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Cytokinin Fluxes during Floral Induction in the Long Day Plant *Sinapis alba* L.[1]

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ABSTRACT

***Sinapis alba* is a long-day (LD) plant that can be induced to flower by a single LD. A number of changes normally occurring in the meristem of plants subjected to the LD can be produced in short day by a single application of a cytokinin to the apical bud. However, flower buds are not produced indicating that evocation by the cytokinin is only partial. In this work, the cytokinin content of root exudate, obtained under vacuum, and of leaf exudate, obtained by the EDTA-method, has been analyzed comparatively in vegetative and induced plants, using reversed-phase HPLC coupled to the *Amaranthus* bioassay. The results show that, as early as 16 hours after the start of the LD, there is an increase of cytokinin activity in both the root and leaf exudates of induced plants. These observations fit nicely with previous results obtained on *Sinapis*, and they indicate that cytokinins are part of the floral stimulus in this species.**

In 2-month-old vegetative plants of *Sinapis alba*, flowering can be induced by a single LD.[2] In response to this treatment, a great variety of changes occurred in the meristem (2), one of the earliest changes being a mitotic wave that always culminated 26 to 30 h after start of the inductive treatment. So far, it has never been possible to dissociate the flowering process from this event, but it was possible to trigger the mitotic rise in the absence of flowering by subjecting the plants to a single day of 11 or 12 h (1). These results suggested that the early mitotic wave was essential to floral evocation in *Sinapis* and that the floral stimulus consisted of more than one component. Defoliation experiments showed that the mitotic activation was controlled by a leaf-generated stimulus moving out of the leaf around the 16th h after start of the LD (3). The nature of this mitotic stimulus was unknown, but evidence suggested that it could be a cytokinin. A single application of an exogenous cytokinin, made directly to the apical bud of vegetative plants maintained in noninductive 8-h SD (at a time corresponding to the movement of the stimulus in plants induced by a single LD), produced a wave of cell divisions identical to that observed after one LD (4). A number of other changes, normally occurring in the meristem of fully induced plants, were also caused by the cytokinin in SD while some events associated with full evocation were not detected and flower primordia were not produced, indicating that the cytokinin caused only partial evocation (12).

If cytokinins were actually one of the controlling factors of flower initiation in *Sinapis*, an inductive LD should produce critical changes in their endogenous status. Preliminary experiments showed that cytokinin levels in the leaves were indeed higher 16 h after the start of the LD in induced plants than in vegetative controls (2).

The aim of this study was to determine whether changes occur in the cytokinin fluxes within the plant in response to an inductive LD. We developed techniques to collect xylem and phloem exudates, and we analyzed their cytokinin content comparatively in vegetative and induced plants.

MATERIAL AND METHODS

Growth Conditions. *Sinapis alba* L. plants were raised in growth cabinets (Conviron, Winnipeg, Canada) from seeds (Maison Job, Nancy, France) sown at random on a mixture of clay and peat. Ten-d-old seedlings were transplanted in 8-cm pots containing a mixture of perlite and vermiculite (1:1). The growth conditions were: day and night temperature, 20°C, RH, 80%; fluorescent white light furnished by V.H.O. Sylvania tubes providing at the top of the plants an irradiance of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$; watering occurred every 2 d with demineralized water and, once a week, with Hoagland solution. Plants were grown for 9 weeks in 8-h SD in which condition they remain strictly vegetative. They were induced to flower by a single 22-h LD and then returned to SD. These plants will be referred to as 'induced' plants in contrast to 'control' plants that were continuously kept in SD.

Collection of Root Exudate. Roots of *Sinapis* do not normally exude when the shoot is excised unless vacuum is applied. This was achieved by fitting a 5 ml disposable syringe with silicon rubber tubing on the cut stump of plants decapitated at the cotyledonary node. By pulling back the piston and maintaining it with a small rod, enough vacuum was provided (about −0.07 to −0.08 MPa) in the syringe to ensure exudation. After 16 h, 3 to 4 ml of exudate were collected per plant. For cytokinin analysis the exudates from 30 plants were pooled. Exudates were immediately frozen after collection and stored at −80°C.

