J. Plant Biol. 39(4) : 257~263 (1996)

Flower-Inducing Activity in the Phloem Exudate and Gene Expression Specific to Photoperiodic Floral Induction in *Pharbitis* Cotyledons

Kim, Kang Chang, Jin Hwan Lee†, Yoonkang Hur[1] and Jueson Maeng*

Department of Life Science, Sogang University, Seoul 100-611, Korea

[1]Department of Biology, Chungnam National University, Daejeon 302-764, Korea

Flower-inducing activity in the phloem exudate of *Pharbitis* cotyledons was investigated using the bioassay of *Pharbitis* and *Lemna*. By SDS-PAGE and 2-D gel electrophoresis of the phloem exudate, two polypeptides of 11 kDa and of approximately 32 kDa (pI 6.9) showing qualitative changes during the flower induction were detected. A polypeptide of approximately 20 kDa (pI 4.8) specifically labeled *in vivo* with [^{35}S]methionine was found during the inductive dark period in *Pharbitis* cotyledon tissues. The polypeptide of the equivalent molecular mass and with the identical pI value was also detected by *in vitro* translation assay. Thus, it is assumed that the 20 kDa polypeptide plays a role in the process of flower induction in *Pharbitis* cotyledons.

Keywords : *Pharbitis nil*, floral induction, gene expression in flowering, phloem exudate

Exposure of the leaves of plants to the appropriate photoperiod induces the production of chemical signal which is transmitted to the shoot apices where meristematic activity for vegetative growth is switched to the reproductive phase for the initiation of flower. There are evidences that promotive and/or inhibitory substances are involved in the induction process (Evans, 1960; Lang *et al.*, 1977; Bernier, 1988) and the activity of transmissible floral stimulus has been confirmed (Purse, 1984; Ishioka *et al.*, 1990, 1991; Kim *et al.*, 1994). However, the attempt to identify molecular components involved in the mechanism of floral induction has not been so successful that chemical identity of floral stimulus synthesized in induced leaves is still unknown. Changes in gene expression are also a component of photoperiodic floral induction (Kimpel and Doss, 1989). Qualitative changes of proteins in *Pharbitis* cotyledons have been detected during the inductive dark period (Stiles and Davies, 1976; Maeng, 1982; Bassett *et al.*, 1991; Li and Tan, 1991; Ono *et al.*, 1991, 1993), and differentiation of RNA populations in the photoperiodically induced *Pharbitis* cotyledons has been studied (Gressel *et al.*, 1978; Warm, 1984; Lay-Yee *et al.*, 1987a,b; Ono *et al.*, 1996).

In the present study, we confirmed the activity of floral stimulus in phloem exudate from photoperiodically induced *Pharbitis* cotyledons by the bioassay using *Pharbitis* and *Lemna* systems, and identified a gene product expressed during floral induction in cotyledons of *Pharbitis nil* Choisy cv. Violet, a short-day plant, by *in vivo* labeling and *in vitro* translation techniques.

MATERIALS AND METHODS

Plant Material 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 acid-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 15 W · m^{-2}.

*Corresponding author: Fax +82-2-705-8455
© 1996 by Botanical Society of Korea, Seoul
†Present address: LG Household & Health Care R & D Institute, Jang-dong, Yusung-gu, Taejeon 305-343, Korea

Collection of Phloem Exudate and Bioassay

Six day- old vegetative plants grown under non-inductive continuous light were exposed to a single 16 h dark period for flower induction. The collection of phloem exudate and the bioassay for measuring the floral stimulus activity at the apices of *Pharbitis* were performed according to Kim *et al.* (1994). *Lemna paucicostata* 6746, a short day plant, was also used in the bioassay to confirm the activity. A 3-frond colony of vegetative *Lemna* which has been aseptically grown under non-inductive continuous light was incubated in 10 mL of 1/10 strength E medium (Cleland and Briggs, 1969) containing the exudate of appropriate concentrations under various photoperiods. The activity of floral stimulus exerted in *Lemna* system was analyzed as described in the previous report (Maeng, 1977).

