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Changes in the Activity of an Enzymatic Marker Bound to Plasmalemma during the Photoperiodic Flowering Induction in Soybean

By

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With 11 Figures

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Summary

CAFFARO S. V., MATEOS J. L. & VICENTE C. 1996. Changes in the activity of an enzymatic marker bound to plasmalemma during the photoperiodic flowering induction in soybean. – Phyton (Horn, Austria) 36 (1): 9–28, 11 figures. – English with German summary.

Soybean plants (*Glycine max* (L.) MERR., cv. Williams), were grown under either non-inductive long day (LD = SD + night break) or inductive short day (SD = 9 h light + 15 h darkness) conditions to study the possible involvement of plasmalemma in the photoperiodic induction of flowering. Pelletable peroxidase activity from three subcellular fractions isolated from both leaves and stem terminal buds was tested for its usefulness as a marker for flowering. Significant differences in values for peroxidase activity (PA) were found among the fractions isolated from leaves. The mitochondria-enriched fraction (MEF) had a rhythmic behaviour of PA with maximal values during light and minimal values during dark periods. In contrast, PA from the plasmalemma-enriched fraction (PEF) continuously increased and that from the microsomal + soluble fraction (MSF) continuously decreased. The inductive SD treatment, which had a clear effect only on PA from PEF, impeded the increase in activity during the first 3 days of SD floral induction. Thereafter, the activity increased to values similar to those for leaves under LD conditions. Electrophoretic analysis of the three subcellular fractions did not reveal an effect of the SD treatment

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either on the pattern of peroxidase isozymes from MEF, PEF or MSF. For terminal buds neither the time-courses of PA found in the subcellular fractions isolated from leaves nor the SD effect on PA from PEF were observed, which indicates that the photoperiodic effect on plasmalemma status is restricted to the leaves.

In vivo application of Ca^{2+} inhibited PEF-PA of LD-treated leaves to mimick the SD effect. A23187, a Ca^{2+} ionophore, also inhibited PEF-PA while Verapamil, a Ca^{2+}-channel blocker, partially reverted the ionophore effect after one SD exposure. In vitro application of Ca^{2+}, as well as Ca^{2+}-chelators (EDTA and EGTA), inhibited both PEF and MSF-PA. These results suggest that a Ca^{2+} uptake into the cell may be triggered as a response to SD application and its possible correlation with perception of the photoperiodic stimulus and the synthesis of the flowering signal by the leaves is discussed.

Zusammenfassung

CAFFARO S. V., MATEOS J. L. & VICENTE C. 1996. Änderungen in der Aktivität eines an das Plasmalemma gebundenen Enzymmarkers während der photoperiodischen Blühinduktion bei Sojabohnen. Phyton (Horn, Austria) 36 (1): 9–28, 11 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Sojabohnenpflanzen (*Glycine max* (L.) MERR., cv Williams) wuchsen entweder unter nicht induktiven Langtag- (LD = SD + Unterbrechung in der Nacht) oder induktiven Kurztagbedingungen (SD = 9 h Licht + 15 h Dunkelheit), um die mögliche Beteiligung des Plasmalemmas an der photoperiodischen Induktion der Blüten zu untersuchen. Die Peroxidaseaktivität dreier subzellulärer Fraktionen, welche sowohl von Blättern als auch von Terminalknospen der Sprosse isoliert wurden, wurden auf ihre Brauchbarkeit als Marker für die Blühinduktion untersucht. Signifikante Unterschiede in den Werten der Peroxidaseaktivität (PA) konnten innerhalb der Fraktionen gefunden werden, welche von den Blättern isoliert wurden. Die mit Mitochondrien angereicherte Fraktion (MEF) zeigte ein rhythmisches Verhalten der PA mit Höchstwerten während der Licht- und Minimalwerten während der Dunkelphasen. Dagegen stieg die PA in der mit Plasmalemma angereicherten Fraktion (PEF) kontinuierlich an und diejenige in der mikrosomalen + löslichen Fraktion (ESF) sank ständig. Die induktive SD-Behandlung, welche nur auf die Peroxidaseaktivität von PEF einen deutlichen Einfluß hatte, verhinderte einen Anstieg in der Aktivität während der ersten drei Tage der SD-Blühinduktion. Die Aktivität stieg danach auf Werte an, die ähnlich jenen von Blättern unter LD-Bedingungen waren. Elektrophoreseuntersuchungen dieser drei subzellulären Fraktionen zeigten keinen Einfluß der SD-Behandlung, weder auf die Muster der Peroxidaseisoenzyme von MEF, PEF noch von MSF. Bei den Terminalknospen konnte weder ein zeitlicher Verlauf der PA gefunden werden, wie sie in den subzellulären Fraktionen der Blätter aufschienen, noch der SD-Effekt auf die PA der mit Plasmalemma angereicherten Fraktion. Dies bedeutet, daß der photoperiodische Einfluß auf den Zustand des Plasmalemmas auf die Blätter beschränkt ist.

Die in vivo-Applikation von Ca^{2+} inhibierte PEF-Peroxidaseaktivität von LD-behandelten Blättern und führte so zu einem ähnlichen Effekt wie unter SD-Bedingungen. Das Ca^{2+} ionophor A23187 inhibierte ebenfalls die PEF-PA während Verapamil, ein Ca^{2+}-Kanalblocker, die ionophoren Effekte nach einer SD-Exposition teilweise wieder zurücknahm. In vitro-Applikationen von Ca^{2+}, gleich wie von Ca^{2+} Komplexbildnern (EDTA und EGTA), inhibierten sowohl PEF als auch

MSF-PA. Diese Ergebnisse lassen erkennen, daß eine Ca^{2+}-Aufnahme in die Zelle als Antwort auf eine SD-Behandlung gehemmt werden könnte. Die mögliche Korrelation zwischen Aufnahme des photoperiodischen Reizes und der Synthese des Blühsignales durch die Blätter wird diskutiert.

Introduction

The photoperiodic induction of flowering was one of the first events in which phytochrome was implicated. However both the mechanism of photoperiodic perception by leaves and the nature of the biochemical pathway for the transduction of the flowering signal from leaves to growing meristems remain obscure. There are no clear evidences for the biochemical events occurring during the first hours of photoperiodic floral induction. Additionally, the necessity of working with small amount of plant tissue presents problems of experimental procedures for identification of endogenous biochemical changes that occur during evocation of buds and transition to flowering.

