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Promotive effect of brassinosteroids on cell division involves a distinct *CycD3*-induction pathway in *Arabidopsis*

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Summary

Brassinosteroids (BRs) are steroid hormones that play an essential role in plant growth and development. However, the contradictory results of previous studies make their role in cell division unclear. Using a cDNA array, we identified genes that respond to BR in the *det2* suspension culture of *Arabidopsis*, and found that epi-brassinolide upregulated transcription of the C*ycD3*, a D-type plant cyclin gene through which cytokinin activates cell division. RNA gel-blot analysis and cell culturing showed that epi-brassinolide may promote cell division through CycD3, and can substitute cytokinin in culturing of *Arabidopsis* callus and suspension cells. The *CycD3* induction by epi-brassinolide was further shown to involve *de novo* protein synthesis, but no protein phosphorylation or dephosphorylation. Induction was also found to occur in cells of a BR-insensitive mutant, *bri1*, suggesting that BR induces *CycD3* transcription through a previously unknown signal pathway in plants.

Keywords: brassinosteroid, *CycD3*, cell division, signal transduction, *Arabidopsis thaliana*.

Introduction

Progression through the eukaryotic cell cycle is regulated at two points, the G1/S and G2/M boundaries. Transition through these points is controlled by distinct families of cyclin-dependent kinases (CDKs), whose activities are determined by co-ordinated binding of different types of cyclins (Pines, 1995). Based on the sequence characteristics and times of action in the cell cycle, cyclins are classified as A, B, D and E types in mammals (Pines, 1995). Cyclin A or B transcript accumulates periodically during the late S to G2 phase, before being destroyed later in mitosis (Pines and Hunter, 1990). The complex formed by cyclin D or E, with their associated CDKs, directly phosphorylates the retinoblastoma (Rb) protein in the mid- to late-G1 phase, thereby driving cells across the G1/S boundary (Sherr, 1996). Among all cyclins, D-type cyclins are particularly important in promoting cell division because they are highly responsive to serum growth factors, rapidly declining on their withdrawal and greatly increasing when quiescent cells are reactivated (Ajchenbaum *et al.*, 1993; Matsushime *et al.*, 1991; Sherr, 1993; Sherr, 1994).

In plants, a number of cyclin homologues have been identified and designated as CycA, CycB and CycD, based on their mammalian equivalents (Mironov *et al.*, 1999; Renaudin *et al.*, 1996). The finding that these cyclins are also associated with their CDKs and then phosphorylate the Rb protein (Ach *et al.*, 1997; Huntley *et al.*, 1998; Mironov *et al.*, 1999; Nakagami *et al.*, 1999) indicates that cell-cycle control in plants may be highly similar to that in mammals. In plants, C*ycA* and C*ycB* are expressed in a cell-cycle-dependent manner, peaking around the G2/M transition (Reichheld *et al.*, 1996). However, C*ycD* genes show a cell-cycle-independent expression pattern, with transcriptions induced by mitogens and remaining at a constant level in actively dividing cells (Fuerst *et al.*, 1996; Huntley and Murray, 1999; Soni *et al.*, 1995). Recent studies have demonstrated that transcriptions of *Arabidopsis* C*ycD2* and C*ycD4* are induced by sucrose (De Veylder *et al.*, 1999; Soni *et al.*, 1995) and *CycD3* by cytokinin or sucrose (Fuerst *et al.*, 1996; Soni *et al.*, 1995). Transgenic *Arabidopsis* leaf explants overexpressing *CycD3* can initiate and maintain cell division in the absence of cytokinin, suggesting that cytokinins activate cell division through induction of *CycD3* at G1/S transition (Riou-Khamlichi *et al.*, 1999). These evidences indicate that plant D-type cyclins may function as mediators of internal and environmental stimuli to drive cell division.

