ERECTA receptor-kinases play a key role in the appropriate timing of seed germination under changing salinity

Amrit K. Nanda, Abdeljalil El Habti, Charles H. Hocart and Josette Masle*

Research School of Biology, The Australian National University, Canberra ACT, Australia

* Correspondence: Josette.Masle@anu.edu.au

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Abstract

Appropriate timing of seed germination is crucial for the survival and propagation of plants, and for crop yield, especially in environments prone to salinity or drought. However, the exact mechanisms by which seeds perceive changes in soil conditions and integrate them to trigger germination remain elusive, especially once the seeds are non-dormant. In this study, we determined that the Arabidopsis ERECTA (ER), ERECTA-LIKE1 (ERL1), and ERECTA-LIKE2 (ERL2) leucine-rich-repeat receptor-like kinases regulate seed germination and its sensitivity to changes in salt and osmotic stress levels. Loss of ER alone, or in combination with ERL1 and/or ERL2, slows down the initiation of germination and its progression to completion, or arrests it altogether under saline conditions, until better conditions return. This function is maternally controlled via the tissues surrounding the embryo, with a primary role being played by the properties of the seed coat and its mucilage. These relate to both seed-coat expansion and subsequent differentiation and to salinity-dependent interactions between the mucilage, subtending seed coat layers and seed interior in the germinating seed. Salt-hypersensitive er105, er105 erl1.2, er105 erl2.1 and triple-mutant seeds also exhibit increased sensitivity to exogenous ABA during germination, and under salinity show an enhanced up-regulation of the germination repressors and inducers of dormancy ABA-insensitive-3, ABA-insensitive-5, DELLA-encoding RGL2, and Delay-Of-Germination-1. These findings reveal a novel role of the ERECTA receptor-kinases in the sensing of conditions at the seed surface and the integration of developmental, dormancy and stress signalling pathways in seeds. They also open novel avenues for the genetic improvement of plant adaptation to changing drought and salinity patterns.

Keywords: Abiotic stress signalling, cell wall, environmental sensing, ERECTA genes, mucilage, osmotic stress, receptor-kinases, salinity, seed dormancy, seed germination, seed size.

Introduction

Seed germination is a vital life-cycle transition in plants. When and under what conditions it occurs largely determine plant survival, reproductive success, yield, and ability to colonise new areas. To maximise the chances of successful completion of the life cycle and production of viable offspring, seeds have evolved mechanisms for the induction of a quiescent, dormant state during late maturation and desiccation on the mother plant. These mechanisms block the capacity of fresh seeds to germinate for a certain period of time under any combination of the same environmental conditions that would be permissive of germination in non-dormant seeds, whether hydric, gaseous, temperature, or light (Baskin and Baskin, 2004). The vast majority of higher plants, including Arabidopsis, are endowed with ‘physiological’ dormancy. The ‘primary’ dormancy acquired on the mother plant gradually declines during dry storage at ambient temperature (a process commonly referred
to as ‘after-ripening’) or under natural conditions as soil temperature increases; the seed becomes increasingly capable of responding to a suite of signals in its surroundings and will eventually germinate (e.g. see Finch-Savage and Footitt, 2017, for a review). In Arabidopsis, this absolutely requires water and occurs in two temporally separate steps, namely the rupture of the testa, or seed coat (a dead tissue), and then the rupture of the endosperm by the radicle of the expanding turgid embryo (Liu et al., 2005; Müller et al., 2006). Embryo reactivation and the weakening of surrounding tissues are tightly coordinated through complex biochemical and hormonal pathways, with a prominent role of abscisic acid (ABA) and gibberellins (GAs) in interaction with ethylene, brassinosteroids, and reactive oxygen species (ROS) (Koomneef and Van der Veen, 1980; Steber and McCourt, 2001; Finkelstein et al., 2008; Liu et al., 2010). ABA inhibits germination whereas GAs promote it through regulation of inter-signalling between the seed coat, endosperm, and embryo in a feedback loop involving DELLA proteins and interactions with cell-wall remodelling enzymes (Müller et al., 2009; Stamm et al., 2012; Graebert et al., 2014; Nonogaki, 2014).

Drought and salinity stress are two inter-related and widespread conditions in natural environments, and are major causes of germination failure, poor crop establishment, and yield loss (Boyer, 1982; Bradford, 1990; Yamaguchi and Blumwald, 2005; Finch-Savage and Leubner-Metzger, 2006; Munns and Tester, 2008). The high vulnerability of seeds to these stresses has long been recognised and yet the molecular controls remain poorly understood, apart from evidence for a deregulation of ABA–GA homeostasis and an impairment of ethylene and ROS signalling (Lopez-Molina et al., 2001; Kim et al., 2008; Yuan et al., 2010; Yu et al., 2016). Natural genetic variation in seed germination under optimal conditions, drought, or salinity has been widely documented, and numerous QTLs have been identified (e.g. Quesada et al., 2002; Clerkx et al., 2004; Galpaz and Reynold, 2010; Wang et al., 2010b; DeRose-Wilson and Gaut, 2011; Yuan et al., 2016). This demonstrates the potential for genetic improvement, but also the complexity of the underlying molecular pathways. While the genetic dissection of seed dormancy has received much attention, very few genes have been demonstrated to control germination in non-dormant seeds to tune it to the prevailing soil conditions (Kim et al., 2008; Ren et al., 2010; Yu et al., 2016). Little is known about how seeds monitor their surroundings, how this information is communicated to their inner compartments, and how the intricate communication between these compartments and the environment that is required for timely germination is modulated (Donohue et al., 2010).

Receptor-like protein kinases (RLKs) at the cell plasma membrane play major roles in signal perception and transduction to downstream intra- and intercellular signalling networks. A vast array of RLKs are encoded by plant genomes (Shiu et al., 2004). Among them are leucine-rich-repeat receptor-like kinases (LRR–RLKs), which form a large family of receptor proteins characterised by an extracellular receptor domain, a trans-membrane domain, and an intracellular kinase domain for signal transduction through phosphorylation cascades. The few that have been characterised provide evidence for their central functions in integrating signalling pathways associated with development, hormones, abiotic stress, and defence (Becraft, 2002; Osakabe et al., 2013). Little information is available on RLKs in seeds, even though developing seeds show a high abundance of secreted peptides and studies have pointed to the importance of peptide-mediated signalling in inter-compartmental coordination during seed development (Ingram and Gutierrez-Marcos, 2015).

The Arabidopsis ERECTA gene family encodes three closely related LRR–RLKs, namely ERECTA (ER), ERECTA-like 1 (ERL1), and ERECTA-like 2 (ERL2), that are known to synergistically regulate many aspects of plant development and morphogenesis and that play prominent roles in organ shape, stomatal patterning, cell proliferation, and meristematic activity (Torii et al., 1996; Shpak et al., 2004, 2005; Pillitteri et al., 2007; Uchida et al., 2012; Bemis et al., 2013; Ettich et al., 2013), as well as being involved in some pathogenic responses (Godiard et al., 2003; Llorente et al., 2005; Jordá et al., 2016). In contrast, little is known of their function in abiotic stress responses, beyond a role in leaf heat tolerance (Shen et al., 2015). We have previously reported a role of ERECTA as a major controller of water-use efficiency, under both well-watered and drought conditions (Masle et al., 2005). This function appears to be broadly conserved in diverse species (Xing et al., 2011; Zheng et al., 2015) and is suggestive of an important adaptive role of the ERECTA family to abiotic stress. Here, we examine the function of the ERECTA family during germination, a key switch that is extremely sensitive to variations in the osmotic and ionic conditions in the soil, both of which vary widely in nature.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana Columbia (Col-0, CS1093) was used as the wild-type (WT), together with the previously described erceta family homozygous mutant lines er105, erl1-2, erl2-1, the double–mutants er105 erl1-2, er105 erl2-1, erl1-2, and the triple-mutant er105 erl1-2 erl2-1 (Torii et al., 1996; Shpak et al., 2004). For simplicity, in figures we refer to er105, erl1-2, and erl2-1 simply as er, erl1, and erl2, respectively. We also used the independent homozygous er2 (Rédei, 1962; Masle et al., 2005), and novel erl1-5 (SALK_019567), and erl2-2 (SALK_015275C) mutants, and a set of double- and triple-mutants that we generated through crosses and PCR genotyping for the presence/absence of TDNA inserts and mutated alleles (primer sequences are listed in Supplementary Table S1 at JXB online). The er105 mutant was obtained by fast-neutron irradiation; it carries a large DNA fragment of unknown origin within the ER gene (At2g26330), inserted between +5 and +1066 (Torii et al., 1996) and has been characterised extensively (Torii et al., 1996; Shpak et al., 2004, 2005; Masle et al., 2005). The er2 mutant (line CS3401 from the Nottingham Arabidopsis Stock Centre) was first identified in an X-ray irradiation mutagenesis experiment for its reduced, compact stature (Rédei, 1962). It carries a frameshift in the middle of the kinase domain, leading to a truncated gene product. The er105 and er2 mutants phenocopy each other and are fully complemented by expression of the native Col-0 ER allele (Masle et al., 2005). The single er2 mutants in the ERECTA-like 1 (ERL1, At5g07180) or ERECTA-like 2 (ERL2, At5g18800) genes are knock-out TDNA insertion mutants that were sourced from the Arabidopsis mutant collections and isolated by PCR genotyping. The location of the single TDNA insertion carried by these mutants was determined by sequencing, and it maps to the LRR–receptor domain in erl1-2, erl2-1 (Shpak et al., 2004), and erl2-2 (SALK_015275C, Supplementary Fig. S3), or to the beginning of the kinase domain in erl1-3 (SALK_019567,
The osmotic pressure (π_e) of the basal medium containing either NaCl of concentrations calculated to provide the same media osmotic pressures. With solutions of either NaCl or PEG8000 dissolved in water at a range KCl at the desired experimental concentrations. For germination assays 0.09 mM Fe-EDTA, and micronutrients) at pH 5.8, and with NaCl or KCl at the desired experimental concentrations. For germination assays under iso-osmotic conditions, seeds were placed on filter paper imbibed with solutions of either NaCl or PEG8000 dissolved in water at a range of concentrations calculated to provide the same media osmotic pressures. The osmotic pressure (π_e) of the basal medium containing either NaCl or KCl was calculated using the van’t Hoff equation and verified experimentally using a VAPRO vapour pressure osmometer (Wescor Inc.). The concentrations of PEG8000 required to obtain a given π_e were determined from a calibration curve of π_e as a function of PEG concentration or KCl was calculated using the van’t Hoff equation and verified experimentally using a VAPRO vapour pressure osmometer (Wescor Inc.).

As the er105 erl1.2 erl1.2+/- erl2.1 and er2 erl1.5 erl2.2 plants are sterile, the segregating progeny of er105 erl1.2+/- erl2.1 or er2 erl1.5+/- erl2.2 were used to investigate the germination of the triple-mutant seeds, and are referred to as er105 erl1.2+/-seg and erl2.1 and er2 erl1.5/-seg erl2.2, respectively.

