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Brassinosteroids Accelerate the Rate of Cell Division in Isolated Petal Protoplasts of *Petunia hybrida*

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Abstract

Brassinosteroids are known to promote cell elongation in a wide range of plant species but their effect on cell division has not been extensively studied. The effect of brassinolide on the kinetics and final division frequencies of regenerating petal protoplasts of *Petunia hybrida* Vilm v. Comanche was examined. Under optimal auxin and cytokinin conditions, 10-100 nM brassinolide not only reduced the time of first cell division by 4.5 days but also altered the final division frequencies after 10 days of culture. One micromolar brassinolide showed the same acceleration of first cell division but inhibited the final division frequency by approximately 9%. Under sub-optimal auxin conditions, 10-100 nM brassinolide accelerated the first cell division, but no significant increase in the 8-10 days final division frequencies. Isolated protoplasts may provide a useful model system for the investigation of the molecular mechanisms of brassinosteroid action on cell division and proliferation in higher plants.

Key words: Brassinosteroids, Brassinolide, Cell division, Petal protoplast

Abbreviation: 2,4-D 2,4-Dichlorophenoxyacetic acid, BAP 6-benzylaminopurine, BR brassinosteroid, IAA indole 3-acetic acid, NAA naphthalene acetic acid

Introduction

Petal cells of flowering plants are highly vacuolate, filled with pigments, and senesce soon after pollination (Landor 1986). The physiological processes that occur in the petal cells are mainly associated with senescence. Therefore, petal cells are notoriously recalcitrant in regaining mitotic actively in cell culture. Oh and Kim (1994) have successfully induced cell division of petunia petal protoplasts and obtained the regeneration of complete plants from petal tissue.

Brassinosteroids (BRs) comprise a class of over 40 polyhydroxylated sterol derivatives that appear to be ubiquitously distributed throughout the plant kingdom. It is known that BRs are widely distributed in the plants as growth-promoting natural products with structural similarity to animal steroid hormones. Recent studies on BR-insensitive and BR-deficient mutants confirmed a role for these compounds in regulating normal plants growth and development (Clouse and Sasse 1998). BRs promotes cell elongation in numerous test systems, and the molecular mechanisms underlying BR-regulated cell elongation are currently being investigated. Besides its known functions in cell elongation, BRs have also been proposed to play a role in cell division. Highly purified brassinolide, a naturally occurring BR, caused not only cell elongation in the second internodes of bean, but also curvature, swelling and splitting of the internodes, which was attributed to increased cell division (Grove et al. 1979). Also, nanomolar concentrations of brassinolide, in the presence of auxin and cytokinin, caused at least a 50% increase in the total number of cells in cultured explants of *Helianthus tuberosus* after 24 hr, suggesting a strong promotive effect of brassinolide on cell division (Clouse and Zurek 1991). Nakajima et al. (1996) also found a significant enhancement of cell-division rates upon the addition of 24-epibrassinolide to the culture medium of Chinese cabbage mesophyll protoplasts.

In contrast to the above findings, several other studies found either no effect or an inhibition of cell division by BRs. Both (22S, 23S, 24S)-(tri-epi)-brassinolide and 24-epibrassinolide

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inhibited the growth of callus and suspension cultures of *Agrobacterium tumefaciens*-transformed of *Nicotiana tabacum* (Roth et al. 1989). In cultured carrot (*Daucus carota*) cells, 24-epibrassinolides promoted cell enlargement but not cell division (Bellincampi and Morpurgo 1988). At the whole plant level, microscopic examination of BR-deficient and BR-insensitive mutants in *Arabidopsis thaliana* showed that the dwarf phenotype was due to reduced cell size, not cell number (Kauschmann et al. 1996). Therefore, the role of BR in cell division remains somewhat confusing. To further investigate the effect of BRs on this process, the kinetics and extent of cell division in BR-treated petal protoplasts of petunia in the presence and absence of auxins and cytokinin were examined in this study.