Peroxidases, which are a useful marker of floral induction in spinach, a long day plant (LDP) (KAREGE & al. 1979, MONTAVON & al. 1988), may provide a good technique for the study of floral induction. It must first be established, however, whether peroxidases also behave as a sensitive enzymatic marker of floral induction in a short day plant (SDP) such as soybean. As peroxidases are widely distributed within the cell and are attached to different subcellular organelles (DE FELIPE & al. 1988, HU & al. 1989), we isolated several subcellular fractions from leaves and the stem apical bud during the first 9 days of floral induction and tested them for changes in peroxidase activity.

Calcium is a cation related to many phytochrome-regulated responses (ROUX 1986, TRETYN & al. 1991). The stimulation of Ca^{2+} uptake and accumulation by red light (RL) is reverted by far-red light (FRL) (BOSSEN & al. 1988). The addition of Ca^{2+} ionophore in darkness can produce physiological effects attributed to RL, while responses to RL can be nullified by Ca^{2+} channel blockers (TRETYN & al. 1990a). Movements of Ca^{2+} also appears to be involved in the photoperiodic induction of flowering. FRIEDMAN & al. 1989 observed that exogenously applied Ca^{2+} chelators and Ca^{2+} channel-blockers inhibited flowering in the SDP *Pharbitis nil* and proposed that cytoplasmic Ca^{2+} concentration increases during photoperiodic floral induction. On this basis, we also analyzed the involvement of Ca^{2+} in the changes on peroxidase activity detected on the subcellular fractions during the first day of short day (SD)-induction of flowering.

Materials and Methods

Abbreviations

D, day; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glycol-bis (-amino-ethylether)N,N'-tetraacetic acid; ER, endoplasmic reticulum; FRL, far-red

light; LD, long day; LDP, long day plant; MEF, mitochondria-enriched fraction; MES, morpholino ethansulfonic acid; MSF, microsomal + soluble fraction; N, night; PA, peroxidase activity; PEF, plasmalemmaenriched fraction; PP, pellet; RL, red light; SD, short day; SDP, short day plant; ST, supernatant; TCA, trichloroacetic acid; Tris, tris (hydroxymethyl)-aminomethane

Plant material and culture conditions

Soybean plants (*Glycine max* (L.) Merr., cv. Williams) were growth-chamber cultivated in pots containing vermiculite watered with Hoagland' solution (HOAGLAND & SNYDER 1933). From sowing to the V_2 phenological stage (plants with a pair of unifoliate leaves and a fully developed trifoliate leaf, according to FEHR & CAVINESS 1977), plants were grown under LD conditions of daily 9 h fluorescent plus incandescent light and 3 h interruption with low-level light from incandescent lamps alone given in the middle of the dark period (CAFFARO & al. 1988, THOMAS & RAPER 1977). Once the V_2 stage was reached, the inductive SD treatment was initiated by eliminating the 3 h interruption of the 15 h dark period.

The combination of fluorescent light from 18W Sylvania F Grolux tubes and incandescent light from 40W Philips lamps provided 80 W m^{-2} of irradiance. The incandescent lamps provided a photomorphogenetic radiation of 34 W m^{-2}. Measurements of irradiance were performed at the surface of the canopy by using a LICOR LI 185B radiometer.

Experimental arrangement

Two experiments were designed in agreement with data of harvest (fig. 1). In the first one, plants were harvested at the end of the last non-inductive nyctoperiod (O_N), and at the end of the 3rd, 6th, and 9th inductive nyctoperiods (3_N, 6_N, and 9_N). In the

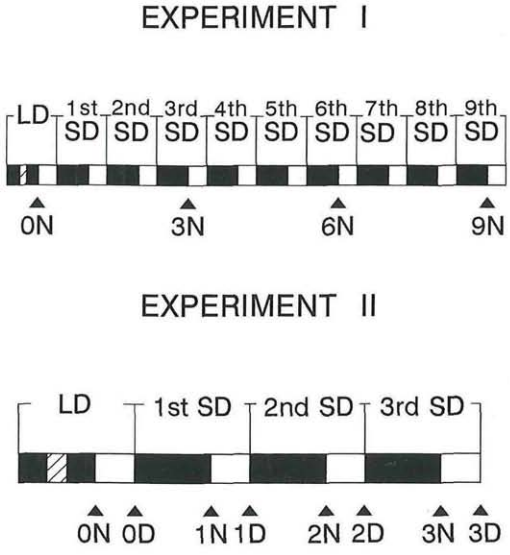

Fig. 1. Arrangement of experiments I and II. Arrows indicate times of harvest. N: night; D: day.

second experiment, plants were harvested twice a day at the end of both nyctoperiod (O_N) and photoperiod (O_D) of the last non-inductive LD and at the ends of the nyctoperiods (1_N, 2_N, 3_N) and photoperiods (1_D, 2_D, 3_D) of the first, second and third inductive SD. An equal number of control plants that had remained under non-inductive LD conditions were harvested for each experiment. Leaf samples included at each harvest fully expanded leaves of each plant, i.e., the pair of unifoliate and the first trifoliolate leaves. Stem terminal bud (c.a. 20 mm length), which comprised at least four to five microscopically differentiated internodes with their corresponding foliar primordia and axillary meristems, was collected from each harvested plant.

All the experiments were replicated and three to four plants were collected per harvest per treatment.

Fractionation of leaf and terminal bud tissues

Harvested tissues were weighed and immediately ground in a mortar, in the dark, with chilled 0.1 mol.l^{-1} phosphate buffer, pH 7.0. Membrane fractions were obtained by successive centrifugations of supernatants at 3,200 × g, 7,000 × g, 13,000 × g and 30,000 × g, at 2 °C for 30 min each. Pellets from 13,000 × g and 30,000 × g were resuspended in 0.1 mol.l^{-1} phosphate buffer, pH 7.0.

Partial characterization of membrane fractions

Membrane fractions were characterized by the specific activity of several enzymes used as organelle markers.

NADH cytochrome c reductase (EC 1.9.2.4), which is an ER marker (Lord 1983), was assayed as the increase in absorbance at 550 nm in a reaction mixture that contained 75 mmol.l^{-1} phosphate buffer, pH 7.4, 1.0 mmol.l^{-1} KCN, 6.0 mmol.l^{-1} NADH, 1.0% (v/v) aqueous ferricytochrome c, and 1.0 mg of membrane protein (Mahler 1955).

