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Isolation of Differentially Expressed Genes by Low Temperature Treatment in Winter Oilseed Rape (*Brassica napus* L. cv. Tammi)

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Abstract - To investigate flowering related genes in winter-type oilseed rape (*Brassica napus* L. cv. Tammi), differentially expressed genes were isolated from leaves of the plant after low temperature treatment which is requirements for floral induction. As a result of suppression subtractive hybridization (SSH), 288 clones were randomly selected from SSH library. Using reverse Northern blot analysis, 150 of 288 clones were identified to be differentially expressed. Out of these 150 clones, 45 clones showed very high identities with the known genes. Four clones showed very high identities over 90% with metallothionein-like gene that is related to flowering-induced genes. Of these 4 clones, the cDNA clone, *rfs-13*, revealed high identity with metallothionein-like protein in *Arabidopsis thaliana* (98%) and *Brassica compestris* (89%). Furthermore, gene expressed in immature flower stages was confirmed by Northern blot analysis.

Key words - *Brassica napus*, cDNA, Flowering, Oil seed rape, Suppression subtractive hybridization (SSH), Transcription

Introduction

The transition from vegetative to reproductive development stage is controlled by both environmental and developmental signals (Blazquez *et al.*, 1998; Levy and Dean, 1998). Environmental factors such as photoperiod, light quality and quantity, temperature, nutrients and water supply affect flowering (Koornneef *et al.*, 1995; Levy and Dean, 1998; Pidkowich *et al.*, 1999). Many plants from temperate regions have a distinct winter requirement for flowering. Therefore, unless exposed to an extended period of low temperature, they flower very late or not at all. Especially, biennials such as cabbage and winter oilseed rape, are require several weeks of exposure to low temperatures (vernalization) to induce flowering (Lardon and Triboi-Blondel, 1995; Michaels and Amasino, 2000).

It is not surprising that a range of different genes have been cloned by low temperature treatment (Goodwin *et al.*, 1996; Michaels and Amasino, 2000; Ok *et al.*, 2004; Ságez-Vásqhez *et al.*, 2000). Also, diverse flowering related genes were reported (Blazquez *et al.*, 1997; Kobayashi *et al.*, 1994; Ok *et al.*, 2003). They reported that multiple genes were associated in the process of flowering.

Winter-type oilseed rape (*Brassica napus* L.) is very important as an oilseed crop, but cultivation areas have been decreased rapidly in the southern regions due to low income and higher production cost in South Korea. However recently, people are interested to cultivate this crop as a garden crop at early spring season. Especially, "cv. Tammi rape" with larger size of flowers has been developed and disseminated. The cultivar will be used as ornamental plant. In order to detect flowering associated new genes in winter oilseed rape, identification of differentially expressed genes after vernalization is required. This will provide basal data for molecular breeders.

A PCR-based cDNA subtraction method, and suppression subtractive hybridization (SSH), have been tried and turned out to be a successful tool for rapid screening of differentially induced genes in many plants (Diatchenko, 1996; Ok *et al.*, 2003; Ok *et al.*, 2004; Shimon *et al.*, 2003). In this paper, to achieve basal information of flowering associated genes induced by low temperature, SSH analysis was executed in winter oilseed rape (*Brassica napus* L. cv. Tammi).

Materials and Methods

Plant materials and low temperature treatment

About 20 seeds of a oilseed rape variety (*Brassica napus* L. Tammi) per treatment were sown in coarse vermiculite in 15 cm pot, and placed in a 20/16℃ (day/night) growth chamber with 16 hr daylength and 250 $\mu mol \cdot m^{-2} s^{-1}$ light intensity for 3 wk for germination and growth(control). The plants were transferred to low temperature growth chamber (5/2℃) for 4 wk with the same daylength and light intensity as control, and moved to the room temperature, for growing un-

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til floral development. The plants were watered weekly with modified Hoagland solution. The second and third leaves per treatment were harvested, and stored at -70℃ freezing chamber for RNA isolation.