Materials and Methods

Plant materials and chemicals

Plants used for protoplast isolation were grown from seeds of a self-fertile tetraploid (2n=4x=28) of *Petunia hybrida* Vilm cv 'Comanche' produced by regeneration of diploid (2n=2x=14) petunia protoplast-derived calli (Oh and Kim 1988). Seeds were shown in Fafard mix no. 4-P in a greenhouse with minimum day/night temperatures of 24°C/18°C under natural lighting conditions. Petal tissues were harvested at appropriate time intervals in the morning. Cellulase Onozuka R-10 and Macerozyme Onozuka R-10 were obtained from Karlan Research (Santa Rosa, Calif.) and other chemicals were purchased from Sigma Chemicals, (St. Louis, MO).

Protoplast isolation and purification

Petal tissues were harvested and immediately surface-sterilized with 70% ethanol for 1 min, followed by immersion in 1% NaOCl for 10 min. The surface-sterilized petals were rinsed three times with sterile distilled water. The lower epidermis of the petals was peeled away with forceps, and the petal pieces were incubated with the cell wall-degrading enzyme solution containing 1% cellulase R-10, 0.3% (w/v) macerozyme R-10, 9% mannitol, and 10 mM $CaCl_2 \cdot H_2O$. The incubation of petal pieces was carried out at 28°C for 4 h in the dark. After incubation with the cell wall-degrading enzymes, the protoplast preparation was filtered through a nylon sieve (45 μM mesh) and centrifuged (100 ¡¿g; 5 min). The enzyme supernatant was removed, and the protoplasts were washed three times by swirling and centrifuging (100 ¡¿g; 5 min) with modified MS liquid medium containing 9% mannitol (described below).

Protoplast culture

Petal protoplasts were plated in modified MS (Murashige and Skoog 1962) liquid medium in which NH_4NO_3 and Fe · EDTA were reduced to 7 mM and 10 μM, respectively, and 1 g/L of sucrose, 100 mg/L of *myo*-inositol, 1 mg/L of thiamine · HCl, and 0.5 mg/L of BAP (6-benzylaminopurine) were added. Brassinolide {2α, 3α, 22R, 23(R)-tetrahydroxy-24(S)-methyl-B7-oxa-5a-cholestan-6-one} was added to autoclaved medium at various concentrations, and approximately 4 mL of protoplast suspension (5 ¡¿10^4 protoplast/ml) was plated in 55 ¡¿10 mm plastic petri dishes, then sealed with parafilm. Protoplasts were incubated in darkness at 28°C for 24 h, followed by exposure for 48 h at 28°C in continuous cool-white fluorescent light (10 μmol $m^{-2}s^{-1}$). Finally, the cultures were maintained at 28°C with a photoperiod of 16 h light (40 μmol $m^{-2}s^{-1}$) and 8 h darkness. After the initial 24 h of incubation in darkness, protoplasts were observed under an inverted microscope every 6-12 h for evidence of cell division. Cell-division frequency was calculated as the number of protoplast that had developed at least one cross wall as a percentage of the total cells. Each data point was replicated three times, and each complete experiment was repeated at least three times.

Results and Discussion

Morphologically normal plants have been regenerated from petal protoplasts of petunia (*Petunia hybrida*) flower (Oh and Kim 1994). Petal protoplasts of *P. hybrida* provide a system in which the cell-division frequency after various growth regulator treatments can be easily monitored by counting dividing and non-dividing cells in an inverted microscope. Oh and Kim (1994) showed a typical *P. hybrida* protoplast undergoing cell division (data not shown).

The effects of various combinations of plant growth regulators in the initial protoplast culture were tested, and the results are summarized in Table 1. When these protoplasts were provided with an optimal combination of 0.2 mg/L 2,4-D (2,4-Dichlorophenoxyacetic acid), 1.0 mg/L NAA (naphthalene acetic acid), 0.2 mg/L IAA (indole 3-acetic acid), and 0.5 mg/L BAP, cells began to divide 6 days after the start of liquid culture and approximately 15% of the cells underwent division (Table 1). The effect of brassinolide on cell division under optimal auxin and cytokinin conditions was monitored by recoding the time of the first detectable cell division and the total division frequency after 4 -12 days of culture. The application of 0.01-1.0 μM brassinolide resulted in an accelerated rate of cell division, with the first detectable division beginning 24-36 hr earlier than the control (Table 1). Although total division frequencies were not dramatically affected by 0.001-0.1 μM brassinolide,

division frequencies were increased by 0.01-0.1 μM brassinolide. But a concentration of 1.0 μM brassinolide caused a substantial reduction in division frequencies at 5 through 10 days (Figure 1).