Protein Extraction from Phloem Exudate

One mL of phloem exudate was precipitated by addition of 0.25 mL of 50% (w/v) trichloroacetic acid followed by incubation at 4°C for 30 min. The pellet was harvested by spinning at 12,000 g for 5 min and resuspended in 1 mL of ice-cold acetone, spun down at 12,000 g for 5 min and was lyophilyzed. The sample was dissolved in 20 μL of SDS gel-loading buffer (0.06 M Tris-HCl (pH 6.8), 10% glycerol, 0.1% (w/v) bromophenol blue, 2% SDS, 5% β-mercaptoethanol) and stored at -70°C.

SDS-Polyacrylamide Gel Electrophoresis

Discontinuous SDS-PAGE (15% polyacrylamide) was performed according to Laemmli (1970) with a slight modification for analysis of protein profiles in the phloem exudate from *Pharbitis* cotyledons. Twenty μL of TCA precipitate was loaded onto the gel and the gel was run at constant current of 15 mA.

In Vivo Labeling and Protein Extraction

To analyze the polypeptides synthesized in intact cotyledons exposed to inductive or non-inductive photoperiods, vegetative seedlings with a pair of fully expanded cotyledons were excised at hypocotyl level and the cut end was kept submerged in 1 mL of HEPES buffer solution containing 100 μCi/mL [^{35}S]methionine (Amersham) for 3 h in the light for preconditioning. At the end of 16 and 24 h incubation in the light or dark periods respectively, labeled cotyledons were harvested, quickly frozen with liquid nitrogen and stored at -70°C.

The labeled cotyledons were ground in liquid nitrogen with a mortar and pestle. The powder of the tissues (1 g) was suspended in 100 μL of a sonication buffer (0.1 M Tris, 50 mL $MgCl_2 \cdot 6H_2O$, pH 7.4) and vortexed. The homogenate was centrifuged at 10,000 g for 1 min. The supernatant was collected and stored at -70°C until use. The concentrations of protein samples were determined by BCA protein assay (Pierce) using BSA as standard.

mRNA Extraction and *In Vitro* Translation

Cotyledons from either non-induced or induced seedlings were ground in liquid nitrogen with a mortar and pestle. Total RNA was extracted from 5 g of the powder using the single-step method of Chomczynski and Sacchi (1987).

Poly(A$^+$)-RNA was isolated from the total RNA dissolved in distilled water using QuickPrep mRNA purification kit (Pharmacia BioTech). One μg of mRNA was translated *in vitro* using rabbit reticulocyte lysate (Promega) in the presence of 20 μCi of [^{35}S]methionine (Amersham).

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed according to O'Farrell (1975) with a slight modification. Tube gels (length, 120 mm; internal diameter, 2.5 mm; 4% (w/v) polyacrylamide, 9 M urea, 2% (v/v) Nonidet P-40, 4% (v/v) Ampholine (pH 3.5-9.5: Pharmacia), 2% (v/v) Ampholine (pH 3.5-10.0: Pharmacia), 0.01% (w/v) ammonium persulfate, 0.1% (v/v) TEMED) were prepared for isoelectric focusing as the first dimension. The gels were loaded with 20 μL of sample solution, sonicated with 20 mg of urea for 30 seconds, overlayered with 10 μL of overlay solution (8 M urea, 0.8% (v/v) Ampholine (pH 3.5-9.5), 0.2% (v/v) Ampholine (pH 3.5-10.0), 5% Nonidet P-40). After isoelectric focusing at 400 V (16 h) and at 800 V (2 h), the gels were subjected to the second-direction electrophoresis at constant current of 15 mA. The second dimensional gels were prepared with 15% SDS- polyacrylamide. The gels were dried and exposed to X-ray film (Hyperfilm-β max, Amersham).

RESULTS AND DISCUSSION

Bioassay for Flower-Inducing Activity

Pharbitis cotyledons were exposed to a 16 h dark period for the induction of flower. At the end of the dark period, cotyledons were excised and phloem sap exuding from the cut ends of cotyledonary petioles was collected. Flower-inducing activity in the exudate was confirmed in the bioassay with *Pharbitis* and *Lemna* systems. A successful inducton of flower was observed in vegetative *Pharbitis* apices with the exudate added to the culture medium (Fig. 1). Flowering response of the plantlets was increased as the exudate in the culture medium was being concentrated; 5% phloem exudate in the medium evoked 20% flowering, while 40% exudate increased the flowering response three times.

