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ORIGINAL ARTICLE

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Brassinosteroids and gibberellins promote tobacco seed germination by distinct pathways

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Abstract Seed germination of *Nicotiana tabacum* L. cv. Havana 425 is determined by the balance of forces between the growth potential of the embryo and the mechanical restraint of the micropylar endosperm. In contrast to the gibberellin GA_4, the brassinosteroid (BR) brassinolide (BL) did not release photodormancy of dark-imbibed photodormant seeds. Brassinolide promoted seedling elongation and germination of non-photodormant seeds, but did not appreciably affect the induction of class I β-1,3-glucanase (βGLU I) in the micropylar endosperm. Brassinolide, but not GA_4, accelerated endosperm rupture of tobacco seeds imbibed in the light. Brassinolide and GA_4 promoted endosperm rupture of dark-imbibed non-photodormant seeds, but only GA_4 enhanced βGLU I induction. Promotion of endosperm rupture by BL was dose-dependent and 0.01 μM BL was most effective. Brassinolide and GA_4 promoted abscisic acid (ABA)-inhibited dark-germination of non-photodormant seeds, but only GA_4 replaced light in inducing βGLU I. These results indicate that BRs and GAs promote tobacco seed germination by distinct signal transduction pathways and distinct mechanisms. Gibberellins and light seem to act in a common pathway to release photodormancy, whereas BRs do not release photodormancy. Induction of βGLU I in the micropylar endosperm and promotion of release of 'coat-enhanced' dormancy seem to be associated with the GA-dependent pathway, but not with BR signalling. It is proposed that BRs promote seed germination by directly enhancing the growth potential of the emerging embryo in a GA- and βGLU I-independent manner.

Keywords Brassinosteroid · Gibberellin · β-1,3-Glucanase · *Nicotiana* (seed germination) · Photodormancy · Seed germination

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Abbreviations ABA: abscisic acid · BL: brassinolide · BR: brassinosteroid · GA: gibberellin · βGLU I: class I β-1,3-glucanase

Introduction

Germinating seed of many endospermic species exhibits 'coat-enhanced' dormancy in which radicle emergence is physically restrained by the endosperm and in some cases additional covering layers (Bewley 1997a; Li and Foley 1997). Seed dormancy and germination are regulated by the interaction of light with several hormones including abscisic acid (ABA), gibberellins (GAs), and ethylene. In the case of tobacco, rupture of the testa (seed coat) precedes rupture of the endosperm (Arcila and Mohapatra 1983). Photodormant tobacco seeds do not germinate in darkness, but treatment with a red-light pulse or with GA is sufficient to release photodormancy, induce testa rupture and subsequent endosperm rupture in the dark (Kincaid 1935; Toole et al. 1955; Ogawara and Ono 1961; Leubner-Metzger et al. 1996). Photodormancy of tobacco is established during seed development, freshly harvested seed is dormant and requires light for its germination (Leubner-Metzger and Meins 2000). After-ripening, i.e. the storage of mature seeds for several months under dry, warm conditions, contributes to the release of photodormancy and this effect varies greatly for different seed batches, as reported for several tobacco cultivars (Kasperbauer 1968; Leubner-Metzger and Meins 2000). Only a little is known on the molecular level about the establishment, maintenance and release of dormancy during after-ripening and imbibition (Bewley 1997a; Li and Foley 1997). Although neither ABA nor ethylene affect photodormancy or testa rupture of dark-imbibed tobacco seeds, ABA delays and ethylene promotes endosperm rupture in the light (Leubner-Metzger et al. 1995, 1998). Class I β-1,3-glucanase (βGLU I) is induced in the micropylar endosperm just prior to its rupture. Physiological studies and recent results obtained with sense transformants strongly

suggest that βGLU I has a causal role in endosperm rupture (Leubner-Metzger and Meins 1999, 2000).