RNA isolation and cDNA library construction

Total RNA was isolated from leaves using TRIZOL® reagent (GibcoBRL, USA) according to the manufacture's instruction. The amount and quality of total RNA was checked by spectrophotometer (O.D. 260/280) and formaldehyde-contained 1% agarose gel electrophoresis. Poly(A)+RNA was extracted from total RNA using the PolyATtract® mRNA isolation system (Promega, USA) according to the manufacture's protocol. A HybrIZAP-2.1 XR Library Construction Kit and a HybriZAP-2.1 XR cDNA Synthesis Kit (Stratagene, USA) were used to construct the cDNA library (Eugentech, Korea). The library was packaged into Gigapack Ⅲ Gold packaging extract and lambda ZAP yielded 2.4×10^7 pfu/ml primary plaques, which were then amplified to a titer of 2×10^{10} pfu/ml. cDNA-inserted pAD-GAL4-2.1 phagemid vectors were excised by mass *in vivo* excision using an ExAssist helper phage system (Stratagene, USA). The titer of the resulting library was as 3×10^8 cfu/ml, and phagemids were used to infect *E. coli* strain XLOLR according to the manufacturer's instructions.

Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization was performed by PCR-select cDNA subtraction Kit user manual (Clontech, USA; Diatchenko *et al*., 1996). Total RNA extracted from normally grown rape, and low temperature stress treated/without treated rapes were used for driver and tester cDNA synthesis, respectively. First strand of cDNA was prepared with 2 mg of poly(A)$^+$RNA by MIMLV reverse transcriptase in final volume of 10μl, using the ancho oligo (dT) primer. Second strand cDNA synthesis was performed with 20X second strand enzyme cocktail, dNTP mix and 5X second strand buffer in total volume of 80μl. Purified tester and driver double-stranded cDNA were digested with restriction endonuclease *Rsa* Ⅰ. The double stranded cDNA was divided into two separated tubes where one aliquot was ligated with adaptor 1 and the other was ligated with adaptor 2R. Adaptor-ligated tester cDNA and RsaI-digested driver cDNA were initially hybridized. Two vials were incubated at 68℃ overnight. The completely hybridized adaptor-ligated double stranded cDNA was used as a template for first round PCR amplification with PCR primer 1, and second round PCR amplification with nested primer 1 and nested primer 2R. Nested PCR was used to further reduce background and enrich for differentially expressed fragments. PCR Products obtained from the second PCT were inserted into PCR2.1 TA vector (Invitrogen, USA).

Reverse Northern analysis

Five microgram of amplified DNAs of each cDNA clones that were randomly selected from subtracted cDNA pools were dot-blotted onto Hybond-XL™ positively charged nylon membrane (Amersham Pharmacia Biotech, UK) by vacuum dot-blot apparatus™ (GibcoBRL, USA) and then immobilzed via UV crosslinking. Radio-labeled cDNA probes were synthesized by reverse transcription of 5g of mRNA that were extracted both from normally grown oilseed rape, and, low temperature treated oilseed rape. Probes were incorporated with 50μCi, [^{32}P]-dCTP for 1 hr using the Superscript™ and the SMART™ PCR cDNA Synthesis Kit (Clontech, USA). Membrane was hybridized with probes at 68℃ for 16hr and then washed with 2xSSC and 0.1% SDS solution at 68℃ for 20 min and three times with 0.1xSSC and 0.1% SDS solution at 68℃ for 20 min. Membrane was exposed to an X-ray film for 2 days.

Sequencing and data analysis

Randomly collected 150 EST(expressed sequence tags) clones from the cDNA library and 48 SSH clones screened by the reverse Northern analysis were sequenced at Green Gene Biotech (Korea). Sequency homology was analyzed using the BLAST program (National Center for Biotechnology Information, USA). Sequences that were less than 200 bp or with more than 5% ambiguity were excluded. Nucleotide sequences and deduced amino acid sequences were compared to previously reported sequences in the EMBL/Gene Bank databases using the BLAST search analysis.

Results and Discussion

Isolation of cDNA Clones

Suppression subtractive hybridization (SSH) was executed to isolate differentially expressed genes after 4-weeks low temperature treatment (vernalization) which is necessary and sufficient for floral development. For SSH, tester (low temperature treatment) and driver (non-treatment) double strand cDNAs were synthesized, and each double strand cDNAs were digested with *RsaI*, resulting in the majority of the double strand cDNA size between 200bp and 600bp. After two steps of hybridization with tester and driver, second round PCR products were subcloned to a T/A cloning vector. More than 1,000 recombinant clones were obtained from the PCR-selected subtraction library. Of those, 288 clones were randomly picked, and their

inserts were amplified using adaptor primers by colony PCR. The size distribution of the 288 clones was 0.2 to 0.5kb, with an average value of 0.35kb. The small insert size is due to the initial digestion of the tester cDNA with *Rsa* I.

