Journal of Photoscience, Vol. 5, No. 3, pp. 131 – 135

CLONING OF GENES EXPRESSED UPON FLORAL INDUCTION IN *PHARBITIS* COTYLEDONS

KANG CHANG KIM[1], YOONKANG HUR[2] and JUESON MAENG*[1]
[1]Department of Life Science, Sogang University, Seoul 100-611, Korea
[2]Department of Biology, Chungnam National University, Taejon 305-764, Korea

(Received 10 *August* 1998; *accepted* 23 *September* 1998*)*

Abstract – Using differential display reverse transcription technique, the present study attempted to isolate and characterize genes specifically expressed in cotyledons of *Pharbitis nil* Choisy cv. Violet during floral induction. A total of 107 bands specific to the inductive condition were initially obtained with 80 primer sets of 20 different arbitrary primers combined with 4 kinds of $T_{12}MN$. In northern blot analysis with reamplified cDNAs as probes, three cDNAs were detected to be expressed specifically in the induced cotyledon tissues, and designated *PnFL-1*, *PnFL-2* and *PnFL-3*, the sizes of which were 228 bp, 317 bp and 272 bp, respectively. A search for sequences similar to the deduced amino acid sequences was conducted using GenBank and EMBL database; sequence encoded by *PnFL-1* had 29% identity with the clone of *Arabidopsis thaliana* similar to reverse transcriptase (GenBank Acc. No. 3047086), *PnFL-2* shared 50% identity with hydroxyproline-rich glycoprotein of *Glycine max* (GenBank Acc. No. 347455), and *PnFL-3* had 46% identity with TAMU *A. thaliana* genomic clone T23E16 (GenBank Acc. No. B67574). None of them was known gene in the plant system up to date, implying that the fragments may comprise parts of genes which are associated with the floral induction in *Pharbitis nil*.

INTRODUCTION

Flower development in photoperiodic plants is evoked by floral stimulus which is transmitted from the leaves or cotyledons where it has been produced in the process of floral induction upon perceiving flower-inductive photoperiod. The induction of flowering, a complex process, is still little understood. The physiological evidences for the existence of both transmissible floral stimuli and inhibitors have been shown[1] and their activity has been investigated by many workers[2-6]. Differential gene expression in leaves and cotyledons during floral induction has been shown to play an important role in the differentiation of the apical buds to form flower buds. Qualitative and quantitative changes in the mRNA populations after floral induction in leaves of the long-day plants, *Hyoscyamus niger* and *A. thaliana* have been detected[7,8]. Qualitative changes in several proteins and mRNAs in *Pharbitis* cotyledons during the inductive dark period have been discussed in relation to floral induction[6,9-19]. Several cDNA clones have isolated from *Pharbitis* cotyledons using different approaches[18-22]. Using differential display reverse transcription (DDRT) -polymerase chain reaction (PCR) technique, Sage-Ono *et al.*[19] recently isolated and characterized a cDNA clone whose expression was closely related to the photoperiodic dark period. However, they did not confirm that the clone is associated with the floral induction.

In this study we describe the isolation and characterization of partial three cDNA clones expressed specifically in the flower-induced cotyledons. As a result of northern blot analysis, these genes were expressed at the very low level in the inductive dark period.

MATERIALS AND METHODS

Plant materials and culture condition. Seeds of *Pharbitis nil* Choisy cv. Violet were stirred with concentrated sulfuric acid for 30 min, rinsed and imbibed overnight in running water. The seeds were sown in prewashed vermiculite in 12 cm diameter pots and grown in a culture room with Hoagland nutrient solution at 26 ± 1°C under continuous illumination for 6-7 days. The irradiance from mixture of cool white fluorescence lamps and incandescence bulbs was adjusted at 15w $\cdot m^{-2}$. Six or seven-day old cotyledons were exposed to 16 h-dark period for one, three or five days for floral induction. Control cotyledons were kept under continuous light for the same period.

Messenger-RNA extraction. Cotyledons from either non-induced or induced seedlings were ground in liquid nitrogen with a mortar and pestle. Total RNAs were extracted from 5 g of the powder using the single-step method by Chomczynski and Sacchi[24]. Poly(A^+)-mRNA was isolated from total RNAs using PolyAtract mRNA isolation II (Promega).