Brassinosteroids (BRs) and GAs interact with light in regulating elongation growth of shoots and photomorphogenesis of seedlings (reviewed in Clouse and Sasse 1998; Altmann 1999; Neff et al. 2000) by what appear to be independent pathways (e.g. Kauschmann et al. 1996; Szerkeres et al. 1996; Nomura et al. 1997; Asami et al. 2000). BR application has been reported to enhance seed germination of certain parasitic angiosperms and cereals (Gregory 1981; Yamaguchi et al. 1987; Takeuchi et al. 1991, 1995), but not that of cress (Jones-Held et al. 1996). The effect of BRs on light-requiring seeds exhibiting endosperm-limited germination has not been investigated. In the present report, the effects of the BR brassinolide (BL) and GA_4 on tobacco seed germination are compared using βGLU I as a molecular marker for endosperm rupture.

Materials and methods

Plant materials and germination experiments

Seed of wild-type *Nicotiana tabacum* L. cv. Havana 425 and of independent kanamycin-resistant empty-vector control lines (TCIB1) (Leubner-Metzger and Meins 2000) after-ripened by storage for ca. 1 year were used. Wild-type and TCIB1 seeds did not differ in their response to the different treatments investigated in the present study. Different batches of after-ripened tobacco seed exhibit variable photodormancy, a phenomenon observed for several tobacco cultivars (Kasperbauer 1968; Leubner-Metzger and Meins 2000). This between-lot variability in photodormancy of after-ripened seeds has been ascribed to a combination of genetic and maternal factors (Kasperbauer 1968). The latter include different environmental conditions during seed development, e.g. harvest time or position of the capsule on the mother plant. Such environmental effects on the mother plant are a likely explanation for the variability in photodormancy of the after-ripened seed batches (Leubner-Metzger and Meins 2000). After 14 days of dark-incubation the photodormant and non-photodormant seed batches used showed germination rates of less than 5% and greater than 95%, respectively.

Germination was analysed as described by Leubner-Metzger et al. (1998). In brief, ca. 100 seeds were sown in plastic Petri dishes containing filter paper wetted with a dilute-salt medium supplemented with 50 μg/ml kanamycin sulfate (Serva), 100 μg/ml Claforan (Hoechst-Pharma AG, Zürich, Switzerland), and further supplemented as indicated with 10 μM *cis*-(±)-ABA (Sigma), 10 μM GA_4 (Sigma), and 10^{-10} to 10^{-6} M BL (CIDtech Research Inc., Mississauga, Ontario, Canada). Petri dishes were incubated at 24°C in continuous white light (3,000 lx; Philips 'TL'D 35 W/33 lamps) or in darkness. After scoring for germination, seeds were stored at –80°C for subsequent analysis.

Analyses of proteins

Procedures for extracting proteins, assays for enzyme activity, immunoblot analysis and protein determination have been described previously (Leubner-Metzger et al. 1995). In brief, βGLU activity was assayed radiometrically using [^{3}H]laminarin as the substrate. The rabbit anti-tobacco βGLU I antibody used for immunoblot analysis detects the class-I, class-II and class-III isoforms of the enzyme (Leubner-Metzger et al. 1998). The ECF Western blotting system (Amersham Pharmacia Biotech) was used for quantitative immunoblot analysis. Quantitation of the fluorescent signals was achieved by using known amounts of purified tobacco βGLU I as standard.

Results

Brassinosteroids do not release photodormancy

Gibberellins can substitute for light in releasing photodormancy and inducing dark-germination of photodormant tobacco seeds (Leubner-Metzger et al. 1996). To determine if BR has a similar effect, photodormant batches of wild-type and TCIB1 seed were imbibed in the dark on filter paper moistened with a dilute solution of nutrient salts supplemented with 10^{-10} to 10^{-6} M BL. No germination or testa rupture was observed after 2 weeks incubation of photodormant seed in the dark (data not shown), indicating that BL does not release photodormancy.

Effects of BR and GA on non-photodormant seeds

The effect of BR on non-photodormant tobacco seeds was investigated by adding 10^{-10} to 10^{-6} M BL to the medium and scoring the percentage of seeds exhibiting testa and endosperm rupture at a fixed time after the start of imbibition. Non-photodormant wild-type and TCIB1 seed batches germinated faster in the light compared to the dark. Germination scoring presented in Fig. 1 was therefore 10 h earlier in the light compared to the dark. Figure 1A shows that BL increased the incidence of endosperm rupture in both dark- and light-imbibed seeds, which was optimal at 10^{-8} M BL. No effect of BL on the incidence of testa rupture was detected (data not shown). After scoring for endosperm rupture, protein extracts were prepared from the seed populations and assayed for βGLU activity and βGLU I antigen content. Immunoblot analysis with antibody that detects all known seed isoforms of tobacco βGLU established that only the 33-kDa βGLU I antigen was present in the extracts (data not shown). Figure 1B shows that βGLU activity and βGLU I antigen content were tightly correlated and were not appreciably increased by BL treatment. Accumulation of enzyme activity and antigen was slightly inhibited at the higher BL concentrations tested.

