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# Polypeptide Profiles from Cotyledons of Developing and Photoperiodically Induced Seedlings of the Japanese Morning Glory (*Pharbitis [Ipomoea] nil*)

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**Abstract.** An unknown substance(s) produced in the cotyledons of seedlings of the Japanese morning glory (*Pharbitis nil*) during a defined period of darkness triggers the subsequent initiation of floral buds at apical and axillary meristems. Recent studies have concentrated on characterizing molecular changes as a possible mechanism associated with its synthesis, but these have failed to eliminate interference due to lack of development unity in the sampled population and to consider different kinetic alternatives of those potential changes. The current study demonstrates that numerous age-related changes occur in polypeptides from cotyledons during growth under noninductive conditions, but that these are minimal in older seedlings selected for improved synchrony of the floral response. Polypeptides from older seedlings sampled at various times during and after a dark inductive period were examined by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). No differences associated with floral induction could be detected. The results indicate that changes in gene expression correlated with floral-induction do not occur in the abundant polypeptide fraction within the limitations of the techniques employed.

Floral initiation in many plants is triggered by changes in day length, a process termed photoperiodic induction, and is believed to result from the synthesis of a substance (or substances) within the leaves and cotyledons of the induced plant (for a review see Kinet et al. 1985). Although many attempts have been made to isolate and identify this floral-inducing substance, no success has been reported to date. Changes in gene expression have been proposed as one possible mechanism regulating or accompanying the synthesis of a floral-inducing substance. Recent studies have supported this hypothesis in a number of experimental systems (Kannangara et al. 1990, Lay-Yee et al. 1987a,b, Warm 1984). However, other researchers have failed to find evidence for rapid changes in gene expression during induction (Friedman et al. 1987, Kimpel and Doss 1989, Ono et al. 1988), suggesting that evidence testing this hypothesis and perhaps the hypothesis itself should be critically reevaluated.

We have examined polypeptides present *in vivo* in cotyledons of the Japanese morning glory cv. Violet during growth and floral induction. The aim of the growth studies was to catalogue age-related differences in polypeptide profiles of seedlings that are capable of responding to photoperiodic induction and to determine morphological markers (after Lamoreaux et al. 1978) that correlate with these differences as criteria for selecting populations with improved synchrony. These populations were then used to examine cotyledon polypeptides during photoperiodic induction. The goal of these studies was to confirm whether or not changes in gene expression accompany this process, as some have reported, including changes that might arise as a consequence of posttranslational modification. We find no evidence of photoinduction-specific changes in the abundant polypeptides from cotyledons.

## Materials and Methods

### *Standard Growth Conditions and Staging Criteria*

Seeds of the Japanese morning glory cultivar Violet (*Pharbitis nil*

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Chois [*Ipomoea nil* Roth]) were obtained from the Marutane Seed Co., Kyoto, Japan and were selected for uniform size. They were imbibed and germinated in the dark for 1 day as previously described (Bassett et al. 1988), except that coarse sand was included in the water wash to improve scarification. At 1 day postimbibition (DPI), well-germinated seeds, with radicles protruding 2–5 mm, were planted in Fafard No. 3 potting soil (Conrad Fafard, Springfield, MA, USA) and placed in continuous light (CL). All light conditions used cool white fluorescent lights (General Electric) at 190 $\mu mol/m^2 s^{-1}$ photosynthetically active radiation (PAR). Seedlings were maintained at 25 ± 3°C throughout. Plants received Peter's 20-20-20 fertilizer (W. R. Grace Co.) at 15 and 22 DPI.

The following criteria were used to define standard seedling age. Only seedlings meeting these criteria were selected for sampling, treatments, or further growth: 2 DPI, no seedlings had emerged; 3 DPI, hypocotyls were erect and the pale, yellow-green cotyledons were vertical and appressed with tightly adhering seed coats (at the distal end of the cotyledon pair) which were removed; 4 DPI, cotyledons were somewhat vertical, and no longer appressed, but were not completely expanded as judged by their wrinkled appearance and were a medium green with no obvious yellow cast; and 5 DPI, cotyledons were horizontal and fully expanded with a smooth, dark green appearance.

### *Standard Floral Induction Conditions*

In order to test the competence of seedlings of various ages to respond to floral-induction conditions, seedlings were subjected to a single inducing short day (SD) by exposure to darkness for 16 h followed by illumination and growth under long day (LD) conditions (16 h light/8 h dark) until 25 DPI. Parallel noninduced controls were treated the same but received 16 h CL instead of 16 h dark. All plants were evaluated at 25 DPI as to the nodes at which floral meristems had developed.

