Original Paper Plant Biotechnology, **16** (4), 279-284 (1999)

Phytochrome B Influences *in-vitro* Shoot Regeneration in *Arabidopsis thaliana* (L.) Heynh.

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Received 28 September 1998; accepted 21 June 1999

Abstract

The effects of *phyA* and *phyB* mutations on shoot bud regeneration in *Arabidopsis thaliana* have been studied. The *phyB* mutant showed a significantly lower frequency of shoot bud regeneration than did the wild-type, indicating a role for phytochrome B in the regulation of *in-vitro* shoot bud regeneration. Interestingly, the shoot bud regeneration frequency of the *phyA* mutant was not significantly different from that of the wild-type in a light and dark , suggesting that phytochrome A may not be involved in the regulation of *in-vitro* shoot bud regeneration. The effects of gibberellin (GA) were also studied because previously we showed that a reduction in endogenous GA level results in a higher shoot bud regeneration frequency. Exogenous paclobutrazol and prohexadione (GA biosynthesis inhibitors) increases, and exogenous GA reduces, the shoot bud regeneration frequency in the *phyB* mutant. Thus shoot regeneration in the *phyB* mutant is still responsive to regulation via the GA signalling system.

Introduction

In-vitro shoot morphogenesis from plant tissue explants is an important technique in modern plant genetic engineering. However, very little is known of the biochemical, physiological or cellular factors which control the morphogenic process. It is well-known that the relative concentration of exogenous auxin and cytokinin influences the regeneration of shoots from tissue explants. For example, shoot regeneration is stimulated by an increase in the cytokinin/ auxin ratio, and inhibited when the cytokinin/auxin ratio is decreased (Flick *et al.* 1983). However, we know little of the other factors with which auxin and cytokinin interact during the regulation of *in-vitro* shoot regeneration.

One of the other factors which regulate *in-vitro* shoot bud regeneration is gibberellin (GA). Comparisons of shoot regeneration from callus of GA-deficient and GA-signalling mutants of *Arabidopsis* showed that callus with reduced levels of endogenous bioactive GAs, or reduced responsivity to GA, regenerates shoot buds more readily than does wild-type (WT) callus (Ezura and Harberd 1995). In addition, characterization of the recently described *pickle* mutant of *Arabidopsis* suggests a role for GA in the regulation of the *in-vitro* regeneration capacity of root cells (Ogas *et al.* 1997).

Another factor that might be expected to influence *in-vitro* shoot regeneration ability is phytochrome. Phytochromes are photoreceptor proteins that mediate plant morphogenesis in response to signals from the light environment (Kendrick and Kronenberg 1994). *Arabidopsis* contains several phytochromes (A-E), the apo-proteins of which are encoded by a small family of divergent genes called *PHYA, PHYB, PHYC, PHYD,* and *PHYE* (Sharrock and Quail 1989; Quail 1991; Clark *et al.* 1994; Cowl *et al.* 1994). Phytochrome-related *Arabidopsis* mutants have been isolated using screens for plants displaying an elongated hypocotyl phenotype in continuous white or continuous far-red light (Koornneef *et al.* 1980; Nagatani *et al.* 1993; Reed *et al.* 1993; Parks and Quail 1993; Whitelam *et al.* 1993). Through the study of these mutants (Reed *et al.* 1994; Johnson *et al.* 1994; Whitelam and Harberd 1994; Yang *et al.* 1995; Devlin *et al.* 1996) and of transgenic plants over-expressing phytochromes A and B (Boylan and Quail 1991; Cherry *et al.* 1991; Wagner *et al.* 1991; Jordan *et al.* 1995), the different functions of the various phytochrome family members have been elucidated.

