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DOI: https://doi.org/10.5169/seals-740198

Archs. Sci. Genève	Vol. 44	Fasc. 2	pp. 227-234	Genève, juin 1991

POLYPEPTIDE PATTERN MODIFICATIONS DURING FLORAL INDUCTION IN LEAVES OF *SPINACIA OLERACEA*

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ABSTRACT

Flowering of spinach plants *(Spinacia oleracea* L. cv. Nobel) was induced by photoperiodic treatment. Leaf epidermis and epidermis-free leaf disks of induced and non-induced plants were incubated in presence of ^{35}S methionine. The proteins were extracted and analysed by two-dimensional (2D) gel electrophoresis. Silver stained 2D gels were exposed to X-ray films. No reproducible differences were found between induced and control plants among accumulated proteins, revealed by silver staining. For newly synthesized proteins revealed by autoradiography, one 30 kDa protein decreased in amount in induced leaves, when another one of 29 kDa increased compared to non-induced leaves. In epidermis the same observation was made, and another protein of 38 kDa was increased. No new protein was reproducibly detected after 24 hours of continuous light, i.e. about 12 hours after the critical photoperiod.

Key words: floral induction, spinach, *Spinacia cleracea*, 2D electrophoresis.

RÉSUMÉ

La floraison a été induite chez des plantes d'épinards (*Spinacia oleracea* L. cv. Nobel) par traitement photopériodique. Des épidermes inférieurs de feuilles et des disques de feuilles sans épidermes inférieurs, provenant de plantes induites et non induites, ont été incubés en présence de méthionine S^{35}. Les protéines ont été extraites et analysées par électrophorèse bidimensionnelle (2D). Les gels de 2D révélés à l'argent ont été ensuite exposés contre des films sensibles aux rayons X. Au niveau des protéines accumulées, la révélation à l'argent n'a pas permis de mettre en évidence de différences reproductibles entre les plantes induites et les plantes témoins. Par contre, parmi les protéines néosynthétisées (révélées par autoradiographie), une protéine de 30 kDa diminuait en quantité dans les feuilles induites, tandis qu'une autre de 29 kDa augmentait par rapport aux feuilles non induites. Dans l'épiderme le même phénomène a été observé. Une autre protéine de 38 kDa augmentait par rapport au témoin. Mais aucune nouvelle protéine n'a été détectée de façon reproductible après 24 heures de lumière, c'est-à-dire environ 12 heures après la photopériode critique.

Mots clés: électrophorèse bidimensionnelle, épinard, induction florale, *Spinacia oleracea*.

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INTRODUCTION

Floral induction is often dependent upon environmental conditions and especially photoperiod (for a review see Vince-Prue *et al.*, 1984; Atherton *et al.*, 1987) in numerous plant species. Although many species are able to flower under almost any conditions compatible with continuous growth, plants are generally classified as long day, day-neutral and short day plants. In spinach, which is a long day plant (LDP), floral induction occurs after an increase of the daylength. Inversely, in short day plants (SDP), induction takes place after a shortening of the daylength. This complex phenomenon in photoperiodically sensitive plants is still little understood, although it has been reported as soon as 1912 (Tournois, 1912; Garner and Allard, 1920).

As leaves have been shown to be the site of perception of the photoperiod (Knott, 1934 and for a review see Evans, 1971), we focused our interest on the primary events that precede the arrival of the inductive stimulus to the apical bud, leading to its floral evocation (Bernier *et al.*, 1981), i.e. the events ocuring in the leaf or in its epidermis.

Two-dimensional (2D) gel electrophoresis of proteins (O'Farrel, 1975) has been adapted to plant material and proved to be useful for genetic (Zivy *et al.*, 1983, Colas des Francs and Thiellement, 1985, Thiellement *et al.*, 1987) as for physiological studies (for a review *see* Damerval *et al.*, 1988), since several hundreds of gene products can be detected at once on a single gel.