The presence of flower-promoting activity in the phloem exudate from florally-induced *Pharbitis* cotyledons was also demonstrated in *Lemna* assay (Figs. 2 and 3). The exudate collected from non-induced cotyledons showed no promotive effect, while the one from induced cotyledons exerted its profound flower-promoting activity in *Lemna paucicostata* 6746, another short-day plant, grown under 15 h light-9 h dark or 14 h light-10 h dark cycles. It was noticed that the promotive activity of *Pharbitis* exudate on *Lemna* flowering was depressed at higher levels of the exudate in the medium, suggesting that each of the two short-day plants, *Pharbitis* and *Lemna*, requires different concentrations of flower-in-

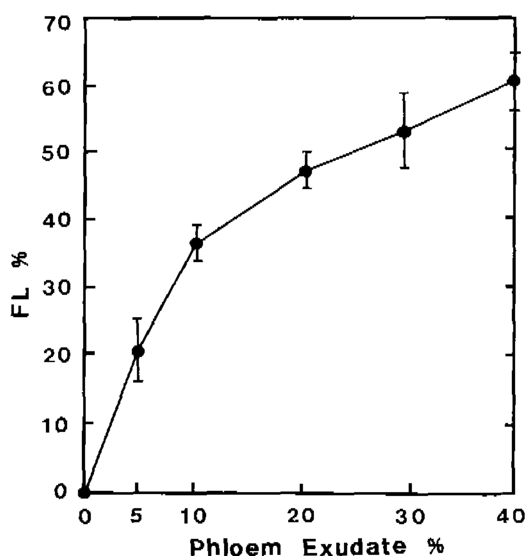

Fig. 1. Flowering responses of vegetative apex cultures of *Pharbitis nil* to the phloem exudate collected from florally-induced cotyledons of *Pharbitis nil* exposed to a single 16 h dark period. Phloem exudate had been lyophilyzed before added to the apex culture medium. Data are shown with standard errors.

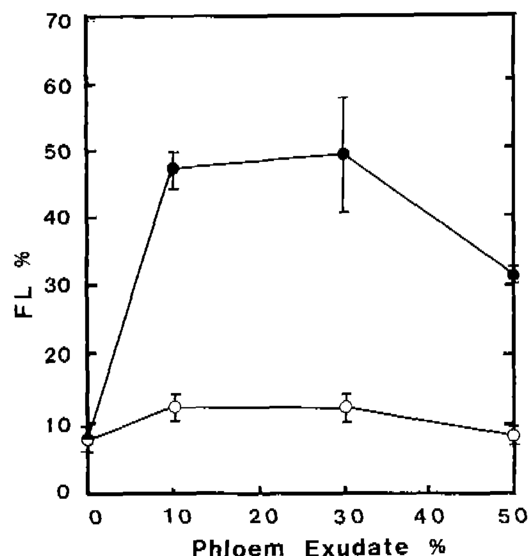

Fig. 2. Effect of phloem exudate of *Pharbitis* cotyledons on flowering of *Lemna paucicostata* 6746 grown under 15 h light -9 h dark cycles. Phloem exudate was collected at the end of a 16 h dark period (●) or collected under continuous light (○). FL%, flowering percentages. Data are shown with standard errors.

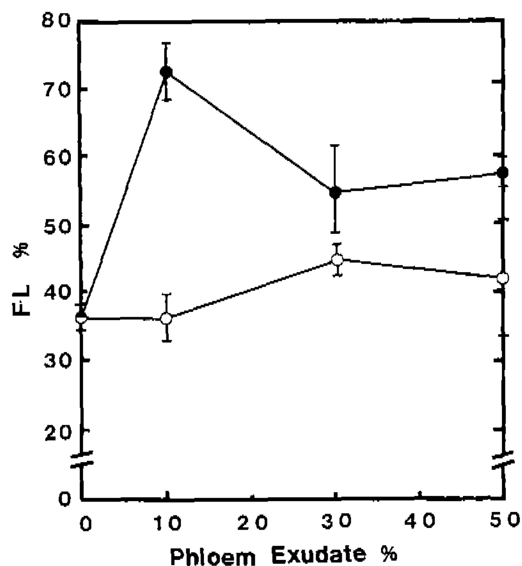

Fig. 3. Effect of phloem exudate of *Pharbitis* cotyledons on flowering of *Lemna paucicostata* 6746 grown under 14 h light-10 h dark cycles. Phloem exudate was collected at the end of a 16 h dark period (●) or collected under continuous light (○). FL%, flowering percentages. Data are shown with standard errors.

ducing substance(s) in *Pharbitis* exudate for the maximum induction of flower.

