Hormonal and molecular events during seed dormancy release and germination

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Introduction

Seed germination of species with 'coat-imposed' dormancy is determined by the balance of forces between the growth potential of the embryo and the constraint exerted by the covering layers, e.g. testa (seed coat) and endosperm. Little is known about the key interconnected molecular processes regulating seed dormancy and germination in response to plant hormones and environmental cues. 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, 1997b; Koornneef *et al.*, 2002). The testa is no hindrance during the germination of some species, such as *Brassica napus* (Schopfer and Plachy, 1984) and *Pisum sativum* (Petruzzelli *et al*., 2000). The process of germination commences with the uptake of water by imbibition of the dry seed, followed by embryo expansve growth. This usually culminates in rupture of the covering layers and emergence of the radicle, generally considered as the completion of germination. Radicle protrusion during seed germination depends on embryo expansion, which is a growth process driven by water uptake.

Abscisic acid (ABA) is known as a positive regulator of dormancy and as negative regulator of seed germination (Hilhorst, 1995; Bewley, 1997b; Koornneef *et al.*, 2002). ABA treatment 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 (Lopez-Molina *et al.*, 2001). In agreement with this, ABA treatment does not inhibit germination scored as initial radicle extension growth of detipped (surgical removal of the micropylar layers covering the radicle tip) seeds of *Lycopersicon esculentum* (e.g. Liptay and Schopfer, 1983; Groot and Karssen, 1992; Bewley, 1997a). Even 1 mM ABA does not inhibit the germination of detipped, whereas 100 µM ABA results in a substantial inhibition of intact tomato seeds (Liptay and Schopfer, 1983). Detipping can also replace the requirement for treatment with gibberellin (GA), a positive regulator of dormancy release and germination, of GA-deficient *gib1* mutant seeds of tomato (Groot and Karssen, 1992). It is therefore the micropylar testa and endosperm tissue of tomato seeds, also termed the micropylar cap, that confers the primary control of germination timing. In many species the seed envelope imposes a physical constraint to radicle protrusion and depending on the species testa, endosperm, perisperm, hull, glumella or the megagametophyte can confer coat-imposed dormancy (e.g. Downie and Bewley, 1996; Benech-Arnold *et al*., 1999). The main focus of this presentation is on testa- and endosperm-imposed dormancy of dicot seeds and on the interactions among ABA, GA, ethylene and brassinosteroids (BR) in regulating the key interconnected molecular processes that determine dormancy and germination.

Testa-imposed dormancy is regulated by ABA-GA interaction

In non-endospermic seeds, as well as in Arabidopsis, which only has a single layer of endosperm, the testa characteristics are responsible for the degree of coat-imposed dormancy (Debeaujon and Koornneef, 2000; Debeaujon *et al.*, 2000). The testa is a diploid and entirely maternal covering tissue that develops from the integuments of the ovule. The testa layers of Arabidopsis, cells of which die during late seed maturation, undergoes considerable developmental changes that are manifested in color, permeability and testa-imposed dormancy. A peak in ABA biosynthesis during seed

development is needed for the induction of primary dormancy of Arabidopsis seeds (Koornneef and Karssen, 1994). Only embryonic ABA, but not maternal or exogenously applied ABA, is able to induce dormancy. ABA deficiency during seed development is associated with absence of primary dormancy of the mature seed. The formation of non-dormant seeds occurs in the ABA-deficient biosynthesis mutant *aba1* of Arabidopsis (Koornneef and Karssen, 1994). However, sensitivity of seeds to ABA is partially maternally controlled and embryonic, maternal, and applied ABA affect other aspects of Arabidopsis seed development.