Only few attempts have been made to detect polypeptide pattern modifications occuring in the leaves during floral induction by the use of this technique (Warm, 1984, Lay-Yee *et al.*, 1987, Kannangara *et al.*, 1990).

Here we report the results obtained by comparing 2D gels from induced and non-induced spinach leaves (lower epidermis and lower epidermis-free leaves).

Abbreviations:

CHAPS. 3- (3-chloroamydopropyl) dimethylammonio] 1-propane-sulfonate, 2D: two-dimensional, DTT: dithiothreitol, HPBS: high salt concentration phosphate buffer saline, LDP: long day plant, MES: 2(N-morpholino) ethanesulfonic acid, MK: MES-potassium buffer, kDa: kilodalton; mRNA: messenger ribonucleic acid, poly (A) RNA: polyadenylated ribonucleic acid, SDS: sodium dodecyl sulfate, SDP: short day plant, TCA: trichloroacetic acid, TEMED: N,N,N',N'-tetramethylethylene- diamine.

MATERIAL AND METHODS

Plant material

Spinach plants (*Spinacia oleracea* L. cv. Nobel) were grown for 4 weeks in a growth chamber under short day illumination (400 μ mol.m^{-2}s^{-1}) of 8h light period. The temperature was maintained at about 20°C. Plants were photoinduced by transfer to

continuous light (400 μ mol.$m^{-2}s^{-1}$) for 24h. The light was provided by white (40W) 24432-0 fluorescent tubes (Sylvania, USA). The relative humidity was maintained at ca. 80%. Control plants remained under short day conditions. The critical photoperiod for flower induction in spinach is about 11-12 hours of continuous light (Greppin et al., 1990).

Incubation and protein extraction

Methionine incorporation in leaf disks was helped by peeling off the lower epidermis. The resulting epidermis were submitted to the same treatment than the leaf disks.

30-50 mg lower epidermis-free leaf disks (diam. 0.5 cm) and lower epidermis were incubated in 250 μ 1 MK buffer (2mM MES, 4.8mM KC1, 8 μ M $MgC1_2$, pH 6.5) containing 20-50 μ Ci of ^{35}S methionine (Amersham, UK, 550 MBq ml^{-1}, > 1000 Ci $mmol^{-1}$) for 3-4 hours at room temperature (ca. 20°C).

After incubation, each sample was extracted with 0.4 ml HPBS buffer (500 mM NaC1, 2.7 nM KC1, 8 mM Na_2HPO_4, 1,5 mM KH_2PO_4, pH 7.4). The slurry was centrifuged for 10 min. at 12,000 g_{max} (10,000 rpm, Sorvall, SS34 rotor) at 4°C and the pellet was discarded.

The resulting supernatant was then transferred to a new Eppendorf tube, precipitated by addition of trichloroacetic acid (TCA) and 2 β mercaptoethanol to a final concentration of 10% (w/v) and 0.07% (v/v) respectively.

The tube was then left at 4°C for at least 1 hour. The precipitate was spun 10 min. at 20,000g_{max} (13,000 rpm, Sorvall, SS34 rotor) at 4°C. The supernatant was removed and the pellet gently covered with 1 ml acetone containing 0.7% (v/v) 2 β mercaptoethanol for 30 min. at -20°C.

Acetone was then drained off with a Pasteur pipette and the pellet dried under vacuum for 5 minutes. The dried pellet was resuspended in the solubilization buffer (9.5 M urea, 5mM K_2CO_3, 1.25% SDS, 0.5% DTT, 6% Triton X-100, 2% ampholines pH 3.5-10) of Zivy (in Damerval *et al.*, 1986). Resuspension was helped by ultrasonication of the sample in an ultrasonic bath (Telsonic, Switzerland). After a quick centrifugation (3 min. at 10,000 g in a Beckman Microfuge), the supernatant was transferred to a new Eppendorf tube. At this point, samples were ready to be immediately used for first dimensional electrophoresis or kept frozen at -20°C for ulterior use. Samples were counted in Beckman Ready value liquid scintillation cocktail in a Beckman LS 1800 scintillator.