Peptide Profiles in Phloem Exudate

Although it was confirmed that the flower-inducing activity was present in the phloem exudate, it was inevitable to investigate the chemical idendity of the activity. As the first step, changes in protein profiles in the process of flower induction in the cotyledon tissues was analyzed. The protein samples were extracted from the concentrated phloem sap exuding out either from induced or non-induced cotyledons and were analyzed by electrophoresis (Fig. 4). In the induced exudate, the quantity of an approximately 40 kDa peptide was increased, while those of about 13, 31 and 43 kDa were reduced. Qualitative change of a peptide of 11 kDa specific to induced exudate indicated that substances of relatively low molecular weight may be involved in the flower-inducing activity in the exudate of *Pharbitis* cotyledons.

Two-dimensional gel electrophoresis was performed to detect the quantitative changes of the peptides in the phloem exudate during flower induction (Fig. 5). The quantity of an approximately 40 kDa peptide (pI 6.4) was increased in the exudate of induced cotyledons, whereas the amounts of six peptides of approximately 31 (pI 6.7), 35 (pI 6.3), 43 (pI 6.9), 48 (pI 5.8), 50 (pI 6.8) and 68 kDa (pI 5.9) were decreased. It was specifically noticed that the peptide of about 32 kDa with pI 6.9 was detected only in the induced exudate. This peptide was not detected by SDS-PAGE but by 2-dimensional gel electrophoresis. The possible explanation of this phenomena is that it might have been masked by peptides of similar size and unable to be detected by SDS-PAGE. The peptide of 11 kDa that appeared only with the inductive treatment on SDS-PAGE could not be detected. It was likely that it had a pI value beyond the range of the two-dimensional gel electrophoresis.

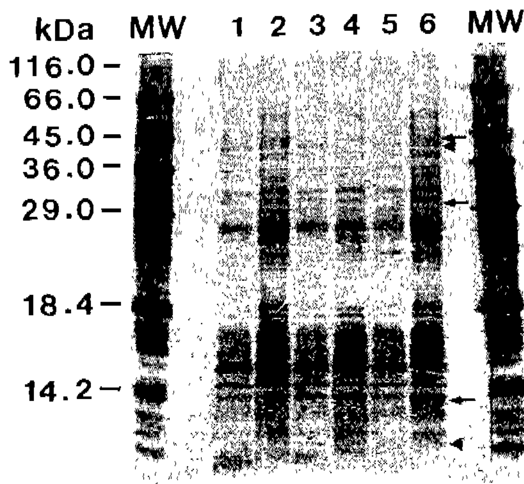

Fig. 4. Silver stained SDS-PAGE of the phloem exudate collected from induced (Lanes 1,3,5) and non-induced (Lanes 2,4,6) *Pharbitis* cotyledons. Each lane was loaded with equivalent amount of protein extracted from a cotyledon. Loci of peptides increased (◀) and decreased (⟵) in the exudate of induced *Pharbitis* cotyeldons are shown.

