20 Emerging and Established Model Systems for Endosperm Weakening

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Abstract

Endosperm rupture is the main germination-limiting process in members of the *Asteraceae* (e.g. lettuce (*Lactuca sativa* L.)) and *Solanaceae* (e.g. tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana tabacum* L.). About four decades ago a 'hatching enzyme' was proposed to cause endosperm weakening (i.e. a decline in the mechanical resistance of the micropylar endosperm), which is likely to be essential for seeds to complete germination. Although, there are established model systems among *Asteraceae* and *Solanaceae* for endosperm weakening, its molecular mechanism(s) still remain(s) a mystery. No single 'hatching enzyme' or universal molecular mechanism has been demonstrated explicitly. For the time being, the provisional conclusion is that endosperm weakening is likely to be achieved by the collaborative or successive action of several distinct molecular mechanisms. The knowledge gained from these established model systems will be compared and discussed. However, consideration of their severe experimental limitations shows that there is an urgent need for novel model systems. Such an optimal system has been recently found within the *Brassicaceae*. In this emerging model system for endosperm weakening, a complete study of the process is possible on each experimental level, from the direct measurement of the weakening by 'puncture force' to molecular investigations (e.g. proteome and genome transcriptome analyses).

Introduction

A major reason for the evolutionary success of the angiosperms is the 'invention' of seeds with double fertilization (Friedman, 1998; Judd *et al.*, 2002). In a typical angiosperm seed the diploid embryo is surrounded by two covering layers: (i) the triploid endosperm (i.e. nutritive tissue, living cells); and (ii) the diploid testa (i.e. the seed coat, maternal tissue, dead cells). Depending on the species, the endosperm is either maintained or obliterated during seed development. The evolutionary trend is towards cotyledon storage and endospermless seeds at maturity. Endosperm

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development and its function as nutritive tissue have been studied thoroughly (Jacobsen *et al.*, 1995; Friedman, 1998; Baskin and Baskin, 2004). In contrast, little is known about the function of the endosperm as a constraint during endosperm-limited germination and coat-imposed dormancy (Bewley, 1997; Leubner-Metzger, 2003). Seed germination is a complex physiological process, water uptake by imbibition is followed by embryo growth, and radicle protrusion through all seed-covering layers is considered as the completion of germination. Environmental factors and plant hormones (e.g. gibberellins (GA), abscisic acid (ABA), brassinosteroids and ethylene) are regulators of germination and/or dormancy (Koornneef *et al.*, 2002; Kucera *et al.*, 2005).

The mature seeds of most angiosperms have a more or less abundant endosperm layer (Fig. 20.1). Mature angiosperm seeds differ in their 'embryo to seed' (E/S) ratios (Martin, 1946; Forbis *et al.*, 2002). Low E/S values, due to abundant endosperm tissue and tiny embryos, are typical for mature seeds of basal angiosperms (Fig. 20.1). High E/S values, due to obliterated endosperm tissue and the predominance of cotyledon storage, are evident in mature seeds of higher angiosperms of the rosid clade. Typical examples are the more or less endospermless *Brassicaceae* seeds with E/S values ~0.9 (Fig. 20.1). Although the mature seeds of *Brassica* species are completely endospermless, a single cell layer of endosperm is present in mature seeds of *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.) (Liu *et al.*, 2005). E/S values between 0.4 and 0.5 are typical for mature seeds of higher angiosperms of the asterid clade (e.g. the *Solanaceae*), with abundant endosperm and additional embryo storage (Fig. 20.1; Bewley, 1997; Hilhorst *et al.*, 1998; Koornneef *et al.*, 2002; Leubner-Metzger, 2003).

