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A prion-like protein regulator of seed germination undergoes hydration-dependent phase separation

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Supplemental Information:
STAR Methods
Figs. S1 to S7
References

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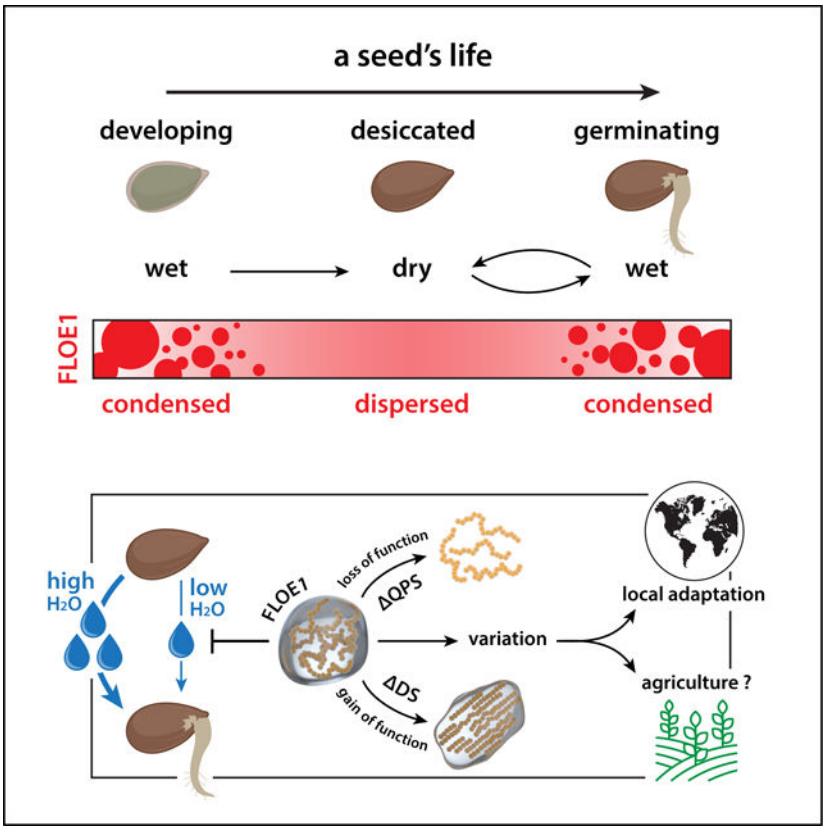
SUMMARY

Many organisms evolved strategies to survive desiccation. Plant seeds protect dehydrated embryos from various stressors and can lay dormant for millennia. Hydration is the key trigger to initiate germination, but the mechanism by which seeds sense water remains unresolved. We identified an uncharacterized *Arabidopsis thaliana* prion-like protein we named FLOE1, which phase separates upon hydration and allows the embryo to sense water stress. We demonstrate that biophysical states of FLOE1 condensates modulate its biological function *in vivo* in suppressing seed germination under unfavorable environments. We find intragenic, intraspecific, and interspecific natural variation in FLOE1 expression and phase separation and show that intragenic variation is associated with adaptive germination strategies in natural populations. This combination of molecular, organismal, and ecological studies uncovers FLOE1 as a tunable environmental sensor with direct implications for the design of drought resistant crops, in the face of climate change.

Graphical Abstract



In Brief:

A previously uncharacterized protein, FLOE1, functions in seeds as a water potential sensor and can undergo reversible phase separation.

Keywords

Phase separation; Prion-like; Intrinsically Disordered Proteins; Seed germination; Water stress; Salt stress; Biomolecular condensate; Bet-hedging; Water sensing; Seed desiccation; Adaptation

INTRODUCTION

Although water is essential for life, numerous organisms developed ways to withstand severe water stress or have quiescent desiccated life stages (Boothby and Pielak, 2017; Esbelin et al., 2018; Giarola et al., 2017). Among the organisms that have come up with such extreme adaptation are the seed plants. Seeds are specialized propagation vectors that can mature to a quiescent, desiccated state, allowing them to remain viable in harsh conditions anywhere from a few years to millennia (Sallon et al., 2020; Sano et al., 2016; Yashina et al., 2012). They can survive extreme desiccation by accumulating protective molecules that profoundly change their cellular biophysical properties (Buitink and Leprince, 2008; Leprince and Buitink, 2015). Upon water uptake, called imbibition, seeds undergo a cascade of biochemical and mechanical events and resume cellular activities (Nonogaki et al., 2018; Rajjou et al., 2012). Seeds can endure multiple hydration-dehydration cycles while

remaining viable and desiccation tolerant (Bai et al., 2012). However, once committed to germination, seeds lose this ability (Rajjou et al., 2012). Thus, poor timing of germination can severely limit seedling survival (Kranner et al., 2010). Despite the fundamental importance of germination control, the molecular details that underpin this decision remain elusive.

Limited water availability dramatically alters protein solubility and induces protein aggregation. To protect their cytoplasmic components, seeds have an arsenal of protective mechanisms. These include the upregulation of osmoprotectants (ElSayed et al., 2014) and chaperones, and a cytoplasmic liquid-to-glass transition during seed maturation (Buitink and Leprince, 2008; Leprince and Buitink, 2015). Given the complex biophysical changes proteins must endure under such extreme conditions, we investigated how seed proteins might have adapted to deal with them. We used transcriptomic data to identify the *Arabidopsis thaliana* seed-enriched proteome. Interestingly, we found an enrichment for protein disorder, among which were several prion-like proteins. These types of proteins have been previously connected to protein phase separation, a physical process that allows cells to compartmentalize biomolecules into membraneless biomolecular condensates (Boeynaems et al., 2018; Shin and Brangwynne, 2017). While this process has been recently implicated in plant biology (Chakrabortee et al., 2016; Fang et al., 2019; Jung et al., 2020; Powers et al., 2019; van Dop et al., 2020; Zhang et al., 2020), it remains unclear how phase separation could play a role in seeds , especially given the challenges regarding protein aggregation under water-limiting conditions.

Here we report the identification of an uncharacterized prion-like protein, FLOE1, which specifically phase separates upon seed imbibition. Through phenotypic and transcriptomic analyses of mutant lines, we provide evidence that the protein allows the embryo to sense water potential and attenuates germination under unfavorable conditions. FLOE1 undergoes phase separation and functions to regulate germination under conditions of water stress. We dissected the molecular grammar of FLOE1 and created a set of mutants that span the material state spectrum. Using these to change FLOE1's biophysical state resulted in altered seed germination, providing evidence for the functional importance of a biomolecular condensate's material properties *in vivo* in a multicellular organism. Finally, we uncovered a vast family of FLOE homologs across green plants. We show that natural sequence variation drives differences in FLOE phase behavior suggesting that FLOE variation might regulate seed germination in the wild.

RESULTS

FLOE1 is an uncharacterized seed-specific prion-like protein

To find seed-enriched proteins, we re-analyzed publicly available *A. thaliana* transcriptomics data and found 449 protein-coding genes that are expressed at higher levels in dry seeds compared to other tissues (Fig. 1A, Table S1) (Austin et al., 2016; Schmid et al., 2005). These proteins had a different amino acid compositional profile (Fig. 1B) and were enriched for regions of structural disorder compared to the rest of the proteome (Fig. 1C). Intrinsically disordered proteins (IDPs) have emerged as key players in cell biology that, among many roles, orchestrate how cells organize themselves and their contingent biochemical reactions

into discrete membraneless compartments by a process called liquid-liquid phase separation (LLPS) (Boeynaems et al., 2018; Shin and Brangwynne, 2017). A subset of IDPs harbors a class of domains known as prion-like domains (PrLD), and we identified 14 proteins with PrLDs in the seed-enriched proteins (Fig. 1D). PrLDs were first identified in fungal prions and later shown to drive reversible protein phase separation in diverse eukaryotic species (Alberti et al., 2009). In yeast, PrLDs give rise to phenotypic diversity via a proposed bet-hedging strategy to help cope with a fluctuating environment (Halfmann et al., 2012). All but one of these PrLD-containing seed-enriched proteins had annotated functions or domains related to nucleic acid metabolism. The one that did not, AT4G28300, was an uncharacterized plant-specific protein, which we named FLOE1, inspired by the second movement of *Glassworks* by Philip Glass as well as the definition of floe being ‘a sheet of floating ice’, which is a phase-separated body of water.