Table 1 shows that in the light 10^{-8} M BL but not 10^{-5} M GA_4 increased the percentage endosperm rupture of non-photodormant seeds. On the other hand, in the dark both hormones increased the incidence of endosperm rupture to roughly the same extent. In the light, βGLU I accumulation was not increased by either GA_4 or BL treatment, whereas in the dark GA_4 but not BL treatment increased βGLU I accumulation. To verify that the hormones at the concentrations used promote elongation growth of tobacco seedlings, we examined the effects of GA_4 and BL treatment on hypocotyl length of de-etiolated seedlings. Treatment with 10^{-8} M BL or

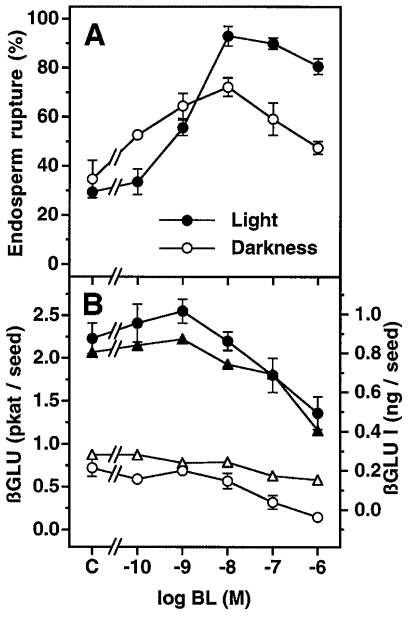

Fig. 1 The effect of different BR concentrations on endosperm rupture (**A**) and βGLU I accumulation (**B**) of germinating non-photodormant tobacco (*Nicotiana tabacum*) seeds. Seeds were imbibed in continuous light (*closed symbols*) or in darkness (*open symbols*) in medium without (*C*) or with 10^{-10} to 10^{-6} M BL added. The incidence of endosperm rupture expressed as percent was scored 55 h (light) and 65 h (darkness) after the start of imbibition. The βGLU enzyme activities are expressed as pkat/seed (●, ○), and the βGLU I protein contents determined by immunoblot analyses are expressed as ng/seed (▲, △). Mean values ± SE of two to three samples each with ca. 100 seeds are presented; SE values ≤ 2.0% and ≤ 0.05 pkat/seed are not drawn; ng βGLU I protein/seed values are from one representative sample for each datum point

10^{-5} M GA_4 increased hypocotyl growth by ca. 28% (Table 1). When added simultaneously, the hormones acted in a roughly additively fashion, resulting in a ca. 50% increase in hypocotyl length. Taken together the results indicate that although BR and GA both promote elongation growth, the actions of the two types of hormone on seed germination differ. Brassinosteroid promotes endosperm rupture in light and dark, but does not increase βGLU activity, whereas, GA only promotes endosperm rupture in the dark and is associated with increased βGLU activity.

Effects of BR and GA on ABA-inhibited seed germination

Treatment with 10 μM ABA greatly delays endosperm rupture and inhibits βGLU I accumulation of photodormant tobacco seed imbibed in the light (Leubner-Metzger et al. 1995, 1996). To compare the effect of BR and GA on ABA-inhibited germination, we measured the time course for endosperm rupture of non-photodormant seed imbibed in continuous light and darkness in the presence of combinations of 10 μM ABA, 0.01 μM BL, and 10 μM GA_4. Treatment of non-photodormant seeds with 10 μM ABA completely blocked endosperm rupture measured after 55 h in continuous light and 65 h in darkness (Table 1). Accumulation of βGLU I was inhibited under both conditions, as described earlier for seeds imbibed in the light (Leubner-Metzger et al. 1995). Figure 2A shows that BL, but not GA_4, promoted ABA-delayed endosperm rupture in continuous light. Treatment with BL decreased the time needed to reach 50% endosperm rupture by ca. 70 h. The delay in endosperm rupture due to ABA was even more pronounced for dark-imbibed non-photodormant seeds (Fig. 2B). Under these conditions, GA_4 decreased the 50% endosperm rupture time by >50 h, BL by >150 h, and GA_4 plus BL by >200 h. Table 1 shows that GA_4 treatment of ABA-imbibed seeds in darkness increased βGLU activity by ca. 3-fold to control levels without ABA added. In contrast, BL treatment of ABA-imbibed seeds in both darkness and light did not