The distribution of the different seed types and the E/S values in a modern phylogenetic tree (Fig. 20.1) support the following evolutionary seed trends (Martin, 1946; Forbis *et al.*, 2002; Baskin and Baskin, 2004): (i) in mature seeds of primitive angiosperms a small embryo is embedded in abundant endosperm tissue, and such seed types are prevalent among basal angiosperms; (ii) the general evolutionary trend within the higher angiosperms (i.e. core eudicots) is by the *Solanaceae*-like endospermic seed types of many asterids towards *Brassicaceae*-like more or less endospermless seed types of many rosids with storage cotyledons; and (iii) in addition to these general seed trends there are clade-specific differences. It has been proposed that endospermless seeds were 'invented' several times independently during evolution (Baskin and Baskin, 2004). Thus, our knowledge on endosperm abundance in mature seeds is quite complete.

In contrast, we do not know how endosperm-limited germination and/or endosperm-enhanced dormancy evolved during angiosperm seed phylogeny. Figure 20.1 shows (in bold) the few clades with at least some experimental evidence for seeds where the endosperm is important as a germination barrier (almost nothing is known for many rosid species). An exception is the work on perisperm weakening of *Cucurbitaceae* seeds (e.g. Welbaum *et al.*, 1995; Yim and Bradford, 1998). Established model systems for endosperm-limited germination (Fig. 20.1) are exclusively asterid species (Bewley, 1997; Hilhorst *et al.*, 1998; Leubner-Metzger, 2003; da Silva *et al.*, 2004, 2005): lettuce (*Lactuca sativa* L., *Asteraceae*, *Asterales*), tomato (*Lycopersicon esculentum* Mill., *Solanaceae*, *Solanales*), tobacco (*Nicotiana tabacum* L., *Solanaceae*, *Solanales*) and coffee (*Coffea arabica* L., *Rubiaceae*, *Gentianales*). Endosperm weakening, a decline

