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SHORT COMMUNICATION

Changes in endogenous hormones and oxidative burst as the biochemical basis for enhanced shoot organogenesis in cold-treated *Saussurea involucrata* explants

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Abstract A low temperature treatment significantly improved shoot morphogenesis of *Saussurea involucrata* Kar. et Kir leaf explants. The biochemical mechanisms underlying the cold-induced shoot organogenesis were investigated by measuring endogenous plant growth hormones, peroxide radicals, and superoxide dismutase (SOD) activity in the cold-treated leaf explants compared to controls. The ratio of zeatin (ZT) to indole-3-acetic acid (IAA) significantly increased in leaf explants subjected to a 5-day treatment at 4 °C compared to controls. The accumulation of $O_2^{\cdot-}$ also rapidly increased in response to cold treatment, and then decreased as SOD activity catalyzed the dismutation of $O_2^{\cdot-}$ to molecular oxygen and H_2O_2; this resulted in a significant increase in H_2O_2 concentrations in the cold-treated explants. We propose that a combination of the increased ZT/IAA ratio and H_2O_2 concentration is the basis for the enhanced shoot morphogenesis in response to cold treatment. These results provide a starting point for an improved understanding of the biochemical mechanisms underlying cold-induced shoot organogenesis of this unique plant species.

Keywords *Saussurea involucrata* · Low temperature · Shoot organogenesis · Superoxide dismutase · Zeatin

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Introduction

Saussurea involucrata Kar. et Kir. (*S. involucrata*), commonly known as snow lotus, is one of the most well-known Chinese medicinal plants. It is commonly used for treating rheumatoid arthritis, gynopathy, and high-altitude diseases (Li and Zhao 1989). It is a perennial herbaceous member of the *Asteraceae* family that grows naturally only in a very specific habitat in the alpine zones of the Tianshan and Kunlun mountain ranges in the Xinjiang province of China (Wu et al. 2010). Due to the combination of overharvesting for commercial uses and the difficulty in cultivating the plant using traditional methods, the plant is listed as a second-grade national protected national wild plant in China (Fu 1992). In vitro propagation techniques provide a powerful system for the mass-multiplication and germplasm conservation of many threatened plant species (Liu et al. 2004a, b) and offer the potential for the propagation of plant species such as *S. involucrata*. In a previous study, we developed an effective in vitro propagation method for *S. involucrata*, and demonstrated that a 5-day pre-treatment at 4 °C significantly improved shoot organogenesis in *S. involucrata* explants (Guo et al. 2007). Low temperature treatment of explants has been utilized in a number of species to enhance the regeneration potential of callus cultures (Hou et al. 1997; Tsugawa and Suzuki 2000); however, the biochemical basis for the enhanced regeneration is unclear.

In plants exposed to low temperatures, one of the fastest kinetic events is oxidative burst; this results in the transient production of reactive oxygen species (ROS) (Apel and Hirt 2004). The antioxidant protection system in plants includes superoxide dismutase (SOD), peroxidase, and catalase; enzymes that are crucial for determining the steady-state level of $O_2^{\cdot-}$ and H_2O_2 in plant cells

(Scandalios 1993). Several reports have suggested a link between ROS and plant developmental physiology, but the molecular mechanism of H_2O_2 and $O_2^{\bullet-}$ signal transduction in plant regeneration is not fully understood (Gupta and Datta 2003; Szechynska-Hebda et al. 2012).

Endogenous plant hormones are also known to play an important role in cell division in tissue culture, and the balance between auxins and cytokinins is an important factor in the morphogenic development of an explant in culture (Skoog and Miller 1957). The objective of this study was to understand the biochemical mechanisms underlying cold-induced shoot organogenesis in *S. involucrata* by measuring endogenous plant hormones, peroxide radicals, and SOD activity.

Materials and methods

Plant material and growth conditions

Seeds of *Saussurea involucrata* Kar. et Kir. were collected from the Xinjiang province in China. These seeds were surface sterilized and germinated as previously described (Guo et al. 2007, 2011). Leaf explants (approximately 0.5 × 0.5 cm) were sectioned from the 30-day-old seedlings and incubated on MS solid medium supplemented with 10 μM 6-benzylaminopurine (BAP) and 2.5 μM 1-naphthalenacetic acid (NAA). For low temperature treatment experiments, the leaf explants were cultivated in an incubator at 4 °C with a 16-h photoperiod under cool-white light (30–40 μmol m^{-2} s^{-1}) for 5 days and then transferred to 25 °C with a 16-h photoperiod under cool-white light (30-40 μmol m^{-2} s^{-1}) for the remaining 35 days. The control explants were cultivated at 25 °C for the full 40 days. The number of regenerated shoots per explant was counted every 5 days starting on culture day 5.