Using reverse Northern blot analysis, we could identify 150 of 288 (52%) clones were expressed differentially (Fig. 1). These clones were serially assigned as *rfs* (rape flowering and low temperature sensitive). In comparison with other SSH experiments (Ok *et al.*, 2003; Ok *et al.*, 2004), 52% is more than enough for analyzing the different expression of genes derived from vernalization treatment in oilseed rape.

(A) (B)

Fig. 1. Reverse Northern screening assay for potentially expressed genes. The cDNA clones in (A) were the forward subtracted cDNAs array (forward subtracted probes) and (B) were the reverse subtracted cDNAs array (reverse subtracted probes).

Fig. 2. Nucleotide sequence alignments of full-length cDNA clones. Comparison of the derived full-length cDNA clones with sequence from the GeneBank/EMBL database.

To characterize the estimated function and the redundancy of these putative positive clones, 150 clones were sequenced. Nucleotide sequences and predicted amino acid sequences were compared to previously reported sequences in the EMBL/Gene Bank databases using the BLAST search analysis. Out of these 150 clones, 48 clones and their putative identities were listed in Table 1. Database comparisons of cDNAs revealed that 45 cDNAs (94%) out of 48 showed homologies to the known genes from other organism and the remaining 3 clones (6%) were no matched (Table 1). Full-length cloning should be necessary to identify ORFs of no-matched clones, because these clones might be the 5' or 3' UTR sequences of the correspondent genes.

A diverse group of genes was identified in this study, although a few number of clones actually were sequenced (Table 1). As expected, many identified clones appeared to be involved in floral induction (*rfs-13, rfs-26,* and *rfs-59*), flower promotion, *rfs-48* (Ryouslke *et al.*, 2002), drought induction (*rfs-10, rfs-28,* and *rfs-50*), disease resistance, *rfs-82*, and *rfs-91* (Madsen *et al.*, 2003) and photosynthesis, *rfs-3, rfs-18, rfs-38, rfs-73, rfs-83,* and *rfs-92* (Baszcynski *et al.*, 1988).

The nucleotide (Fig. 2) and the deduced amino acid sequences (Fig. 4) of the putative gene products were compared. Especially, three clones (*rfs-13, rfs-26,* and *rfs-59*) showed very high identities over 90% with metallothionein-like protein (gene) that related to flowering-induced genes (Fig. 2). Of these 3 clones, the cDNA clone, *rfs-13*, revealed high identity with metallothionein-like protein in *Arabidopsis thaliana* (98%) and *Brassica compestris* (89%), which is expressed greatly at transition apex floral apex (Kawashima *et al.*, 1991; Kitashiba *et al.*, 1996; Zhou and Glodsbrough, 1995; Fig. 4). Of defense-related genes, *rfs-82*, and *rfs-91* revealed high identity (98%) with disease associated gene of *Oryza sativa* (Madsen *et al.*, 2003). Also, *rfs-4*, and *rfs-76* revealed high identity (98%) with glutathione S-transferase protein (accession number; AAM 12488, AAP54712) of *Oryza sativa*. The results indicate that transition of diverse genes as well as flowering associated genes is required for floral development. Other reports (Kole, *et al.*, 2001; Levy and Dean, 1998; Ok *et al.*, 2003; Robert *et al.*, 1998) showed the same tendency.

rfs-4 C 1 2 3 4 5
rfs-10 C 1 2 3 4 5
rfs-16 C 1 2 3 4 5
rfs-26 C 1 2 3 4 5
rfs-80 C 1 2 3 4 5
rfs-91 C 1 2 3 4 5

Fig. 3. Northern blot analysis using six different development stages of oilseed rape leaves. About 30 *ug* of total RNA from expanded leaves was fractionated by denaturing agarose gel electrophoresis, blotted onto nylon membrane, and hybridized with biotinylated cDNA probe. Membrane were washed and exposed to X-ray film. Line C; 1 weeks (20/16℃), Line 1; 1 day after 4 weeks vernalization (5/2℃), Line 2; 2 days after 4 weeks vernalization, Line 3; 3 days after 4 weeks vernalization, floral apex stage, Line 4; 4 days after 4 weeks vernalization, Line 5; 5 days after 4 weeks vernalization, immature flower bud stage.