Table 1 Effect of BR and GA on seed germination, seedling growth and βGLU activity induction of non-photodormant tobacco seeds

Treatment	Germinating seeds				Seedlings
	Light		Darkness		Light
	Endosperm rupture	βGLU activity	Endosperm rupture	βGLU activity	Hypocotyl growth
Control	29.4 ± 1.6[a]	2.2 ± 0.2[b]	34.7 ± 2.9	0.7 ± 0.1	100 ± 3[c]
0.01 μM BL	93.0 ± 0.4	2.2 ± 0.1	72.1 ± 3.8	0.6 ± 0.1	127 ± 2
10 μM GA_4	27.6 ± 2.4	2.1 ± 0.2	80.2 ± 1.4	2.3 ± 0.3	128 ± 2
0.01 μM BL + 10 μM GA_4	94.1 ± 0.9	n.d.[d]	84.8 ± 2.2	n.d.	146 ± 3
10 μM ABA	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	n.d.
0.01 μM BL + 10 μM ABA	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	n.d.
10 μM GA_4 + 10 μM ABA	0.0 ± 0.0	n.d.	0.0 ± 0.0	0.6 ± 0.1	n.d.

[a]Mean ± SE of % endosperm rupture of at least two populations of ca. 100 non-photodormant seeds imbibed for 55 h in continuous light or for 65 h in darkness
[b]βGLU activity expressed as pkat per seed
[c]Mean ± SE of % control hypocotyl growth of at least 20 de-etiolated seedlings germinated for 150 h in darkness and then grown for 100 h in continuous light
[d]Not determined

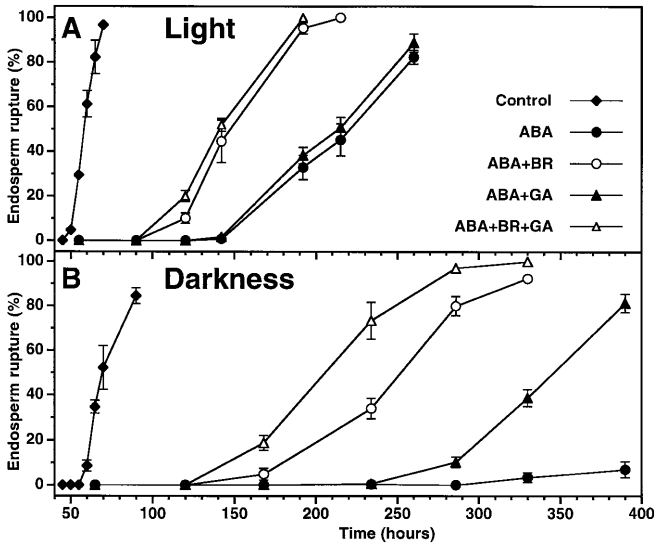

Fig. 2 The effect of BR and GA on the time courses of ABA-inhibited endosperm rupture of non-photodormant tobacco seeds imbibed in continuous light (**A**) or in darkness (**B**). The incidence of endosperm rupture is expressed as percent of seeds scored with time in medium supplemented with 10 μM ABA and, as indicated, with 0.01 μM BL and 10 μM GA_4. Results obtained with seeds not treated with hormones are presented as control. Mean values ± SE of two samples each with ca. 100 seeds are presented; SE values ≤ 2.0% are not drawn

increase βGLU activity. BL treatment in the dark of ABA-imbibed seeds did not promote βGLU I accumulation, but GA_4 caused ca. 3-fold higher values (Table 1). Taken together the results show that BR promotes ABA-inhibited endosperm rupture in the light and in darkness without affecting βGLU I accumulation. Gibberellin can partially replace light in the promotion of ABA-inhibited endosperm rupture and βGLU I accumulation in dark-imbibed seeds, but has no effect in the light.

