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Comparison of endogenous cytokinins and cytokinin oxidase/dehydrogenase activity in germinating and thermoinhibited *Tagetes minuta* achenes

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ABSTRACT

Tagetes minuta L. achenes are thermoinhibited at temperatures above 35 °C and have accelerated radicle emergence (germination) when subsequently transferred to an optimal temperature (25 °C). Endogenous cytokinins and cytokinin oxidase/dehydrogenase (CKX) activity were compared in normally germinating (25 °C) and thermoinhibited (72 h at 36 °C then transferred to 25 °C) *T. minuta* achenes. Following imbibition, endogenous cytokinin concentrations changed in normally germinating *T. minuta* achenes, with a gradual decrease in dihydrozeatin-type (DHZ) cytokinins, a large increase in *cis*-zeatin-type (*cZ*) cytokinins, a smaller increase in N^6-(2-isopentenyl)adenine-type (iP) cytokinins and a peak of *trans*-zeatin-type (*tZ*) cytokinins at 13 h. These changes in the isoprenoid cytokinin profile were similar in the thermoinhibited achenes imbibed at 36 °C, despite the thermal block preventing radicle emergence. The exception was the iP-type cytokinins that only increased when transferred to 25 °C. Profiles of the physiologically active free bases showed an increase in *tZ* prior to radical emergence in both normally germinating (13 h) and thermoinhibited achenes. A large transient peak in aromatic cytokinins [N^6-benzyladenine-type (BA)] occurred during early seedling establishment in normally germinating achenes (40 h) while a transient maximum in BA-type cytokinins was found prior to radicle emergence in the thermoinhibited achenes (24 h). The CKX activity was enhanced in normally germinating achenes as the cytokinin concentration increased following imbibition. In thermoinhibited achenes, an elevated temperature negatively affected the CKX activity that only increased when the achenes were transferred to 25 °C, corresponding to an increase in iP-type cytokinins. However, the favored cytokinin deactivation pathway in *T. minuta* appears to be 9-glycosylation, as 9-glucosides accounted for over 50% of the total cytokinin pool in both normal and thermoinhibited achenes.

Introduction

Viable seeds are considered dormant if they fail to complete germination in favorable conditions. Temperature is an important environmental factor, with the optimal temperature range for germination varying from species to species. Thermodormancy is the suspension of germination at an elevated temperature with a dormancy-breaking treatment required prior to germination resuming at an optimal temperature. In contrast, thermoinhibited seeds only require a shift to within the optimal temperature range to immediately induce germination to resume (Horowitz and Taylorson, 1983). Thermoinhibition can be either coat-imposed by mechanical restraints, permeability barriers or inhibitory substances in the seed coat or embryo-imposed by endogenous chemicals (Hills et al., 2001). Endogenous hormones, especially abscisic acid (ABA), are responsible for the imposition and

Abbreviations: ABA, abscisic acid; Ade, adenine; BA, N^6-benzyladenine; BAR, N^6-benzyladenosine; BARMP, N^6-benzyladenosine-5′-monophosphate; BA9G, N^6-benzyladenine-9-glucoside; CKX, cytokinin oxidase/dehydrogenase; *cZ*, *cis*-zeatin; *cZOG*, *cis*-zeatin-*O*-glucoside; *cZR*, *cis*-zeatin riboside; *cZRMP*, *cis*-zeatin riboside-5′-monophosphate; *cZROG*, *cis*-zeatin riboside-*O*-glucoside; *cZ9G*, *cis*-zeatin-9-glucoside; DHZ, dihydrozeatin; DHZOG, dihydrozeatin-*O*-glucoside; DHZR, dihydrozeatin riboside; DHZRMP, dihydrozeatin riboside-5′-monophosphate; DHZROG, dihydrozeatin riboside-*O*-glucoside; DHZ9G, dihydrozeatin-9-glucoside; iP, N^6-(2-isopentenyl)adenine; iPR, N^6-(2-isopentenyl)adenosine; iPRMP, N^6-(2-isopentenyl)adenosine-5′-monophosphate; iP9G, N^6-(2-isopentenyl)adenine-9-glucoside; *m*T, *meta*-topolin; *m*TR, *meta*-topolin riboside; *o*T, *ortho*-topolin; *tZ*, *trans*-zeatin; *tZOG*, *trans*-zeatin-*O*-glucoside; *tZR*, *trans*-zeatin riboside; *tZ9G*, *trans*-zeatin-9-glucoside.