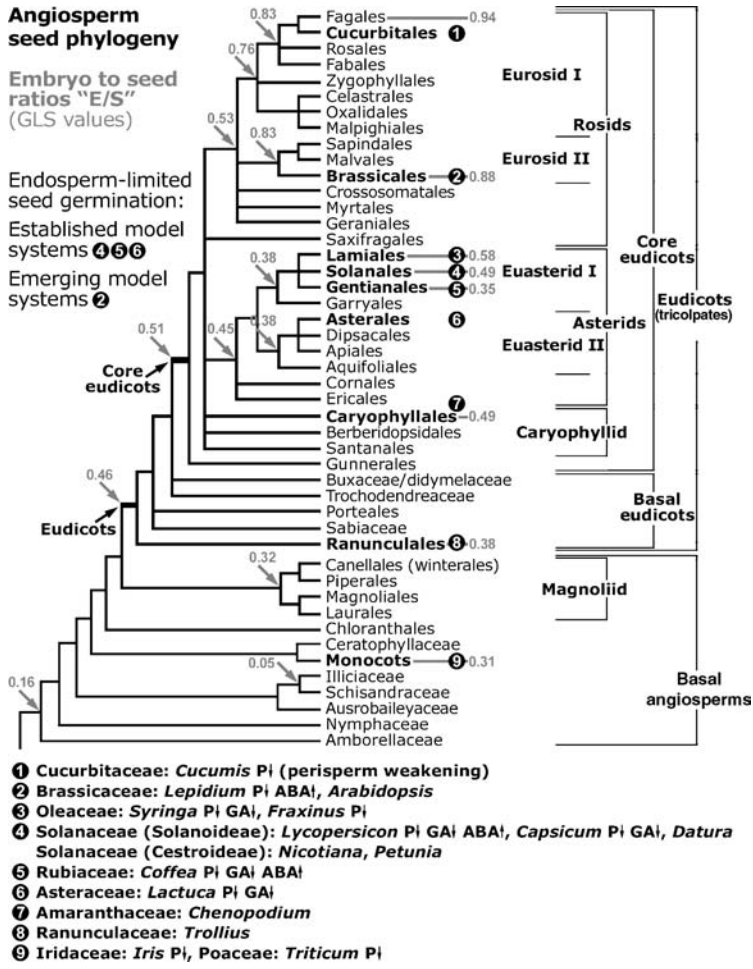

Fig. 20.1. Angiosperm seed phylogeny. Grey numbers in the phylogenetic tree represent 'embryo to seed' (E/S) ratios expressed as generalized least squares (GSL) values (Forbis *et al*., 2002). Clades with experimental evidence for endosperm weakening are in bold and numbered; some of these provide model systems. P↓: endosperm weakening during germination was measured as decreasing puncture force; GA↓: endosperm weakening promoted by GA; and ABA↑: endosperm weakening inhibited by ABA.

in the mechanical resistance of the micropylar endosperm (i.e. endosperm covering the radicle tip), appears to be a prerequisite for the germination of these species. Endosperm weakening can be measured directly by the puncture-force method (see Müller *et al*., Chapter 30, this volume). Puncture-force experiments are not possible with seeds as small as tobacco or *Arabidopsis* (Fig. 20.2), but have been performed for larger asterid seeds of the *Asterales* (Pavlista and Haber, 1970; Tao and Khan, 1979), *Gentianales* (da Silva *et al*., 2004, 2005), *Solanales* (Watkins and Cantliffe, 1983;

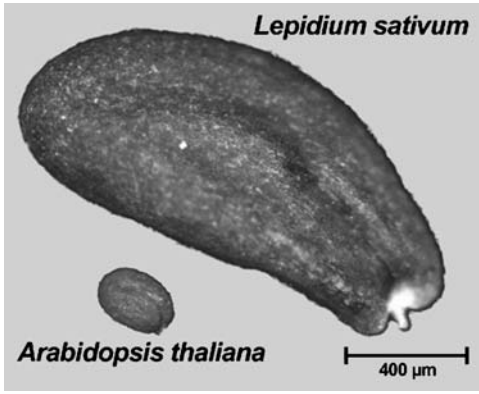

Fig. 20.2. Size comparison of dry seeds of *Lepidium sativum* and *Arabidopsis thaliana*, two closely related species of the *Brassicoideae* subfamily of the *Brassicaceae*.

Toorop *et al.*, 2000; Wu *et al.*, 2000) and *Laminales* (Junttila, 1973; Finch-Savage and Clay, 1997). All these experiments showed a decline in the puncture force of the micropylar endosperm prior to endosperm rupture (P↓ in Fig. 20.1). Promotion of endosperm weakening by GA appears to be a general phenomenon (GA↓ in Fig. 20.1). Thus, endosperm weakening prior to endosperm rupture seems to be widespread among asterid seeds, but has not been investigated in rosid seeds.

ABA Inhibition of Seed Germination: ABA Inhibits Endosperm Rupture, but not Testa Rupture

Another phenomenon of endospermic seeds is that endosperm rupture is inhibited by ABA (Karssen, 1976; Finch-Savage and McQuistan, 1991; Toorop *et al.*, 2000; Petruzzelli *et al.*, 2003). In the established model systems of the asterid clade it has been shown that ABA inhibits endosperm rupture, at least in part, by acting in an inhibitory manner on the micropylar endosperm (Ni and Bradford, 1993; Leubner-Metzger, 2003; da Silva *et al.*, 2004). The germination of intact tomato seeds is inhibited by 10–100 µM ABA, but surgical removal of the micropylar cap permits germination (i.e. initial embryo elongation) even in the presence of 1000 µM ABA (Liptay and Schopfer, 1983). The *Solanaceae* family can be divided into two large subgroups (Judd *et al.*, 2002; Petruzzelli *et al.*, 2003): (i) the *Solanoideae* (e.g. *Capsicum*, *Lycopersicon* and *Datura*); and (ii) the *Cestroideae* (e.g. *Nicotiana* and *Petunia*). In *Solanoideae*-type seeds, the micropylar covering layers, testa and endosperm form a caplike structure (i.e. a micropylar cap) (Hilhorst *et al.*, 1998; Toorop *et al.*, 2000). A visible distinction between testa rupture and endosperm rupture is not possible for *Solanoideae*-type seeds. A typical feature of *Cestroideae*-type seeds like tobacco is a two-step germination with a visible distinction between testa rupture and endosperm rupture (Fig. 20.3; Leubner-Metzger, 2003; Petruzzelli *et al.*, 2003).

