

1 **REVIEW PAPER**

2 **The biomechanics of seed germination**

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35 **Abstract**

36 From a biomechanical perspective the completion of seed (and fruit) germination  
37 depends on the balance of two opposing forces: The growth potential of the  
38 embryonic axis (radicle-hypocotyl growth zone) and the restraint of the seed covering  
39 layers (endosperm, testa, pericarp). The diverse seed tissues are composite  
40 materials which differ in their dynamic properties based on their distinct cell-wall  
41 composition and water uptake capacities. The biomechanics of embryo cell growth  
42 during seed germination depends on irreversible cell-wall loosening followed by water  
43 uptake due to the decreasing turgor and this leads to embryo elongation and  
44 eventually radicle emergence. Endosperm weakening as a prerequisite for radicle  
45 emergence is a widespread phenomenon among angiosperms. Research into the  
46 biochemistry and biomechanics of endosperm weakening has demonstrated that the  
47 reduction in puncture force of a seed's micropylar endosperm is environmentally and  
48 hormonally regulated and involves tissue-specific expression of cell-wall remodelling  
49 proteins such as expansins, diverse hydrolases, and the production of directly acting  
50 apoplastic reactive oxygen. The endosperm weakening biomechanics and its  
51 underlying cell-wall biochemistry differs between the micropylar (ME) and chalazal  
52 (CE) endosperm domains. In the ME it involves cell-wall loosening, cell separation  
53 and programmed cell death to provide decreased and localised ME tissue resistance,  
54 autolysis and finally the formation of an ME hole required for radicle emergence.  
55 Future work will further unravel the molecular mechanisms and environmental  
56 regulation of the diverse biomechanical cell-wall changes underpinning the control of  
57 germination by endosperm weakening.

58

59 **Introduction**

60 All living organisms and processes are bound by the laws of physics and chemistry.  
61 Understanding these fundamental mechanisms is key to elucidating the roles of  
62 biological materials and structures in life. Plant biomechanics has risen to a topical,  
63 multidisciplinary and expanding field of science (Moullia, 2013; Niklas *et al.*, 2006).  
64 The application of new techniques previously only used in material science are  
65 leading to new advances and insights in biological materials (Cranford and Buehler,  
66 2010; Ebenstein and Pruitt, 2006; Walters *et al.*, 2010). The mechanical properties of  
67 plants are an interplay of cell wall, whole cell, tissue and organ properties and are  
68 highly dependent on water content (Fratzl and Weinkamer, 2007; Jeronimidis, 1980;  
69 Vogler *et al.*, 2015). A plant's life cycle depends on biomechanics at several stages.  
70 Starting with the fertilisation and the mechanics of pollen tube formation (Gossot and  
71 Geitmann, 2007; Zonia and Munnik, 2009) up to the seed or fruit propagation  
72 (Elbaum and Abraham, 2014; Hofhuis *et al.*, 2016; Nathan *et al.*, 2002; Witztum and  
73 Schulgasser, 1995). The vulnerable and complex process of seed germination also  
74 depends on decisive and specific changes in tissue and cell properties. Per  
75 definition, seed germination starts with the uptake of water by the quiescent, dry seed  
76 followed by the elongation of the embryonic axis (Bewley, 1997b). This usually  
77 culminates in the rupture of the covering layers and emergence of the radicle,  
78 generally considered as the completion of germination (Finch-Savage and Leubner-  
79 Metzger, 2006). From a mechanical point of view, the germination process can be  
80 seen as an interplay between two opposing forces: the growth potential of the  
81 embryo and the restraining force of the seed covering layers. While the physiological,  
82 biochemical and molecular mechanisms of seed germination have been summarised  
83 in numerous reviews (see for example Bewley, 1997b; Finch-Savage and Leubner-  
84 Metzger, 2006; Koornneef *et al.*, 2002; Linkies and Leubner-Metzger, 2012),  
85 integrated works in which an interdisciplinary effort has been made to combine them  
86 with methods from biophysics, engineering and mathematical sciences are rare. In  
87 this review paper we are focusing on biological materials and seeds in particular from  
88 a mechanical perspective.

89

90 **Biological materials**

91 Biological materials and structures are normally composites which are mainly made  
92 up from polymeric fibres embedded in a protein matrix (Vincent, 1990; Vincent and  
93 Currey, 1980; Wainwright *et al.*, 1982). Considering these weak individual building  
94 blocks, it is striking that many biological systems exhibit mechanical properties  
95 beyond what can be achieved using the same synthetic materials (Chen *et al.*, 2008;  
96 Srinivasan *et al.*, 1991; Vincent, 1992). Plant cell walls consist of cellulose,  
97 hemicellulose, pectin, lignin, and protein. This rigid structure together with the  
98 osmotic characteristics of the protoplast, govern the mechanical properties of cells,  
99 tissues and organs (Brett and Waldron, 1996; Cosgrove, 2005). In contrast to this,  
100 animal tissue protoplasts are in most cases not surrounded by such a rigid  
101 compartment (Meyers *et al.*, 2008; Vincent and Wegst, 2004). It is not so much the  
102 material properties of the individual components determining the mechanical  
103 behaviour but rather their specific arrangement within a structure. Also, based on the  
104 fibre orientations and the amount of the constituents, the mechanical properties of  
105 the various material systems or structures are different (Burgert, 2006; Wegst and  
106 Ashby, 2004). The exceptional mechanical performance of biological materials  
107 resides in their hierarchical organisation at multiple levels, from the molecular to the  
108 macroscopic scale (Aizenberg *et al.*, 2005; Currey, 2005; Gibson, 2012; Gordon *et al.*,  
109 1980; Jeronimidis and Atkins, 1995; Mann and Weiner, 1999; Rüggeberg *et al.*,  
110 2009). Wood, for example, is one of the most widely distributed high-performance  
111 materials with a specific strength comparable to steel (Gordon *et al.*, 1980). Its  
112 optimisation is achieved by the arrangement of components on at least five structural  
113 levels: integral (geometrical make-up of axes), macroscopic (tissue structure),  
114 microscopic (cell structure), ultra-structural (cell wall structure) and biochemical (cell  
115 wall components) (Jeronimidis, 1980). As shown by Ji and Gao (2004) and Gao *et al.*  
116 (2003) the smallest hierarchical level is on the nanoscale and intricately linked to  
117 higher levels.

118 Materials respond to external stresses. Engineers describe the mechanical behaviour  
119 of materials by loading a sample and measuring the force and displacement of the  
120 material as it deforms. This results in force-displacement curves, which can be  
121 converted into typical stress-strain curves. These stress-strain curves have several  
122 regions of interest and reveal several of the properties of a material (Figs. 1, 2A).  
123 Stress (or pressure) is defined as the force per area and strain (or deformation) is

124 defined as the amount of elongation or contraction (increase or decrease in length)  
125 caused by the stress.

126 Stress  $\sigma = \frac{F}{A}$  (where F is the force and A is the cross section)

127 Strain  $\epsilon = \frac{\Delta L}{L}$  (where  $\Delta L$  is the change in length and L is the original length)

128 Some characteristic responses that materials exhibit are shown in Figure 1 and are  
129 defined as follows:

130 *Elastic behaviour:* recoverable deformation; Stress is proportional to strain.  
131 Deformation occurs instantly and the material returns to its original shape after load is  
132 removed. For an ideal elastic material, no energy is lost during the loading and  
133 unloading.

134 *Plastic behaviour:* non-recoverable deformation; Plastic deformation occurs after a  
135 certain threshold (Yield stress) is reached. An increase in strain leads to a non-linear  
136 change in load.

137 *Viscoelastic behaviour:* time-dependent deformation; The word viscoelasticity  
138 originates from viscosity and elasticity. The rate of deformation is a function of the  
139 stresses. That means the deformation depends on how quickly load is applied.  
140 Viscoelastic materials will return to their original shapes after a certain amount of time  
141 after load is removed.

142 Biological materials are structurally complex and show a complex mechanical  
143 behaviour in response to external loading (Fratzl and Weinkamer, 2007; Speck and  
144 Burgert, 2011). Most biological materials (if not all) show a viscoelastic behaviour to a  
145 greater or lesser extent (Sasaki, 2012). They do have a viscous component and do  
146 show time-dependent behaviour. Therefore, the strain or loading rate (change in  
147 strain or stress with respect to time) needs to be taken into account. The higher the  
148 strain or loading rate, the larger a peak strain/stress will be. Another characteristic a  
149 viscoelastic material can possess is creep. Creep is a slow plastic (permanent)  
150 deformation that occurs when a constant load is applied over time. Most biological  
151 materials operate within the elastic region under normal loading conditions.  
152 Furthermore, biological materials are anisotropic. This means that the mechanical  
153 properties differ for different directions of loading. Wood for example does behave

154 differently if tested along or perpendicular to the grain (Burgert, 2006; Salmén, 2004).  
155 The same holds true for diverse seed or fruit coats.