All the seeds in any given experiment were of the same age, and were stored together under the same conditions after being harvested from plants that were grown together in the same growth chamber, which was set at 21 °C constant temperature with a 12-h or 16-h light period depending on experiment, at 120–130 µmol quanta m⁻² s⁻¹ light intensity. As the effect of parent-of-origin on seed germination and seed size, seeds were manually excised from mature siliques of the same age, produced from flowers that were tagged at fertilisation at similar positions on the primary inflorescence.

Germination assays
All assays were done using seeds stratified by moist-chilling at 4 °C to remove residual dormancy. Seeds were surface-sterilised and sown on 0.7% agar media supplemented with Hoagland’s nutrient solution [2 mM KNO₃, 5 mM Ca(NO₃)₂, 4H₂O, 2 mM MgSO₄·7H₂O, 2 mM KH₂PO₄, 0.09 mM Fe-EDTA, and micronutrients] at pH 5.8, and with NaCl or KCl at the desired experimental concentrations. For germination assays under iso-osmotic conditions, seeds were placed on filter paper imbibed with solutions of either NaCl or PEG8000 dissolved in water at a range of concentrations calculated to provide the same media osmotic pressures. The osmotic pressure (π_e) of the basal medium containing either NaCl or KCl was calculated using the van’t Hoff equation and verified experimentally using a VAPRO vapour pressure osmometer (Wescor Inc.). 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Seeds were individually scored for both testa and endosperm rupture (germination sensu stricto) under a binocular microscope within the growth chamber, at 1–3-day intervals until all seeds on the control plates (0 mM NaCl) had germinated (30 h at most), or 1–3 times daily on the NaCl, KCl, or PEG plates as appropriate, until no change in scores were observed. Data are represented either as percentages of seeds exhibiting testa or endosperm rupture as a function of incubation time post-germination, or as T₅₀ values, which corresponds to the time (h) post-germination when 50% of seeds showed testa or endosperm rupture (Bewley et al., 1999). Ruthenium red staining of the mucilage was performed as described by James et al. (2011). The embryos were placed in 1.5-ml Reacti-Vials (ThermoFisher Scientific) fitted with Teflon-lined caps. To each vial 50 µl CHCl₃ was added followed by the internal standard, heptadecaenoic acid (C₁₇:0, 15 µl, 9.66 mg in 25 µl CHCl₃), and methanoic HCl (3 M, 500 µl). The samples were mixed and heated at 90 °C for 60 min, and then allowed to cool before being washed into glass tubes with CHCl₃. Water (1 ml) was added to each tube and the FAMEs were extracted using hexane:chloroform (4:1 v/v, 3×1 ml). The extracts were combined and washed with water (200 µl). The organic phase was then dried with anhydrous Na₂SO₄, decanted, and evaporated under nitrogen. The residue was dissolved in CH₂Cl₂ (150 µl) and transferred to GC/MS auto-sampler vials for analysis.

Mucilage extraction and analysis
Mucilage extraction was performed on 40-mg samples of dry seeds. Each sample (n=4 per genotype per experiment) was suspended in 1 ml of milliQ water, shaken at 500 rpm for 24 h at 4 °C, then vortexed for 5 s, and centrifuged at 8000 g for 3 min. A 600-µl volume of the supernatant was recovered. The seeds were then rinsed twice with 200 µl water, each time followed by vortexing, centrifuging, and recovery of 200 µl of the supernatant each time. The pooled supernatants (1 ml total volume) were flash-frozen in liquid nitrogen and immediately lyophilised. The mucilage thus recovered was weighed on a 10⁻⁶ g high-precision micro-balance. Because of the degree of genetic variation in seed size that we observed, sub-samples of a known number of seeds (at least 500) were weighed,
imaged at high resolution, and analysed for size using ImageJ prior to mucilage extraction, thus allowing the average amount of mucilage per seed to be calculated. Reductions of uronic acid methyl-esters and free uronic acids in the extracted mucilage were carried out following established protocols (Kim and Carpita, 1992; Petolino et al., 2012). The reduced polysaccharides were then hydrolysed, reduced, acetylated, and subjected to GC/MS analysis as described by Peng et al. (2000).

**Analysis of seed sodium content**

Dry seeds (three biological replicates of 10 mg each per genotype and treatment) were imbibed and stratified at 4 °C in the dark in 0 mM or 150 mM NaCl for 2 d followed by 24 h at room temperature with shaking. The seeds were rinsed three times with 2 ml water, freeze-dried, weighed, and microwave-digested for 2 h in 4 ml of 20% nitric acid at 175 °C (Method 3051; US EPA, 2007). The digests were diluted to a final volume of 5 ml and sodium ions were measured using inductively coupled plasma optical emission spectrometry (Vista-Pro CCD Simultaneous ICP-OES,Varian).

**Quantitative RT-PCR**

Total RNA was extracted from dry, imbibed or germinating seeds using TRIzol reagent (Invitrogen). mRNA isolation and reverse-transcription were done as described by Branco and Masle (2019); primer sequences are given in Supplementary Table S1. Analyses were carried out on samples of 300 seeds, with four biological replicates per genotype, time-point, and treatment (media with 0 mM or 150 mM NaCl). The seeds were sampled from four plates where all the genotypes were present together. The expression levels were normalised to the geometric mean of the expression levels of four reference genes, namely APT1 (At1g27450), PDF2 (At1g13320), bHLH (At4g38070), and PPR (At5g55840), which were chosen on the basis of their very stable expression across tissues, developmental stages, and growth conditions, including abiotic stresses (Czechowski et al., 2005). Gene expression was measured just before sowing (‘dry’ seeds), at the end of seed imbibition and stratification (germination stage I), 20 h post-stratification (stage II, testa rupture), and then 72 h post-stratification (stage III-G, when endosperm rupture had completed on control media; seeds on 150 mM NaCl that had not germinated at this time were analysed separately and are referred to as stage III-NG). Seeds were sampled within the cold room or the growth room (dry seeds and stages I–III, respectively) within 5 min from start to finish for each plate, and were immediately flash-frozen in liquid nitrogen. The experiment was repeated three times.

**Statistical analysis**

Statistical analysis was performed using the Statistix 9 software (Analytical Software, Tallahassee, USA). For multivariate comparisons of the mucilage composition profiles, discriminant orthogonal projected latent structure (OPLS) analysis was carried out using the SIMCA software (Umetrics, www.umetrics.com) with salinity as a quantitative variable.

**Accession numbers**

The accession numbers of the genes used in this study are as follows: AtER (At2g26330), AtERL1 (At5g62230), AtERL2 (At5g07180), AtPDF2 (At1g13320), AtbHLH (At4g38070), and AtPPR (At5g55840).

**Results**

The ERECTA genes control the timing and speed of germination in response to changing salinity and osmotic conditions

Loss of function of ER/ERL had no effect on testa or endosperm rupture in 0 mM NaCl media except for er105 erl1.2 seeds, which showed a small but consistent lag in testa rupture (Fig. 1, Supplementary Fig. S1A, B) that persisted through to the next germination phase leading to radicle protrusion. Salinity delayed germination in a dose-dependent manner, as expected (Supplementary Fig. S2A, B), but with striking differences among the genotypes (Fig. 1, Supplementary Fig. S1C, D). Wild-type (WT), er11.2, erl2.1, and erl1.2 erl2.1 seeds germinated first, ahead of er105, er105 erl2.1, er105 erl1.2, and finally er105 erl1.2/seg erl2.1 seeds, due to both delayed testa rupture and slower progression to endosperm rupture. As the ERECTA family has never previously been implicated in the control of

**Fig. 1.** The three Arabidopsis ERECTA family members synergistically control the timing and speed of seed germination under salinity. **A, B** T50 values (h post-stratification to rupture in 50% of seeds) for testa rupture (A) and endosperm rupture (B). **C** Time interval between testa rupture and endosperm rupture. The experiment was repeated five times with different seed batches and similar results were obtained. WT, wild-type (Col-0). As the triple-mutant is sterile, the segregating progeny of er105 erl1.2/+− erl2.1 plants were used to investigate germination, and is referred to as er11.2/seg erl2.1 (note that for simplicity, er105, er11.2, and erl2.1 are abbreviated to er, er11, and erl2, respectively, in the figure). Data are means (±SE) from n=4 plates, with 30 seeds per genotype per plate. Different letters indicate significant differences as determined by two-way ANOVA and Tukey’s HSD pair-wise tests (P<0.001).
seed germination, the experiments were repeated with an independent set of knock-out mutants carrying the er2, erl1-5, and erl2-2 null mutations (see Methods and Supplementary Fig. S3), alone or in combination, and similar results were obtained (Supplementary Fig. S4A). Taken together, these results unambiguously established that the observed genetic differences in seed germination in response to external NaCl concentrations were causally related to disruption of the ERECTA genes.

We next examined the effects of imposing salinity stress after stratification or after germination. Similar germination kinetics and differential sensitivity to NaCl among the genotypes were observed regardless of whether seeds were subjected to salinity stress post-stratification or directly from sowing (Fig. 2A–D). However, when exposed to 150 mM NaCl after radicle protrusion, all the genotypes displayed similar sensitivity to the salinity stress (Fig. 2E). These data demonstrate a germination-specific

![Figure 2](image-url)

**Fig. 2.** Germination-specific functions of the ERECTA genes in the control of germination sensitivity to salinity. (A–D) Time-course of endosperm rupture for the wild-type (WT), er105, er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 seeds over a 10-d incubation period on agar media containing either 0 mM NaCl (A, C) or 150 mM NaCl (B, D) following imbibition and stratification either directly on the media (A, B) or in water prior to plating (C, D). (E) Seedling relative expansion rates on 0 mM or 150 mM NaCl media. Seeds were first germinated on NaCl-free media and then transferred to fresh 0 mM or 150 mM NaCl plates and seedling expansion was then measured over the next 72 h. Measurements of the whole-seedling projected area were made on captured images using the ImageJ software. Note that for simplicity, er105, erl1-2, and erl2-1 are abbreviated to er, erl1, and erl2, respectively, in the figure. All data are means (±SE), n=7. Different letters indicate significant differences as determined by two-way ANOVA and Tukey’s HSD pair-wise tests (P<0.001).
function of the ERECTA genes in the sensing and signalling of salinity. This function requires ER but involves the three family members in a non-totally redundant manner.

ERECTA family expression during seed germination has not previously been reported. We therefore examined promoter activity in transgenic seeds expressing proERf::GUS constructs (Supplementary Fig. S5). The expression patterns of the ERECTA gene family did not appear to be influenced by salinity but they differed among members, with expression of ERL2 only seen in the cotyledons and the shoot apical meristem, while ER and ERL1 promoter activities were also detected in the hypocotyl. Measurements of transcript abundance by RT-qPCR (Fig. 3) confirmed the presence of transcripts of the ERECTA genes in dry seeds and showed a strong and early induction of ER and ERL1 expression during stratification and imbibition (germination phase I) and during the next phase (stage II) leading to testa rupture, while ERL2 had low expression. Salinity induced expression of ER, especially during germination phase III leading to radicle protrusion, but it had little influence on ERL1 or ERL2. These results support a role of the three ERECTA family members throughout germination, with specificity among them.