Collection of Leaf Exudate. The EDTA method of King and Zeevaart (19) was adapted to *Sinapis*. The optimum conditions for exudation were determined by measuring the dry weight of exudates obtained from 30 leaves in various conditions. The exudation procedure selected for subsequent analysis was as follows: 200 adult leaves (the first 5 leaves below the half-expanded leaf of 40 plants) were placed together in a 500 ml beaker containing 50 ml of 20 mM EDTA (pH 7.5) after the petioles had been recut under distilled water. The beaker was enclosed in an airtight vessel containing water to ensure maximum RH and to prevent EDTA uptake by the leaves. The leaves were maintained in the EDTA solution until the end of the exudation period,

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[2] Abbreviations: LD, long day; MeOH, methanol; TEAA, triethylammonium-acetate; AcCN, acetonitrile; Z, trans-zeatin; ZR, trans-zeatin riboside; 2-iP, isopentenyladenine; 2-iPA, isopentenyladenine riboside.

since transferring them to distilled water after 1 or 2 h in EDTA reduced exudation by 90 to 95%. During exudation, the leaves were subjected to a light-dark cycle identical to that experienced by intact induced and control plants, since exudation rate was similar in light and darkness.

As we noticed that petioles became leaky after a time in the EDTA solution, the possibility of exudate contamination by leakage of the petiole cells was examined by dipping small pieces of petioles in the EDTA for the same time as entire leaves. We estimated that at least 80% of the dry weight recovered after exudation originated from the leaf blades. After TLC analysis of carbohydrates, sucrose, which is the major sugar in phloem exudate of most species (10), was detected in the exudates from entire leaves only, indicating its phloem origin. After 16 h in EDTA, the leaves were removed and the exudates were immediately frozen at −80°C.

Exudate Purification and Cytokinin Analysis. In preliminary experiments the exudates were fractionated in aqueous and butanolic fractions, and the aqueous fraction was treated with alkaline phosphatase and reextracted with butanol before assaying as described below. As no cytokinin activity could be detected in the aqueous fraction, this purification step was finally omitted. Root exudates were purified in a single step using C18 cartridges (Baker 10-SPE, C18, 3 ml). Ten ml of MeOH, followed by 10 ml of 1 mM TEAA solution (pH 7), were passed through the cartridge before up to 100 ml of root exudate were loaded and washed through with 10 ml of TEAA. Cytokinins retained on the cartridge were eluted with 3 times 1 ml of AcCN:water (35:65). Recovery of cytokinins with this method was found to be >95% when pure standard cytokinins (purchased from Sigma) were passed through the cartridge and estimated by analytical HPLC. The AcCN fraction was evaporated under vacuum to 300 μl, and filtered on Millipore HV 0.45 μm before HPLC.

Leaf exudates were similarly purified except that EDTA contained in the exudates had to be eliminated first. This was done by drying the exudates under vacuum and redissolving them into 1 ml of water followed by 20 ml of MeOH. Most of the EDTA precipitated in MeOH and was eliminated by centrifugation (15,000 rpm; 5 min). MeOH was eliminated under vacuum and the exudates dissolved into 20 ml of water before passing through the C18 cartridge.

Separation of cytokinins was performed by HPLC. The equipment used consisted of a LKB 2150 HPLC pump (LKB, Bromma, Sweden) with a LKB 2152 gradient controller. Samples were loaded using a Rheodyne injector fitted with a 250-μl sample loop. Reverse-phase chromatography with gradient elution was used to separate the different cytokinins (Fig. 1). Absorbancy at 268 nm was monitored by an LKB 2151 variable wavelength detector. A mixture of pure standard cytokinins was injected regularly during work to estimate the variations of retention times. These variations never exceeded 2%. Fractions were collected every minute during 20 min and the cytokinin content of each fraction was estimated by the *Amaranthus* bioassay as described by Biddington and Thomas (5). The activity in each fraction was compared with a standard curve made by serial dilution of a solution of BA, and the results were expressed as equivalent quantities of BA.

