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Liquid–liquid phase separation in plants: Advances and perspectives from model species to crops

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ABSTRACT

Membraneless biomolecular condensates play important roles in both normal biological activities and responses to environmental stimuli in living organisms. Liquid–liquid phase separation (LLPS) is an organizational mechanism that has emerged in recent years to explain the formation of biomolecular condensates. In the past decade, advances in LLPS research have contributed to breakthroughs in disease fields. By contrast, although LLPS research in plants has progressed over the past 5 years, it has been concentrated on the model plant *Arabidopsis*, which has limited relevance to agricultural production. In this review, we provide an overview of recently reported advances in LLPS in plants, with a particular focus on photomorphogenesis, flowering, and abiotic and biotic stress responses. We propose that many potential LLPS proteins also exist in crops and may affect crop growth, development, and stress resistance. This possibility presents a great challenge as well as an opportunity for rigorous scientific research on the biological functions and applications of LLPS in crops.

Key words: biomolecular condensates, crops, intrinsic disordered proteins, LLPS potential prediction

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INTRODUCTION

Eukaryotic cells use membrane-bound organelles and membraneless biomolecular condensates to compartmentalize biomolecules and ensure that biochemical reactions operate efficiently in a specific time and space (Weber and Brangwynne, 2012; Hyman et al., 2014; Banani et al., 2017). In cells, micrometer-scale compartments composed of concentrated proteins and nucleic acids that lack surrounding membranes are called "biomolecular condensates" (Banani et al., 2017). Biomolecular condensates are distributed throughout the cell nucleus, cytoplasm, and membrane and are involved in many biological processes, including regulation of gene transcriptional activation or repression, remodeling of chromatin structure, the DNA damage response (DDR), RNA splicing and processing, ribonucleoprotein formation, stress granule (SG) formation, small peptide and protein transport, and protein degradation pathways (Sabari et al., 2018; Shin et al., 2018; Gibson et al., 2019; Fujioka et al., 2020; Noda et al., 2020; Strickfaden et al., 2020; Zhang et al., 2020; Wang et al., 2021a; Guo et al., 2021; Peng et al., 2021; Wagh et al., 2021; Ravindran et al., 2023). Condensates that participate in specific biological processes are also given different names, such as nucleoli (Boisvert et al., 2007), SGs (Buchan and Parker, 2009; Decker and Parker, 2012), photobodies (Van Buskirk et al., 2012), and dicing bodies (Fang and Spector, 2007; Xie et al., 2021). In recent years, the biological functions and organization of biomolecular condensates have become an emerging topic of research, especially in animals and yeast. However, long growth cycles and technical limitations have kept the study of biomolecular condensates in plants from developing past its infancy (Emenecker et al., 2021; Solis-Miranda et al., 2023).

Despite the lack of a membrane barrier, biomolecular condensates remain in high concentrations in the cellular environment under certain conditions. Dense biomolecular condensates exhibit diverse states, including liquid, gel, and solid states (Field et al., 2023). How these condensates are formed is one of the mysteries of biology. Studies have shown that phase separation is a major driving force for formation of biomolecular condensates. Because many biomolecular condensates have liquid-like properties, liquid–liquid phase separation (LLPS) is predominant in research (Hyman et al.,

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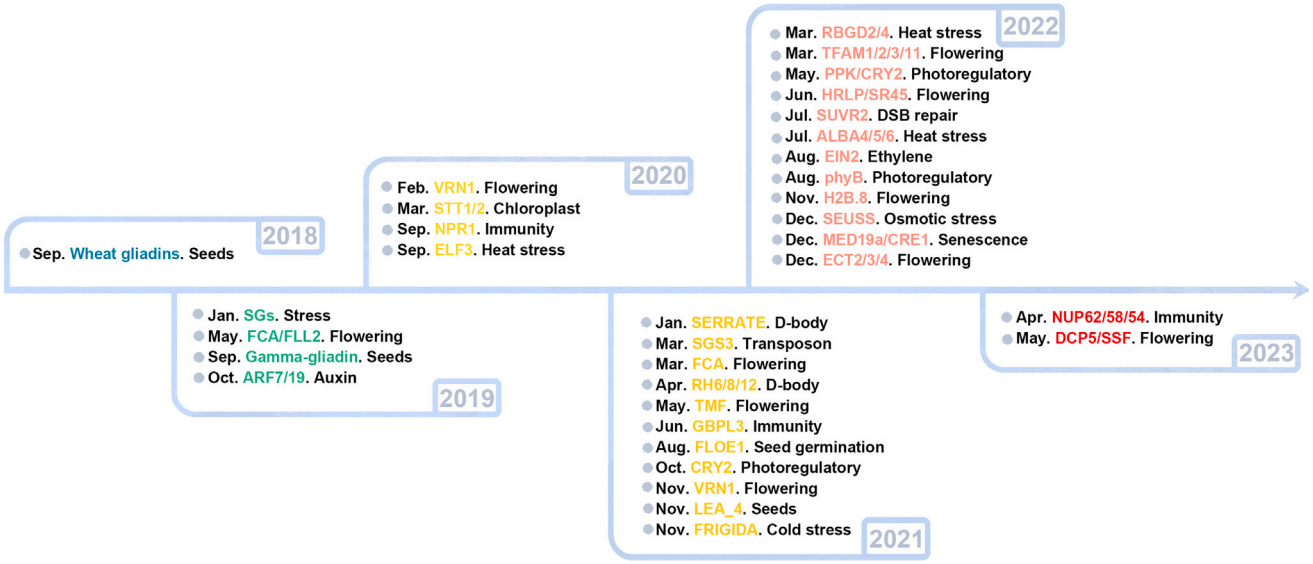

Figure 1. Reports of LLPS in plants are increasing yearly.
Since 2018, it has gradually been discovered that plant proteins undergo phase separation to form biomolecular condensates involved in plant growth, development, and stress resistance. Most advances were reported from 2021 to 2022. The first column in each frame refers to the month. The colored second column refers to the phase-separation protein. The third column refers to the biological process involved.

2014; Gao et al., 2022). In cell biology, LLPS refers to the condensation of macromolecules such as proteins or nucleic acids into a dense phase, usually similar to a liquid droplet, in a specific cellular environment; this dense phase coexists with the dilute phase (Shin and Brangwynne, 2017; Alberti et al., 2019). In 2009, Brangwynne et al. proposed that P granules in embryonic cells of *Caenorhabditis elegans* are liquid droplets formed by phase transitions. Since then, LLPS research has proliferated in the biological field (Brangwynne et al., 2009). In general, the basic functions of LLPS are as follows: rapidly sensing environmental changes, storing macromolecules, increasing local concentrations to accelerate biochemical reactions, creating isolation barriers, influencing subcellular localization, shaping morphological structures, and forming filtering devices (Alberti et al., 2019; Bergeron-Sandoval et al., 2021; Bouchard et al., 2018; Dao et al., 2018; Di Matteo et al., 2017; Feric et al., 2016; Lee et al., 2016; Nott et al., 2015; Riback et al., 2017; Schmidt and Gorlich, 2016; Sheu-Gruttadauria and MacRae, 2018; Van Buskirk et al., 2012). Most of the phase-separated biomolecular condensates reported in plants to date have liquid properties, and a few exhibit hydrogel states (Wang et al., 2023a). To our knowledge, no solid-state particles have been observed. Here, we focus on the biological functions and formation conditions of biomolecular condensates formed via LLPS in plants.