FLOE1 undergoes hydration-dependent phase separation

FLOE1 accumulates during embryo development and its expression peaks in the mature desiccated state (Fig. S1A–B). We generated transgenic *A. thaliana* lines expressing *FLOE1*’s genomic region—from its predicted promoter to its last coding codon—fused to *GFP*. FLOE1 formed cytoplasmic condensates during embryonic development (Fig. 1E, Fig. S1C) and in embryos dissected in water from dry seeds (Fig. 1F, Fig. S1D). However, when we dissected dry seeds in glycerin instead of water (to mimic the desiccated environment), FLOE1 did not form condensates and was diffusely localized (Fig. 1F, Fig. S1E–F). When we transferred these embryos from glycerin to water, FLOE1 condensates spontaneously appeared (Fig. 1F) and were fully reversible with repeated hydration-dehydration cycles (Fig. 1F, Fig. S1K). Pre-treating seeds with the translation inhibitor cycloheximide did not affect the formation of FLOE1 condensates, indicating that they are distinct from stress granules and processing bodies (Gutierrez-Beltran et al., 2015), and that their emergence did not depend on FLOE1 translation upon imbibition (Fig. S1G). In line with this, FLOE1 condensates appeared rapidly upon hydration (in less than a minute), arguing against the involvement of biosynthetic processes and for a rapid biophysical response to water. To directly test whether FLOE1 forms condensates in response to changes in water potential, we dissected embryos in solutions of varying concentrations of salt, mannitol, or sorbitol (Fig. 1G–I, Fig. S1H–I). High concentrations of salt resembled dry conditions–embryos lacked visible FLOE1 condensates (Fig. 1G–I). Lowering the salt concentration resulted in a gradual emergence of condensates, which was highly variable at the cell-to-cell (Fig. 1G–H) and tissue levels (Fig. 1I). In intermediate concentrations, we observed a small number of cells with apparent nuclear localization of FLOE1 (Fig. 1J, Fig. S1J), suggesting this could be a behavior associated with early steps of imbibition, before the majority of the protein condenses in the cytoplasm. Like our observations with repeated hydration-dehydration cycles, FLOE1 condensation was also reversible by moving seeds back and forth between solutions of high or no salt (Fig. 1K, Fig. S1K). Thus, FLOE1 forms cytoplasmic condensates in response to changes in water potential *in vivo* and these are fully reversible (Fig. 1L).

FLOE1 attenuates germination under water stress

We next asked whether FLOE1 functions in germination. Lines carrying the knockout allele *floe1-1* did not show any obvious developmental defects, and *floe1-1* seeds had the same size, shape, and weight as wildtype seeds (Fig. S2A). *floe1-1* seeds germinated indistinguishably from wildtype seeds under standard conditions (Fig. S2B) but, intriguingly, had higher germination levels under conditions of water deprivation induced by salt (Fig. 2A, Fig. S2C) or mannitol (Fig. S2C). We confirmed that these phenotypes were caused by mutations in *FLOE1* using independent lines carrying CRISPR-Cas9 deletion alleles and *floe1-1* lines complemented with the wildtype allele (Fig. S2C–F). Germination during stressful environmental conditions is risky for a plant and can reduce fitness. Indeed, both wildtype and *floe1-1* seedlings displayed developmental defects or eventually died under these conditions (Fig. S2G), whereas ungerminated seeds retained their full germination potential upon stress alleviation (Fig. 2B), in line with bet-hedging strategies utilized by stressed seeds (Gremer and Venable, 2014; Johnston and Bassel, 2018; Villa Martin et al., 2019). To define the role of FLOE1 condensates in stress-mediated attenuation of germination, we first asked whether the condensates would reform upon stress alleviation. Whereas ungerminated salt-stressed seeds were largely devoid of FLOE1 condensates, even after 15 days of incubation, alleviating salt stress robustly induced their appearance (Fig. 2C, Fig. S2H). Moreover, the extent of FLOE1 phase separation correlated with germination levels (Fig. S3). Importantly, in contrast to the hydration experiments we performed on naked embryos by submersion (Fig. 1F–K), we performed these experiments by incubating intact seeds on agar plates with the indicated salt concentrations–representing more physiologically relevant conditions (Fig. S3D). These results show that FLOE1 phase separates *in vivo* and that this event coincides with the onset of germination. Moreover, under water limitation, FLOE1 attenuates germination, thereby enabling a better chance of survival (Fig. 2D).

To place FLOE1's role in the context of other germination pathways, we performed transcriptomics on wildtype and *floe1-1* plants. RNA-seq analysis suggests that FLOE1 functions upstream of key germination pathways. *floe1-1* seeds had only few changes in gene expression compared to wildtype seeds in both their desiccated state and under normal imbibition. But under salt stress, loss of FLOE1 caused a significant change in the transcriptome (Fig. 2E). In line with the observation that *floe1-1* seeds germinate more under water stress, their salt stress-responsive transcriptome was marked by an upregulation of metabolic genes, consistent with promoting germination, and stress-response genes, as the seeds exit their stress-tolerant desiccated state (Fig. 2F, Fig. S2I, Table S2). Interestingly, *floe1-1* seeds showed relatively lower transcript levels of genes involved in ribosome biogenesis compared to wildtype seeds under salt stress (Fig. 2F, Fig. S2I). We attribute the apparent downregulation of these housekeeping genes in *floe1-1* seeds under salt stress to the initiation of gene expression upon germination. Finally, since the ratio of the phytohormones abscisic acid (ABA) and gibberellin (GA) is a major regulator of germination under unfavorable conditions, including salinity (Shu et al., 2017), we explored whether FLOE1 is involved in this hormonal regulation. Disruption of the ABA/GA balance through supplementation of ABA or inhibition of GA synthesis did not induce any germination differences between *floe1-1* and wildtype seeds (Fig. S2J–K). Together, these

results suggest that FLOE1 regulates germination through a distinct process that involves the direct sensing of water potential.

Synergistic and opposing molecular forces regulate FLOE1's phase behavior

Numerous yeast proteins undergo oligomerization or phase separation upon stress-induced quiescence (Munder et al., 2016) but FLOE1 undergoes biomolecular condensation upon *release* from the quiescent state. To define the mechanism by which FLOE1 undergoes this switch, we dissected the molecular grammar underlying this behavior. We first expressed and purified MBP-tagged FLOE1 protein from *E. coli* to determine if it can undergo phase separation in isolation. Upon cleavage of the MBP tag, FLOE1 spontaneously demixed to form droplets (Fig. 3A, Fig. S4A–B) that enriched FLOE1 but excluded MBP (Fig. S4C), similar to the behavior of other phase-separating proteins (Burke et al., 2015). To test if FLOE1 can phase separate in the range of physiological protein concentrations, we set out to estimate the local protein concentration of FLOE1 *in vivo* and performed dilution series *in vitro*. By calculating the volume of the cytoplasmic fraction of *A. thaliana* embryos (see STAR Methods), we found that FLOE1's *in vivo* concentration is within the same range it can phase separate *in vitro* (Fig. S4D, Fig. S5A–G, Movie S1, Table S3). Because FLOE1 can phase separate in isolation, we next defined which protein domains drive phase separation. FLOE1 harbors a predicted coiled-coil domain and a conserved plant-specific domain of unknown function (DUF1421) (Fig. 3B). Disorder prediction algorithms identified another predicted folded region and two different disordered regions, one enriched for aspartic acid and serine (DS-rich) and the other enriched for glutamine, proline, and serine (QPS-rich). We heterologously expressed FLOE1 in two orthogonal systems, tobacco leaves (Fig. 3C, Fig. S1L–M, Movie S2) and the human osteosarcoma cell line U2OS (Fig. 3D). In these two systems, like in *A. thaliana* (Fig. 1), FLOE1 formed spherical condensates, providing independent platforms for interrogating the molecular drivers of condensation. We deleted each domain of FLOE1 and assayed the impact on cytoplasmic condensation (Fig. 3C–E). In both tobacco and human cells, mutants lacking either the short coiled-coil domain or DUF1421 behaved identically to the wildtype protein (Fig. 3C–E). In contrast, deletion of the other domains altered FLOE1 condensation (Fig. 3C–E). Deletion of the predicted folded domain, which we refer to as the nucleation domain, abolished cytoplasmic condensation, resulting in a fraction of the protein redistributing to the nucleus. Folded oligomerization domains play important roles in nucleating phase separation of several IDPs (Boeynaems et al., 2018). Indeed, expression of chimeric fusion proteins revealed that this domain is sufficient to nucleate phase separation of the FLOE1 PrLD (i.e., QPS) and the PrLD derived from the human FUS protein, which has been extensively studied for its phase separation behavior (Patel et al., 2015) (Fig. 4A). All-atom simulations and homology-based modeling suggest that the nucleation domain adopts a trimeric coiled coil conformation (Fig. S4E), providing the multivalent interactions required for nucleating protein phase separation.

