

## CHAPTER-5

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### BRASSINOSTEROIDS PROMOTE SEED GERMINATION

Seed germination of *Arabidopsis thaliana*, *Nicotiana tabacum*, and of parasitic angiosperms (*Orobranche* and *Striga* species) is determined by the balance of forces between the growth potential of the embryo and the mechanical restraint of the micropylar testa and/or endosperm tissues. Brassinosteroids (BR) and gibberellins (GA) promote seed germination of these species and counteract the germination-inhibition by abscisic acid (ABA). Severe mutations in GA biosynthetic genes in *Arabidopsis*, such as *gal-3*, result in a requirement for GA application to germinate, but germination in this phenotype can also be rescued by BR. Germination of both the BR biosynthetic mutant *det2-1* and the BR-insensitive mutant *bril-1* is more strongly inhibited by ABA than is germination of wild type. In contrast to GA, BR does not release tobacco photodormancy; i.e. seed germination in darkness remains blocked. BR promotes germination of non-photodormant tobacco seeds, but did not appreciably affect the induction of class I  $\beta$ -1,3-glucanase ( $\beta$ Glu I) in the micropylar endosperm. BR and GA promote tobacco seed germination by distinct signal transduction pathways and distinct mechanisms. Xyloglucan endo-transglycosylase (XET) enzyme activity accumulates in the embryo and the endosperm of germinating tobacco seeds and this appears to be partially controlled of BR. GA and light seem to act in a common pathway to release photodormancy, whereas BR does not release photodormancy. Induction of  $\beta$ Glu I in the micropylar endosperm and promotion of release of 'coat-imposed' dormancy seem to be associated with the GA-dependent pathway, but not with BR signaling. It is proposed that BR promote seed germination by directly enhancing the growth potential of the emerging embryo in a GA-independent manner.

#### HORMONAL REGULATION OF SEED DORMANCY AND GERMINATION

Brassinosteroids (BR) and gibberellins (GA) both regulate elongation growth of shoots and photomorphogenesis of seedlings and seem to antagonize the growth-inhibiting actions of abscisic acid (ABA) (Altmann, 1999; Neff *et al.*, 2000; Bishop and Koncz, 2002). Only little is known about the interconnected molecular key processes regulating seed dormancy and germination in response to plant hormones and environmental causes. Seed germination of species with 'coat-imposed' dormancy is determined by the balance of forces between the growth potential of the embryo and the constrain, exerted by the covering layers, e.g. testa (seed coat) and endosperm. Seed dormancy can be coat-imposed and/or determined by the embryo itself and is a temporary failure or block of a viable seed to complete germination under physical conditions that normally favor the process (Hilhorst, 1995; Bewley,

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1997b; Koornneef *et al.*, 2002). The process of germination commences with the uptake of water by imbibition of the dry seed, followed by embryo expansion growth. This usually culminates in rupture of the covering layers and emergence of the radicle is generally considered as the completion of germination. The testa is no hindrance during the germination of *Pisum sativum* (Petruzzelli *et al.*, 2000) and *Brassica napus* (Schopfer and Plachy, 1984). *Arabidopsis thaliana* seeds have only a remainder of the endosperm and the testa characteristics are responsible for the degree of coat-imposed dormancy (Debeaujon and Koornneef, 2000; Debeaujon *et al.*, 2000). In tomato seeds the micropylar testa and endosperm tissues, also termed the micropylar cap, confer the primary control of germination timing (Liptay and Schopfer, 1983; Toorop *et al.*, 2000; Wu *et al.*, 2000). Radicle protrusion, during seed germination depends on embryo expansion, which is a growth process driven by water uptake. Treatment with ABA of non-endospermic, non-dormant *B. napus* seeds has no effect on the kinetics of testa rupture, but it inhibits the post-germinational extension growth of the radicle (Schopfer and Plachy, 1984). Thus, ABA does not inhibit initial imbibition of water (water uptake phases 1 and 2) needed for initial embryo extension growth. ABA inhibits the transition to the seedling growth phase (water uptake phase 3) after radicle emergence. In contrast, ABA inhibits the germination of seeds with testa- and/or endosperm-imposed dormancy including *A. thaliana* (Beaudoin *et al.*, 2000; Debeaujon and Koornneef, 2000; Steber and McCourt, 2001), and tobacco (Leubner-Metzger *et al.*, 1995; Leubner-Metzger and Meins, 2000). In the Solanaceae this is achieved by an inhibitory action of ABA on the final step of radicle emergence through the micropylar endosperm (Liptay and Schopfer, 1983; Toorop *et al.*, 2000). ABA treatment does not inhibit the germination of tomato scored as initial radicle extension growth of detipped (surgical removal of the micropylar layers covering the radicle tip) seeds (Liptay and Schopfer, 1983; Groot and Karssen, 1992; Bewley, 1997a). Even 1000  $\mu\text{M}$  ABA does not inhibit the germination of detipped, whereas 100  $\mu\text{M}$  ABA results in a substantial inhibition of the germination of intact tomato seeds (Liptay and Schopfer, 1983).