RESULTS

Root and leaf exudates were collected at the same time on plants induced by a 22-h LD and on control plants kept in 8-h SD, during a 16-h period starting 16 h after start of the LD or SD. The results presented were obtained over five independent experiments for root exudate and four experiments for leaf exudate.

Effect of Induction on Root and Leaf Exudation Rate. The volume of root exudate collected from induced plants was slightly, but consistently, increased in all experiments (Table I). However, owing to the large variability between individuals, the differences were not significant at $P = 0.05$. Leaf exudation was markedly stimulated, in all experiments, by the extension of the photoperiod (Table I).

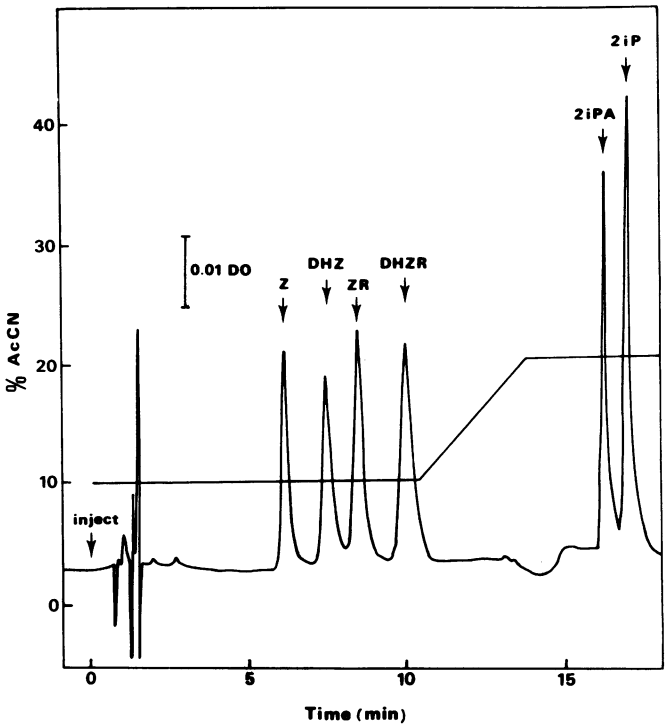

FIG. 1. UV-Absorbance trace from reverse-phase HPLC of a standard mixture of cytokinins. Stationary Phase: Nucleosil 5 μm C18, 100 mm long, 4 mm diameter; mobile phase: AcCN 10%, pH = 7 with TEAA, for 11 min, 1 ml/min, then linear gradient to 25% AcCN after 3 min, AcCN 25% for 20 min. Sample: ±400 ng of each cytokinin in 250 μl of water. Detection: 268 nm. Z, zeatin; ZR, zeatin riboside; DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; 2-iP, isopentenyladenine; 2-iPA, isopentenyladenine riboside.

Effect of Induction on Cytokinin Content of Root and Leaf Exudates. Typical HPLC-bioassay profiles of root and leaf exudates in induced and control plants are shown (Fig. 2).

Quantitative results obtained over several independent experiments are summarized in Tables II and III.

For root exudates, the main peak of cytokinin activity co-chromatographed with ZR in the five experiments. In two experiments, a lower, but significant amount of activity co-chromatographying with Z was detected, while activity co-chromatographying with 2-iP-type cytokinins was also found twice. An increase in the total cytokinin activity of the root exudate of induced plants was observed in all experiments and in all fractions where activity was detectable.

In leaf exudate, the relative distribution of the cytokinin activity on the chromatogram was quite variable from experiment to experiment. In all cases, cytokinin activity co-chromatographying with 2-iP + 2-iPA was detected, while activity having the retention times of Z and ZR was detected in three out of the four experiments. As for the root exudate, an important increase in the cytokinin activity occurred in the induced plants; it is observed in all experiments and in all fractions exhibiting a detectable activity, except in the Z and ZR fractions of experiment 4 where activity in induced plants was a bit lower than in the control.