### *Protocol for Floral Induction-Specific Development*

Floral induction requires a single minimum 11 h dark period uninterrupted by exposure to light (termed a night break, NB). Photoinduction-related changes are thus predicted to be reversed by a NB (Imamura 1967) and can be distinguished from the mere dark-related changes a SD photoinduction protocol may cause. Therefore, in order to identify potential induction-specific changes, 5 DPI seedlings with plumules 3–5 mm long (grown as usual under CL conditions) were divided into three treatment groups that varied only in light treatment during the next 16 h. Representatives of each group were sampled immediately before these differential treatments.

The CL treatment group was than transferred to another growth chamber and maintained under continuous illumination for 16 h. The SD and SD + NB treatment groups were left in the original growth chamber and the lights extinguished at 3 p.m. All lights in the room were also extinguished and the windows covered with black plastic to prevent light leaks during sampling. Representatives of the SD and SD + NB groups were sampled under green safe-lights at the indicated times, immediately before NB treatments. For NB treatments, cardboard boxes covered in black plastic were placed over the trays containing the SD seedlings, and the SD + NB seedlings were illuminated for 15 min, after which the lights were again extinguished and the boxes removed from the SD plants. After a total of 16 h dark for the SD, the remaining seedlings of all three groups were transferred to the same growth chamber, illuminated, and grown under LD for 20 days. Samples were taken at the indicated times, and plants remaining after all samples had been taken were scored for floral development as described above.

### *Extraction of Cotyledon Proteins*

To the extent possible with careful dissection, only cotyledons were collected. Cotyledon pairs from at least three seedlings were pooled, wrapped in tared aluminum foil, immersed in liquid nitrogen, and stored at −80°C prior to analysis. In preparation for extraction, they were transferred from −80°C to liquid nitrogen, quickly weighed to minimize thawing, and immediately returned to liquid nitrogen. Sodium dodecyl sulfate–soluble protein was isolated, quantified, and stored as previously described (Bassett et al. 1988), except that 40 μg/ml bestatin and 0.7 μg/ml pepstatin were included in the extraction buffer of samples from one of the experiments (the one whose results are reported here) to analyze age-related changes during growth under noninductive conditions.

### *Electrophoresis and Staining of Polypeptides*

Standard equilibrium two-dimensional gel electrophoresis (2-D PAGE) was performed as described (Bassett et al. 1988). Isoelectric focusing (IEF) marker proteins (BioRad) were included in one gel of each batch of IEF gels, and the pH gradient was confirmed in each run by slicing and eluting a control gel. Molecular mass marker proteins (Sigma MW-SDS-70 kit supplemented with phosphorylase B [$M_r$ = 92.5 kDa], β-galactosidase [$M_r$ = 116 kDa], and carbonic anhydrase [$M_r$ = 29 kDa]) were included in all 2-D gels. Polypeptides were visualized using a silver-based staining procedure described by Oakley et al. (1980) that produces a wide range of polypeptide-specific colors. Polypeptide spots were evaluated by visual inspection with the aid of a clear plastic grid as a reference guide.

Each sample was electrophoresed at least two times in order to check for potential electrophoretic or staining artifacts. Samples from each treatment group were electrophoresed in parallel in the first dimension (IEF) and on the same day in identical slab gel boxes for the second dimension and were compared to samples from at least two independent experiments to verify the consistency of constituent polypeptides and putative differences observed between samples.

## Results

### *Synchrony of Floral Response*

To examine the response of young seedlings to an inductive dark treatment and to determine their relative synchrony, seedlings 3, 4, and 5 DPI were subjected to a 16 h dark period followed by growth in LD for 20–22 days. The magnitude of the flowering response in each age group was determined by the percentage of individuals flowering and forming

**Table 1.** Synchrony of flowering response.

| | No. of plants scored | Flowering[b] (%) | Terminal floral buds[c] (%) | Lowest floral bud at node[d] 1 (% of plants) | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|
| Seedling age (DPI)[a] | | | | | | | |
| 3 | 42 | 100 | 100 | 95 | 5 | 0 | 0 |
| 4 | 43 | 100 | 100 | 60 | 40 | 0 | 0 |
| 5[e] | 138 | 100 | 100 | 6 | 51 | 42 | 1 |
| Plumule length at 5 DPI | | | | | | | |
| <2 mm | 40 | 100 | 100 | 17 | 78 | 5 | 0 |
| 2–2.9 mm | 55 | 100 | 100 | 2 | 60 | 38 | 0 |
| 3–5 mm | 43 | 100 | 100 | 0 | 16 | 82 | 2 |
| No induction[f] | 152 | 0 | 0 | — | — | — | — |

