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Brassinolide affects the rate of cell division in isolated leaf 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 as extensively studied. We examined the effect of brassinolide on the kinetics and final division frequencies of regenerating leaf mesophyll protoplasts of *Petunia hybrida* Vilm v. Comanche. Under optimal auxin and cytokinin conditions, 10–100 n*M* brassinolide accelerated the time of first cell division by 12 h but had little effect on the final division frequencies after 72–120 h of culture. One micromolar brassinolide showed the same acceleration of first cell division but inhibited the final division frequency by approximately 20%. Under sub-optimal auxin conditions, 10–100 n*M* brassinolide both accelerated the time of first cell division and dramatically increased the 72- to 120-h final division frequencies. Isolated protoplasts may provide a useful model system to investigate the molecular mechanisms of brassinosteroid action on cell proliferation.

Key words Brassinosteroid · Brassinolide · Protoplast · Cell division

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *BAP* 6-benzylaminopurine · *BR* brassinosteroid · *DAPI* 4,6-diamidino-2-phenylindole · *IAA* indole acetic acid · *NAA* naphthalene acetic acid

Introduction

Brassinosteroids are widely distributed plant growth-promoting natural products with structural similarity to animal steroid hormones, and recent studies on BR-insensitive and -deficient mutants has confirmed a role for these compounds in the regulation of normal plant growth and development (Clouse and Sasse 1998). BRs promote cell elongation in numerous test systems, and the molecular mechanisms underlying BR-regulated cell elongation are currently being investigated (reviewed in Clouse 1997). Besides their known function 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 internode, which was attributed to increased cell division (Grove et al. 1979). Clouse and Zurek (1991) found that 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 h, suggesting a strong promotive effect of brassinolide on cell division. 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 inhibited the growth of callus and suspension cultures of *Agrobacterium tumefaciens*-transformed, hormone-autonomous *Nicotiana tabaccum* (Roth et al. 1989). In cultured carrot (*Daucus carota* L.) cells, 24-epibrassinolide promoted cell enlargement but not cell division (Sala and Sala 1985; 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). Thus, the role of BR in cell division remains somewhat confusing. In order to further investigate the effect of BRs on this process, we examined the kinetics and extent of cell division in BR-treated leaf mesophyll protoplasts of petunia, in the presence and absence of auxins and cytokinin.

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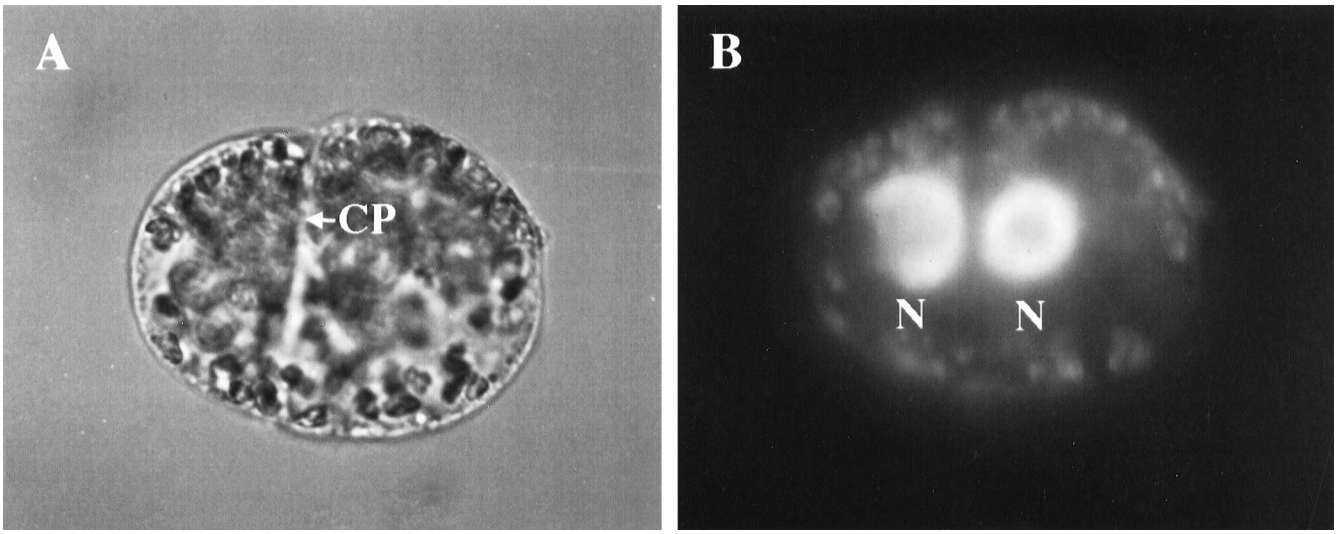