In line with their role in driving phase separation of other prion-like proteins, deletion of the QPS PrLD reduced condensate formation in cells (Fig. 3C–E) and in the test tube (Fig. S4F). Consistent with the emerging sticker-spacer framework for PrLDs (Martin et al., 2020; Wang et al., 2018), the QPS PrLD has regularly spaced aromatic tyrosine residues along its sequence that may act as attractive stickers. Substituting tyrosine residues for serines

(Y-S) decreased condensate formation in both human and tobacco cells in a dose-dependent manner (Fig. 4B, Table S3). By mapping out a phase diagram (Fig. 4C) and probing the molecular dynamics using fluorescence recovery after photobleaching (Fig. 4D) of Y-S and S-Y mutants, we confirmed that the number of tyrosines determines both the saturation and gelation concentrations of FLOE1 condensates, consistent with what has been shown for other PrLDs (Martin et al., 2020). These findings provide further evidence that FLOE1 condensates form via LLPS, and increasing its multivalency drives gelation into more solid-like irregular assemblies. While changing the number of stickers can drive a liquid-to-gel transition, altering sticker strength may also alter the gelation concentration. Substituting tyrosines for weaker (phenylalanine) or stronger (tryptophan) aromatic residues affected both condensate morphology and intracondensate dynamics in a predictable manner, in which increasing the stickiness of the QPS PrLD induced gelation of FLOE1 (Fig. 4E–F, Table S3).

Surprisingly, removing the N-terminal DS domain also induced FLOE1 gelation (Fig. 3C–E, Fig. 4G). Serine substitution of aromatic residues in the DS domain had a similar effect as DS deletion (Fig. 4G), suggesting that the aromatic residues in the DS and QPS domains have opposing functions. Intriguingly, electron microscopy revealed that the gel-like condensates formed via DS-8xY/F-S (8 phenylalanine and tyrosine residues changed to serine in the DS region) had a regular striated substructure and were strikingly distinct from the homogeneous wildtype condensates (Fig. 4H, Fig. S5I–M, Movie S3), consistent with their reduced dynamics and change in morphology (Fig. 4I–J). This striated substructure is reminiscent of synaptonemal complexes that have also been proposed to form through phase separation of a coiled coil protein (Rog et al., 2017). Thus, synergistic and opposing molecular forces tightly regulate FLOE1's biophysical phase behavior, and changing this balance allows its properties to be experimentally toggled between dilute, liquid droplet, and solid gel states (Fig. 4K).

FLOE1's condensate material properties modulate its function in germination

To directly test whether FLOE1's role in seed germination is a function of its biophysical state, we generated *A. thaliana* lines carrying wildtype or different FLOE1 domain deletion mutants in the null background (Fig. 5A–B). These mutants behaved consistently in *A. thaliana* embryos as they did in human and tobacco cells (Fig. 3C–E). The ΔQPS mutant was unable to phase separate upon imbibition (Fig. 5B), whereas the ΔDUF mutant formed condensates that were indistinguishable from the wildtype (Fig. 5B–D). In contrast, the ΔDS mutant formed condensates that were larger than those formed by the wildtype protein (Fig. 5B–D), consistent with what we observed in tobacco and human cells. The ΔDS mutant has lost its hydration-dependency and seems to exist in a constitutively condensed state (Fig. 5E). This is important because now we have a way to eliminate the reversibility of FLOE1 condensate formation. We assayed germination levels under salt stress and found that removing the DUF domain resulted in phenotypes that were indistinguishable from those of knockout lines complemented with wildtype FLOE1 (Fig. 5F, Fig. S6A–D). However, removing the QPS domain resulted in phenotypes indistinguishable from those of knockout mutants (Fig. 5F, Fig. S6A–D), suggesting that this domain is required for FLOE1 function. Surprisingly, removing the DS domain resulted in greatly enhanced germination levels under

salt stress, surpassing even that of the null mutant, indicating that ΔDS likely functions as a gain-of-function mutation (Fig. 5F, Fig. S6A–D). These data provide evidence that the reversibility of FLOE1 condensate formation is important for its function, but it remains possible that the ΔQPS and ΔDS mutations have additional effects on FLOE1 function independent of phase separation (see Discussion).

Assaying the transcriptome of ΔDS seeds showed large changes in gene expression compared to wildtype complemented lines (Fig. S6F–G, Table S2). In contrast to *floe1-1* seeds, where gene expression changes were limited to salt stress (Fig. 2E), in ΔDS seeds, we observed significant changes in both dry and non-stress imbibition conditions (Fig. S6F–G), suggesting the possibility of non-stress related phenotypes. Indeed, ΔDS seeds displayed faster germination rates under standard conditions (Fig. S6D–E). These findings indicate that switching the material properties of a biomolecular condensate can have broad phenotypic effects that can impact organismal fitness in a variety of environmental conditions (Fig. 5G).

Variation in the FLOE1 DS domain regulates variation in phase behavior, seed dormancy and germination in natural populations

To investigate the role of the material properties of FLOE1 in an ecological and evolutionary context, we first examined FLOE1's intragenic variation in phase behavior, and explored variation in expression level and seed dormancy across hundreds of *A. thaliana* ecotypes. *FLOE1* encodes two isoforms from alternatively spliced transcripts (6A). Besides the full-length isoform (FLOE1.1), which is the dominant form in all ecotypes (Fig. S7A), *FLOE1* encodes a shorter splice isoform that lacks the majority of the DS domain (6A). This shorter isoform (FLOE1.2) forms larger condensates (Fig. 6B, Fig. S6H) that can recruit the longer isoform (Fig. 6C). We next asked whether this intragenic variation in material properties of FLOE1.1 and FLOE1.2, which, based on our mutant studies, are associated with attenuating and promoting germination, respectively, could tune germination in natural populations. To explore this question, we generated a set of *floe1-1* lines complemented with different expression levels of FLOE1, and found that FLOE1 transgene expression inversely correlated with germination levels under salt stress (Fig. S2D, Fig. S6B–C). This provided an opportunity to look for evidence for such a tuning mechanism in wild populations, and to see if FLOE1 isoforms elicited differential effects in nature. We took advantage of publicly available transcriptomic data for a wide array of *A. thaliana* ecotypes (1001 Genomes Consortium, 2016), with a final set of 478 ecotypes (Table S4), and several published phenotypic studies (Martinez-Berdeja et al., 2020; Togninalli et al., 2020). Consistent with our findings from manipulating gene expression levels and FLOE1 mutants, seed dormancy across ecotypes was positively correlated with *FLOE1.1* expression, but not with *FLOE1.2* expression (Fig. S7B). Since *FLOE1.1* is the dominant isoform (Fig. S7A), this observation is in line with our results showing that FLOE1 dose-dependently attenuates germination in lab strains (Fig. S2D). By contrast, *FLOE1.2* expression across ecotypes was strongly correlated with promoting germination, whereas this correlation was less pronounced for *FLOE1.1* expression (Fig. S7C–F). Given that FLOE1.2 seems to mimic the phase behavior of our ΔDS mutant, the increased germination levels coinciding with its increased expression are consistent with the gain-of-function phenotype of ΔDS seeds (Fig. 5F, Fig. S6B,E).

While these correlations corroborate our experimental data, they do not address whether the expression of FLOE1 isoforms is tuned to the specific ecological niche, and whether this variation could drive phenotypic adaptation in wild populations. To explore this question, we examined the isoform expression across ecotypes with climate data from their respective GPS coordinates of collection. In accordance with the hypothesis that *FLOE1* expression is tuned by local environmental conditions, its expression was correlated with precipitation and temperature (Fig. S7G–L). Surprisingly, *FLOE1.1*/*1.2* expression was positively correlated with summer precipitation, while negatively correlated with temperature, indicating this dormancy/germination switch may be a mechanism more prevalent in colder regions of the distribution. Since bet-hedging strategies are often employed by organisms to cope with changing environments, we asked how expression of the isoforms behaved in the context of variability in precipitation by calculating the coefficient of variation of July rainfall at the geographic locations of origin for a time-series from 1958–2017 (Abatzoglou et al., 2018). Strikingly, *FLOE1.2* (putative promoter of germination) expression negatively correlated with the yearly variation in precipitation, whereas this was far less pronounced for *FLOE1.1* (putative attenuator of germination) (Fig. S7M–N), indicating that natural populations living in environments with unpredictable precipitation express lower levels of *FLOE1.2*. This suggests that expression of *FLOE1.2* to control germination/dormancy may not be an advantageous strategy for plants living in environments with unpredictable rainfall such as the Mediterranean basin, where temperature may be a more reliable cue to fine-tune dormancy, as shown by other studies of temperature sensing germination delayers (Exposito-Alonso, 2020). In summary, natural variation in the DS domain between FLOE1 isoforms may fine-tune FLOE1 function in seed dormancy and germination across ecotypes for local adaptation.