In Vivo Labeled Proteins

In leaves and cotyledons that photoperiodically in-

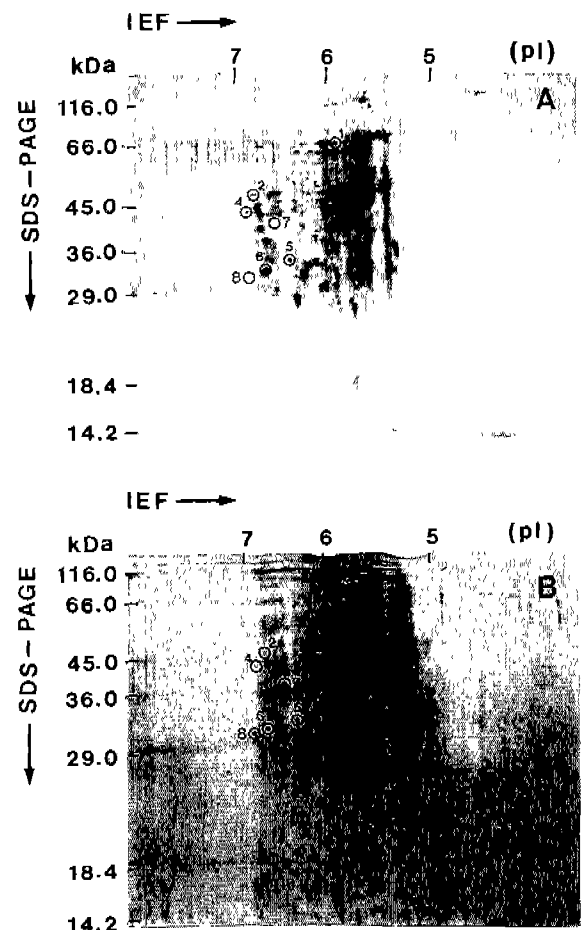

Fig. 5. Two-dimensional gel electrophoretic comparison of protein profiles in the phloem exudate of *Pharbitis* cotyledons. Exudates were collected in non-inductive continuous light (A), or at the end of a 16 h inductive dark period (B). Proteins with significant changes in their radioactive label are indicated by circles with numbers.

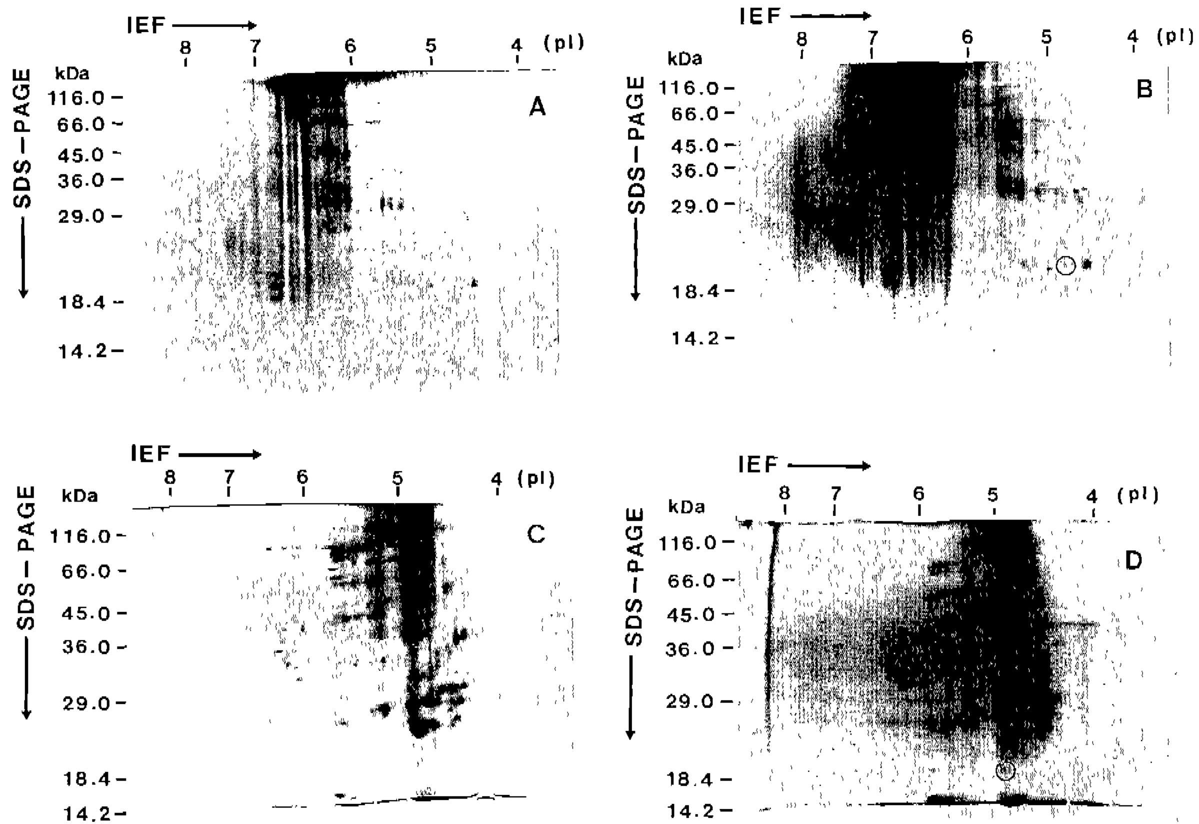

Fig. 6. Fluorograms of *in vivo* labeled proteins on 2-D gel electrophoresis extracted from *Pharbitis* cotyledons. Panels A, *in vivo* labeled proteins from non-induced cotyledons exposed to a 16 h light period; B, proteins from cotyledons induced by 16 h dark; C, proteins from non-induced cotyledons kept in a 24 h light period; D, proteins from cotyledons induced by 24 h dark. A circle in B or D indicates a locus of a protein synthesized specifically under the inductive condition.