156 Figure 2 shows stress-strain diagrams, which enable us to derive several key  
157 parameters of the tested material. Typically, materials exhibit an initial linear stress-  
158 strain response where the slope corresponds to the Elastic Modulus  $E$  (or stiffness)  
159 of the material. A flexible material is characterised by a low Elastic Modulus whereas  
160 a high Elastic Modulus correlates to a stiff material. If a test were stopped within the  
161 linear (elastic) region the material it would return to its initial shape. At higher forces,  
162 above a certain threshold, the elastic limit (Yield point) is reached and plastic  
163 deformation occurs. Another important variable obtained from the stress-strain curve  
164 is the maximum strength of the material under a load such as tension, compression,  
165 torsion or bending. The area underneath the curve corresponds to the energy  
166 absorbed by the material and equals the toughness. Stiffness and strength are often  
167 used by biologists in the wrong context as they describe very different characteristics  
168 of a material. A material can be stiff but weak (e.g. a cookie) or flexible but strong  
169 (e.g. leather) (Fig. 2B). An excellent overview about the mechanical properties of  
170 materials and their failure is given by Mattheck (2004).

171 Combining a biologist's and a material scientist's point of view on structure and  
172 mechanics is a timely approach to advance our understanding of plants and provide  
173 new insights on biomaterials. Recently, engineering tools have been applied to  
174 describe seed deterioration and the in engineering well known material property  
175 charts have been extended to include seeds (Fig. 3) (Walters *et al.*, 2010). The idea  
176 of material property charts was coined by Ashby and compares mechanical  
177 properties by plotting one property against another (Ashby, 1989; Ashby *et al.*, 1995;  
178 Wegst and Ashby, 2004). They are a sophisticated graphical way of presenting and  
179 comparing material property data. Two properties are plotted; one on each axis of the  
180 graph, while common combinations are for example: Strength vs. Density, Modulus  
181 vs. Density, Modulus vs. Strength, Fracture Toughness vs. Modulus. Figure 3  
182 illustrates schematically a material property chart where the Elastic Modulus ( $E$ ) is  
183 plotted against the density ( $\rho$ ) (Ashby *et al.*, 1995). The scales are logarithmic  
184 showing a wide range of materials on just one chart. For the comparison of different  
185 materials, the material indices  $E/\rho$ ,  $E^{1/2}/\rho$ , and  $E^{1/3}/\rho$  are plotted onto the figure as  
186 guidelines for minimum mass design. Materials which lie on a line perform equally,

187 those above the line are better with respect to lightweight structures and those below  
188 are worse. It is observable that biological materials are relatively light materials with  
189 low density yet providing a relatively high elastic-modulus. According to Walters *et al.*  
190 (2010), the Elastic Modulus of seeds vary by one order of magnitude, and depends  
191 on the species and environmental factors. The material density is centred near  
192 1000 kg/m<sup>3</sup>. The Elastic Modulus within the seed material family lies within the range  
193 of polymers and foams and other natural materials wherever the density is similar to  
194 wood, polymers and elastomers (Fig. 3) (Walters *et al.*, 2010).

195

### 196 **Biophysical aspects of seed germination**

197 Seeds, and in many cases also seed-harboring fruits, evolved as the typical  
198 dispersal and propagation units of the angiosperms and gymnosperms (Linkies *et al.*,  
199 2010). Structurally distinct seed and embryo types have been defined (Baskin and  
200 Baskin, 2014; Martin, 1946) and their distinct compartments and tissues serve  
201 important roles during germination and seedling establishment. In the mature seeds  
202 of most angiosperm species the diploid embryo is enclosed by one or more layers of  
203 seed “coats” or coverings. These “coats” typically consist of a more or less abundant  
204 living triploid endosperm and a diploid dead maternal testa (seed coat) which both  
205 play key roles in the control of germination (Finch-Savage and Leubner-Metzger,  
206 2006; Weitbrecht *et al.*, 2011; Yan *et al.*, 2014). In cases where dry fruits are  
207 dispersed, the seed is in addition encased by pericarp (fruit coat) layers (Hermann *et*  
208 *al.*, 2007; Olsen, 2004; Psaras, 1984).

209 Mechanical properties of whole seeds or parts of seeds have mainly been examined  
210 in food science, especially the fracture toughness, impact damage, tensile and  
211 compression strength. Measurements have mainly been carried out with seeds or  
212 fruits of beans (Altuntaş and Yıldız, 2007; Bartsch *et al.*, 1986; Bay *et al.*, 1996;  
213 Davies and Zibokere, 2011; Fahloul *et al.*, 1996; Ogunjimi *et al.*, 2002; Ozturk *et al.*,  
214 2009; Shahbazi *et al.*, 2011), olives (Georget *et al.*, 2001; Kılıçkan and Güner, 2008),  
215 walnuts (Altuntas and Erkol, 2011; Altuntas and Özkan, 2008), sunflower (Gupta and  
216 Das, 2000), cumin (Saiedirad *et al.*, 2008) and wheat (Mabille *et al.*, 2001). In large  
217 parts, these measurements determined the influence of different moisture contents  
218 on the mechanical properties. In general, an increase in moisture content causes a  
219 decrease in fracture toughness. The major mechanical entities and associated



220 features which control seed germination are the properties of the seed/fruit coats, the  
221 endosperm weakening and the embryo growth potential.

222 The outer seed coverings consist mostly of dead tissues (testa, pericarp) and  
223 represent the seed's interface with the external environment. Their roles include  
224 protecting the embryo against adverse ambient conditions. In addition they serve a  
225 mechanical purpose in coat imposed seed dormancy to control germination timing  
226 (Bewley, 1997b; Debeaujon *et al.*, 2000; Kelly *et al.*, 1992; Werker, 1980). In many  
227 species a living layer of more or less abundant endosperm is interposed between  
228 these dead outer tissues and the embryo (Finch-Savage and Leubner-Metzger,  
229 2006; Yan *et al.*, 2014). In addition to providing mechanical restraint, coat-associated  
230 mechanisms of the endosperm, testa and/or pericarp are to control or even prevent  
231 water uptake, to interfere with leaching of inhibitors of embryo elongation such as  
232 abscisic acid (ABA), or gaseous exchanges which may cause oxygen deficiency  
233 within the embryo (Bewley and Black, 1994). It has for example been shown for  
234 *Lepidium sativum* seeds prior to testa/endosperm rupture that the testa and  
235 endosperm interfere with oxygen uptake required for ethylene production (Linkies *et*  
236 *al.*, 2009). The same is true for sugar beet fruits where the pericarp confers the major  
237 restraint (Hermann *et al.*, 2007).

238

### 239 **Biomechanics of embryo growth during seed germination**

240 Plant cells possess a rigid cell-wall which together with the turgor pressure from  
241 water uptake into the vacuole provides stability to the plant. In order to grow, the  
242 plant cells need to expand in a controlled manner. A good overview on the process is  
243 given in a review by Cosgrove (2005). The primary cell-walls of plants are  
244 presumably a nonlinear viscoelastic material which can expand plastically (Niklas,  
245 1992; Schopfer, 2006). The irreversible cell expansion is produced by creating a  
246 driving force for water uptake by decreasing the turgor through stress relaxation in  
247 the cell-wall (Fry, 2004; Schopfer, 2006). Upon cell wall loosening the polymers in the  
248 cell-wall move apart from each other (creep) and allow expansion growth of the cell  
249 due to water influx into the vacuole. Candidates proposed to be involved in the cell-  
250 wall loosening include expansins (Cosgrove, 2000a; Cosgrove, 2000b), xyloglucan  
251 endotransglycolases/hydrolases (Fry *et al.*, 1992; Van Sandt *et al.*, 2007), endo-(1,4)-  
252  $\beta$ -D-glucanases (Inukai *et al.*, 2012; Nicol *et al.*, 1998), as well as apoplastic reactive

253 oxygen species (aROS) (Müller *et al.*, 2009; Schopfer, 2001; Schopfer *et al.*, 2002).  
254 Upon imbibition of a quiescent seed the low water potential (“dry” state) causes rapid  
255 water uptake driven by the matrix potential (Schopfer, 2006; Weitbrecht *et al.*, 2011).  
256 The osmotic water uptake eventually leads to a turgid state, to the activation of the  
257 metabolism and to cell expansion growth in the embryo axis. Specific embryo growth  
258 zones have been identified (Bassel *et al.*, 2014; Sliwinska *et al.*, 2009). While this cell  
259 expansion growth is associated with endoreduplication, only the cell growth but not  
260 cell division is required for the embryo to complete germination through radicle  
261 emergence (Oracz *et al.*, 2012; Sliwinska *et al.*, 2009; Weitbrecht *et al.*, 2011). In  
262 order to complete germination the embryo growth potential must increase and  
263 exceed the restraint. The mechanism by which this occurs is through an increase in  
264 the embryo cell-wall extensibility which enables plastic rather than merely elastic wall  
265 extension, and by simultaneously decreasing the restraints of the embryo covering  
266 layers (Fig. 4). These changes are inhibited by ABA which thereby lowers the embryo  
267 growth potential and cell expansion growth (Da Silva *et al.*, 2008; Schopfer and  
268 Plachy, 1985) and inhibits the restraint weakening of the endosperm (Linkies and  
269 Leubner-Metzger, 2012; Müller *et al.*, 2006). Similar biochemical mechanisms in the  
270 cell-walls of micropylar endosperms are also underpinning endosperm weakening  
271 required for endosperm rupture during germination. However, cell separation  
272 (disrupting cell adhesion) and localised programmed cell death (PCD) are additional  
273 features of endosperm weakening (Bethke *et al.*, 2007; Morris *et al.*, 2011).