Variation in the DS domain resides not only intragenically, but also intergenically. The *A. thaliana* genome also encodes two *FLOE1* paralogs, which we named *FLOE2* (AT5G14540) and *FLOE3* (AT3G01560). When expressed in tobacco cells, FLOE2 and FLOE3 also formed larger condensates compared to FLOE1.1 (Fig. 6D, Fig. S6H). FLOE2 and FLOE3 have expanded DS regions with lower overall structural disorder, while their QPS domains were very similar to FLOE1's (Fig. 6E). To see if FLOE2 and FLOE3 could recruit FLOE1.1 similar to FLOE1.2, we coexpressed them in tobacco. Surprisingly, coexpression of FLOE1 with FLOE2 or FLOE3 resulted in the formation of demixed condensates, illustrating immiscibility of FLOE1 with its paralogs (Fig. 6F, Fig. S6I). Such complex topologies are common among biomolecular condensates, and have been linked to differences in surface tension or viscosity between the different phases (Boeynaems et al., 2019; Feric et al., 2016). Since the DS domain is a key tuner of FLOE1 material properties, we wondered how deletion of this domain would impact condensate mixing. Surprisingly, while FLOE1/ΔDS FLOE1 condensates showed weak ΔDS substructures, consistent with the more gel-like nature of this mutant, combining the ΔDS FLOE1 mutant with FLOE2 or FLOE3 condensates lead to complete condensate mixing (Fig. 6F). Since the ΔDS FLOE1 mutant had such a pronounced phenotype compared to *floe1-1* lines in promoting germination under salt stress, we hypothesize that the aberrant interaction of ΔDS FLOE1 with endogenous FLOE2/3 paralogs may be in part driving its gain-of-function phenotype (Fig. 6G).

Wide variety in FLOE phase behavior across the plant kingdom

To assess whether phase separation is conserved in the FLOE family, we explored the diversity of this protein family in nature. We found FLOE homologs in all green plant lineages, even in those preceding seed evolution (Fig. 7A–B, Fig. S6J). Phylogenetic analysis revealed the emergence of two major clades, FLOE1-like and FLOE2-like. While the latter clade includes the algal lineage, the FLOE1-like clade is restricted to seed plants. Both clades show conserved variation in the length of the two disordered domains (Fig. 7C). By testing a set of FLOE homologs across the plant kingdom, we find wide phenotypic variation in phase separation (Fig. 7D, Fig. S6K) that involves differences in condensate sizes and nuclear localization (Fig. 3C). These findings indicate there exists a wealth of natural sequence variation that might give rise to these different behaviors. Given our observations linking the variation in FLOE1 isoforms and paralogs in *A. thaliana* to phenotypic variation in both engineered and natural strains, this wide diversity of FLOE paralogs with differential phase separation behaviors suggests the possibility that this gene family could be a driver of functional phenotypic variation across the plant lineage.

DISCUSSION

Phase separation is emerging as a universal mechanism to explain how cells compartmentalize biomolecules. Despite advances involving the widespread identification of novel biomolecular condensates and elucidating their underlying molecular grammar, many questions remain. Chief among these is whether protein phase separation is truly functional for cellular physiology or whether it is an unintended consequence of protein evolution (Martin and Holehouse, 2020). Recent work in yeast shows that phase separation of prion-like and related proteins is important for their function (Franzmann et al., 2018; Riback et al., 2017), suggesting that these protein domains may indeed have evolved to drive protein condensation. Interestingly, amyloid-based aggregation of the same prion-like proteins has also been shown to confer fitness advantages to yeast under certain environmental conditions (Halfmann et al., 2012). This observation indicates that the material properties of biomolecular condensates may be important in regulating protein function. Yet, this picture is less clear for multicellular organisms, especially since aggregation of several of these phase separation-prone proteins is implicated in human disease (King et al., 2012; Ramaswami et al., 2013; Scheckel and Aguzzi, 2018). The question therefore still remains whether this process is truly functional in multicellular eukaryotes. Emerging evidence suggests the functionality of protein condensates in plants (Chakrabortee et al., 2016; Fang et al., 2019; Jung et al., 2020; Powers et al., 2019; van Dop et al., 2020; Zhang et al., 2020) and flies (Bakthavachalu et al., 2018). However, *in vivo* evidence for a functional role of the emergent properties of phase separation is lacking. Here we demonstrate that conformational switches between liquid and solid-like states of FLOE1 can drive functional phenotypic variability via bet-hedging in a multicellular organism, similar to the prion-based bet-hedging strategies described in yeast (Halfmann et al., 2012).

Seed germination follows a bet-hedging strategy by spreading the risk of potentially lethal conditions, such as drought, across different seasons or years (Gremer and Venable, 2014; Johnston and Bassel, 2018; Villa Martin et al., 2019). While seeds withstand many stresses

in their desiccated dormant state, seedlings are at the mercy of their environment and present the most vulnerable stage in a plant's life. Therefore, precise timing of germination is crucial for plant fitness. Seeds should germinate under conditions that ensure the highest chance of seedling survival, yet a trade-off is that if they wait too long, they may miss the window of good conditions, increase their risk to be eaten or buried too deeply, or ultimately exhaust their viability. Importantly, rainfall cue sensing that drives seed behavior remains largely unresolved, and not only poses an interesting biological problem, but also an outstanding agricultural challenge of great societal importance (Baskin and Baskin, 2004; Johnston and Bassel, 2018).

To identify factors that could regulate seed timing and bet-hedging strategies, we investigated the seed-enriched proteome. These proteins were overrepresented for disorder and involved a set of prion-like proteins. Among them was an uncharacterized prion-like protein that we named FLOE1. Interestingly, FLOE1 formed reversible condensates in *A. thaliana* embryos that were hydration-dependent. Seed desiccation presents a peculiar biological challenge to proteins as they have to switch between a completely desiccated state and a 'wet' state that supports cellular biochemistry, all while preventing irreversible protein aggregation. FLOE1 undergoes hydration-dependent phase separation in plant seeds and it is likely that similar processes occur in a wide variety of organisms with quiescent desiccated life stages, including human pathogens, nematodes, and fungal spores (Boothby and Pielak, 2017; Esbelin et al., 2018; Giarola et al., 2017).

Given that water uptake is the key initiating event for germination, seeds must have evolved a water sensor that allows them to measure water potential. By characterizing *FLOE1* knock-out and complemented lines, we provide evidence that FLOE1 attenuates germination under water-limiting conditions in a dose-dependent manner and that its condensation coincides with imbibition. Moreover, the extent of FLOE1 condensation is directly correlated with seed germination. An interesting observation is that despite germination being a binary decision for a seed, FLOE1 condensation displays large variation at the cellular and tissue levels. The link between cellular variability and organism-scale variability in seeds is still unclear (Mitchell et al., 2017), and future work should aim to elucidate how embryos integrate a distribution of cellular phenotypes within a tissue into an ON/OFF switch decision.

To test whether FLOE1's biophysical state is important for its function as a germination regulator, we dissected its molecular grammar and interrogated mutants for their ability to rescue the *floe1-1* phenotype. First, we found that FLOE1's phase behavior is tuned by opposing forces exerted by two disordered domains. While the prion-like QPS domains drives condensation, its DS domain tunes the material state by fluidizing FLOE1 condensates. This information allowed us to engineer two key mutants: one that can no longer phase separate (ΔQPS) and one that presents solid-like condensates (ΔDS). Second, by complementing *floe1-1* with these mutants, we find that the QPS domain is required for its function, whereas the ΔDS mutant acts in a gain-of-function manner leading to a dramatic germination phenotype. In the evolutionary game theory framework (Gremer and Venable, 2014; Johnston and Bassel, 2018; Villa Martin et al., 2019), the ΔDS mutant behaves like a "high-stakes gambler"—it perceives the risk of germination under stress (e.g.,

seedling dying) to be lower than the chance of there being a change in the environment (e.g., increased rainfall). Thus, our work shows that the emergent properties of a biomolecular condensate can tune fitness *in vivo*, by tuning bet-hedging strategies at a crucial step in a seed's life (Fig. 5G).

