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Possible Role of Cytokinins in Flower Induction in Strawberry

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Abstract: During inductive conditions, biochemical or physiological changes are recognized. One possible change that might occur is hormone content. In this study, changes in endogenous cytokinins in the shoot tips, leaves and roots of strawberry plants (*Fragaria* × *ananassa* Duch cv. Kordestan) under flower bud inductive conditions were measured and compared with non-induced plants. Runner plants were potted and grown in non-inductive conditions (high temperature; long-day). Half of the plants were then put under inductive conditions (low temperature; short-day) for 3 weeks. Samples for cytokinin analysis were taken from induced and non-induced plants every 3 days during 3 weeks. Results indicated that free cytokinin in shoot tips of induced plants were significantly higher than in non-induced plants. Free cytokinin in shoot tips and leaves of induced plants increased from 3 to 12 days After the Start of the Short-day Treatment (DASST) and thereafter decreased. Bound cytokinin level in shoot tips of induced plants decreased from 3 to 12 DASST. Free and bound cytokinin in roots of induced plants decreased from 3 to 12 DASST and increased afterward. The results suggest that the changes in free cytokinin during the process of flower induction play an important role in flower induction in strawberries.

Key words: Flower induction, free and bound cytokinins, strawberry

INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is a widely adapted small fruit grown from the low-latitude of tropics and subtropics to high-latitude, cold continental areas (Hancock, 1999). It is well known that night chilling, short daylength, or restriction of nitrogen supply effectively induce strawberry flower buds (Guttridge, 1985). Flower induction is the event that initiates the transition of a vegetative apex to a floral apex in response to an environmental or development cue. In photoperiodically sensitive plants, the flowering signal is translocated from the perceiving organs (leaves) to the apex. In many species, however, flowering can be induced by a variety of environmental and/or chemical signals (Darnell *et al*., 2003). Flower induction in June-bearing strawberry is controlled by photoperiod and temperature and a period of time of inductive conditions is required (Guttridge, 1985; Serce and Hancock, 2005). During inductive conditions, biochemical and/or physiological changes are recognized. One of the possible changes that might occur is in hormone content (Bernier *et al*., 1993). Changes in endogenous plant growth regulators during flower induction are still unclear; although it has been pointed out that the regulators could be closely related to reproductive growth.

Although cytokinins (CK) are suspected to be involved in the process of flower initiation in strawberry and other species, evidence for this involvement is minimal. Yamasaki and Yamashita (1993) reported that the CK composition changes in strawberry crowns during flower initiation. Zeatin increased just before flower initiation then decreased. Exogenous CK increased inflorescence number in both SD and LD strawberry cultivars, presumably by increasing branch crown formation (Weidman and Stang, 1983). In other species, changes in CK composition during the flowering event

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have been reported. For instance, isopentenyladenine (ip), isopentenyladenosine (2iPA) and ZR concentrations were lower before flower induction than after in *Litchi chinensis* Sonn. (Chen, 1991), *S. alba* (Bernier *et al*., 1993), *Aranda* (Zhang *et al*., 1995), *Euphorbia longana* (Chen *et al*., 1997a), *Phalaenopsis hybrida* (Chou *et al*., 2000), pear (Ito *et al*., 2001), *Arabidopsis thaliana* (Corbesier *et al*., 2003b) and *Olea europaea* L. (Ulger *et al*., 2004). Chen *et al*. (1997b) showed that the floral transition (trigger spiking) in *Phalaenopsis hybrida* requires a period of low temperature. On the other hand, high temperature reduces the endogenous levels of auxins, cytokinins and gibberellins, but increases ABA and ethylene levels (Yakushikina and Tarasov, 1982). Chou *et al*. (2000) suggested that high temperature leads to an accumulation of glucoside cytokinins and a reduction of free base and riboside cytokinins in *Phalaenopsis hybrida*.