Here we describe experiments which compare the

shoot regeneration ability of tissue from *Arabidopsis phyA* and *phyB* mutants with that of WT. *phyA* mutant is deficient for phytochrome A function, display an elongated hypocotyl in continuous far-red light (but not in continuous white light) and do not exhibit an obvious adult mutant phenotype when grown in standard greenhouse conditions (Dehesh *et al.* 1993; Nagatani *et al.* 1993; Parks and Quail 1993; Whitelam *et al.* 1993). *phyB* mutant is deficient for phytochrome B function, display an elongated hypocotyl in continuous white light and continuous red light, but not in continuous far-red light, and exhibit a pale-green, elongated adult plant phenotype in standard greenhouse conditions (Reed *et al.* 1993; Bradley *et al.* 1995, 1996). Phytochrome A and B have overlapping but distinct functions in *Arabidopsis* development (Johnson *et al.* 1994; Reed *et al.* 1994). Our experiments show that *phyB* mutant root explants (but not *phyA* mutant explants) display a significantly lower frequency of shoot bud regeneration than do WT explants. Furthermore, shoot regeneration in the *phyB* mutant is still responsive to regulation via the GA signalling system, because the GA biosynthesis inhibitors, paclobutrazol and prohexadione increase, and supplementary GA reduces, the frequency of shoot bud regeneration in *phyB*.

Materials and methods

Plant materials and growth conditions

Seeds of *phyA-1* (Whitelam *et al.* 1993; Quail *et al.* 1994), *phyB-1* (Reed *et al.* 1993; Quail *et al.* 1994) and wild-type *Arabidopsis thaliana* (L.) Heynh. Landsberg *erecta* (WT) were freshly harvested for use. Seed sterilization, and plating of seeds on GM medium was as previously described (Ezura and Harberd 1995). The seeds were chilled at 4 ℃ for 4 d, and then incubated at 20 ℃ in a growth room (16 hr light/ 8 hr dark cycle). Illumination was from fluorescent lamps (FLR40S W/N, Toshiba) at a fluence rate of 50 μmol $m^{-2} s^{-1}$.

Shoot bud regeneration

Four weeks after the commencement of the 20 ℃ incubation, seedling roots were excised and transferred onto the callus-inducing medium (CIM, see Ezura and Harberd 1995). The roots were then incubated in the growth room (same temperature and light conditions as above) for 5 d. The roots were then cut into 5-mm length segments, starting from the basal end of the hypocotyl. Five root explants were prepared per seedling root. The explants were then transferred onto shoot-inducing medium (SIM) with or without paclobutrazol (PAC), prohexadione or gibberellin (GA_3). GA_3, PAC and prohexadione were added to the media just before pouring. Thirty root explants, 10 explants each for WT, *phyA* and *phyB* (Table 1), twenty root explants (Table 2) and twenty five root explants (Table 3 and 4) were inoculated per Petri dish, each dish containing 30 m*l* of SIM medium.

Evaluation of shoot bud regeneration

Shoot bud regeneration from root explants was evaluated, using a stereomicroscope, three weeks following the commencement of incubation of the explants in the growth room. The frequency of shoot bud regeneration was measured by determining the number of root explants displaying differentiating shoot buds and/or the regeneration index (RI), the average of the regeneration factors (RF) of the five root explants originated from a single seedling. RFs were put into the following categories: Explants with no shoot buds were designated as 1; those with one shoot bud as 2; those with 2-4 shoot buds as 3; those with 5-9 shoot buds as 4; those with more than 10 shoot buds as 5.

Results and discussion

The process of shoot bud regeneration from *Arabidopsis* root explants (WT and mutants) can be summarized as follows. Root segments are first incubated on CIM (5 days) and then transferred to SIM (see Materials and methods). There are no detectable changes in root morphology whilst the material is incubated on CIM. However, following a week incubation on SIM, callus tissue is clearly visible at both cut ends of the root segment, and at the sites of the epidermal root hairs, (H.E. and K.H., unpublished observation). As described previously (Ezura and Harberd 1995), foci of regeneration first appear as clusters of green cells. These clusters differentiate into shoot buds and develop leaves within three weeks of culture on SIM.

Root explants from WT, *phyA*, and *phyB* plants were cultured as described above, and shoot bud regeneration was observed after 3 weeks of culture (**Table 1**). There were no significant differences in the number of root explants differentiating shoot buds, or in regeneration index, between the WT and *phyA* samples when they were cultured both in a light and dark. However, *phyB* root explants regenerate shoot buds at a frequency significantly lower than that is seen with *phyA* and WT root explants. These observations suggest that phytochrome B is involved in the regulation of *in-vitro* shoot bud regeneration in *Arabidopsis*, but phytochrome A may not be.