Discussion

The most important observation of this study was that BR promoted endosperm rupture of germinating tobacco seeds, but did not release photodormancy. In photodormant tobacco seeds, both the testa and the endosperm remain intact during dark-imbibition and even prolonged incubation in darkness does not induce seed germination (Kasperbauer 1968; Leubner-Metzger et al. 1996). A light pulse or GA treatment can release photodormancy, whereas BR (this study), ABA and ethylene (Leubner-Metzger et al. 1996, 1998) cannot release photodormancy of tobacco. Germination of non-photodormant tobacco seeds was faster in the light compared to the dark. Brassinosteroid promoted endosperm rupture of light- and dark-imbibed non-photodormant seeds, but did not appreciably affect testa rupture. Thus, BR seems to accelerate endosperm rupture by acting on processes associated with the non-photodormant state of tobacco seeds.

A concentration of 10 nM BL was most effective in promoting endosperm rupture of non-photodormant tobacco seeds imbibed in the light and in the dark. Nanomolar concentrations of endogenous BR have been identified in seed of several species (e.g. Adam and Marquardt 1986; Schmidt et al. 1997). Treatment of cereal caryopses with similarly low concentrations of BR accelerated seedling establishment (Gregory 1981) and increased the low germination rate of aged kernels (Yamaguchi et al. 1987). Germination of the endospermic seeds of parasitic *Orobranche* and *Striga* species is, in contrast to tobacco, inhibited by light (Takeuchi et al. 1991, 1995). Neither BR nor GA can substitute for the conditioning treatment with strigol, which is needed for inducing germination of unconditioned (i.e. dormant) seed. Treatment with BR promotes the germination of conditioned (i.e. non-dormant) *Orobranche* and *Striga* seeds imbibed in the light and in the dark. In contrast to the non-dormant endospermic seeds of tobacco and parasitic angiosperms, BR application did not affect germination of non-photodormant, non-endospermic cress seeds imbibed in the dark (Jones-Held et al. 1996). This suggests that BR may in general promote germination of endospermic seeds, but does not affect testa rupture and therefore does not promote the germination of non-endospermic seeds.

The release of photodormancy and promotion of seed germination of light-requiring species is regulated by the phytochrome system (reviewed in Furuya and Schäfer 1996; Neff et al. 2000). Phytochrome B mediates red/far-red light photoreversible responses and principally regulates germination, whereas phytochrome A and an unknown phytochrome mediate additional light-regulation (e.g. Toole et al. 1955; Adam et al. 1994; Shinomura et al. 1994, 1998; Kretsch et al. 1995; Yang et al. 1995; Poppe and Schäfer 1997). GA can substitute for the red-light trigger needed to release photodormancy and to induce dark-germination of tobacco seeds (Ogawara and Ono 1961; Leubner-Metzger et al. 1996). Red light has been shown to up-regulate the biosynthesis of bioactive GA_1 and GA_4 by inducing GA 3β-hydroxylase genes in germinating seeds of lettuce and *Arabidopsis* (Toyomasu et al. 1993, 1998; Yamaguchi et al. 1998; Kamiya and Garcia-Martinez 1999). GA_1 and GA_4 are also the major bioactive GA of tobacco and GA 3β-hydroxylase does not accumulate in developing tobacco seed (Jordan et al. 1995; Kusaba et al. 1998; Itoh et al. 1999). Saturating endogenous GA_1 and GA_4 levels in light-imbibed tobacco seeds seem therefore to be the obvious explanation for the failure of GA treatment to promote tobacco germination in the light, while it promotes dark-germination of non-photodormant seeds. Promotion of ABA-delayed seed germination of *Nicotiana plumbaginifolia* by light or GA involves stimulation of ABA degradation and inhibition of ABA synthesis (Kraepiel et al. 1994; Grappin et al. 2000). ABA inhibits endosperm rupture of photodormant tobacco seeds imbibed in the light (Leubner-Metzger et al. 1995). The present study with non-photodormant

