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Effect of heat stress on polyamine metabolism in proline-over-producing tobacco plants

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ABSTRACT

The effect of heat stress on the accumulation of proline and on the level of polyamines (PAs) in tobacco plants was investigated. Responses to heat stress were compared in the upper and lower leaves and roots of tobacco plants that constitutively over-express a modified gene for the proline biosynthetic enzyme Δ1-pyrroline-5-carboxylate synthetase (*P5CSF129A*) and in the corresponding wild-type. In the initial phases of heat stress (after 2 h at 40 °C), the accumulation of proline increased in the wild type but slightly decreased in the transformants. The response to heat stress in proline-over-producing tobacco plants involved a transient increase in the levels of free and conjugated putrescine (Put) and in the levels of free spermidine (Spd), norspermidine (N-Spd) and spermine (Spm) after a 2-h lag phase, which correlated with stimulation of the activity of the corresponding biosynthetic enzymes. Diamine oxidase (DAO) activity increased in both plant genotypes, most significantly in the leaves of WT plants. Polyamine oxidase (PAO) activity increased in the roots of WT plants and decreased in the leaves and roots of the transformants. After 6 h of heat stress, proline accumulation was observed in the transformants, especially in the lower leaves; much more modest increase was observed in the WT plants. A decrease in the levels of free and conjugated Put coincided with down-regulation of the activity of ornithine decarboxylase and marked stimulation of DAO activity in the leaves and roots of the transformants. PAO activity increased in the roots of the transformants but decreased in the leaves. Conversely, in WT tobacco subjected to 6 h of heat stress, slight increases in free and conjugated PA levels were observed and the activity of DAO only increased in the roots; PAO activity did not change from the value observed during the initial phase of heat stress. 6 Hours' heat stress had no effect on the level of malondialdehyde (MDA; a product of lipid peroxidation), in the upper leaves of either genotype. After a recovery period (2 h at 25 °C), most of the studied parameters exhibited values comparable to those observed in untreated plants. The coordination of the proline and polyamine biosynthetic pathways during heat stress conditions is discussed.

1. Introduction

Temperatures above the normal optimum are sensed as heat stress by all living organisms. Heat stress disturbs cellular homeostasis and can lead to severe retardation of growth and development, and even to plant death [1]. High temperature stress induces the rapid production and accumulation of reactive oxygen species (ROS) [2,3]. These high levels of ROS seem to function as a signal, triggering protective responses; however, they are also potentially harmful to all cellular compounds and negatively influence cellular metabolic processes [4]. The detoxification of these ROS is consequently very important and plants have evolved complex strategies to deal with them [5]. Thus, plant cells typically respond to increases in ROS levels by increasing the expression and activity of ROS-scavenging enzymes and increasing their production of antioxidants in order to maintain redox homeostasis. Environmental stresses such as heat stress induce the accumulation of proline in many plant species [6]. Proline plays a role in cellular osmoregulation and also exhibits many protective effects; plants with elevated proline levels were reported to exhibit enhanced tolerance to abiotic stresses [7]. Levels of proline can be increased either by stimulation of its biosynthesis by Δ1-pyrroline-5-carboxylate synthetase(s) (P5CS) or by inhibition

Abbreviations: ADC, arginine decarboxylase; DAO, diamine oxidase; DM, dry mass; FM, fresh mass; MDA, malondialdehyde; N-Spd, norspermidine; N-Spm, norspermine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; PAs, polyamines; Put, putrescine; ROS, reactive oxygen species; RWC, relative water content; SAMDC, S-adenosylmethionine decarboxylase; SM, saturated mass; Spd, spermidine; Spm, spermine.

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of its degradation by proline dehydrogenase(s). A common method for obtaining plants with elevated proline levels involves overexpression of the P5CS gene. However, native P5CS is subject to feed-back inhibition by its product (proline). This feed-back loop can be circumvented by site-directed mutagenesis [8]. In P5CS from *Vigna aconitifolia*, this was achieved by substitution of the phenylalanine at position 129 for alanine. Using this construct, we prepared tobacco plants with significantly elevated proline content [9].

Polyamines (PAs) are also important in protecting plants against abiotic stress due to their roles in osmotic adjustment, maintenance of membrane stability and free-radical scavenging [10]. Enhanced production of PAs is one of the responses to abiotic stress [11]. Most of the biological functions of PAs are dependent on their polycationic nature, which facilitates interactions with anionic macromolecules (such as DNA and RNA) and negatively charged groups of membranes. In plant cells, PAs occur as free bases but may also be covalently linked to either small molecules (especially to hydroxycinnamic acids) to give soluble conjugated PAs, to high molecular-mass substances such as hemicelluloses and lignin, or (in small amounts) to proteins to give insoluble conjugated PAs. In plants, biosynthesis of the three most common PAs – putrescine (Put), spermidine (Spd) and spermine (Spm) – is initiated either by direct decarboxylation of ornithine by the enzyme ornithine decarboxylase (ODC) or by decarboxylation of arginine by arginine decarboxylase (ADC) via agmatine and N-carbomoylputrescine intermediates. Another essential enzyme for PA synthesis is S-adenosylmethionine decarboxylase (SAMDC), which is required for production of the aminopropyl group used in the biosynthesis of Spd and Spm. Diamine oxidase (DAO) and polyamine oxidase (PAO) are thought to play a major role in the production of H_2O_2 via catabolism of PAs in plant tissues. DAO catalyzes the oxidative deamination of diamines (Put, cadaverine) to produce Δ-pyrroline with the release of ammonia and H_2O_2. Oxidation of PAs (Spd, Spm) by PAO yields 1,3-aminopropylpyrroline, along with H_2O_2 and diaminopropane. H_2O_2 may have roles in structural defense and in signalling. However, it is also cytotoxic and causes oxidative stress when present at high levels (for a review see [12]). Changes in the levels of free PAs and in the rates of their catabolism have been observed in plants subjected to abiotic stress [13].