Specificity of Gene Transcription

Transcription of some of the genes identified above was examined with respect to time specificity of expression after low temperature treatment. To confirm whether the reverse Northern results of sequenced clones were related to flowering or not, 6 of 48 clones were analyzed by Northern blot analysis (Fig. 3). These 6 genes could be

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                     *        20         *        40         *        60         *        80
B. juncea   : msccqqncgcgcaqckcvqcqqckmvpdllsfsqetetcetlvlqlapamnsqfeasgetfvaendackcgsdckcnpctck---- : 80
A.thaliana  : msccqqscgcgsackcqqcqqckrvpdl----endatetlvlqvapamnsqveasgetfvaendackcgsdckcnpctck---- : 76
rfs-13      : -------------------------------------------------------EASCETFVAENDACKCRSDCKCNPCTCK*RII : 31
              msccqq cqcq  ckc qcqqck vpdl    e t tetlvlq apamnsq EASGETFVAENDACKCqSDCKCNPCTCK

                     *       100         *       120         *       140         *
B. juncea   : ------------------------------------------------------------ :  -
A.thaliana  : ------------------------------------------------------------ :  -
rfs-13      : *DPKTISNISNVV**WLISPIVLLAMLVFFVLC-----FMVWS*DISLTRYINQKYYGFETSAATT : 88
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Fig. 4. Protein sequence alignments of full-length cDNA clones. Comparison of the derived protein of the full-length cDNA clones with sequences from the GeneBank/EMBL databases. Multiple alignment the metallothionein-like protein sequences shows similarity to *rfs-13*. Filled boxes indicate the majority of the genes showing the same sequence.

Table 1. Putative identity of genes expressed in leaf of Brassica napus L. cv. Tammi after low temperature treatment