Cytochrome c oxidase (EC 1.9.3.1), which is a mitochondrial marker, was determined as described by Moore & Proudlove 1983. After incubating 0.1-1.0 mg protein with 0.6 mg digitonin for 1 min at 25 °C, 1.1 ml of 10 mmol.l^{-1} phosphate buffer, pH 7.0, and 0.32 mmol. l^{-1} reduced cytochrome c were added; the decrease in absorbance was determined at 550 nm.

Mg^{2+} -dependent ATPase activity (EC 3.6.1.3), which is a plasmalemma marker (Hall 1983), was assayed by the production of inorganic phosphate. Fractions were incubated for 30 min at 30 °C in 30 mmol.l^{-1} Tris-MES buffer, pH 7.5, containing 30 mmol.l^{-1} $MgSO_4$, 30 mmol.l^{-1} K_2SO_4 and 30 mmol.l^{-1} ATP (modified from Wang & al. 1989). The reaction was stopped by the addition of 5% TCA (w/v). Samples were then centrifuged at 10,000 × g for 20 min at 2 °C and the supernatants were used to estimate inorganic phosphate by formation of phosphomolibdate and measuring absorbance at 700 nm (Lowry & López 1950).

Total protein content was determined by the method of Lowry & al. 1951 by using bovine serum albumin as a standard.

Peroxidase assay

Peroxidase (EC 1.11.1.7) activity (PA) was assayed by using reaction mixtures containing 0.6 mmol phosphate, pH 7.0, 81.6 µmol guaiacol, 0.032% hydrogen peroxide (v/v) and 1.0 mg membrane protein in a final volume of 6.0 ml. One unit of specific activity was defined as a 1.0 unit increase in absorbance at 470 nm per mg protein per min.

Electrophoretic assay

Small pieces of filter paper (10 mm × 2 mm) were soaked with the subcellular fraction obtained by each successive centrifugations as described above and then inserted into 180 × 280 × 2 mm starch gels. Gels contained 12% (w/v) starch in Poulik's buffer (POULIK 1957). Three specific staining systems were assayed: O-dianisidine, 3-amino-9-ethylcarbazole, and aluminium lactate. Anodic and cathodic isozymes were revealed by using 3-amino-9-ethylcarbazole, as described by SHAW & PRASAD 1970.

In vivo application of Ca^{2+}

To study the effect of calcium in vivo, three different Ca^{2+} solutions containing either 1.5 mmol.l^{-1} $CaCl_2$, 1.5 mmol.l^{-1} $CaCl_2$ + 10 µmol.l^{-1} A23187 ionophore, or 1.5 mmol.l^{-1} $CaCl_2$ + 10 µmol.l^{-1} verapamil were applied to leaves at the end of the last non-inductive photoperiod. Leaf samples were collected at the end of the first inductive photoperiod.

In vitro application of Ca^{2+}

PEF and MSF-PA from leaves of one SD-induced and LD-non induced plants were assayed in reaction mixtures containing various concentrations of $CaCl_2$ from 0.01 to 10 mmol.l^{-1} to determine the pattern of Ca^{2+} inhibition, or, alternatively, preincubated for 20 s with 1.0 mmol.l^{-1} $CaCl_2$, 0.5 mmol.l^{-1} EGTA or 0.5 mmol.l^{-1} EDTA before substrate addition.

Results

Marker enzyme activities of the different membrane fractions are shown in figure 2. The 7,000 × g pellet contained very low activity of any marker enzymes. The 13,000 × g pellet (MEF) was enriched in mitochondrial membranes, as demonstrated by its high cytochrome c oxidase activity. The 30,000 × g pellet (PEF) contained high activity for NADH cytochrome c reductase, which is indicative of ER, and for Mg^{2+}-dependent ATPase, which is a marker for plasmalemma. High NADH cytochrome c reductase activity also was found in the supernatant of 30,000 × g centrifugation (MSF). Essentially, the plasmalemma was contained in the 30,000 × g pellet whereas the ER was distributed between both pellet and supernatant.

Negligible differences in PA were detected between inductive and non-inductive treatments for MEF and MSF (fig. 3, 5). PEF was the only fraction that exhibited a significant decrease in PA during the first three days of floral induction (fig. 4 B), but this effect was transient and, after c.a. 6 days, PA again reached similar values for both treatments (fig. 4 A). Although these three leaf subcellular fractions were enriched rather than pure, their PA were different during the experiment. MEF showed a rhythmic activity with maxima during day (light) and minima during night (dark) periods (fig. 3 B), PEF showed a pattern of increasing activity (fig. 4), and MSF showed a pattern of decreasing activity (fig. 5).

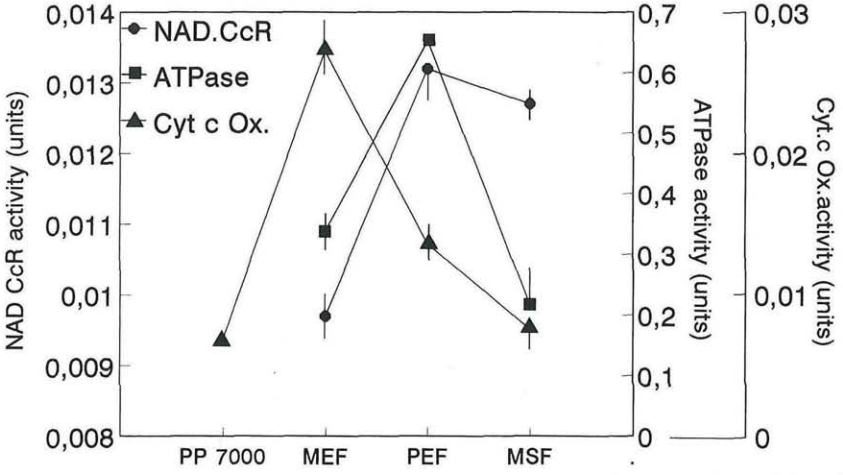

Fig. 2. Characterization of membrane fractions on the basis of the specific activity of marker enzymes: 1) cytochrome c oxidase (Cyt. c Ox.) for mitochondria; 2) Mg^{2+}-dependent ATPase (ATPase) for plasmalemma; 3) NADH cytochrome c reductase (NAD CcR) for endoplasmic reticulum. Values are the mean of three replicates. Vertical bars give standard error when bigger than the symbols.