Salinity induces both osmotic and ionic stress (Munns and Tester, 2008). To investigate the contributions of these two components, we next examined germination responses to polyethylene glycol (PEG)8000, a high molecular weight non-permeating osmoticum that mimics drought-induced osmotic stress, in the salinity hyper-sensitive mutants er105, er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1. Under equivalent external osmotic conditions, seed germination was significantly less inhibited by PEG than by NaCl. The effect of PEG was innocuous up to an osmotic pressure in the medium (πe) of 0.5 MPa (Fig. 4A). When PEG was present at higher concentrations (πe = 0.74–0.99 MPa; equivalent to 150 mM and 200 mM NaCl, respectively), germination was slowed down, and to a greater extent in the double- and triple-mutants than the WT. However, the delay in germination was mild, of the order of 1 d. By Day 3 post-stratification, even under 0.99 MPa with PEG, germination was either complete (WT, er105, er105 erl1.2, and er105 erl2.1 seeds) or nearly complete (90%, er105 erl1.2/seg erl2.1 seeds), (Fig. 4A, B), which was in contrast to the strong inhibition observed at the same πe with 200 mM NaCl (Fig. 4C, Supplementary Fig. S2A, B). Such severe inhibition was only observed at much higher PEG concentrations, and even then some seeds still germinated (Fig. 4B). Taken together, these data indicate that within the germination-permissive range of NaCl concentrations, the ERECTA genes modulated the germination sensitivity to salinity mostly via interactions with the ionic effects of NaCl, but are also involved in the control of germination sensitivity to osmotic and hypersmotic stress.

The NaCl-hypersensitive mutants also exhibited increased sensitivity to KCl, but to a much lower extent under equivalent osmotic conditions (Fig. 5). This indicates that the function of the ER genes in seed germination under salinity was predominantly related to effects of the sodium ion.

It was notable that while all or the vast majority of WT, erl1.2, erl2.1, and erl1.2 erl2.1 seeds plated on NaCl medium eventually germinated (90–100%, similar to salt-free media), a significant proportion of er105, er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 seeds failed to do so, even after a lengthy incubation period (Fig. 6, Supplementary Fig. S1). Among those, the majority (up to 70%) did not even exhibit testa rupture. To determine whether they were damaged or dead seeds, we transferred them to NaCl-free media. Most then germinated readily, within 20–25 h (Fig. 6), bringing the final percentage of germinated seeds to similar levels as observed for control seeds that had not been exposed to salt. Failure to germinate on saline media was therefore not due to irreversible cellular damage and loss of seed viability, but rather to a slower or halted progression of the germination process. Consistent with their maintained viability and fast germination upon release from salinity stress, seeds with arrested germination on saline media showed similar expression levels of the ERECTA genes to germinated seeds (ERL1 and ERL2) or even higher (ER) (Fig. 3, comparison of III-NG to III-G).
Fig. 4. The Arabidopsis ERECTA family regulates seed germination sensitivity to salinity mostly via interactions with ionic effects, but is also involved in the control of germination under osmotic stress. (A) Percentage of seeds with endosperm rupture for the wild-type (WT) and the salt-hypersensitive mutants er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 at different times post-stratification as a function of the osmotic potential of the media (\(\pi_e\)), which was varied using PEG8000 at concentrations ranging from 0–171 \(\text{g} \text{L}^{-1}\). Note that for simplicity, er105, erl1-2, and erl2-1 are abbreviated to er, erl1, and erl2, respectively, in the figure. Data are means, \(n=3\) plates. (B) Germination response over an extended range of PEG concentrations, in an independent experiment with a different seed batch. Data are means of \(n=3\) plates, and show the percentage of seeds exhibiting endosperm rupture at 6 d post-stratification. (C) Kinetics of seed germination under 0.99 MPa \(\pi_e\) induced by NaCl, using the same seed batch as in (B). The arrow indicates germination scores on day 6 when at least 90% of seeds had germinated under the same osmotic conditions induced by PEG, as shown in (B). Data are means of \(n=3\) plates, with 30 seeds per plate and per genotype. The experiments were repeated three times with similar results.
Fig. 5. Seed germination in the salt-hypersensitive Arabidopsis mutants er105, er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 is more sensitive to external NaCl than KCl under iso-osmotic conditions. Time-courses of germination under two different osmotic potentials (\(\pi_s\)) are shown, induced by supplementation of the media with either NaCl or KCl. Data are means (±SE) of \(n=5\) plates, with 30 seeds per plate and per genotype. The experiments were carried out twice with similar results. Note that for simplicity, er105, erl1-2, and erl2-1 are abbreviated to er, erl1, and erl2, respectively, in the figure.

Fig. 6. Seed germination in the salt-hypersensitive Arabidopsis mutants er105, er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 readily resumes upon removal of the stress. Time-course of seed germination on media containing 150 mM NaCl (0–490 h) followed by transfer to NaCl-free media (arrow). The experiment was repeated three times with similar results. Data are means (±SE) of \(n=5\) plates, with 30 seeds per genotype per plate. Different letters indicate significant differences as determined by one-way ANOVA and Tukey’s HSD pair-wise tests (\(P<0.05\); NS, not significant). Note that for simplicity, er105, erl1-2, and erl2-1 are abbreviated to er, erl1, and erl2, respectively, in the figure.

The ERECTA genes affect the regulation of seed germination by ABA and GA

Salinity and osmotic stress promote ABA signalling and biosynthesis during germination (Seo et al., 2006; Piskurewicz et al., 2008; Yuan et al., 2010), and ABA is a strong inhibitor of germination. We therefore compared the germination kinetics of WT seeds and ERECTA-family mutants on media supplemented with ABA. ABA treatment consistently had a mild delaying effect on testa rupture, and this was most pronounced for er105 erl1.2 seeds (Fig. 7A). ABA strongly inhibited endosperm rupture in a genotype-dependent manner (Fig. 7B). Germination of er105 erl1.2 was the most sensitive to ABA, and was slower than for WT seeds even in the 1 \(\mu\)M ABA range. Under higher concentrations, seeds of the other two salt-hypersensitive mutants, er105 erl2.1 and er105 erl1.2/seg erl2.1, but not er105, also showed differences from the WT, with enhanced ABA sensitivity; interestingly, this was also the case for seeds of erl1.2 erl2.1, which is not hypersensitive to salinity (Fig. 7B). These data indicate the involvement of both ABA-dependent and ABA-independent pathways in the ERECTA family-mediated sensitivity of seed germination to salinity.

The inhibitory effect of ABA on germination is antagonised by GAs (Koornneef et al., 1982; Holdsworth et al., 2008; Weitbrecht et al., 2011; Liu et al., 2016). Rather than the absolute levels, the balance of ABA/GA is key to the commitment of seeds to germinate. The DELLA RGL2 protein plays a pivotal role in the cross-talk between ABA and GA signalling in the imbibed seed. RGL2 acts as the main GA signalling repressor in germinating seeds, through activation of a number of transcriptional regulators, including ABI3 and ABI5 that are central effectors of ABA signalling, establishment of dormancy, and repression of seed germination (Lopez-Molina et al., 2001, 2002; Lee et al., 2002; Piskurewicz et al., 2008, 2009; Liu et al., 2016). ABI3 and ABI5 are also involved in the regulation of early seedling arrest of growth under water stress in Arabidopsis (Lopez-Molina et al., 2001, 2002) and in the reversible inhibition of germination under salinity in its halophytic relative Eutrema salsugineum (Kazachkova et al., 2016). To better understand the interaction of the ERECTA genes with ABA regulation of seed germination, we examined the expression of ABI3, ABI5, and RGL2 in WT and er105 erl1.2/seg erl2.1 seeds. We also examined DELAY OF GERMINATION1 (DOG1), a pivotal seed dormancy gene that genetically interacts with ABI3 and with...
Fig. 7. The Arabidopsis ERECTA genes interact with the sensitivity of seed germination to exogenous ABA and with the expression of major ABA and GA signalling genes. Wild-type (WT) and the salt-hypersensitive Arabidopsis mutants er105, er105 erl1-2, er105 erl2-1, and er105 erl1.2/seg erl2.1 were examined (note that for simplicity, er105, erl1-2, and erl2-1 are abbreviated to er, erl1, and erl2, respectively, in the figure). (A) Germination response to exogenous ABA application as indicated by T$_{50}$ values (h post-stratification to rupture in 50% of seeds) for the testa (A) and the endosperm (B). Data are means (±SE) of $n=3$ plates, with 30 seeds of each genotype per plate. The experiment was repeated three times with similar results. (C) Expression of ABI3, ABI5, RGL2, and DOG1 genes in dry seeds and seeds sampled at the end of stratification (stage I), then 20 h later (stage II, testa rupture), and then after an additional 52 h (stage III-G, when endosperm rupture had completed on control media). Seeds on 150 mM NaCl that had not germinated by stage III were sampled and analysed separately, and are labelled as III-NG. Different letters indicate significant differences as determined using two-way ANOVA and Tukey’s HSD pair-wise tests ($P<0.05$).
a central type 2C protein phosphatase of the ABA signalling pathway during germination, and that also regulates ABI5 expression (Dekkers et al., 2016; Née et al., 2017; Nishimura et al., 2018). Constitutive expression levels were similar in the WT and mutant seeds (Fig. 7C). Salinity consistently caused an up-regulation of expression, and this was stronger in er105 erl1.2/seg erl2.1 than in the WT. This indicated that the salinity signalling cascade mediated by the ERECTA genes interacted with the ABA–GA signalling network of germination and dormancy. We also examined the expression of the ABA biosynthesis genes ABA2 and NCDE4 and the GA biosynthesis genes GA3OX1 and GA2OX2, and found that none of them showed a differential response to salinity between the mutants and the WT (Supplementary Fig. S6).

The role of the ERECTA genes in seed germination partly overlaps with a role in determining seed size and is primarily maternally controlled

Seed germination occurs when the pressure exerted by the turgid expanding radicle of the embryo overcomes the mechanical resistance of the surrounding testa and micropylar endosperm (Linkies et al., 2009; Nonogaki, 2014). As mature embryos of the er105 erl1.2 erl2.1 mutant have smaller cotyledons (Uchida et al., 2013), we reasoned that reduced growth potential could be a factor in the observed delay in the emergence of their radicle under salinity and osmotic stress, and possibly that of the other salt-hypersensitive mutants. We first measured seed size as a surrogate for embryo size, since the Arabidopsis embryo occupies most of the seed volume. The seeds of all the salt-hypersensitive genotypes (i.e. er105, er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1) were significantly smaller than those of the WT, erl1.2, and erl2.1 (Fig. 8A). Remarkably, however, the seeds of the non-salt-hypersensitive mutant erl1.2 erl2.1 were larger than those of the WT. These data revealed a novel function of the ERECTA4 genes in the determination of seed size, with specificity among them. Moreover, they suggested a link between the function of the ERECTA gene family in germination sensitivity to salinity and its influence on seed size. However, the fact that erl1.2 erl2.1 seeds germinated simultaneously with those of the WT in the presence or absence of salt despite their significant difference in size indicated that the link was not absolute.