Differential display reverse transcription. Differential display reverse transcription was performed by using RNA map kit (GeneHunter)[23]. Reverse transcription was done in 4

* To whom correspondence should be addressed.

independent reactions using $T_{12}MA$, $T_{12}MC$, $T_{12}MT$ and $T_{12}MG$ primers, where M represents a degenerated mixture of dA, dG and dC. Each reaction mixture was composed of 0.1 μg of mRNA, 2.5 μM $T_{12}MN$ primer, 20 μM dNTP and 300 U MMLV reverse transcriptase, and incubated at 37°C for 60 min. The reaction was stopped by incubation at 95°C for 5 min. One tenth volume of the mixture was subjected to PCR amplification in the presence of α-^{35}S dATP using Taq polymerase (AmpliTaq, TAKARA). Amplification was carried out for 40 cycles of 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s. and an additional extension period at 72°C for 5 min. The 80 different primer sets used for PCR amplification were the combinations of twenty 5'-AP (arbitrary primer) with the 3'-$T_{12}MN$ primers that were used for initial cDNA synthesis. The amplified cDNA fragments were seperated by 6 % denaturating polyacrylamide gel electrophoresis in TBE buffer at 60 W. The gel was dried and exposed to X-ray film (Hyperfilm-β max, Amersham).

Reamplification of cDNA. The differentially expressed cDNA bands were selected after autoradiography and excised from the dried polyacrylamide gel. The gel slices along with the 3M paper were soaked in 100 μL dH_2O for 10 min, boiled for 15 min and spun for 2 min. The supernatant was transferred to a new microfuge tube, added with 10 μL of 3 *M* sodium acetate, 5 μL of glycogen (10 mg/mL) and 450 μL of 100 % EtOH, and precipitated for 30 min at -80°C. The cDNA pellets were harvested after spinning for 10 min at 4°C. Pellets were rinsed with 200 μL ice-cold 85 % EtOH and dissolved in 10 μL dH_2O. Four microliters of each of the pellets was used for reamplification with the same primer set and PCR condition.

Northern blot analysis. To confirm whether differentially expressed cDNAs obtained are correct, northern blot analysis was carried out using the radioisotope-labeled cDNA as probes. Five micrograms of mRNA were denatured in 2.2 *M* formaldehyde and 50 % formamide for 10 min at 65°C. The denatured mRNA was separated on 1 % agarose gel containing 6.7 % formaldehyde, 2.0 *M* MOPS, pH 7.0 and 2 m*M* EDTA, transferred to a nylon membrane by capillary transfer with SSPE buffer. The nylon membrane was UV-cross-linked for 10 s. Reamplified cDNAs were labeled with α-^{32}P dCTP by PCR method. The membranes were prehybridized at 42°C for 4 h in 10 mL hybridization buffer cotaining 50 % formamide, 5X Denhardt's reagent, 5X SSPE buffer, 0.1 % SDS and 1 mg salmon sperm DNA. The probes were boiled at 100°C for 10 min, added to fresh hybridization buffer and hybridized at 42°C for 16 h. Following hybridization, the membranes were washed twice for 15 min at room temperature in 6X SSPE and 0.1 % SDS, then twice washed for 30 min at 42°C in 1X SSPE and 0.1 % SDS, and were finally washed for 30 min at 42°C in 0.1X SSPE and 0.1 % SDS. The membranes were exposed to X-ray films at -70°C

Cloning of PCR products and preparation of plasmid DNA. *PnFL-1* and *PnFL-3* cDNA fragments which were inserted in pMOSBlue vector (Amersham) transformed competent *E. coli* strain MOSBlue, and *PnFL-2* cDNA fragment inserted into pT-Adv vector (Clonetech) transformed *E. coli* strain TOP10F'. The process was performed in accordance with instructions from the manufacturers. Alkali lysis method[25] was used to isolate the plasmid DNAs from the transformed bacterial cells.