The patterns of PA in fractions of the stem terminal bud were distinct from those observed in the comparable leaf subcellular fractions (fig. 6). Also the SD effect of the photoperiodic treatment on PEF-PA of leaves was not observed in the PEF-PA of terminal buds.

Electrophoretic analysis of leaf peroxidase revealed a similar isozymatic pattern among the three subcellular fractions. At least five anodic and three cathodic peroxidase isozymes were observed in both MEF and PEF (fig. 7), whereas eleven anodic and four cathodic isozymes were detected in MSF. The higher PA in MSF relative to MEF and PEF may have contributed to this additional number of isozymes found in MSF by facilitating a better detection of clearer bands. However, different patterns did not occur among the isozymatic forms detected from the three subcellular fraction that could be correlated to the differential patterns of peroxidase activity observed either among these three subcellular fractions or in the SD response of PEF.

Total protein content per gram fresh weight followed a similar pattern for both treatments (data not shown).

In vivo addition of Ca^{2+} produced no changes in PEF-PA after the first inductive photoperiod (fig. 8 A). However, a clear inhibition of PEF-PA by Ca^{2+} was obtained for LD-treated leaves. The A23187 ionophore slightly increased Ca^{2+} promoted-inhibition in leaves after the first SD, whereas verapamil slightly reversed this inhibition. Both compounds did not significantly affect PEF-PA inhibited by Ca^{2+} in LD-treated leaves.

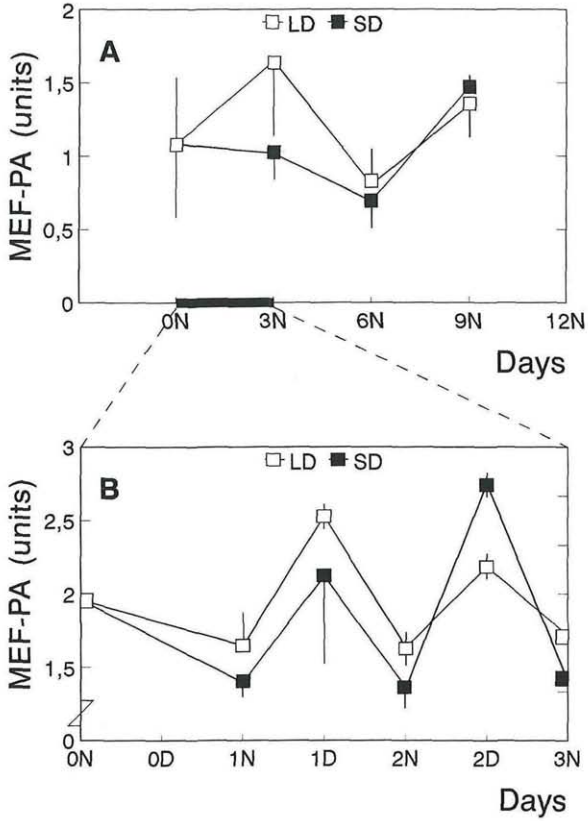

Fig. 3. Peroxidase activity of MEF from leaves collected during 9 days in Experiment I (A) and 3 days in Experiment II (B) of SD applications. N: night; D: day. Values are the mean of six replicates. Vertical bars give standard error when bigger than the symbols.

MSF-PA from both LD and SD-growing plants was inhibited by applying Ca^{2+} to leaves. This inhibition by Ca^{2+} was reversed by A23187 and verapamil (fig. 8 B).

When progressively increasing concentrations of Ca^{2+} were included in the reaction mixtures, maximal in vitro-inhibition of PEF-PA was obtained for the range from 1.0 mmol.l-1 (65.1% maximal inhibition for LD-treated plants) to 2.5 $mmol.l^{-1}$ Ca^{2+} (57.2 % of maximal inhibition for SD-treated plants) (fig. 9 A). The maximal inhibition of MSF-PA was obtained by adding 2.5 $mmol.l^{-1}$ Ca^{2+} (fig. 9 B) in which the pattern of enzyme inhibition was similar for peroxidases extracted from both LD- and SD-treated leaves.

The in vitro addition of Ca^{2+}, EGTA or EDTA to MSF resulted in a clear inhibition of the specific enzyme activity in the LD (fig. 10 A) as well

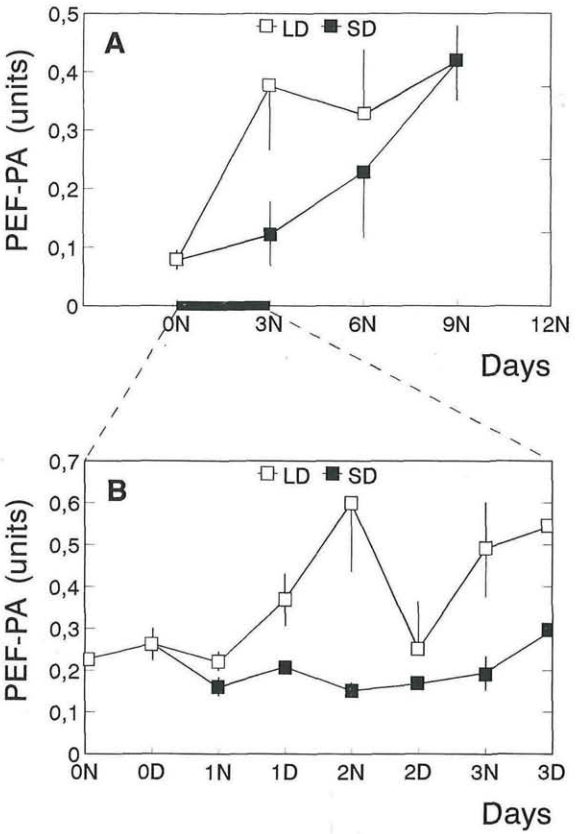

Fig. 4. Peroxidase activity of PEF from leaves collected during 9 days in Experiment I (A) and 3 days in Experiment II (B) of SD applications. N: night; D: day. Values are the mean of six replicates. Vertical bars give standard error when larger than the symbols.

as in the SD-treated leaves (fig. 10 B). However, the addition of either EGTA or EDTA 20 s before addition of Ca^{2+} to the MSF not only prevented the inhibition of PA by Ca^{2+} but also enhanced PA to a higher value than that observed for the control. In contrast, adding Ca^{2+} to the MSF 20 s before addition of either EGTA or EDTA did not prevent inhibition of PA by Ca^{2+}, although the inhibition was less than when the compounds were added alone. PEF-PA showed a similar but not identical pattern to that observed for MSF, since no activation over the control took place by any combined treatment (fig. 11 A and B).