Analytical methods

Plant tissue was extracted in 50 mM potassium phosphate buffer (pH 7.8, containing 0.1 mM EDTA and 4 % (w/v) insoluble polyvinylpolypyrrolidone), and the supernatant was used for enzymatic assay to measure the SOD activity. One unit of SOD activity is defined as a 50 % decrease of the SOD-inhibitable NBT reduction per gram fresh weight (Beaucham and Fridovich 1971). The endogenous H_2O_2 and $O_2^{\bullet-}$ levels were measured using previously reported methods (Wang and Luo 1990).

The endogenous plant growth hormones were quantified using an Agilent 1100 HPLC system equipped with variable-wavelength UV detector, a quaternary pump, an online solvent vacuum degasser, and an auto-sampler with 20 μl injection loop. An Alltech analytical column (250 × 4.6 mm I.D., 5 μm) fitted with an Alltech C18 guard cartridge (8 × 4.6 mm I.D., 5 μm) was used at a column temperature of 25 °C. Ultra pure water containing MeOH (A) was used as chromatographic eluent with a flow rate of 0.5 ml/min. The endogenous plant growth hormones were analyzed separately using a linear gradient as follows: (1) zeatin (ZT): 0–20 min, 10–30 % A; 20–30 min, 30 % A; 268 nm; (2) indole-3-acetic acid (IAA): 0–20 min, 30–70 % A (pH 3.5), 215 nm (Ma and Chu 1994). Reference standards of ZT and IAA were purchased from Sigma-Aldrich.

Statistical analyses

All experiments were conducted using a completely randomized design, and each experiment consisted of five explants per culture dish and ten replicate dishes per treatment. Each experiment was repeated twice. The data were subjected to a one-way analysis of variance (ANOVA), and the Tukey-HSD test was used to calculate significant differences. SPSS for Windows (SPSS Inc. Chicago, version 7.5.1) was used for all statistical analyses, and a value of $P < 0.05$ was considered to be significant.

Results and discussion

Shoot organogenesis significantly increased when the leaf explants were incubated at 4 °C for 5 days (Fig. 1). In both the control and the cold-treated explants, the number of regenerated shoots increased from day 10 to 25 and remained steady from day 25 to 40. However, the number of shoots in the cold treated explants increased more rapidly and achieved a number nearly twice that of the control plants (5.4 ± 0.4 vs. 9.3 ± 0.8); this response was similar to our previously published results (Guo et al. 2007). Similar results have been found in other tissue types; in callus with reduced differentiation efficiency following 6 months in culture, a low temperature treatment at 5 °C for 10 days increased the callus differentiation rate up to fourfold (Hou et al. 1997).

The balance between auxin and cytokinin is known to influence the morphogenic development of an explant in tissue culture; a high cytokinin to auxin ratio promoted shoot formation in a number of plant species (Sarul et al. 1995; Yoshimatsu and Shimomura 1994; Zhang et al. 2005). To determine if the cold treatment influenced the concentrations of endogenous hormones, we measured the concentrations of the ZT and IAA every 5 days. At day 5, the ZT concentration was similar in both the cold-treated and control explants; however, by day 10, the ZT concentration in the cold-treated explants was nearly double that of the control. At each subsequent timepoint tested, the

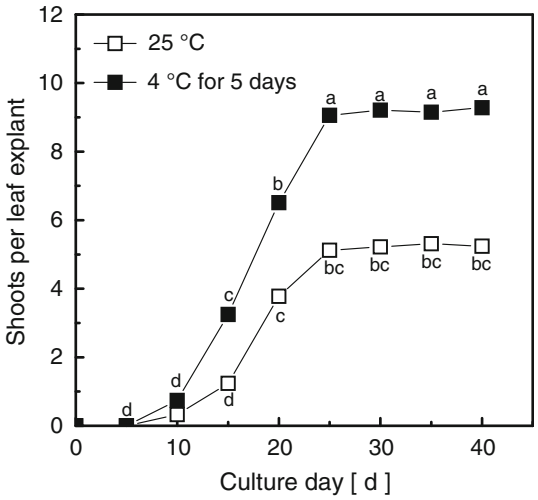

Fig. 1 Effect of cold treatment compared to controls on the shoot organogenesis of *S. involucrata* leaf explants. Means with common letters are not statistically different at $p \leq 0.05$ according to Tukey's HSD test

cold-treated explants continued to have almost twice the level of ZT of the control explants (Fig. 2a). The IAA concentration showed a very different pattern; the IAA concentration was similar in the cold-treated and control explants at day 5, however by day 10, the IAA concentration significantly increased in the control explants, while only increasing only slightly in the cold-treated explants (Fig. 2b). The IAA concentration in the control explants was slightly higher on day 15, then decreased on days 20 and 25 to reach a similar concentration as the cold-treated explants (which slowly increased at each time point) by day 25. Overall, the combination of increased ZT and decreased IAA concentrations in the cold-treated explants resulted in a significantly higher ZT/IAA ratio in the cold-treated explants (Fig. 2c); on day 10, the ZT/IAA ratio was nearly threefold higher than the ratio in the control explants. It has been clear since the classic discovery of Skoog and Miller that the ratio of cytokinin to auxin is crucial for shoot organogenesis (high ratio) and root development (low ratio) (Skoog and Miller 1957); therefore, the alteration of the endogenous plant growth hormone balance in response to low temperature is a key factor in the enhanced shoot morphogenesis in *S. involucrata*.