The study described in this paper was undertaken in order to examine the role of proline and PAs in the heat stress response of tobacco plants. The relationship between the concentrations of PAs and proline seems to be rather complex. The levels of PAs and proline could be interrelated because they share certain common intermediates in their biosynthetic and catabolic pathways [14]. Oxidative deamination of Put generates a pyrroline that can act as a substrate for proline synthesis after further metabolic processing [15]. Conversely, accumulated proline can be catabolized to glutamate, which is a substrate in PA synthesis [16]. It has been suggested that transgenic plants with elevated levels of proline and PAs exhibit enhanced stress tolerance [17].

Comparison of the stress responses of plants with significantly enhanced proline levels using a tobacco line that over-expresses a modified P5CS gene (*P5CSF129A*) to that of the corresponding wild type (WT) tobacco plants allowed us to estimate the impact of increased proline levels on the dynamics of PA levels under stress conditions. The extent of lipid peroxidation (as indicated by MDA levels) in the wild type and transformant was also investigated.

2. Materials and methods

2.1. Plant material and stress application

Wild-type (*Nicotiana tabacum* L. cv. M51) and transgenic *35S:P5CSF129A* tobacco plants (for detailed information see [9]) were grown in soil in a growth chamber (SANYO MLR 350H, Osaka, Japan) for 6 weeks with a 16-h photoperiod at 130 μmol m^{-2} s^{-1}, day/night temperatures of 25/23 °C and a relative humidity (RH) of ca. 80%. The plants were watered by placing their pots in a tray containing water (ca. 0.2–0.8 cm). After 6 weeks, one half of the plants were transferred to another chamber in which they had no external water source and were subjected to heat stress at 40 °C for 2 h or 6 h. At the end of stress treatment some of the heat-stressed plants were transferred to a 25 °C chamber and stored there for a 2 h 'recovery' period. After this, samples from all three groups of plants (control, treatment, and recovery) were harvested for analysis. Samples of the upper leaves (specifically, the two youngest unfolded leaves) and lower leaves were collected, cut into pieces and immediately frozen in liquid nitrogen after removal of the main vein. Root samples were shaken to remove the soil, washed briefly with cold tap water (ca. 1 min), dried with filter paper and frozen in liquid nitrogen. For each treatment a total of two (control) or four (stress) plants were harvested. Three independent experiments were performed.

2.2. Determination of water potential, osmotic potential and relative water content

The leaves' water potentials were determined using a pressure chamber (Labio a.s., Czech Republic), using an established method [18]. The water potential of the entire leaf was measured. After determination of the balancing pressure in MPa, the leaf was inserted in a syringe and quickly frozen by immersion in liquid nitrogen. The leaf's osmotic potential was determined psychrometrically from the leaf sap, using a Wescor HR-33 micrometer (USA) with C52 sample chambers. The results were converted to MPa by reference to those obtained using calibration solutions of NaCl. The relative water content (RWC) of leaves was measured as follows: individual cut leaves were weighed to determine their fresh mass (FM), saturated with water in beakers for 12 h, re-weighed to give the water-saturated mass (SM), dried at 80 °C, and finally weighed again to give the dried mass (DM). Their RWC was then calculated as: $\mathrm{RWC}\ (\%) = [(\mathrm{FM} - \mathrm{DM})/(\mathrm{SM} - \mathrm{DM})] \times 100$.

2.3. Determination of free proline levels

The levels of free proline in the samples were determined according to the method of Bates et al. [19].

2.4. Ornithine decarboxylase, arginine decarboxylase and S-adenosylmethionine decarboxylase assays

The activities of ornithine decarboxylase (ODC; EC 4.1.1.17), arginine decarboxylase (ADC; EC 4.1.1.19) and S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) activities were determined by a radiochemical method according to [20].

The protein contents of the samples were measured according to Bradford's method using bovine serum albumin as a standard [21].

2.5. Diamine oxidase and polyamine oxidase assays

The activities of diamine oxidase (DAO; EC 1.4.3.6) and polyamine oxidase (PAO; 1.5.3.11) were estimated by a modification of the radiometric method of Paschalidis and Roubelakis-Angelakis [22], using [1,4-^{14}C] putrescine (1.92 GBq mmol^{-1}, Amersham Pharmacia Biotech, UK) or [1,4-^{14}C] spermidine (4.37 GBq mmol^{-1}, Amersham Pharmacia Biotech, UK).

Samples for the measurement of DAO and PAO activity were extracted in 3 volumes of ice-cold 0.1 M phosphate buffer (pH 8) and 0.1 M Tris–HCl buffer (pH 8.5), respectively, containing 2 mM β-mercaptoethanol, and centrifuged at 20,000 × *g* for 30 min at