Original Paper

Brassinolide as a modulator of the activities of cell wall loosening proteins

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Abstract The role of brassinolide (BR) in acid-induced cell wall loosening was studied using *lkb* dwarf pea (*Pisum sativum* L.), a BR mutant. Cell wall loosening at pH4.5 was analyzed by creep measurements of internode segments from *lkb* and *LKB* (wild type) seedlings stored in 50% glycerol at $-15°C$ (G-segments). Although cell wall extensibility (ϕ) was not significantly different between *LKB* and *lkb*, the yield threshold (y) was distinctly lower in *LKB* than in *lkb*, suggesting that some load-bearing bonds in the cell wall of *LKB* are more easily broken than in *lkb*. In G-segments from BR-treated, growth stimulated *lkb* seedlings y was lowered, and the pattern of the creep rate change was similar to that of *LKB*. BR applied *in vitro* to *lkb* G-segments also lowered y. In heat-treated *LKB* G-segments no acid-induced cell wall loosening was observed. However, it was restored by addition of a crude cell wall protein fraction. In heat-treated *lkb* G-segments, acid-induced cell wall loosening could be observed only in the presence of both the protein fraction and BR. It is suggested that BR is necessary for acid-induced cell wall loosening and that BR acts as a modulator of the activity of wall loosening proteins which are limiting the yield threshold.

Key words: Brassinolide, cell wall-bound proteins, cell wall extensibility, cell wall loosening, dwarf pea, *Pisum sativum* L.

Brassinolide (BR) is an essential plant hormone required for normal growth (Clouse and Sasse 1998; Li and Chory 1999; Sasse 2003). Mutations impairing in its biosynthesis or perception result in dwarfism (Schumaker and Chory 2000; Bishop 2003). Stem growth is accomplished by cell proliferation and cell expansion, and stem elongation is usually due to cell elongation. A key control part of the cell in its expansion is the cell wall. The kinetics of cell wall expansion (extension) under the steady state condition is expressed by an equation, $v = \phi (P-y)$, where v, ϕ, P and y respectively represents the rate of cell expansion, cell wall extensibility, turgor pressure and the yield threshold of the cell wall (Lockhart 1965). The parameters, ϕ and y are believed to be under the control of cell wall loosening proteins, ϕ and y proteins, which are associated with the cell wall (Okamoto and Okamoto 1995). The activity of these proteins is pH- and temperature-dependent (Taguchi et al. 1999). They are most active under acidic conditions. Recently, y proteins named yieldins were isolated from *Vigna unguiculata* L. hypocotyls (Okamoto-Nakazato et al. 2000a) and their genes were cloned (Okamoto-Nakazato et al. 2000b). The acidic environment is thought to activate cell wall loosening enzymes including ϕ and y proteins (Taguchi et al. 1999), resulting in cell wall loosening and hence cell wall expansion under the effective turgor pressure (P-y). Beside ϕ and y proteins expansins are known to intimately involve in the process of cell wall expansion (Cosgrove 1998, 1999, 2000a, b; Cosgrove et al. 2002). The mechanisms of these cell wall loosening proteins are still enigmatic. BR has also been reported to induce cell wall loosening in pakchoi hypocotyls (Wang et al. 1993), soybean epicotyls (Zurek et al. 1994) and squash hypocotyls (Tominaga et al. 1994). Clouse (1997) has suggested that BR is involved in the modification of the cell wall through the activation of wall enzymes. Enzymes which control the modification of the cell wall have been reported to be influenced by BR (Mølhøj et al. 2002; Rose et al. 2002).

We present in this paper evidence that BR is necessary for cell wall loosening and suggest that BR may act as a modulator of the activities of cell wall loosening proteins.

Materials and methods

Preparation of plant materials

Seeds of garden pea (*Pisum sativum* L., cv. *Torsdag*, *LKB*) and one of its isogenic dwarf mutants, *lkb* (NGB5862) were provided by Prof. T. Yokota, Teikyo University, Japan, and propagated at the former National

Institute of Agrobiological Resources, Japan, by the courtesy of Dr. N. Katsura, the then Director General. The seeds had originally been supplied by Prof. J. B. Reid, University of Tasmania, to Prof. Yokota. Seeds soaked in tap water overnight at 25°C were sown in wet vermiculite contained in a wooden box (25×25×5 cm), and germinated in a growth chamber (Koitotron FR-525A, Koito Industries Co., Ltd.) at 25±1°C under continuous light of 42.8 $Jm^{-2}s^{-1}$. Water was supplied once a day.