Translating scientific discoveries into innovative applications has always been the aim of scientists. Currently, LLPS is being used to treat many diseases and cancers, and it is gradually being applied to clinical medicine (Molliex et al., 2015; Patel et al., 2015; Zhu et al., 2020; Ahn et al., 2021; Portz et al., 2021; Boyko and Surewicz, 2022; Mehta and Zhang, 2022; Xie et al., 2022). By contrast, although LLPS research in plant systems has made some progress, LLPS is still far from being used in production applications. In 2021, two reviews systematically introduced the concept of phase separation and pointed out that studies on LLPS in plants were lacking (Emenecker et al., 2020, 2021). Nevertheless, the findings are very exciting for scholars in the plant field, and bodies once found in plant cells have been reinterpreted as evidence of LLPS (Cuevas-Velazquez and Dinneny, 2018; Wang and Liu, 2019; Kim et al., 2021b; Xu et al., 2021b; Lei et al., 2021; Maruri-Lopez et al., 2021; Field et al., 2023). From 2018 to 2023, a growing number of studies have shown that LLPS has crucial effects on plant development, flowering, photomorphogenesis, and stress resistance (Figure 1). Most of these studies have targeted *Arabidopsis thaliana*. The roles of LLPS in other plants, especially crops, have apparently been neglected. Both a previous study (Zhang et al., 2022a) and our proteomic data analysis (Figure 5) have shown the existence of many potential phase-separated proteins in crops. Substantive research in this area is almost nonexistent, and exciting discoveries are expected in the coming years.

In this paper, we first review the many potential phase-separation proteins in plants, which are involved in diverse pathways. Second, we classify and discuss recently reported biomolecular condensates formed by phase separation according to their effects on plant growth, development, and stress resistance. Third, we propose that studying biomolecular condensates in crops will be valuable for improving resistance and yield. Finally, we review some analysis and prediction tools suitable for plant proteins with potential LLPS to help plant researchers quickly determine the properties of target proteins.

IDENTIFICATION OF INTRINSICALLY DISORDERED PROTEINS IN PLANTS

Multivalent interactions play a central role in driving biomolecules to undergo LLPS (Lyon et al., 2021). Proteins with multivalency are usually the dominant targets of phase-separation studies.

These multivalent proteins typically contain multivalent tandem structural domains, low-complexity domains (LCDs), intrinsically disordered regions (IDRs), prion-like domains (PrLDs), or RNA-binding domains (RBDs). These domains are likely to overlap and to be found in the same protein. In some cases, LLPS of proteins requires one independent domain, but more often, it requires the interplay of multiple domains (Wang et al., 2018; Emenecker et al., 2021; Gao et al., 2022). Some proteins are composed of multiple tandem repeat domains that interact with ligands to increase overall mutual affinity. Modular multivalent proteins often undergo phase separation only in the presence of ligand partners (Li et al., 2012). LCDs, IDRs, PrLDs, and RBDs are usually enriched in charged amino acids, polar amino acids, and aromatic amino acids. These amino acid residues generate intra- and intermolecular electrostatic attraction, cation–π, dipole–dipole, and π–π stacking, and other weak multivalent interactions to promote the nucleation and growth of biomolecular condensates (Brangwynne et al., 2015). Protein posttranslational modifications (PTMs) are also critical for phase separation. PTMs affect phase transition behavior by changing the structure, charge, hydrophobicity, and other properties of proteins involved in phase separation (Luo et al., 2021). Proteins that contain IDRs are called intrinsically disordered proteins (IDPs) and are very popular topics in current phase-separation studies. Here, we summarize the proteins that contain disordered regions in plants. Although these proteins are not definitively potential LLPS proteins, they are highly relevant to phase separation.

In 2013, approximately 30% of the proteins in *A. thaliana* were found to show a >50% disorder rate, and these IDPs were highly correlated with the environmental stress response (Pietrosemoli et al., 2013). In 2016, a study identified nearly 500 PrLD-containing proteins in *A. thaliana* that were involved mainly in transcription, RNA binding, RNA metabolic processes, reproductive development, and flower development (Chakrabortee et al., 2016). Among them, Luminidependens (LD), Flowering Locus CA (FCA), Flowering Locus PA (FPA), and Flowering Locus Y (FY) in the autonomous flowering pathway all contain PrLDs (Chakrabortee et al., 2016). Since then, it has been confirmed that FCA can indeed undergo LLPS to form droplets that affect the flowering pathway (Fang et al., 2019). In 2020, 836 RNA-binding proteins (RBPs) were identified in *A. thaliana*, and 1865 proteins were classified as candidate RBPs; these RBPs were involved mainly in RNA processing and metabolism (Marondedze, 2020). Phase-separation proteins have also been identified from eight species (*Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Oryza sativa*, *Triticum aestivum*, *Zea mays*, *A. thaliana*, *Brassica rapa pekinensis*, and *Solanum lycopersicum*) via biotinylated isoxazole (b-isox) precipitation experiments. Several of these potential LLPS proteins are associated with gene transcription, RNA processing and splicing, ribosome assembly, ribosome biosynthesis, RNA transport, and translation regulation. Notably, many of the enriched proteins are involved in responses to environmental stimuli such as heat, cold, salt, and oxidative stress (Zhang et al., 2022a). These findings suggest that there are many potential LLPS proteins in crops. Whether LLPS can enhance crop yield and resistance by regulating growth, development, and stress responses warrants further exploration.

LLPS PLAYS IMPORTANT ROLES IN PLANT GROWTH, DEVELOPMENT, AND STRESS RESISTANCE

Photomorphogenesis

To fully utilize natural light as energy, plants have evolved a series of photoreceptors to recognize light ranging from UV to far-red light (wavelengths 380–735 nm). Upon exposure to light, photoreceptors and related proteins rapidly translocate from the cytoplasm to the nucleus to form bodies called photobodies (Van Buskirk et al., 2012; Cuevas-Velazquez and Dinneny, 2018; Pardi and Nusinow, 2021). The blue-light receptor cryptochrome 2 (CRY2) and red-light receptor phytochrome B (phyB) are the most well-known receptors. CRY2 is induced by blue light to undergo LLPS to help the transcription factor TEOSINTE BRANCHED1-CYCLOIDEA-PCF 22 (TCP22) and the transcriptional regulator LWD bind to the TBS motif of the *CCA1* promoter, promoting the transcription of the central component of the circadian oscillator *CCA1*. The PPK kinases enhance the interaction between TCP22 and LWD through phosphorylation modifications to promote the formation of photobodies (Figure 2A) (Mo et al., 2022). Light also induces CRY2 to undergo LLPS with the N^6-methyladenosine RNA methyltransferases MTA, MTB, and FIP37 to form photobodies, enhancing the methyltransferase activity of MTA and thus increasing N^6-methyladenosine (m^6A) deposition on the mRNA of CCA1. This m^6A deposition stabilizes CCA1 mRNA against degradation, which in turn positively regulates the circadian clock. In addition, the IDR and phosphorylation modification of CRY2 play an important role in maintaining the liquid phase of photobodies (Figure 2B) (Wang et al., 2021b).