274

## 275 **Endosperm weakening in Asterid clade seeds and fruits**

276 In the case of endosperm-limited germination, the endosperm acts, at least in part, as  
277 a mechanical barrier for radicle protrusion (Linkies and Leubner-Metzger, 2012). It  
278 has been reported for many species that a decline in the mechanical resistance of  
279 the micropylar endosperm (the endosperm covering the radicle tip) appears to be a  
280 prerequisite for radicle protrusion (Table 1 and associated references). From a  
281 mechanistic point of view, seed germination is determined by the interaction of two  
282 antagonistic forces: the increase of the embryo growth potential and the decrease in  
283 the resistance of the covering layers (Fig. 4). The direct evidence for the endosperm  
284 weakening (PF↓ in Table 1) has been obtained by puncture force measurements, i.e.  
285 the direct quantification of the force needed for puncturing the micropylar endosperm

286 by a metal probe (Fig. 5). This was first achieved with larger seeds from the Asterid  
287 clade (Table 1), and was only recently accomplished with tiny (< 1 mm length)  
288 tobacco (*Nicotiana tabacum*, Solanaceae) seeds (Lee *et al.*, 2012). Mature tobacco  
289 seeds exhibit 3-5 layers of rather thick-walled living endosperm cells (Fig. 4B). The  
290 endosperm is enclosed by a thin testa, which consists of an outer layer of dead cells  
291 and a living inner parenchyma layer (Avery, 1933; Leubner-Metzger, 2003). Rupture  
292 of the testa (TR) and the endosperm (ER) are temporally well separated successive  
293 events during the germination of tobacco seeds (Arcila and Mohapatra, 1983;  
294 Leubner-Metzger *et al.*, 1995). The testa rupture starts near the funiculus and  
295 progresses along the ridges of the testa, leaving a dome-shaped endosperm  
296 structure covering the radicle. Tobacco is not only the smallest seed for which  
297 endosperm weakening was directly quantified by the puncture force method (Lee *et al.*,  
298 2012), but also the smallest seed for which the spatiotemporal patterns of water  
299 uptake were investigated by <sup>1</sup>H-nuclear magnetic resonance (NMR) microimaging  
300 (Manz *et al.*, 2005). This non-destructive method revealed a non-uniform water  
301 uptake and distribution as the micropylar end of the seed is the major entry point of  
302 water. Micropylar endosperm and the radicle show the highest water content in the  
303 TR stage prior to ER (Fig. 4A). The spatial analysis even revealed that already prior  
304 to TR these compartments have a significantly higher water content compared to the  
305 non-micropylar endosperm and the cotyledons. It is therefore obvious to assume that  
306 the processes associated with the tobacco seed's late TR stage also include  
307 biomechanical and biochemical cell-wall alterations.

308 To investigate the underpinning biomechanical mechanisms of tobacco endosperm  
309 weakening, comparative puncture force analysis of the micropylar endosperm (ME)  
310 and the chalazal endosperm (CE) were conducted (Lee *et al.*, 2012). To achieve this  
311 with such a tiny seed as tobacco a thin needle and a special sample holder filled with  
312 water is required (Fig. 5B). Figure 6 shows that TR is associated with a significant  
313 decrease in ME resistance which coincides with TR. A further decrease in ME  
314 resistance was just prior to ER. Most strikingly, this TR-associated endosperm  
315 weakening was only evident in the ME. In contrast to the ME, there was no significant  
316 endosperm weakening in the CE associated with TR, and the slight decrease in CE  
317 resistance just prior to ER was considerably smaller compared to the ME (Fig. 6).  
318 The major conclusion from this is that the mature tobacco seed exhibits an  
319 endosperm polarity in which the ME and CE have distinct roles: The CE does not

320 weaken to serve as “anchor” or “holding structure” for the embryo to support that the  
321 elongation growth due to the increasing embryo growth potential is directed towards  
322 the micropylar seed end. The ME weakens, at least partially, by biochemical cell-wall  
323 changes allowing enhanced water uptake into the embryonic axis growth zone cells.  
324 Also allowing ER and radicle protrusion at a defined location, namely at the  
325 weakened ME (Fig. 6). The ME weakening is therefore a key biomechanical and  
326 biochemical process which controls tobacco germination timing.

327 In agreement with this conclusion, microscopic studies showed that storage reserves  
328 are degraded in the ME cells prior to ER and to radicle protrusion (Arcila and  
329 Mohapatra, 1983; Leubner-Metzger *et al.*, 1995). The microscopy also shows that the  
330 endospermic hole which is always formed at the micropylar end of the germinating  
331 tobacco seed, has a smooth outline and therefore seems to result from biochemical  
332 tissue dissolution rather than from the pushing action of the protruding radicle. These  
333 processes leading to ER and radicle emergence require transcription and translation  
334 (Arcila and Mohapatra, 1992). The endosperm cell-walls of solanaceous seeds are  
335 known to be rich in mannan ( $\beta$ -1,4-linked D-mannose) and heteromannans (gluco-  
336 and galactomannans, glucose or galactose  $\alpha$ -1,6-linked to the main mannan chain)  
337 (Bewley, 1997a; Buckeridge, 2010; Lee *et al.*, 2012; Morris *et al.*, 2011; Rodríguez-  
338 Gacio *et al.*, 2012). These cell-wall mannans are rigidity- and mechanical strength-  
339 conferring cross-linking hemicellulosic matrix polysaccharides. In some species they  
340 serve as endosperm storage reserves, and due to their viscosity and solubility in  
341 water may also have roles during seed imbibition. In *Solanum* spp. seeds (Table 1)  
342 the second step of the biphasic ME weakening is controlled by ABA and is  
343 associated with endo- $\beta$ -1,4-mannanase accumulation in the ME (Gong and Derek  
344 Bewley, 2007; Nonogaki *et al.*, 2000; Pinto *et al.*, 2007; Toorop *et al.*, 2000). The  
345 hypothesis that hydrolytic enzyme accumulation in the ME is required for endosperm  
346 weakening and radicle protrusion was first proposed by Ikuma and Thimann (1963).  
347 Tobacco endosperm monosaccharide linkage analysis of neutral sugars shows that  
348 ca. 65% are heteromannans (>90% of these constitute  $\beta$ -1-4-mannan linkages) (Lee  
349 *et al.*, 2012). In situ localisation of heteromannan cell-wall epitopes by  
350 immunofluorescence microscopy using a specific antibody demonstrated that  
351 heteromannan was specifically degraded in the ME at TR, but not at earlier time  
352 points and not in the CE (Fig. 6). This spatiotemporal heteromannan-degradation  
353 pattern in the ME cell-walls suggests that endo- $\beta$ -1,4-mannanase accumulation in the

354 ME contributes to the ME weakening during tobacco seed germination (Fig. 6). Other  
355 cell-wall hydrolases, including endo- $\beta$ -1,3-glucanase, were also proposed to  
356 contribute to ME weakening (Leubner-Metzger *et al.*, 1995; Leubner-Metzger and  
357 Meins, 2000; Manz *et al.*, 2005). To further study endosperm weakening tobacco is  
358 an ideal Asterid system due to the separate TR and ER, and because it has  
359 abundant endosperm and a straight embryo, which make it structurally a typical and  
360 simple system with a clearly expressed endosperm polarity.

361 In lettuce (*Lactuca sativa*, Asteraceae) fruits the embryo is completely enclosed by a  
362 living endosperm composed of 2-3 cell layers which is a mechanical constraint to  
363 embryo growth and the completion of germination (Bewley, 1997a; Halmer *et al.*,  
364 1975; Ikuma and Thimann, 1963). In the intact lettuce fruit (achene) the embryo and  
365 endosperm are enclosed by a testa (seed coat) and pericarp (fruit coat) covering  
366 (Fig. 7). Lettuce micropylar (ME) and chalazal (CE) endosperm cell-walls differ  
367 considerably in their composition. Indirect biomechanical measurements showed that  
368 lettuce endosperm weakening precedes endosperm rupture in the light, but not in  
369 darkness (photoinhibition) and gibberellin (GA) treatment can replace the light to  
370 induce endosperm weakening (Tao and Khan, 1979). To conduct the biomechanical  
371 work on lettuce these authors used an indirect measurement method of the forces,  
372 i.e. by calculating them as the difference between puncturing embryo plus  
373 endosperm and embryo alone, perpendicular to the seed axis of radicle elongation.  
374 As a technical advance, Zhang *et al.* (2014) provided a new method to measure  
375 solely the endosperm using adhesive tape to hold the soft and delicate endosperm  
376 tissue in place (Fig. 7B, C). A decrease in the ME puncture force was evident in  
377 association with endosperm rupture while the CE did not weaken (Zhang *et al.*,  
378 2014). Further to this, ABA inhibits and ethylene promotes the lettuce endosperm  
379 weakening and ER (Fig. 7C) (Chen *et al.*, 2016; Zhang *et al.*, 2014).