[a] Age of seedling at induction in days postimbibition (DPI).
[b] [No. individuals flowering/no. individuals] × 100.
[c] [No. individuals with terminal floral buds/no. individuals] × 100.
[d] Nodes are numbered acropetally from the cotyledons (node 0).
[e] Data from the groups selected by plumule length were combined and represent the relative synchrony of an unselected population at 5 DPI.
[f] Noninduced controls combined from 30 3 DPI, 31 4 DPI, and 91 5 DPI seedlings.

terminal floral buds; synchrony was measured as a function of the lowest node (numbered acropetally from the cotyledons) bearing a floral bud in its axil (Table 1). Seedlings at all ages examined were capable of maximum flowering response (100%) to a 16 h dark period in terms of both the percentage of the population flowering and percentage forming terminal floral buds. With increasing seedling age, the position of the lowest node bearing a floral bud shifted from the 1st and 2nd to the 2nd and 3rd nodes. Seedlings at 3 DPI appeared to be more synchronous by this criterion than either 4 DPI or unselected 5 DPI seedlings. Ninety-five percent of 3 DPI seedlings flowered at the first node, whereas the lowest flowering nodes were equally distributed between the first and second or second and third for 4 DPI and unselected 5 DPI seedlings, respectively. Synchrony in the 5 DPI population could be improved by selecting seedlings with plumule lengths between 3 and 5 mm (Table 1). In some experiments synchrony in this group has approached 100% with nearly all individuals having the 1st axillary flower in the 3rd node position, while synchrony of the 5 DPI plants with plumules <2 mm has been similar to that of 4 DPI seedlings (data not shown).

### *Age-Related Changes in Cotyledon Protein Profiles*

An average of 229 polypeptide spots were resolved by 2-D PAGE of proteins from pooled cotyledons of three to four seedlings at 3, 4, 5, and 6 DPI grown in continuous light, which is noninductive for flowering. These polypeptides range in size from about 14–80 kDa with pIs between 3.1 and 6.5 (Fig. 1A–D). The differences observed among profiles were for the most part quantitative and progressive. For example, a decrease in the abundance of polypeptides in the $M_r$ range of approximately 14–20 kDa was observed comparing cotyledons from 3 DPI through 6 DPI seedlings (Fig. 1A and B, circled spots absent in C and D), whereas other polypeptides increased in abundance in these seedlings (Fig. 1A–D, open arrows and the large subunit of rubisco). In addition, two polypeptides were reproducibly observed to decrease in 4 and 5 DPI seedlings before increasing again in 6 DPI seedlings (Fig. 1A–D, closed arrows). By 6 DPI the profiles were relatively stable, as fewer differences were noted among 7 and 8 DPI cotyledons examined in another experiment (data not shown).

### *Induction-Related Changes in Cotyledon Protein Profiles*

Separate experiments were designed to examine cotyledon polypeptide profiles at various times after the beginning of dark induction using 5 DPI seedlings selected for enhanced apical synchrony (plumules 3–5 mm in length). After the seedlings had been segregated into three treatment groups, pooled samples taken from three to four seedings of each group prior to treatment (0 h) were extracted