When protoplasts were subjected to suboptimal auxin conditions resulting from the elimination of NAA from the medium, a total division frequency at 10 days was reduced to approximately 4.0 % (Table 1). Under these conditions, 0.01-1.0 μM brassinolide again resulted in an accelerated rate of cell division when compared with the control, with the same (4 days) reduction in time of first cell division as seen with optimal auxin. In contrast to the optimal medium under suboptimal auxin conditions, 0.01-1.0 μM brassinolide caused an increase in cell-division frequencies at 6-10 days (Figure 2). One micromolar brassinolide showed a significant inhibition of cell-division frequency under these conditions when compared with 0.1 μM brassinolide. Elimination of both IAA and NAA or 2,4-D and IAA from optimal medium containing 0.1 μM brassinolide resulted in a delay of 4-5 days in the onset of cell division and reduced the 5-day division frequency from 25% in the optimal medium down to approximately 5.0% (Table 1). Takematsu et al. (1983) reported that BR and auxin in combination promoted growth in various plant callus cultures more effectively than auxin and BAP. In petunia petal protoplasts, however, elimination of BAP from the optimal medium containing 0.1 μM brassinolide resulted in a complete absence of cell division after 5 days of culture. BAP plus 0.1 μM brassinolide in the absence of auxins also failed to promote cell division, as did 0.1 μM brassinolide alone (Table 1).

These results, like those of Nakajima et al. (1996) on Chinese cabbage protoplasts, showed an acceleration of the cell division rate by BR treatment. The results also suggested that the kinetics as well as a wide range of growth regulator concentrations/combinations should be considered in the studies of the effects of BR on cell division. It is apparent that high concentration of BR are inhibitive rather than promotive on cell division, and that the auxin and cytokinin level with the cells is also critical in determining the BR effect. The differences among the published results on the effect of BRs on cell divi-

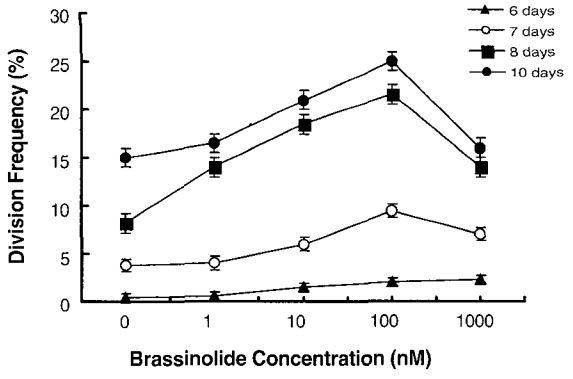

Figure 1. Effect of brassinolide on cell-division frequency of regenerating *P. hybrida* petal protoplasts under optimal auxin conditions. Protoplasts were isolated and cultured in modified MS medium containing 0.9 μM 2,4-D, 1.14 μM IAA, 5.37 μM NAA, 2.22 μM BAP, 0.6M mannitol and various concentrations of brassinolide. Each data point represents the percentage of 2,500 microscopically examined protoplasts that were undergoing cell division (±SE).

Table 1. Effect of different growth regulator combinations on cell division in regenerating *Petunia hybrida* petal protoplasts.

Plant growth regulator concentrations (μM)					First detectable cell division(h)	2nd/3rd (h)	Division Frequencies[a]
2,4-D	IAA	NAA	BAP	BR			
0.90	1.14	5.37	2.22	0.0	144	192-216	15.0±2.0
0.90	1.14	5.37	2.22	0.001	144	192-216	16.5±2.2
0.90	1.14	5.37	2.22	0.01	108	168-180	21.0±2.0
0.90	1.14	5.37	2.22	0.1	108	168-180	25.0±2.1
0.90	1.14	5.37	2.22	1.0	108	168-180	16.0±1.5
0.90	1.14	0.0	2.22	0.0	204	240-264	4.0±0.5
0.90	1.14	0.0	2.22	0.001	204	240-264	4.5±0.7
0.90	1.14	0.0	2.22	0.01	192	216-240	5.2±0.3
0.90	1.14	0.0	2.22	0.1	192	216-240	9.0±1.0
0.90	1.14	0.0	2.22	1.0	192	216-240	8.0±1.2
0.90	0.0	0.0	2.22	0.1	240	280-312	5.3±0.4
0.0	0.0	5.37	2.22	0.1	240	280-312	4.5±0.6
0.0	1.14	0.0	2.22	0.1	-[b]	-	-
0.90	1.14	5.37	0.00	0.1	-	-	-
0.0	0.0	0.0	2.22	0.1	-	-	-
0.0	0.0	0.0	0.0	0.1	-	-	-
0.0	0.0	0.0	0.0	0.1	-	-	-