Discussion

Although the method used for the isolation of subcellular fractions produced only enriched and not pure fractions, distinctive time-courses of

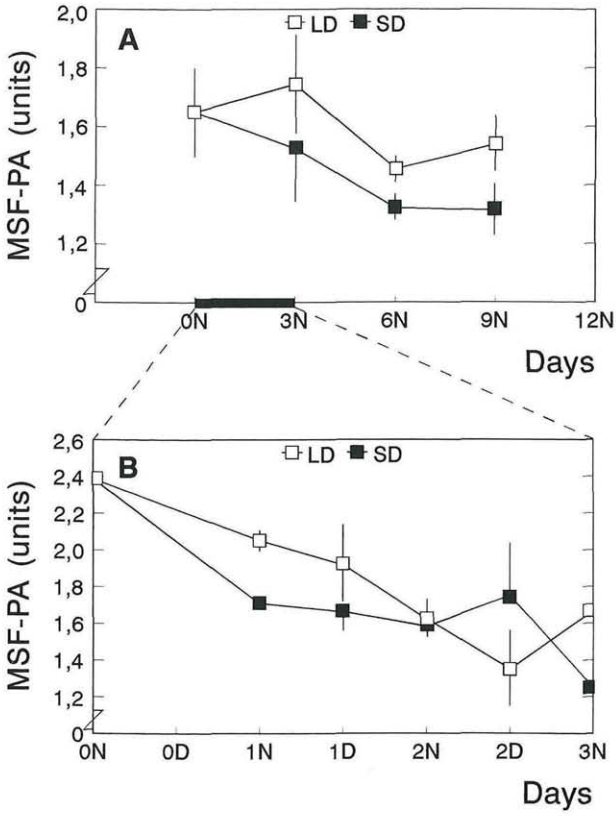

Fig. 5. Peroxidase activity of MSF from leaves collected during 9 days in Experiment I (A) and 3 days in Experiment II (B) of SD applications. N: night; D: day. Values are the mean of six replicates. Vertical bars give standard error when bigger than the symbols.

peroxidase activity occurred in each one. Moreover, while both the 30,000 × g pellet and supernatant contained ER membranes, only the peroxidase activity in the 30,000 × g pellet respond to SD treatment. Since only the 30,000 × g pellet contained plasmalemma, the response to SD appears to involve plasmalemma and this simple method of isolation of subcellular fractions is sufficient to work with peroxidase activity as a flowering marker in a SDP, such as soybean. In addition, ASKERLUND & al. 1987 reported that peroxidase activity found in a plasmalemma fraction is truly membrane-bound because this binding is not supressed by washes with high ionic strength solutions. Therefore, the photoperiodic response observed in the peroxidase activity of the PEF suggests to be due to peroxidase strongly bound to plasmalemma and not to a binding derived from experimental manipulation.

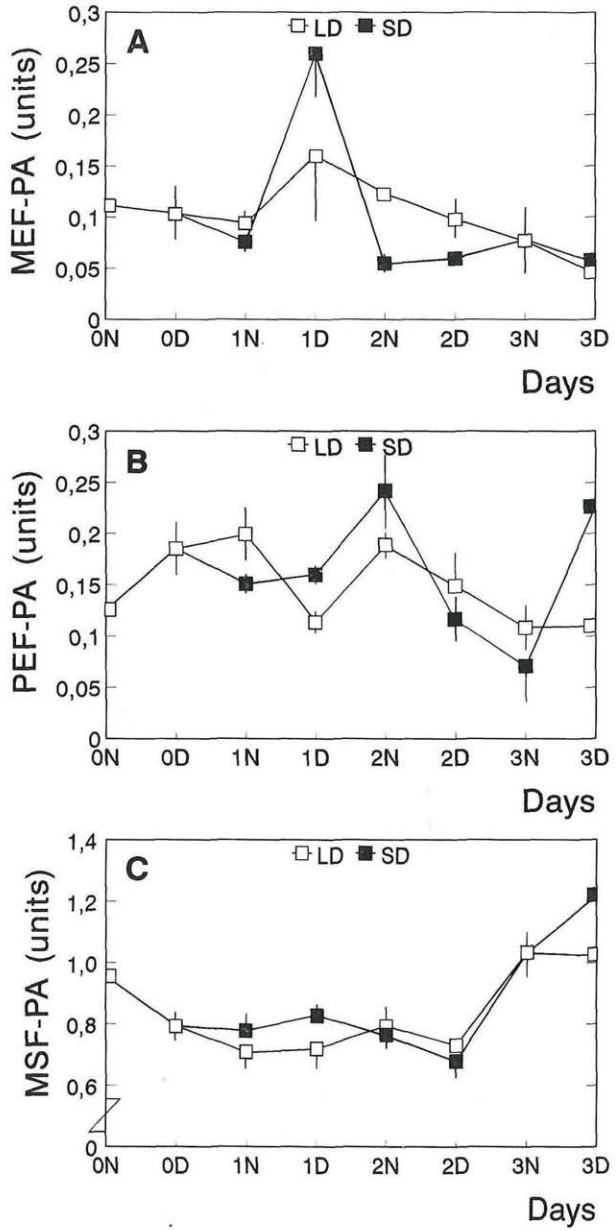

Fig. 6. Peroxidase activity of MEF (A), PEF (B), and MSF (C) from terminal stem buds during the first 3 days of SD applications in Experiment II. N: night; D: day. Values are the mean of six replicates. Vertical bars give standard error when bigger than the symbols.