The observation that variation in FLOE1 sequence and its expression level tune germination, make it a prime candidate as a seed bet-hedging gene. We made use of available transcriptomic, physiological, and climatologic data of *A. thaliana* ecotypes to test this hypothesis and found evidence that wild populations might use FLOE1 variation to tune their bet-hedging strategies according to their environment's predictability. By analyzing the conservation of FLOE1, we find a vast repertoire of FLOE sequence diversity and phase separation behavior, suggesting the possibility that other species may also use this gene family to fine-tune germination as a local adaptation mechanism. Of note, the FLOE family precedes the origin of seed plants, and even in *A. thaliana*, FLOE1 paralogs—FLOE2 and FLOE3—show a broader tissue expression (Waese et al., 2017), pointing to potential functions beyond seed germination.

In conclusion, we have identified a putative plant water potential sensor that undergoes reversible hydration-dependent phase separation and regulates seed germination. Our findings have direct implications for not only cell biology and ecology, but also agriculture. By providing a set of guiding principles to engineer a novel biomolecular condensate (Hastings and Boeynaems, 2021), we hope to inspire the development of designer crops engineered to withstand the effects of climate change.

LIMITATIONS OF THE STUDY

The precise molecular function of FLOE1 remains unresolved. Our data are consistent with FLOE1 functioning at the transition between the desiccated state of the dry embryo and the reactivated hydrated state – when the protein undergoes reversible condensation. But there are alternative interpretations. It is possible that FLOE1 might have a function independent of phase separation. The ΔQPS mutant that we made to prevent condensation might disrupt this other FLOE1 function in the dispersed state independent of its ability to form a condensate. It is also formally possible that the ΔDS mutant, despite being in a constitutively condensed state, might have an enhanced function in a very minor dispersed fraction that we cannot readily detect. Future work will be needed to precisely define contributions of FLOE1 function in the disperse vs. condensed state. Because FLOE1 is dispersed in the dry state of the embryo, these analyses represent technical challenges and new experimental methods will be needed. Currently, the tools to investigate dry biological matter are limited. Perhaps techniques can be borrowed from the material science field to study the dry state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Dorone et al. (2021): HIGHLIGHTS

- *FLOE1* is a plant-specific gene that regulates seed germination under water stress
- FLOE1 undergoes reversible hydration-dependent phase separation
- Ecological data suggests that FLOE1 variation is involved in local adaptation
- FLOE1 can be engineered to tune seed germination with potential use for agriculture

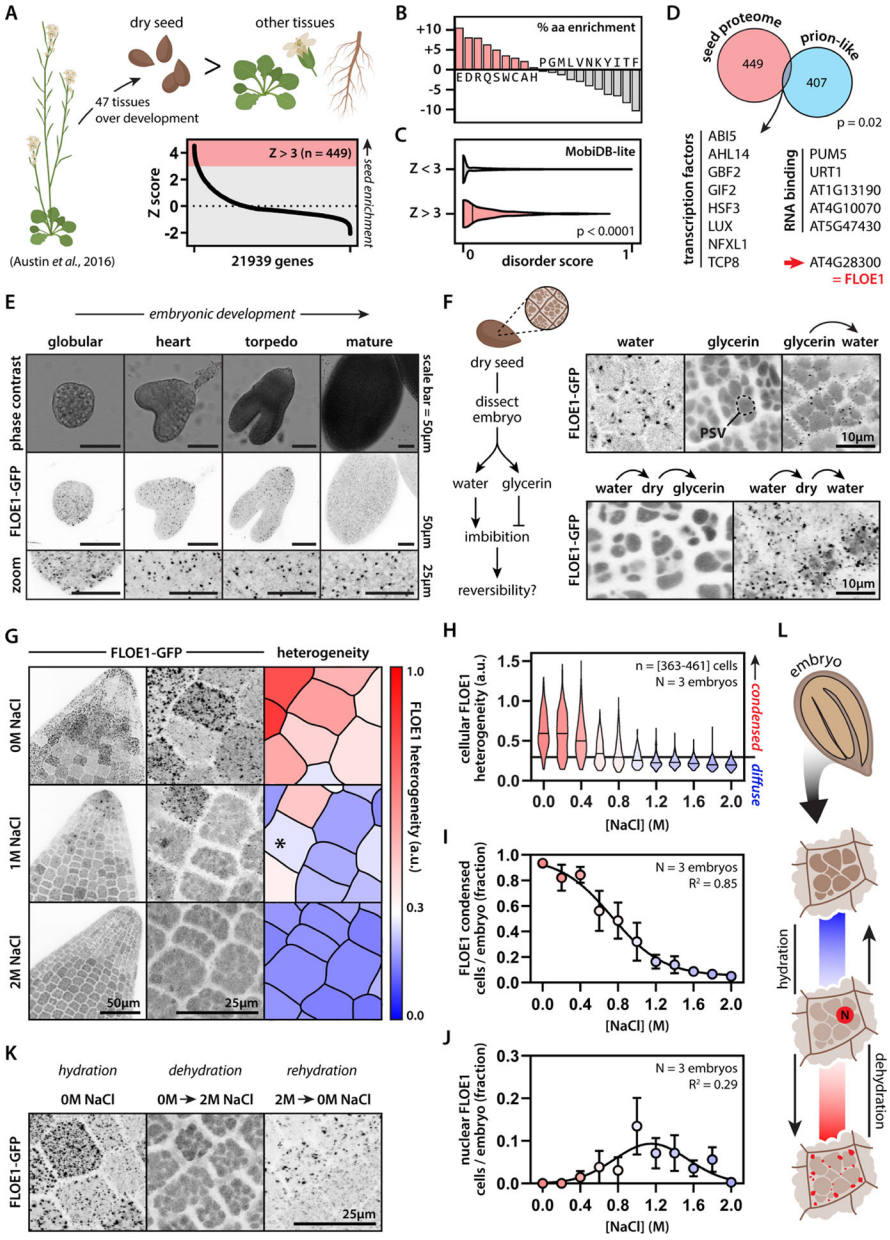

Figure 1: FLOE1 is an uncharacterized seed protein that undergoes biomolecular condensation in a hydration-dependent manner.

(A) Identification of genes enriched in dry *Arabidopsis* seeds. (B–C) The seed proteome is enriched for specific amino acids (B) and intrinsic disorder (C). Mann-Whitney test. (D) The seed proteome is enriched for prion-like proteins. Binomial test. AT4G28300 is an uncharacterized prion-like protein, which we named FLOE1. (E) FLOE1p:FLOE1-GFP is expressed during embryonic development and forms condensates. (F) FLOE1-GFP forms condensates in embryos dissected from dry seed in a hydration-dependent and reversible manner. Embryonic cotyledons are shown. PSV denotes autofluorescent protein storage vacuoles that are more prominent in the dry state than in the hydrated state (see Fig. S1E). (G) Cell-to-cell variation in subcellular FLOE1-GFP heterogeneity in response to salt. Radicles are shown. * denotes nuclear localization. (H) Quantification of cellular FLOE1

heterogeneity as a function of salt concentration. Black line denotes the 95th percentile of the 2M NaCl heterogeneity distribution. (I) Quantification of the percentage of cells per radicle that show FLOE1 condensation as a function of salt concentration. Mean ± SEM. Four-parameter dose-response fit. (J) Quantification of the percentage of cells per radicle that show FLOE1 nuclear localization as a function of salt concentration. Mean ± SEM. Gaussian fit. (K) FLOE1-GFP condensation exhibits reversibility between high and no salt treatment. Radicles are shown. (L) Scheme highlighting different FLOE1 behaviors upon imbibition. Fluorescence microscopy images are maximum projections. GFP signal is displayed as an inverted gray scale.