Table 1. Frequency of *in-vitro* shoot bud regeneration from root explants of phytochrome mutants of *Arabidopsis thaliana*. Regeneration index of shoot buds is expressed as the average of regeneration factors per 5 explants, taken from a data set consisting of a total of 50 explants. Values from the same column are not significantly different from one another (Duncan's multiple range test; 5% level) when followed by the same letter. Experiments in a light and dark condition were performed independently.

Line	No. of explants differentiating shoot buds/ 5 explants	Regeneration index of shoot buds/ 5 explants
(Light)		
WT	4.1a	3.8a
phyA	4.5a	3.7a
phyB	2.9b	2.4b
(Dark)		
WT	4.5a	3.0a
phyA	4.2a	3.0a
phyB	1.8b	1.6b

Previously, we reported that GA-deficiency and GA-signalling mutations affect shoot regeneration frequency: reduced endogenous GA levels and reduced GA responsiveness result in increased shoot bud regeneration frequencies (Ezura and Harberd 1995). Accordingly, we observed the effects of the GA-biosynthesis inhibitors paclobutrazol (PAC) and prohexadione, and of GA_3, on shoot bud regeneration frequency in *phyB* (**Table 2**). PAC acts by inhibiting the enzymatic oxidation of ent-kaurene to ent-kaurenoic acid, an early step in GA-biosynthesis, thus reducing endogenous GA levels (Rademacher 1989). Prohexadione acts by inhibiting the enzymatic oxidation of GA_{20} to GA_1, a late step in GA-biosynthesis, thus reducing endogenous GA levels (Miyazawa *et al.*, 1997). As shown previously for WT (Ezura and Harberd 1995), the number of *phyB* explants differentiating shoot buds was significantly increased by the addition of 0.01 or 0.1 mg/*l* PAC to the regeneration medium (**Table 2**). The regeneration index was also significantly increased by the addition of 0.01 or 0.1 mg/*l* PAC to the regeneration medium. The highest PAC dose used in these experiments (1.0 mg/*l*) is associated with a lower regeneration frequency than that is seen with the 0.01 and 0.1 mg/*l* doses. This could be due to the toxic effects of higher doses of PAC, although, if so, it is occurring at a lower concentration than seen in previous experiments (Ezura and Harberd 1995). Prohexadione showed similar effects with PAC on shoot regeneration from *phyB* explants (**Table 3**), suggesting the involvement of GA on this system. As expected, the increase in shoot bud regeneration frequency caused by the addition of 0.1 mg/*l* PAC was reversed by the addition of GA_3 to the regeneration medium in a dose dependent manner (**Table 4**). These results show that *in-vitro* shoot bud regeneration from *phyB* explants, although less frequent than that of WT, can still be influenced by the GA-signalling system.

Previous experiments have implicated the phytochromes in the regulation of *in-vitro* morpho genesis. Phytochrome deficient mutants of *Nicotiana plumbaginifolia* exhibit altered *in-vitro* morphogenetic processes. This change in regenerative behavior is correlated with an increase in indole-3-acetic acid (IAA, auxin) levels (Kraepiel *et al.* 1995). Since auxin/cytokinin concentration ratio is so crucial to *in-vitro* regeneration (Flick *et al.* 1983), it could be that altered auxin levels in phytochrome mutants are responsible for altered *in-vitro* regeneration properties. Our experiments make use of *Arabidopsis* mutants which are specifically deficient for one or the other of phytochrome A or

Table 2. Effects of paclobutrazol on *in-vitro* shoot bud regeneration from root explants of *phy B* mutant of *Arabidopsis thaliana*. Regeneration index of shoot buds is expressed as the average of regeneration factors per 5 explants, taken from a data set consisting of a total of 80 explants. Values from the same column are not significantly different from one another (Duncan's multiple range test; 5% level) when followed by the same letter.

Paclobutrazol mg/ *l*	No. of explants differentiating shoot buds/ 5 explants	Regeneration index of shoot buds/ 5 explants
0	2.6b	2.3b
0.001	2.8b	2.4b
0.01	3.5a	3.1a
0.1	3.6a	3.0a
1.0	2.7b	2.2b

Table 3. Effects of prohexadione *in-vitro* shoot bud regeneration from root explants of *phy B* mutant of *Arabidopsis thaliana*. Regeneration index of shoot buds is expressed as the average of regeneration factors per 5 explants, taken from a data set consisting of a total of 75 explants. Values from the same column are not significantly different from one another (Duncan's multiple range test; 5% level) when followed by the same letter.