Sequencing and analysis of cDNA fragments. Nucleotide sequencing of the cDNA fragments was performed using T7 sequenase version 2.0 sequencing kit (Amersham) according to the protocols provided by the manufacturer. The individual DNA sequences of cDNA fragments were analyzed with Blast DNA search program through GenBank and EMBL database.

RESULTS AND DISCUSSION

To isolate genes expressed specifically during floral induction, mRNAs were extracted from cotyledons previously exposed either to non-inductive continuous light or to one to three inductive cycles of 8 h light - 16 h dark period. The mRNAs were then subjected to DDRT experiment. One hundred and seven bands specific to the inductive condition were initially obtained by the experiment with 80 primer sets (4 $T_{12}MN$ with 20 APs). Figure 1 showed three bands among 107. These cDNA fragments (or bands) were extracted from squencing gel, reamplified and run on 1% agarose gel.

Using the reamplified cDNA fragments as probes, northern blot analyses were performed to examine whether

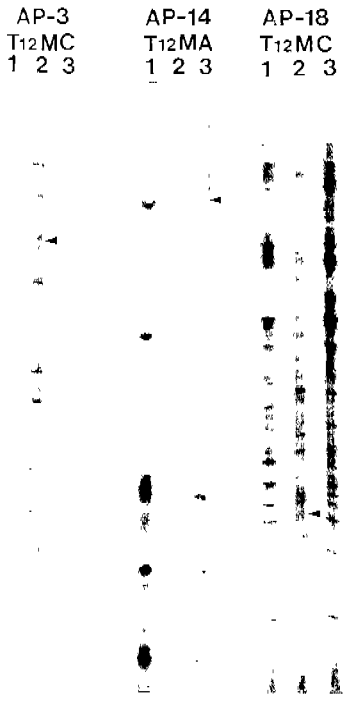

Figure 1. Differential display of DDRT-cloned cDNA fragments. mRNAs isolated from cotyledons exposed to continuous light (Lane 1), a cycle (Lane 2) or three cycles (Lane 3) of 16 h dark period were reverse-transcribed and PCR amplified using primer sets of $T_{12}MA$, $T_{12}MC$ and arbitrary primers, AP-3, AP-14 and AP-18. Arrowheads indicate cDNA fragments detected only in induced tissues.

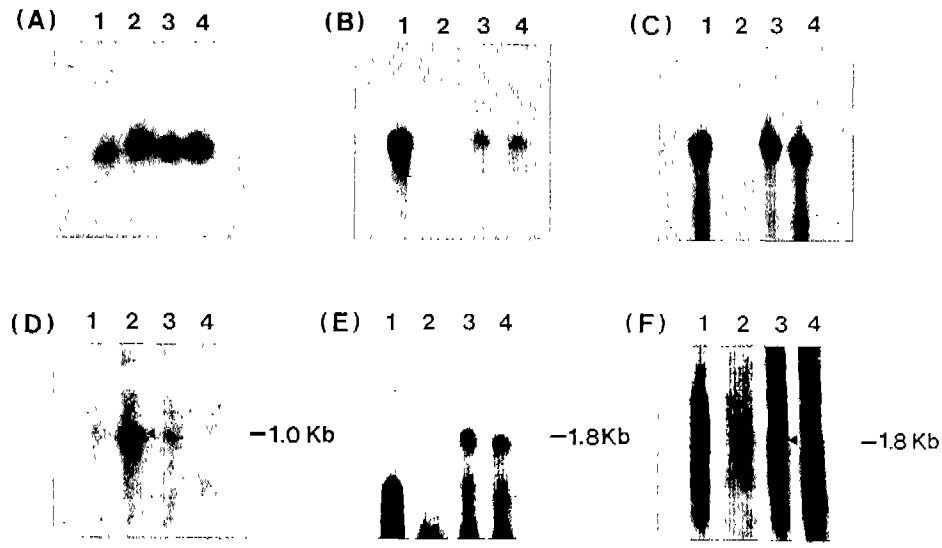

Figure 2. Northern blot analysis of mRNAs. mRNAs isolated from cotyledons exposed to continuous light (Lane 1), a cycle (Lane 2), three cycles (Lane 3) or five cycles (Lane 4) of 16 h dark period were hybridized with reamplified cDNA fragments. A, a cDNA expressed both in induced and in non-induced tissues; B and C, cDNAs expressed less in induced tissues than in non-induced tissues; D ~ F, cDNAs expressed only in induced tissues. Arrowheads indicate *PnFL-1*, *PnFL-2* and *PnFL-3* in D, E and F, respectively.

