = presence → absence → absence; III = absence → presence → presence; IV = absence → absence → presence. A7T13, A7T14, A7T15, and A7T16 are the primer combinations.

Functional classification of differentially expressed TDFs

A BLAST search in the NCBI database revealed corresponding homologous sequences for all of the 54 TDFs, 29 of which corresponded to genes with known functions (Table 2). Using Gene Ontology, these 29 genes could be classified into 4 groups based on the biological functions of their encoded putative proteins: metabolism (25.9%), stimulus response (13.0%), transcription regulation (7.4%), and transportation (5.6%). Nevertheless, the functions for almost half of the TDFs (48.2%) were unconfirmed (Figure 3).

Table 2. Nucleotide homology of the transcript-derived fragments with known gene sequences in the database using BLASTN algorithm along with their expression patterns.

TDF clone	Length (bp)	Similarity	E value	Phenotype
2	252	<i>Arabidopsis thaliana</i> protein kinase domain-containing protein (AT3G01300) mRNA, complete cds	8e-66	α
4	107	<i>Arabidopsis thaliana</i> sodium: hydrogen antiporter 1 (NHD1) mRNA, complete cds	1e-19	-
12	135	<i>Carludovica palmata</i> 26S ribosomal RNA gene, partial sequence	1e-43	β
14-1	172	<i>Hippophae rhamnoides</i> subsp <i>sinensis</i> 26S ribosomal RNA gene, partial sequence	7e-69	β
14-3	109	<i>Arabidopsis thaliana</i> ATCS; ATP binding/ATP citrate synthase/citrate (SI)-synthase (ATCS) mRNA, complete cds	5e-27	β
18-1	134	<i>Brassica rapa</i> clone 457 R2R3-MYB transcription factor mRNA, complete cds	9e-29	α
20-2	124	<i>Arabidopsis thaliana</i> AT4G25910 mRNA, complete cds, clone: RAFL25-34-K14	3e-28	α
22	119	<i>R. sativus</i> prxK1 gene	4e-43	-
26-2	171	<i>Arabidopsis thaliana</i> At1g73980/F2P9_15 mRNA, complete cds	2e-51	-
28	213	<i>Arabidopsis thaliana</i> putative cystathionine gamma-synthase (At3g01120) mRNA, complete cds	5e-70	β
30-2	130	<i>Brassica oleracea</i> var. <i>gemmifera</i> MYC mRNA, complete cds	2e-06	-
38	209	<i>Brassica juncea</i> gamma-tocopherol methyl transferase mRNA, complete cds		
45-3	121	<i>Brassica juncea</i> trans-membrane water channel protein mRNA, complete cds	2e-27	χ
47	123	<i>Brassica rapa</i> subsp <i>pekinensis</i> epithiospecifier modifier (ESM) gene, complete cds	7e-23	χ
48-2	101	<i>Arabidopsis thaliana</i> photosystem II Psb27 protein (PSB27) mRNA, complete cds	5e-18	-
64	356	<i>Arabidopsis thaliana</i> AT5g66760/MSN2_16 mRNA, complete cds	2e-136	-
72	189	<i>Arabidopsis lyrata</i> subsp <i>lyrata</i> gamma-VPE, mRNA	5e-50	-
78	344	<i>Arabidopsis lyrata</i> subsp <i>lyrata</i> auxin-responsive GH3 family protein, mRNA	2e-86	χ
85-1	287	<i>Arabidopsis thaliana</i> CYP71B14; electron carrier/heme binding/iron ion binding/monooxygenase/oxygen binding (CYP71B14) mRNA, complete cds	4e-82	-
86	603	<i>Arabidopsis lyrata</i> subsp <i>lyrata</i> zinc finger family protein, mRNA	3e-127	α
87	338	<i>Brassica juncea</i> APETALA3 (AP3) mRNA, complete cds	2e-150	β
89	327	<i>Arabidopsis thaliana</i> elongation factor 1-alpha/EF-1-alpha (AT1G07920) mRNA, complete cds	5e-107	-
92-2	118	<i>Brassica napus</i> mTERF-like protein mRNA, complete cds	5e-12	-
105	313	<i>Arabidopsis lyrata</i> subsp <i>lyrata</i> ribosomal protein S6 family protein, mRNA	5e-97	-
118	332	<i>Arabidopsis lyrata</i> subsp <i>lyrata</i> glyoxalase 2-1 (GLX2-1), mRNA	5e-107	β
119-1	145	<i>Arabidopsis thaliana</i> MA3 domain-containing protein (AT3G48390) mRNA, complete cds	2e-19	β
124	319	<i>Arabidopsis thaliana</i> GGT4 (At4g29210) mRNA, complete cds	2e-71	-
128	216	<i>Arabidopsis thaliana</i> mtHsc70-1 (mitochondrial heat shock protein 70-1); ATP binding (mtHsc70-1) mRNA, complete cds	1e-62	β

Figure 3. Distribution of the differentially expressed genes involved in floral bud abortion in radish.