duced to flower, genes specific to flower induction are expressed (Stiles and Davies, 1976; Lay-Yee *et al.*, 1987a,b). The proteins specifically synthesized during the process of flower induction in cotyledon tissues were examined by *in vivo* labeling with [^{35}S] methionine (Fig. 6). Within the range of molecular mass of approximately 14 to 120 kDa and with pI range between 4.0 and 8.0, more than 150 spots were located. A labeled polypeptide of about 20 kDa with pI value of approximately 4.8 was found in the fluorograms of the induced cotyledons. An equivalent spot could not be detected for non-induced vegetative cotyledons. It was observed that during inductive 16 h and 24 h dark periods, the protein specific to flower induction in cotyledons was synthesized. Bassett *et al.* (1991) could not find any differences in soluble proteins extracted from *Pharbitis* cotyledons by 2-D PAGE, while Li and Tan (1991) observed the appearance of a specific polypeptide spot of 19 kDa (pI 4.5) at 24 hours after 16 h dark period. Presumably 20 kDa protein that we detected would be identical to this 19 kDa peptide. Ono *et al.* (1993) also examined proteins labeled *in vivo* with [^{35}S]methionine extracted from *Pharbitis* cotyledons. They found no qualitative difference between induced and non-induced treatments. However, they found that the intensity of one polypeptide of 22 kDa with pI 7.5 showed quantitative changes. We were not able to find 22 kDa (pI 7.5) on the fluorograms. The discrepancy among the reports mentioned above may result from the differences of the experimental techniques.

In Vitro Translation Product

To examine whether the 20 kDa protein detected by *in vivo* labeling technique was a translation product from mRNA expressed during flower induction in cotyledons, mRNAs were extracted from induced and non-induced *Pharbitis* cotyledons and translated *in vitro* in the presence of [^{35}S]methionine using rabbit reticulocyte lysate. Two-dimensional polyacryla-

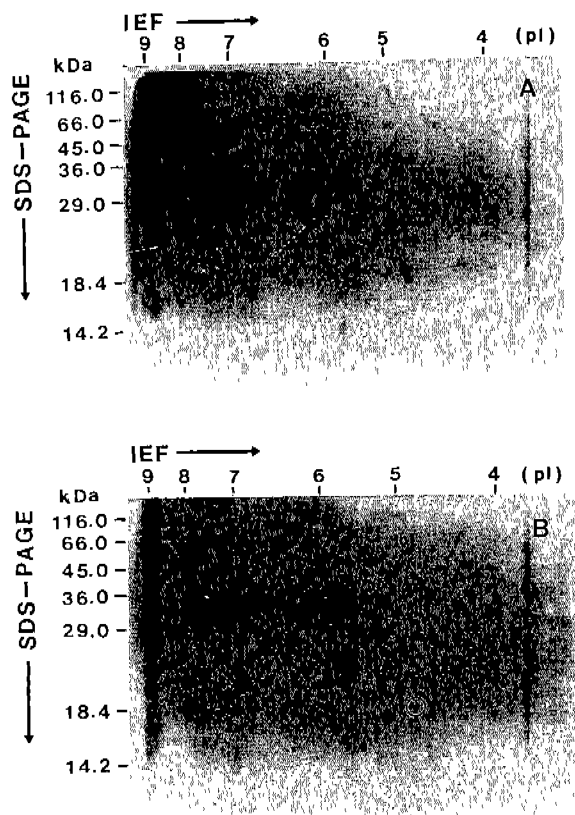

Fig. 7. Fluorograms of *in vitro* translation products on 2-D gel electrophoresis from mRNAs isolated from *Pharbitis* cotyledons treated with non-inductive 16 h light (Panel A), or from cotyledons exposed to an inductive 16 h dark period (Panel B). A circle in B shows a locus of a protein synthesized specifically during flower induction.