the cDNAs are expressed only in cotyledons under the inductive condition. No signal was detected when we used the blot containing 50 μg of total RNA (Data not shown). All blots used in this experiment contain 5 μg of poly (A^+) mRNA which was equivalent to over 150 μg of total RNA, suggesting that very low levels of the transcript had been produced. Seventy two cDNAs out of 107 were expressed both in the induced cotyledons and non-induced cotyledons (Fig. 2A), while 32 cDNAs were found to be expressed at the lower level in the induced cotyledons than non-induced ones (Fig. 2B and 2C). And only three cDNAs obtained with AP-3, AP-14 and AP-18 were expressed only in the induced cotyledons and were designated *PnFL-1*, *PnFL-2* or *PnFL-3*, respectively (Fig. 2D - F). These results imply that although the DDRT method is theoretically very sensitive, its practical outcome is not so promising. *PnFL-1* mRNA, approximately 1.0 kb, was expressed only in

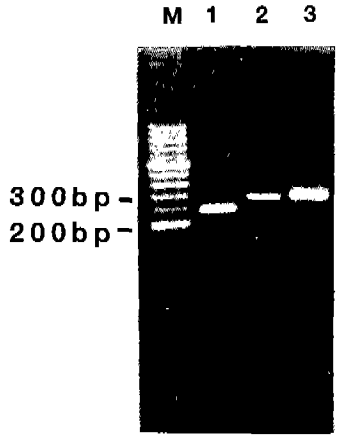

Figure 3. Agarose gel electrophoresis of cDNA fragments reamplified by PCR. M, 50 bp DNA ladder; Lanes 1, 2 and 3 show *PnFL-1*, *PnFL-2* and *PnFL-3* cDNA fragments, respectively.

(A) *PnFL-1*

```
   CCGATAGGTCACCGTATTAAGCAGCTGCGTATGAAACAGGATGCTATTAGGCACATACGC
→  P  I  G  H  R  I  K  Q  L  R  M  K  Q  D  A  I  R  H  I  R
GATCCAGATGCGGTAGCTGAGTTTCAAAATCTCGAAATCCAGTTGTCCCAGCTGGAAGCCTAA
 D  P  D  A  V  A  E  F  Q  N  L  E  I  Q  L  S  Q  L  E  A  *
GAGGATATTTTCTGGAGGCAGCGACCAAAGCAACACTGGCTACGTGGAGCTTATGCTAATACA
 E  D  I  F  W  R  Q  R  P  K  Q  H  W  L  R  G  A  Y  A  N  T
AAATTTTATCACAGGTATGCCTCTGTCCGC
 K  F  Y  H  R  Y  A  S  V  R  →
```

(B) *PnFL-2*

```
   AGCCGAATTACCCGACTATCTACACCTTCTTTCGGAAACTACCTAGTGTGGAAGTTAATT
→  S  R  I  T  R  L  S  T  P  S  F  G  N  Y  L  V  W  K  L  I
TCCTGCTATTGATCATTGACACAGACTGCCACCTTTAAGACTAGACTCAATGTGTGTTTTTTT
 S  L  T  *  S  P  T  Q  T  A  T  P  K  T  R  L  N  V  C  P  P
CTTGTCATCACCGATCCCAAACCTCCAACGTCTTACCCATTACTTTATGATGTTAGTATCTTC
 L  V  I  T  D  P  K  P  P  T  S  Y  P  L  L  Y  D  Y  S  I  F
TACCCACCACCTTCTGCTTCACCTTCTCCTCAACTCCACCGGCCTTTTGGAAGGTCAGGTTCA
 Y  P  P  P  S  A  S  P  S  P  Q  L  H  R  P  F  G  R  S  G  S
CAGCGCGGCGGTGGGGTCTTAACCAACAAAGGGTCTTTGACGGAACAACTTAACGACAAGTCG
 Q  R  G  G  G  V  L  T  T  K  R  S  L  T  E  Q  L  N  D  K  S
GGTAA
 G  →
```