In general, in photoperiodic plants, daylengths favorable for flowering are essentially perceived by expanded leaves in which they cause floral induction. This process then results in the production and export of a floral stimulus which moves in the phloem and eventually reaches the Shoot Apical Meristem (SAM) where it causes floral evocation followed by flower formation (Thomas and Vince-Prue, 1997). In *Sinapis* plants induced to flower by a single long day, the cytokinin content increases significantly in the leaves and in the leaf phloem sap at the time of movement of the floral stimulus (Bernier *et al*., 1993; Lejeune *et al*., 1994). Subsequently, the cytokinin content increases in the SAM, at the time of early mitotic activation (Jacqmard *et al*., 2002). In addition, an exogenous cytokinin application to vegetative plants grown in short days can induce various cellular and molecular changes in the SAM that are normally associated with floral transition (Bernier *et al*., 2002). On account of a positive effect of cytokinin on flowering, the late flowering *uniflora* mutant of tomato shows a marked acceleration of flowering when supplied with a cytokinin (Dielen *et al*., 2001). Corbesier *et al*. (2003) found that, in both the leaf tissues and leaf exudates of *Arabidopsis thaliana*, isopentenyladenine forms of cytokinins increased from 16 h after the start of the long day. At 30 h, the shoot apical meristem of induced plants contained more isopentenyladenine and Zeatin than vegetative controls. These cytokinin increases were well correlated with the early events of floral transition. Though changes in endogenous cytokinins have an important role in flower induction in some plants, however, it has not been thoroughly investigated in strawberry. In this study, free, bound and total cytokinin contents in the shoot tips, leaves and roots of June-bearing strawberry plants during flower induction were determined and compared with cytokinin content in non-induced plants.

MATERIALS AND METHODS

Plant Material

Runner plants of cv. Kordestan were potted in 18 cm diameter pots and grown under non-inductive conditions (28±3°/22±3°C day/night; 16 h daylength) using supplemental light from a combination of fluorescent and incandescent lamps. After the establishment of plants, the plants were transferred to flower-inducing conditions (23±3°/13±3°C day/night 8 h daylength). Samples for cytokinin analysis were taken from induced and non-induced plants between 10.00-12.00 h on each sampling date to minimize diurnal effects. Four replicate plants were sampled every 3 days over a 3 weeks period of flower induction. Shoot tips, leaves and roots were sampled. The apex was cut 1 mm below the growth point, with four-to-five leaf primordia as the shoot tip.

All samples were washed immediately in cold water and packed in ice for transfer to the laboratory. Samples were then freeze-dried at -50°C for 48 h, ground using a Wiley Mill, passed through a 40-mesh screen and stored at -20°C until analyzed for cytokinin contents.

Cytokinin Analysis

Extraction, purification and quantitative determination of free and bound cytokinin in samples were done, with minor modifications, according to the Unyayar *et al*. (1996) and Ergun *et al*. (2002).

One gram dry weight (Freeze-dried) of each sample was taken and combined with 60 mL of methanol: chloroform: 2N ammonium hydroxide (12:5:3 v/v/v). Each combined extract (60 mL) was kept in a bottle at -20°C in deep freeze for further analysis. Combined extract was treated with 25 mL distilled water. The chloroform phase was discarded. The water-methanol phase was evaporated using a vacuum rotary evaporator (Quickfit, Instrumentation, England). The water phase was adjusted to the extract pH value of 7 with 2N HCl and 15 mL ethyl acetate was added at each of the three steps. This procedure provided the isolation of free-form cytokinin from the extraction solvent. After adjusting the extract pH value to 11 with 2N NaOH and an incubated for 1 h at 70°C, the same procedure was used for the isolation of bound-form cytokinin from the extraction solvent. Evaporation of ethyl acetate was performed at 45°C using a vacuum rotary evaporator (Quickfit, Instrummentation, England). Thin-layer Chromatography (TLC) was done using silica gel 60 (Merck Chemicals, Germany).

TLC-Separated cytokinin was isolated from the aluminum plates according to the standard synthetic benzyladenine RF values. Giving cytokinin RF was dissolved with 2 mL of methanol for filtration and separation from silica using plunger-operated pipetter (Transferpette, Brand, Germany).

Spectrophotometric assay was done using 269 nm wave length for standard synthetic benzyladenine and isolated samples. Total cytokinin was then calculated as the sum of free and bound cytokinin. The amount of cytokinin in samples was expressed as standard synthetic benzyladenine equivalent. Cytokinin contents were expressed as $\mu g\ g^{-1}$ d.w.