Prohexadione mg/*l*	No. of explants differentiating shoot buds/ 5 explants	Regeneration index of shoot buds/ 5 explants
0	1.8c	1.7c
0.001	2.5b	2.0b
0.01	2.8b	2.2b
0.1	3.5a	2.6a
1.0	2.1c	2.1b
10.0	1.6d	1.5d

Table 4. Effects of paclobutrazol and GA_3 on *in-vitro* shoot bud regeneration from root explants of *phy B* mutant of *Arabidopsis thaliana*. Regeneration index of shoot buds is expressed as the average of regeneration factors per 5 explants, taken from a data set consisting of a total of 50 to 75 explants. Values from the same column with/without paclobutrazol are not significantly different from one another (Duncan's multiple range test; 5% level) when followed by the same letter.

Paclobutrazol mg/*l*	GA_3 mg/*l*	No. of explants differentiating shoot buds/ 5 explants	Regeneration index of shoot buds/ 5 explants
0	0	2.2a	1.9a
0	0.01	1.6b	1.5b
0	0.1	1.2b	1.5b
0	1.0	0.9c	1.3b
0	10.0	0.0d	1.0c
0.1	0	2.5a	2.4a
0.1	0.001	2.5a	2.1a
0.1	0.01	1.6b	1.7b
0.1	0.1	1.4b	1.4c
0.1	1.0	1.1b	1.3c
0.1	10.0	0.1c	1.0d

B. We show that, whilst deficiency for phytochrome A has no obvious effect on shoot bud regeneration, deficiency for phytochrome B has a marked effect. Phytochrome B also plays the predominant role in the regulation of growth in light-grown plants (Reed *et al.* 1993; Johnson *et al.* 1994; Reed *et al.* 1994; Whitelam and Harberd 1994). It remains to be tested if phytochrome B deficient *Arabidopsis* plants have elevated IAA levels. If phytochrome B deficient mutants were found to have elevated IAA levels, whilst phytochrome A deficient mutants were not, this result would imply a specific effect of phytochrome B on auxin accumulation.

Our previous data implicated GA in the regulation of *in-vitro* shoot bud regeneration (Ezura and Harberd 1995). Here, we show that in a phytochrome B deficient background, changes in GA level affect changes in *in-vitro* shoot bud regeneration frequency. It could be that phytochrome mediates its effects via the GA-signalling system, either by alteration of GA amount, or by alteration of GA responses. For example, previous studies of phytochrome-deficient elongated hypocotyl mutants of cucumber and *Arabidopsis* have suggested that phytochrome B down-regulates responsiveness to GA (Lopez-Juez *et al.* 1995; Reed *et al.* 1996). Effective concentrations of paclobutrazol for improving shoot bud regeneration was ranged from 0.01 to 0.1 mg/*l* in *phyB* mutant (**Table 2**) and from 0.1 to 1 mg/*l* in WT (**Table 4**; Ezura and Harberd 1995), indicating that endogenous GA levels in *phyB* mutant was lower than that in WT. Thus, it is

possible that the reduced frequency of shoot bud regeneration observed in *Arabidopsis phyB* mutant is due to increased responsiveness to GA. However, it is equally possible that phytochrome and GA affect common processes independently of one another (Peng and Harberd 1997). The same analysis between *phyB* and GA insensitive mutants using a double mutant will provide an answer to this question.

In conclusion, we show here that phytochrome B, but not phytochrome A, plays an important role in the regulation of *in-vitro* shoot bud regeneration. Since phytochrome detects changes in the light environment, it might be useful for those seeking to optimize conditions for *in-vitro* regeneration to pay attention to the effects of light quantity and quality on the regeneration process.

Acknowledgments

We thank to Hiroshi Kamada, and Toshiyuki Ogawara for useful discussion of this manuscript. Paclobutrazol and Prohexadione were supplied by Zeneca Co., Tokyo, Japan and Kumiai Chemical Industry Co., Ltd., Shizuoka, Japan, respectively. Work in N.P.H.'s laboratory is supported by the Core Strategic grant from the BBSRC to the John Innes Centre.

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