According to the revised hormone-balance hypothesis for seed dormancy proposed by Karssen and Laçka (Karssen and Laçka, 1986), ABA and GA act at different times and sites during "seed life". ABA induces dormancy during maturation and GA plays a key role in the promotion of germination. Seed germination of the GA-deficient biosynthesis mutant *ga1* of Arabidopsis depends on the addition of GA to the medium during imbibition (Koornneef and Karssen, 1994). Seed dormancy of Arabidopsis appears to be a quantitative trait and can be released by chilling or light treatment of imbibed seeds or by after-ripening, i.e. a period of dry storage at room temperature for several months. Numerous Arabidopsis mutants that affect the development of the testa without impairing the viability of the seeds have been isolated (e.g. Debeaujon and Koornneef, 2000; Debeaujon *et al.*, 2000). In general, the alterations in the testa characteristics of these mutants caused decreased testa-imposed dormancy. Freshly harvested seeds of testa mutants germinate more readily and are more sensitive to dormancy releasing treatments compared to wild-type seeds. Seed germination of testa mutants is more sensitive to GA treatment, and reciprocal crosses with wild type demonstrated that this effect is determined by the altered testa characteristics. Comparative studies that also include reciprocal crosses between testa and hormone-deficient mutants support the view that dormancy and germination are probably the net result of a balance between many promoting and inhibiting factors including GA and ABA that target the embryo and the testa. Debeaujon and Koornneef (2000) concluded that the GA requirement for dormancy release and germination is determined by (1) ABA produced in the developing seeds and/or the state of dormancy set by ABA and (2) ABA produced upon imbibition especially in dormant seeds. When the restraint to radicle protrusion imposed by the seed envelopes is weakened by the testa mutations, the embryo growth potential threshold required for germination is lowered. Thus, the GA requirement for Arabidopsis seed germination is determined both by testa characteristics and by embryonic ABA. Therefore, testa-imposed dormancy of Arabidopsis appears to be regulated by an indirect ABA-GA interaction.

In endospermic seeds the contributions of both the testa and the endosperm layers to the degree of coat-imposed dormancy have to be considered (Hilhorst, 1995; Bewley, 1997a). The testa accounts for approximately 20% of the mechanical resistance during the early phase of tomato seed imbibition (Groot and Karssen, 1987) and the mechanical resistance of the testa appears to decrease only during the late phase just prior to radicle protrusion. In agreement with this, a much thinner testa (one cell layer) of the ABA-deficient *sit*^{w} mutant compared to wild-type (4-5 cell layers) tomato is correlated with faster seed germination of the mutant seeds (Hilhorst and Downie, 1995). Germination of intact *sit*^{w} seeds occurred at lower external osmotic potentials, and removal of the micropylar testa did not affect seed germination of the *sit*^{w} mutant, but significantly promoted wild-type seed germination. Hilhorst and Downie (1995) concluded that, although the testa resistance is smaller compared to the endosperm resistance, it is the micropylar testa that finally controls the completion of tomato seed germination, i.e. radicle emergence. Interestingly, faster seed germination of the ABA-deficient *sit*^{w} mutant is also associated with significantly increased ßGlu I expression compared to wild-type tomato (Leubner-Metzger, unpublished results). ABA-deficiency causes altered testa characteristics during

seed maturation, i.e. altered testa-imposed dormancy, whereas GA positively regulates tomato germination during seed imbibition. Thus, the ABA-deficient *sit*^{w} mutant is also a testa mutant and, as in Arabidopsis, testa-imposed dormancy of tomato appears to be regulated by an indirect ABA-GA interaction.

Testa rupture of *Nicotiana* seeds appears also to be regulated by an indirect ABA-GA interaction. In the mature seed of tobacco, three to five layers of rather thick-walled endosperm cells surround the embryo. The periphery of the endosperm is pressed against the thin testa, which consists of an outer layer of cutinized and lignified dead cells and a living inner parenchyma layer (Leubner-Metzger, 2001). 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 *et al.*, 1995; Web: http://www.leubner.ch/). Testa rupture starts near the funiculus and spreads in random directions along the ridges on the testa. Channels underlying the ridges facilitate progress of testa rupture. When seeds reach the advanced testa rupture stage the micropylar endosperm covering the radicle tip is exposed as a dome-shaped structure. Microscopic studies showed that storage reserves are degraded in the micropylar endosperm cells prior to protrusion by the radicle; and that the endospermic hole, which has a smooth outline and is always formed at the micropylar end of germinating tobacco seeds, results from tissue dissolution rather than from the pushing action of the protruding radicle (Arcila and Mohapatra, 1983; Leubner-Metzger *et al.*, 1995). Surgical removal of the micropylar testa and the endosperm tissues permits radicle growth under conditions that inhibit germination of intact seeds of tobacco (Bihlmeier, 1927; Kincaid, 1935), demonstrating that regulation of germination by the micropylar covering layers is a common characteristic of Solanaceous seeds (e.g. (Liptay and Schopfer, 1983; Hilhorst, 1995; Bewley, 1997a). ABA-deficiency during seed development of *Nicotiana* seeds is also associated with absence of primary dormancy of the mature seed. In the *aba2* mutant of *N. plumbaginifolia* ABA deficiency is due to a mutation in the *ABA2* gene, encoding zeaxanthin epoxidase, a key step in ABA biosynthesis (Marin *et al.*, 1996). Antisense- and sense-*ABA2* transformation of *N. plumbaginifolia* resulted in decreased and increased ABA biosynthesis and seed dormancy, respectively (Frey *et al.*, 1999). The onset of dormancy in *Nicotiana tabacum* is correlated with a peak in ABA content at approximately 15-20 days after pollination (DAP); a rapid decline in ABA content follows during further seed maturation; and dormancy has been established when seeds are harvested after DAP 25 (Phillips *et al.*, 1997; Leubner-Metzger and Meins, 2000). Seed dormancy is not established and precocious germination occurs in transgenic tobacco expressing an anti-ABA antibody that causes deficiency in free ABA (Phillips *et al.*, 1997).