Statistical Analysis

The experiment was arranged in a completely randomized design with four replications. Statistical analysis was performed using MSTATC software. Standard error in individual determinations was calculated.

RESULTS

Free, Bound and Total Cytokinin Levels in Shoot Tips

Time of sampling, inducing state and interaction of time of sampling and inducing state had a significant effect on free, bound and total cytokinin contents in shoot tips (Table 1).

Free cytokinin in shoot tips of induced plants increased from 3 to 12 days after the start of the short-day treatment (DASST) and thereafter decreased (Fig. 1). Free cytokinin concentration in shoot tips of induced plants at 3, 6, 9, 12 and 15 DASST were significantly higher than those of non-induced plants. In non-induced plants, there were no significant differences in free cytokinin level among sampling dates (Fig. 1).

Bound cytokinin level in shoot tips of induced plants decreased from 3 to 12 DASST and then increased from 15 to 21 DASST. The lowest level was at 12 DASST. In induced plants, bound cytokinin level from 3 to 21 DASST was significantly lower than in those of non-induced plants (Fig. 1). Total cytokinin level in induced plants decreased from 3 to 21 DASST and in these sampling dates they were significantly lower than those of non-induced plants. There were no significant differences in total cytokinin level of non-induced plants among different sampling dates (Fig. 1).

Table 1: Statistical significances effects of inducing state of strawberry plants (Induced or non-induced) and time of sampling, as a factorial experiment, on cytokinin contents during flower induction in shoot tips

	Source of variation		
Cytokinin	Time of sampling	Inducing state	Time of sampling × Inducing state
Free cytokinin	*	*	*
Bound cytokinin	*	*	*
Total cytokinin	*	*	*

*p<0.05

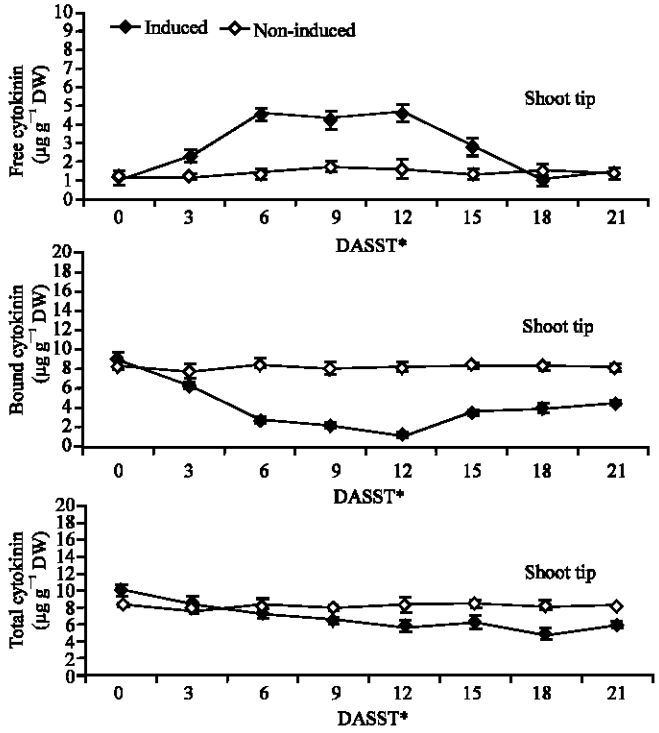

Fig. 1: Changes in free, bound and total cytokinin levels in shoot tips of induced and non-induced strawberry plants during flower induction period. Data are means±SE (n = 4). * Days after the start of the short-day treatment

Table 2: Statistical significances effects of inducing state of strawberry plants (Induced or non-induced) and time of sampling, as a factorial experiment, on cytokinin contents during flower induction in leaves

	Source of variation		
Cytokinin	Time of sampling	Inducing state	Time of sampling × Inducing state
Free cytokinin	*	ns	*
Bound cytokinin	*	*	ns
Total cytokinin	*	*	*

*p<0.05, ns = not significant

Free, Bound and Total Cytokinin Levels in Leaves

Time of sampling had a significant effect on free, bound and total cytokinin contents in leaves. Bound and total cytokinin contents were significantly affected by inducing state. Free and total cytokinin contents were significantly affected by the interaction of sampling time and inducing state (Table 2).