The reasons underlying the changes in endogenous hormones are unclear. An early study on winter wheat seedlings demonstrated that 5 days at 2 °C increased auxin oxidase activity by more than fourfold (Bolduc et al. 1970). In available studies in which cytokinins were analyzed, they were generally analyzed directly following cold-treatment; in our study, the cytokinin concentration did not increase immediately in response to low temperature, rather, the highest ZT concentration was obtained 5 days following the return to cultivation at 25 °C. Several studies have demonstrated that auxin may negatively regulate cytokinin biosynthesis; for example, in the nodal stem of *P. sativum*, Tanaka et al. found that auxin negatively regulates local cytokinin biosynthesis by controlling the expression level of *PsIPT*, a gene encoding a key enzyme in cytokinin biosynthesis (Tanaka et al. 2006). This opens the possibility that perhaps in the control explants, as auxin levels increased, cytokinin levels were regulated, while in the cold-treated explants the lowered auxin levels may have allowed for the increase in ZT. The crosstalk between auxin and cytokinins is complicated and is still being investigated, and it is likely that a combination of factors led to the increase in ZT. Although our understanding of the biosynthesis of cytokinins and their interactions with other hormones is still unclear, it is known that the precise interplay of auxin and cytokinin signaling pathways is central to direct cells towards de novo shoot organogenisis, but thus far there is no clear molecular model, in particular during the early stages of shoot organogenesis (Duclercq et al. 2011).

Oxidative burst occurs in response to cold stress and is another factor that we hypothesized may affect shoot organogenesis in the cold-treated leaf explants. In response to stress, the superoxide radical $O_2^{\cdot-}$ is produced, which is then converted to molecular oxygen and H_2O_2 by superoxide dismutase (SOD) (Mittler 2002; Neill et al. 2002). To investigate oxidative burst in response to cold treatment, we measured the levels of $O_2^{\cdot-}$, SOD activity, and H_2O_2 in the cold-treated and control explants every 5 days. In the cold-treated explants, the level of $O_2^{\cdot-}$ spiked on day 5 in response to cold stress (Fig. 2d), and then decreased at each subsequent time point, resulting in a concentration lower than in the control explants on days 20 and 25. This decrease is likely to be a result of the increasing activity of SOD in the cold treated explants; the SOD activity increased in both the cold-treated and the control explants, but it increased more sharply and to a higher level in the cold treated explants, reaching the highest levels on days 20 and 25 (Fig. 2e). As the levels of $O_2^{\cdot-}$ decreased and SOD activity increased in the cold-treated explants, the production of H_2O_2 increased; it dramatically increased from days 5 to 20, then leveled out, while it stayed level throughout the cultivation period in the control explants (Fig. 2f).

Although in the past ROS have been characterized simply as a factor causing cellular damage, it is now known that ROS play a pivital role in plant growth and development (Gupta 2011). Recent research has focused on the potential of ROS as signaling molecules that regulate growth and morphogenesis and act as second messengers. Although some findings indicate that there is a relationship between free radical damage and plant recalcitrance in