[a]Each data point represents the percentage of 2,500 microscopically examined protoplasts that were undergoing cell division after 10 days of culture (±SE).
[b]No dividing cells were evident after regular microscopic evaluation of 2,500 protoplasts for 10 days.

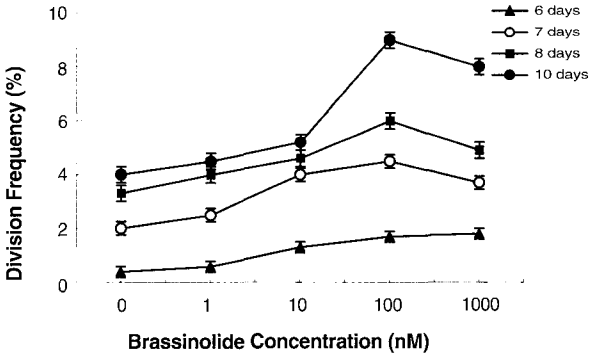

Figure 2. Effect of brassinolide on cell-division frequency of regenerating *P. hybrida* petal protoplasts under sub-optimal auxin conditions. Protoplasts were treated as described in Figure 1 except that NAA was eliminated from all media.

sion may be due to limited number of timepoints or concentrations used. For example, Sala and Sala (1985) concluded with insufficient experimental evidences that 24-epibrassinolide prompoted elongation but not cell division in cell suspension cultures of carrot.

Much remains to be learned about the mechanisms of BR effects, both positive and negative, on cell division. Two reports (Bach et al. 1991; Gaudinova et al. 1995) have indicated that BR treatment alterd the endogenous auxin and cytokinin levels in callus cultures, thus impacting on the control of cell division. In petal protoplasts including mesophyll protoplasts, regeneration of the wall was required before division proceeds (Mayer and Herth 1978), and it is possible that the acceleration in initial division seen in *P. hybrida* petal protoplasts is due to a promotive effect on wall regenaration by BR. Such a possible effect on cell-wall synthesis would not be surprising given that BRs are known to regulate genes encoding wall-modifying enzymes (Zurek and Clouse 1994; Oh et al. 1998; Xu et al. 1995) and to alter wall properties during elongation (Wang et al. 1993; Tominaga et al. 1994; Zurek et al. 1994). It has also been proposed that BR promote dedifferentiation of mesophyll protoplasts, which accelerates division (Nakajima et al. 1996).

As preliminary results, brassinolide affects the kinetics of the cell cycle in synchronized cell cultures of tobacco and also regulates the expression of genes associated with the S phase, including histone H_2B and High Mobility Group-1 protein (Jiang J, Clouse S.D., unpublished). A promotive role for BRs in Arabidopsis cell division has been implicated by the recent finding that 24-epibrassinolide treatment of *det2* cell suspension cultures increases transcript levels of the gene encoding cyclinD3(CycD3), a protein involved in the regulation of G1/S transition in the cell cycle. CycD3 is also regulated by cytokinins. It is interesting to note that 24-epibrassinolde could effectively substitute for zeatin in the growth of *Arabidopsis* callus and cell suspension cultures (Hu et al. 2000). Our results, coupled with those of Nakajima et al. (1996), suggest that protoplasts may represent excellent model systems for studying BR effects on cell division. It would be of interest to investigate cell-division rates in protoplasts isolated from BR-deficient and BR-insensitive mutants, and to study the effect of BR on the expression of cyclins and mitotically associated protein kinases.

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