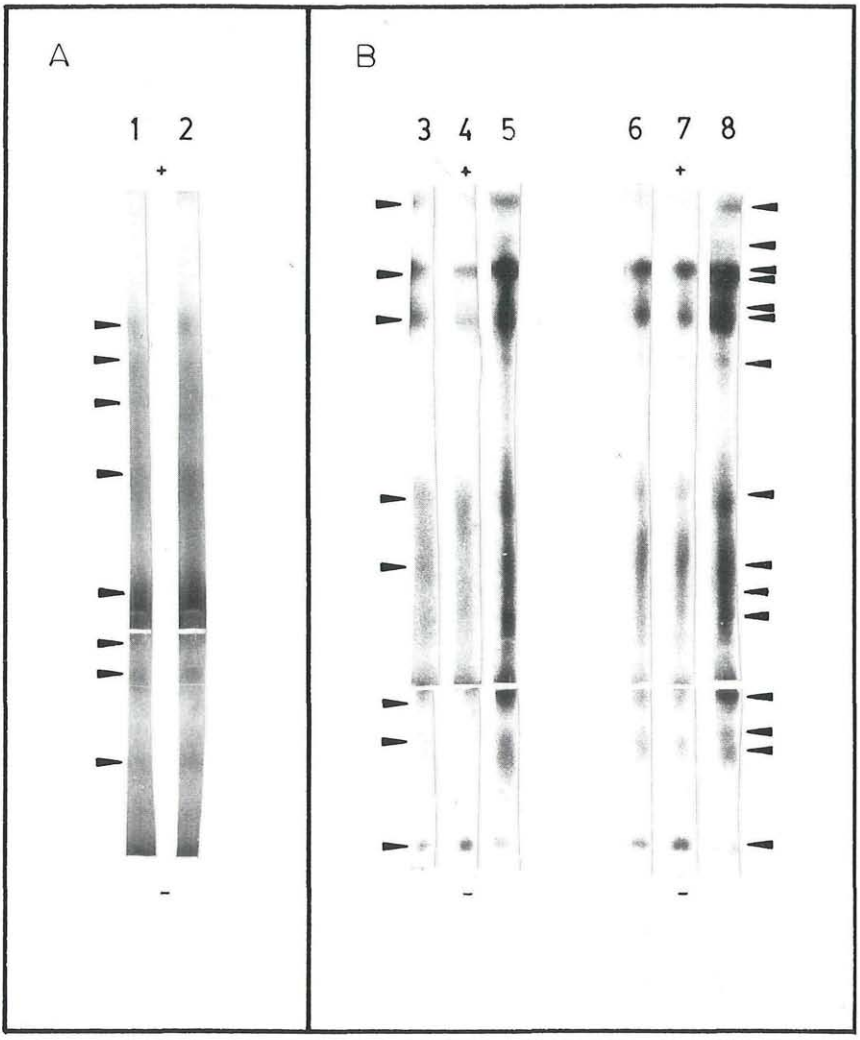

Fig. 7. Electrophoretic analysis of isoperoxidases in leaves. A) Isoperoxidases from PEF isolated from leaves growing under LD (lane 1) and after one SD (lane 2). B) Isoperoxidases from MEF (lanes 3 and 6), from PEF (lanes 4 and 7) and from MSF (lanes 5 and 8) after 11 SDs (lanes 3, 4 and 5) or 17 SDs (lanes 6, 7 and 8). (+) anode; (-) cathode.

KIEFER & al. 1985 showed that Ca^{2+} promotes a strong binding of basic, cathodic peroxidases to a crude microsomal suspension of *Pharbitis* and that these basic peroxidases are found to change during floral induction in LDPs (GASPAR & al. 1982). In spinach (a LDP), the basic peroxidase activity

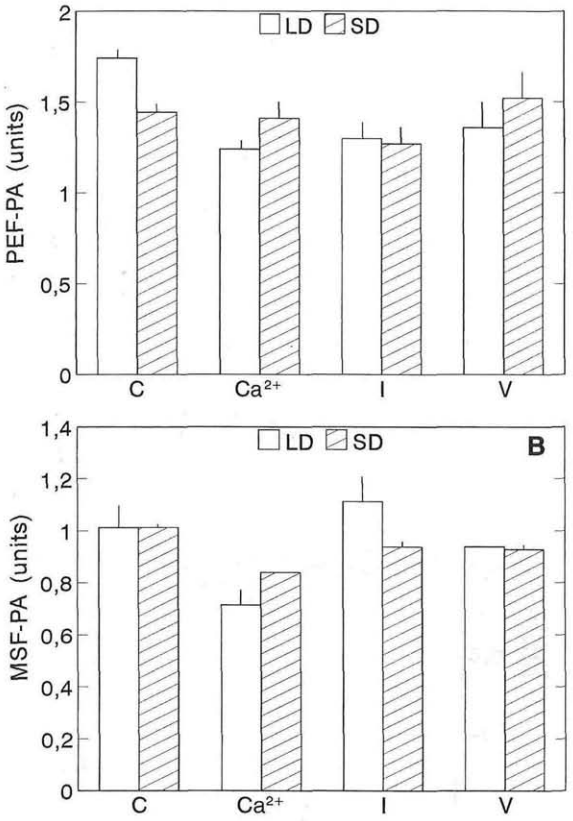

Fig. 8. Peroxidase activity of A) leaf PEF and B) leaf MSF obtained after in vivo application of Ca^{2+}, Ca^{2+} ionophore (I = A23187) or a Ca^{2+}-channel blocker (V = Verapamil). C = control set. Values are the mean of six replicates. Vertical bars give standard error when bigger than the symbols.

obtained from the 10,000 × g fraction of leaves decreased under inductive LD conditions of continuous light (PENEL & GREPPIN 1975).

Despite these previous findings, we found no differences in the eight isoforms of peroxidase, three cathodic and five anodic isozymes, bound to the PEF in leaves of soybean (a SDP), during LDs or during the first 17 SDs (fig. 7). This implies that SD conditions inhibit activation of total peroxidase attached to the PEF whereas LD conditions promote activation without changes in the isozyme pattern.