Rupture of the testa and the endosperm are distinct and temporally separate events during the germination of tobacco seeds (Arcila and Mohapatra, 1983; Leubner-Metzger, 2003). ABA treatment of tobacco seeds does not appreciably affect the kinetics of testa rupture, but it delays endosperm rupture and results in the formation of a novel structure, consisting of the enlarging radicle with a sheath of greatly elongated endosperm tissue. Class I  $\beta$ -1,3-glucanase ( $\beta\text{Glu I}$ ) is induced after testa rupture and just prior to endosperm rupture. This induction is exclusively localized in the micropylar endosperm at the site where the radicle will emerge. ABA inhibits the induction of the  $\beta\text{Glu I}$  genes during tobacco seed germination and specifically delays endosperm rupture (Leubner-Metzger *et al.*, 1995). The close correlation between  $\beta\text{Glu I}$  induction and the onset of endosperm rupture under a variety of physiological conditions support the hypothesis that  $\beta\text{Glu I}$  contributes to endosperm rupture. Direct evidence for a causal role of  $\beta\text{Glu I}$  during endosperm rupture comes from sense-transformation with a chimeric ABA-inducible  $\beta\text{Glu I}$  transgene (Leubner-Metzger and Meins, 2000; Leubner-Metzger, 2003). This has

been achieved by transformation of tobacco with a sense- $\beta$ Glu I construct consisting of the genomic DNA fragment of the tobacco  $\beta$ Glu I B gene regulated by the castor bean *Cat1* gene promoter, which is known to confer ABA-inducible, endosperm-specific transgene expression in germinating tobacco seeds. ABA down-regulates the  $\beta$ Glu I host genes in wild-type seeds, but due to the ABA-inducible  $\beta$ Glu I-transgene it causes high-level  $\beta$ Glu I expression in sense- $\beta$ Glu I- seeds. ABA treatment delays endosperm rupture of after-ripened seeds, but due to  $\beta$ Glu I over-expression this delay is significantly reduced in sense- $\beta$ Glu I- seeds.  $\beta$ Glu I over-expression reduces the ABA-mediated delay in endosperm rupture of seeds, but ABA treatment does not effect the kinetics of testa rupture. Taken together, these results support the view that a threshold  $\beta$ Glu I content is necessary, but not sufficient, for endosperm rupture (Leubner-Metzger *et al.*, 1995; Leubner-Metzger, 2003). In the presence of ABA  $\beta$ Glu I becomes a limiting factor for endosperm rupture, and removal of this block due to expression of the ABA-inducible  $\beta$ Glu I-transgene in sense- $\beta$ Glu I- seeds promotes endosperm rupture until other ABA-sensitive processes become limiting (Leubner-Metzger and Meins, 2000). While these results do not exactly show how  $\beta$ Glu I promote endosperm rupture, they directly show that  $\beta$ Glu I is causally involved and that it substantially contributes to endosperm rupture. Possible mechanisms of  $\beta$ Glu I-action have been discussed in a recent review (Leubner-Metzger, 2003).