Brassinosteroids (BRs) are steroid hormones that are widely distributed in the plant kingdom, with a regulatory function in normal plant growth and development (Grove *et al.*, 1979; Mandava, 1988). The biosynthesis of BR in plants has been well studied, and at least eight loci have been found to lead to BR deficiency (Bishop *et al.*, 1999; Choe *et al.*, 1998; Choe *et al.*, 1999; Ephritikhine *et al.*, 1999; Kauschmann *et al.*, 1996; Klahre *et al.*, 1998; Li *et al.*, 1996; Nomura *et al.*, 1997; Szekeres *et al.*, 1996). Analysis of BR-deficient and -insensitive mutants confirmed its essential role for cell elongation, male fertility, senescence and xylem differentiation (Altmann, 1998; Clouse and Sasse, 1998). However, whether BR plays a role in cell division is still an open question, and contradictory results have been reported. For example, application of nanomolar BR to parenchyma cell cultures stimulated cell division, showing at least a 50% increase in total cell number in the presence of auxin and cytokinin (Clouse and Zurek, 1991). In the culture of Chinese cabbage protoplasts, BR promoted cell division in a dose-dependent manner and enhanced cell-cluster formation when applied with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Nakajima *et al.*, 1996). Similar results were also reported for *Petunia* protoplast cultures (Oh and Clouse, 1998). However, studies with carrot cell cultures, and with hormone autonomous callus or suspension cultures of *Agrobacterium*-transformed tobacco, showed that BR had no effect on promoting cell division (Roth *et al.*, 1989; Sala and Sala, 1985). Moreover, microscopic examination of BR-deficient and BR-insensitive mutants indicated that the dwarf phenotype mainly resulted from a reduction in cell size rather than in cell number (Kauschmann *et al.*, 1996). Recently, an *Arabidopsis* cell-cycle-dependent kinase-related gene, *CDC2b*, was found to be induced by BR in darkness; however it was shown to play a role in hypocotyl elongation, cotyledon enlargement, and apical hook formation, rather than in cell-cycle control (Yoshizumi *et al.*, 1999).

In order to understand whether BR plays a role in promoting cell division, we identified genes that respond to BR by cDNA array. Here we report that *CycD3* is upregulated in response to epi-brassinolide treatment, and that epi-brassinolide can substitute cytokinin in promoting growth of callus and suspension cultures of *Arabidopsis*. We also report evidence that *CycD3* induction by BR is through an unidentified BR-signalling pathway.

Results

Transcription of CycD3 *is induced by epi-brassinolide*

To identify genes responsive to BR, we used cDNA array to monitor gene expression of *det2* suspension cultures treated with 24-epi-brassinolide (BL). The *det2* mutant was used because it is defective in the early step of BR biosynthesis, and has very low level of endogenous BR (Fujioka *et al.*, 1997; Li *et al.*, 1996). In a total of 13 000 arrayed cDNA clones, 53 were found to be BR-responsive (BRRs) and were designated as *BRR1–BRR53*. Sequencing and homology analyses indicated that these genes are mainly involved in signal transduction, RNA splicing, ion transportation, and stress response (data not shown). *BRR36,* an apparently BR-induced clone (Figure 1a,b), was found to be identical to *Arabidopsis CycD3* (EMBL accession number X83371).

To confirm and further understand *CycD3* induction by BR, RNA gel-blot analysis was performed with *det2* and wild-type (Columbia ecotype, Col-0) suspension cultures. The *det2* cells incubated with different concentrations of BL for 4 h accumulated *CycD3* transcripts in a dose-dependent manner, and showed the highest induction with 5 μM BL treatment (Figure 2a). Kinetic studies showed that *CycD3* transcription was apparently induced within 1 h, and reached the maximum (approximately fourfold compared to control) at 8 h when *det2* cells were cultured with 5 μM BL (Figure 2b). However, *CycD3* transcripts started to accumulate in Col-0 suspension cells after 2 h treatment, and peaked at 8 h with an up to 2.5-fold increase (Figure 2b). The lagged response time and lower level of increase suggest that wild-type cells are less sensitive to exogenous BR than the deficient mutant.