Photoperiod may have an action similar to RL-FRL phytochrome-modulated responses (BOSSEN 1990), in which the binding of Pfr to plasmalemma after Pr to Pfr phototransformation (NAPIER & SMITH 1987a and b, LAMPARTER & al. 1992, SINESHCHEKOV & al. 1994) induces changes in

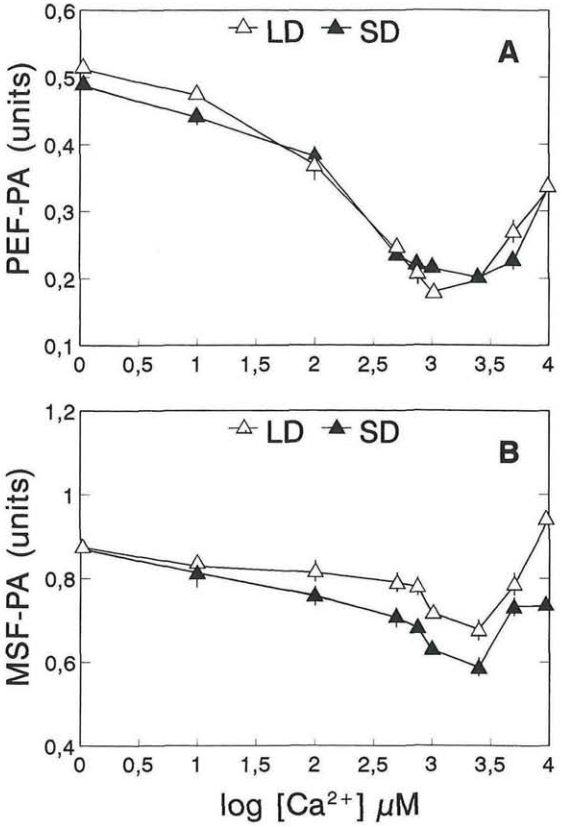

Fig. 9. Peroxidase activity of A) leaf PEF and B) leaf MSF obtained after in vitro addition of increasing concentrations of Ca^{2+}. Values are the mean of three replicates. Vertical bars give standard error when bigger than the symbols.

membrane density, polarity and permeability (ROUX 1986). If so, perhaps the photoperiodic effect we observed on the activation-inhibition process of plasmalemma-bound peroxidases is dependent on the plasmalemma status as follows: SD induction of flowering produces changes in the status of plasmalemma that in turn results in modifications in the ionic equilibrium of the cell. Indeed, the thickness of the plasmalemma increases during floral induction and this thickening apparently is a consequence of a conformational change in the arrangement of constituents within the membrane rather than changes in composition of the membrane (PENEL & al. 1988).

Ca^{2+} seems to be implied directly in the photoperiodic response in leaves. An exposure to one SD produces an inhibition of PEF but not MSF peroxidase. TRETYN & al. 1992 found that localization of intracellular Ca^{2+}

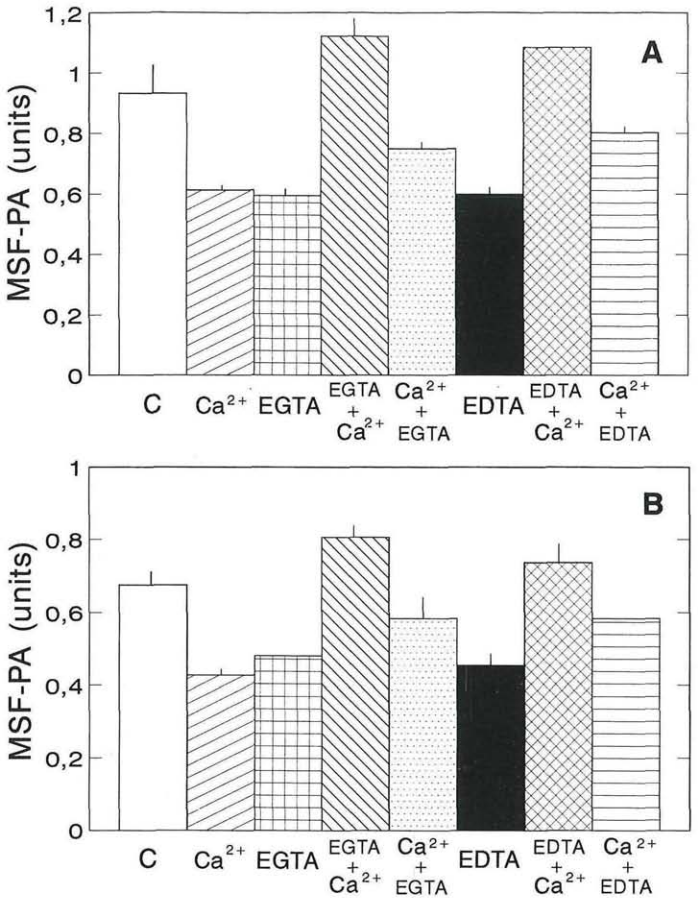

Fig. 10. Peroxidase activity of leaf MSF obtained after in vitro application of Ca^{2+}, EGTA, or EDTA. A) LD-treated plants; B) SD-treated plants. 1) CONTROL; 2) 1 mmol.l^{-1} Ca^{2+} ; 3) 0.5 mmol.l^{-1} EGTA; 4) 0.5 mmol.l^{-1} EGTA + 1 mmol.l^{-1} Ca^{2+}; 5) 1 mmol.l^{-1} Ca^{2+} + 0.5 mmol.l^{-1} EGTA; 6) 0.5 mmol.l^{-1} EDTA; 7) 0.5 mmol.l^{-1} EDTA + 1 mmol.l^{-1} Ca^{2+}; 8) 1 mmol.l^{-1} Ca^{2+} + 0.5 mmol.l^{-1} EDTA. Values are the mean of three replicates. Vertical bars give standard error when bigger than the symbols.

in coleoptiles of etiolated oat seedlings is mediated by phytochrome. Ca^{2+} is located at the outer and the inner surfaces of plasmalemma and at the cisternae of endoplasmic reticulum after R exposure. After the first inductive SD, Ca^{2+} might become associated with plasmalemma to inhibit PEF but not MSF peroxidase. This postulate would presume that cytoplasmic Ca^{2+} concentration normally is too low to inhibit plasmalemma-bound peroxidase except when stimulate by SD to become concentrated on the surface of the plasmalemma. Supply of exogenous