In contrast to the inhibition by ABA, seed dormancy release and germination are promoted by GA (Hilhorst and Karssen, 1992; Hilhorst, 1995; Bewley, 1997b; Koornneef *et al.*, 2002; Leubner-Metzger, 2003). Seeds of GA-deficient mutants do not germinate without exogenous treatment with GA. This is also the case for the GA-deficient *gib1* mutant of tomato (Groot and Karssen, 1992; Wu *et al.*, 2000), but detipping can replace the requirement for the GA-treatment and induce germination of *gib1* seeds. 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 (Leubner-Metzger *et al.*, 1996; Leubner-Metzger, 2001, 2002). Endosperm rupture during dark-germination of tobacco is accompanied by GA-enhanced expression of  $\beta$ Glu I in the micropylar endosperm. Thus, GA can release coat-imposed dormancy, induce testa rupture, enhance  $\beta$ Glu I expression in the endosperm, promote endosperm rupture and counteract the inhibitory action of ABA on seed germination. Red light has been shown to up-regulate the biosynthesis of bioactive GA<sub>1</sub> and GA<sub>4</sub> by inducing GA biosynthetic genes in germinating seeds of lettuce and Arabidopsis (Toyomasu *et al.*, 1993, 1998; Yamaguchi *et al.*, 1998, 2001). Recent publications demonstrate that BR also interact with light and ABA in regulating seed germination of Arabidopsis and tobacco (Leubner-Metzger, 2001; Steber and McCourt, 2001). The role of BR in seed germination and the interactions of BR with other plant hormones during this process are the focus of this review.

## PROMOTION OF SEED GERMINATION BY BRASSINOSTEROIDS

Brassinosteroids (BR) and GA interact with light in regulating elongation growth of shoots and photomorphogenesis of seedlings by what appear to be independent pathways (Altmann, 1999; Neff *et al.*, 2000; Bishop and Koncz, 2002). Endogenous BRs have been identified in the seeds of several species, including pea (Yokota *et al.*, 1996), *A. thaliana* (Schmidt *et al.*, 1997) and *Lycchnis viscaria* (Friebe *et al.*, 1999). BR application has been reported to enhance germination of certain parasitic angiosperms (Takeuchi *et al.*, 1991, 1995), cereals (Gregory, 1981; Yamaguchi *et al.*, 1987), *Arabidopsis* (Steber and McCourt, 2001), and tobacco (Leubner-Metzger, 2001). Pretreatment with brassinolide stimulates the germination and seedling emergence of aged rice seeds (Yamaguchi *et al.*, 1987) and seed treatment of barley accelerated subsequent seedling growth (Gregory, 1981). It is, however, not known, whether the promoting effect of BR on cereal grains is manifested only on the level of seedling growth or also on the level of germination *per se*. While BR treatment was found to affect the early seedling growth of cress, in the same publication was stated (based on data not shown) that BR do not affect the germination of non-photodormant, non-endospermic cress seeds imbibed in the dark (Jones-Held *et al.*, 1996). In contrast to cereals and cress, the effects of BR on germination *per se* were studied at length in experiments in seeds of parasitic angiosperms, *Arabidopsis* and tobacco.

Germination of the endospermic seeds of parasitic *Orobranche* and *Striga* species is, in contrast to *Arabidopsis* and tobacco, inhibited by light (Takeuchi *et al.*, 1991, 1995; Babiker *et al.*, 2000). Neither BR, ethylene nor GA can substitute for the conditioning treatment with strigol, which is needed for inducing germination of unconditioned (i.e. dormant) seed. Conditioning removes the restriction on the ethylene biosynthetic pathway and increases the capacity to produce ethylene (Babiker *et al.*, 2000). Treatment with BR promotes the germination of conditioned (i.e. non-dormant) *Orobranche* and *Striga* seeds, imbibed in the light and in the dark. These results demonstrate that BR alone is not able to replace strigol and release the dormancy of these seeds. BR promotes the seed germination of parasitic angiosperms after dormancy has been released by counteracting the inhibitory effects of light, acting independently of GA and possibly by promoting ethylene action.