Table I. *Volumes of Root Exudates and Dry Weights of Leaf Exudates Collected from Induced and Control Plants during a 16 h Period Starting 16 h after Beginning of the LD or SD*

Data are means ± SE for root exudate. Statistical treatment could not be applied in the case of leaf exudate since it was not possible to measure leaf exudation for each plant individually

Experiment No.	Root Exudate		Leaf Exudate	
	Induced	Control	Induced	Control
	ml per plant		*mg per leaf*	
1	4.28 ± 0.32	3.99 ± 0.32		
2	3.34 ± 0.21	2.81 ± 0.29	2.70	1.70
3	4.12 ± 0.22	3.67 ± 0.21	3.18	1.72
4	3.03 ± 0.28	2.84 ± 0.24	3.40	2.40
5	4.11 ± 0.26	3.35 ± 0.23	3.30	1.49

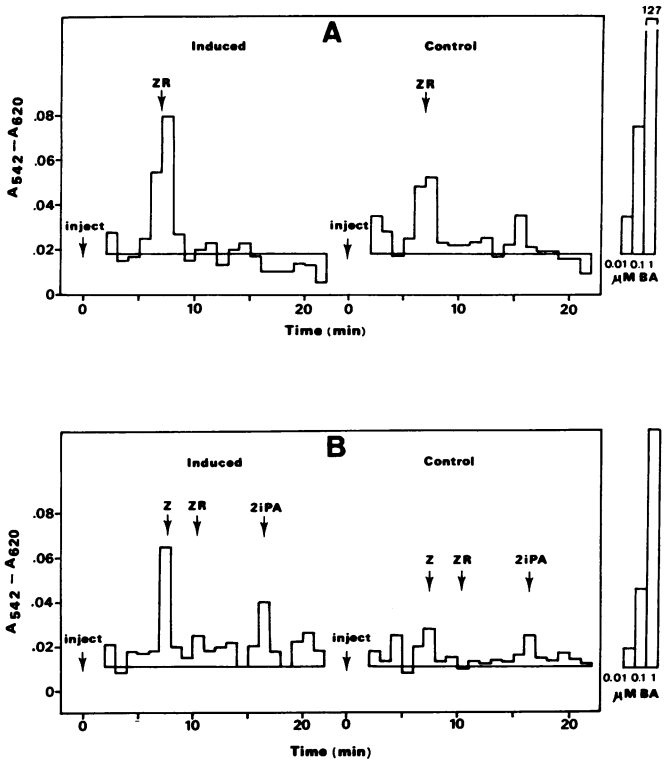

FIG. 2. Typical HPLC-bioassay profile of root (A) and leaf (B) exudates collected from induced and control plants during a 16-h period starting 16 h after beginning of the LD or SD.

DISCUSSION

Our results concerning the identity of the cytokinins in the exudates are consistent with data from the literature. Compounds with chromatographic properties similar to Z and ZR have been found to be major cytokinins in the xylem exudate of a number of species, such as *Populus robusta, Lycopersicon esculentum, Helianthus annuus, Bougainvillea* sp. *Glycine max*, and *Phaseolus vulgaris* (11, 13, 17, 20, 23, 24). The presence of 2-iP and/or its riboside in the xylem exudate of *Bougainvillea* was also suggested (24), and, recently, 2-iP-type cytokinins were detected by immunoassay in xylem exudate collected from *Eucalyptus* (8). In contrast, owing to the difficulties encountered to collect large quantities of reasonably pure phloem sap, little is known about the cytokinin composition of phloem exudate, and the major forms of cytokinins found in phloem sap seem to vary from one species to another. In the bleeding sap of *Yucca* inflorescence stalks, cytokinins occurred mainly as nucleotides of Z and 2-iP (25, 26), while in *Xanthium strumarium*, Phillips and Cleland (21) found that phloem sap obtained by the aphid technique contained cytokinins co-chromatographying with Z and its riboside. The presence of large amounts of cytokinin glucosides was also reported in the honeydew from senescing leaves of *Salix babylonica* (23). Finally, high levels of 2-iP-type cytokinins, as compared with the zeatin-types, were found to occur in the phloem exudate of a number of tree species (29). Unfortunately, the radioimmunoassay technique used in the latter study did not permit one to differentiate between free bases, ribosides, ribotides, or glucosides.