Application of BR to lkb seedlings

BR was applied in a 10 μl drop of ethanol containing 0.1% Tween 20 to the surface of the 6th leaf of *lkb* seedlings with 5 mm long 7th internodes (about 14 days old). Control *lkb* and wild (*LKB*) seedlings were treated with the same solvent only. The treated seedlings were grown under the same conditions before treatment. The length of the 7th internode were measured with a ruler at certain time intervals over a period of 6 days.

Preparation of glycerinated stem segments for creep measurements

From the upper region of the 7th internode of each seedling, an 8 mm segment was excised 24 h after BR treatment at which growth was linear (see Figure 1). Seedlings were selected for uniformity in internode diameter of about 1.3 mm for *LKB* seedlings and 2.0 mm for *lkb* seedlings. Excised segments were immediately immersed in ice-cold 50% glycerol aqueous solution and then stored at −15°C for more than 2 weeks. This treatment is known to eliminate membrane permeability without affecting cell wall function (Hager et al. 1971; Okamoto and Okamoto 1994; Taguchi et al. 1999).

Creep measurements

The method of creep measurement of G-segments with an extensiometer followed that of Taguchi et al. (1999). G-segments were incubated in 1mM McIlvaine buffer (1 mM citric acid, 2 mM Na_2HPO_4) adjusted to pH4.5 at 25°C for 1 h before creep measurements. After removing glycerol remaining on the surface with filter paper, each G-segment was fixed between two clamps (about 5 mm apart) with an adhesive, Alon Alpha (Konishi Co., Ltd.), and the lower clamp was connected to an acrylite incubation vessel filled with 0.5 ml of 1 mM McIlvaine buffer. Extension of the segment was measured by the upward movement of the upper clamp which was detected by a differential transformer (DTD-3, Hoshidenki Co., Ltd.), and recorded by a pen recorder (3057 portable recorder, Yokokawa Co., Ltd.). Prior to creep measurements the sensitivity of the differential transformer was calibrated with a dial gauge (TM-110, Teclock Co., Ltd.). All measurements were made at 25±1°C under pulling tensions applied to the segment.

The tension was increased by 10 gf every 30 min up to a maximum of 120 gf. The creep rate was determined 20–30 min after application of the tension by dividing the length of the segment (Taguchi et al. 1999). Measurements for the *in vitro* effect of BR on G-segments were also made in the same manner as described above except the presence of BR in the buffer.

Relative creep rates (%h^{-1}) were plotted against applied load per area of the cut surface of each segment (gfmm^{-2}). The average of ϕ values were obtained from the regression line of the second phase by the least square method, and y values were obtained from the cross point of the two regression lines.

Extraction cell wall proteins

Procedures for the extraction of crude cell wall proteins from pea seedlings followed those of Taguchi et al. (1999). Whole shoots (approximately 120 g) of light-grown 12-day-old Alaska pea seedlings were harvested and immediately frozen in liquid N_2. The frozen material was powdered and suspended in 10 mM phosphate buffer adjusted to pH6.0, containing 1 μM PMSF (Sigma Chemical Co., Ltd.), a proteinase inhibitor. A cell wall fraction was obtained by repeated centrifugation at 26,000 g for 20 min at 4°C. The cell wall fraction was suspended in 1 M NaCl solution overnight at 4°C to release ionically bound cell wall proteins. Cell wall proteins were precipitated by ammonium sulfate, washed and concentrated with an Ultrafree C15 centrifugal unit (10,000 molecular weight limit, Millipore Co., Ltd.) The obtained protein fraction was stored as 1 mgl^{-1} in 50% glycerol solution at −15°C. The cell wall protein was quantified by Bio-Rad Protein Dye Assay Reagent (Bio-Rad Lab. Co., Ltd.).

Reconstitution experiments

For reconstitution assays, G-segments were heat-inactivated in distilled water at 90°C for 20 sec, and then incubated in the protein solution for 20 h at 4°C under shaking. The creep of the reconstituted segments was measured in the presence or absence of BR in the same bathing buffer as in the preceding measurements.