In *A. thaliana* BR promotes the germination of pre-chilled (i.e. non-dormant) seeds of the BR-deficient biosynthesis mutant *det2-1* and the BR-insensitive response mutant *bri1-1* imbibed in the light (Steber and McCourt, 2001). Seed germination of *det2-1* and *bri1-1* is more strongly inhibited by ABA than is germination of the wild type and BR is, therefore, able to partially overcome the inhibition of germination by ABA. BR treatment rescues the germination phenotype of the severe GA-deficient biosynthesis mutant *gal-3*, which normally requires GA treatment for dormancy release and germination. BR treatment also partially rescues the germination phenotype of the severe GA-insensitive response mutant *sly1* (*sleepy1*), which can not be rescued by treatment with GA. Interestingly, a new allele for *sly1* was identified in a screen for BR-dependent germination and suggests

interactions between BR and GA signaling in seeds (Steber *et al.*, 1998; Steber and McCourt, 2001). This is further supported by the germination phenotype of the *gpa1* mutant of *Arabidopsis* (Ullah *et al.*, 2002). The *GPA1* gene encodes the alpha subunit of a heterotrimeric G protein. Seeds with the *gpa1* null mutation are 100-fold less responsive to GA and *GPA1* overexpressing seeds are hypersensitive for GA. The *gpa1* mutant seeds are also completely insensitive to BR rescue of germination when the level of GA in seeds is reduced. These results point to a role for BR in stimulating germination of *Arabidopsis* seeds via embryo expansion and this effect is likely to be specific to germination. The interactions between hormonal signaling pathways appear to be of utmost importance for the regulation of germination and the inhibitory effects of ABA are counteracted by BR, GA and ethylene (Beaudoin *et al.*, 2000; Debeaujon and Koornneef, 2000; Steber and McCourt, 2001). The *Arabidopsis sax1* (*hypersensitive to abscisic acid and auxin*) dwarf mutant is impaired in BR biosynthesis and exhibits pleiotropic seedlings effects with respect to ABA, auxin, GA, ethylene and BR, but seed germination of *sax1* and wild-type is not differentially inhibited by ABA (Ephritikhine *et al.*, 1999).

BR promotes seedling elongation and germination of non-photodormant tobacco seeds, but do not appreciably affect testa rupture and the subsequent induction of  $\beta$ Glu I in the micropylar endosperm (Leubner-Metzger, 2001). Treatment with BR, but not GA, accelerates endosperm rupture of tobacco seeds imbibed in the light. BR and GA promote endosperm rupture of dark-imbibed non-photodormant seeds, but only GA enhances  $\beta$ Glu I induction. Promotion of endosperm rupture by BR is dose-dependent and 0.01  $\mu$ M brassinolide is most effective. BR and GA promote ABA-inhibited dark-germination of non-photodormant seeds, but only GA replaces light in inducing  $\beta$ Glu I. These results indicate that BR and GA promote tobacco seed germination by distinct signal transduction pathways and distinct mechanisms. GA and light act in a common pathway to release photodormancy, whereas BR does not release photodormancy.  $\beta$ Glu I induction in the micropylar endosperm and release of coat-imposed dormancy seem to be associated with the GA/light pathway, but not with BR signaling. These findings suggest as a model for the endosperm-limited germination of tobacco: (1) Photodormancy is released exclusively by the GA/light-pathway; (2) Promotion of subsequent endosperm rupture by the BR and the GA/light signal transduction pathways is achieved by independent and distinct mechanisms; (3) ABA inhibits endosperm rupture by interfering with both pathways; (4) The GA/light pathway regulates  $\beta$ Glu I induction in the micropylar endosperm and seems to control endosperm weakening; (5) It is proposed that the BR pathway promotes endosperm rupture of non-dormant seeds by enhancing the growth potential of the embryo (Leubner-Metzger, 2001, 2003).