Dormancy of *Nicotiana* seeds can be released during after-ripening, i.e. a period of dry storage of freshly harvested, mature seeds (Grappin *et al.*, 2000; Leubner-Metzger and Meins, 2000, 2001). The work of Grappin et al. (2000) demonstrated that a further decline in ABA content and decreased sensitivity to ABA are involved in the after-ripening-mediated transition from the dormant to the non-dormant state of *N. plumbaginifolia*. In addition, *de novo* ABA biosynthesis occurs in imbibed fresh (dormant) seeds, but not in after-ripened (non-dormant) seeds. The after-ripening-mediated promotion of *N. tabacum* germination is due to the promotion of both testa and subsequent endosperm rupture (Leubner-Metzger and Meins, 2000, 2001). Addition of ABA to the medium during imbibition resembles the effects of maternal ABA during seed development and residual ABA in mature seeds. Imbibition of fresh or after-ripened tobacco seeds in medium with 10 µM ABA greatly delays endosperm rupture, but does not affect the kinetics of testa rupture of fresh or after-ripened tobacco seeds.

Involvement of ß-1,3-glucanase in the after-ripening-mediated promotion of tobacco testa and endosperm rupture

Class I ß-1,3-glucanase (ßGlu I) is transcriptionally induced in germinating tobacco seeds just prior to endosperm rupture, but after testa rupture (Leubner-Metzger *et al.*, 1995; Leubner-Metzger *et al.*, 1998). ßGlu I induction is highly localized in the micropylar endosperm at the site of radicle emergence. Light, GA and ethylene promote ßGlu I expression and endosperm rupture. ABA inhibits ßGlu I expression and endosperm rupture of wild-type seeds and transgenic TCIB1 seeds, which originate from empty-vector-transformed tobacco lines (TCIB1) and serve as proper controls in sense- and antisense-experiments (Leubner-Metzger and Meins, 2000, 2001). A chimeric ABA-inducible ßGlu I transgene was used for the transformation of tobacco and yielded independent sense-ßGlu I lines (TKSG7). Sense-ßGlu I transformation caused over-expression of ßGlu I in TKSG7 seeds and promoted endosperm rupture of mature seeds and of ABA-treated after-ripened seeds.