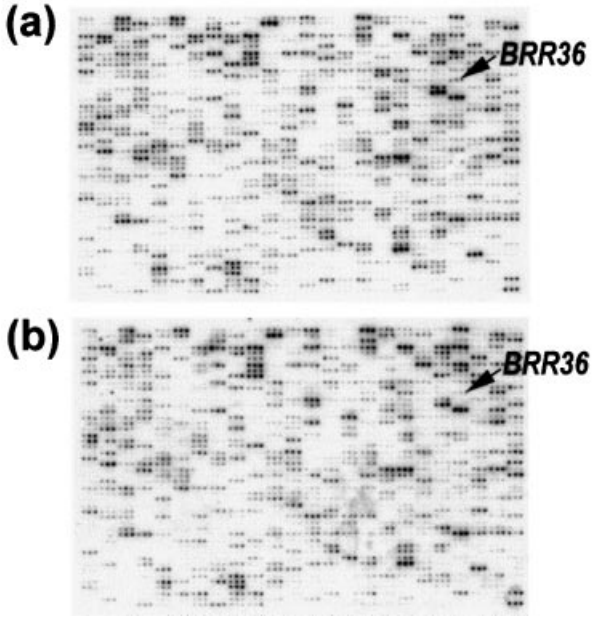

Figure 1. Gene expression pattern of *det2* suspension cultures treated with epi-brassinolide.
The cDNA array was performed by hybridizing the cDNA clones arrayed in triplicate on filters with first-strand cDNA probes. RNA for reverse-transcribed probes was isolated from *det2* cells treated with 5 μM 24-epi-brassinolide (BL) for 2 h (a) or without BL treatment (b). The differentially expressed clones identified were sequenced and blasted against GenBank. The *BRR36* (arrow) clone was identified as *cycD3*.

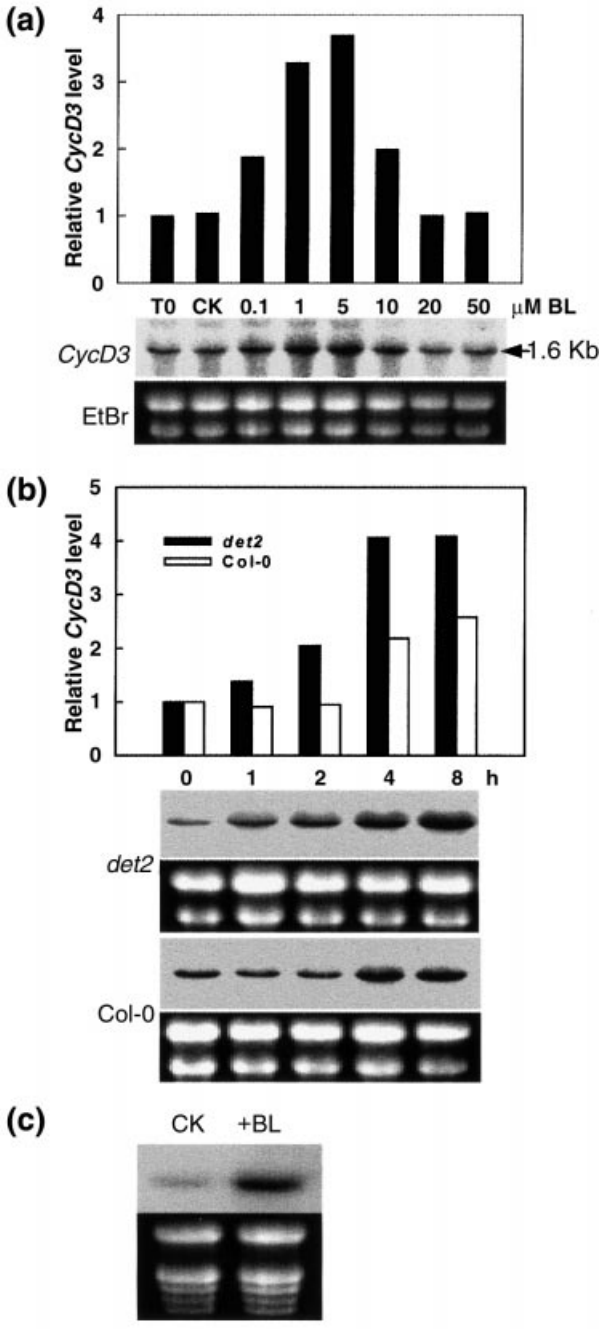

Figure 2. Induction of *CycD3* by epi-brassinolide.
Total RNA (≈15 μg) was loaded in each lane and RNA blots were probed with *cycD3*. All data shown were calibrated against the RNA loading.
(a) Dose dependence. The *det2* cells were treated for 4 h with different concentrations of BL or with DMSO (CK) after incubating in hormone-free medium for 48 h. A sample (T0) was taken before treatment.
(b) Time course of induction in deficient mutant and wild-type cells. The *det2* and Col-0 cells were treated with 5 μM BL after hormone starvation.
(c) Induction in plants. RNA was prepared from 30-day Col-0 plants at 24 h after spraying with water (CK) or 1 μM BL (+BL).