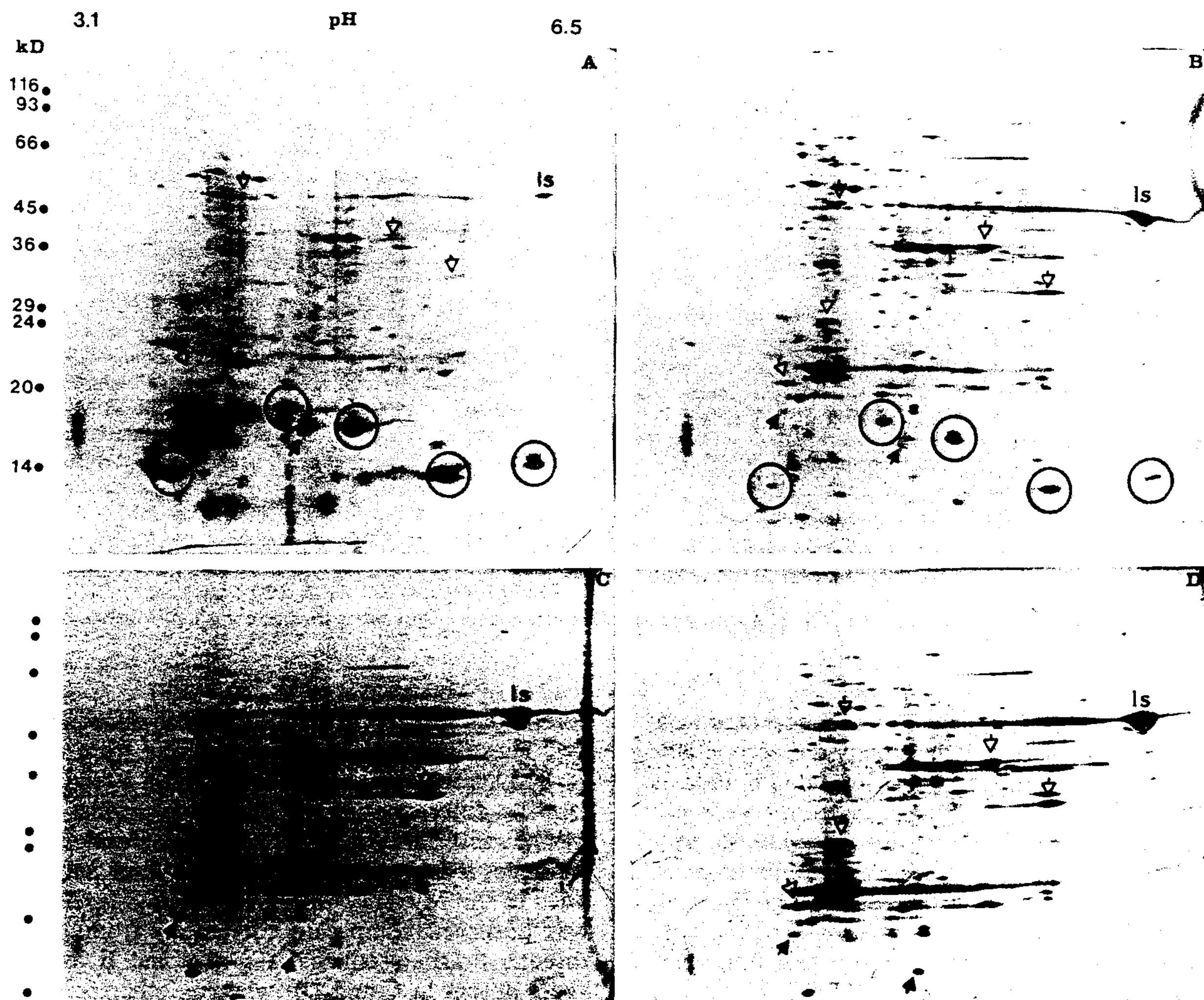


**Fig. 1.** 2-D PAGE profiles of cotyledon polypeptides during growth under noninductive conditions. Cotyledons were sampled and the proteins extracted, electrophoresed, and visualized as described in Materials and Methods. Cotyledon polypeptide profiles from seedlings 3 DPI (A), 4 DPI (B), 5 DPI (C), and 6 DPI (D). Molecular mass markers (kDa) are indicated to the left in A; pH of the IEF gels is indicated at the top of A. Polypeptides that increase in abundance between 3 and 6 DPI are indicated by open arrows; polypeptides that decrease in abundance between 3 and 4 DPI are circled. Closed arrows indicate polypeptides that decline from 3 to 4 DPI and then increase again from 5 to 6 DPI. ls, large subunit of rubisco.

and the polypeptide profiles analyzed for uniformity. The overall profiles were essentially identical with respect to the number of spots resolved and the positions of individual polypeptides (Fig. 2), thus confirming that the three populations were developmentally synchronous with respect to polypeptide profiles, as desired.