(C) *PnFL-3*

```
   GCTGCCGGTCACTTAAGCTCGAGCATGGCCCTAGGCTAGACTCGATCCCACGTACACATAC
←  V  A  L  S  N  S  S  T  G  P  D  S  R  L  *  P  A  H  T  H
CAAACCCCCGATCCCTAAATAACATATATTACATTGTTAAATATATTTAAGCATACTTATCCC
 N  P  P  *  P  N  I  T  Y  L  T  V  I  *  I  F  E  Y  S  Y  P
CTCCTACAAGATATCCGAGTGTTCAAATATGTGTATATATATCTAAATTCGCATATCCCTGCA
 L  I  N  *  L  S  V  L  K  Y  V  Y  I  Y  I  *  A  Y  L  S  T
TGGCATGTACTGCGTTGGTAAAAATTCTAATCTGTTCCACTAAATCCTAATCCCACGTTTTTT
 G  Y  M  V  C  G  N  K  L  N  S  L  T  I  *  S  *  P  A  F  F
TTTTTTTTGTATTTGATCTCTTCTCGATTTAGACTCGATCCCAACCCG
 F  F  V  Y  V  L  S  S  R  F  R  L  *  P  Q  A  ←
```

Figure 4. Nucleotide and deduced amino acid sequences of cDNA fragments. Single letters below the nucleotide sequences represent abbreviations of deduced amino acids. Arrows indicate the directions of translation. Asterisks (*) indicate stop codons.

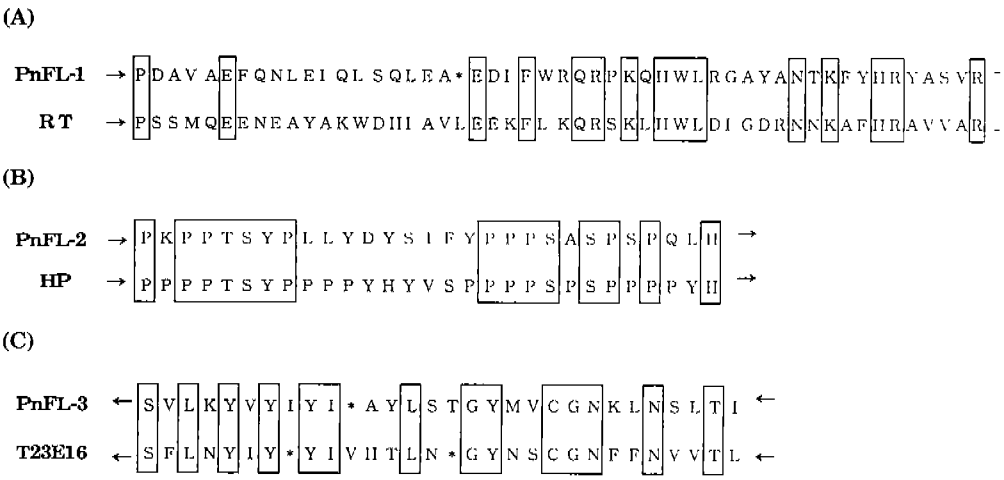

Figure 5. Comparison of amino acid sequences of PnFL-1, PnFL-2 or PnFL-3 with known proteins. Amino acid residues identical to each other are enclosed in boxes. RT, a clone of *Arabidopsis thaliana* similar to reverse transcriptase; HP, hydroxyproline-rich glycoprotein of *Glycine max*; T23E16, TAMU *Arabidopsis thaliana* genomic clone T23E16. Asterisks (*) indicate stop codons.

cotyledons exposed to a single inductive cycle (Fig. 2D). The level of *PnFL-2* mRNA, about 1.8 kb, was highest when three inductive cycles were imposed (Fig. 2E). *PnFL-3* mRNA of approximately 1.8 kb was detected only in cotyledons exposed to three cycles of the inductive dark period (Fig. 2F).