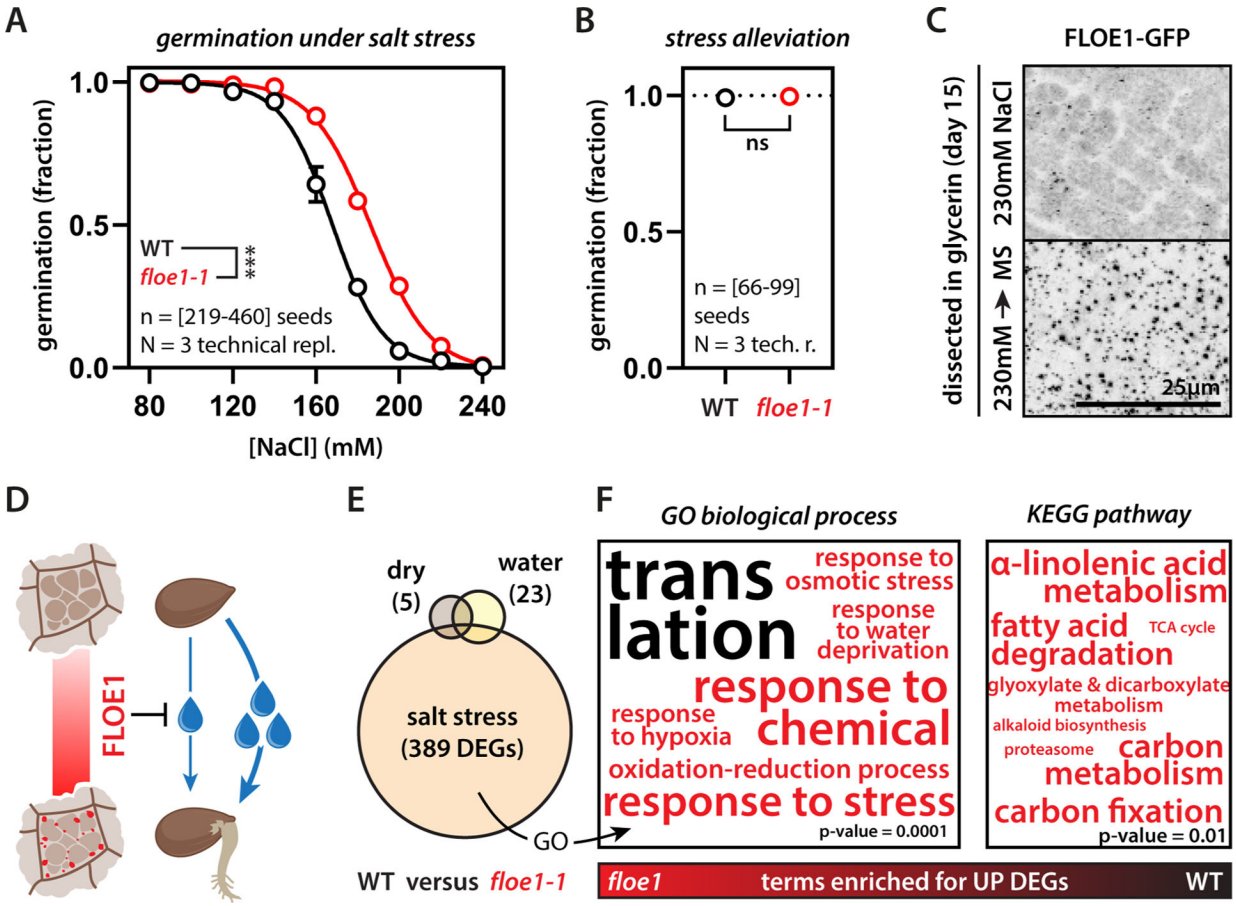

Figure 2: FLOE1 attenuates germination under water stress.

(A) *floe1-1* seeds show higher germination levels under salt stress. Two-way ANOVA, four-parameter dose-response fit, *** p-value < 0.001. Representative of three independent experiments. ΔIC_{50} is 18.5mM. (B) Seeds retain full germination potential under standard conditions after a 15-day 230mM salt stress treatment. Representative of three independent experiments. (C) Condensates are largely absent in ungerminated seeds after 15 days of incubation under salt stress. FLOE1 condensates appear within two hours after transfer to standard conditions (MS medium). Maximum projection images of radicle cells. GFP signal is displayed as an inverted gray scale. (D) Scheme highlighting the potential function of FLOE1 in attenuating germination when water potential is low. Droplets indicate water availability. (E) *floe1-1* seeds show high numbers of differentially expressed genes (DEGs) after imbibition under salt stress, as opposed to unimbibed (dry) and normally imbibed (water) seeds. Imbibition was performed on MS medium by first stratifying for 5 days followed by 4h incubation in a growth cabinet. (F) *floe1-1* seeds upregulate stress response genes and genes implicated in metabolism compared to wildtype seeds, and have relatively lower expression of genes involved in ribosomal biogenesis. The only KEGG pathway enriched for the WT was “ribosome” (p-value = 3.88E-17, not shown). See also Table S2. Font size correlates to −log10 (p-value). P-values at bottom-right for scale.

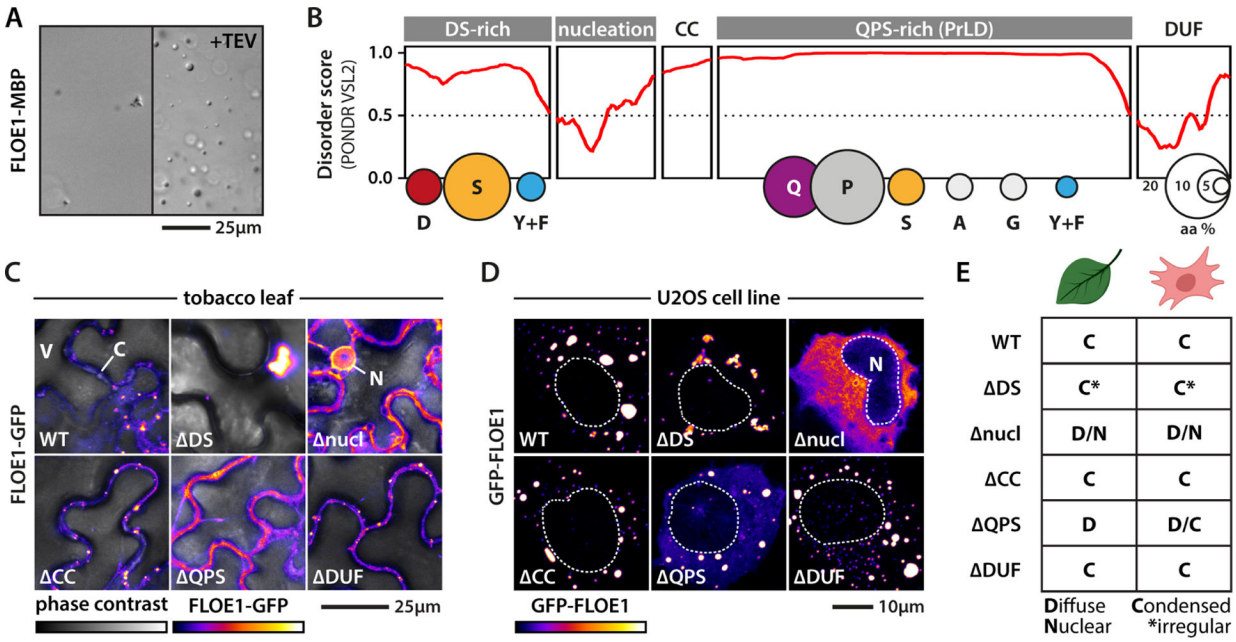

Figure 3: Molecular dissection of FLOE1 phase separation.

(A) Recombinant MBP-FLOE1 phase separates in the test tube upon MBP cleavage with TEV protease. Irregular small aggregates can be seen pre-cleavage highlighting FLOE1 aggregation-propensity. DIC imaging. (B) FLOE1 domain structure. CC = predicted coiled coil, DUF = DUF1421. Balloon plots show amino acid composition of the disordered domains. (C–D) Expression of FLOE1 domain deletion mutants in tobacco epidermal pavement cells (C) and human U2OS cells (D). V = vacuole, C = cytoplasm, N = nucleus. (E) Summary of FLOE1 behavior in tobacco and human cells. Fluorescence microscopy images are single optical sections. GFP signal is displayed as a false-colored intensity scale.