After the seedlings had been sampled at 0 h, they were subjected to one of the following three treatments: a 16 h dark inductive period (SD), a 16 h dark inductive period interrupted by three 15 min light exposures at 4, 8, and 12 h after the start of the dark treatment (SD + NB), or continued growth in the light (CL). Cotyledon pairs of three to four seedlings from each treatment were sampled at 4, 8, and 12 h after beginning the dark treatment. Assessment of unsampled plants (scoring controls) for floral response to treatment (Table 2) indicated maximal flowering and terminal floral bud formation in the SD group; no flowering was observed in the SD + NB or CL groups. Synchrony of the floral response in the SD group was determined to be 78% in this experiment (see Table 1, 5 DPI plants with plumules 3–5 mm).

Representative profiles from cotyledons sampled at 8 and 12 h after the start of induction are shown in Fig. 3. Although minor quantitative differences can be identified among treatments (e.g., Fig. 3,

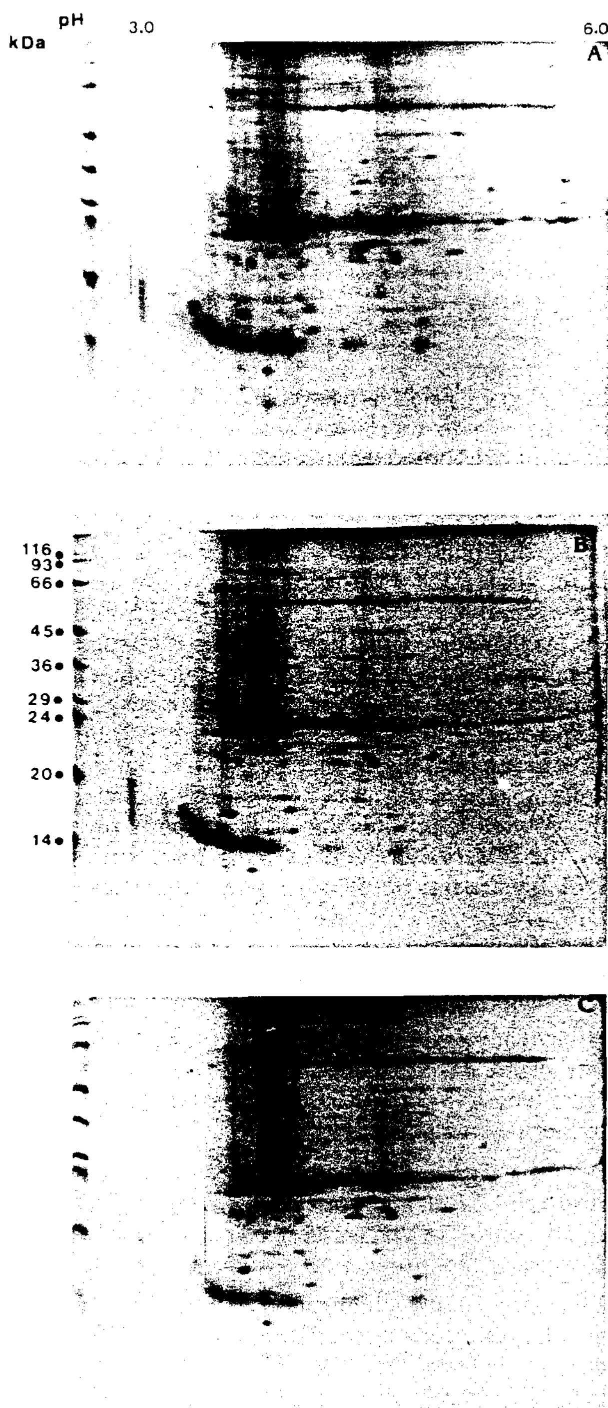


**Fig. 2.** 2-D PAGE profiles of cotyledon polypeptides from 5 DPI seedlings selected prior to photoperiodic treatment. Selected 5 DPI seedlings grown in continuous light were separated into three groups and each group sampled prior to treatment. (A) Seedlings designated for SD treatment; (B) seedlings designated for SD + NB treatment; (C) seedlings designated for CL treatment. Molecular mass markers (kDa) are indicated to the left of B; pH of the IEF gels is indicated at the top of A.

arrows), these differences were either not reproducible or, if reproducible, were not consistent with subsequent flowering behavior of the population. For example, one polypeptide was routinely observed to be more abundant in the 12 h CL controls than in the SD treatment (Fig. 3, circles), but this suppression of its accumulation by SD treatment is not related to subsequent flowering since its suppression also occurred in the SD + NB group which, like the CL controls, remained vegetative (Table 2). Samples taken at 4 h (data not shown) were essentially identical to those at 8 h shown in Fig. 3. Furthermore, in a separate experiment profiles from samples taken at 16 h after the beginning of the dark treatment, and at 8, 16, 24, 32, and 56 h after transfer to LD were not significantly different with respect to photoperiodic treatment (data not shown). In the latter experiments, samples were also separated by nonequilibrium pH gradient gel electrophoresis to resolve the more basic polypeptides and again no differences were noted between treatments. Therefore, the profiles within each time point sampled are essentially identical with respect to treatment up to 72 h after the start of induction.