Early in 2010, researchers demonstrated the mobility of phyB-composed photobodies through fluorescence recovery after photobleaching experiments (Rausenberger et al., 2010). It has been suggested that the red-light receptor phyB also undergoes LLPS to form photobodies upon red-light irradiation or at low temperature and reverts to a diffuse state under far-red light or high-temperature conditions. Photobodies composed of phyB selectively absorb transcription factors and regulate the light signal transduction pathway. The N-terminal NTE and C terminus of phyB are the key regions for phase separation, and the NTE region specifically senses temperature (Figure 2C) (Chen et al., 2022). In addition, phyB has been found to interact with CRY2 (Más et al., 2000). Given the complexity and diversity of components in the droplets formed by phase separation, it is hypothesized that the two photoreceptors might act in coordination in the same photobody (Quail, 2021). However, whether the two different light receptors can appear in the same photobody remains to be clarified, and the specific biological functions of the photobody require further investigation.

Chloroplasts are the photosynthetic factories of plants, and biomolecular condensates are essential in these organelles. In 2020, chloroplast proteins were shown to undergo LLPS to participate in the sorting of nuclear-encoded proteins. In the corresponding study, the researchers identified two ankyrin-repeat proteins, STT1 and STT2, which are specifically involved in sorting chloroplast twin arginine translocation (cpTat) substrates to

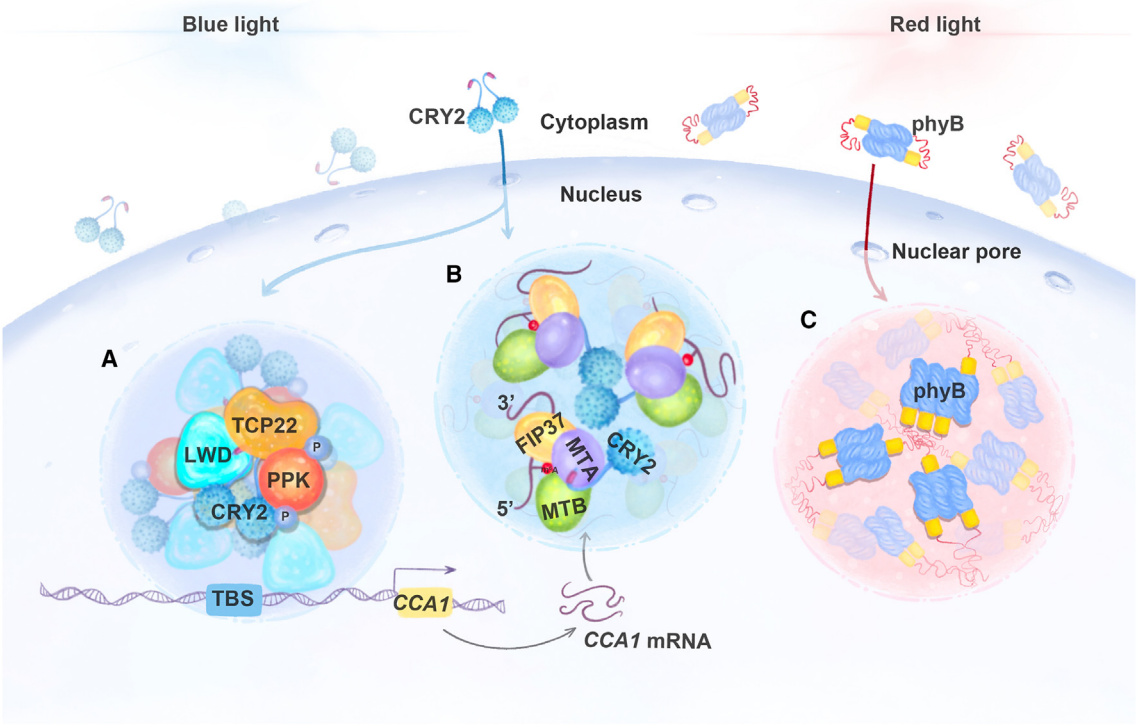

Figure 2. Photobodies generated via LLPS in plants.

(A and B) CRY2 is induced by blue light to move from the cytoplasm into the nucleus to form photobodies. **(A)** CRY2 undergoes LLPS with TCP22, LWD, and PPK to form photobodies that promote CCA1 transcription. **(B)** CRY2 undergoes LLPS with MTA, MTB, and FIP37 in the nucleus to form photobodies. The photobodies promote deposition of m^6A on the mRNA of CCA1, which in turn prevents mRNA degradation.

(C) The red-light receptor phyB is induced by red light to enter the nucleus from the cytoplasm and undergoes LLPS to form photobodies to regulate the light signal transduction pathway.

the thylakoid membranes. STT1 and STT2 form heterodimers through C-terminal interactions, and the N-terminal IDR binds to substrate proteins in the cpTat pathway to initiate LLPS. The droplets formed by LLPS facilitate translocation of the cpTat substrate to the thylakoid membranes. Phase separation is subsequently reversed by interaction of the STT protein with the membrane protein Hcf106, a major component of the thylakoid receptor complex, which allows the proteins completing the transport of the client protein to be released from the thylakoid (Ouyang et al., 2020). In the same year, another study revealed that snowy cotyledon 1 (SCO1), a nuclear-encoded protein localized in chloroplasts, is induced by heat stress to form foci with SG-like properties. Exposure to 33°C for 30 min induced the formation of SCO1-GFP foci in chloroplasts, which dispersed as the temperature recovered. Treatment with the hydrophobic interaction–disrupting reagent 1,6-hexanediol decreased the size of the SCO1-GFP foci. This is another example of condensation occurring in plastids (Chodasiewicz et al., 2020). Overall, evidence suggests that chloroplasts and biomolecular condensates are used jointly by cells to efficiently complete biochemical reactions.

Flowering

Flowering time is a key agronomic trait with a strong effect on crop yield and quality. Several biomolecular condensates that affect flowering have been identified, all of which have been found to repress the transcription of floral repressor C (*FLC*). Six of these biomolecular condensates regulate *FLC* expression by targeting its antisense transcript (Figure 3A and 3B), transcript intron region (Figure 3C), promoter (Figure 3D and 3E), and/or genomic region (Figure 3F). In 2019, it was discovered that both the *A. thaliana* RBP FCA and the coiled-coil protein FLL2 have PrLDs and exhibit LLPS potential. FCA, which interacts with RNA 3′-end processing factor, is driven by FLL2 to form nuclear bodies through LLPS. These nuclear bodies facilitate polyadenylation of the *FLC* antisense transcript (*COOLAIR*), thereby inhibiting FLC transcription. Mutation of FLL2 blocks the phase separation of FCA, resulting in late flowering and defective petal development in *A. thaliana* (Fang et al., 2019) (Figure 3A). Furthermore, a 2021 study showed that the nuclear bodies formed by FCA phase separation contain not only the 3′-end processing factors but also the components of the m^6A methyltransferase complex, MTA, MTB, and FIP37. m^6A indirectly contributes to phase separation of FCA in *COOLAIR*, and FCA nuclear bodies in turn promote m^6A deposition (Xu et al., 2021a; Lee et al., 2022) (Figure 3B). Recently, researchers identified a new RBP, hnRNP R-LIKE PROTEIN (HRLP), which interacts with the mRNA alternative splicing factor serine/arginine-rich 45 (SR45) (Ali et al., 2007) in *A. thaliana*. HRLP and SR45 both form biomolecular condensates on FLC transcript intron I, which promote the formation of DNA:RNA hybrids (R-loops), thereby inhibiting polymerase II (Pol II)-mediated *FLC* transcription (Zhang et al., 2022c) (Figure 3C).