Although whole-plant responses to hormones are complex and do not necessarily reflect those produced in culture (Davies, 1995; Krikorian, 1995), the induction of

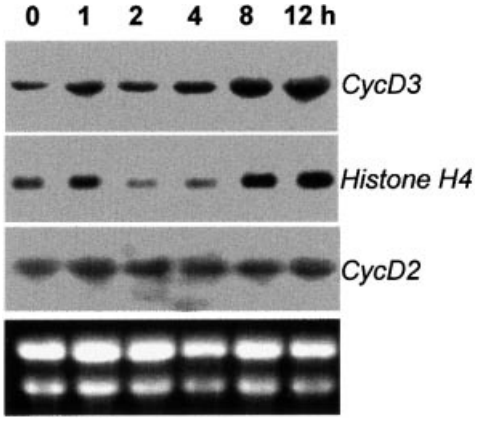

Figure 3. Expression of *CyCD3 histone H4 and cycD2* in the suspension cells treated with epi-brassinolide.
RNA was from *det2* cells incubated in medium with 5 μM BL after hormone starvation.

CycD3 by BL was also found to occur in plants. Treatment with 1 μM BL for 24 h caused a sizable increase of *CycD3* mRNA levels in Col-0 plants (Figure 2c).

Epi-brassinolide may promote cell division through induction of CycD3

D-type cyclins have been shown to drive cells through the G1/S boundary in the plant cell cycle (Huntley and Murray, 1999; Mironov *et al.*, 1999), and the *CycD3* mRNA was found mainly in proliferating tissues such as shoot meristem, young leaf primodia and axillary buds (Riou-Khamlichi *et al.*, 1999). The finding of *CycD3* induction by BL suggests that BR may play a promotive role in cell division through CycD3. We therefore carried out a further investigation on the expression of *histone H4*, a gene whose transcription was regarded as a marker of the S phase in cell division (Reichheld *et al.*, 1995). When *Arabidopsis det2* cells were incubated with 5 μM BL, *histone H4* expression started to increase at 4–8 h, shortly after induction of *CycD3* (Figure 3), indicating that quiescent cells were reactivated to enter the cell cycle. However the expression of C*ycD2*, another D-type cyclin gene that is inducible by sucrose (Soni *et al.*, 1995), showed no obvious alteration (Figure 3). These results suggest that the promotive effect of BR on cell division is through *CycD3* induction.

Epi-brassinolide can substitute cytokinin in callus and suspension cultures in Arabidopsis

Our finding that BL induces *CycD3* transcription, and the observation that cytokinin also induces *CycD3* transcription (Riou-Khamlichi *et al.*, 1999), led to a comparison of the kinetics of RNA induction. Treated with 5 μM BL or 1 μM zeatin (ZT), *det2* cells showed a similar RNA induction profile, except that induction by BL is weaker (Figure 4). These findings suggest that BR may function similarly

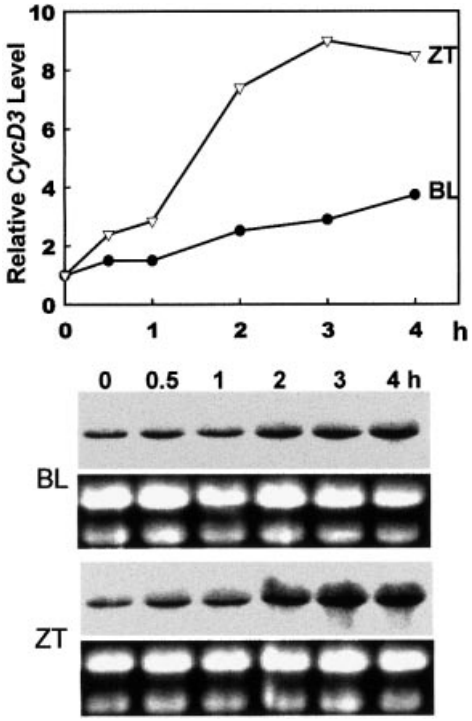

Figure 4. Induction kinetics of *CycD3* by epi-brassinolide and zeatin.
RNA samples were prepared from *det2* cells incubated with 5 μM BL or 1 μM zeatin (ZT) for 0, 0.5, 1, 2, 3 and 4 h after hormone starvation. All data shown were calibrated against the RNA loading.