## POSSIBLE MECHANISMS OF BR-PROMOTED SEED GERMINATION

Taken together, the findings in parasitic angiosperms, *Arabidopsis* and tobacco suggest that GA and BR act in parallel to promote cell elongation and germination and to counteract the inhibitory action of ABA on seeds. Since BR stimulates germination of the GA-insensitive mutant *sly1*, it is unlikely that BR acts by increasing GA sensitivity. It is possible that BR acts by stimulating GA biosynthesis in *Arabidopsis* seeds imbibed in the light (Steber and McCourt, 2001). BR action via stimulation of GA biosynthesis is however unlikely for tobacco, because BR does not promote the expression of  $\beta$ Glu I, which is induced by GA in the dark (Leubner-Metzger, 2001). It is known that BR can stimulate ethylene production and ethylene treatment can rescue the germination phenotype of the GA-deficient *Arabidopsis gal-1* mutant (Karssen *et al.*, 1989; Koornneef and Karssen, 1994; Steber and McCourt, 2001). However, there are several arguments against the hypothesis that BR acts via ethylene: (1) Ethylene levels are not increased in cress seedlings following BR treatment of seeds (Jones-Held *et al.*, 1996). (2) Endogenous ethylene promotes  $\beta$ Glu I accumulation in the micropylar endosperm of tobacco, but BR treatment promotes endosperm rupture without enhancing  $\beta$ Glu I accumulation (Leubner-Metzger *et al.*, 1998; Leubner-Metzger, 2001). (3) Ethylene rescue of

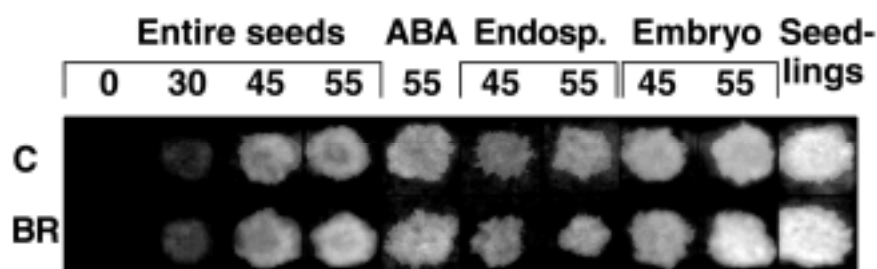


Figure 1. The accumulation of XET enzyme activity in germinating seeds of *Nicotiana tabacum* cv. Havana 245. Tobacco seeds were imbibed without (Control; C) and with 10 nM brassinolide (BR) in the medium and incubated for the times indicated (hours) in continuous light; in addition 10  $\mu$ M ABA was added to the medium of one series (ABA) (Leubner-Metzger, 2001). Protein extracts from entire seeds, seed tissues (Endosperm, Embryo), or seedlings were used. Testa rupture in the populations of ca. 150 seeds was 0 % at 30 h and 100 % at 45 h. Endosperm rupture was 0 % (45 h), ca. 0 % (Control, 55 h) and ca. 30 % (BR, 55 h); only seeds without endosperm rupture were used for the extracts. Tissues were homogenized and XET enzyme activities were determined by the XET 'dot blot' assay as described by Fry (1997). For the semiquantitative XET assay ca. 3  $\mu$ l (60  $\mu$ g protein) were applied onto the XET 'dot blot' test paper and incubated for 7 h at 25 °C. Elevated fluorescence under the UV lamp is indicative for accumulating XET enzyme activity.