## Discussion

In well-synchronized morning glory seedlings at 5 DPI, we have failed to detect any significant floral induction-specific changes in the more abundant polypeptides of the responding cotyledons during the period 4–72 h after the start of induction. Our approach differed from earlier ones in several respects that make this negative observation a significant result. To insure that sampling was representative, we have (1) delayed induction until after most developmental-specific changes have occurred, (2) improved the synchrony of the seedlings so that remaining developmental changes should be seen in all individuals of the population, (3) confirmed the uniformity of the population before treatment, (4) pooled three to four seedlings per time point and treatment, (5) used a color-producing silver stain to aid in polypeptide identification, and (6) repeatedly performed the electrophoretic separations to isolate artifacts from any true differences in the polypeptides. We also employed a control (SD + NB) treatment that reverses floral induction, in order to distinguish the result of simple dark induction from that associated with floral induction. In order to detect changes in gene expression at both the translation and posttranslation level we examined polypeptides directly. Finally, we examined the cotyledons over a time period appropriate to any plausible model of the kinetics of accumulation

**Table 2.** Subsequent flowering behavior of selected 5 DPI seedlings receiving SD, SD + NB, or CL treatment.[a]

| Treatment[b] | Total floral buds per plant | Flowering (%) | Terminal floral buds (%) | Lowest floral bud at node 1 (% of plants) | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|
| SD | 7 | 100 | 100 | 0 | 22 | 78 | 0 |
| SD + NB[c] | 0 | 0 | 0 | — | — | — | — |
| CL | 0 | 0 | 0 | — | — | — | — |

[a] 5 DPI seedlings were selected by plumule length (3–5 mm) prior to treatment.
[b] SD = 16 h dark treatment; SD + NB = 15 min light interrupting dark treatment; CL = continuous light (no dark treatment given).
[c] Night breaks were given at 4, 8, and 12 h after the start of induction; N = 9 seedlings.

of a floral inducer. Several of these points are discussed separately below.

*Influence of Seedling Age on the Synchrony of the Floral Response and on Polypeptide Profiles from Uninduced Cotyledons*

The response of seedlings of different ages (3–5 DPI) to SD treatment was maximal with respect to the percentage of individuals flowering and setting terminal floral buds. In similar experiments, maximal expression of terminal flower buds was not observed until seedlings were 6 days old (equivalent to 5 DPI in our experiments) at the time of induction (King and Evans 1969) or 4 DPI according to Owens and Paolillo (1986b). Likewise, maximal flowering (100% of the individuals flowering) was obtained only with seedlings equivalent to 4 DPI or older when grown under CL prior to a 16 h dark inductive period (Marushige and Takimoto 1967).

The relative developmental state of the apex correlated with seedling age at the time of floral induction (measured by the position of the first axillary floral bud), as has been demonstrated previously in morning glory (Imamura 1967, King and Evans 1969, Owens and Paolillo 1986a), However, the synchrony of the population of induced individuals with respect to the lowest axillary floral bud was only established in our study and the study by Owens and Paolillo (1986a). Their results with seedlings 5 DPI selected solely by cotyledon expansion and/or position (vertical or horizontal) were similar to ours for unselected 5 DPI seedlings. This indicates that improved synchrony of seedlings 5 DPI can only be achieved with an additional selection by plumule length (M. Christianson, personal communication, after Lamoreaux et al. 1978) prior to induction.