380 A crucial role of hormonal regulation of endosperm weakening and cell-wall  
381 remodelling during lettuce germination in light and temperature responses was  
382 established (Bewley, 1997a; Chen *et al.*, 2016; Huo *et al.*, 2013). The endosperm  
383 weakening precedes the completion of lettuce germination by typical ER and radicle  
384 emergence (Fig. 7A). If the endosperm weakening is inhibited by treatment of lettuce  
385 seeds with sodium dichloroisocyanurate (SDIC) the embryo expands but cannot  
386 protrude through the endosperm (Pavlišta and Haber, 1970). Thus the embryo starts

387 to buckle within its hull and may eventually germinate despite an atypical ER (Fig.  
388 7A). Lettuce endosperm cell-walls contain L-arabinofuranose and evidence was  
389 provided to propose that  $\alpha$ -L-arabinofuranosidase accumulates and causes the  
390 endosperm weakening during lettuce germination (Liu *et al.*, 2015; Zhang *et al.*,  
391 2014). SDIC treatment inhibited the enzyme accumulation in association with  
392 inhibited endosperm weakening. SDIC was also instructive to establish a role for  
393 apoplastic reactive oxygen species (aROS) in lettuce endosperm weakening as well  
394 as in lettuce embryo expansion growth (Zhang *et al.*, 2014). Further to this, the  
395 accumulation of cellulase activity in the lettuce ME and its regulation by ABA and  
396 ethylene was proposed to play a role in both processes (Chen *et al.*, 2016; Zhang *et al.*,  
397 2014). The current findings from various endospermic species from the Asterid  
398 clade (Table 1) therefore support the view that endosperm weakening resulting in a  
399 decreased ME resistance as quantified by puncture force analysis is mediated  
400 through the combined or successive action of several cell-wall-modifying hydrolases,  
401 transglycolases, expansins and directly acting aROS. While biochemical mechanisms  
402 mediating cell-wall loosening such as aROS seem to be shared between embryo  
403 expansion growth and endosperm weakening, the differences in cell-wall composition  
404 and the spatiotemporal accumulation patterns of specific cell-wall modifying proteins  
405 or aROS may provide in addition cell separation as a hallmarks of the endosperm  
406 weakening process (Bethke *et al.*, 2007; Lee *et al.*, 2012; Morris *et al.*, 2011).

407

#### 408 **Endosperm weakening and embryo growth in Rosid clade seeds**

409 While there are, besides tobacco, tomato and lettuce several other species from the  
410 Asterid clade for which endosperm weakening has been directly demonstrated by the  
411 puncture force method (Table 1), garden cress (*Lepidium sativum*, Brassicaceae) has  
412 emerged as an established model system for endosperm weakening in the Rosid  
413 clade (Linkies and Leubner-Metzger, 2012). There is in addition plenty of indirect  
414 evidence in strong support for the view that endosperm weakening is a widespread  
415 phenomenon in the Rosid clade and also, for example, controls *Arabidopsis thaliana*  
416 seed germination (Bethke *et al.*, 2007; Denay *et al.*, 2014; Linkies *et al.*, 2009; Müller  
417 *et al.*, 2006; Penfield *et al.*, 2006; Scheler *et al.*, 2015; Yang *et al.*, 2008). This  
418 includes microscopically visible early reserve breakdown in the ME including  
419 vacuolation of protein storage vacuoles which is promoted by GA and inhibited by

420 ABA (Bethke *et al.*, 2007), altered seed germination and dormancy responses of  
421 mutants and transgenic lines (Bentsink and Koornneef, 2008; Debeaujon *et al.*, 2000;  
422 Denay *et al.*, 2014), as well as local cell separation at the site radicle protrusion in the  
423 *A. thaliana* ME (Bethke *et al.*, 2007). Scarification (“embryo rescue”) by removing the  
424 testa and endosperm, results in embryo growth from dormant *A. thaliana* seeds  
425 (Graeber *et al.*, 2014). Figure 8 shows that the endosperm is sufficient to prevent  
426 germination when the testa is removed from dormant *A. thaliana* seeds (Bethke *et al.*,  
427 2007). Treatment with dormancy releasing compounds induces endosperm rupture  
428 and radicle emergence (Fig. 8D). This demonstrates that the physiological dormancy  
429 (PD) of *A. thaliana* seeds is coat dormancy imposed by the endosperm (Bethke *et al.*,  
430 2007) and the testa (Debeaujon *et al.*, 2000). Both species, *A. thaliana* and *L.*  
431 *sativum*, have, as lettuce, a thin living endosperm encasing the embryo, its 1 and 2-3  
432 cell layers respectively (Bethke *et al.*, 2007; Müller *et al.*, 2006). Besides seed size, a  
433 major difference between the two species is that while *A. thaliana* seeds have PD, *L.*  
434 *sativum* are non-dormant (ND), they belong to the ND class of seed dormancy (Willis  
435 *et al.*, 2014). Overexpression of the *A. thaliana* dormancy gene DOG1 resulted in  
436 establishing PD in transgenic *L. sativum* seeds (DOG1-OE in Fig. 8). This PD of  
437 DOG1-OE *L. sativum* seeds is coat dormancy imposed by the altered endosperm,  
438 the excised embryos grow and exhibit no difference in their embryo growth potential  
439 when compared to the wild type (Graeber *et al.*, 2014). The physiological coat  
440 dormancy therefore means that it prevents the progression of endosperm weakening  
441 in the DOG1-OE *L. sativum* seeds (Fig. 8C). The endosperm weakening in ND *L.*  
442 *sativum* wild-type seeds has roles in regulating the speed, uniformity, and response  
443 of seed germination towards environmental cues.

444 For *Lepidium sativum* (Morris *et al.*, 2011) and *Lactuca sativa* (Dutta *et al.*, 1994)  
445 incubation of weakening-induced isolated endosperms undergo hormonally regulated  
446 cell-wall autolysis and eventually may form a hole in the ME. The possible relation of  
447 the cell-wall autolysis to endosperm weakening is supported by its hormonal  
448 regulation, and for the cell-wall autolysis it is clear that transcription and translation  
449 are both required (Morris *et al.*, 2011). Due to its large seed size, direct  
450 measurements of different seed compartments by the puncture force method are  
451 possible, while direct puncture force measurements of the closely related tiny  
452 *Arabidopsis* seed have not yet been achieved. Direct biomechanical measurement of  
453 *L. sativum* endosperm weakening by the puncture force method demonstrated that

454 an early signal from the embryo is required to induce it (Müller *et al.*, 2006). When  
455 MEs were isolated very early during imbibition, i.e. prior to their induction (for *L.*  
456 *sativum* before 5h), they did not weaken. When however, 8h-isolated MEs were  
457 incubated further, the weakening, hole formation and autolysis proceeded in an  
458 organ-autonomous process (Linkies *et al.*, 2009; Morris *et al.*, 2011; Müller *et al.*,  
459 2006). Further experimentation has shown that in isolated *L. sativum* MEs, GA can  
460 replace the embryo signal, that de novo GA biosynthesis occurs in the endosperm,  
461 and that the weakening is regulated, at least in part, by the GA-ABA ratio. Treatment  
462 of seeds with ABA caused a delayed onset and slower rate of ME weakening. The  
463 ER of seeds without and with ABA treatment exhibited a very similar relationship to  
464 the decreasing ME puncture force (Linkies *et al.*, 2009). While the absolute puncture  
465 force values differed by a factor two between the ME resistances of two *L. sativum*  
466 cultivars at 8h, a similar ca. two-fold relative reduction in the resistance was evident  
467 at 18h, and this ME weakening was in both cases inhibited by ABA (Graeber *et al.*,  
468 2010). As GA, also ethylene promotes *L. sativum* ME weakening and counteracts the  
469 ABA inhibition. Ethylene signalling is required and during the late phase of  
470 germination the oxygen-requiring production of ethylene from its precursor 1-  
471 aminocyclopropane-1-carboxylic acid (ACC) by ACC oxidase (ACO) activity  
472 accumulation enhances the progression of ER (Linkies *et al.*, 2009) These findings  
473 for the hormonal regulation of *L. sativum* ME weakening are summarised in Figure  
474 8E and in a review by Linkies and Leubner-Metzger (2012).

475 The endosperm cell-wall composition of the Brassicaceae *L. sativum* and *A. thaliana*  
476 indicated conserved architectures with cellulose, unesterified homogalacturonan and  
477 arabinan being major components (Lee *et al.*, 2012). In contrast to the endosperm of  
478 Solanaceae seeds which are rich in heteromannans (ca. 65% in tobacco), the  
479 endosperm of *L. sativum* contains only 3.5% heteromannans (Lee *et al.*, 2012).  
480 Despite the low heteromannan content regulated endo- $\beta$ -1,4-mannanase gene  
481 ortholog expression was evident in the endosperm of *L. sativum* and *A. thaliana* and  
482 together with the knockout-mutants are in agreement with roles during germination  
483 (Iglesias-Fernández *et al.*, 2011; Morris *et al.*, 2011). The spatiotemporal regulation  
484 of their gene expression and possible roles in *L. sativum* and *A. thaliana* endosperm  
485 weakening of cell-wall remodelling proteins targeting the cellulose microfibrils or the  
486 matrix polysaccharides in which they are embedded, namely hemicelluloses and  
487 pectins, is described in detail in Morris *et al.* (2011) and Scheler *et al.* (2015). Recent



488 work by Graeber *et al.* (2014) shows that GA metabolism itself and the expression of  
489 GA-regulated cell-wall remodelling genes including expansins and xyloglucan  
490 endotransglycolases/hydrolases are severely altered in DOG1-OE *L. sativum* seeds  
491 (Fig. 8). The DOG1-overexpression did not result in an altered embryo growth  
492 potential, but blocked ME weakening in a temperature-dependent manner.