Results

Effect of BR on lkb growth

The growth of the 7th internode of *lkb* seedlings was much less than that of *LKB* seedlings (Figure 1A). BR (10 ng/plant) applied to the leaf at the 6th node of *lkb* seedlings stimulated the growth of the 7th internode (Figure 1A). The stimulation reached 48% after 144 h. The growth rate (growth increase per hour) increased linearly during the first 36 h in *LKB*, *lkb* and BR-treated *lkb* seedlings (Figure 1B). The growth rate of *LKB* was approximately 5 times higher than that of *lkb*. In BR-
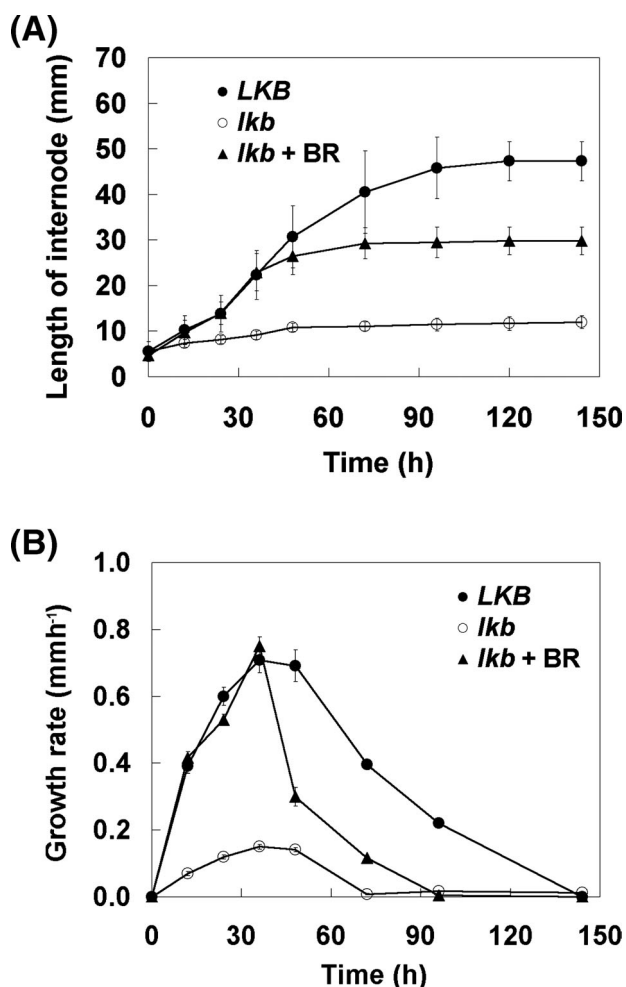
(A)

(B)

Figure 1. Growth curves (A) and growth rate curves (B) of the 7th internode of *LKB*, *lkb* and BR-treated *lkb* seedlings. Each point represents the mean of 10 seedlings with its standard deviation indicated by a vertical bar. BR was applied at the dosage of 10 ng/plant in an aliquot of 10 μl of 50% ethanolic solution containing 0.1% Tween 20 to the leaf at the 6th node.

treated *lkb*, the growth rate was restored to the same degree as in untreated *LKB*.

In vivo effect of BR on lkb creep

Creep rate changes in G-segments proportionally increased with an increase in applied tension (load), giving two distinct phases (Figure 2). In the first phase, creep rate increased slowly up to a critical load (y) which represents the yield threshold of the cell wall. In the second phase, creep rate increased sharply, the slope of which is represented by cell wall extensibility (ϕ). In *LKB* y was lower than in *lkb*. But in BR-treated *lkb*, y was almost the same as in *LKB*. Creep rate changes of G-segments taken from BR-treated seedlings showed patterns very similar to those of *LKB*. On the other hand, there seemed to be statistically no significant difference among the ϕ values obtained for *LKB*, *lkb* and BR-treated *lkb*. Their y and ϕ values are summarized in
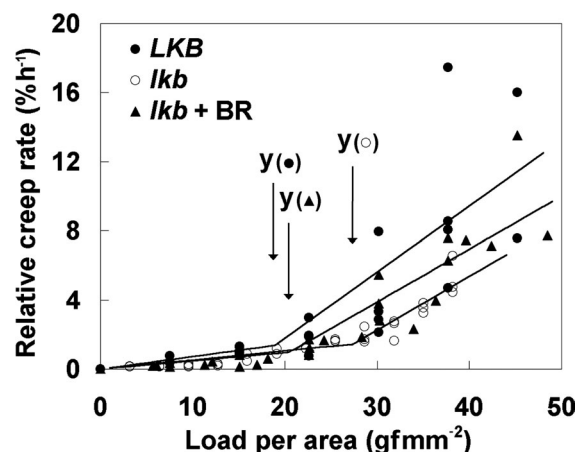

Figure 2. Relationship between creep rate and load applied in glycerinated internode segments of *LKB*, *lkb* and BR-treated *lkb* seedlings. Relative creep rate is expressed as the creep rate divided by the initial length of the segment. Load per area was obtained by dividing each applied load by the square of the diameter of each segment.