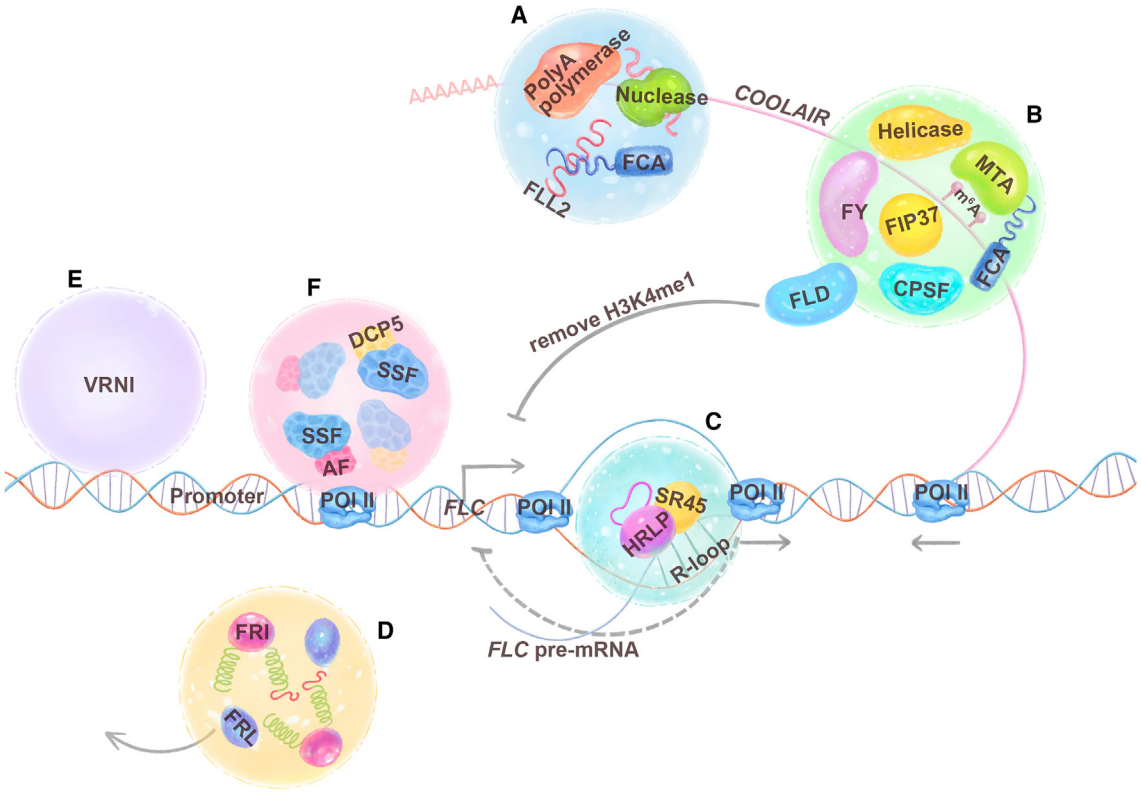

Figure 3. Biomolecular condensates that regulate *FLC* transcription.

(A) Biomolecular condensates formed by FLL2-driven phase separation promote polyadenylation of *COOLAIR*.

(B) The m^6A methyltransferase complex contained in droplets formed by FCA phase separation promotes m^6A deposition on *COOLAIR*. In addition, the droplets contain 3′-end processing factors that recruit the histone demethylase FLD to remove H3K4me1 from the chromatin tangled by *FLC*, resulting in *FLC* silencing.

(C) Biomolecular condensates formed by phase separation of HRLP and SR45 promote R-loop formation and repress Pol II-mediated *FLC* transcription.

(D) Induced by cold, FRI forms biomolecular condensates with FRL, causing FRI to no longer localize to the transcriptional activation site of *FLC* to promote transcription.

(E) Droplets formed by VRN1 phase separation inhibit *FLC* transcription.

(F) SSF undergoes LLPS in the genomic region of *FLC* to regulate *FLC* transcription. SSF acts as a scaffolding protein that interacts with activating factors to promote *FLC* transcription and with DCP5 to repress *FLC* transcription. AF, activating factors.

In addition to biomolecular condensates that cluster on *FLC* transcripts to repress *FLC* expression (Figure 3A–3C), several other biomolecular condensates are associated with *FLC* promoter binding (Figure 3D and 3E). For example, FRIGIDA (FRI), the transcriptional activator of *FLC* in *A. thaliana*, causes an early-flowering phenotype when mutated. Induced by cold, FRI undergoes reversible LLPS to form nuclear condensates that isolate FRI from the *FLC* transcriptional activation region. When the temperature rises, FRI nuclear condensates return to a diffuse state, allowing FRI to bind to the *FLC* promoter to promote expression (Csorba et al., 2014; Yang et al., 2014; Zhu et al., 2021) (Figure 3D). The *FLC* transcriptional repressor Vernalization 1 (VRN1), which contains two B3 DNA-binding domains, also displays LLPS potential. Experiments have shown that the VRN1 protein forms droplets upon binding to *FLC* promoter sequences *in vitro*. In particular, the charge segregation of the IDR of VRN1 plays an important role. However, the relationship between the LLPS of VRN1 and the transcriptional repression of *FLC* remains to be clarified (Levy et al., 2002; Zhou et al., 2019; Wang et al., 2021c) (Figure 3E).

A recent study has shown that the processing body (P-body) component DECAPPING5 (DCP5) and the flowering repressor SISTER OF FCA (SSF) both contain PrLDs and exhibit LLPS properties. DCP5 interacts with SSF in the nucleus and represses *FLC* transcription by inhibiting Pol II enrichment in an LLPS-related manner. Enrichment of DCP5 in the *FLC* genomic region depends on SSF. In that study, the authors found that SSF and DCP5 exhibit opposite effects on *FLC* transcription and flowering-time regulation. They proposed that SSF may act as a scaffold protein anchored at the *FLC* locus to fine-tune *FLC* transcription by interacting with positive and negative regulators (Wang et al., 2023b) (Figure 3F).

A similar biomolecular condensate that affects flowering and also functions as a transcriptional repressor in the promoter region is TERMINATING FLOWER (TMF) in tomato. Previous studies have found that TMF restricts early flowering in tomato by repressing transcription of the shoot apical meristem maturation gene *ANANTHA* (*AN*) (MacAlister et al., 2012). In 2021, the TMF protein was shown to undergo phase separation after ROS treatment, forming condensates that bind to the promoter

region of *AN* to inhibit *AN* expression. Interestingly, in addition to the IDRs of TMF, the cysteine residues between the two IDRs are also critical for phase separation. The intra- and intermolecular disulfide bonds of TMF increase after H_2O_2 treatment, which enhances molecular multivalency to facilitate condensate formation (Huang et al., 2021b). Huang et al. have also reported that TMF FAMILY MEMBERs (TFAMs) in tomato all contain IDRs and can undergo LLPS. The ALOG transcription factor family contains genes duplicated in multiple rounds; these duplications have expanded the genes into TMF and TFAMs, which jointly regulate stem-cell fate transition in the shoot apical meristem. TMF and TFAM1/2/3 undergo liquid phase co-separation to form heterotypic transcriptional condensates that precisely regulate spatiotemporal expression of the flowering gene *AN* (Huang et al., 2022). Whether members of the same family of phase-separated proteins also have phase-separation potential should not be ignored.