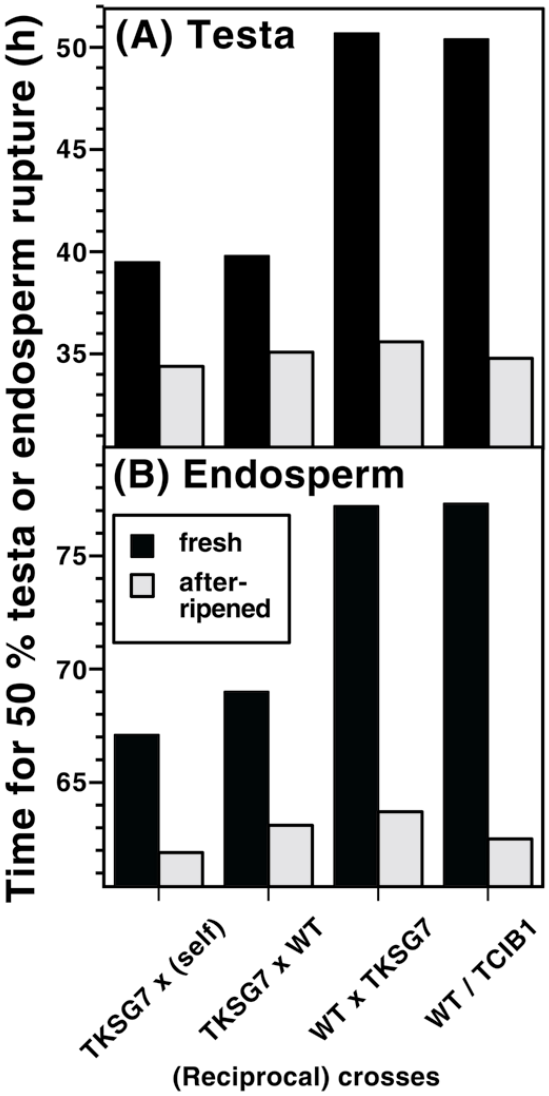

Figure 1. The effect of after-ripening and sense-ßGlu I transformation on testa rupture **(A)** and endosperm rupture **(B)** during the germination of tobacco seeds. Fresh (*filled columns*) or after-ripened (*stippled columns*) progeny seeds from reciprocal crosses of a homozygous monogenic sense-ßGlu I lines (TKSG7) or an empty-vector (TCIB1) line with wild-type (WT) were compared. The female parent in cross is on the left and the male parent is not shown in self crosses. The incidence of testa and endosperm rupture expressed as percentage was scored over time from the start of imbibition in continuous light and the time needed for 50% rupture was determined. Mean values presented are based on the results from three independent TKSG7 lines; mean values ± SE for each single TKSG7 line and further details are published in Leubner-Metzger (2002).