493 That the endosperm is a mediator of communication between the embryo and its  
494 environment has been summarised by Yan *et al.* (2014). In *L. sativum* DOG1 exerts  
495 its temperature-dependent control of germination timing exclusively via the control of  
496 ME weakening: In DOG1-OE *L. sativum* the weakening occurs at 18°C, but is  
497 inhibited at 24°C (Graeber *et al.*, 2014). Interestingly, thermoinhibition of wild-type *L.*  
498 *sativum* seeds is also mediated by inhibiting ME weakening (Fig. 8E). In addition to  
499 temperature as an abiotic environmental cue, biotic environmental cues such as the  
500 allelochemical myriganone A (MyA) also exerts germination-inhibiting effects, at least  
501 in part, by inhibiting ME weakening (Fig. 8E). As for DOG1-overexpression, MyA has  
502 the seed's GA metabolism as a target (Oracz *et al.*, 2012; Voegelé *et al.*, 2012). In  
503 addition to this MyA also interferes with the production of aROS required to mediate  
504 embryo expansion growth and ME weakening. Figure 9 shows that aROS is  
505 produced in the growth zone (hypocotyl/radicle) of the *L. sativum* embryo and this  
506 production is inhibited by ABA and promoted by GA and ethylene (Linkies *et al.*,  
507 2009; Müller *et al.*, 2009). While ABA inhibits the ME weakening, the artificial  
508 production of aROS in the presence of ABA caused endosperm weakening (Figs. 8E,  
509 9). Müller *et al.* (2009) showed that aROS-mediated germination is caused by direct  
510 scissoring of cell-wall polysaccharides. Distinct and tissue-specific target  
511 polysaccharides were evident, and the hormonally regulated aROS production serves  
512 important roles in embryo expansion growth and in ME weakening.

513

## 514 **Biomechanics of cereal grain endosperm weakening and germination**

515 A mature cereal grain is a single-seeded fruit (caryopsis) with several major  
516 compartments and bran tissues (Burton and Fincher, 2014; Domínguez and Cejudo,  
517 2014; Fath *et al.*, 2000). The highly differentiated embryo is, with its scutellum, in  
518 direct proximity of the large starchy endosperm storage compartment (dead tissue)  
519 which is encased by the aleurone layer (living endosperm tissue) and the dead bran  
520 layers (testa and pericarp tissues). *In vivo* 1H-NMR microimaging during cereal grain

521 imbibition suggests several preferred pathways for water uptake which include the  
522 micropyle as an opening, the embryo and scutellum as water-distribution organs, and  
523 parts of the bran layers which allow fast water uptake during the very early phases of  
524 wheat imbibition (Rathjen *et al.*, 2009). The ratio between the hormones ABA  
525 (inhibiting) and GA (promoting) control germination and post-germination reserve  
526 mobilisation of cereal grains in which GA serves as a signal produced by the embryo  
527 to induce the aleurone layer to express and/or secrete hydrolytic enzymes into the  
528 starchy endosperm (Burton and Fincher, 2014; Domínguez and Cejudo, 2014; Fath  
529 *et al.*, 2000). In agreement with this role the cereal aleurone is a living tissue layer of  
530 the wheat grain, but undergoes PCD during germination and seedling establishment.  
531 Tensile tests have been carried out to determine the mechanical properties of the  
532 various wheat grain bran layers (Antoine *et al.*, 2003). In agreement with these  
533 observations and the PCD of the aleurone layer during germination and starch  
534 mobilisation, we recently showed by puncture force measurements that GA treatment  
535 of isolated aleurone layers promotes the weakening of this living endosperm tissue,  
536 while GA does not affect the dead intermediate (testa and inner pericarp) layers of  
537 wheat grains (Hourston *et al.*, unpublished). Novel tools are required to further  
538 investigate the biomechanical changes of cereal grain tissues including the  
539 coleorhiza covering the radicle for which a similar ABA-regulated role for dormancy  
540 and germination timing as for the eudicot seed ME (Millar *et al.*, 2006).

541

542 *Mechanosensing in seeds*

543 Sensing mechanical forces to control gene expression, tissue growth and fate is an  
544 essential part of plant life (Monshausen and Haswell, 2013). We propose that seeds  
545 constitute an excellent system for studying mechanosensing due to the striking  
546 interactions between seed covering layers and the distinct fates either leading to  
547 growth (embryo) or to death (ME) of tissues. Mechanical signalling involved in seed  
548 coat expansion has been postulated by Creff *et al.* (2015). Their study with *A.*  
549 *thaliana* seeds showed that mechanical stress exerted by the embryo and  
550 endosperm is perceived in a mechanosensitive layer in the seed coat. Recently  
551 nano-indentation has been used to measure the stiffness of the endosperm of  
552 developing *A. thaliana* seeds (Fourquin *et al.*, 2016). A stiffer endosperm was found  
553 in *zou* mutants compared to wild-type seeds and embryo growth was inhibited as the  
554 stiff covering layer presumably prevents its expansion (Fourquin *et al.*, 2016; Yang *et*  
555 *al.*, 2008). In agreement with the postulation of these mechanosensitive tissues is the  
556 “touch”-gene hypothesis (Monshausen and Gilroy, 2009; Nonogaki, 2013) stating that  
557 the induction of ME gene expression is caused by the pushing force of the elongating  
558 radicle. This could be in an interplay with their hormonal regulation. Among the  
559 “touch”-genes are those encoding cell wall remodelling proteins such as expansins.  
560 Direct evidence for the ME mechanosensing and signalling of this gene induction in  
561 seeds is however still lacking. Furthermore, seed osmosensing and signalling and its  
562 interplay with plant hormones might play a key role during germination, as the water  
563 uptake and the water content plays major roles in seed germination for the  
564 mechanical properties of cell walls. The combination of molecular and biomechanical  
565 work is promising to unravel the underpinning mechanisms of the germination  
566 process and the endosperm weakening. Unravelling the complex regulation of seed  
567 germination and its molecular basis to understand the cell-wall related changes in  
568 tissue mechanics in a manifold of species and with integrative approaches is needed  
569 to gain a comprehensive view on the germination process. Despite a strong  
570 enthusiasm to understand the vital process of seed germination there are still open  
571 questions (Nonogaki *et al.*, 2010). The acquired evidence reveals that endosperm  
572 weakening involves evolutionary conserved as well as species-specific molecular,  
573 biochemical and biomechanical mechanisms. These mechanisms have the  
574 endosperm cell-wall properties as target and strongly suggest that further integrative  
575 and interdisciplinary studies with several seeds from distinct phylogenetic clades are

576 required. The consideration of crop seeds in these future studies is of utmost  
577 relevance to seed industry. It also extends the investigations of the biomechanical  
578 seed properties of the natural seed “coats” to artificial seed “coats” and the  
579 mechanical properties of pellet materials.

580

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586

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Table 1: Endosperm weakening in the Angiosperm clades. Puncture force measurements: PF↓ = Endosperm weakening (EW); GA↓ = EW promoted by GA; Ethylene↓ = EW promoted by ethylene; ACC↓ or ethephon↓ = EW promoted by ACC or ethephon (via conversion to ethylene); ABA↑ = EW inhibited by ABA; \*OH↓ EW promoted by apoplastic reactive oxygen species (aROS);

<b>Rosid clade:</b>	Cucurbitaceae	<i>Cucumis</i>	PF↓ (perisperm)	Welbaum, 1999; Welbaum <i>et al.</i> , 1995; Yim and Bradford, 1998
	Brassicaceae	<i>Lepidium</i>	PF↓ GA↓ Ethylene↓ ACC↓ ABA↑ *OH↓	Graeber <i>et al.</i> , 2010; 2014; Linkies <i>et al.</i> , 2009; Morris <i>et al.</i> , 2011; Müller <i>et al.</i> , 2006; 2009; Oracz <i>et al.</i> , 2012; Voegele <i>et al.</i> , 2012
		<i>Arabidopsis</i>		Bethke <i>et al.</i> , 2007; Creff <i>et al.</i> , 2015; Fourquin <i>et al.</i> , 2016
<b>Asterid clade:</b>	Oleaceae	<i>Syringa</i>	PF↓	Junttila, 1973
		<i>Fraxinus</i>	PF↓ GA↓	Finch-Savage and Clay, 1997
	Solanaceae	<i>Solanum</i>	PF↓ GA↓ ABA↑ Priming↓	Anese <i>et al.</i> , 2011; Chen and Bradford, 2000; Groot and Karssen, 1987, 1992; Pinto <i>et al.</i> , 2007; Toorop <i>et al.</i> , 2000; Wu <i>et al.</i> , 2000
		<i>Capsicum</i>	PF↓ GA↓	Petruzzelli <i>et al.</i> , 2003; Watkins and Cantliffe, 1983
		<i>Datura</i>		Arana <i>et al.</i> , 2005; 2007

		<i>Nicotiana</i>	PF↓	Lee <i>et al.</i> , 2012; Leubner-Metzger, 2003
		<i>Petunia</i>		Petruzzelli <i>et al.</i> , 2003
	Rubiaceae	<i>Coffea</i>	PF↓ GA↓ ABA↑	da Silva <i>et al.</i> , 2004; 2005
		<i>Genipa</i>	PF↓ ABA↑	Queiroz <i>et al.</i> , 2012
	Asteraceae	<i>Lactuca</i>	PF↓ GA↓ *OH↓ Etephon↓	Chen <i>et al.</i> , 2016; Tao and Khan, 1979; Zhang <i>et al.</i> , 2014
<b>Monocots:</b>	Iridaceae	<i>Iris</i>	PF↓	Blumenthal <i>et al.</i> , 1986
	Poaceae	<i>Triticum</i>	PF↓ GA↓ ABA↑	Benech-Arnold, 2004; Hourston <i>et al.</i> , unpublished

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## Figure legends:

**Fig. 1.** Stress strain curves illustrating different types of material behaviour. For an elastic behaviour, loading and unloading paths coincide (no energy lost). Elastic-plastic materials undergo a non-reversible plastic deformation after a threshold is reached, while the unloading includes elastic elements. Plastic materials undergo a non-reversible deformation. Energy is lost during the deformation and correspond to the area underneath the curve. Viscoelastic materials show a time-dependent behaviour and dissipate energy during loading/unloading. The amount of energy absorbed by the material is equal to the area between the loading and unloading curve (hysteresis).