In addition to their critical role in flowering-time regulation, biomolecular condensates also have indispensable functions in flower development. Inspired by the phase separation of chromatin (Larson et al., 2017; Strom et al., 2017; Gibson et al., 2019; Strickfaden et al., 2020), some researchers have speculated that the condensation of chromatin structure in male germ cells of flowering plants is correlated with phase separation. They found that the sperm-specific histone variant H2B.8 colocalizes with condensed chromatin. In contrast to H2B, H2B.8 contains an additional IDR at the N terminus that can drive H2B.8 and chromatin to undergo phase separation to form droplets. H2B.8 clusters mainly in AT-rich euchromatin regions with low transcriptional activity. This not only ensures transcriptional activity but also reduces the volume of the sperm nucleus, which is essential for successful fertilization in flowering plants (Buttress et al., 2022). In summary, plants regulate flowering-related gene transcription and chromatin remodeling by forming biomolecular condensates to ensure that plants flower at the appropriate time.

Abiotic stress

To respond in a timely manner to unknown stress challenges that can occur at any time in the natural environment, immobile plants employ the phase-separation mechanism to rapidly organize relevant biomolecules within the cell to form membraneless "resistance teams." These intracellular stress-induced membraneless condensates, also known as SGs, serve mainly to balance the storage, translation, and degradation of RNA (Protter and Parker, 2016; Kearly et al., 2022). Figure 4 summarizes condensates that are recognized for their roles in plant cells in response to cold stress, heat stress, osmotic stress, hydrated conditions, nutrient stress, and stress-induced DNA damage. The transcriptional repression of *FLC* mentioned above is usually closely associated with low-temperature stress (Helliwell et al., 2015), and the cold-induced droplets formed in the nucleus by FRI can isolate FRI from the transcriptional activation region of *FLC* (Zhu et al., 2021) (Figures 3D and 4A).

Currently known condensates whose formation is induced by heat stress are classified into two types: receptors that sense temperature changes and SGs that protect transcripts. In 2020, researchers identified EARLY FLOWERING 3 (ELF3), a core component of temperature sensing in *A. thaliana* (Box et al., 2015; Nieto et al., 2015), as a novel thermoreceptor. ELF3 undergoes reversible phase separation at high temperature to form deactivated droplets, then reverts to an active and diffuse state after a temperature decrease. The prion-like domain of ELF3, which contains a polyglutamine (polyQ) repeat, plays key roles in temperature sensing and droplet formation (Jung et al., 2020) (Figure 4B). Early studies proposed ELF3 as a multifunctional protein with two separable roles, one as a component of the evening complex affecting the circadian network and the other as a restrictor of light input to the oscillator by regulating phyB signaling (Kolmos et al., 2011). In the circadian network, ELF3 acts as a scaffold protein that interacts with EARLY FLOWERING4 (ELF4) and the transcription factor LUX ARRYTHMO (LUX) at dusk to form the evening complex, which represses the transcription of multiple circadian clock–related genes, thus inhibiting hypocotyl elongation and flowering (Nusinow et al., 2011). ELF4 causes ELF3 to form bodies in the nucleus (Herrero et al., 2012). It has also been reported that ELF3 interacts with the red-light receptor phyB and that phyB is required for accumulation of ELF3 in the light (Liu et al., 2001; Nieto et al., 2015). PhyB is not only a photoreceptor but also a thermosensor, integrating light- and temperature-sensing pathways (Jung et al., 2016; Legris et al., 2016). As mentioned above, phyB is induced by red light to undergo LLPS to form photobodies but reverts to a diffuse state upon heating (Chen et al., 2022). In short, ELF3 interacts with different proteins involved in pathways such as the rhythm, light-signaling, and temperature-sensing pathways. Whether the proteins mentioned above appear in the same or different bodies formed by phase separation is a question worthy of discussion and exploration.

Other biomolecular condensates whose formation is induced by heat stress are called SGs. SGs are conserved, cytoplasmic messenger ribonucleoprotein particles that form from pools of mRNAs stalled in translation initiation. SGs also contain various translation initiation factors, RBPs and non-RBPs (Jain et al., 2016; Protter and Parker, 2016; Kosmacz et al., 2019). The expression of heat shock transcription factors (HSFs) and heat shock proteins (HSPs) is rapidly induced in plants to cope with heat stress. These molecules work in concert to improve thermotolerance (Ohama et al., 2017; Ren et al., 2019; Zhao et al., 2020). Therefore, it is critical to protect HSF and HSP mRNAs from degradation at high temperatures. When *A. thaliana* is exposed to heat stress, acetylation lowers binding affinity (ALBA4/5/6) proteins in the cytoplasm undergo phase separation to form SGs, which protect the HSF mRNAs from degradation by the exoribonuclease XRN4 (Tong et al., 2022). Two functionally redundant RNA-binding glycine-rich group D2 proteins (RBGD2 and 4) undergo LLPS in response to heat stress to form SGs, which enhance plant heat tolerance by protecting heat-responsive transcripts (e.g., HSFA2, HSP70) (Zhu et al., 2022) (Figure 4B).

Biomolecular condensates in plants can also act as receptors to sense water and osmotic stress. The transcriptional regulator SEUSS, which regulates the growth and development of *A. thaliana* under normal conditions (Franks et al., 2002, 2006; Pfluger and Zambryski, 2004; Bao et al., 2010; Lee et al., 2014; Zhai et al., 2020), rapidly phase separates and forms droplets