Two-step germination with separate testa and endosperm rupture is widespread over the entire phylogenetic tree and has been described for many species, including *Trollius* (*Ranunculaceae*, *Ranunculales*, basal eudicots) (Hepher and Roberts, 1985), *Chenopodium* (*Amaranthaceae*, *Caryophyllales*, caryophyllids) (Karssen, 1976) and

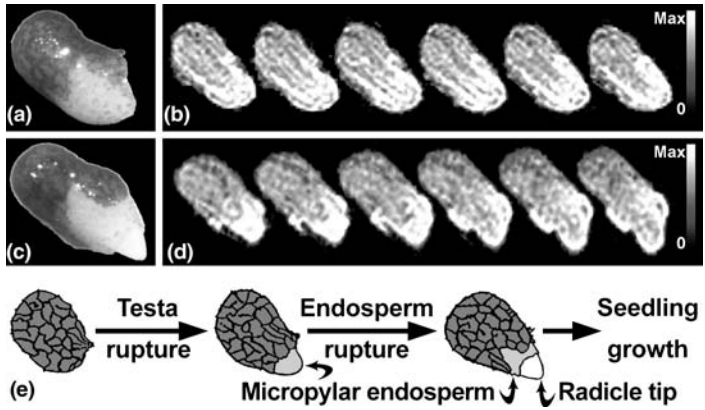

Fig. 20.3. Two-step germination and water uptake of tobacco seeds. (a, b and e) Testa rupture is followed by (c, d and e) endosperm rupture and subsequent seedling growth. (b and d) Nuclear magnetic resonance (NMR) images show that the micropylar endosperm and the radicle are major sites of water uptake. White: maximum water abundance; black: minimum water abundance (see NMR images in Manz *et al*., 2005 for details).

Nicotiana (*Cestroideae*, *Solanaceae*, *Solanales*, asterids) (Leubner-Metzger, 2003). Recently, it has also been found in the *Brassicaceae* species, *Arabidopsis thaliana* L. (Heynh.) (Liu *et al*., 2005) and lepidium (*Lepidium sativum* L.) (see Müller *et al*., Chapter 30, this volume). Separate testa and endosperm rupture is therefore a feature of endospermic seeds within the rosid clade. It was also found that ABA inhibits endosperm rupture, but not testa rupture, of *Arabidopsis* and lepidium (see Müller *et al*., Chapter 30, this volume). In agreement with this, ABA also does not inhibit testa rupture of endospermless *Brassica* seeds (Schopfer and Plachy, 1984). Separate testa rupture and endosperm rupture are an important experimental advantage of tobacco seeds when compared with tomato seeds. It has helped considerably to assign different enzymes, transcription factors and plant hormones to their target sites (Leubner-Metzger, 2003).

Although ABA inhibits the embryo growth potential and the transition to postgermination growth, it does not inhibit initial water uptake by imbibition or initial embryo elongation (Liptay and Schopfer, 1983; Schopfer and Plachy, 1984; Homrichhausen *et al*., 2003; Manz *et al*., 2005). We measured the regulation of water uptake of germinating tobacco seeds spatially and temporally by *in vivo* ^{1}H nuclear magnetic resonance (^{1}H-NMR) microimaging and ^{1}H-MAS NMR spectroscopy (Manz *et al*., 2005). These non-destructive state-of-the-art methods show that water distribution in the water uptake phases II and III is inhomogeneous (Fig. 20.3). The micropylar seed end is the major entry point of water. The micropylar endosperm and the radicle show the highest hydration. ABA specifically inhibits endosperm rupture and phase III water uptake, but does not alter the spatial and temporal pattern of phase II water uptake. Taken together, these findings demonstrate that the micropylar endosperm is a main target for the ABA inhibition of endosperm rupture.