We next considered the possibility of developmental defects in the smaller, salinity hypersensitive mutant seeds. As expected, homozygous er105 erl1.2 erl2.1 segregants displayed reduced, rounder cotyledons and a broader shoot apical meristem, as previously reported (Uchida et al., 2013). However, their hypocotyls and embryonic roots were similar to the WT in length, number, and the size of constitutive cells (Supplementary Fig. S7).

Seeds reserves are essential for successful germination and are mostly stored in cotyledons in Arabidopsis. Smaller seeds and cotyledons suggest less reserves, which could be responsible for hypersensitivity to salinity and osmotic stress. To examine this, we quantified fatty acid methyl esters (FAMEs) derived from embryo lipids, which constitute the major fraction of Arabidopsis seed reserves (Penfield et al., 2004; Lionen and Schwender, 2009). There were no significant differences across the range of genotypes except for er105 erl1.2/seg erl2.1 seeds (15% decrease) and hence, apart from this one genotype, no correlation with germination sensitivity to salt (Supplementary Fig. S8A). The relative proportions of FAME species were also similar across genotypes (Supplementary Fig. S8B, C). Taken together, these results indicated that the delayed or arrested germination of er105, er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 seeds on saline media was not likely to be due to reduced embryo size and growth potential per se.

Germination involves complex communication between the embryo, seed coat, and the intermediate endosperm, which is a single-cell layer in the Arabidopsis seed. We therefore next considered a potential role of the ERECTA genes on seed germination via effects on the tissues surrounding the embryo. We took advantage of the different contributions of the maternal and paternal genomes to the genetic make-up of the three seed compartments (seed coat ♀♀, endosperm ♂♂, embryo ♀♂) and performed reciprocal crosses between the WT and the salt-hypersensitive er105 erl1.2 and er105 erl2.1 mutants. These generated F1 seeds with the same embryo genotype but with either WT or mutant seed coat, and with predominantly WT or mutant endosperm. The two groups of F1 seeds germinated synchronously on NaCl-free media, but showed significantly different kinetics when subjected to salinity stress (Fig. 8C). Remarkably, for each cross, F1 seed germination occurred synchronously with seeds of the maternal parent. This demonstrates that the function of the ERECTA genes in the regulation of germination sensitivity to salinity was primarily maternally controlled and mediated by the tissues surrounding the embryo, in particular the seed coat. Supporting this, when excised from their covering layers, ‘naked’ er105 erl1.2, er105 erl2.1, and er105 erl1.2 erl2.1 mature embryos grew at similar rates to WT embryos, whether cultured with or without salt (Fig. 8B). F1 seeds also clustered with their maternal parent with respect to seed size (Fig. 8D), showing that the ERECTA genes effect on seed size was also of maternal origin, and strengthening the case for overlap of the control of seed size and germination response to salinity by the ERECTA genes.

The regulation of germination mediated by the ERECTA genes involves the seed-coat mucilage

We examined which properties of the seed coat might be controlled by the ERECTA4 genes in response to salinity. To examine seed-coat permeability, seeds were incubated in tetrazolium red, a cationic dye classically used to detect seed-coat defects and abnormal permeability (Wharton, 1955; Molina et al., 2008). Similar staining and tetrazolium salt reduction rates were observed across the genotypes except for significant increases in er105 erl1.2 and to a lesser extent in erl1.2 seeds (Fig. 9A), which was suggestive of increased seed-coat permeability or NADPH-dependent reductase activity in these two mutants. We next measured seed sodium contents after 24 h stratification with or without salt and found no differences between the genotypes (Fig. 9B). A thick cuticle lining the outer side of the endosperm has been described in mature Arabidopsis seeds, which constitutes a barrier protecting the inner living tissues of the seed (De Giorgi et al., 2015;
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Loubéry et al., 2018). Furthermore, this cuticle is maternally inherited, similar to the differential salt sensitivity of germination that we observed. We therefore examined the possibility that its permeability may have differed between the WT and salt-hypersensitive er105 erl1.2, er105 erl2.1, and triple-mutant endosperms by using Toluidine Blue permeability tests. Embryos were excised from seeds incubated either in the presence of paclobutrazol (PAC) to inhibit both endosperm and testa rupture or in the presence of ABA to allow only testa rupture and hence expose the endosperm cuticle. Following incubation with Toluidine Blue, no staining was apparent in WT embryos from seeds treated with either PAC or ABA.
The Arabidopsis ERECTA genes are involved in the control of seed-coat permeability and mucilage composition, and play a salinity-dependent role in the regulation of germination speed. The wild-type (WT) and the mutants er105, erl1.2, erl2.1, er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 were examined (note that for simplicity, er105, erl1-2, and erl2-1 are abbreviated to er, erl1, and erl2, respectively, in the figure). (A) Seed-coat permeability to Tetrazolium Red. Data are means (±SE) of n=4 replicates of 100 seeds each. Significant differences were determined using two-way ANOVA and Scheffe's post hoc test (*P<0.05; **P<0.01). (B) Seed sodium content of seeds at 24 h post-stratification on 0 mM or 150 mM NaCl media. Data are means (±SE) of n=3 pools of 10 mg mature seeds each. Different letters indicate significant differences as determined using two-way ANOVA and Tukey's HSD pair-wise tests; P=0.42 for genotype effect under control conditions and P=0.39 under salt treatment. (C) Correlations between mass of water-soluble mucilage per seed and seed size. Data for mucilage are means of n=4 seed samples of 40 mg per genotype and data for weight are means of n=5 pools of a known number of seeds (20–40) corresponding to seeds from five siliques of same age. Regression lines: 0 mM NaCl, y=36.6x–0.20, r² =0.84; 150 mM NaCl, y=36.3x+0.002, r² =0.81. The experiment was repeated three times with similar results. Similar results were also obtained with size expressed as area (data not shown). (D) Relationship between ratios of galacturonic acid/galactose (GalUA/Gal) and rhamnose/xylose (Rhm/Xyl). Different letters next to data points indicate significant differences in GalUA/Gal as determined using one-way ANOVA and Tukey's post-hoc tests, compared to all unlabelled data points (*P<0.05). The difference in Rhm/Xyl between er erl1.2/seg erl2.1 and the WT is significant at P=0.08. (E) T50 values (h post-stratification to rupture in 50% of seeds) for testa rupture (TeR) and endosperm rupture (EnR) for intact seeds and seeds with the outer water-soluble mucilage removed (‘demucilaged’). Data are means of n=3 plates, with 30 seeds per genotype per plate. Labelled points highlight genotypes where removal of the mucilage significantly advanced germination on 150 mM NaCl media. The 1:1 line represents no effect of mucilage removal. (F) Expression of TCH3 in the WT and er erl1.2/seg erl2.1 in dry and imbibed seeds during the three germination phases Data are means (±SE) of n=4 samples of 300 seeds each per genotype and treatment.
(Supplementary Fig. S9A), which was consistent with previous reports (De Giorgi et al., 2015; Loubéry et al., 2018). This was also the case for embryos from the mutants, apart from er105 erl1.2 treated with ABA (i.e. exhibiting testa rupture), which frequently showed some faint staining in parts of the superficial cell layers of the hypocotyl and in the very tip of cotyledons. To test for possible interactions between salinity and genotype on endosperm cuticle permeability, the assays were repeated in the presence of 100 mM NaCl (Supplementary Fig. S9B). Similar results were obtained, with no sign of staining in PAC-treated WT or mutant seeds, and no marked staining in ABA-treated seeds. Overall, these results provide no indication for a role of differential seed-coat or endosperm permeability in the dramatic differences in germination sensitivity to NaCl between the WT and the three mutants examined.

During seed-coat differentiation on the mother plant, the specialised epidermal cells secrete mucilage polysaccharides that line their inner walls and build a central volcano-shaped columnella (Beekman et al., 2000; Western et al., 2000; Haughn and Western, 2012). The desiccated, highly hydropobic mucilage rapidly swells upon hydration and ruptures the enclosing outer primary wall, wrapping the seed in a gelatinous capsule traversed by cellulose rays radiating from the columnella. Mutant seeds affected in mucilage synthesis or extrusion have been reported to be more sensitive to low water potential during germination (Penfield et al., 2001; Yang et al., 2010). This prompted us to next examine mucilage release during imbibition by the WT and mutant seeds in our study. We collected the loosely adhering mucilage that can easily be detached from the seed surface, as opposed to the inner fraction that is bound to the cell wall. Large variation was observed in the amounts recovered between the genotypes, but that correlated with the genetic variation in seed size (Fig. 9C). Salinity caused large increases in mucilage extrusion, but to a similar extent in all genotypes and so the relationship with seed size was unchanged. Staining with ruthenium red, which binds to pectin, consistently showed a thicker and often darker halo of mucilage under saline conditions, but with no indication of variation among genotypes within the saline and control treatments (Supplementary Fig. S10A, B).

Previous studies have suggested the importance for germination of the physico-chemical properties of the mucilage and of its attachment to the seed, rather than simply the amount present (Rautengarten et al., 2008; Saez-Aguayo et al., 2013). We therefore analysed mucilage composition. The expected sugars were detected, mostly rhamnose (Rhm) and galacturonic acid (GalUA) derived from rhamnogalacturonans type I (RG I), the major pectin of Arabidopsis seed mucilage (Macquet et al., 2007; Arsovsky et al., 2009), and low amounts of other neutral and acidic sugars derived from RG I side-chains (Supplementary Fig. S10C). When analysed individually, these sugars showed no significant variations among genotypes; however, examination of compositional profiles by multivariate analysis suggested genetic variation in the relative abundance of backbone sugars (Rhm and GalUA) and some side-chain sugars (Xyl and GaI), which lead us to compare their ratios across the full range of genotypes (Fig. 9D). This revealed dramatically increased GalUA/Gal ratios in the mucilage of er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 seeds compared to that of WT and other mutant seeds (P=0.027) and, apart from erl1.2 erl2.1, a trend for higher rhamnose to xylose ratios in mutant seeds, especially of the er105 erl1.2 and er105 erl1.2/seg erl2.1 genotypes (P=0.08). These results suggest that the ERECTA genes play a role in the control of mucilage composition and architecture via interactions with the mechanisms controlling the abundance of carboxyl sites (i.e. the potential sites for pectin cross-linking) and perhaps also pectin branching. Moreover, they indicate a link between this role and the function in the regulation of seed germination.

To examine this in more detail, we took an indirect, holistic approach, and compared the germination kinetics of intact seeds and ‘demucilaged’ seeds from which we removed the shell of loosely adherent mucilage extruded during imbibition. Demucilaged seeds consistently germinated more slowly than intact seeds on salt-free media (Fig. 9E), as is common. This was also the case under saline conditions for WT, er1.2, erl1.2, and erl1.2 erl2.1 seeds, but remarkably, mucilage removal had the opposite effect in er105 erl1.2, er105 erl2.1, and er105 el1.2/seg el2.1 seeds, where the delay in germination relative to the WT was 66% to 74% shorter than observed for intact seeds (Fig. 9E), dropping from 39 h to 13 h for er105 erl2.1, 68 h to 23 h for er105 erl1.2, and 153 h to 39 h for er105 el1.2/seg el2.1. This was due to faster progression from testa rupture to endosperm rupture. These results demonstrate a critical role of the water-soluble mucilage in mediating the function of the ERECTA genes in controlling the germination response to salinity.