**Fig. 2.** Schematic diagram showing typical stress–strain curves. (A) The material exhibits an elastic and plastic region. Several key parameters can be derived from the diagram: Elastic Modulus  $E$ , Yield strength (point of elastic limit) and the maximum strength of the material. (B) Typical curves for stiff, strong, weak or flexible materials.

**Fig. 3.** Material property chart plotting Young's modulus  $E$  against density  $\rho$ . The heavy envelopes enclose data for a given class of material. The guidelines of constant  $E/\rho$ ,  $E^{1/2}/\rho$  and  $E^{1/3}/\rho$  allow to identify structurally efficient materials which are light and stiff (after Ashby (2007); Ashby *et al.* (2013). Properties for seeds inserted as determined by Walters *et al.* (2010).

**Fig. 4.** (A) Noninvasive in vivo  $^1\text{H}$ -nuclear magnetic resonance (NMR) microimaging analysis of water uptake and distribution during tobacco seed germination. The spatial distribution of proton mobility within the seed tissues is visualised by false colours (relative scales from zero [0, black] to maximum signal strength [max, white]). Microimages of the testa rupture stage are shown with a resolution of approx.  $30\ \mu\text{m}$  (after Manz *et al.* (2005)). (B) Seed structure of tobacco (*Nicotiana tabacum*). (C) Schematic of the micropylar endosperm (ME) and the radicle tip of a tobacco seed. Gibberellins (GA) promote the induction of cell wall hydrolases, expansins and apoplastic reactive oxygen species (aROS), thereby promoting endosperm weakening and endosperm rupture. Abscisic acid (ABA) inhibits the induction of cell wall hydrolases and aROS, thereby inhibiting endosperm weakening and endosperm rupture. GA promotes and ABA inhibits the embryo growth potential.

**Fig. 5.** Puncture force device to measure endosperm weakening. (A) Example of a custom-made puncture force machine consisting of a force and displacement (metering axis) sensor, a camera, LED lights and a xy positioning stage. A measuring tip (needle) with chosen tip diameters / geometry is driven into the sample while force and displacement were recorded. (B) Example of a sample holder for tobacco seeds (schematic and photograph). Tobacco seeds were cut in half and the embryo and testa removed, which left the empty but intact endosperm into which the metal probe could be lowered. Delicate material is kept hydrated by adding water to the sample holder.



**Fig. 6.** Time course analyses of endosperm weakening and germination kinetics of *Nicotiana tabacum*. The micropylar (ME) and chalazal (CE) endosperm weakening and rupture of seeds are shown over time. The weakening was determined by measuring the tissue resistance via puncture force measurements at the times indicated. Testa rupture (TR) begins at 28 h, and endosperm rupture (ER) at 60 h, respectively. *In situ* localization of cell wall epitopes in longitudinal sections of tobacco seeds. LM21 HM binds to abundant heteromannans in the endosperm. The immunolabeling of germinating tobacco seeds with LM21 HM revealed a specific degradation of heteromannan (HM) at the micropylar endosperm (ME) after testa rupture. Calcofluor White is a non-specific fluorochrom that binds to cellulose in cell walls and was used as control. R, radicle; C, cotyledons; T, testa; PE peripheral endosperm; Bars = 50  $\mu$ m. Modified from Lee *et al.* (2012).

**Fig. 7.** Lettuce (*Lactuca sativa*) endosperm weakening and germination. (A) Lettuce fruit/seed morphology, endosperm rupture, and seedling growth. Typical and atypical endosperm rupture (buckling) is shown. Typically the endosperm is ruptured at the micropylar end of the endosperm. Rarely or if endosperm weakening is prevented lettuce shows atypical endosperm rupture. (B) Puncture force method for lettuce. The lettuce endosperm is placed on top of a thin steel needle and is lowered (punctured) through adhesive tape. (C) The endosperm weakening of the micropylar and the chalazal endosperm is shown versus time. The micropylar endosperm (ME) shows a weakening during germination. The force to rupture the ME is lowered by the addition of ethephon, an ethylene-releasing compound, and the weakening is inhibited by ABA. The chalazal endosperm (CE) shows a higher resistance compared to the ME and does not appreciably weaken (water). Treatment with sodium dichloroisocyanurate (SDIC) causes an initial CE stiffening which is weakened during imbibition. Note that SDIC treatment is associated with the inhibition of ME weakening and with embryo buckling. B and C modified from Zhang *et al.* (2014) and Chen *et al.* (2016).

**Fig. 8.** Coat-imposed dormancy and control of Brassicaceae germination timing by the endosperm. (A) Image analysis of *Lepidium sativum* embryo growth (after Voegelé *et al.*, 2012). (B) Embryo growth potential and (C) micropylar endosperm weakening of *L. sativum* wild type and a transgenic line overexpressing the DOG1 dormancy gene (DOG1-OE, after Graeber *et al.*, 2014). (D) Endosperm-mediated coat dormancy of *Arabidopsis thaliana* seeds revealed by testa removal (after Bethke *et al.*, 2007). (E) Summary of control of *L. sativum* germination timing by micropylar endosperm weakening. Note that *L. sativum* wild type seeds are non-dormant, but that DOG1-OE establishes physiological dormancy mediated by the inhibition of endosperm weakening. The regulation of *L. sativum* wild type seed endosperm weakening by abiotic (temperature) and biotic (allelochemical) factors as well as by hormones and apoplastic reactive oxygen species is presented.

**Fig. 9.** Accumulation of apoplastic reactive oxygen species (aROS) during *Lepidium sativum* germination (adapted from Müller *et al.* 2009). (A) Apoplastic superoxide ( $O_2^{\cdot -}$ ) in the embryos and the micropylar endosperm of seeds imbibed in continuous white light. NBT (nitroblue tetrazolium) histostaining shows production of apoplastic  $O_2^{\cdot -}$ . (B) In vivo detection of apoplastic  $\cdot OH$  production in the micropylar endosperm (ME) and the radicle of *L. sativum* during seed germination without and with ABA added. Note the different scales of the y axes for the ME and the radicle.

Figures:

Figure 1

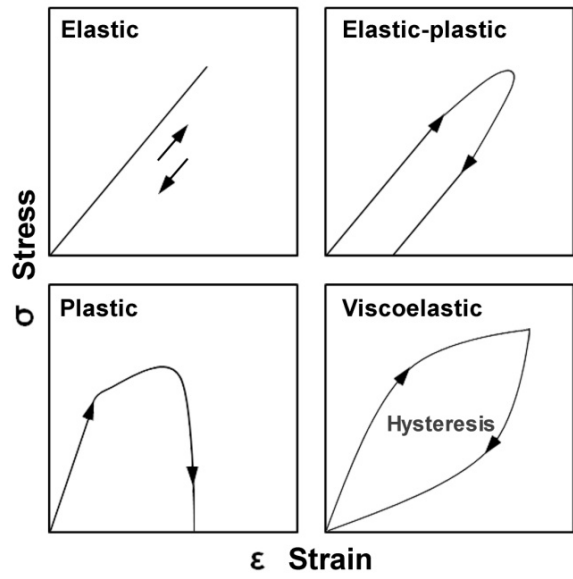


Figure 2

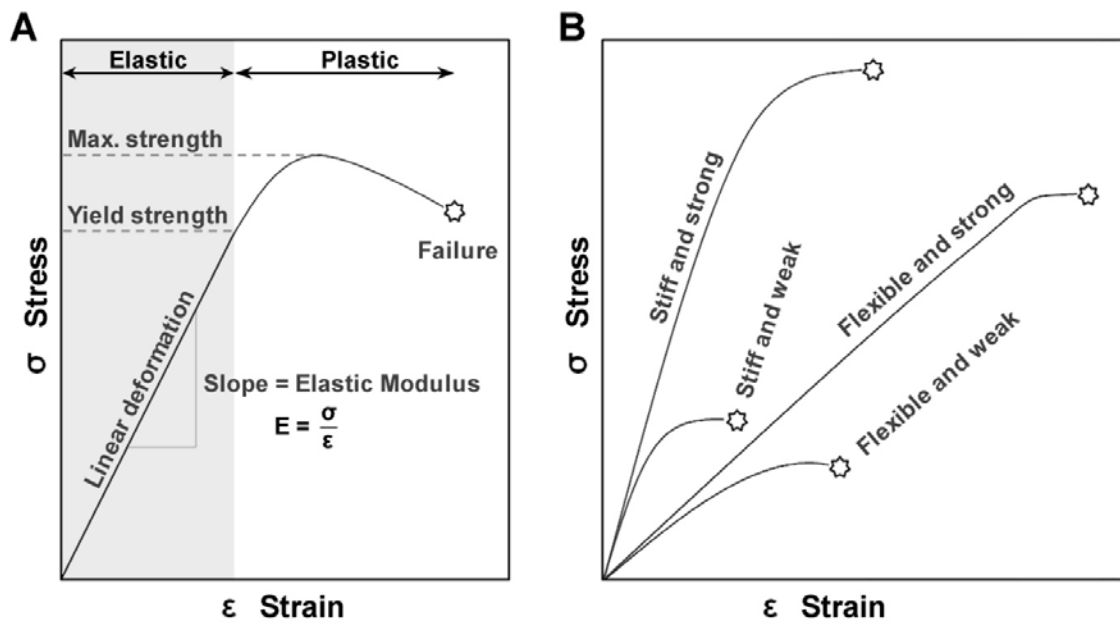


Figure 3

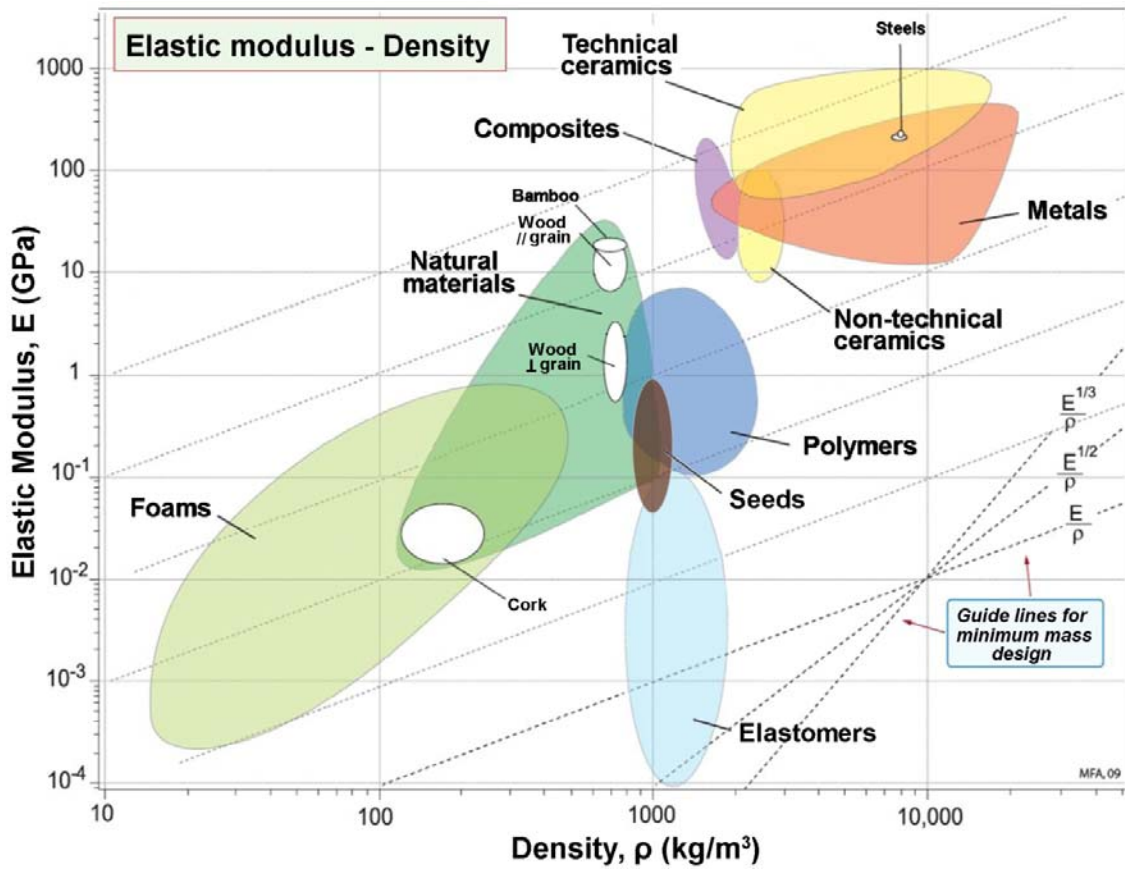


Figure 4

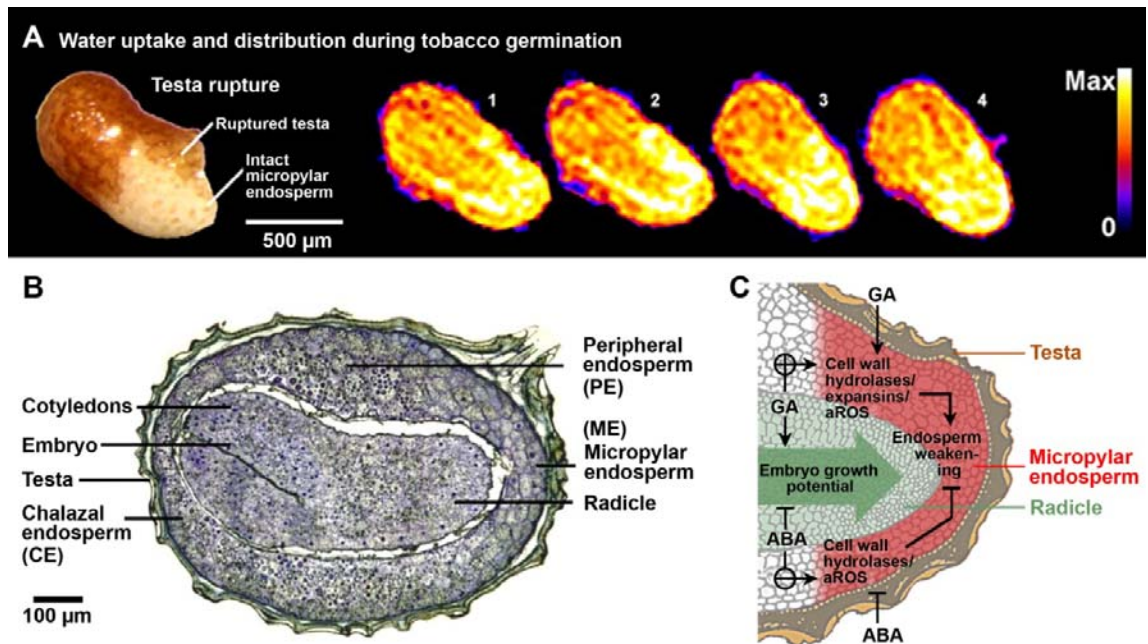


Figure 5