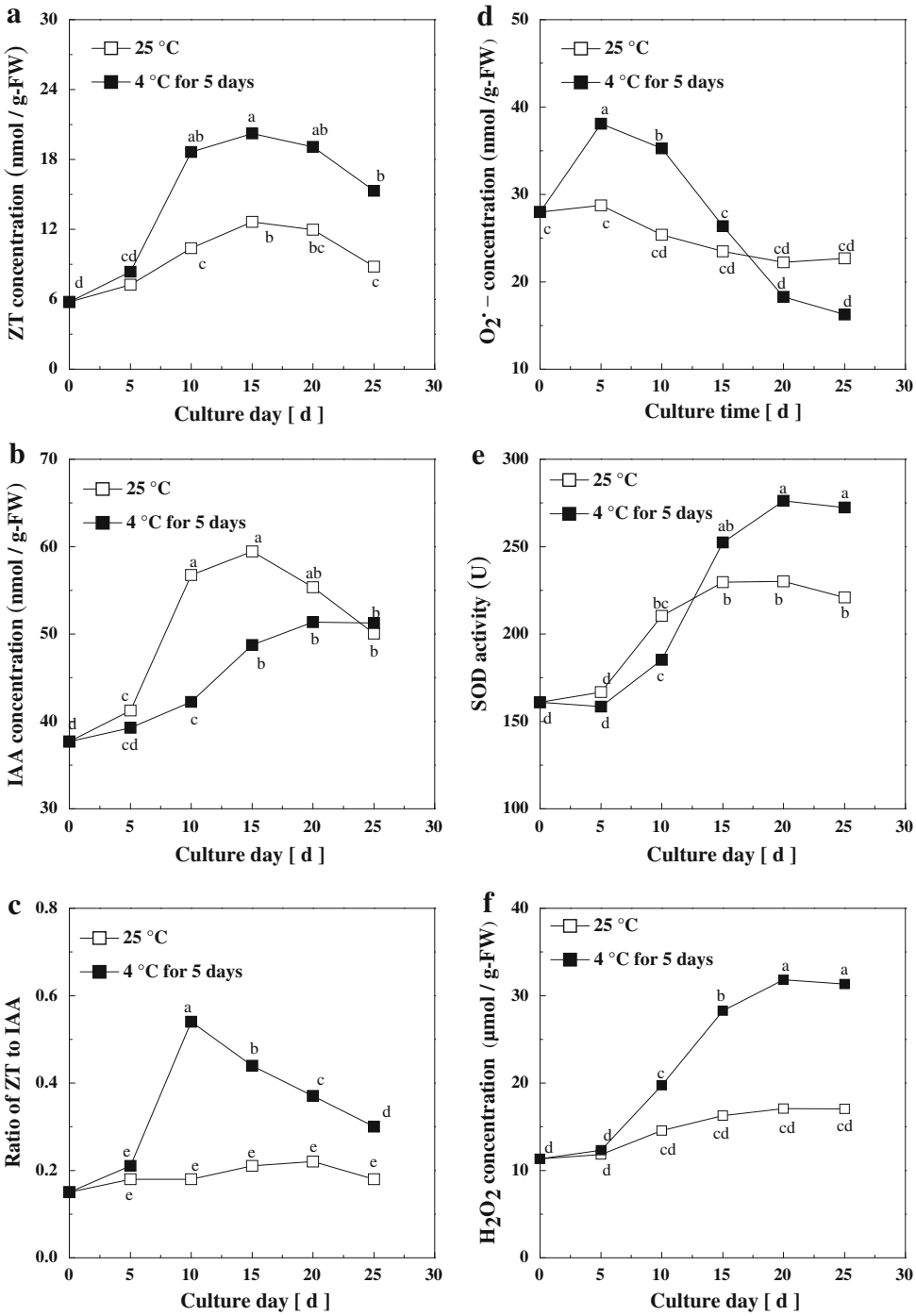

Fig. 2 Effect of cold treatment compared to controls on zeatin (ZT) (**a**), indole-3-acetic acid (IAA) (**b**) and ZT/IAA ratio (**c**), $O_2^{\cdot-}$ (**d**), superoxide dismutase (SOD) activity (**e**), and H_2O_2 (**f**) levels in *S. involucrata* leaf explants. Means with common letters are not statistically different at $p \leq 0.05$ according to Tukey's HSD test

culture (Papadakis and Roubelakis-Angelakis 2002), it is also clear that ROS are involved in shoot organogenesis. For example, it has been suggested that H_2O_2 may function as a messenger in the bud primordium formation process (Tian et al. 2003), in shoot primordium development (Gupta 2011), and exogenous H_2O_2 stimulated shoot organogenesis in both winter wheat and strawberry callus (Szechynska-Hebda et al. 2012; Tian et al. 2003). Szechynska-Hebda et al. (2012) examined the effect of endogenous H_2O_2 induced by cold on the improvement of tissue regeneration efficiency and concluded that mild oxidative stress and H_2O_2 dependent metabolic pathways are crucial for in vitro shoot formation. A higher concentration of cellular H_2O_2 was observed in the immature embryos, despite an increase in the response of antioxidative enzymes. Therefore, is likely that the increased concentration of H_2O_2 contributed to the enhanced shoot morphogenesis in the cold-treated explants.

Overall, the biochemical basis for cold-induced shoot morphogenesis in *S. involucrata* is complex, but this study demonstrates that two key factors, the changes in the ratio of endogenous auxin and cytokinin, and oxidative burst, specifically the increase in H_2O_2, are likely to play a role in the enhanced shoot morphogenesis. Future research will focus more heavily on elucidating the transcriptional regulation, role of other hormones, and understanding the interplay between the endogenous hormones and the oxidative pathway.

Authors' contributions The work presented here was carried out in collaboration between all authors. BG and CZL defined the research focus and designed the experiments. BG carried out the laboratory experiments. BG, ARS, and CZL analyzed the data, interpreted the results, and wrote the manuscript. All authors have contributed to and approved the manuscript.

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