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maintenance of embryo-imposed thermoinhibition while gibberellins, ethylene and cytokinins are associated with the alleviation of thermoinhibition (Taylor et al., 2005).

Tagetes minuta L. is an example of a thermoinhibited species. Optimal conditions for germination of *T. minuta* achenes in the laboratory are 25 °C in continuous low light. Radicle emergence is first observed after 14 h imbibition and maximum radicle emergence (germination) achieved after 30 h (Forsyth and van Staden, 1983; Hills et al., 2001). Achenes are thermoinhibited when imbibed at 35 °C (Drennan and van Staden, 1989). When there is a temperature shift to 25 °C, the thermoinhibited achenes have accelerated germination with radicle emergence within 2 h and maximum germination within 24–26 h (Forsyth and van Staden, 1983; Drennan and van Staden, 1989; Hills et al., 2001). Removal of the fruit coat and leaching of the achenes prior to imbibition had no effect on the alleviation of thermoinhibition, showing that thermoinhibition in *T. minuta* is not coat-imposed (Taylor et al., 2005). Exogenous cytokinins had an inhibitory effect on germination of *T. minuta* (Taylor et al., 2005) while gibberellins had a positive effect and could partially overcome thermoinhibition (Drewes and van Staden, 1990), especially when applied in combination with an ABA biosynthesis inhibitor (fluridone) and ethylene (ethephon; Taylor et al., 2005). These results show that control of thermoinhibition in *T. minuta* is due to the interaction of a number of endogenous factors, mainly hormonal, that are actively imposed at the embryo level (Taylor et al., 2005).

Hills et al. (2001) distinguished approximately 200 polypeptides in dry *T. minuta* achenes. The level of expression of some of these polypeptides did not change, while others either decreased or increased during germination. Other polypeptides only appeared after 12 h imbibition, and few were synthesized between 12 and 36 h. This polypeptide pattern during germination under optimum conditions was similar in the thermoinhibited achenes, with the only major difference consisting in *de novo* synthesis of ten thermoinhibition-specific polypeptides. The concentration of these ten polypeptides decreased rapidly within 2–20 h of a temperature shift to 25 °C, suggesting they may actively repress germination at high temperatures (Hills et al., 2001). cDNA clones of these genes expressed specifically in thermoinhibited *T. minuta* achenes were isolated, providing evidence that thermoinhibition in *T. minuta* is under positive genetic control (Hills et al., 2005).

Although apparently not directly involved in germination, developing seeds represent a rich source of cytokinins, being a site of cytokinin biosynthesis (Emery and Atkins, 2006). Seeds also have relatively high levels of the only known cytokinin degrading enzyme, cytokinin oxidase/dehydrogenase (CKX; Galuszka et al., 2000). We previously reported on the changes in the endogenous cytokinin profile during germination and early seedling development in *T. minuta* when germinated at 25 °C. Cytokinin concentrations increased following imbibition with highest concentrations detected at 48 h during early seedling establishment. Cytokinin concentrations gradually decreased as the seedling continued growing. The prevalent isoprenoid cytokinin types were dihydrozeatin- (DHZ), *cis*-zeatin- (*c*Z) and N^6-(2-isopentenyl)adenine-types (iP) with very low concentrations of *trans*-zeatin-type (*t*Z) cytokinins and high concentrations of the aromatic N^6-benzyladenine (BA) in the dry achenes (Stirk et al., 2005).