EST ID	Insert size (bp)	Putative identity	Origin of matching sequence (accession)	S(%)[a]	Homology species
rfs-3	818	Chlorophyll a/b binding protein	AY086307	89	*Arabidopsis thaliana*
rfs-4	447	Putative glutathione S-transferase	AAM12488	98	*Oryza sativa*
rfs-5	388	hypothetical protein	NP_950502	66	Onion yellows phytoplasma
rsf-6	490	Proline-rich protein	AAF28388	93	*Arabidopsis thaliana*
rsf-9	554	Cytochrome P450-like protein	AAP54709	98	*Oryza sativa*
rsf-10	663	Drought-induced protein	S71562	83	*Helianthus annuus*
rfs-12	456	ATP-dependent protease subunit	BAA84410	95	*Arabidopsis thaliana*
rfs-13	339	Metallothionein-like protein	CAA71805	96	*Brassica juncea*
rfs-18	680	Ribulose bisphosphate carboxylase	CAA30290	88	*Brassica napus*
rfs-19	347	Hypothetical protein	AAP53228	98	*Oryza sativa*
rfs-21	391	Putative 1,4-beta-xylanase	AAM10751	98	*Oryza sativa*
rfs-25	322	Hypothetical protein	AAM48251	85	*Oryza sativa*
rfs-26	486	Metallothionein-like protein	D78491	89	*Brassica campestris*
rfs-28	285	Drought-induced-19-like 1 protein	CAB80713	88	*Arabidopsis thaliana*
rfs-30	314	Hypothetical protein	AAP54715	98	*Oryza sativa*
rfs-33	391	Ribosomal protein	BAA84367	96	*Arabidopsis thaliana*
rfs-38	268	Ribulose bisphosphate carboxylase.	CAA43410	100	*Brassica napus*
rfs-43	524	Hypothetical protein	NP_193095	89	*Arabidopsis thaliana*
rfs-45	442	ATPase beta subunit	CAB92327	97	*Raphanus sativus*
rfs-46	395	Unnamed protein	CAA34015	95	*Oryza sativa*
rfs-48	792	Fructose-1,6-bisphosphate aldolase	X66814	82	*Spinacia oleracea*
rfs-50	284	Drought-induced-19-like 1 protein	CAB80713	88	*Arabidopsis thaliana*
rfs-52	650	Auxin-independent growth promoter	AAD39288	83	*Arabidopsis thaliana*
rfs-56	554	Unknown protein	AAL24136	96	*Arabidopsis thaliana*
rfs-58	651	Expressed protein	NP_563934	86	*Arabidopsis thaliana*
rfs-59	290	Metallothionein-like protein	AAB53104	98	*Brassica napus*
rfs-60	665	Putative plastocyanin	AAG50089	88	*Arabidopsis thaliana*
rfs-64	454	Oxygen-evolving enhancer protein 3 precursor-like protein	AAN18214	88	*Arabidopsis thaliana*
rfs-73	538	Chlorophyll a/b-binding protein	CAA34459	92	*Mustard*
rfs-76	313	Putative glutathione S-transferase	AAP54712	98	*Oryza sativa*
rfs-77	555	Expressed protein	AV522860	96	*Arabidopsis thaliana*
rfs-78	733	Reverse transcriptase	AAP5327	98	*Oryza sativa*
rfs-80	737	Omega-6 fatty acid desaturase	AAG51042	84	*Arabidopsis thaliana*
rfs-82	313	Putative disease resistance protein	AAP54740	98	*Oryza sativa*
rfs-83	816	Chlorophyll a/b binding protein	AAP44089	94	*Brassica oleracea*
rfs-84	290	Metallothionein-like protein	AAB53104	98	*Arabidopsis thaliana*
rfs-85	650	Expressed protein 341	AV530357	82	*Arabidopsis thaliana*
rfs-86	538	Maturase	CAA77339	95	*Nicotiana tabacum*
rfs-87	737	Fiber protein	AAQ84318	79	*Gossypium barbadense*
rfs-88	737	Unknown protein	AAO6393	86	*Arabidopsis thaliana*
rfs-89	733	SIAH1 protein	CAB89182	85	*Brassica napus*
rfs-91	388	Putative disease resistance protein	AAL79344	97	*Oryza sativa*
rfs-92	764	Chlorophyll a/b binding protein	AAL67432	92	*Broccoli floret*
rfs-93	557	Putative alliinase	BAB68042	85	*Oryza sativa*
rfs-97	423	Protein kinase	NP_188090	88	*Arabidopsis thaliana*

[a]Similarity of isolated EST sequence with pre-determined gene sequence of GeneBank.

classified into two groups. One (*rfs-4*) revealed the same signals of expression from control to immature flower bud stages. The other group (*rfs-10*, *rfs-16*, *rfs-26*, *rfs-80* and *rfs-91*) having unique expression after low temperature treatments. Of these 5 genes, interestingly, expression of disease-related *rfs-91* gene was rapidly increased just in immature flower bud stage. On the other hand, transcripts of *rfs-26* gene was gradually decreased at immature flower bud stages. The finding was that disease-related proteins which were accumulated during flower development supported the likelihood and these diverse plant gene function under low temperature stress as well as flowering associated genes (Blazquez *et al.*, 1997; Kobayashi *et al.*, 1994; Ok *et al.*, 2003; Robert *et al.*, 1998). Expression of *rfs-80* gene that revealed high identity (84%) with omega-6 fatty acid desaturase protein of *Cyanobacterium* (Hitz *et al.*, 1994) showed just 2 days after vernalization but not appeared from 3 days. The diverse expression pattern under stress treatment was reported in other researches (Lardon and Triboi-Blondel, 1995; Michaels and Amasino, 2000). This expression also, includes floral homoeotic genes (Levin and Meyerowitz. 1995; Mandel *et al.*, 1992). Therefore, it is inferred that diverse genes are functioning in the process of floral induction through the vernalization in winter type oilseed rape.

Flower associated genes and diverse genes were successfully detected after low temperature treatment by SSH analysis in this study. In particular, these genes and the different gene expression pattern data make it feasible to develop new varieties for molecular breeders in the future. Further study require structural analysis and function analysis of the flowering related genes (Robert *et al.*, 1998).

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