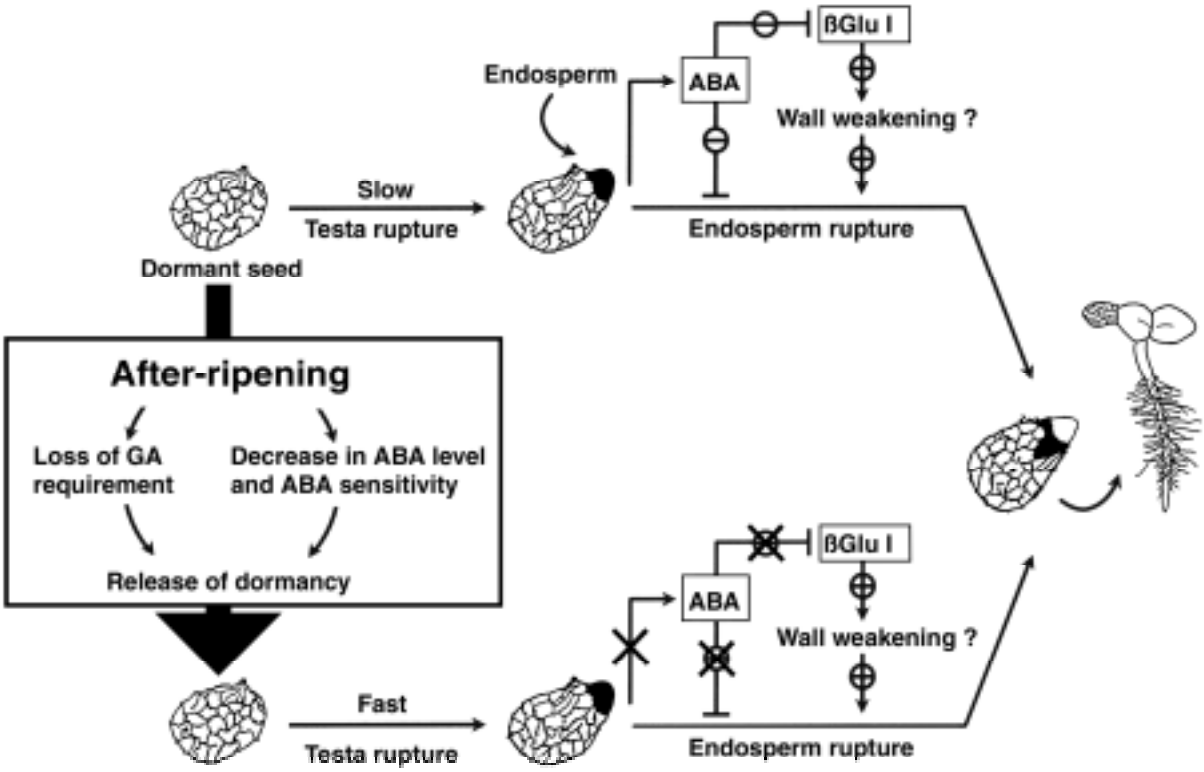

Figure 2. A speculative model relating afterripening, ßGlu I and endosperm rupture of tobacco. According to the model, ßGlu I, which is transcriptionally down-regulated by ABA, contributes to endosperm wall weakening and breaking of coat-imposed dormancy. ABA accumulated during seed maturation is sufficient to delay ßGlu I induction and endosperm rupture. Afterripening decreases ABA levels and possibly sensitivity to ABA allowing the induction of ßGlu I during imbibition, which helps modulate coat-imposed dormancy. After-ripening promoted testa rupture of seeds imbibed in the light and is also correlated with loss of GA requirement for dark-germination. The model does not exclude the possibility that other factors contribute to afterripening and modulation of coat-imposed dormancy, or that ABA has additional functions, e.g. in photodormancy. (Modified after G. Leubner-Metzger and F. Meins, 2000; © Blackwell Publishing, reprinted with permission.)

Recently, we discovered that the after-ripening-mediated promotion of tobacco seed germination is mainly due to a promotion of testa rupture and a similar promotion of subsequent endosperm rupture (Leubner-Metzger, 2002). Furthermore, over-expression of ßGlu I in sense-ßGlu I-transformed TKSG7 seeds replaces the after-ripening effects and promotes testa rupture and endosperm rupture of fresh TKSG7 seeds. Reciprocal crosses between wild-type tobacco and sense-ßGlu I transformant lines showed that ßGlu I over-expression in the seed covering layers can replace the promoting effect of after-ripening on testa rupture and endosperm rupture, but only if the mother plant is a sense-ßGlu I line (Fig. 1). This maternal effect supports a model of two sites for ßGlu I action: (1) ßGlu I contribution to the after-ripening-mediated release of dormancy in the dry seed state, which is manifested in the promotion and ABA-insensitivity of testa rupture during imbibition (Fig. 2); (2) ABA-sensitive expression of ßGlu I in the micropylar endosperm, which contributes to endosperm rupture (Fig. 2). In contrast to endosperm rupture, which seems to be caused by enzymatic degradation of the micropylar

endosperm tissue during radicle extension, testa rupture appears to be achieved by a different mechanism that is characterized by a spread of increasing cracks in random directions along the ridges of the testa. In agreement with a role of ßGlu I during after-ripening, a delay in testa rupture and a similar delay of subsequent endosperm rupture was found in after-ripened antisense-ßGlu I seeds (Leubner-Metzger and Meins, 2001). Fresh TCIB1 and antisense-ßGlu I seeds do not differ in their kinetics of testa rupture. The delay in testa rupture of after-ripened antisense-ßGlu I seeds must be established during after-ripening of the dry, mature seed. These findings support the view that ßGlu I is expressed and susceptible to antisense inhibition in antisense-ßGlu I seeds. The after-ripening-mediated release of tobacco dormancy is also correlated with a decrease in GA requirement for testa rupture during dark imbibition (Leubner-Metzger, 2002). Thus, as in Arabidopsis and tomato, testa-imposed dormancy of tobacco appears to be regulated by an indirect ABA-GA interaction. The importance of testa characteristics appears to be a common feature during the after-ripening-mediated release of coat-enhanced dormancy in endospermic and non-endospermic seeds.