Although they appear as distinct layers in the imbibed seed, the mucilage and epidermal cell walls are tightly bound. The subersised seed coat and underlying endosperm constitute a mechanically strong barrier that needs to be weakened to enable radicle emergence. The micropylar endosperm that surrounds the radicle tip is thought to be the major source of mechanical resistance to radicle protrusion (Linkies et al., 2009; Dekkers et al., 2013). Endosperm weakening is effected by cell wall-modifying enzymes in interaction with ROS and hormonal signals from the embryo, especially GA (Finch-Savage and Leubner-Metzger, 2006; Müller et al., 2006; Penfield et al., 2006). We therefore hypothesised that the importance of the mucilage and seed coat in mediating delayed or arrested germination in the er105, er105 erl1.2, er105 erl2.1, and er105 el1.2/seg el2.1 mutants on saline media could in part be related to ERECTA family-dependent differences in the mechanical properties of the endosperm and seed coat. The Arabidopsis seed is too small for direct measurement of the forces involved in testa and endosperm rupture, as is possible in other species (Linkies et al., 2009), leading us instead to examine the expression of the Arabidopsis TOUCH (TCH) gene TCH3, which encodes a calmodulin-like protein and is greatly up-regulated in response to a range of mechanical signals in other tissues (Braam and Davis, 1990). Comparison of TCH3 expression in the WT and er105 erl1.2/seg erl2.1 seeds showed the presence of transcripts at similar, low levels in dry seeds (Fig. 9F). Imbibition triggered de novo transcription of TCH3 on 150-mM NaCl media in both the WT and mutant seeds, consistent with the known role of calcium in salinity signalling (Munns...
and Tester, 2008). Induction was significantly enhanced in er105 er1.2/seg erl2.1 seeds and was transient, preceding testa rupture and then disappearing. These results are suggestive of enhanced mechanical constraints imposed on er105 er1.2/seg erl2.1 embryos before endosperm rupture compared with the WT. On salt-free media, de novo transcription of TCH3 did not occur before the final phase of germination, and again it was enhanced in er105 er1.2/seg erl2.1 seeds compared to the WT.

Discussion

The mechanisms by which seeds monitor conditions in their immediate surroundings in order to optimise the timing of the initiation and completion of germination are mostly unknown. In this study, we demonstrated that the Arabidopsis ERECTA family acts to control the timing of seed germination according to external salinity and osmotic levels (Figs 1, 4). Loss of function of ER, or of ER and its paralogs, slows down germination or even prevents it under increasing salinity and osmotic stress, although seed viability is not compromised as germination readily resumes upon the return of favourable conditions (Fig. 6). The sensing of changing salinity levels mediated by the ERECTA gene family involves interactions with the ABA–GA signalling network of germination and dormancy, and is primarily controlled by the endosperm and testa surrounding the embryo, with a critical but not exclusive role of the testa and its mucilage (Figs 8, 9). These findings reveal previously unsuspected regulators of the interactions between the seed and its environment, and a novel function of the three ER family receptor-like kinases in controlling these interactions, together with cryptic genetic variation in seed germination.

The ERECTA gene family regulates germination in saline conditions via maternally controlled effects on the tissues surrounding the embryo

The seed coat derives from the maternal ovule integuments, which expand and undergo profound developmental and biochemical transformations following fertilisation, resulting in a highly differentiated, impermeable and mechanically strong tissue (Beeckman et al., 2000; Western et al., 2000). Mucilage is secreted and deposited in its outer, epidermal layer concomitantly with embryo morphogenesis, following the cessation of integument expansion (reviewed by Haughn and Western, 2012; North et al., 2014), but its physiological roles have remained elusive. Apart from anchoring the imbibed seed to its physical substrate, the gelatinous mucilage is generally thought to facilitate germination, especially under osmotic stress, through sequestering water and keeping the seed hydrated (Penfield et al., 2001; Arsovski et al., 2010; Yang et al., 2010). However, several studies have suggested that mucilage can also inhibit germination under unsuitable conditions, perhaps through limiting water and oxygen diffusion to the embryo (Western, 2012, and references therein). Our current study has shed some light on the poorly understood genetic control of the context-dependent role of the seed mucilage in germination, and revealed that the ERECTA genes are key players. We observed a promoting role of the mucilage on the speed of germination in WT, erl1.2, erl2.1, and erl1.2 erl2.1 seeds, under both saline and non-saline conditions (Fig. 9E). However, in the salt-hypersensitive er105 er1.2, er105 erl2.1, and er105 er1.2/seg erl2.1 seeds this role was only expressed under non-saline conditions: under salinity, it was lost (in er105 er1.2, er105 erl2.1) or even reversed (in the triple-mutant). These results link, for the first time, the seed mucilage and the ERECTA genes in the regulation of seed germination sensitivity and response to environmental variations at the seed surface.

By what mechanisms could the ERECTA genes control the salinity-dependent properties of the seed mucilage that regulate the germination process? The mucilage is like a pectin-rich secondary cell wall (Haughn and Western, 2012). The degrees of pectin branching and cross-linking (with calcium ions in particular) are known to greatly influence their hydrophilicity, adsorption to cellulose microfibrils, and partitioning between loose outer mucilage and adherent inner mucilage (Willats et al., 2006; North et al., 2014; Ralet et al., 2016). It is also well established that the small monovalent Na+ ions have the general capacity to easily displace the larger divalent Ca2+ ions that cross-link the carboxyl residues of adjacent pectin molecules (Fry, 1986; Willats et al., 2006; Ghanem et al., 2010), thus leading to looser, more hydrophilic mucilage upon imbibition with saline compared to salt-free water, and also to greater abundance of mucilage (Fig. 9C; Ghanem et al., 2010) due to increased release of pectin molecules from the cellulose matrix. The enrichment in uronic acids in the seed mucilage of er105 er1.2, er105 erl2.1, and er105 er1.2/seg erl2.1 potentially increases the sites for Ca2+-Na+ exchange, and the trend of reduced xylose content relative to backbone rhamnose is suggestive of altered branching (Fig. 9D). We propose that this has the potential to significantly modify the swelling properties of the mucilage and of the subtending walls, and to change their osmotic potential, conformation, and rigidity upon imbibition with a saline or high osmolarity solution (Willats et al., 2006; Ghanem et al., 2010; Ralet et al., 2016). It may also significantly modify the rearrangement of mucilage- and wall components that occurs as pectin molecules are released (Rautengarten et al., 2008), and it perhaps also affects Ca2+ influx to the adjoining inner endosperm and embryo. Thus, the increase in uronic acids and decrease in xylose/rhamnose ratio would modify the overall chemical and mechanical interactions between the seed environment, seed coat, and interior compartments, and thereby the perception and early signaling events of salinity.

The ERECTA gene family controls seed germination sensitivity to salinity through mechanisms partially overlapping with those regulating seed coat enlargement during embryogenesis

The mutant seeds with enhanced sensitivity to salt and hyperosmotic stress during germination were smaller than those of the WT (Fig. 8A). Seed size in Arabidopsis is controlled by complex interactions of zygotic and maternal factors, and by signalling between the integuments and endosperm (Garcia et al., 2003; Luo et al., 2005; Day et al., 2008; Dilkes...
Our reciprocal crosses showed that the variation in final seed size among the mutants and WT is of maternal origin (Fig. 8D). Final seed size is reached early in seed development, through a first phase of active cell proliferation in both the integuments and the endosperm, triggered by fertilisation, followed by a period of mostly cell expansion. Expansion ceases 5–6 d post-anthesis, concomitantly with the endosperm switching from syncitial development to cellularisation (Garcia et al., 2005) and with the start of starch and mucilage synthesis. Variation in maximum cell elongation appears to be the main driver of maternal variations in seed size, such as observed here, through a so-called ‘compensatory’ growth mechanism (Garcia, 2005). The ER gene has been implicated in a compensatory mechanism between cell number and size in Arabidopsis leaves (Ferjani et al., 2007), and comparison of the seed epidermis in Ler and Columbia accessions suggests ‘compensation’ may take place in the seed integuments too (Garcia et al., 2005). Interestingly, the progression and completion of integument growth during ovule development has previously been reported to require a minimum level of ERECTA family signalling (Pillitteri et al., 2007), which was ascribed to a role of ERECTA genes in cell proliferation activity through interactions with cell-cycle regulators. However, the final cell number in the mature ovule was unchanged, making it unlikely that the reduced size of the seed cavity and less-expanded seed coat that we observed in the er105, er105 erl1.2, er105 erl2.1, and er105 erl1.2/sig erl2.1 seeds (Fig. 8A) were pre-determined prior to fertilisation. Moreover, no ovule integument growth defects were reported by Pillitteri et al. (2007) other than in the triple mutant er105 erl1.2 erl2.1+/−. In the present work, we found that loss of ER alone was sufficient to cause reduced seed size, and further loss of ERL1 or ERL2 had only a small additional effect. Furthermore, when it occurred in an ER background, loss of ERL1 and ERL2 instead caused an increase of seed size beyond that observed in the WT (Fig. 8A). This supports the idea that partly different mechanisms are involved in the ERECTA-mediated control of seed size and germination sensitivity to salinity. Given the role of the ERECTA family in the composition of the mucilage (Fig. 9D) and reported increases in uronic acids and cellulose in leaves of two er mutants (Sánchez-Rodriguez et al., 2009), an intriguing hypothesis is that the ERECTA family may regulate cell wall formation and assembly, not only during mucilage and secondary cell wall deposition but also prior to that, during seed-coat enlargement and formation of the seed cavity. This would provide a unifying explanation for the link that we found between the ERECTA family-mediated regulation of seed germination sensitivity to salinity, salinity-dependent mucilage properties in germinating seeds, and seed size.

**ERECTA family-mediated salt signalling in germinating seeds involves a complex regulatory network**

The Arabidopsis seed coat is in immediate contact with the single-cell layer endosperm, which itself is in direct contact with the embryo. Although it is less well-documented than in humans, there are demonstrated cases of the ability of plant membrane receptors or mechano-sensitive channels to monitor cell wall integrity, physical interactions between the membrane and wall, wall deformation, and wall rheology (Hamann, 2012; Monshausen and Haswell, 2013; Hamilton et al., 2015; Haswell and Verslues, 2015). The ERECTA family proteins belong to the class XIII of leucine-rich repeats receptor-kinases. Interestingly, among its other four members, this class includes FEI1 and FEI2 (Shiu et al., 2004) that have been shown to interact with the SOS5 arabinogalactan protein to influence cellulose production and pectin assembly in the Arabidopsis seed mucilage, as well as mucilage adherence (Harpaz-Saad et al., 2011; Griffiths et al., 2014). In addition, based on analysis of disease resistance in two er mutants, the ER protein has been suspected of interacting with wall-associated kinases (WAKs) during defence against some pathogens via effects on cell wall composition (Sánchez-Rodriguez et al., 2009). WAKs are known to be tightly bound to pectins, especially galacturonic acids, in a Ca2+-dependent manner (Wagner and Kohorn, 2001; Decreux and Messaen, 2005), and several WAK/WAK-Like proteins have been implicated in responses to mineral ions, including Na+ (Sivaguru et al., 2003; Hou et al., 2005; de Lorenzo et al., 2009), and to osmotic stress (SOS6/AtCSLD5; Zhu et al., 2010), through unknown mechanisms. Modifications of mucilage and bound cell walls mediated by the ERECTA family may thus be perceived and signalled to the seed interior by the ERECTA proteins themselves either directly or through modified interactions with cell wall-associated proteins, osmo-sensors, or mechano-sensors (Dekkers et al., 2013; Nonogaki, 2014). The induction of TCH3 in the er105 erl1.2/seg erl2.1 seeds supports this hypothesis (Fig. 9F).