Analysis of differentially expressed TDFs by real-time PCR

To confirm the cDNA-AFLP results at the mRNA level, we performed real-time PCR analysis. Six differentially expressed TDFs were chosen: TDF₁₇₋₁ (function unknown), TDF₂₂ (encoding isoperoxidase), TDF₈₅₋₁ (encoding cytochrome P450 71B14), TDF₈₄ (encoding the zinc finger protein), TDF₁₂₄ (encoding gamma-glutamyl transpeptidase 4, GGT4), and TDF₇₈ (encoding the auxin-responsive GH3 family protein). Among these 6 TDFs, all but TDF₇₈ were upregulated in the aborted buds, and the upregulated level was higher in the fertile plants than in the sterile plants. In contrast, TDF₇₈ was downregulated in the aborted buds, and the downregulated level was lower in the fertile plants than in the sterile plants. The changes in expression varied among the 6 TDFs. TDF₂₂ showed the most upregulation, in which expression increased 818.9-fold in the fertile plants and 130-fold in the sterile plants. TDF₁₇₋₁ showed the least upregulation, in which expression only increased 10.9-fold in the fertile plants and 3.7-fold in the sterile plants. TDF₇₈ showed 17.5- and 69.6-fold downregulation in fertile and sterile plants, respectively (Figure 4). These real-time PCR data are consistent with the cDNA-AFLP results, which confirmed the differential expression of these TDFs.

Figure 4. Real-time PCR analysis of interested differentially expressed transcript-derived fragments. NB = normal buds; AB = aborted buds; F = fertile plants; S = sterile plants.

DISCUSSION

FBA is a widespread phenomenon in plant reproduction and evolution. However, only a few studies have been conducted so far regarding the mechanism of FBA, and most of these studies have only focused on the effects of exogenous hormones and environmental stress on FBA (Ahmed and Hall, 1993; Bosac et al., 1994; Mouhouche et al., 1998; Krupnick et al., 2000; Warner and Erwin, 2005). Only a few studies have investigated FBA at the molecular level, such as gene expression patterns (Jia and Zhang, 2008; Zhang et al., 2010). In this study, we analyzed gene expression differences between aborted and normal buds of radish, using cDNA-AFLP and real-time PCR. Fifty-four TDFs were obtained and classified into 4 categories according to their putative encoded proteins. Half of the TDFs' functions are unknown, since there is a lack of information in the radish genome. A large proportion of the TDFs obtained were involved in metabolism and stimulus response, which is in accordance with results of Jia and Zhang (2008), suggesting that FBA may be caused by abnormal metabolism and responses to stimuli. Moreover, our real-time PCR data of 6 differentially expressed TDFs show that expression levels of these TDFs differ between fertile and sterile plants.

TDF₂₂ exhibits 100% sequence homology with the *prxK1* gene, one of the members of the radish peroxidase gene family, and its expression is upregulated when FBA occurs. Plant-specific heme peroxidase belongs to a superfamily that contains 3 different classes of peroxidases (Welinder, 1992): intracellular class I (EC1.11.1.5/6/11), class II released by fungi (EC1.11.1.13/14), and secreted class III plant peroxidases (EC 1.11.1.7). Class III plant peroxidases are involved in a broad range of physiological processes throughout the plant life cycle (Passardi et al., 2005). They are, for example, involved in the removal of H₂O₂, oxidation of toxic reductants, cell growth, and senescence (Santos et al., 2001). In addition, class III peroxidases can generate highly reactive oxygen species (Liszkay et al., 2003; Passardi et al., 2004), which can also act as part of signal transduction pathways (Laloi et al., 2004) during certain processes, including biotic and abiotic stress responses, allelopathic plant-plant interactions, cell division/elongation, and programmed cell death (Bethke and Jones, 2001; Foreman et al., 2003; Apel and Hirt, 2004; Foyer and Noctor, 2005).

TDF₇₈ shows 85% sequence homology with the *Arabidopsis* GH3 family protein (XM_002871520) and is downregulated in aborted buds. An *in vitro* study revealed that most of the members of GH3 family proteins, including AtGH3-9, have indole-3 acetic acid adenylation enzymatic activity, so the GH3 protein may be involved in auxin homeostasis by reducing the free auxin (Staswick et al., 2002, 2005; Khan and Stone, 2007; Wang et al., 2008).

TDF₈₆ exhibits 93% sequence homology with the *Arabidopsis* zinc finger protein (AT5G25560) and is upregulated in aborted buds. Recent studies have confirmed that the zinc finger protein can promote the transition from vegetative to reproductive growth in plants (Wu et al., 2008). Furthermore, zinc finger proteins are also involved in the development of flowers, ovules, and seeds (Sakai et al., 1995; Huang et al., 2006; Sicard et al., 2008).

TDF₁₂₄ has 83% sequence homology with the *Arabidopsis* GGT4 protein (At4g29210) and its expression is upregulated in aborted buds. Like most GGTs from animal tissues, the GGTs from some plants, including tomato, onion, and radish, exhibit broad substrate specificity and have been shown to hydrolyze glutathione (GSH) and several glutathione S-conjugates (Keillor et al., 2005; Zhang et al., 2005; Grzam et al., 2007). Pharmacologically induced GSH deficiencies in newborn mammals, such as rats and guinea pigs, lead to rapid multi-organ fail-

ure and death within a few days (Meister, 1994), and GSH depletion causes embryo lethality in *Arabidopsis* knockouts that lack the first enzyme of the committed pathway in GSH synthesis (Cairns et al., 2006). Thus, both plant and mammalian cells rely on at least some of the multi-functional properties of GSH for their vigor and survival (Noctor et al., 2012).

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