In leaves of induced plants, free cytokinin level increased at 3, 6 and 9 DASST and thereafter decreased (Fig. 2). In non-induced plants, there were no significant differences among different sampling dates. Free cytokinin concentration in leaves of induced plants at 6 and 9 DASST were significantly higher and at 12, 15, 18 and 21 DASST lower than in those of non-induced plants (Fig. 2). In induced plant, bound cytokinin level in leaves decreased at 3 DASST and thereafter remained at constant level. In non-induced plants, there were no significant differences among sampling dates (Fig. 2).

Total cytokinin level in leaves of induced plants decreased from 12 to 21 DASST and in these sampling dates they were significantly lower than in those of non-induced plants (Fig. 2). There were no noticeable changes in total cytokinin level in leaves of non-induced plants (Fig. 2).

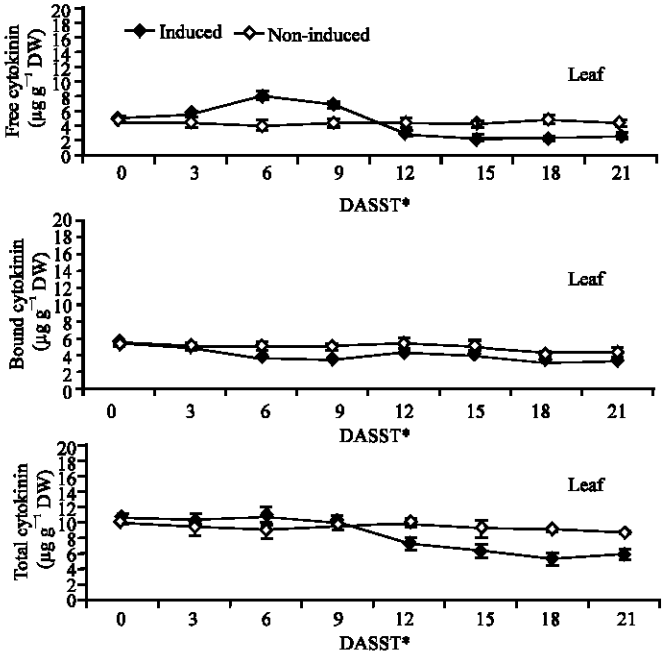

Fig. 2: Changes in free, bound and total cytokinin levels in leaves of induced and non-induced strawberry plants during flower induction period. Data are means±SE (n = 4) * Days after the start of the short-day treatment

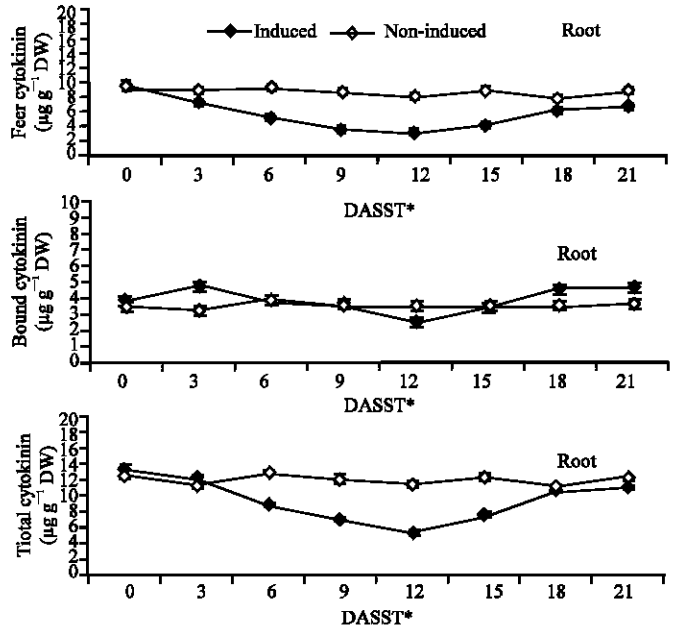

Fig. 3: Changes in free, bound and total cytokinin levels in roots of induced and non-induced strawberry plants during flower induction period. Data are means±SE (n = 4) * Days after the start of the short-day treatment