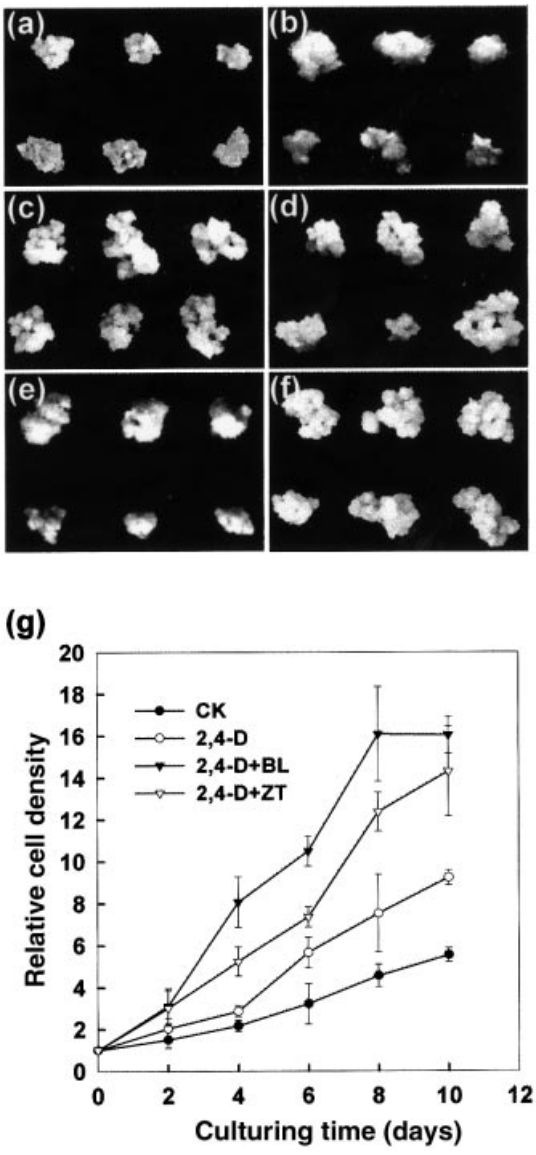

Figure 5. Epi-brassinolide substitutes cytokinin in *Arabidopsis* cell culture.
(a–f) Growth status of calli in media containing different hormone combinations. Calli of similar size to *det2* (above) and Col-0 (below) were cultured for 20 days in B5 media containing various combinations of 2,4-D (4.5 μM); BL (5 μM); and ZT (1 μM). (a) 2,4-D; (b) BL; (c) 2,4-D/BL; (d) 2,4-D/ZT; (e) BL/ZT; (f) 2,4-D/BL/ZT.
(g) Cell densities of *det2* suspensions cultured in media containing various hormones. Cells were diluted and cultured in triplicate in liquid media with hormones as in (a,c,d), or without hormone (CK). Relative density (1.0) represents cell density at 0 days (1.74×10^4 cells ml^{-1}). Error bars show standard error.

to cytokinin in *Arabidopsis* cell cultures. In the culture of many plants, maintenance of cell division and callus propagation normally requires the simultaneous presence of both auxin and cytokinin (Krikorian, 1995). We therefore transferred calli of similar size of *det2 and* Col-0 to the media containing different combinations of 2,4-D, BL and/or ZT. After culturing for 20 days, calli of *det2* and Col-0 were found in a good status with normal cell division on media containing 2,4-D/BL, 2,4-D/ZT or 2,4-D/ZT/BL (Figure 5c,d,f, respectively). However, calli cultured on media containing 2,4-D, BL or BL/ZT were much smaller or more condensed (Figure 5a,b,e, respectively). To further confirm the promotive effect of BL on cell division, we quantified cell densities of *det2* suspensions cultured in the three media described above. When cultured in fresh media, *det2* cells divided even more rapidly in the medium containing 2,4-D/BL than in 2,4-D/ZT (Figure 5g). Greening of calli cultured in constant light on the medium containing 5.0 μM BL plus 0.45 μM 2,4-D was also observed (data not shown). These results showed that BR could substitute cytokinin in cell cultures of *Arabidopsis*.

Epi-brassinolide induces CycD3 *transcription through a distinct signalling pathway*

To investigate the pathway leading to the induction of *CycD3* by BR, we examined *CycD3* expression in *det2* cell cultures treated with inhibitors of the signal transduction pathway. Okadaic acid (OA), a protein phosphatase inhibitor, showed no inhibition of *CycD3* induction by BL (Figure 6a). Staurosporine (St), a broad-range inhibitor of protein kinases, also showed no effect on *CycD3* induction by BL (Figure 6a). These results indicate that phosphoryl-