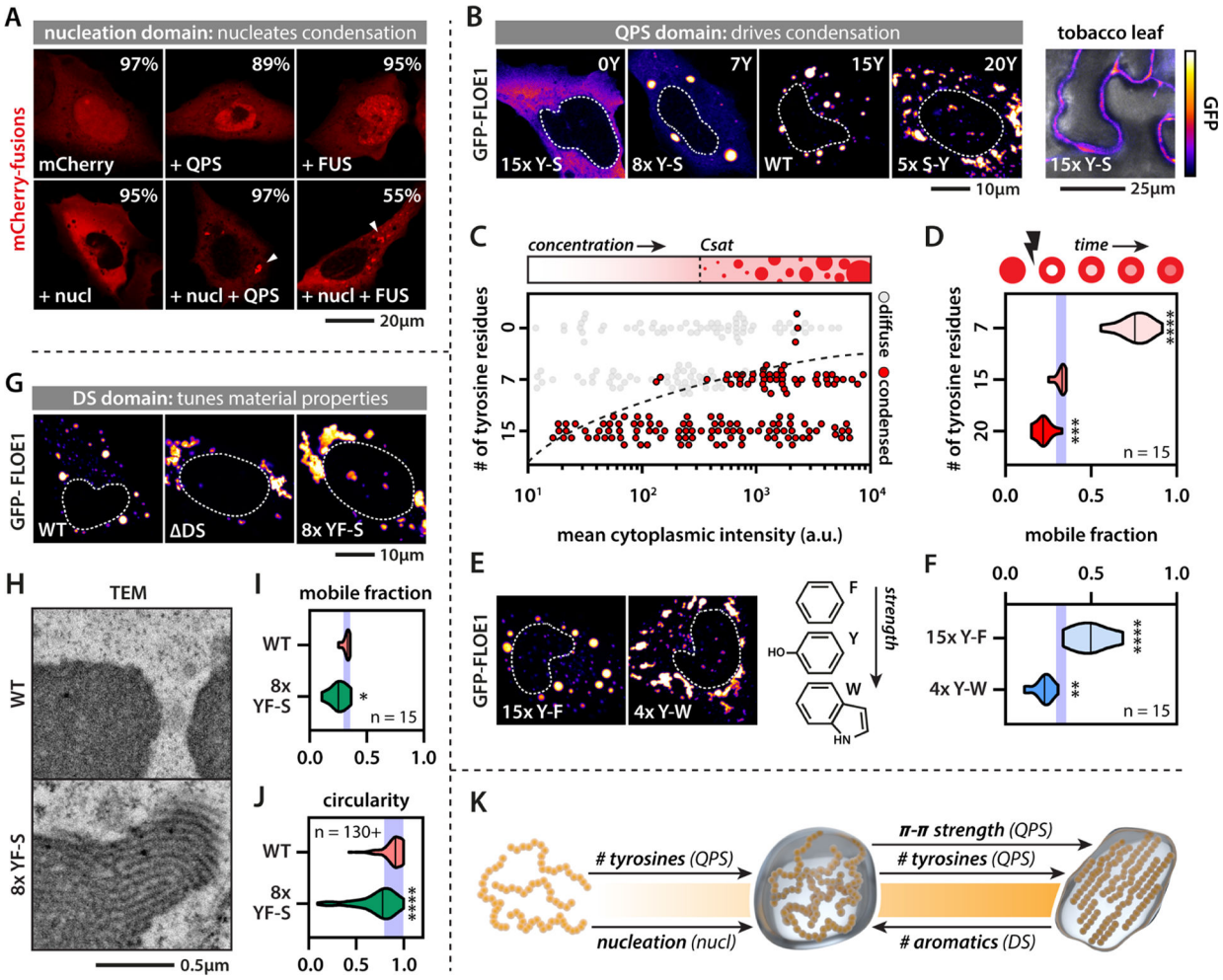

Figure 4: Molecular dissection of FLOE1 phase separation.

(A) Chimeric proteins containing both the FLOE1 nucleation domain and the PrLDs from FLOE1 (QPS) or the human FUS protein form cytoplasmic condensates. Percentages display number of cells lacking or containing condensates. Average of three experiments. Arrowheads point at cytoplasmic condensates. (B) The number of QPS tyrosine residues alters FLOE1 phase separation in human and tobacco cells. (C) FLOE1 phase diagram as a function of concentration and number of QPS tyrosines. (D) Number of QPS tyrosines affects intracondensate FLOE1 dynamics. Mobile fraction as assayed by FRAP is shown. One-way ANOVA. (E–F) QPS tyrosine-phenylalanine and tyrosine-tryptophan substitutions alter condensate morphology (E) and intracondensate dynamics compared to WT (F). One-way ANOVA. (G) DS deletion or DS tyrosine/phenylalanine-serine substitutions alter condensate morphology. (H) TEM shows that mutant DS FLOE1 condensates have filamentous substructure that is absent in the WT. U2OS cells. (I) DS tyrosine/phenylalanine-serine substitutions alter intracondensate dynamics. Student's t-test. (J) DS tyrosine/phenylalanine-serine substitutions alter condensate morphology. Mann-Whitney. (K) Scheme summarizing synergistic and opposing roles of FLOE1 domains on the material property spectrum. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001. Purple band denotes WT mean ± SD (D, F, I, J). Fluorescence microscopy images

are single optical sections. GFP signal is displayed as a false-colored intensity scale (scale in panel B).

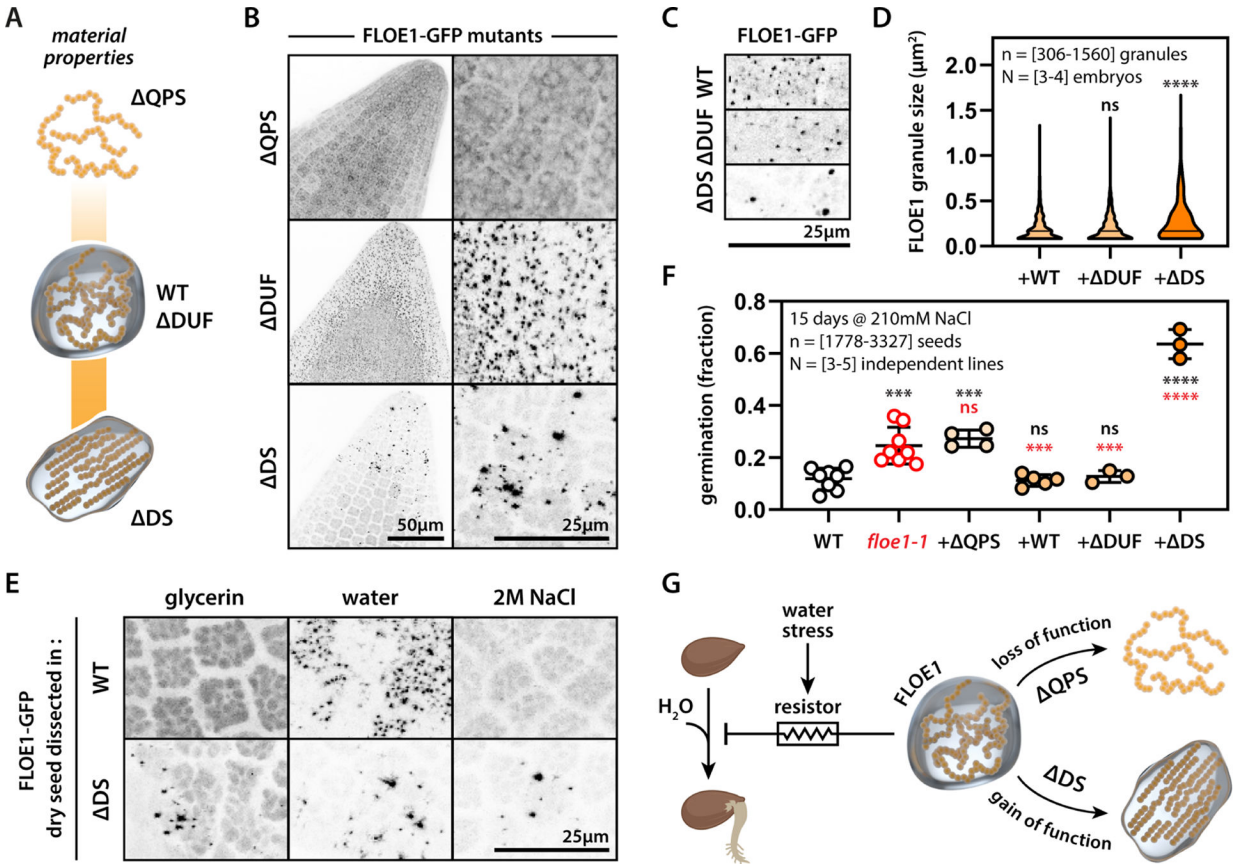

Figure 5: FLOE1 condensate material properties regulate its role in seed germination under salt stress.

(A) Scheme highlighting position of tested FLOE61 mutants on the material properties spectrum. (B) Representative images of *floe1-1* mutants complemented with ΔQPS, ΔDUF and ΔDS forms of FLOE1 upon dissection in water. Maximum projection images from embryo radicles. (C) Close-up images of WT and mutant FLOE1 condensates. Single optical sections from embryo radicles. (D) Quantification of FLOE1 condensate size. One-way ANOVA. (E) ΔDS FLOE1 condensates are not dependent on hydration. Maximum projection images from embryo radicles. (F) Germination levels of WT, *floe1-1* and complemented lines. One-way ANOVA. Representative of three independent experiments. (G) Scheme highlighting FLOE1's role in regulating germination and the effect of mutants with altered material properties. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001. GFP signal is displayed as an inverted gray scale.