Table 3: Statistical significances effects of inducing state of strawberry plants (Induced or non-induced) and time of sampling, as a factorial experiment, on cytokinin contents during flower induction in roots

	Source of variation		
Cytokinin	Time of sampling	Inducing state	Time of sampling × Inducing state
Free cytokinin	*	*	*
Bound cytokinin	*	*	*
Total cytokinin	*	*	*

$p<0.05$, ns = not significant

Free, Bound and Total Cytokinin Levels in Roots

Time of sampling, inducing state and interaction of time of sampling and inducing state had a significant effect on free, bound and total cytokinin contents in roots (Table 3).

Free cytokinin in roots of induced plants decreased from 3 to 12 DASST and increased afterward, so that the lowest level was at 12 DASST (Fig. 3). In non-induced plants free cytokinin level in roots did not significantly change in different sampling dates. In induced plants, bound cytokinin level in roots decreased at 9 and 12 DASST and increased at 3, 18 and 21 DASST (Fig. 3).

Total cytokinin in roots of induced plants decreased from 3 to 15 DASST and increased at 18 and 21 DASST. The lowest level in induced plants was at 12 DASST. During the period of experiment total cytokinin level showed no significant differences among different sampling dates in non-induced plants (Fig. 3).

DISCUSSION

Results in Fig. 1-3 shows that free cytokinin in leaves and shoot tips of induced plants increased noticeably at 3, 6 and 9 DASST. While leaves bound cytokinin in these sampling dates decreased. Free cytokinin in roots of induced plants decreased from 3 to 12 DASST and thereafter increased. However, bound cytokinin in roots of induced plants did not noticeably change. These findings are in agreement with those obtained by Yamasaki and Yamashita (1993), who observed an increase in zeatin riboside before flower initiation followed by a specific increase of zeatin riboside just after the initiation occurred in the crowns of strawberry plants. In addition, cytokinins have been shown to occur in higher concentration in short-day (Henson and Wareing, 1974) and their application can induce flowering in some SD plants (Kinet *et al*., 1985). Chang *et al*. (1999) observed that cytokinin levels in tuberose corms during floral induction increased and suggested that cytokinins have a role in tuberose apex evocation. Ulger *et al*. (2004) suggested that an increase in cytokinins during the flower induction period in olive may have a positive role on floral formation. Cytokinin increases were well correlated with the early events of floral transition (Corbesier *et al*., 2003).

In photoperiodic plants, well expanded leaves are essential for perceiving light, to cause floral induction. This process then results in the production and export of a floral stimulus which moves in the phloem and eventually reaches the Shoot Apical Meristem (SAM) where it causes floral evocation followed by flower formation (Thomas and Vince-Prue, 1997). In *Sinapis alba* exposure to a single LD has caused the rapid signal probably sucrose production, in mature leaves that was transported quickly to the root system, presumably in the phloem. The level of cytokinins in the root exudates has been reported to increase early and transiently in response to the inductive LD (Bernier *et al*., 1993). This increase might be due to either an enhanced cytokinin biosynthesis by roots or an increased release of preexisting cytokinins by roots. In *Sinapis* plants induced to flower by a single long day, the cytokinin content increased significantly in the leaves and in the leaf phloem sap at the time of movement of the floral stimulus (Bernier *et al*., 1993; Lejeune *et al*., 1994). Subsequently, the cytokinin content increased in the SAM, at the time of early mitotic activation (Jacqmard *et al*., 2002). In addition, an exogenous cytokinin application to vegetative *Sinapis* plants grown in short days can induce various

cellular and molecular changes in the SAM that are normally associated with floral transition (Bernier *et al*., 2002). It was found that the increased root-to-shoot flux of cytokinins were essential for flowering in *Sinapis alba* (Bernier *et al*., 1993).

The possible role of cytokinins in flower induction and development is not quiet clear; however the results reported here suggest that the changes in free cytokinin during the process of flower induction play an important role in flower induction in strawberries.

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