It would be intriguing to unravel the downstream cascade. The salt-hypersensitive er105 erl1.2, er105 erl2.1, and er105 erl1.2/seg erl2.1 seeds showed enhanced sensitivity to exogenous ABA, and enhanced up-regulation of ABI3, ABI5, and RGL2 under saline conditions compared to the WT (Fig. 7). These genes are emerging as important mediators of salinity and osmotic stress and as controllers of ABA–GA homeostasis in imbibed seeds. ABA synthesised in the endosperm and released to the embryo activates the abundance and activity of the ABI3 and ABI5 transcription factors, and triggers an auto-feedback loop that maintains RGL2 mRNA at high levels and represses cell-wall modifying enzymes (Giraudat et al., 1992; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001, 2002; Lee et al., 2002, 2010; Piskurewicz et al., 2008, 2009; Kang et al., 2015). Our data indicate that the regulation of germination sensitivity to changing salinity levels mediated by the ERECTA4 genes interferes with that signalling loop.

A well-documented adaptive mechanism that seeds have evolved to withstand unfavourable conditions such as high temperatures, cold, osmotic or salinity stress, and to maintain embryo viability is secondary dormancy (Bewley, 1997), a reversible, transient quiescent state induced and released in adaptation to fluctuating environmental conditions (Koornneef et al., 1982; Giraudat et al., 1992; Léon-Kloosterziel et al., 1996; Finch-Savage and Leubner-Metzger, 2006; Lefebvre et al., 2006; Weitbrecht et al., 2011; Ibarra et al., 2016). ABI3, ABI5, and RGL2 are prominent players in the regulation of secondary dormancy and increased sensitivity to ABA, and
up-regulation of AB13, AB15 and RGL2 has been reported during early growth arrest in newly germinated Arabidopsis seedlings under water stress and salinity (Lopez-Molina et al., 2001, 2002). Here, we found that loss of various combinations of ERECTA genes sensitised seed germination to salinity and frequently arrested it (Fig. 6). This arrest was reversible, as germination readily resumed upon stress release and progressed to completion as quickly as in seeds that were never exposed to stress. Arrested seeds showed up-regulation of DOG1 (Fig. 7), a major controller of the coat- and endosperm-mediated dormancy that occurs in Arabidopsis seeds. DOG1 interacts with GA and ABA signalling upstream of AB15, and appears to be an agent of environmental adaptation of germination among Arabidopsis accessions (Dekkers et al., 2013; Graeber, 2014; Née et al., 2017; Nishimura et al., 2018). Taken as a whole, these observations suggest that the ERECTA family interacts with the molecular controls of dormancy to appropriately cue and pace germination. While promotion of fast germination under stress may be seen as desirable, it can also expose the newly germinated seedling to the risk of death should the adverse conditions persist or worsen as the embryo becomes directly exposed to the external environment with all its reserves already depleted. In such circumstances, delay or arrest of germination may be a useful protective strategy to maximise the chances of survival by temporarily safeguarding the embryo against such a fate. In that context, the environment-dependent function of the ERECTA family with regards to germination speed and temporary arrest would perform a vital adaptive function. Interestingly, the loss of ER and ERL1 and/or ERL2 caused the arrest of germination in absolutely all seeds within a cohort only under extremely severe stress (~200 mM NaCl) (Supplementary Fig. S2). Under milder stress, some seeds did germinate at the same time as the WT, some showed increasing delays, and others were arrested until the stress was released, thus demonstrating a mixed response that might balance the risks of death against loss of fitness or ability to complete the life cycle in time.

There is increasing agreement that the endosperm plays a prominent role in the regulation of dormancy and germination in Arabidopsis seeds, in particular through its role in modulating the ABA–GA balance that is central to the control of these processes. The endosperm is a predominantly maternal tissue that is in direct contact with the seed coat, and it is of utmost importance in the determination of seed size (Garcia et al., 2005) and in the de novo synthesis of ABA and its transport to the embryo (Lee et al., 2010; Kang et al., 2015). It is also an important site of interactions between hormones, ROS, and cell-wall remodelling enzymes that weaken the mechanical resistance to radicle protrusion (Lee et al., 2012; Dekkers et al., 2013; Nonogaki, 2014). According to our permeability tests, there was no apparent association between the differential germination sensitivity to salinity among the genotypes and the variations in endosperm permeability, apart from possibly the er105 erl1.2 mutant (Supplementary Fig. S9). However, this requires further detailed examination, as more broadly does the role of the ERECTA family on the biochemical and mechanical properties of the endosperm, and on the upstream perception events of changes in the seed environment.

Conclusions

Plants need to be endowed with a ‘surveillance’ system for the perception of external environmental cues, their transduction to internal compartments, and their integration with developmental pathways. This study has demonstrated a key role of the ERECTA family in the integrative network in seeds that controls the most critical decision in the life cycle, namely when to initiate a new plant. Given the evolutionary conservation of the ERECTA receptor-kinases across a broad range of plant species, and an increasing interest in mucilage as a model for cell wall studies and as an important adaptive feature, our findings open new avenues for determining the mechanisms that seeds have evolved to control germination and to tune it to local conditions in order to maximise the chances of survival.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Germination dynamics of the wild-type and ERECTA mutants in response to NaCl.

Fig. S2. Loss of ER alone or in combination with ERL1 and ERL2 sensitises seed germination to salinity in a dose-dependent manner.

Fig. S3. Phenotypes of selected erecta mutants.

Fig. S4. Increased sensitivity of seed germination to salinity in the er2, er2 erl1-5, er2 erl2-2, and er2 erl1-5 erl2-2 mutants.

Fig. S5. Time-course of promoter activity of ERECTA genes in mature dry seeds and germinating seeds.

Fig. S6. Expression levels of ABA and GA biosynthesis genes in response to salinity in WT and er105 erl1.2/seg erl2.1 seeds.

Fig. S7. Mature er105 erl1.2 erl2.1 embryos exhibit similar radicle size and patterning to WT embryos.

Fig. S8. Relative abundance of total fatty acid methyl-esters and relative proportions of individual species in embryos of the WT and ERECTA family mutants at full seed maturity.

Fig. S9. Toluidine Blue tests for seed-coat and endosperm cuticle permeability in response to salinity in the WT and salt-hypersensitive er105 erl1.2, er105 erl2.1, and er105 erl1.2 erl2.1 seeds.

Fig. S10. Characteristics of the seed mucilage in the WT and erecta mutants.

Table S1. List of primers used for genotyping and RT-qPCR.

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References

Arsovski AA, Haughn GW, Western TL. 2010. Seed coat mucilage cells of Arabidopsis thaliana as a model for plant cell wall research. Plant Signaling & Behavior 5, 796–801.

Arsovski AA, Popma TM, Haughn GW, Carpita NC, McCann MC, Western TL. 2009. ATBXL1 encodes a bifunctional beta-D-xylansidase/alpha-L-arabinofuranosidase required for pectic arabinan modification in Arabidopsis mucilage secretory cells. Plant Physiology 150, 1219–1234.

Baskin JM, Baskin CC. 2004. A classification system for seed dormancy. Seed Science Research 14, 1–16.

Becraft PW. 2002. Receptor kinase signaling in plant development. Annual Review of Cell and Developmental Biology 18, 163–192.

Beeckman T, De Rycke R, Viane R, Inzé D. 2000. Histological study of seed coat development in Arabidopsis thaliana. Journal of Plant Research 113, 139–148.

Bemis SM, Lee JS, Shpak ED, Torii KU. 2013. Regulation of floral patterning and organ identity by Arabidopsis ERECTA-family receptor kinase genes. Journal of Experimental Botany 64, 5323–5333.

Bewley JD. 1997. Seed germination and dormancy. The Plant Cell 9, 1055–1066.

Bewley JD, Bradford K, Hilhorst H, Nonogaki H. 2013. Seeds: physiology of development, germination and dormancy. Springer Science+Business Media, 133–181.

Boyer JS. 1982. Plant productivity and environment. Science 218, 443–448.

Braam J, Davis RW. 1990. Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell 60, 357–364.

Bradford KJ. 1990. A water relations analysis of seed germination rates. Plant Physiology 94, 840–849.

Branco R, Masle J. 2019. Systemic signalling through translationally controlled tumour protein controls lateral root formation in Arabidopsis. Journal of Experimental Botany 70, 3927–3940.

Clerx EJ, El-Lithy ME, Vierling E, Ruys GJ, Blankestijn-De Vries H, Groot SP, Vreugdenhil D, Koornneef M. 2003. Arabidopsis haku mutants reveal new controls of seed size by the endosperm. Plant Physiology 131, 1661–1670.

Czechowski T, Stitt M, Alltmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiology 139, 5–17.

Day RC, Herridge RP, Ambrose BA, Macknight RC. 2008. Transcriptome analysis of proliferating Arabidopsis endosperm reveals biological implications for the control of syncytial division, cytokinin signaling, and gene expression regulation. Plant Physiology 148, 1964–1984.

De Giorgi J, Piskurewicz U, Loubery S, Utz-Pugin A, Bailly C, Mène-De Giorgi J, Piskurewicz U, Loubery S, Utz-Pugin A, Bailly C, Mène-Laporte P, Thompson R, Clarke J, Sousa C, De la Sen P, Sanemoto H, Kato K, Ishiguro M. 2000. Analysis of natural allelic variation of Arabidopsis seed germination and seed longevity traits between the accessions Landsberg erecta and Shakdara, using a new recombinant inbred line population. Plant Physiology 135, 432–443.

Degier J, Piskurewicz U, Loubery S, Utz-Pugin A, Bailly C, Mène-Laporte P, Thompson R, Clarke J, Sousa C, Crespi M. 2009. A novel plant leucine-rich repeat receptor kinase regulates the response of Madicago truncatula root tips to salt stress. The Plant Cell 21, 668–680.

Debeaujon I, Koornneef M. 2000. Gibberellin requirement for Arabidopsis seed germination is determined both by testa characteristics and embryonic abscisic acid. Plant Physiology 122, 415–424.

Decreux A, Messiaen J. 2005. Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. Plant & Cell Physiology 46, 268–278.