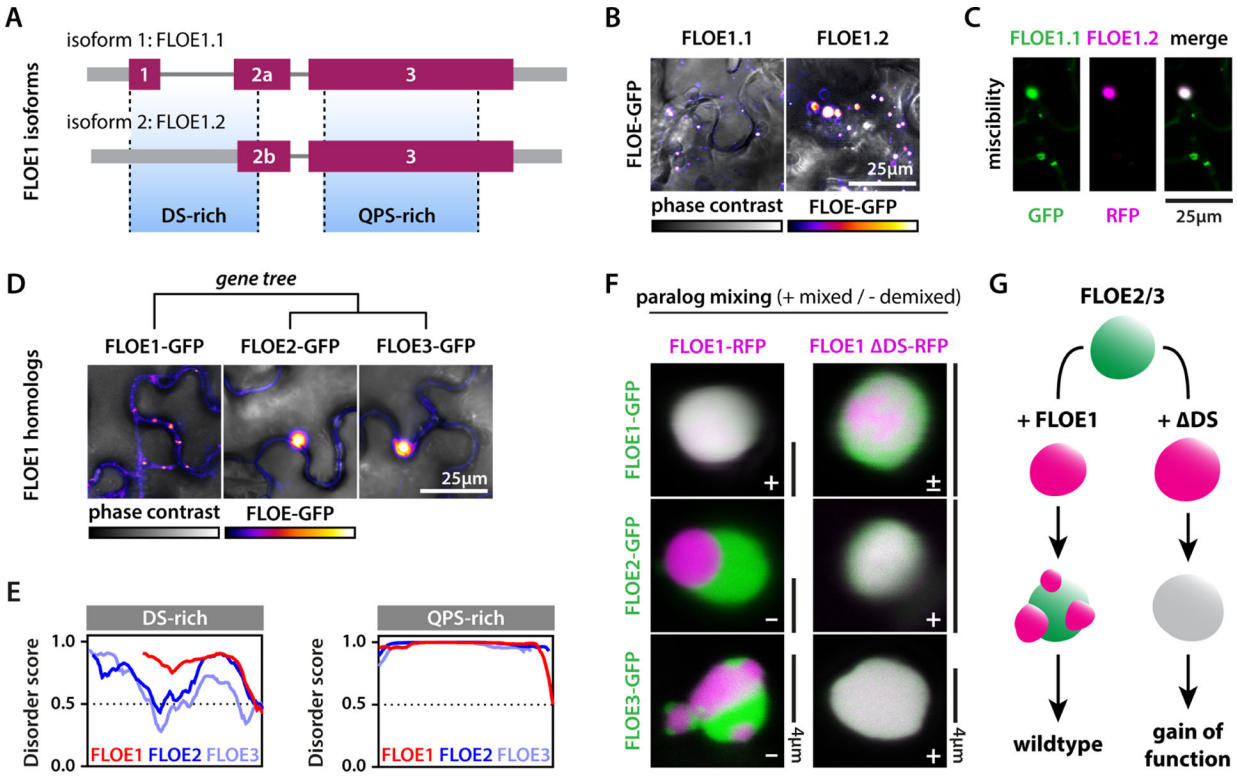

Figure 6: The DS-rich domain drives variation in condensate properties of FLOE1 isoforms and paralogs.

(A–B) *A. thaliana* has two FLOE1 isoforms. FLOE1.2 is missing most of the DS-rich region (A) and forms larger condensates than FLOE1.1 in tobacco leaves (B). (C) The large FLOE1.2 condensates recruit FLOE1.1. (D) FLOE1 has two *A. thaliana* paralogs that form larger condensates in tobacco leaves. (E) Disorder plots show strong length- and disorder variation between FLOE1 and FLOE2/3 in their DS-rich domains, but not in their QPS-rich domains. (F) FLOE1 condensates do not mix with FLOE2 and FLOE3 condensates. Deletion of the FLOE1 DS-rich domain partially disrupts mixing with wildtype FLOE1, but drives uniform mixing with FLOE2/3 condensates. (G) Scheme highlighting the switch-like role of the FLOE1 DS-domain in condensate mixing and the corresponding phenotypes. All images are single optical sections of tobacco epidermal pavement cells. (B, D) GFP signal is displayed as false-colored intensity scale (scale in panel D). (C, F) GFP and RFP signal are false-colored green and magenta.

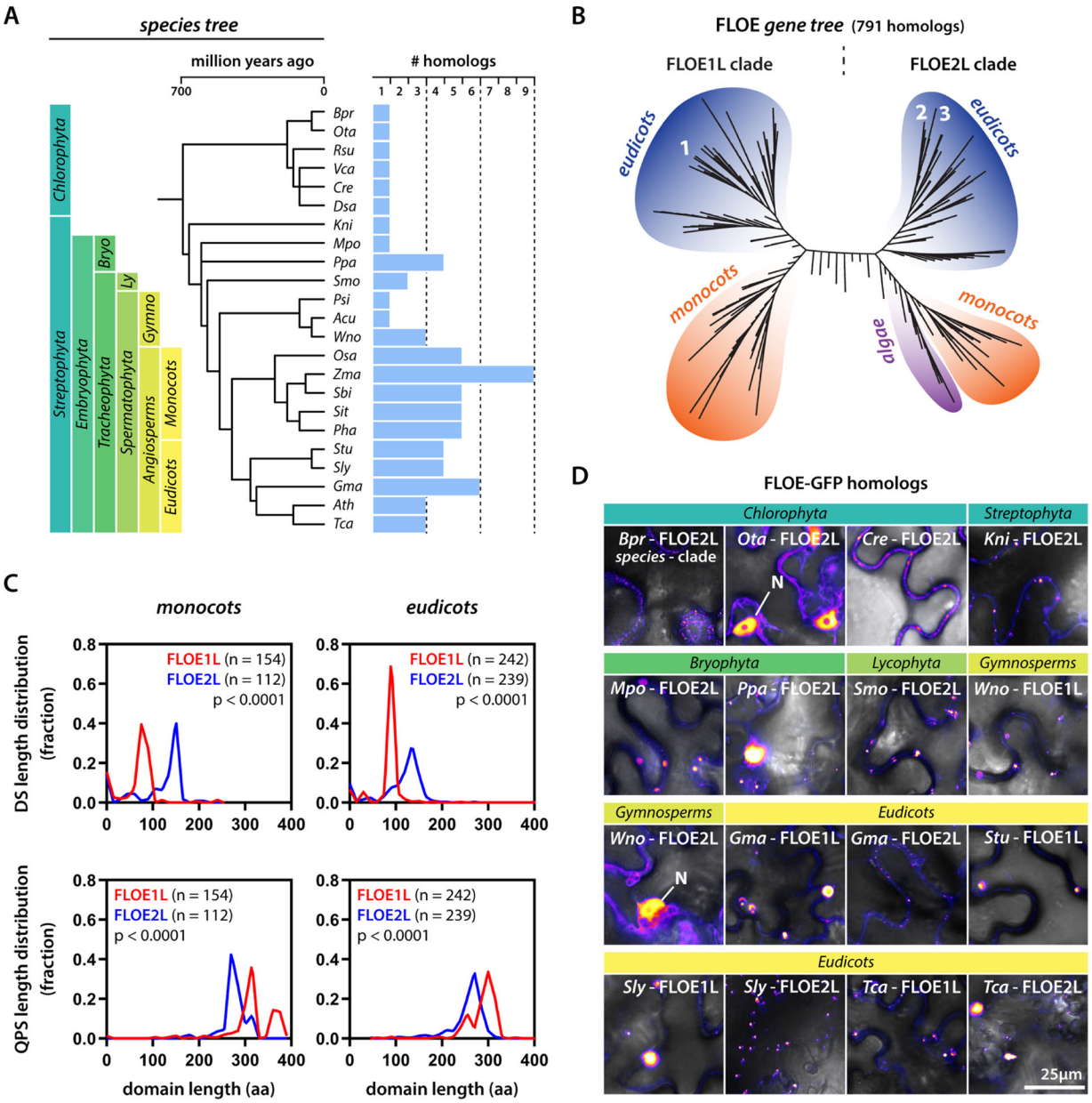

Figure 7: Natural sequence variation tunes FLOE phase separation.

(A) A species tree of the plant kingdom with example species and their number of FLOE homologs. (B) Gene tree of FLOE homologs. Numbers correspond to FLOE1, FLOE2, and FLOE3. (C) Distribution of DS and QPS length differences between the FLOE1-like (FLOE1L) and FLOE2-like (FLOE2L) clades among monocots and eudicots. Mann-Whitney. (D) Examples of FLOE homologs from across the plant kingdom. N denotes nuclear localization. Single optical sections of tobacco epidermal pavement cells. GFP signal is displayed as false-colored intensity scale. Full species names for (B,D) in Fig. S6J. Each panel indicates the specific clade of the homolog (FLOE1L or FLOE2L).