The increased flux of cytokinin observed in the root exudate following induction by LD results mainly from a rise in cytokinin concentration, since exudate volumes collected from both induced and vegetative plants are very similar. This increase suggests the existence of a shoot-to-root signal under daylength control that modifies the export of cytokinins from the roots via the xylem. The possibility of such a signal has been discussed in *Xanthium strumarium* (14, 15, 28), a SDP, in which a decline of the cytokinin content of root exudate was detected after only one long night that was sufficient to cause flower initiation. In *Xanthium*, floral initiation and decrease in cytokinin level seem closely related, as both can be reversed by interrupting the long night with a light break (15). Although these changes in cytokinin level of the root exudate are opposite in *Xanthium* and *Sinapis*, they suggest that the roots, as a major source of cytokinin, have an important role to play in the induction of flowering in these two species.

A puzzling observation is that flowering can be induced in *Sinapis* plants that have been derooted and placed on distilled water 1 or 2 d before receiving a LD (2) (P Lejeune *et al.* unpublished result). This finding does not rule out, however, the possibility that cytokinins are essential to the reproductive transition since the roots are not the only source of cytokinin in the plant (9, 22, 27). In derooted *Sinapis* plants, shoot parts could thus substitute for roots in producing or releasing cytokinins, although, in intact plants, roots could be the main site of production of this growth regulator.

The changes in the leaf exudate are of special interest. The increase in dry weight occurring in response to daylength extension is probably due to an increased sucrose content as suggested by TLC analysis of exudates for carbohydrates. Increased levels in starch and/or soluble sugars were also recorded in leaves and apical buds of induced plants from the 10th h of the LD (6, 7, 18). The rise in cytokinin export from the leaves fits well with the hypothesis that cytokinins represent the mitotic trigger which moves out of the leaves 16 h after start of the LD (2). The only other plant studied so far in this respect is *Xanthium* in which a rise in cytokinin activity in the honeydew of aphids feeding on induced plants has been reported (21). This result in *Xanthium* is difficult to integrate with the observations of Henson and

Table II. *Cytokinin Content of Root Exudates Produced by Induced and Control Plants during a 16-h Period Starting 16 h after Beginning of the LD or SD*

Experiment Number	Z		ZR		2iP + 2iPA	
	Induced	Control	Induced	Control	Induced	Control
	equivalents of pM of BA produced per plant					
1	2.80	0.51	3.82	1.80	1.97	1.60
2	0.40	0.31	1.08	0.22	ND[a]	ND
3	ND	ND	1.85	0.57	ND	ND
4	ND	ND	3.77	1.71	ND	ND
5	ND	ND	0.80	ND	0.82	ND

[a] Not detectable.

Table III. *Cytokinin Content of Exudates Collected from Leaves of Induced and Control Plants during a 16-h Period Starting 16 h after Beginning of the LD or SD*

Experiment No.	Z		ZR		2iP + 2iPA	
	Induced	Control	Induced	Control	Induced	Control
	equivalents of pM of BA produced per leaf					
1	0.97	0.27	0.46	0.19	0.48	0.34
2	ND[a]	ND	ND	ND	2.80	0.13
3	0.62	0.05	3.98	0.99	1.47	0.30
4	0.17	0.25	0.20	0.21	0.46	0.18

[a] Not detectable.

Wareing (16) indicating that after a single inductive SD the cytokinin content of *Xanthium* plants decreases markedly. It must be noted, however, that because of the rather long time needed with the aphid method to collect sufficient quantities of phloem sap, the plants used by Phillips and Cleland may have not been at the same developmental state as those used by Henson and Wareing.

In view of the present results we suggest that, following exposure to an inductive LD, the leaves of intact *Sinapis* plants generate a factor that modifies the export of cytokinins from the roots and increases their concentration in the transpiration stream. Since the leaves drain most of the xylem content, this may explain the enhanced cytokinin level in the leaves at the 16th h (2). Cytokinins would then be re-exported from the leaves to the apical bud where they induce a mitotic wave and some other events normally associated with the floral transition.

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