Fig. 1A, B Cell division in isolated mesophyll protoplasts of *P. hybrida*. Protoplasts were isolated as described in Materials and methods and cultured for 5 days in modified MS medium containing 0.9 μ*M* 2,4-D, 1.14 μ*M* IAA, 5.37 μ*M* NAA, 2.22 μ*M* BAP and 0.6 *M* mannitol. **A** The regenerated cell wall and the cell plate (*CP*) are clearly evident. **B** DAPI staining of the same cell followed by epi-fluorescence microscopy shows nuclear (*N*) division

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 sown in Fafard mix no. 4-P in a greenhouse with minimum day/night temperatures of 24°/18 °C under natural lighting conditions. Cellulase Onozuka R-10 and Macerozyme Onozuka R-10 were from Karlan Research (Santa Rosa, Calif.); other chemicals were from Sigma (St. Louis, Mo.).

Protoplast isolation and purification

Fully expanded leaves were harvested and immediately surface-sterilized with 70% ethanol for 1 min, followed by immersion in 1% NaOCl for 10 min. After three rinses in sterile water, the lower epidermis of the leaves was peeled away with fine forceps, and the leaf pieces were incubated in darkness for 5 h at 28 °C in a solution containing 1% (w/v) Cellulase R-10, 0.3% (w/v) Macerozyme R-10, 0.6 *M* mannitol and 10 m*M* $CaCl_2 \cdot H_2O$. Following incubation, protoplasts were filtered through a nylon sieve (52-μm mesh) and centrifuged (60 *g*, 5 min). The supernatant was removed, and protoplasts were washed three times by resuspending and centrifuging (60 *g*, 5 min) in modified MS liquid medium (described below).

Protoplast culture

Protoplasts were plated in modified MS (Murashige and Skoog 1962) liquid medium in which NH_4NO_3 and Fe · EDTA were reduced to 7 m*M* and 10 μ*M*, respectively, and the medium was supplemented with sucrose (1 g/l), myo-inositol (100 mg/l), thiamine · HCl (1 mg/l), mannitol (0.6 *M*), 2,4-D (0.2 mg/l), IAA (0.2 mg/l), NAA (1.0 mg/l), and BAP (0.5 mg/l). Brassinolide [2α,3α,22(R),23(R)-tetrahydroxy-24(S)-methyl-B-homo-7-oxa-5α-cholestan-6-one] was added to autoclaved medium at various concentrations, and approximately 4 ml of protoplast suspension (5×10^4 protoplasts/ml) was plated in 55×10-mm plastic petri dishes and sealed with parafilm. Protoplasts were incubated in darkness at 28 °C for 24 h, followed by 48 h at 28 °C in continuous cool-white fluorescent light (10 μmole m^{-2} s^{-1}). Finally, the cultures were maintained at 28 °C with a photoperiod of 16 h light (40 μmole m^{-2} s^{-1}) and 8 h darkness. After the initial 24 h of dark incubation, 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 protoplasts 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

Mesophyll 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. Figure 1 shows a typical *P. hybrida* protoplast undergoing cell division. The cell plate is clearly evident and staining with DAPI verifies nuclear division. When these protoplasts are provided with an optimal combination of 2,4-D, NAA, IAA and BAP, cells begin to divide 60 h after the start of liquid culture and by 5 days, over 80% of the cells have undergone division (Table 1). The effect of brassinolide on cell division under optimal auxin and cytokinin conditions was then monitored by recording the time of the first detectable cell division and the total division frequency after 2–5 days of culture. Brassinolide application of 0.01–1.0 μ*M* resulted in an accelerated rate of cell division, with the first detectable division beginning 12 h earlier than the control (Table 1). Total division frequencies were not dramatically affected by 0.001–0.1 μ*M* brassinolide, but a concentration of 1.0 μ*M* brassinolide caused a substantial (nearly 20%) reduction in division frequencies at 72 through 120 h (Fig. 2).