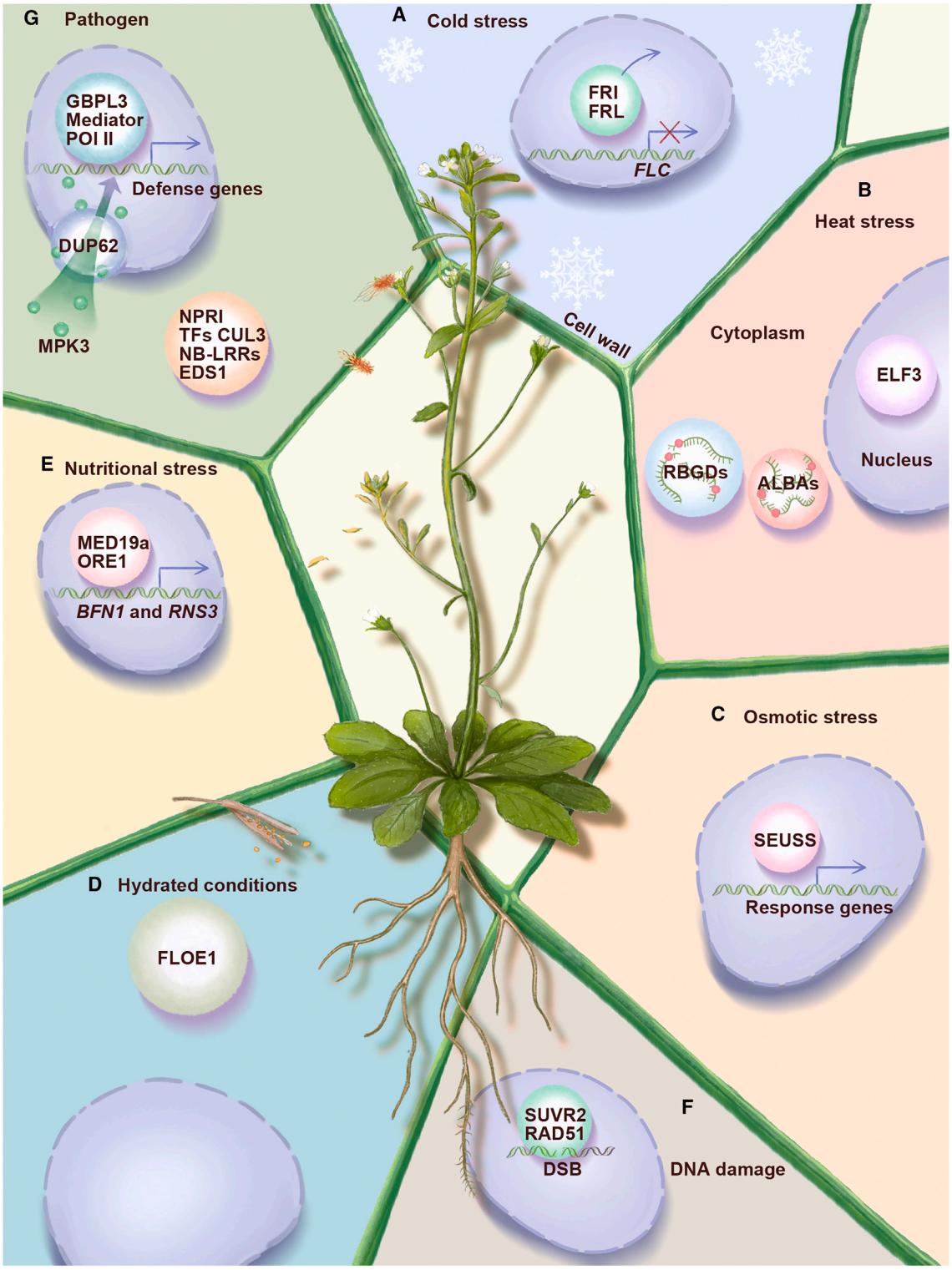

Figure 4. Biomolecular condensates involved in plant stress resistance.

(A–G) Biomolecular condensates that appear in plants under different conditions. Each unit in the figure represents a plant cell. In the face of **(A)** cold stress, **(B)** heat stress, **(C)** osmotic stress, **(D)** hydrated conditions, **(E)** nutrient stress, **(F)** DNA damage caused by stress, and **(G)** pathogen infection, biomolecular condensates appear in cells of flowers, stems, leaves, roots, and seeds to improve resistance.

upon sensing osmotic stress caused by high salt or drought, promoting the expression of osmotic-stress-related genes and thus enhancing plant stress resistance. Conformation of the two α helices on IDR1 of SEUSS plays an important role in sensing the crowding effect caused by osmotic stress (Wang et al., 2022) (Figure 4C). The prion-like protein FLOE1 in *A. thaliana* seeds regulates the expression of genes related to seed germination through reversible phase separation in response to hydrated conditions, allowing seeds to germinate in a suitable environment. Two special disordered regions have a significant effect on the phase separation of FLOE: one enriched in glutamine, proline, and serine (QPS rich) and the other enriched in aspartic acid and serine (DS rich). The QPS region of FLOE1 is a prion-like domain that promotes phase separation, and mutation of QPS inhibits seed germination. The DS region adjusts the fluidity of FLOE1 condensates. Mutation of DS causes FLOE1 condensates to exhibit a solid-like state and leads to a dramatic germination phenotype (Dorone et al., 2021) (Figure 4D). Late embryogenesis abundant (LEA) family proteins are also thought to play an important role in seed development. LEA proteins act as molecular chaperones and protective proteins to resist stresses in plants. Because of their many hydrophobic amino acid residues, LEA proteins are typically in a fully stretched state (Boudet et al., 2006; Battaglia et al., 2008; Hanin et al., 2011; Chatelain et al., 2012). The high frequency of interactions of disordered LEA proteins has led to speculation that they may function as protective proteins through the formation of isolation barriers via LLPS (Dirk et al., 2020). One study has shown that *A. thaliana* seed-specific LEA9/42/48 proteins undergo LLPS in cells, but their biological functions require further investigation (Ginsawaeng et al., 2021).

Biomolecular condensates also participate in nutrient stress and the DDR pathway. Under nitrogen deficiency, *A. thaliana* Mediator subunit 19a (MED19a) is deacetylated, resulting in increased multivalency and promoting LLPS. The senescence-related transcription factor ORESARA1 (ORE1) is abundantly expressed in response to nutrient stress, and ORE1 enters the condensates by interacting with MED19a. Condensates containing these two proteins bind to the promoters of the senescence-related genes BFN1 and RNS3 to promote gene transcription (Cheng et al., 2022) (Figure 4E). Various types of environmental stress can cause many kinds of DNA damage, even lethal damage. Thus, the DDR is also a large and complex mechanism that requires orderly organization in the nucleus to work efficiently (Vitale et al., 2017; Kim, 2019). When severe DNA double-strand breaks (DSBs) occur in *Medicago truncatula* nuclei, the histone methyltransferase MtSUVR2 drives LLPS of MtRAD51, a key enzyme of the homologous recombination repair pathway, forming DNA repair bodies to facilitate homologous recombination repair. The IDR1 and LCD of MtSUVR2 are indispensable for phase separation, and IDR2 is the key region for interaction with MtRAD51 (Liu et al., 2022) (Figure 4F).

Biotic stress

Plants also employ LLPS machinery to resist diseases when faced with biotic stresses. In *A. thaliana*, non-expresser of pathogenesis related (PR) 1 (NPR1) often participates in disease resistance through multiple pathways, including regulation of downstream disease-resistance gene transcription, activation of systemic acquired resistance by binding of salicylic acid (SA), and participation in protein degradation pathways to maintain disease resistance protein homeostasis (Clarke et al., 2004; Wang et al., 2005; Fu and Dong, 2013; Üstün et al., 2017; Jin et al., 2018; Lai et al., 2018; Olate et al., 2018; Gómez-Díaz and Ikeda, 2019). A 2020 study showed that NPR1 is induced by SA to undergo LLPS to form SA-induced NPR1 condensates (SINCs) in the cytoplasm. SINCs contribute to the formation of the E3 ubiquitin ligase complex, which in turn promotes the degradation of many apoptosis-related proteins, such as NB-LRRs, EDS1, and WRKY54/70, by the ubiquitination pathway in SINCs, thereby ensuring the survival of uninfected cells without effector-triggered immunity (Zavaliev et al., 2020) (Figure 4G).

Biomolecular condensates induced by biotic stresses also regulate disease-resistance gene transcription to enhance immunity. GBPL3, a guanylate-binding protein (GBP)-like GTPase (GBPL) family member, is normally inhibited by the pseudo-GTPase GBPL1 in an inactive state. Upon application of SA, pipecolic acid, or *Psm* ES4326 to trigger the defense response, GBPL3 undergoes LLPS and forms defense-activated condensates (GDACs). Within the GDACs, GBPL3 directly binds to the promoters of defense genes and recruits Mediator complex subunits and RNA polymerase II to promote disease-resistance gene transcription (Huang et al., 2021a) (Figure 4G). Interestingly, higher growth temperatures have been found to inhibit the formation of partial GDACs. Two GDAC subpopulations appear to exist in plants. One is sensitive to 28°C and is associated with recruitment of GBPL3 to the promoter of the master immune transcription factor CBP60g. The other is insensitive to 28°C and is associated with recruitment of GBPL3 to the NPR1 promoter (Kim et al., 2022). Higher growth temperatures can negatively affect plant disease resistance (Li et al., 2020b; Bruessow et al., 2021). Breeding climate-adapted, disease-resistant plants based on the temperature-sensing property of biomolecular condensates is also important for future agricultural production.