Analysis of polypeptide changes occurring in the cotyledons following germination and growth under uniform conditions noninductive for flowering (CL) demonstrate that numerous changes occur during the 5 days following imbibition, with a gradual stabilization of profiles between 5 and 6 DPI. These changes (both increases and decreases in abundance) are consistent with the physiological changes thought to occur in this organ during the transition from storage to photosynthetic function (Marushige and Marushige 1966, Riedell 1983), the latter illustrated by the dramatic increase in the large subunit of rubisco between 3 and 5 DPI (Fig. 1). They are also consistent with those changes seen in polypeptides and mRNAs in cotyledons of other germinating embryos, for instance those of cotton (Dure et al. 1981, Hughes and Galau 1989) and *Brassica napis* (Harada et al. 1988). That the developmental state of the apex correlates with changes in the cotyledon profiles during this time is supported by the observation that extracts from seedlings 3 DPI (synchrony ~95%, Table 1) and seedlings 5 DPI with plumules between 3 and 5 mm (synchrony 78–82%, Tables 1 and 2) are more reproducible between experiments than those of seedlings 4 DPI (synchrony ~60%) or unselected seedlings 5 DPI (synchrony ~51%) and by the fact that the polypeptide profiles from seedlings 5 DPI selected by plumule length (3–5 mm) are virtually identical prior to inductive treatment.

*Analysis of Polypeptide Profiles During Floral Induction of 5 DPI Seedlings Selected for Enhanced Developmental Synchrony*

Developmentally synchronous populations of 5 DPI seedlings subjected to a SD, SD + NB, or CL treatment were analyzed for differences in cotyledon proteins by 2-D PAGE. By examining the extant polypeptides present in cotyledons, these experiments should have revealed both qualitative and quantitative differences in proteins which would reflect direct and indirect alterations in gene expression provided (1) that such changes were associated with the abundant polypeptide population and (2) that such differences were of a magnitude sufficient to distinguish from those due to technical limitations (i.e., loading, staining, etc). In addition, these experiments were designed to accommodate sev-