When protoplasts were subjected to suboptimal auxin conditions resulting from the elimination of NAA from the medium, total division frequency at 5 days was reduced to approximately 5% (Table 1). Under these conditions, 0.01–1.0 μ*M* brassinolide again resulted in an accelerated

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

Plant growth regulator concentrations (μ*M*)					First detectable cell division (h)	Second/third cell division (h)	Division frequencies[a]
2,4-D	IAA	NAA	BAP	BR			
0.90	1.14	5.37	2.22	0.0	60	72–96	81.3±2.0
0.90	1.14	5.37	2.22	0.001	60	72–96	81.5±1.9
0.90	1.14	5.37	2.22	0.01	48	60–96	83.3±1.5
0.90	1.14	5.37	2.22	0.1	48	60–96	85.5±1.8
0.90	1.14	5.37	2.22	1.0	48	72–96	62.1±2.4
0.90	1.14	0.0	2.22	0.0	84	120–144	5.2±0.3
0.90	1.14	0.0	2.22	0.001	84	120–144	5.4±0.4
0.90	1.14	0.0	2.22	0.01	72	96–120	9.6±0.2
0.90	1.14	0.0	2.22	0.1	72	96–120	21.3±0.5
0.90	1.14	0.0	2.22	1.0	72	96–120	10.4±0.8
0.90	0.0	0.0	2.22	0.1	84	120–144	5.4±0.3
0.0	0.0	5.37	2.22	0.1	84	120–144	4.5±0.2
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	0.0	–	–	–

[a] Each data point represents the percentage of 2,100 microscopically examined protoplasts that were undergoing cell division after 5 days of culture (±SE)
[b] No dividing cells were evident after regular microscopic evaluation of 2,100 protoplasts for 5 days

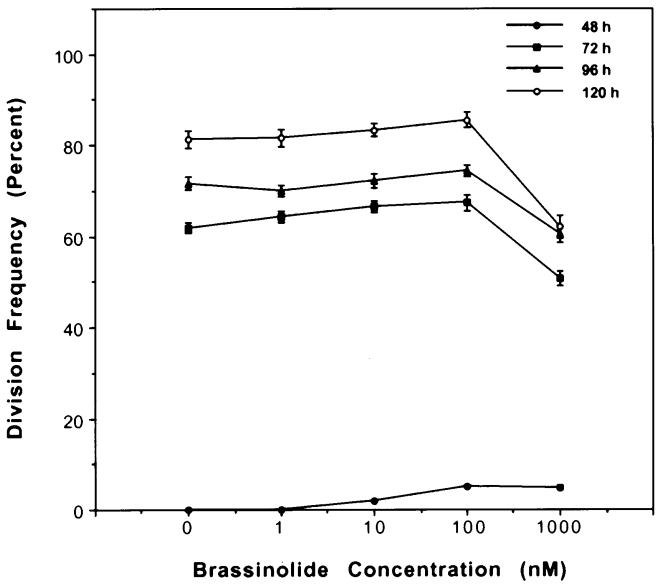

Fig. 2 Effect of brassinolide on cell-division frequency of regenerating *P. hybrida* protoplasts under optimal auxin conditions. Protoplasts were isolated as described in Materials and methods and cultured for the indicated period in modified MS medium containing 0.9 μ*M* 2,4-D, 1.14 μ*M* IAA, 5.37 μ*M* NAA, 2.22 μ*M* BAP, 0.6 *M* mannitol and various concentrations of brassinolide. Each *data point* represents the percentage of 2,100 microscopically examined protoplasts that were undergoing cell division (±SE)