Cytokinins play a role in mediating heat stress responses in plants. For example, there was a decrease in *t*Z-type cytokinins and an increase in *c*Z- and iP-type cytokinins in the leaves, and to a lesser extent, the roots of heat-stressed (40 °C) tobacco plants. In addition, CKX activity was suppressed within 2 h of exposure to elevated temperatures (Dobra et al., 2010). Thermoinhibition can be broadly viewed as a heat stress response by viable seeds. As thermoinhibition in *T. minuta* is a complex interactive process under the control of a combination of plant hormones, it provides an ideal system to investigate the role of cytokinins in thermoinhibition. The aims of the present study were to compare the cytokinin profiles and CKX activity in normally germinating and thermoinhibited *T. minuta* achenes.

Materials and methods

Plant material and germination conditions

Partially dried inflorescences of *Tagetes minuta* L. were collected from a single site in Pietermaritzburg, South Africa (29°36′S; 30°23′E). To allow for after-ripening, the inflorescences were placed on open trays in the laboratory for 6 weeks until fully dried. Plant debris was removed by sieving and the achenes collected and stored in a brown paper bag at 4 °C. *T. minuta* achenes were incubated in 90 mm Petri dishes lined with two layers of Whatman's No. 1 filter paper and wetted with 7 mL distilled water. Incubation took place in a controlled-environmental chamber at 25 ± 1 °C with a light intensity of 14 μmol m^{-2} s^{-1}. This is referred to as "normal germination". Additional achenes were thermoinhibited by imbibing them at 36 ± 1 °C for 72 h. The Petri dishes were placed in sealed plastic bags (Jiffy bags) to reduce moisture loss. Following the 36 °C treatment, the Petri dishes were removed from the plastic bags and transferred to 25 °C until germination was completed.

Germination trials were conducted using four replicates of 25 achenes each of normal and thermoinhibited achenes. Achenes were monitored every 2 h and radicle emergence was recorded. Once the radicle was visible, germination was considered complete. In addition, 1 g FW samples with additional distilled water added as required to keep the filter paper moist, were collected at various times during germination for both normal and thermoinhibited achenes. Samples were immediately frozen in liquid nitrogen, ground and lyophilized. Samples were stored at −70 °C until analyzed for endogenous cytokinins and CKX activity.

Quantification and identification of endogenous cytokinins

Samples were collected to include dry quiescent achenes (0 h), a stage just prior to radicle emergence (13 h), at approximately 50% radicle emergence (26 h) and at two points a few hours after maximum germination was achieved (early seedling establishment; 40 h and 48 h). Thermoinhibited achenes were collected after 24 h, 48 h and 72 h at 36 °C and at approximately 50% radicle emergence (6 h at 25 °C) and again at two points after maximum germination was achieved (12 h and 18 h at 25 °C). Duplicate samples were analyzed for their endogenous cytokinin content using the method described by Novák et al. (2003, 2008) where samples were extracted in 70% ice-cold ethanol and a cocktail of deuterium labeled cytokinins added to check recovery during purification. These extracts were purified by combined anion (DEAE-Sephadex-C18 cartridge), octadecylsilica column and immunoaffinity chromatography based on a generic monoclonal cytokinin antibody. This method resulted in three fractions that were analyzed by UPLC (Waters Acquity UPLC™ System) linked to a Xevo™ triple quadrupole mass spectrometer (UPLC-MS/MS) equipped with an electrospray interface [ESI(+)] and photodiode array detector (Waters PDA 2996). The concentration of the various cytokinins was calculated based on the recovery values of the appropriate internal standards (Novák et al., 2003, 2008).

Determination of CKX activity and substrate specificity

Duplicate samples were collected of normally germinating achenes to include dry quiescent achenes (0 h), as the first radicles