Dekkers BJ, He H, Hanson J, Willems LA, Jamar DC, Cuff G, Rajou L, Hilhorst HW, Bentsink L. 2016. The Arabidopsis DELAY OF GERMINATION 1 gene affects ABSICIC ACID INSENSITIVE 5 (ABI5) expression and genetically interacts with ABI5 during Arabidopsis seed development. The Plant Journal 85, 451–465.

Dekkers BJ, Pearce S, van Bolderen-Veldkamp RP, et al. 2013. Transcriptional dynamics of two seed compartments with opposing roles in Arabidopsis seed germination. Plant Physiology 163, 205–215.

DeRose-Wilson L, Gaut BS. 2011. Mapping saline tolerance during Arabidopsis thaliana germination and seedling growth. PLoS ONE 6, e122832.

Dilkes BP, Spielman M, Weizbauer R, Watson B, Burkart-Waco D, Scott RJ, Comai L. 2008. The maternally expressed WRKY transcription factor TTG2 controls lethality in interploidy crosses of Arabidopsis. PLoS Biology 6, 2707–2720.

Donohue K, de Casas RR, Burghardt L, Kovach K, Willis CG. 2010. Germination, postgermination adaptation, and species ecological ranges. Review of Ecology 41, 293–319.

Etchells JP, Provost CM, Mishra L, Turner SR. 2013. WOX4 and WOX14 act downstream of the PXY receptor kinase to regulate plant vascular proliferation independently of any role in vascular organisation. Development 140, 2224–2234.

Ferjani A, Horiguchi G, Yano S, Tsukaya H. 2007. Analysis of leaf development in fugu mutants of Arabidopsis reveals three compensation modes that modulate cell expansion in determinate organs. Plant Physiology 144, 988–999.

Finch-Savage WE, Footitt S. 2017. Seed dormancy cycling and the regulation of dormancy mechanisms to time germination in variable field environments. Journal of Experimental Botany 68, 843–856.

Finch-Savage WE, Leubner-Metzger G. 2006. Seed dormancy and the control of germination. New Phytologist 171, 501–523.

Finkelstein RR, Lynch TJ. 2000. The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. The Plant Cell 12, 599–609.

Fry SC. 1986. Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annual Review of Plant Biology 37, 165–186.

Galpaz N, Reymond M. 2010. Natural variation in Arabidopsis thaliana revealed a genetic network controlling germination under salt stress. PLoS ONE 5, e15198.

Garcia D, Gerald JNF, Berger F. 2005. Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in Arabidopsis. The Plant Cell 17, 52–60.

Garcia D, Saingery V, Chambrier P, Mayer U, Jürgens G, Berger F. 1999. Arabidopsis haku mutants reveal new controls of seed size by the endosperm. Plant Physiology 130, 1661–1670.

Ghanem ME, Han RM, Classen B. 2010. Muclage and polysaccharides in the halophyte plant species Kosteletzkya virginica: localization and composition in relation to salt stress. Journal of Plant Physiology 167, 382–392.

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM. 1992. Isolation of the Arabidopsis ABI3 gene by positional cloning. The Plant Cell 4, 1251–1261.

Godiard L, Sauviciu L, Torii KU, Grenon O, Mangin B, Grimsley NH, Marco Y. 2003. ERECTA, an LRR receptor-like kinase protein controlling development pleiotropically affects resistance to bacterial wilt. The Plant Journal 36, 353–365.

Greaber K, Linkies A, Steinbrecher T, et al. 2014. DELAY OF GERMINATION 1 mediates a conserved coat-dormancy mechanism for the temperature- and gibberellin-dependent control of seed germination. Proceedings of the National Academy of Sciences, USA 111, E3571–E3580.

Griffiths JS, Tsai AY, Xue H, Voiniciuc C, Sola K, Seifert GJ, Mansfield SD, Haughn GW. 2014. SALT-OVERLY SENSITIVE5 mediates Arabidopsis seed coat mucilage adherence and organization through pectins. Plant Physiology 165, 991–1004.

Hamann T. 2012. Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms. Frontiers in Plant Science 3, 77.

Hamilton ES, Schlegel AM, Haswell ES. 2015. United in diversity: mechanism-sensitive ion channels in plants. Annual Review of Plant Biology 66, 113–137.

Hanke DE, Northcote DH. 1975. Molecular visualization of pectin and DNA by ruthenium red. Biopolymers 14, 1–17.

Harpaz-Saad S, McFarlane HE, Xu S, Divi UK, Forward B, Western TL, Kieber JJ. 2011. Cellulose synthesis via the FEI2 RLK/SOS5 pathway and cellulose synthase 5 is required for the structure of seed coat muclage in Arabidopsis. The Plant Journal 68, 941–953.

Haswell ES, Verslues PE. 2015. The ongoing search for the molecular basis of plant osmosensing. The Journal of General Physiology 145, 389–394.
Haughn GW, Western TL. 2012. Arabidopsis seed coat mucilage is a specialized cell wall that can be used as a model for genetic analysis of plant cell wall structure and function. Frontiers in Plant Science 3, 64.

Holdway-Edmunds MD, Edsitsk M, Soppe WJ. 2008. Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytologist 179, 33–54.

Hou XW, Tong HY, Selby J, DeWitt J, Peng XX, He ZH. 2005. Involvement of a cell wall-associated kinase, WAKL4, in Arabidopsis mineral responses. Plant Physiology 139, 1704–1716.

Ibarra SE, Tognacca RS, Dave A, Graham IA, Sanchez RA, Botto JF. 2016. Molecular mechanisms underlying the entrance in secondary dormancy of Arabidopsis seeds. The Plant Cell & Environment 39, 213–221.

Ingram G, Gutierrez-Marcos J. 2015. Peptide signalling during angio- sperm seed development. Journal of Experimental Botany 66, 5151–5159.

James GO, Hocart CH, Hillier W, Chen H, Kordbacheh F, Price GD, Djordjevic MA. 2011. Fatty acide profiling of Chlamydomonas reinhardtii under nitrogen deprivation. Bioresource Technology 102, 3343–3351.

Jiang WB, Huang HY, Hu YW, Zhu SW, Wang ZY, Lin WH. 2013. Brassinosteroid regulates seed size and shape in Arabidopsis. Plant Physiology 162, 1955–1977.

Jordá L, López-Torres S, Escudero V, Núñez-Corcuera B, Delgado-Cerezo M, Torri KU, Molina A. 2016. ERECTA and BAK1 receptor-like kinases interact to regulate immune responses in Arabidopsis. Frontiers in Plant Science 7, 897.

Kang J, Yim S, Choi H, Kim A, Lee KP, Lopez-Molina L, Martinoia E, Lee Y. 2015. Abscisic acid transporters cooperate to control seed germination. Nature Communications 6, 8113.

Kazachkova Y, Khan A, Acuña T, López-Díaz I, Carrera E, Khozin-Goldberg I, Fait A, Barak S. 2018. A seed coat bedding assay shows that RGL2-dependent release of abscisic acid to regulate endosperm rupture during germination: a comparison of Arabidopsis thaliana seed coat mucilage. Plant & Cell Physiology 48, 984–990.

Masle J, Gilmore SR, Farquhar GD. 2005. The ERECTA gene regulates plant transpiration efficiency in Arabidopsis. Nature 436, 866–870.

McFarlane HE, Gendre D, Western TL. 2014. Seed coat ruthenium red staining assay. Bio-protocol 4, e1096.

Molina I, Ohrogue JB, Pollard M. 2008. Deposition and localization of lipid polymer in developing seeds of Brassica napus and Arabidopsis thaliana. The Plant Journal 53, 437–449.

Monshausen GB, Haswell ES. 2013. A force of nature: molecular mechanisms of mechanocoeptation in plants. Journal of Experimental Botany 64, 4663–4680.

Müller K, Linkies A, Vreeburg RAM, Fry SC, Krieger-Liszay A, Leubner-Metzger G. 2009. In vivo cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. Plant Physiology 150, 1865–1865.

Müller K, Tintelnot S, Leubner-Metzger G. 2006. Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of Lepidium sativum (cress) and endosperm rupture of cress and Arabidopsis thaliana. Plant & Cell Physiology 47, 864–877.

Muñns R, Tester M. 2008. Mechanisms of salinity tolerance. Annual Review of Plant Biology 59, 651–681.

Nanda N, Kramer K, Nakabayashi K, Yuan B, Xiang Y, Miatton E, Finkemeier I, Soph 3. 2017. DELAY OF GERMINATION1 requires PP2C phosphate starvation of the ABA signalling pathway to control seed dormancy. Nature Communications 8, 72.

Nishimura T, Tsuchiya W, Moresco JJ, et al. 2018. Control of seed dormancy and germination by DOG1-AHK1 PP2C phosphatase complex via binding to heme. Nature Communications 9, 2132.

Nonogaki H. 2005. Arabidopsis enhancer-trap lines for seed germination-associated genes. The Plant Journal 41, 936–944.

Liu X, Hu P, Huang M, Tang Y, Li Y, Li L, Hou X. 2016. The NF-YC-RGL2 module integrates GA and ABA signalling to regulate seed germination in Arabidopsis. Nature Communications 7, 12768.

Liu Y, Ye N, Liu R, Chen M, Zhang J. 2010. H2O2 mediates the regulation of ABA catabolism and GA biosynthesis in Arabidopsis seed dormancy and germination. Journal of Experimental Botany 61, 2979–2990.

Llorente F, Alonso-Blanco C, Sánchez-Rodríguez C, Jordà L, Molina A. 2005. ERECTA receptor-like kinase and heterotrimic G protein from Arabidopsis are required for resistance to the necrotrophic fungus Pectobacterium carboxytophilum. The Plant Journal 43, 165–180.

Lopez-Molina L, Mongrand S, Chua NH. 2001. A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proceedings of the National Academy of Sciences, USA 98, 4782–4787.

Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT, Chua NH. 2002. ABI5 acts downstream of ABI3 to execute an ABI3-dependent growth arrest during germination. The Plant Journal 32, 317–328.

Loubéry S, De Giorgi J, Uzt-Pugin A, Deninois L, Lopez-Molina L. 2018. A maternally deposited endosperm cuticle contributes to the physiological defects of transparent testa seeds. Plant Physiology 177, 1218–1233.

Luo M, Dennis ES, Berger F, Peacock WJ, Chaudhury A. 2005. MINISEED3 MIN3, a WRKY family gene and HAUKIZ (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis. Proceedings of the National Academy of Sciences, USA 102, 17531–17536.

Macquet A, Ralet MC, Kronenberger J, Marion-Poll A, North HM. 2007. In situ, chemical and macromolecular study of the composition of Arabidopsis thaliana seed coat mucilage. Plant & Cell Physiology 48, 984–990.

McFarlane HE, Gendre D, Western TL. 2014. Seed coat ruthenium red staining assay. Bio-protocol 4, e1096.

Molina I, Ohrogue JB, Pollard M. 2008. Deposition and localization of lipid polymer in developing seeds of Brassica napus and Arabidopsis thaliana. The Plant Journal 53, 437–449.