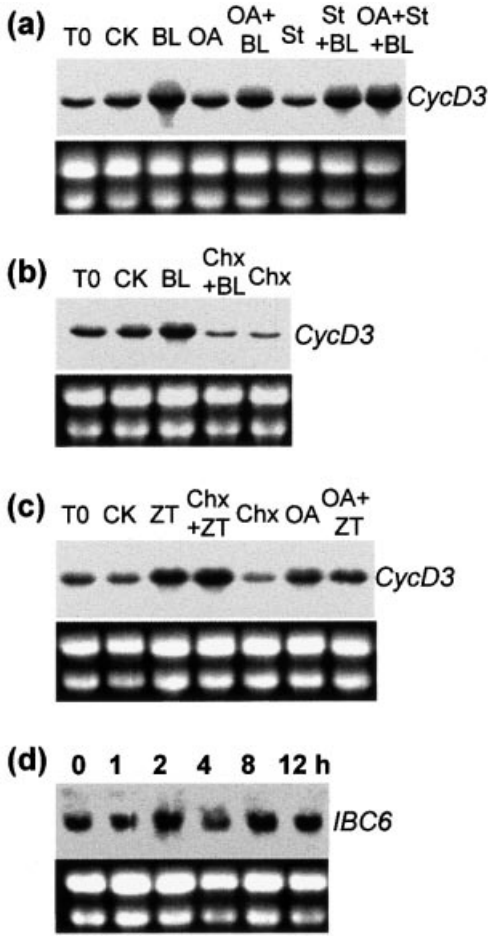

Figure 6. Analysis of *CycD3* induction pathway of epi-brassinolide.
(a) Protein phosphatase inhibitor okadaic acid (OA) and kinase inhibitor staurosporine (St) showed no effect on *CycD3* induction by BL. The *det2* cells were incubated for 4 h with DMSO (CK), BL (5 μM), OA (0.1 μM), St (1 μM) and their combinations as indicated.
(b) Protein synthesis inhibitor cycloheximide (Chx) blocked *CycD3* induction by BL. The *det2* cells pretreated with ethanol (CK and BL) or 100 μM Chx for 1 h were treated for additional 4 h with 5 μM BL (Chx + BL) or with equal volume DMSO (CK and Chx).
(c) *CycD3* induction by ZT was inhibited by OA but not by Chx. The *det2* cells pretreated with 100 μM Chx for 1 h were treated for an additional 4 h with 1 μM ZT (Chx + ZT) or with equal volume DMSO (Chx), and the untreated *det2* cell were treated with DMSO (CK), OA (0.1 μM) and OA (0.1 μM)/ZT (1 μM).
(d) *IBC6* showed no response to BL. The *det2* cells were treated with 5 μM BL after hormone starvation.

ation and dephosphorylation may not be involved in the BR-induced *CycD3* pathway. However, when cells were pretreated with the protein synthesis inhibitor cycloheximide (Chx) for 1 h, *CycD3* induction by BL was nearly completely inhibited (Figure 6b), suggesting that *CycD3* induction by BR requires *de novo* protein synthesis and *CycD3* is not a primary BR response gene. The induction of *CycD3* by ZT, however, was blocked by OA but not by Chx in *det2* cell (Figure 6c), which is consistent with the results reported for *Arabidopsis* ecotype Landsberg cells (Riou-Khamlichi *et al.*, 1999). Furthermore, the expression of

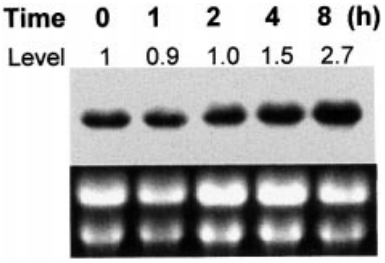

Figure 7. *CycD3* induction by epi-brassinolide in *bri1* mutant cells.
The *bri1* cells were treated with 5 μM BL for 0, 1, 2 or 4 h after hormone starvation. All data shown were calibrated against the RNA loading.

IBC6, a gene specifically induced by cytokinin (Brandstatter and Kieber, 1998), showed no response to BL treatment (Figure 6d). We conclude that the pathway of *CycD3* expression induced by BR is different from that induced by cytokinin.

The finding that *CycD3* induction by BR involves no protein phosphorylation or dephosphorylation is apparently inconsistent with the BRI1 signal pathway (see Discussion), the only pathway so far identified to transduce BR signals (Li and Chory, 1997; Schumacher and Chory, 2000). It is possible that BR induces *CycD3* transcription through an unidentified signal pathway in plants. To confirm our hypothesis, an RNA gel-blot analysis was performed in the suspension culture of *bri1*, a BR-insensitive mutant in which the BRI1 pathway is blocked (Li and Chory, 1997). When treated with 5 μM BL, *bri1* cells accumulated *CycD3* mRNA after 2 h incubation in a dose-responsive manner (Figure 7) similar to that of wild-type Col-0. This evidence suggests that *CycD3* induction by BR involves another BR signalling pathway that is different from the BRI1 pathway.