tobacco seeds shows that ABA-delayed endosperm rupture is more pronounced in the dark than in the light and that GA can replace light in decreasing this delay. Thus, light and GA seem to act in a common pathway that involves phytochrome, counteracts ABA effects and serves two functions, it releases photodormancy and it promotes endosperm rupture of non-photodormant tobacco seeds. In contrast, BR cannot substitute for light in releasing photodormancy and BR treatment of non-photodormant tobacco seeds promotes endosperm rupture and counteracts ABA effects also in the light. Furthermore, light does not appear to regulate BR biosynthetic genes (Choi et al. 1996; Neff et al. 1999, 2000). Their distinct action on photodormancy and their distinct interaction with light- and ABA-effects on endosperm rupture of non-photodormant seeds strongly suggest that GA and BR affect tobacco seed germination by independent pathways and by different mechanisms.

Completion of radicle emergence during seed germination essentially depends on embryo extension, which is a turgor-driven growth process. In addition, in seeds with 'coat-enhanced' dormancy the covering layers are a mechanical constraint that has to be overcome by the growth potential of the embryo (reviewed in Bewley 1997b). Endosperm rupture is the germination-limiting process in members of the Asteraceae (e.g. lettuce) and Solanaceae (e.g. tomato, tobacco, pepper and *Datura* spp.) and a decline in the mechanical resistance of the micropylar endosperm is necessary for germination to be completed. This process is likely to be achieved by cell wall weakening due to digestion of cell-wall material by specific hydrolases (Bewley 1997b). In the case of tobacco we proposed this function for βGLU I, which is induced in the micropylar endosperm just prior to its rupture and is tightly linked with altered endosperm rupture in response to light, GA, ABA, and ethylene (reviewed in Leubner-Metzger and Meins 1999). ABA delayed endosperm rupture of seeds imbibed in the light and inhibited βGLU I accumulation in a concentration-dependent manner (Leubner-Metzger et al. 1995). Recent results obtained by sense transformation of tobacco with a chimeric ABA-inducible βGLU I transgene resulted in overexpression of βGLU I in seeds and promoted endosperm rupture of ABA-treated seeds (Leubner-Metzger and Meins 2000). These results provided direct evidence for a causal role of βGLU I during endosperm rupture and suggested that regulation of βGLU I by ABA signalling pathways might have a key role in the release of 'coat-enhanced' dormancy. The present study with non-photodormant tobacco seeds shows that GA substitutes for light and counteracts ABA in inducing βGLU I and promoting endosperm rupture during dark-germination. GA can promote embryo extension, but the major site of GA action during seed germination appears to be the endosperm (reviewed in Bewley 1997b). GA induces hydrolases, cell wall digestion, weakening and rupture in the micropylar endosperm of lettuce, tomato, pepper and other species. Microscopic studies have revealed structural changes in the micropylar endosperm prior and during endosperm rupture of tobacco (Arcila and Mohapatra 1983). These findings suggest for tobacco that the GA/light pathway interacts with ABA in controlling micropylar endosperm-specific βGLU I expression, followed by endosperm weakening. In contrast, the BR pathway promoted tobacco seed germination in a βGLU I-independent fashion, which suggests that endosperm weakening is not the major site of BR action. This supports the view that the net result of multiple promoting and inhibiting factors regulates seed germination (Debeaujon and Koornneef 2000). Future experiments will focus on the hypothesis that BR promotes germination of non-dormant seeds by a direct mechanism that affects embryo extension growth.

In conclusion, BR and GA promote tobacco seed germination by distinct signal transduction pathways. GA and light act in a common pathway to release photodormancy, whereas BR does not release photodormancy. βGLU I induction in the micropylar endosperm and release of 'coat-enhanced' dormancy seem to be associated with the GA/light pathway, but not with BR signalling. Taken together, our findings suggest the following as a model for the endosperm-limited germination of tobacco:

i. Photodormancy is released exclusively by the GA/light-pathway.
ii. Promotion of subsequent endosperm rupture by the BR and the GA/light signal transduction pathways is achieved by independent and distinct mechanisms.
iii. ABA inhibits endosperm rupture by interfering with both pathways.
iv. The GA/light pathway regulates βGLU I induction in the micropylar endosperm and seems to control endosperm weakening.
v. It is proposed that the BR pathway promotes endosperm rupture of non-dormant seeds by enhancing the growth potential of the embryo.

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