ABA, Endosperm Hydrolases and Endosperm Weakening in Established Asterid Model Systems

Direct measurements of the effect of ABA on endosperm weakening by puncture-force experiments have been published only for the asterid species like coffee and tomato (Toorop *et al.*, 2000; Wu *et al.*, 2000; da Silva *et al.*, 2004, 2005). The coffee embryo is enveloped by an endosperm tissue and surrounded by an endocarp (da Silva *et al.*, 2004). The endosperm is composed of a hard greenish tissue with polyhedral cells, is isodiametrically divided into a hard external endosperm and a soft internal endosperm, and belongs to the nuclear type. Endosperm weakening was measured in imbibed seeds with the endocarp mechanically removed. Endosperm weakening in coffee is biphasic. The first phase of endosperm weakening is ABA-insensitive, which is followed by the second phase that is inhibited by ABA. This second phase accounts for ~53% (420 mN) of the total difference in puncture force (da Silva *et al.*, 2004). Endosperm weakening of tomato is also biphasic, with a first phase that is ABA-insensitive. In this case the second phase, which is inhibited by ABA, has been reported to account for ~6% (30 mN; Wu *et al.*, 2000) or ~24% (80 mN; Toorop *et al.*, 2000) of the total tomato micropylar cap weakening. These results were obtained by incubating whole seeds in a medium with and without ABA, and dissecting the seeds when puncture-force measurements were performed (Groot and Karssen, 1987, 1992; Toorop *et al.*, 2000; Wu *et al.*, 2000). If micropylar caps are dissected from tomato seeds prior to the onset of endosperm weakening (3 h), a further 24 h incubation of isolated micropylar caps in medium with GA results in endosperm weakening (i.e. puncture force decreases by 170 mN), whereas incubation of isolated micropylar caps in a GA- plus ABA-containing medium inhibits endosperm weakening completely (Groot and Karssen, 1992). Although ABA clearly inhibits the second-phase endosperm weakening of coffee, the situation in tomato is less clear.

The micropylar cap of tomato seeds consists of endosperm and testa. No visible distinction between testa rupture and endosperm rupture is possible in tomato. Although the micropylar testa of tomato seeds accounts for only 20% of the initial puncture force and does not weaken during the first phase of the biphasic micropylar cap weakening, a significant decline in testa puncture force occurs during the second (i.e. ABA-sensitive) phase of tomato micropylar cap weakening just prior to radicle protrusion (Groot and Karssen, 1987). Tomato micropylar cap weakening is also highly dependent on the physiological seed stage. Endosperm weakening and endosperm rupture were delayed in freshly harvested tomato seeds when compared with after-ripened seeds (Groot and Karssen, 1992). ABA deficiency of the *sit*^{w} tomato mutant replaced this after-ripening effect, and micropylar cap weakening and endosperm rupture of freshly harvested and after-ripened *sit*^{w} seeds were equal. The ABA-deficient *sit*^{w} tomato mutant has a thinner testa (i.e. one cell layer thick) compared with wild-type seeds (i.e. 3–4 cell layers thick) and is therefore a testa mutant (Hilhorst and Downie, 1995). A species with separate testa rupture and endosperm rupture would provide a seed model system with experimental advantages for studying endosperm weakening (see Müller *et al.*, Chapter 30, this volume).