To determine whether these clones are a part of new gene identified to date, three cDNA fragments were reamplified by PCR and subjected to nucleotide sequence analysis. The size of *PnFL-1, PnFL-2* and *PnFL-3* cDNA fragments were found to be 228, 317 and 272 bp, respectively (Fig. 3).

Figure 4 shows nucleotide sequences and deduced amino acids of the cDNA fragments. To examine similarity the cDNA fragments have with any genes cloned to date, the sequences were analyzed with Blast DNA search program through GenBank and EMBL database. As shown in Fig. 5, *PnFL-1* had 29 % identity with the clone of *Arabidopsis thaliana* similar to reverse transcriptase (GenBank accession number 3047086), *PnFL-2* shared 50 % identity with hydroxyproline-rich glycoprotein of *Glycine max* (GenBank accession number 347455), and *PnFL-3* had 46 % identity with TAMU *Arabidopsis thaliana* genomic clone T23E16 (GenBank accession number B67574).

Despite many attempts so far have been focused on gene expression in the process of flower induction, a very limited number of genes and polypeptides have been found to be somewhat related to floral induction. A small heat-shock protein gene has been found to be regulated by a photoperiod that promoted flowering in *Pharbitis*[21], and a flower-inducing substance of high molecular mass has been extracted from *Lemna, Pharbitis, Brassica* and *Glycine* spp., which was a polypeptide of about 120 kDa[26]. A polypeptide that induced flowering in *Lemna paucicostata* 151 with amino-terminal sequence of Leu-Val-Gly-Asn-Thr was extracted[27]. Accumulation of germin-like protein mRNA in cotyledons of *Pharbitis* was found to increase transiently during flower-inductive darkness[17]. In recent reports of Sage-Ono *et al.*[18,19], they isolated from cotyledons of *Pharbitis* two cDNA clones; one designated *PnC401* and the other encoding a homolog of human translationally controlled tumor protein, and they observed fluctuations of the transcripts were significantly relevant to the flower- inductive process. The fact that cDNAs isolated in the present study do not show any homology to any known clones which are related to flowering leads to postulation that *PnFL-1, 2* and *3* are likely novel genes which are functionally associated with the floral induction process in *Pharbitis nil*. Further investigation is needed for full sequence analysis and characterization.

Acknowledgement – This work was supported by a grant from the Basic Science Research Institute Program, the Ministry of Education, Republic of Korea (BSRI-96-4420) to JM.

REFERENCES

1. Bernier, G. (1988) The control of floral evocation and morphogenesis. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 175-219.
2. Purse, J. G. (1984) Phloem exudate of *Perilla crispa* and its effects of flowering of *P. crispa* shoot explants. *J. Exp. Bot.* **35**, 227-238.
3. Ishioka, N., S. Tanimoto and H. Harada (1990) Flower-inducing activity of phloem exudate in cultured apices from *Pharbitis* seedlings. *Plant Cell Physiol.* **31**, 705-709.
4. Ishioka, N., S. Tanimoto and H. Harada (1991) Flower-inducing activity of phloem exudates from *Pharbitis* cotyledons exposed to various photoperiods. *Plant Cell Physiol.* **32**, 921-924.
5. Kim, S. H., M. W. Yi and J. Maeng (1994) Effects of electric current on flowering in *Pharbitis* and floral stimulus activity in the phloem exudate of cotyledons. *J. Plant Biol.* **37**, 159-166.