mide electrophoresis was performed to analyze the translation products (Fig. 7). More than 100 polypeptide spots were located on 2-D gels, most of which were found to have molecular mass of approximately 14-120 kDa and with pI values ranging 4.0-9.0. As shown in Figure 7, most of the translation products showed quantitative differences between induced and non-induced conditions. However, one spot of approximate molecular mass of 20 kDa with pI 4.8 could be identified only in induced system. This polypeptide which is identical to the one from the *in vivo* labeled polypeptides is assumed to be a product of a gene expressed specifically during the flower induction in *Pharbitis* cotyledons. However, it is not certain yet whether the peptide of 11 kDa detected in the phloem exudate (Fig. 4) was a processed form of 20 kDa protein or a product from 20 kDa-mediated gene expression.

Using a wheat germ system, Lay-Yee *et al.* (1987b) found a 28 kDa polypeptide with pI of 6.6 which was specific to the induced *Pharbitis* cotyledons. Using a rabbit reticulocyte lysate, Ono *et al.* (1991) failed to detect the spot (28 kDa) that Lay-Yee *et al.* had previously found. We were not able to spot the 28 kDa polypeptide either, since systems and methods we adopted were different from them used by Lay-Yee *et al.* The experimental systems used in the present study have some limitations that relatively smaller polypeptides of less than 14 kDa are beyond the ranges of detection. It can be suggested that *in vitro* translation will be performed with mRNAs of 500 bp or shorter to detect small sized-polypeptides specific to the flower induction in *Pharbitis* cotyledons, and the product will be separated on the high percentage gradient gel.

ACKNOWLEDGEMENTS

The present work was supported by a grant from the Basic Science Research Institute Program, the Ministry of Education, 1995 (BSRI-95-4220) to J. Maeng.

LITERATURE CITED

Bassett, C.L., C.P. Mothershed and G.A. Galau. 1991. Polypeptide profiles from cotyledons of developing and photoperiodically induced seedlings of Japanese morning glory (*Pharbitis(Ipomoea) nil*). *Plant Growth Reg.* **10**: 147-155.

Bernier, G. 1988. The control of floral evocation and morphogenesis, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39**: 175-219.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156-159.

Cleland, C.F. and W.R. Briggs. 1969. Gibberellin and CCC effects on flowering and growth in the long-day plant *Lemna gibba* G3. *Plant Physiol.* **44**: 503-507.

Evans, L.T. 1969. The nature of flower induction. *In* The Induction of Flowering. L.T. Evans (ed.). Macmillan, Australia, pp. 457-480.

Gressel, J., A. Zilberstein, L. Strausbauch and T. Arzee. 1978. Photoinduction of *Pharbitis* flowering, relationship to RNA synthesis and other metabolic events. *Photochem. Photobiol.* **27**: 237-240.

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.

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.

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.

Kimpel, J.A. and R.P. Doss. 1989. Gene expression during floral induction by leaves of *Perilla crispa*. *Flowering Newlett.* **7**: 20-25.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lang, A., M.KH. Chailakhyan and I.A. Frolova. 1977. Promotion and inhibition of flower formation in a day-neutral plant in grafts with a short-day plant and a long-day plant. *Proc. Natl. Acad. Sci. USA* **74**: 2412-2416.

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.

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.

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.

Maeng, J. 1977. Studies on the effect of 2-chloroethylphosphonic acid (Ethephon) on the floral induction in photoperiodic plants. *Korean J. Bot.* **20**: 77-82.

Maeng, J. 1982. Immunological analysis of proteins in the leaf of *Pharbitis nil* during photoinduction of flowering. *Korean J. Bot.* **4**: 167-174.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**: 4007-4021.

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.

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.

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.

Purse, J.G. 1984. Phloem exudate of *Perilla crispa* and its effects on flowering of *P. crispa* shoot explants. *J. Exp. Bot.* **35**: 227-238.

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.

Warm, E. 1984. Changes in the composition of *in vitro* translated leaf mRNA caused by photoperiodic flower induction of *Hyoscyamus niger*. *Physiol. Plantarum.* **61**: 344-350.

(Received December 5, 1996)