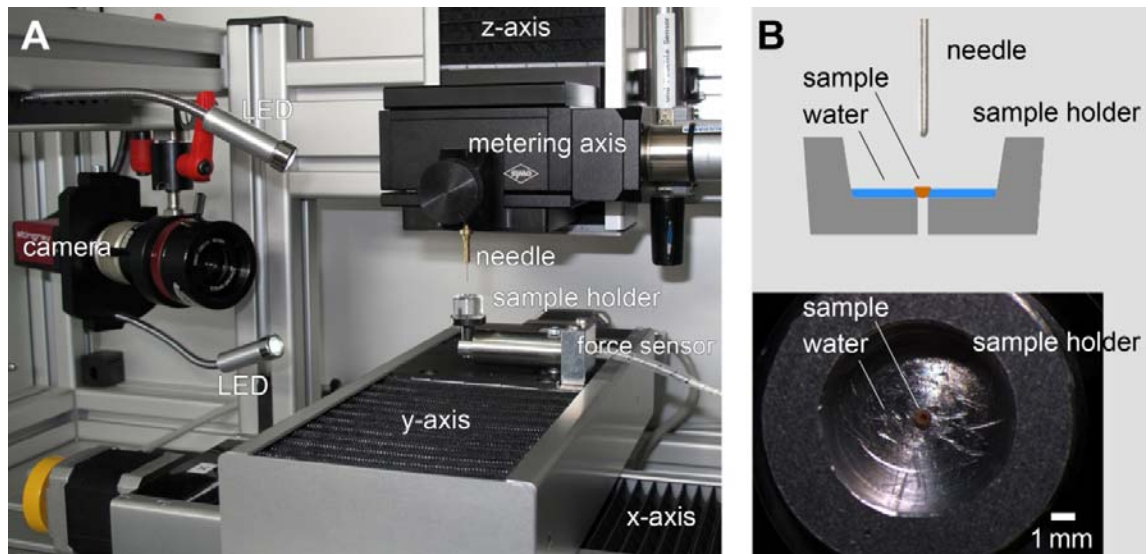


Figure 6

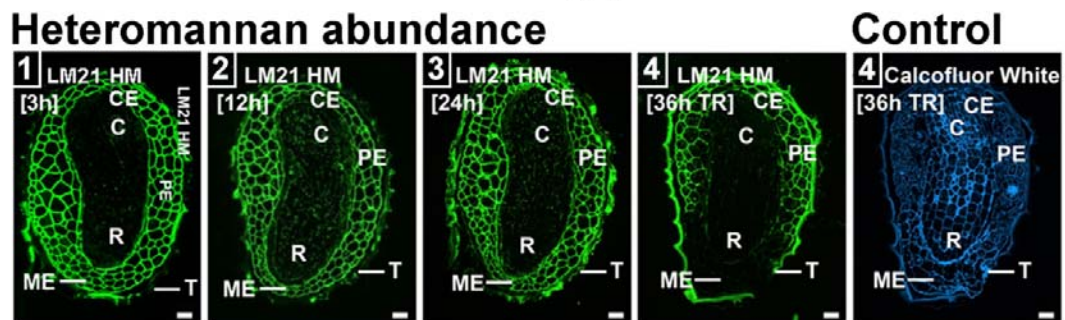
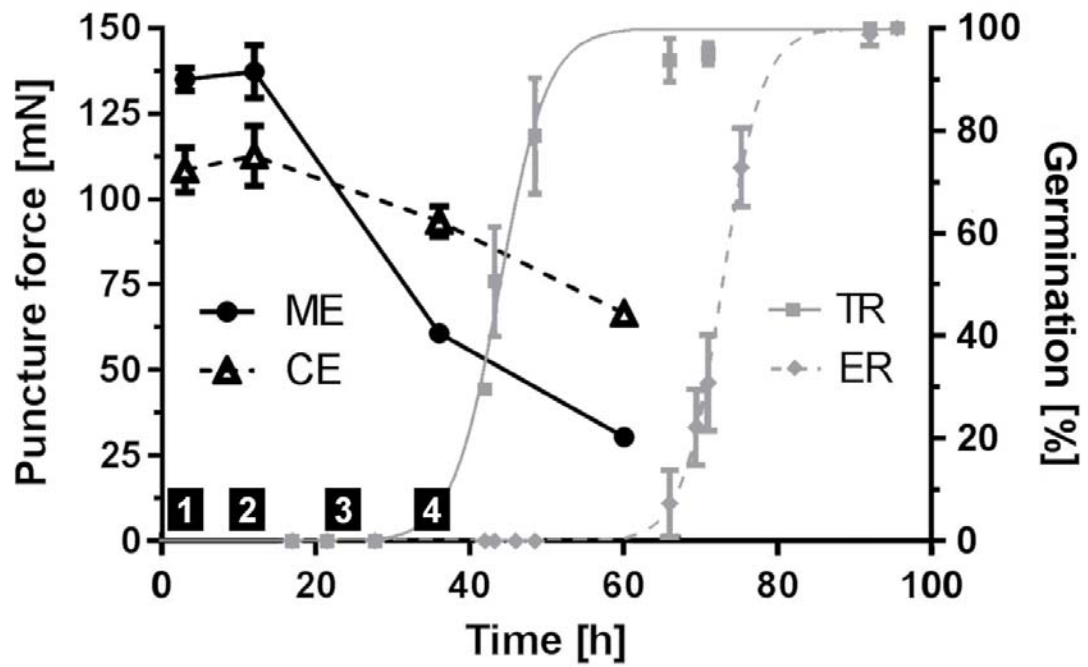




Figure 7

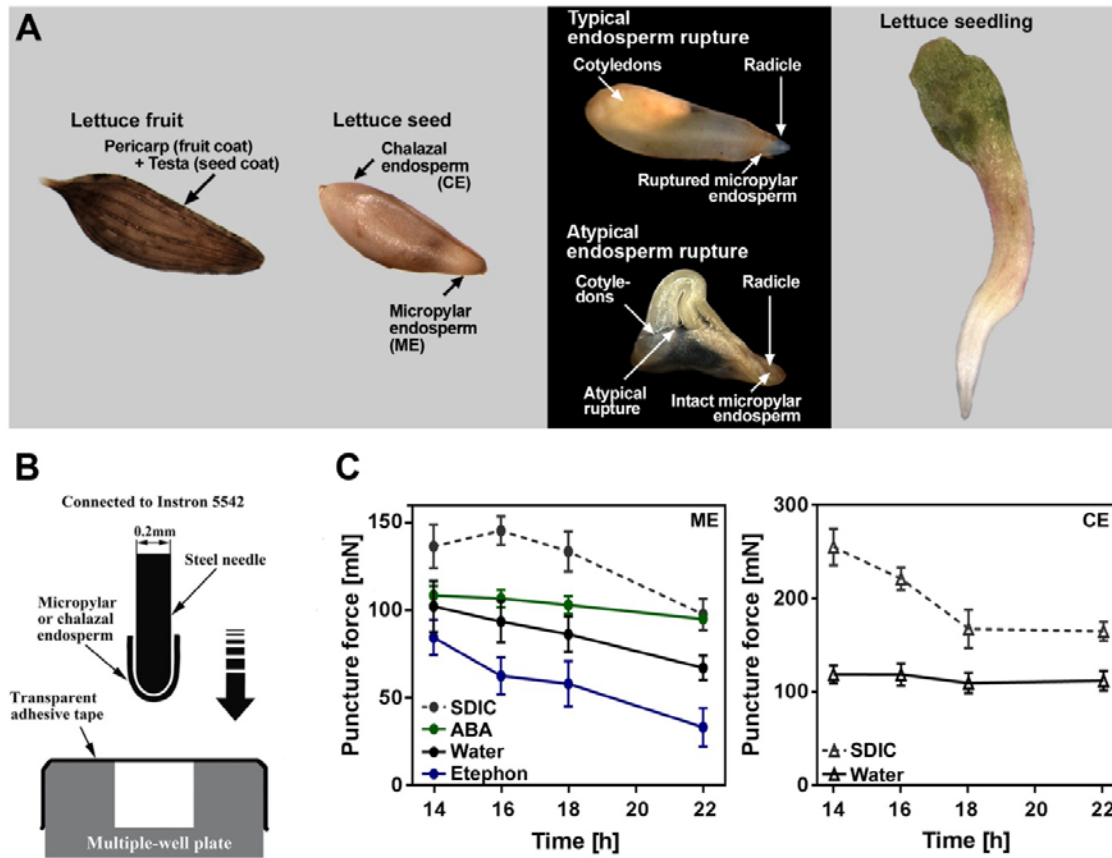


Figure 8

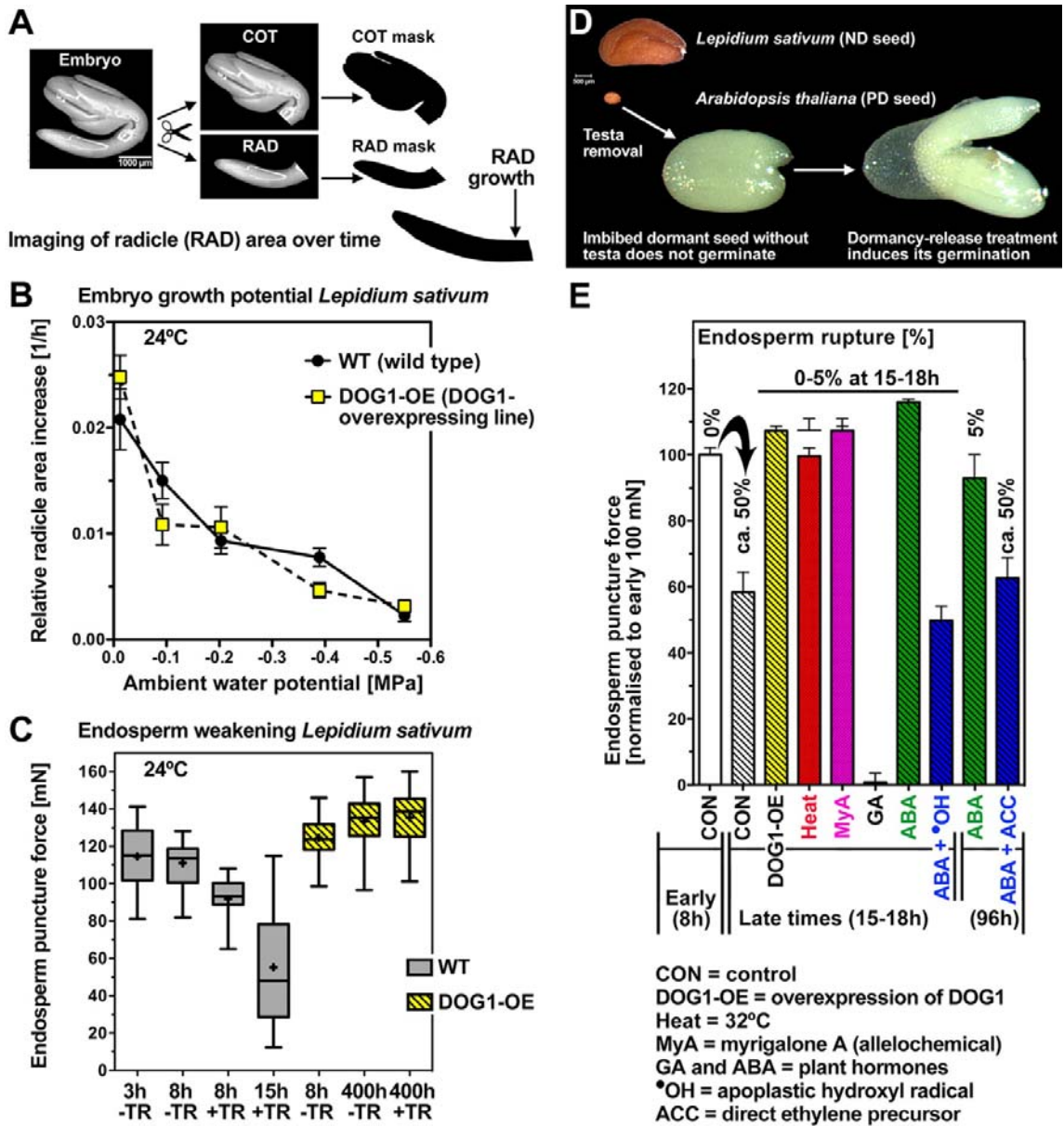


Figure 9