Monshausen GB, Haswell ES. 2013. A force of nature: molecular mechanisms of mechanocoeptation in plants. Journal of Experimental Botany 64, 4663–4680.

Müller K, Linkies A, Vreeburg RAM, Fry SC, Krieger-Liszay A, Leubner-Metzger G. 2009. In vivo cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. Plant Physiology 150, 1865–1865.

Müller K, Tintelnot S, Leubner-Metzger G. 2006. Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of Lepidium sativum (cress) and endosperm rupture of cress and Arabidopsis thaliana. Plant & Cell Physiology 47, 864–877.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. Annual Review of Plant Biology 59, 651–681.

Née G, Kramer K, Nakabayashi K, Yuan B, Xiang Y, Miatton E, Finkemeier I, Soph 3. 2017. DELAY OF GERMINATION1 requires PP2C phosphatases of the ABA signalling pathway to control seed dormancy. Nature Communications 8, 72.

Nishimura T, Tsuchiya W, Moresco JJ, et al. 2018. Control of seed dormancy and germination by DOG1-AHK1 PP2C phosphatase complex via binding to heme. Nature Communications 9, 2132.

Nonogaki H. 2014. Seed germination – emerging mechanisms and new hypotheses. Frontiers in Plant Science 5, 233.

North HM, Berger A, Saez-Aguayo S, Ralet MC. 2014. Understanding polysaccharide production and properties using seed coat mutants: future perspectives for the exploitation of natural variants. Annals of Botany 114, 1251–1263.

Osakabe Y, Yamaguchi-Shinozaki K, Shinozaki K, Tran LS. 2013. Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress. Journal of Experimental Botany 64, 445–458.

Penfield S, Li Y, Gilday AD, Graham S, Graham IA. 2006. Arabidopsis ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination by the endosperm. The Plant Cell 18, 1887–1899.
Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW. 2001. MYB61 is required for mucilage deposition and exclusion in the Arabidopsis seed coat. The Plant Cell 13, 2777–2791.

Penfield S, Ryott EL, Gildey AD, Graham S, Larson TR, Graham IA. 2004. Reserve mobilisation in the Arabidopsis endosperm fuels hypocotyl elongation in the dark, is independent of abscisic acid, and requires PHOSPHOLIPIDYRUVATE CARBOXYKINASE1. The Plant Cell 16, 2705–2718.

Peng LC, Hocart CH, Redmond JW, Williamson RE. 2000. Fractionation of carbohydrates in Arabidopsis root cell walls shows that three radial swelling loci are specifically involved in cellulose production. Planta 211, 406–414.

PetitoLNA, Walsh C, Fincher GB, Bacic A. 2012. Determining the poly-saccharide composition of plant cell walls. Nature Protocols 7, 1590–1607.

Pillitteri LJ, Bemis SM, Shpak ED, Torii KU. 2007. Haploinsufficiency after successive loss of signaling reveals a role for ERECTA-family genes in Arabidopsis ovule development. Development 134, 3099–3109.

Piskuriewicz U, Jikumaru Y, Kinoshita N, Namba E, Kamiya Y, Lopez-Molina L. 2008. The gibberellin acid signaling repressor RGL2 inhibits Arabidopsis seed germination by stimulating abscisic acid synthesis and ABI5 activity. The Plant Cell 20, 2729–2745.

Piskuriewicz U, Turecková V, Lacombe E, Lopez-Molina L. 2009. Far-red light inhibits germination through DELLA-dependent stimulation of ABA synthesis and ABI3 activity. The EMBO Journal 28, 2259–2271.

Quesada V, García-Martínez S, Piqueras P, Ponce MR, Miclo JL. 2002. Genetic architecture of NaCl tolerance in Arabidopsis. Plant Physiology 130, 951–963.

Ralet MC, Crépeau MJ, Vigouroux J, Tran J, Berger A, Sallé C, Granier F, Botran L, North HM. 2016. Xylanis provide the structural driving force for mucilage adhesion to the arabidopsis seed coat. Plant Physiology 172, 167–168.

Rautengarten C, Usadel B, Neumetzler L. 2008. A subtilisin-like serine protease essential for mucilage release from Arabidopsis seed coats. The Plant Journal 54, 466–480.

Rédei JP. 1962. Single locus heterosis. Zeitschrift für Vererbungsgeschichte 93, 164–170.

Ren Z, Zheng Z, Chinnusamy V, Zhu J, Cui X, Iida K, Zhu JK. 2010. RAS1, a quantitative trait locus for salt tolerance and ABA sensitivity in Arabidopsis. Proceedings of the National Academy of Sciences, USA 107, 5669–5674.

Raz JC, Berger A, Botran L, Popratz D, Marion-Poll A, North HM. 2013. PECTIN METHYLESTERASE INHIBITOR6 promotes Arabidopsis mucilage release by limiting methylesterification of homogalacturonan in seed coat epidermal cells. The Plant Cell 25, 308–323.

Sánchez-Rodríguez C, Estévez JM, Llorente F, Hernández-Blanco C, Jordà L, Pagán I, Berrocal M, Marco Y, Somerville A, Molina A. 2009. The ERECTA receptor-like kinase regulates cell wall-mediated resistance to pathogens in Arabidopsis thaliana. Molecular Plant-Microbe Interactions 22, 953–963.

Seo M, Hanada A, Kuwahara A, et al. 2006. Regulation of hormone metabolism in Arabidopsis seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. The Plant Journal 48, 354–366.

Sessions A, Weigel D, Yanofsky MF. 1999. The Arabidopsis thaliana MERISTEM-LAYER1 promoter specifies epidermal expression in meristems and young primordia. The Plant Journal 20, 259–263.

Shen H, Zhong X, Zhao F, et al. 2015. Overexpression of receptor-like kinase ERECTA improves thermotolerance in rice and tomato. Nature Biotechnology 33, 996–1003.

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF, Li WH. 2004. Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. The Plant Cell 16, 1220–1234.

Shpak ED, Berthiaume CT, Hill EJ, Torii KU. 2004. Synergistic interaction of three ERECTA-family receptor-like kinases controls Arabidopsis organ growth and flower development by promoting cell proliferation. Development 131, 1491–1501.

Shpak ED, Mabee JM, Pillitteri LJ, Torii KU. 2005. Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science 309, 290–293.

Sivaguru M, Ezaki B, He ZH, Tong H, Osawa H, Baluska F, Volkmann D, Matsumoto H. 2003. Aluminum-induced gene expression and protein localization of a cell wall-associated receptor kinase in Arabidopsis. Plant Physiology 132, 2256–2266.

Stamm P, Ravindran P, Mohanty B, Tan EL, Yu H, Kumar PP. 2012. Insights into the molecular mechanism of RGL2-mediated inhibition of seed germination in Arabidopsis thaliana. BMC Plant Biology 12, 179.

Steger CM, McCourt P. 2001. A role for brassinosteroids in germination in Arabidopsis. Plant Physiology 125, 763–769.

Tori KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF, Komedya Y. 1996. The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. The Plant Cell 8, 735–746.

Uchida N, Lee JS, Horst RJ, Lai H-H, Kajita R, Kakimoto T, Tasaka M, Torii K. 2012. Regulation of inflorescence architecture by intertissue layer ligand–receptor communication between endodermis and phloem. Proceedings of the National Academy of Sciences, USA 109, 6337–6342.

Uchida N, Shimada M, Tasaka M. 2013. ERECTA-family receptor kinases regulate stem cell homeostasis via buffering its cytokinin responsiveness in the shoot apical meristem. Plant & Cell Physiology 54, 343–351.

US EPA. 2007. Method 3051A (SW-846): microwave assisted acid digestion of sediments, sludges, and oils, Revision 1. Washington DC: US Environmental Protection Agency.

Wagner TA, Kohorn BD. 2001. Wall-associated kinases are expressed throughout plant development and are required for cell expansion. The Plant Cell 13, 303–318.

Wang A, Garcia D, Zhang H, Feng K, Chaudhury A, Berger F, Peacock WJ, Dennis ES, Luo M. 2010a. The VQ motif protein IKU1 regulates endosperm growth and seed size in Arabidopsis. The Plant Journal 63, 670–679.

Wang Z, Wang J, Bao Y, Wu Y, Zhang H. 2010b. Quantitative trait loci controlling rice seed germination under salt stress. Euphytica 178, 297–307.

Weibrecht K, Müller K, Leubner-Metzger G. 2011. First off the mark: early seed germination. Journal of Experimental Botany 62, 3289–3309.

Western TL. 2012. The sticky tale of seed coat mucilages: production, genetics, and role in seed germination and dispersal. Seed Science Research 22, 1–26.

Western TL, Skinner DJ, Haughn GW. 2000. Differentiation of mucilage secreting cells of the Arabidopsis seed coat. Plant Physiology 122, 345–356.

Wharton MJ. 1955. The use of tetrazolium test for determining the viability of seeds of the genus Brassica. Proceedings of the International Seed Test Association 20, 81–88.

Willats W, Knox JP, Mikkelsen JD. 2006. Pectin: new insights into an old polymer are starting to gel. Trends in Food Science and Technology 17, 97–104.

Xing HT, Guo P, Xiao XL, Yin WL. 2011. PdERECTA, a leucine-rich repeat receptor-like kinase of poplar, confers enhanced water use efficiency in Arabidopsis. Plant Physiology 154, 229–241.

Yamaguchi T, Blumwald E. 2005. Developing salt-tolerant crop plants: challenges and opportunities. Trends in Plant Science 10, 615–620.

Yang X, Dong M, Huang Z. 2010. Role of mucilage in the germination of Artemisia sphaerocephala (Asteraceae) achenes exposed to osmotic stress and salinity. Plant Physiology and Biochemistry 48, 131–138.

Yu Y, Wang J, Shi H, Gu J, Dong J, Deng XW, Huang R. 2016. Salt stress and ethylene antagonistically regulate nucleocytoplasmic partitioning of COP1 to control seed germination. Plant Physiology 170, 2340–2350.

Yuan K, Rashotte AM, Wysocka-Diller JW. 2010. ABA and GA signaling pathways interact and regulate seed germination and seedling development under salt stress. Acta Physiologiae Plantarum 33, 261–271.

Yuan W, Flowers JM, Sahraie DJ, Purugganan MD. 2016. Cryptic genetic variation for Arabidopsis thaliana seed germination speed in a novel salt stress environment. Gnes Genomes Genetics 8, 3129–3138.

Zheng J, Yang Z, Madgwick PJ, Carmona-Silva E, Parry MA, Hu YG. 2015. TaER expression is associated with transpiration efficiency traits and yield in bread wheat. PLoS ONE 10, e0128415.

Zhou Y, Zhang X, Kang X, Zhao X, Zhang X, Ni M. 2009. SHORTHYPOCOTYL UNDER BLUE1 associates with MINISEED3 and HAUK2 promoters in vivo to regulate Arabidopsis seed development. The Plant Cell 21, 96–117.

Zhu J, Lee B-H, Dellinger M, Cui X, Zhang C, Wu S, Nothnagel EA, Zhu JK. 2010. A cellulose synthase-like protein is required for osmotic stress tolerance in Arabidopsis. The Plant Journal 63, 128–140.