Two-dimensional electrophoresis

The procedure was according to Hochstrasser *et al.* (1988) using the following devices: a Model 175 Tube Cell (Bio-Rad, USA) coupled to an electrophoresis constant voltage power supply ECPS 3000/150 (Pharmacia, Sweden) for the first dimension and

a Protean II 2-D Multi-Cell (Bio-Rad, USA) coupled to a constant voltage power supply Model 1000/500 (Bio-Rad, USA).

Isoelectrophoresis was conducted in Bio-Rad 1.5 mm inner diameter and 20 cm long capillary tubes.

The mixture was as follows: 10 g urea, 7 ml deionized water, 2.5 ml acrylamide/ diacrylpiperazine (30% / 0.8%), 0.4 ml ampholines pH 3.5-10, 0.6 ml ampholines pH 4-8, 1ml of a solution containing 0.3 g CHAPS, 0.1 ml Nonidet P-40 and 0.9 ml deionized water, 20 µ 1 TEMED and 40 µ 1 of a 10% ammonium persulfate solution.

Twelve samples were usually run overnight for 30,000 volts. hours. Each sample loaded contained 300,000 cpm of incorporated ^{35}S methionine. The "SDS" dimension was conducted in batches of 6 gels according to Hochstrasser *et al.* (1988), except we used uniform 12% total acrylamide concentration gels. The gels were silver stained according to Oakley *et al.* (1980) modified by Hochstrasser *et al.* (1988). After drying, the gels were exposed on Hyperfilm β Max (Amersham, UK) for 10 days and developed. Chemicals were obtained from Bio-Rad (USA) and Fluka (Switzerland), ampholines were Resolyte from BDH (UK).

Each experiment, from extraction to electrophoresis of induced and non-induced plants, was repeated at least 3 times.

RESULTS

Examples of protein patterns obtained from spinach leaf lower epidermis and leaves without lower epidermis are shown in fig. 1 and fig. 2. No reproducible difference was found between induced and non-induced spinach plants among more than 1000 protein spots revealed by silver staining (accumulated proteins).

Concerning the newly synthesized proteins, among more than 500 spots revealed by autoradiography, only two show a reproducible detectable quantitative difference on the lower epidermis-free leaf pattern (fig. 3). One spot of 30 kDa apparent molecular mass is decreased when another one, located just below, and of an apparent molecular mass of 29 kDa, is increased after 24h of continuous light.

On the lower epidermis autoradiograms, the same two spots behave in the same manner, although they are both more intense in this fraction. One additional 38 kDa spot is more abundant in induced conditions (fig. 4). These spots easily detected on autoradiograms can hardly be seen on the silver stained gels themselves, where they are too faint for any reproducible variation to be ascertained.

This can be noticed on the figures where the same 2D gels are shown, revealed either by silver staining or by autoradiography.

Thus, after 24 hours of continuous light, only three polypeptides are quantitatively modified in the spinach leaves at the level of newly synthesised proteins. No new

protein is reproducibly found in the induced plant leaves and no protein disappears, compared to the non-induced plants.

DISCUSSION

Despite numerous attempts, all experiments devoted to isolate in leaves a protein specifically related to the floral stimulus have failed (CLELAND, 1978). However, recently TAKEBA *et al.* (1890) report the finding of a flower inducing substance of 120 kDa.

In the recent years few authors have studied the polypeptide changes associated with the floral induction in the leaves. WARM (1984) detected at least 10 modifications concerning polypeptides ranging from 4 to 48 kDa with pIs between 4.9 and 6.4 when comparing in vitro translated mRNA from induced and non-induced *Hyoscyamus niger* L. (annual strain, LDP) leaves. But this experiment has been made with 10 week old plants, induced during three days by exposition to continuous light and returned 14 hours to darkness before mRNA extraction. So the newly synthesized polypeptides could not be considered as early products of the induction, since *H. niger* needs a critical photoperiod of 12-13 hours under the fluorescent light used in this experiment and at least two days of continuous illumination to be induced (LANG, 1986).