Endosperm-imposed dormancy is regulated by antagonistic interactions of GA, ethylene and BR with ABA

In addition to the testa, in many endospermic species the usually triploid (two-thirds of its genome originates from the mother plant) micropylar endosperm confers coat-imposed dormancy. Thus, in endospermic seeds the contributions of both the testa and the endosperm layers to the degree of coat-imposed dormancy have to be considered (Hilhorst, 1995; Hilhorst and Downie, 1995; Bewley, 1997a; Nonogaki *et al.*, 2000; Toorop *et al.*, 2000; Wu *et al*., 2000). Endosperm rupture is the main germination-limiting process in members of the Asteraceae (e.g., lettuce) and Solanaceae (e.g., tomato and tobacco). In these cases of endosperm-limited germination, weakening of the micropylar endosperm surrounding the radicle tip seems to be required for radicle protrusion and is likely to involve cell-wall hydrolysis by the action of GA-induced hydrolytic enzymes. It is possible, but not proven, that tomato endosperm weakening is a biphasic process and only the second phase is inhibited by ABA (Hilhorst and Downie, 1995; Bewley, 1997a; Nonogaki *et al.*, 2000; Toorop *et al.*, 2000; Wu *et al.*, 2000). ßGlu I is induced by GA just prior to tomato endosperm rupture and is inhibited by ABA, which also inhibits germination (Wu *et al.*, 2000). The close correlation between ßGlu I induction and the onset of endosperm rupture under a variety of physiological conditions support the hypothesis that ßGlu I contributes to endosperm rupture of tobacco. ABA inhibits the induction of the ßGlu I genes, specifically delays endosperm rupture (Fig. 3) and results in the formation of a novel structure, consisting of the enlarging radicle with a sheath of greatly elongated endosperm tissue (Leubner-Metzger *et al*., 1995). Direct evidence for a causal role of ßGlu I during endosperm rupture comes from sense-transformation with a chimeric ABA-inducible ßGlu I transgene (Leubner-Metzger and Meins, 2000). ABA down-regulates the ßGlu I host genes in TCIB1 and wild-type seeds, but due to the ABA-inducible ßGlu I-transgene it causes high-level ßGlu I expression in TKSG7 seeds. ABA treatment delays endosperm rupture of after-ripened TCIB1 and TKSG7 seeds, but due to the sense-ßGlu I transformation this delay is significantly reduced in TKSG7 seeds. ßGlu I over-expression reduces the ABA-mediated delay in endosperm rupture of fresh and after-ripened seeds. These results support the view that a threshold ßGlu I content is required, but not sufficient, for endosperm rupture. In the presence of ABA ßGlu I becomes a limiting factor for endosperm rupture, and removal of this block due to expression of the ABA-inducible ßGlu I-transgene in TKSG7 seeds promotes endosperm rupture until other ABA-sensitive processes become limiting. While these results do not

show how ßGlu I promotes endosperm rupture, they directly show that ßGlu I is causally involved and that it substantially contributes to endosperm rupture.