Discussion

The promotive role of brassinosteroids on cell division

BR is a widely distributed phytohormone that is essential for plant growth and development (Clouse and Sasse, 1998). Both promotive and inhibitory effects of BR on cell division have been reported (Clouse and Zurek, 1991; Grove *et al.*, 1979; Nakajima *et al.*, 1996; Oh and Clouse, 1998; Roth *et al.*, 1989; Sala and Sala, 1985). In this paper we present evidence that BR functions in promoting plant cell division through *CycD3* induction, which was suggested by recent studies on the cell division activated by cytokinins (Riou-Khamlichi *et al.*, 1999). Because the effects of mitogenic factors on cell division and their interaction are very complex, the contradictory results of previous studies may be caused by the unbalanced concentration or combination of phytohormones in the media (Nakajima *et al.*, 1996). It is apparent that a high BR concentration in the culture medium is less effective, or even inhibitory

rather than promotive for cell division, and that the auxin and cytokinin status is also critical in determining BR effect (Oh and Clouse, 1998).

As both BR and cytokinin promote cell division through *CycD3* induction, they should play a similar role in cell division. Our finding that BR can substitute cytokinin in callus and suspension cultures of *Arabidopsis* strengthens this point. A similar result was reported by Takematsu and co-workers, who found that brassinolide and auxin in combination promoted growth in various plant callus cultures more effectively than auxin and 6-benzylaminopurine (Takematsu *et al.*, 1983). Because BR occurs endogenously at a very low level in plant cells (Mandava, 1988), it is possible that its effect on promoting cell division is curtained or substituted by intracellular cytokinins in whole plant. This might explain why the dwarf phenotype of BR-deficient and -insensitive mutants is mainly due to the reduced cell size rather than cell number (Kauschmann *et al.*, 1996). Therefore BR-deficient mutants provide an invaluable system for the study of BR functions.

A distinct CycD3-induction pathway of brassinosteroids

Cell division is a fundamental event in plant growth and development, and is affected by many mitogenic factors such as light, temperature, nutrients and phytohormones. The mitogenic factors activate a largely unknown signal transduction cascade leading to the activation of cell division. In a recent study, it has been shown that CycD3 is a mediator of plant mitogenic signals (Riou-Khamlichi *et al.*, 1999; Soni *et al.*, 1995; Sorrell *et al.*, 1999). We demonstrate here that BR induces *CycD3* transcription and promotes cell division, and that CycD3 also mediates the BR signal.

Induction of *CycD3* by cytokinin involves protein phosphorylation and dephosphorylation, but no newly synthesized protein is required (D'Agostino and Kieber, 1999; Kakimoto, 1998; Riou-Khamlichi *et al.*, 1999). On the contrary, the process of *CycD3* induction by BR involves no protein phosphorylation and dephosphorylation, but the synthesis of new protein is required. These results, together with the observation that *IBC6* showed no response to BL, indicate that BR regulates *CycD3* transcription through a particular signalling pathway. In this pathway, the newly synthesized protein should be essential for *CycD3* induction.

Brassinosteroid signal transduction

Observation of the C*ycD3*-induction pathway of BR raises a question concerning BR signal transduction. The BR-insensitive mutant *bri1* shows severe pleiotropic effects on plant development, including dwarfism, de-etiolation, male sterility and altered leaf morphology (Kauschmann *et al.*, 1996; Li and Chory, 1997). The *BRI1* gene has been cloned and shows strong sequence homology to leucine-rich receptor kinases that function in transducing extracellular signals. Moreover, sequence analysis of *bri1* alleles confirms that the putative ligand-binding and kinase domains are essential for function *in vivo* (Fredrichsen *et al.*, 2000; Li and Chory, 1997). BRI1 is a putative plasma-membrane-located BR receptor that can perceive BR by the extracellular domain (Fredrichsen *et al.*, 2000; He *et al.*, 2000), and is the only important component identified so far in BR signal transduction (Schumacher and Chory, 2000). As BRI1 is homologous to the leucine-rich receptor kinases, protein phosphorylation and dephosphorylation should be a part of the signalling cascades in the BRI1 pathway. The BR-induced *CycD3* transcription is apparently inconsistent with this pathway. The occurrence of *CycD3* induction by BR in the *bri1* mutant cells suggests that another BR signalling pathway may exist in plants. This hypothesis is strengthened by our recent finding that the *BRR8*, a gene downregulated by BR, also responded to inhibitors in a similar manner to *CycD3* (unpublished results).