Table 1.   *In vivo* effect of BR on *lkb* creep.

	ϕ (kgf^{-1}h^{-1})	y(gfmm^{-2})	n
LKB	4.31±2.29 [a]	19.3±1.5 [a]	4
lkb	3.18±0.30 [a]	27.2±1.4 [b]	3
lkb+BR	3.35±0.12 [a]	21.4±2.1 [a]	4

Data were taken from Figure 2. ϕ: the average of ϕ values obtained by the least square method for each segment. BR: 10 ng/plant. n: sample number. Each value represents the mean with its standard deviation. Within the same row, different letters indicate significance at P<0.05.

Table 2.   *In vitro* effect of BR on *lkb* creep.

	ϕ (kgf^{-1}h^{-1})	y(gfmm^{-2})	n
Control (−BR)	3.18±0.30 [a]	27.2±1.4 [a]	3
+10nM BR	4.05±1.24 [a]	19.3±4.1 [b]	5
+100nM BR	4.40±1.20 [a]	16.5±4.2 [b]	5

BR was included in the bathing buffer for creep measurements. Control (−BR): the same as in Table 1; ϕ: the average of ϕ values obtained by the least square method for each segment. n: sample number. Within the same row, different letters indicate significance at P<0.05.

Table 1.

In vitro effect of BR on lkb creep

G-segments of *lkb* were treated with 10 nM and 100 nM BR solutions for 1 h, and then creep was measured in the presence of the respective concentration of BR. As summarized in Table 2, y was lower in the presence of BR. The decrease in y value was more conspicuous with 100 nM BR. Although, higher values of ϕ were obtained with BR treatments, these are statistically insignificant.
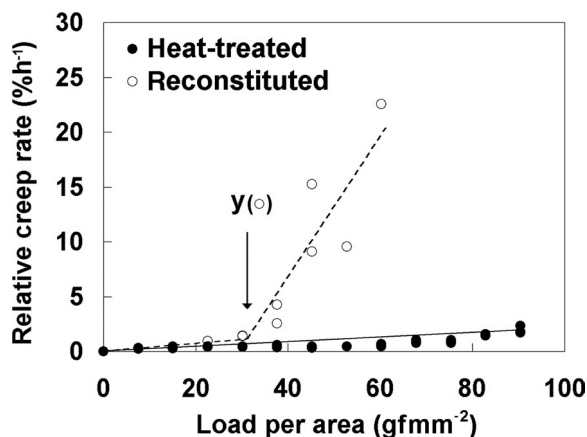
Figure 3. Effect of the cell wall protein fraction on the creep rate change in heat-treated glycerinated *LKB* segments. Segments were heated in distilled water at 90°C for 20 s. Relative creep rate is expressed as the creep rate divided by the length of the segment before the measurement. Load per area was obtained by dividing each applied load by the square of the diameter of each segment.
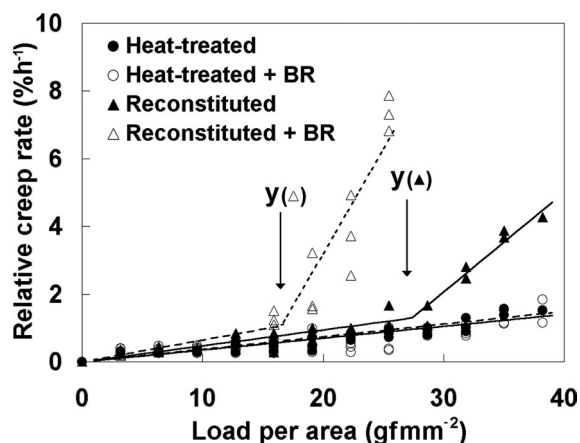

Figure 4. *In vitro* effect of BR and effect of the cell wall protein fraction on the creep rate change in glycerinated *lkb* segments. Segments were heated in distilled water at 90°C for 20 s and then incubated with 1 mg ml^{-1} protein solution at 4°C for 20 h under shaking. BR was included in the bathing buffer for creep measurements. Relative creep rate is expressed as the creep rate divided by the length of the segment before the measurement. Load per area was obtained by dividing each applied load by the square of the diameter of each segment.