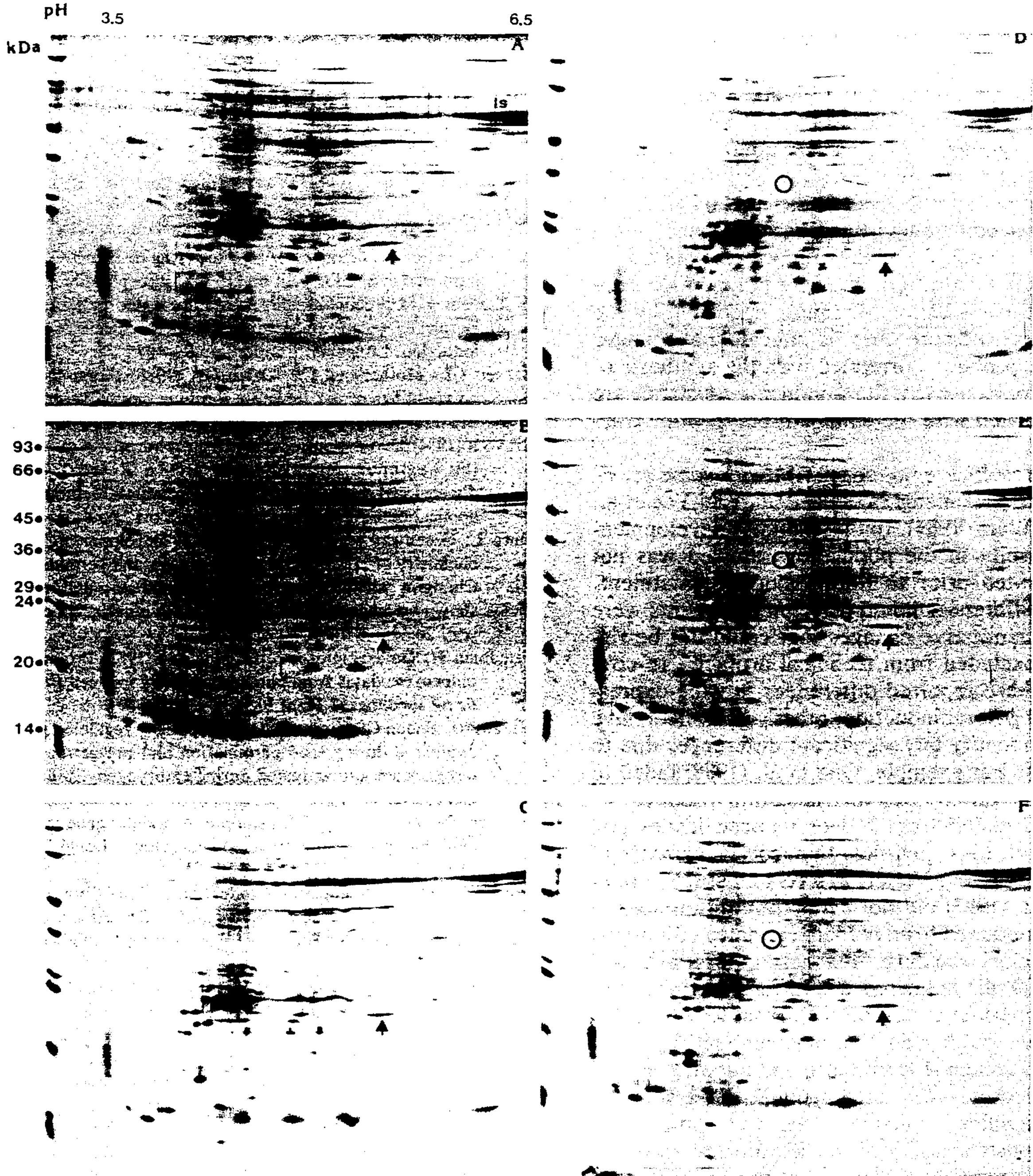


**Fig. 3.** 2-D PAGE profiles of cotyledon polypeptides during photoperiodic induction of selected 5 DPI seedlings. Proteins from cotyledons of SD-, SD + NB-, and CL-treated seedlings were isolated and electrophoresed as described in Materials and Methods. (A–C) Seedlings 8 h after the beginning of treatment. (A) SD; (B) SD + NB; (C) CL. (D–F) Seedlings 12 h after the beginning of treatment. (D) SD; (E) SD + NB; (F) CL. Samples A–F were electrophoresed together (IEF and slab gels). Molecular mass markers (kDa) are indicated to the left of B; pH of the IEF gels is indicated at the top of A. Closed arrows indicate a polypeptide that varies in abundance as a result of loading/electrophoresis artifacts; circles indicate a polypeptide that reproducibly is observed to be more abundant in CL-treated seedlings than in the SD or SD + NB groups. ls, large subunit of rubisco.

eral kinetic alternatives that might be associated with putative changes in gene expression as a mechanism to explain floral induction. For example, the critical night length for flowering in "Violet" is 10–11 h after the start of a single inductive dark period (Imamura 1967), i.e., interruption of the dark period before this time prevents flowering. The earliest documented apical response to floral induction in morning glory (an increase in DNA synthesis in the central zone) occurs approximately 48 h after the start of the inductive dark period (Arzee et al. 1970). Therefore, if changes in gene expression reg-

ulate or accompany synthesis of a floral-inducing substance, they must begin before the 10th h and be at maximal or nearly maximal levels some time prior to 48 h after the start of induction, assuming that neither transport (King et al. 1968, Wardlaw 1972) nor synthesis of a possibly nonproteinaceous hormone are rate-limiting. Our experiments included sampling times before, during, and after this period to accommodate the range of kinetic possibilities. Although the results do not rule out possible changes occurring in the rare polypeptide population at levels below the limits detectable by the techniques used here, they do indicate that changes in gene expression correlated with the synthesis of or *in situ* response to a floral-inducing substance are not associated with the abundant polypeptide fraction.

These results are not consistent with some previous reports (Kannangara et al. 1990, Lay-Yee et al. 1987a,b, Warm 1984). In these studies developmental uniformity in the population sampled was not demonstrated prior to floral-inductive treatment, and the differences noted between treatments were subtle, quantitative changes that could not be rigorously excluded from technical artifacts. In contrast to these reported differences in gene expression during floral induction, other researchers have failed to identify any significant differences due to treatment. For example, Ono et al. (1988) failed to detect any differences in translation products of mRNAs isolated from NB or induced leaves (1st leaf), cotyledons, petioles, hypocotyls, or roots of morning glory cv. Violet. In a related study, Friedman et al. (1987) did not detect any differences in proteins obtained from phloem exudates of induced morning glory seedlings. Furthermore, Kimpel and Doss (1989) did not detect any differences in *in vitro* translation products of mRNAs from leaves of *Perilla* grown under inductive or noninductive conditions. We conclude from these and our studies that floral induction does not appear to be associated with a significant change in the concentration or obvious posttranslational modification of an abundant polypeptide. Thus, the hypothesis that changes in gene expression regulate or accompany synthesis of a floral-inducing substance must apply only to gene products of the rare abundance class. Alternatively, synthesis of a floral-inducing substance may rely on biochemical alterations that change the activity of preexisting enzymes which themselves are most likely associated with the low abundance class polypeptides.

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