In animals, there are two major paradigms of steroid hormone signal transduction. The first involves a membrane-located receptor with an extracelluar ligand-binding domain, and an intracellular domain responsible for transducing the signal to the next member, often a kinase or G protein. The signal is amplified through cascades of phosphorylation/dephosphorylation, and involves second messengers such as calcium, cyclic AMP and diacyl glycerol (Mendona *et al.*, 1995). The second pathway involves an intracellular receptor complex that recognizes steroid ligands to directly affect transcription of specific genes by binding to the respective promoters (Beato *et al.*, 1995). In plants no evidence has been found so far for the existence of an intracellular BR signal pathway, and no gene has been identified that encodes a candidate protein for an intracellular receptor of BR. However, a chaperone heterocomplex, similar to the intracellular steroid receptor in animals, has been identified in wheat germ lysate (Owens-Grillo *et al.*, 1996; Reddy *et al.*, 1998; Stancato *et al.*, 1996). Therefore it is likely that in plants there may be an intracellular signal pathway through which BR regulates the transcription of some genes, in a similar fashion to the steroid hormone in animals.

Experimental procedures

Callus, suspension culture and plant growth

Seeds of *Arabidopsis thaliana* wild type (ecotype Columbia, Col-0), BR-deficient mutant *det2*, and -insensitive mutant *bri1* were sterilized and cultured to induce callus on a B5 medium containing 2% glucose, 4.5 μM 2,4-D and 0.45 μM kinetin in the dark at 25°C. The induced calluses were propagated every 30 days. The suspension culture was established by suspending calli in the

liquid medium as described above, and maintained in constant low light with orbital shaking at 130 rpm. The culture medium was exchanged weekly. Well dispersed suspension culture of *det2* was used for determination of cell density. The Col-0 plants for 24-epi-brassinolide (BL) treatment were grown on vermiculite saturated with 0.3 × B5 medium under continuous illumination at 23°C, as described previously (Mou *et al.*, 2000).

cDNA array

The cDNA array procedures were performed as previously described (Hu *et al.*, 1999), with some modifications. A total of 13 000 cDNA clones from a cDNA library constructed in λYES were arrayed in triplicate on filters using the Biomek 2000 HDRT system (Beckman, Fullerton, CA, USA), and the filters were probed with α-^{32}P-dCTP-labelled cDNA prepared from the total RNA of *det2* suspension culture, untreated or treated with 5 μM BL for 2 h.

Treatment with epi-brassinolide and inhibitors

On the seventh day after medium exchange, suspension cultures were washed three times with B5 medium, and maintained in B5 medium for 48 h for hormone starvation. A sample was taken before treatment (T0) and the remaining cells were treated with BL, zeatin (ZT) and/or inhibitors, according to the times and concentrations indicated. To effectively inhibit protein synthesis, cycloheximide (Chx, Sigma, St Louis, MO, USA) was added into suspension cultures 1 h before BL or ZT treatment. Okadaic acid (OA) and staurosporine (St) were purchased from CalBiochem (La Jolla, CA, USA). To test the response of whole plants to BL, 30-day plants of Col-0 were sprayed with 1 μM BL and harvested after 24 h.

Cell density

Suspension cultures at stationary phase (7 days after subculture) were diluted 10-fold in triplicate with fresh media containing different hormones, as indicated. Cell density was measured by haemocytometry after maceration (Sorrell *et al.*, 1999). Cell suspension (1 ml) was taken and then added to an equal volume of 15% (w/v) CrO_3 and left at room temperature overnight. Cells were dispersed by repeatedly passing through a 1 ml disposable pipette tip. Cell number measurements were performed at an interval of 2 days.

RNA extraction and RNA gel-blot analysis

All suspension cultures and plants harvested were immediately frozen in liquid nitrogen and stored at –70°C. Total RNA was extracted using guanadine thiocyanate extraction buffer (Wadsworth *et al.*, 1988), precipitated by ethanol, and purified with LiCl and chloroform. For RNA gel-blot analysis, 15 μg total RNA was fractionated in a formaldehyde agarose gel, blotted onto nylon filters (Hybond-N$^+$, Amersham, Little Chalfont, UK), and immobilized in vacuum baker at 80°C for 2 h. Probes were prepared using a random labelling system (Amersham), and hybridization was carried out in Church buffer as described (Church and Gilbert, 1984). Quantification of radioactivity was performed with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) and calibrated against RNA loading stained with ethidium bromide (EtBr).

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