Reconstitution experiments with cell wall protein fraction

By heat-treatment the ability of G-segments of *LKB* to show creep was completely diminished. However, it recovered by the addition of a crude cell wall protein fraction (Figure 3). In G-segments of *lkb*, ϕ and y were also completely lost by heat-treatment, and the loss of the ability to show creep was not restored by BR alone (Figure 4). However, the pattern of the creep rate change was also restored by the addition of the protein fraction (Figure 4). In the presence of both the protein fraction and 10 nM BR, the pattern of the creep rate change was

similar to that of unheated G-segments of *lkb* in the presence of 10 nM BR as shown in Table 2 (Figure 4).

Discussion

The present study has confirmed that exogenously applied BR can stimulate the internode growth of dwarf garden pea, *lkb*, which is a BR biosynthesis mutant (Nomura et al. 1997, 1999). The growth rate of the *lkb* 7th internode can be normalized at a dosage of 10 ng/plant BR (Figure 1B). Similar BR dwarf mutants have been discovered in *Arabidopsis thaliana* L. (Chory et al. 1991; Koncz et al. 1992; Kauschmann et al. 1996; Li et al. 1996; Szekeres et al. 1996; Takahashi et al. 1995), and in *Lycopersicon esculentum* L. (Chala et al. 2000; Yokota 1997). It is obvious that BR is necessary for normal stem growth.

How is BR involved in the stimulation of stem growth? Our results using *lkb* strongly suggest that BR is involved in the process of cell elongation through affecting acid-induced cell wall loosening. The basic patterns of the creep rate change in G-segments of *LKB* (Figure 2, Table1) are similar to those of pumpkin hypocotyls (Taguchi et al. 1999) and cowpea hypocotyls (Okamoto and Okamoto 1994). The degree of cell wall extensibility is expressed by ϕ, and ϕ value is higher, the extensibility is larger. A lower y value means that some load-bearing bonds in the cell wall which are limiting the yield threshold are more easily broken. In G-segments of *lkb*, a similar pattern of creep rate change can also be obtained (Figure 2). However, y is higher than that of *LKB*. This indicates that in the G-segments of *lkb*, some load-bearing bonds in the cell wall may be stronger than in *LKB*, and therefore, acid-induced cell wall loosening is not easily established. Since *lkb* lacks endogenous BR (Nomura et al. 1997), it is suggested that the BR deficiency is related to the lower ability of *lkb* cell walls to respond to cell wall acidification. In fact, G-segments of *lkb* sampled from BR-treated seedlings show patterns of the creep rate change similar to those of *LKB* (Figure 2, Table1). It is likely that BR is necessary for a change in y of the cell wall to cause wall loosening. On the other hand, the present results indicate that BR is not directly control cell wall extensibility itself, because BR does not affect ϕ.

How is the action of BR related to these proteins? Two possible roles are assumed. One is that BR acts at the transcriptional level. BR may regulate expression of the genes of such proteins, and *lkb* seedlings cannot produce these proteins in a sufficient quantity. In support of this model, BR regulation of genes encoding xyloglucan endotransglucosylases and expansins has been demonstrated (Clouse 1997). Another possible role of BR is that it acts at post-transcriptional levels.

One of the most interesting findings in the present

study is the *in vitro* effect of BR (Figure 4). In the presence of exogenous BR supplied directly to G-segments of *lkb*, they behave like those of *LKB* in the creep rate change in response to acidity. This *in vitro* effect of BR indicates that the presence of BR is required in the process of acid-induced cell wall loosening. The results of the reconstitution experiments (Figure 4) provide further supporting evidence. Heat-treatment of G-segments at 90°C for 20 sec deprives their ability to respond to acidity. It is suggested that the cell wall proteins responsible for y changes are inactivated by the heat-treatment. This ability can be recovered by the addition of a crude cell wall protein fraction extracted from *Alaska* pea seedlings. The result confirms previous similar reports by Okamoto and Okamoto (1995) and Taguchi et al. (1999). In the present study, it was demonstrated that BR alone could not restore the ability of G-segments of *lkb* to respond to acidity, but it could do so in the presence of the crude protein fraction.

In conclusion, BR is required directly for the process of acid-induced cell wall loosening. It is likely that BR is an indispensable factor or modulator for the action of cell wall proteins responsible for at least y changes. Although this study supports the possibility that BR acts at a post-transcriptional level, this conclusion does not exclude the role of BR at the transcriptional level in the process of cell elongation.

Acknowledgements

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