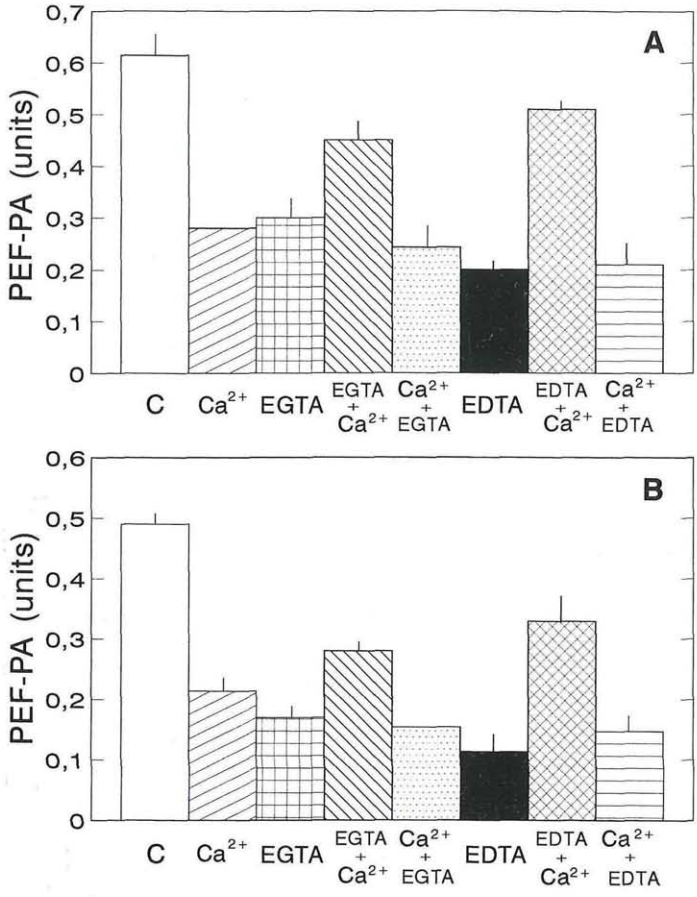

Fig. 11. Peroxidase activity of leaf PEF obtained after in vitro application of Ca^{2+}, EGTA, or EDTA. A) LD-treated plants; B) SD-treated plants. 1) CONTROL; 2) 1 mmol.l^{-1} Ca^{2+}; 3) 0.5 mmol.l^{-1} EGTA; 4) 0.5 mmol.l^{-1} EGTA + 1 mmol.l^{-1} Ca^{2+}; 5) 1 mmol.l^{-1} Ca^{2+} + 0.5 mmol.l^{-1} EGTA; 6) 0.5 mmol.l^{-1} EDTA; 7) 0.5 mmol.l^{-1} EDTA + 1 mmol.l^{-1} Ca^{2+}; 8) 1 mmol.l^{-1} Ca^{2+} + 0.5 mmol.l^{-1} EDTA. Values are the mean of three replicates. Vertical bars give standard error when bigger than the symbols.

Ca^{2+} to soybean leaves apparently increased the internal (cytoplasmic) concentration; hence, when the cytoplasmic concentration is increased, both MSF and PEF-PA are inhibited. Exposure to SD after Ca^{2+} supply does not further inhibit PEF peroxidase. Only when calcium ionophore was also applied, there was a small increase of peroxidase inhibition. Perhaps, SD inducing-Ca^{2+} uptake do not exert an additive inhibitory effect over the already Ca^{2+} inhibited-PA. Only enhancement of PEF-PA inhibition may be caused by a higher intracellular Ca^{2+} concentration caused by ionophore

application. Similar results were obtained from in vitro Ca^{2+} experiments in which, in comparison with LD-treated leaves, a higher Ca^{2+} concentration was needed to cause maximal PEF-PA inhibition after one SD exposure.

EGTA strongly inhibited flowering in the SDP *Pharbitis nil* when applied before the start of the inductive dark period (FRIEDMAN & al. 1989, TRETYN & al. 1990b). Calcium-channel and calmodulin inhibitors also inhibited flowering. As discussed by TRETYN & al. 1990b, direct application of these compounds to the surface of plants can cause damage and perturbations not related to floral induction. The possibility of such anomalies were avoid by TAKENO 1993 who used a perfusion method to apply EGTA, $LaCl_3$ and chlorpromazine and reported a strong inhibition of flowering with lower applied concentrations. In the present study, the lack of a clear effect of the Ca^{2+} ionophore or the Ca^{2+} channel-blocker on the flowering marker perhaps is attributable to anomalies of direct application. In addition, the failure of in vitro EGTA (or EDTA) to reverse Ca^{2+} inhibition possibly was the result of particular interactions between these compounds and the peroxidase moiety. Specially, EGTA (or EDTA) perhaps chelated the constitutive Ca^{2+} into the peroxidase moiety to change the protein structure and inhibit its activity. However, when exogenous Ca^{2+} is bound to the peroxidase molecule, its removal by EGTA or EDTA seems to be very difficult since peroxidase inhibition is maintained by preincubation of Ca^{2+} followed by treatment with the chelatory agent.

The time moment of Ca^{2+} application is important since the inhibitory effect on flowering was detected only when compounds were applied before the beginning of the inductive night and not when applied immediately after the end of the first inductive night (TAKENO 1993). This suggests that the increase in Ca^{2+} uptake is stimulated by the first inductive night, an event for which the flowering marker (PA) was sensitive. However, an increase in the intracellular Ca^{2+} concentration does not imply an increase either in the endogenous Ca^{2+} content of the leaf (HAVELANGE 1989) or in the quantity of Ca^{2+} exported from the leaves (HAVELANGE & BERNIER 1993). Thus, perception of the photoperiodic stimulus by leaves appears to involve a remobilization of intracellular Ca^{2+} as a consequence of plasmalemma status, rather than transport from other parts of the plant (e.g. the roots).

The changes in the plasmalemma status that occur as a result of the photoperiodic perception seem to be located only in the leaf. The plasmalemma is not affected in the same way in apical bud cells. Although, CRÈVECOEUR & al. 1992 found a slight increase in the plasmalemma thickness for apical bud cells of the LDP spinach, this change seemed to be related to evocation and not to the transduction of the inductive signal itself.

All these observations indicate that the electrical hypothesis of the floral signal transduction across plasmalemma and the symplastic organization of higher plants (PENEL & al. 1985, WAGNER & al. 1985) must be considered as a more complicated process. Although differences in peroxidase activity between dark-cultured and red-irradiated leaves support the idea of a rapid inter-organ communication across the plasma membrane (KAREGE & al. 1979), this mechanism seems to be different from that of the flowering signal transduction from the leaves to the apical bud.

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