LAY-YEE *et al.* (1987) have found one 28 kDa polypeptide more abundant in induced leaves from in vitro translation of poly (A) RNA isolated from induced and non-induced *Pharbitis nil* cv. Violet (SDP).

KANNANGARA *et al.* (1990) found that three polypeptides of 16, 15 and 9 kDa were not more detectable in induced leaf blades of *Xanthium strumarum* (SDP) compared to non-induced ones.

In the experiments reported in the results section, plants were induced to flower by lengthening the light period. It is likely that other metabolic effects were also induced, not related to the floral induction process.

Suggestions and experiments proposed to encompass this problem do not seem fully satisfactory (return to SD after 3 days light exposure, gibberellic acid treatment, etc...) This beeing especially true in LDP. However we only observed a very small number of changes on the polypeptide patterns.

Only the more abundant proteins are dectected by silver staining of the gels, and only the ones whose isoelectric points lie approximately between pH 4 and pH 9 and whose apparent molecular masses range from 100 to 10 kDa. Thus, even if a very great number of proteins are examined, we cannot discard the possibility of appearance, disappearance or quantitative modification of proteins. Those can be either more acidic or basic than the pH range used. Those can also be hidden under the very abundant spots due to the different forms of the large sub unit of the ribulose bisphosphate carboxylase oxygenase (EC 4.1.1.39) which represents about 50 per cent of the leaf proteins.

As discussed before and elsewere (ZIVY *et al.*, 1984, BAHRMAN *et al.*, 1985), any disappearance or appearance of a spot revealed by silver staining may in fact reflect quantitative modification of a polypeptide whose amount is close to the level of detection. Thus any qualitative difference is very difficult to ascertain except if genetical studies are undertaken, with mutually exclusive allelic forms for instance. The experiment described in this paper show that only quantitative modifications of three polypeptides are found to be associated with the induced conditions in *Spinacia oleracea* leaves. The close location on the 2D gels of two of them, the 30 and the 29 kDa spots, and their relative quantitative variation may indicate that they are structurally related. They can be for instance the precursor and the mature forms of a same protein, or the result of another kind of post-transcriptional or post-translational modification. One may also notice the similarity in molecular mass with the polypeptide reported to be increased in induced conditions by LAY-YEE *et al.* (1987).

Thus, in our experimental conditions, no new specific protein synthesis appears to be associated with the first events of floral induction. Or, at least, no new polypeptide is synthesized in a sufficient amount to be detected in spinach leaves 11 to 12 hours after the critical photoperiod. This result relies with the idea that the floral stimulus does not necessarily require new gene activation to be integrated by the leaf tissues and transmitted to the apical meristems. Accordingly, it is only when the signal reaches the apices that new sets of genes (for instance homeotic genes according to COEN *et al.*, 1990), corresponding to the new developmental program, are activated.