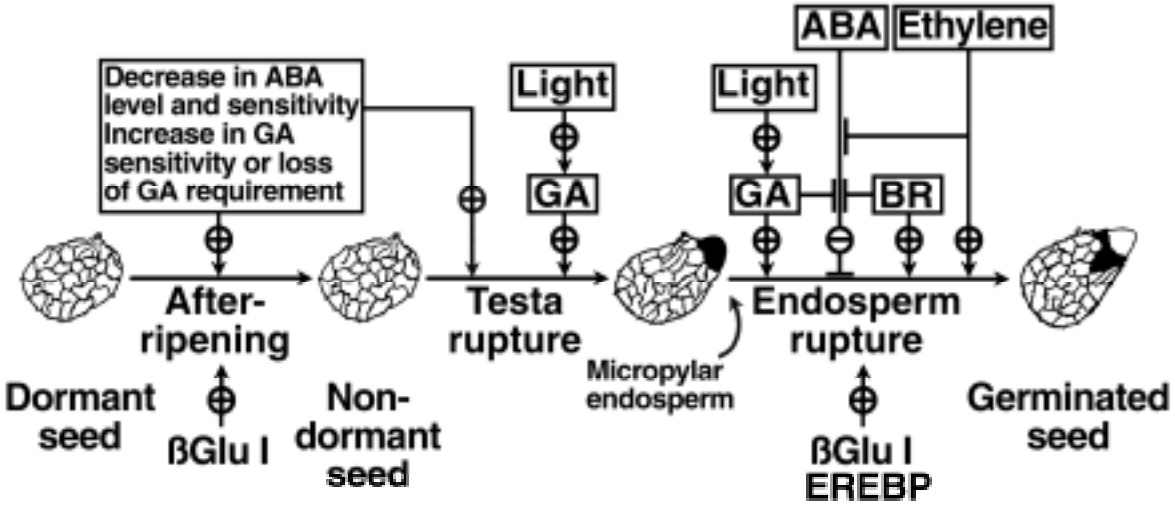

Figure 3. Hormonal interactions during tobacco seed after-ripening, dormancy release and germination and their effects on testa rupture and endosperm rupture. Expression of the abscisic acid (ABA)-inhibited ßGlu I genes contribute to the release of coat-imposed dormancy and the promotion of germination by acting at two sites. First, decrease in ABA level and sensitivity eventually permit ßGlu I expression in seeds during after-ripening. This ßGlu I contributes to the release of coat-imposed dormancy and promotes testa rupture in the light. Second, ßGlu I is induced by the light/gibberellin (GA) pathway in the micropylar endosperm and facilitates endosperm rupture. Endosperm-specific ßGlu I expression and endosperm rupture are inhibited by ABA and promoted by light, GA and ethylene. The light/GA pathway also counteracts ABA effects by promoting ABA degradation. Ethylene and brassinosteroids (BR) counteract ABA effects and promote endosperm rupture, but do not affect testa rupture. EREBPs (ethylene responsive element binding proteins) are transcription factors that mediate hormonal regulation of ßGlu I expression and endosperm rupture. BR and light/GA promote tobacco endosperm rupture by distinct signal transduction pathways. A 'plus' sign means promotion and a 'minus' sign inhibition of a process. (After G. Leubner-Metzger, "Hormonal interactions during seed dormancy release and germination", in: "Handbook of Seed Science", A. Basra, ed., The Haworth Press, Inc., Binghamyon, NY, USA, © 2002, reprinted with permission.)

Tobacco germination is accompanied by ethylene evolution and endogenous ethylene is required for the promotion of endosperm rupture and high-level ßGlu I expression of light-imbibed seeds (Fig. 3) and of non-photodormant dark-imbibed seeds (Leubner-Metzger *et al.*, 1998). Ethylene does not affect the spatial and temporal pattern of ßGlu I expression, and does not break photodormancy or affect the kinetics of testa rupture. A promoter deletion analysis of a tobacco ßGlu I gene in germinating tobacco seeds suggests that the distal region, which contains the positively-acting ethylene-responsive element (ERE), is required for high-level, ethylene-sensitive expression; that the proximal region is necessary and sufficient for low-level micropylar-endosperm specific expression; and, that both regions contribute to down-regulation by ABA (Leubner-Metzger *et al.*, 1998). These promoter regions contain several highly conserved *cis*-acting elements for the regulation by tissue-

specific factors, GA, ABA and ethylene (Leubner-Metzger *et al.*, 1998; Leubner-Metzger, 2001). Enhancer activity and ethylene responsiveness of ßGlu I depend on the AGCCGCC box present as two copies in the ERE. They are the binding site of ERE binding proteins (EREBPs), which are transcription factors mediating ethylene responses. Transcripts of the EREBPs showed a novel pattern of expression during tobacco seed germination (Leubner-Metzger *et al.*, 1998). A direct antagonistic interaction of ethylene and GA with ABA in regulating ßGlu I accumulation in the micropylar endosperm and endosperm rupture (Fig. 3) may therefore be mediated, at least in part, by the EREBP-type transcription factors.

Finally, brassinosteroids (BR) and GA seem to promote tobacco (Fig. 3) and Arabidopsis seed germination by distinct signal transduction pathways and distinct mechanisms (Leubner-Metzger, 2001; Steber and McCourt, 2001). GA and light act in a common pathway to release tobacco photodormancy, whereas BR does not release photodormancy. ßGlu 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 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) Ethylene promotes endosperm rupture by enhancing ßGlu I expression; (4) ABA inhibits endosperm rupture by interfering with these three hormones; (5) The GA/light pathway and ethylene regulate ßGlu I induction in the micropylar endosperm and seem to control endosperm weakening; (6) The BR pathway seems to promote endosperm rupture of non-dormant seeds by directly enhancing the growth potential of the embryo.

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