Little is known about the molecular mechanisms of endosperm weakening (Bewley, 1997; Toorop *et al.*, 2000; Leubner-Metzger, 2003; da Silva *et al.*, 2004,

2005). Ikuma and Thimann (1963) proposed the 'hatching hypothesis' of seed biology as 'the final step in the germination control process is the production of an enzyme whose action enables the tip of the radicle to penetrate through the coat'. Experiments to identify this 'hatching enzyme' have been conducted in a variety of species and have provided evidence for the contribution of various cell wall–modifying proteins (e.g. endo-β-1,4-mannanases, endo-β-1,3-glucanases and expansins) (Bewley, 1997; Hilhorst *et al.*, 1998; Koornneef *et al.*, 2002; Leubner-Metzger, 2003). Expression of endo-β-1,4-mannanase in the micropylar endosperm of coffee is associated with the second phase of endosperm weakening and is inhibited by ABA (da Silva *et al.*, 2004). In contrast, endo-β-1,4-mannanase in tomato seeds is associated with the first phase of endosperm weakening and is not inhibited by ABA (Nonogaki *et al.*, 2000; Toorop *et al.*, 2000). Expression of endo-β-1,3-glucanase in the micropylar endosperm, its inhibition by ABA and the inhibition of endosperm rupture by ABA is widespread among the *Solanaceae* (Wu *et al.*, 2000; Leubner-Metzger, 2003; Petruzzelli *et al.*, 2003). The ABA inhibition of endosperm rupture is partially reverted in transgenic tobacco seeds that overexpress endo-β-1,3-glucanase in the seed-covering layers under the control of an ABA-inducible transgene promoter (Leubner-Metzger and Meins, 2000; Leubner-Metzger, 2002; Manz *et al.*, 2005). This directly proves that endo-β-1,3-glucanase is causally involved in promoting endosperm rupture, but it is not the sole 'hatching enzyme' (Leubner-Metzger, 2003). Conclusive evidence for a sole 'hatching enzyme' has not yet been found. In his review, Bewley (1997) stated that 'endosperm weakening is likely to be essential for some seeds to complete germination, how it is achieved remains a mystery'. Taken together, these findings support the view that germination control by the seed-covering layers is achieved by the collaborative or successive action of several cell wall–modifying proteins and various molecular mechanisms (Leubner-Metzger, 2003). The intriguing issue that arises is that there might be evolutionary conserved molecular mechanisms as well as species-specific adaptations for endosperm weakening.

ABA, Endosperm Weakening and Novel Molecular Mechanisms Studied in Emerging Rosid Model Systems

Within the rosid clade, there is prevalence for more or less endospermless seeds (Fig. 20.1). There are also several examples of endospermic rosid seeds (O'Brien and McCully, 1969), but not a single publication about rosid endosperm weakening. A new model system for endosperm-limited germination with molecular phylogenetic placement within the rosid clade is therefore a necessity (Mandoli and Olmstead, 2000; Soltis and Soltis, 2003). The genome of the rosid model plant *Arabidopsis* is completely sequenced. The placement of a novel rosid model system in close proximity with this 'molecular' model plant allows successful utilization of the *Arabidopsis* databases for modern molecular methods like transcriptomics and proteomics (Mandoli and Olmstead, 2000; Hall *et al.*, 2002).

Lepidium is closely related to *Arabidopsis*; both belong to *Brassicoideae* subfamily of *Brassicaceae*. Both species have separate testa and endosperm rupture, and ABA inhibits endosperm rupture, but not testa rupture (Liu *et al.*, 2005; see Müller *et al.*,

Chapter 30, this volume). *Arabidopsis* seeds are too small to perform puncture-force measurements (Fig. 20.2). These measurements show that endosperm weakening occurs prior to lepidium endosperm rupture (see Müller *et al.*, Chapter 30, this volume). This endosperm weakening is inhibited by ABA. In future, we want to exploit these experimental advantages to investigate the molecular mechanisms that regulate lepidium endosperm weakening. Lepidium is an emerging model system for studying endosperm weakening. Reactive oxygen species are a novel molecular mechanism for endosperm weakening and embryo expansion during seed germination (see Müller *et al.*, Chapter 30, this volume).

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