6. Kim, K. C., J. H. Lee, Y. Hur and J. Maeng (1996) Flower-inducing activity in the phloem exudate and gene expression specific to photoperiodic floral induction in *Pharbitis* cotyledons. *J. Plant Biol.* **39**, 457-463.
7. Warm, E. (1984) Changes in the composition of *in vitro* translated leaf mRNA caused by photoperiodic flower induction of *Hyoscyamus niger*. *Physiol. Plant.* **61**, 344-350.
8. Lechner, F. J. and W. Rau (1993) A complex pattern of changes in polysomal mRNA populations is evident in the leaves of *Arabidopsis thaliana* (L.) Heynh. during photoperiodic induction of flowering. *Planta* **189**, 522-532.
9. Stiles, J. I. and P. J. Davies (1976) Qualitative analysis by isoelectric focusing of the protein content of *Pharbitis nil* apices and cotyledons during floral induction. *Plant Cell Physiol.* **17**, 855-857.
10. Maeng, J. (1982) Immunological analysis of proteins in the leaf of *Pharbitis nil* during photoinduction of flowering. *Korean J. Bot.* **4**, 167-174.
11. Lay-Yee, M., R. M. Sachs and M. S. Reid (1987a) Changes in cotyledon mRNA during ethylene inhibition of floral induction in *Pharbitis nil* strain Violet. *Plant Physiol.* **84**, 545-548.
12. Lay-Yee, M., R. M. Sachs and M. S. Reid (1987b) Changes in cotyledon mRNA during floral induction of *Pharbitis nil* cv. Violet. *Planta* **171**, 104-109.
13. Bassett, C. L., C. P. Mothershed and G. A. Galau (1991) Polypeptide profiles from cotyledons of developing and photoperiodically induced seedlings of the Japanese morning glory (*Pharbitis*[*Ipomoea*]*nil*). *Plant Growth Reg.* **10**, 147-155.
14. Li, L. W. and K. H. Tan (1991) Specific protein occurrence related with photoperiodic flower induction in the cotyledons of *Pharbitis nil* cv. Violet. *Sci. in China* **34**, 570-575.
15. Ono, M., K. Yamada, K. Sage, M. Okazaki and H. Harada (1991) Changes in *in vitro* translated polypeptides of *Pharbitis nil* Chois., strain Violet cotyledons during the first flower-inductive and non-inductive photoperiods. *Plant Sci.* **78**, 11-18.
16. Ono, M., K. Sage-Ono, M. Yasui, M. Okazaki and H. Harada (1993) Changes in polypeptides in *Pharbitis* cotyledons during the first flower-inductive photoperiod. *Plant Sci.* **89**, 135-145.
17. Ono, M., K. Sage-Ono, M. Inoue, H. Kamada and H. Harada (1996) Transient increase in the level of mRNA for a germin-like protein in leaves of the short-day plant *Pharbitis nil* during the photoperiodic induction of flowering. *Plant Cell Physiol.* **37**, 855-861.
18. Sage-Ono, K., M. Ono, H. Harada and H. Kamada (1998a) Dark-induced accumulation of mRNA for a homolog of transcriptionally controlled tumor protein (TCTP) in *Pharbitis*. *Plant Cell Physiol.* **39**, 357-360.
19. Sage-Ono, K., M. Ono, H. Harada and H. Kamada (1998b) Accumulation of a clock-regulated transcript during flower-inductive darkness in *Pharbitis nil*. *Plant Physiol.* **116**, 1479-1485.
20. Felsheim, R. F. and A. Das (1992) Structure and expression of a heat-shock protein 83 gene of *Pharbitis nil*. *Plant Physiol.* **100**, 1764-1771.
21. Krishna, P., R. F. Felsheim, J. C. Larkin and A. Das (1992) Structure and light-induced expression of a small heat-shock protein gene of *Pharbitis nil*. *Plant Physiol.* **100**, 1772-1779.
22. O'Neill, S. D., X. S. Zhang and C. C. Zheng (1994) Dark and circadian regulation of mRNA accumulation in the short-day plant *Pharbitis nil*. *Plant Physiol.* **104**, 569-580.
23. Liang, P. and A. B. Pardee (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.
24. Chomczynski, P. and N. Sacchi (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.
25. Sambrook, J., E. F. Fritsch and T. Maniatis (1989) *Molecular Cloning: A Laboratory Manual.* 2nd ed. pp. 1.25-1.28. Cold Spring Harbor Laboratory Press, U.S.A.
26. Takeba, G., Y. Nakajima, A. Kozaki, O. Tanaka and Z. Kasai (1990) A flower-inducing substance of high molecular weight from higher plants. *Plant Physiol.* **94**, 1677-1681.
27. Kozaki, A., G. Takeba and O. Tanaka (1991) A polypeptide that induces flowering in *Lemna paucicostata* at a very low concentration. *Plant Physiol.* **95**, 1288-1290.