rate of cell division when compared with the control, with the same 12-h reduction in time of first cell division as seen with optimal auxin. However, the actual time of first cell division was 24 h later than that observed under optimal auxin conditions (Table 1). In contrast to the optimal medium, under suboptimal auxin conditions 0.01–1.0 μ*M* brassinolide caused a marked increase in cell-division frequencies at 72 through 120 h (Fig. 3). One micromolar brassinolide showed a significant inhibition of cell-division frequency under these conditions when compared with 0.1 μ*M* brassinolide but still promoted a twofold increase when compared to the control (Table 1). Elimination of both IAA and NAA or 2,4-D and IAA from the optimal medium containing 0.1 μ*M* brassinolide resulted in a delay of 36 h in the onset of cell division and reduced the 5-day division frequency from 85% in the optimal medium down to approximately 5% (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 *P. hybrida* protoplasts, however, elimination of BAP from the optimal medium containing 0.1 μ*M* brassinolide resulted in a complete absence of cell division after 5 days of culture. BAP plus 0.1 μ*M* brassinolide in the absence of auxins also failed to promote cell division, as did 0.1 μ*M* brassinolide alone (Table 1).

Our results, like those of Nakajima et al. (1996) on Chinese cabbage protoplasts, show an acceleration of the rate of cell division by BR treatment. They also point to the importance of considering kinetics as well as a wide range of growth regulator concentrations/combinations in any study of the effects of BR on cell division. It is apparent that high concentrations of BR are inhibitory rather than promotive of cell division and that the auxin and cytokinin status of the cells is also critical in determining the BR effect. Some of the differences in the published results on the effect of BRs on cell division may be due to examining a limited number of timepoints or concentrations. For example, Sala and Sala (1985) concluded that 24-epibrassinolide promoted elongation but not cell division in cell suspension cultures of carrot. However, only a single, rather high concentration of 3 μ*M* 24-epibrassinolide was employed, and it would be of interest to determine whether

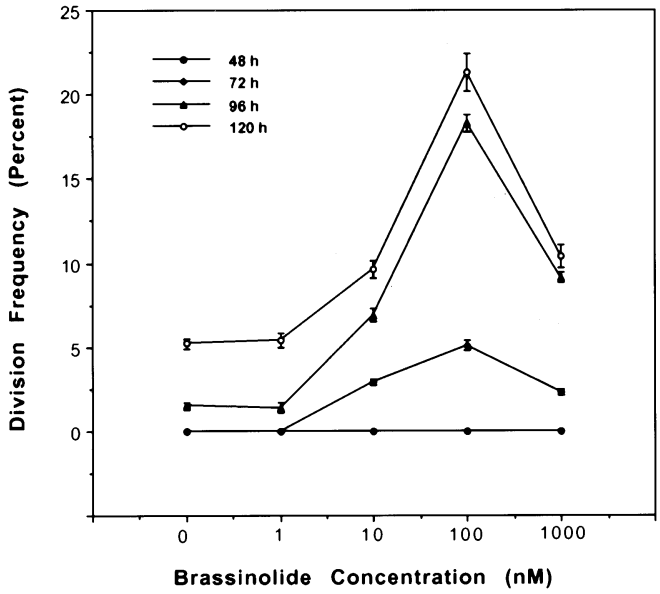

Fig. 3 Effect of brassinolide on cell-division frequency of regenerating *P. hybrida* protoplasts under sub-optimal auxin conditions. Protoplasts were treated exactly as described in Fig. 2 except that NAA was eliminated from all media

lower BR concentrations might have a stimulatory effect on cell division in this system.

Much remains to be learned about the mechanisms underlying BR effects, both positive and negative, on cell division. Two studies (Bach et al. 1991; Gaudinova et al. 1995) have indicated that BR treatment alters the endogenous auxin and cytokinin levels in callus cultures, thus influencing the control of cell division. In protoplasts, regeneration of the wall is required before division proceeds (Mayer and Herth 1978), and it is possible that the acceleration in initial division seen in *P. hybrida* protoplasts is due to a promotive effect on wall regeneration by BR. Such an 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; 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 BRs promote dedifferentiation of mesophyll protoplasts, which accelerates division (Nikijama et al. 1996). Our results, coupled with those of Nikijama 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, as would studies of the effect of BR on the expression of cyclins and mitotically associated protein kinases.

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