*gal-1* seed germination results in seedlings exhibiting triple response, but BR rescue of *gal-3* seed germination results in seedlings that do not exhibit triple response (Steber and McCourt, 2001). Another possibility would be BR-action via auxin. Auxin also stimulates cell elongation but it does not rescue germination of *gal-3* (Koorneef and Karssen, 1994). Thus, if BR stimulates germination via embryo expansion, this effect is likely to be specific to seed germination.

Xyloglucan is a major structural polysaccharide in the cell walls of higher plants and modification of xyloglucan bondages is proposed to be involved in cell expansion growth (Fry, 1995; Campbell and Braam, 1999). Xyloglucan endotransglycosylases (XET) are enzymes with potential wall-modifying functions and are thought to be involved in the regulation of cell wall loosening necessary for cell expansion growth. XETs cleave a xyloglucan chain and then conserve the energy of the cut bond by synthesizing a new bond on another xyloglucan chain. XETs are encoded by a multigene family and their expression is highly regulated by plant hormones and environmental factors. Gene expression in vegetative tissues of several XETs is known to be induced by BR, auxin and/or GA (Fry, 1997; Campbell and Braam, 1999). In seeds XETs are involved in the post-germinational mobilization of cell wall xyloglucan reserves (Edwards *et al.*, 1985; De Silva *et al.*, 1993; Fanutti *et al.*, 1993; Tine *et al.*, 2000). The transcripts of the GA-regulated XET gene LeXET4 is expressed in the endosperm cap during tomato seed germination (Chen *et al.*, 2002). LeXET4 mRNA was strongly expressed in germinating seeds, was much less abundant in stems, and was not detected in roots, leaves or flower tissues. During germination, LeXET4 mRNA was induced prior to endosperm rupture and was localized exclusively to the endosperm cap region. Expression of LeXET4 was dependent on exogenous GA in GA-deficient *gib-1* mutant seeds. ABA had no effect on LeXET4 mRNA expression in wild-type tomato seeds. The temporal, spatial and hormonal regulation pattern of LeXET4 gene expression suggests that LeXET4 has a role in endosperm cap weakening, a key process regulating tomato seed germination (Chen *et al.*, 2002). In tomato seeds it was not tested whether XET activity accumulates during germination and whether the LeXET4 mRNA or any other tomato seed XET is induced by BR. Steven Fry introduced a specific XET 'dot-blot' assay for the semiquantitative determination of XET enzyme activity (Fry, 1997). This 'XET test paper' was used to detect XET-catalyzed transglycosylation in protein extracts of germinating tobacco seeds (Figure 1). XET enzyme activity in dry tobacco seeds was very low and accumulated during imbibition. The onset of this accumulation was already visible at the onset of testa rupture (30 h) and further accumulation to high specific XET enzyme activities was detected prior to the onset of endosperm rupture (45 h). XET enzyme activity accumulation was detected in the endosperm and in the embryo and ABA did not appreciably affect the accumulation. Treatment of tobacco seeds with BR enhanced the accumulation of XET enzyme activity especially in the embryo (Fig. 1) and BR is known to promote endosperm rupture of tobacco (Leubner-Metzger, 2001). It is, therefore, possible that XETs in the endosperm and in the embryo play roles in mediating the promotion of seed germination by BR via cell wall loosening. However, this can only be a part of the story, since the counteracting

effects of BR on ABA-mediated inhibition of endosperm rupture are not correlated with inhibited XET enzyme activity accumulation. After cloning of the corresponding cDNAs the regulation of specific XETs in the endosperm and the embryo will be investigated in future experiments.

#### CONCLUSION

The results obtained with *Arabidopsis*, tobacco and parasitic angiosperms suggest that the BR pathway promotes germination of non-dormant seeds by directly enhancing the growth potential of the embryo (Takeuchi *et al.*, 1991, 1995; Leubner-Metzger, 2001; Steber and McCourt, 2001). Exploring the molecular mechanisms of the BR-mediated promotion of cell extension growth and seed germination is a challenging field for future experiments.

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