A recent study identified *A. thaliana* genes encoding the central subcomplex of the nuclear pore, NUP62, NUP58 and NUP54, that can undergo phase separation to form hydrogels. The disordered N-terminal FG-rich region is critical for phase separation of NUP62. The phase separation of NUP62 mediates transport of the key immune regulator MPK3 from the cytoplasm to the nucleus, enabling plants to have a positive resistance response (Figure 4G). Notably, NUP62 does not undergo LLPS but rather phase separation on the nuclear pore to form a hydrogel, which is rare in the study of plant biomolecular condensates. The phase separation of NUP62 modulates broad-spectrum plant resistance to a variety of pests and diseases, such as the fungal pathogen *Sclerotinia sclerotiorum*, the bacterial pathogen *Pseudomonas syringae* pv. *tomato,* and the pest *Spodoptera exigua*, motivating studies on applications in crop resistance (Wang et al., 2023a).

Hormone signaling

Plant hormone signaling pathways are also closely related to LLPS, and studies to date have focused on auxin and ethylene. The auxin response factor (ARF)-family transcriptional activators ARF7 and ARF19 (Ulmasov et al., 1999; Tiwari et al., 2003) are localized mainly in the nucleus in highly auxin-responsive tissues to activate auxin-related gene expression and promote plant growth. By

contrast, in less auxin-responsive tissues, the PB1 domain and intrinsically disordered middle region of ARF7/19 drive the proteins to phase separate into condensates that localize in the cytoplasm, isolating transcriptional activation (Powers et al., 2019). Endoplasmic reticulum membrane-bound ETHYLENE-INSENSITIVE 2 (EIN2) in the *A. thaliana* ethylene signaling pathway has also been analyzed for its phase-separation potential. The PrLDs, IDR, and RNA-binding sites all suggest that EIN2 can undergo LLPS in cells (Lu et al., 2022). The underlying mechanisms and biological functions require further exploration.

RNA processing

The multivalent weak interactions of biomolecules are an important prerequisite for phase separation, in which multivalent macromolecular RNA plays a key role in formation and dissolution of biomolecular condensates. These biomolecular condensates, also known as RNA granules, are formed by the phase separation of RNA and proteins and regulate the transcription, splicing, processing, and translation of RNA (Anderson and Kedersha, 2006; Drino and Schaefer, 2018; Lin and Fang, 2021). Currently, the best-known RNA granules in plants are the processing factory microRNAs (miRNAs), also called dicing bodies (D-bodies). The core components of plant D-bodies include RNase III DICER-LIKE 1 (DCL1), the double-stranded RBP HYPONASTIC LEAVES 1 (HYL1), and the C2H2 zinc-finger protein SERRATE (SE) (Song et al., 2007; Spector, 2007; Dong et al., 2008). The IDR and LCD of the SE protein jointly drive DCL1 and HYL1 to undergo LLPS to form D-bodies, effectively enhancing primary (pri)-miRNA processing to generate miRNAs. HYL1 then carries mature miRNAs released from the D-bodies (Xie et al., 2021). In addition to the three proteins mentioned above, D-bodies also contain the DEAD-box helicases RNA helicase 6 (RH6), RH8, and RH12, all of which exhibit phase-separation properties. Under normal conditions, RH6, RH8, and RH12 interact with SE to promote the formation of D-bodies. When the *Plum pox virus* infects plant cells, these three proteins are hijacked by viral protein linked to the genome (VPg) to the viral replication site, promoting phase separation of the virus into virus bodies and helping it to proliferate (Li et al., 2021).

In plants, small interfering RNA (siRNA)-regulated transposon silencing mechanisms (Ito, 2012) are also closely associated with the LLPS machinery. As early as 2009, suppressor of gene silencing 3 (SGS3) was shown to interact with RNA-dependent RNA polymerase 6 (RDR6) in the nonclassical RNA-directed DNA Methylation (RdDM) pathway to form SGS3/RDR6 bodies in the cytoplasm, thereby regulating double-stranded RNA synthesis (Kumakura et al., 2009). The increasing focus on phase separation in biology has led to new insights into the formation of puncta in cells. A 2021 study reported that SGS3 has the potential to phase separate and drive LLPS of RDR6 in the cytoplasm to form condensates named siRNA bodies. Furthermore, transposon RNA containing nonoptimal codons undergoes RNA truncation due to ribosomal arrest, and the truncated RNA enters the siRNA bodies, thus resulting in transposon silencing (Kim et al., 2021a).

LLPS RESEARCH IN CROPS

To date, most biomolecular condensate studies in plants have focused mainly on *A. thaliana*. Although there have been few related reports in crops, the future research potential of this field is limitless. In *M. truncatula*, MtSUVR2 promotes DSB repair by driving LLPS of the homologous recombinase MtRAD51. An evolutionary tree and predictions of phase-separation potential indicate that SUVR-family proteins of soybean and *Lotus corniculatus* also exhibit phase-separation potential, suggesting that similar mechanisms may exist in soybean and *L. corniculatus* (Liu et al., 2022). The functions of proteins in this family tend to be both conserved and divergent in evolution. In tomato, the ALOG DNA-binding domain is highly conserved in TMF and TFAM1/2/3/11, but there are numerous variations in the IDRs of these proteins, resulting in different abilities of phase separation and transcriptional regulation (Huang et al., 2022). This suggests that multicopy proteins may vary in their IDRs, diversifying their families with different functions. Some researchers have found that wheat gliadins also undergo LLPS to form condensates. These condensates may be precursors of protein bodies, membrane-free organelles that perform storage functions in wheat (Boire et al., 2018; Sahli et al., 2019). Whether all seed-type crops employ the LLPS mechanism for protein storage and the role played by LLPS remain to be determined.

An analysis of potential phase-separation proteins by Zhang et al. was an important starting point in crop LLPS research (Zhang et al., 2022a). However, this study focused more on IDPs than on other types of proteins. The multivalent tandem repeat structural domain is also an important driver of LLPS. To gain a more comprehensive understanding of potential LLPS proteins in crops, we analyzed proteins containing tandem repeat domains in *Gossypium hirsutum*, *M. truncatula*, *O. sativa*, *T. aestivum*, and *Z. mays*. We downloaded proteomic data separately for the five species. The Python programming language was used to write scripts for the statistical analysis of characteristic amino acids. The hash value of each protein sequence of specified length (n = 10 or 20) was calculated by the sha256 hashing algorithm, and data with duplicate hash values greater than or equal to 3 were retained. The hash algorithm is widely used in the computer field for fast retrieval of large-scale data (Yu et al., 2022a). We quickly retrieved proteins with more than three repetitive amino acid sequences (sequence length of 10 or 20 amino acids) from the five proteomic databases for statistical analysis. The results showed that the proportion of proteins with more than three sequence tandem repeats was highest in *T. aestivum* and *M. truncatula* (Figure 5).