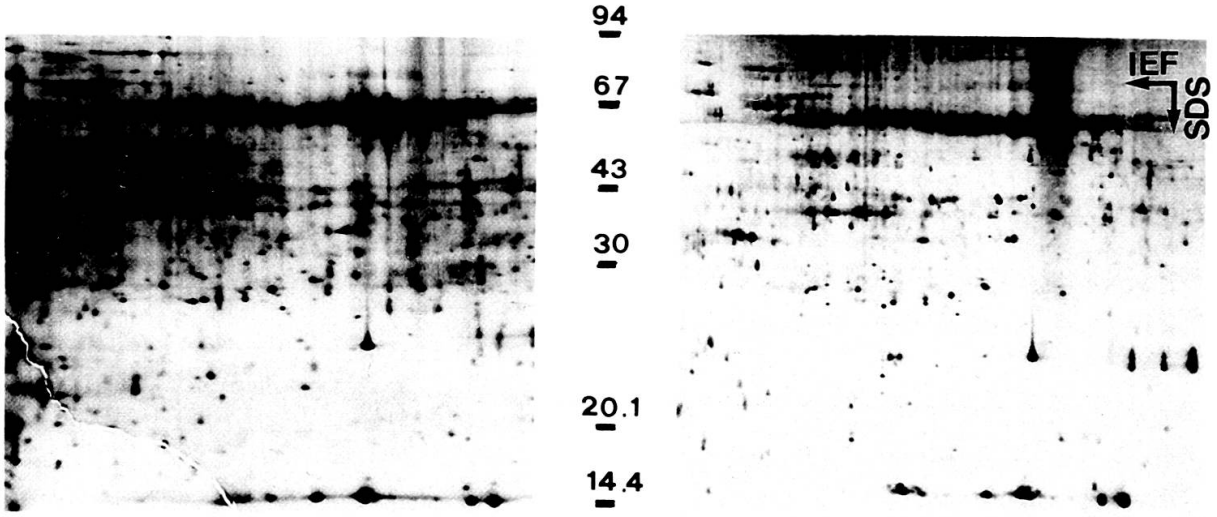

FIG. 1
Silver stained 2D gel from lower epidermis-free spinach leaves in non-induced conditions (SD, 8 hours light).

FIG. 2.
Silver stained 2D gel from lower epidermis of spinach leaves in induced conditions (LD, 24 hours light).

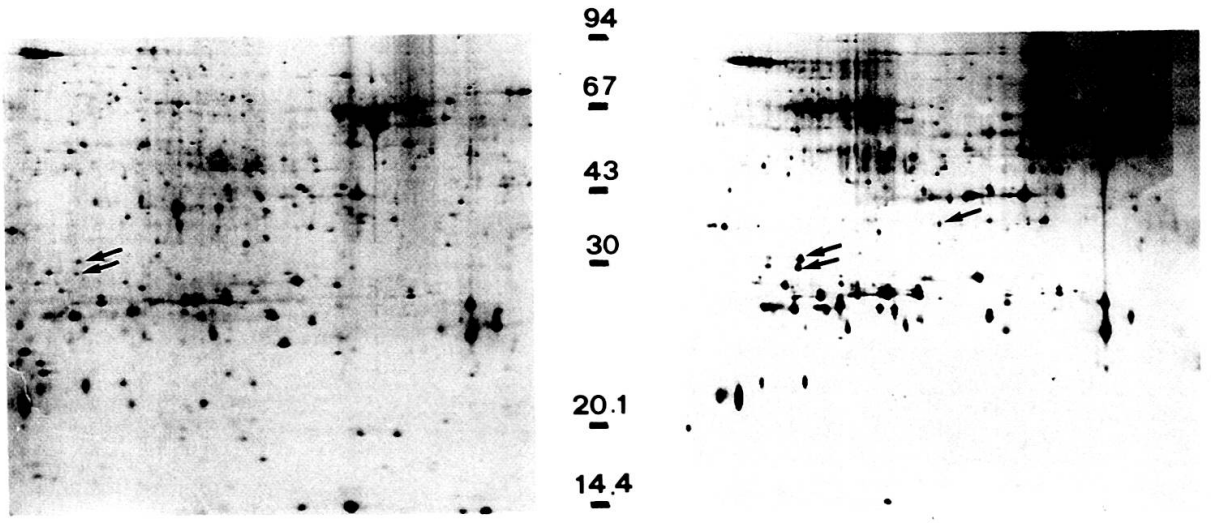

FIG. 3.
Autoradiogram from the 2D gel shown in Figure 1.

FIG. 4.
Autoradiogram from the 2D gel shown in Figure 2.

Arrows indicate the polypeptides that are quantitatively modified between induced and non-induced conditions. Standards of molecular mass in kilodaltons are indicated.

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