TOOLS FOR ANALYSIS AND PREDICTION OF PROTEINS WITH LLPS POTENTIAL

The multivalency of biomacromolecules enables proteins, DNA, or RNA to nucleate under specific conditions and then grow into biomolecular condensates that are isolated from the surrounding environment to form a specific reaction environment. Elucidation of basic protein properties is crucial for the study of phase separation. IDRs, PrLDs, RBDs, DNA-binding regions, amino acid types, charge distributions, hydrophobicity, and PTMs must all be considered. To support further analysis of plant proteins with phase-separation potential, we have summarized relevant websites that are applicable to plants (Table 1).

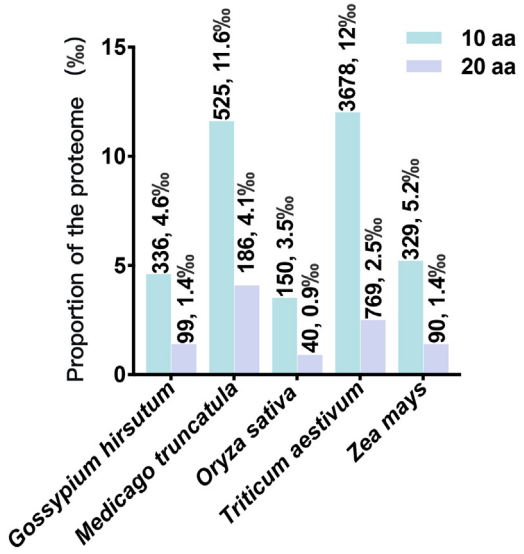

Figure 5. Prediction of potential LLPS proteins based on crop proteomes.

The proportion of proteins that contain multivalent tandem structural domains in five species (*G. hirsutum*, *M. truncatula*, *O. sativa*, *T. aestivum*, and *Z. mays*). Numbers above the bars represent the total number of proteins and the number per thousand.

The FuzDrop and dSCOPE websites provide comprehensive analysis results (Yu et al., 2022b; Hatos et al., 2022). LLPSDB, PhaSepDB2.1, DisProt, and PhaSePro are all integrated databases of proteins related to phase separation that have been identified to date; these proteins can be used to analyze target proteins by searching and comparison (Xue et al., 2010; Li et al., 2020a; Mészáros et al., 2020; Hou et al., 2022). The PSPredictor and Deephase websites score protein phase-separation potential and enable import of multiple protein sequences at once, making them suitable for rapid analysis of large numbers of proteins (Saar et al., 2021; Chu et al., 2022). PONDR, IUPred3, PrDOS, ESpritz, and PLAAC are websites for analysis of disordered sequences in proteins; each server uses a different calculation method (Bomma et al.; Erdos et al., 2021; Ishida and Kinoshita, 2007; Lancaster et al., 2014; Walsh et al., 2012). Because the predictions obtained are slightly different, it is necessary to combine the predictions with experimental results. CIDER and ProtScale focus on charge distribution, hydrophilicity, hydrophobicity, and other characteristics of amino acids (Kyte and Doolittle, 1982; Wilkins et al., 1999; Uversky, 2002; Campen et al., 2008; Das and Pappu, 2013; Martin et al., 2016). SGnn is dedicated to the predictive analysis of heat-stress-induced SGs (Iglesias et al., 2021). Certainly, no matter how clear and detailed the results, no database analysis can directly confirm that target proteins can undergo LLPS to form biomolecular condensates; thus, appropriate biological experiments are also necessary.

CHALLENGES AND FUTURE PERSPECTIVES

Biomolecular condensates are ubiquitous in plants, and proteins with phase-separation potential are abundant

Phase separation regulates plant growth and resistance

(Pietrosemoli et al., 2013; Chakrabortee et al., 2016; Zhang et al., 2022a). This year, research progress on phase separation in plants seems to have slowed, with only a few research articles published in the first half of 2023 (Wang et al., 2023a, 2023b) (Figure 1). The discovery of phase separation was initially exciting, but biologists have become divided as more and more studies have been published. Some suggest that more care should be taken in identifying examples of phase separation in cells, and some claim that phase separation is far less important than many scientists assert. Notably, the criteria for judging phase separation should not be limited to shape observation and fluorescence recovery after photobleaching experiments (Leslie, 2021). To establish a better system for phase-separation studies, some principles and guidelines for biological phase-separation studies have been proposed (Gao et al., 2022). Gao et al. have drawn a detailed map of requirements for phase-separation studies, including information analysis and experimental requirements *in vivo* and *in vitro*, emphasizing the need to closely link findings to biological functions when studying biomolecular condensates. To ensure the rigor of *in vitro* experiments, Brangwynne et al. developed the Corelet oligomerizing biomimetic system. This technique can be used to map the complete intracellular phase diagram, indicating the concentration and transition mechanism by which phase separation occurs (Bracha et al., 2018). To study the effect of biomolecular condensates formed in the nucleus through LLPS on chromatin, Brangwynne et al. also developed the CasDrop tool for observing the mechanistic relationship between chromatin and droplets (Shin et al., 2018). This is very helpful for studying the biological functions of transcription factors through LLPS. In conclusion, the current system of phase-separation studies is not yet well established, and more rigorous experiments are needed to demonstrate the existence and biological functions of LLPS in cells.

The function of the LLPS machinery in plants has both common and distinct features compared with that in animals and yeast. Thus far, research has indicated that droplets formed via LLPS in plants function to establish a compartmentalized microenvironment, protect or store macromolecules, increase local concentrations, accelerate biochemical reactions, and translocate proteins. These functions are consistent with previously reported functions (Alberti et al., 2019). Biomolecular condensates play important roles in plant-specific physiological processes such as photosynthesis, flowering, and seed germination (Fang et al., 2019; Ouyang et al., 2020; Xu et al., 2021a; Dorone et al., 2021; Zhu et al., 2021; Zhang et al., 2022c). Because of their immobility, plants require precise and efficient regulatory mechanisms to respond to harsh natural environments in a timely and flexible manner. Biomolecular condensates formed by LLPS are typically characterized by rapid aggregation (seconds to hours) and dissolution when not needed to reduce resource occupancy (Wang et al., 2021b; Zhang et al., 2022b; Chen et al., 2022; Liu et al., 2022), making them a powerful means for rapid adaptation to environmental stimuli. The LLPS mechanism contributes to good fitness for plants with high environmental dependence. Which plant proteins are responsible for sensing stress, transducing signals, and performing functions through LLPS when facing stress? Are

Website	URL	Function	Reference
FuzDrop	https://fuzdrop.bio.unipd.it	comprehensive analysis	Hatos et al., 2022
dSCOPE	http://dscope.omicsbio.info	comprehensive analysis	Yu et al., 2022b
LLPSDB	http://bio-comp.org.cn/llpsdb/home.html	integrated data base	Li et al., 2020a
PhaSepDB2.1	http://db.phasep.pro	integrated data base	Hou et al., 2022
DisProt	https://disprot.org	integrated data base	Xue et al., 2010
PhaSePro	https://phasepro.elte.hu	integrated data base	Mészáros et al., 2020
PSPredictor	http://www.pkumdl.cn/PSPredictor	LLPS potential rating	Chu et al., 2022
Deephase	https://deephase.ch.cam.ac.uk	LLPS potential rating	Saar et al., 2021
PONDR	http://www.pondr.com	IDR prediction	Bomma et al.
IUPred3	https://iupred.elte.hu	IDR prediction	Erdos et al., 2021
PrDOS	http://prdos.hgc.jp	IDR prediction	Ishida and Kinoshita, 2007
ESpritz	http://protein.bio.unipd.it/espritz/	IDR prediction	Walsh et al., 2012
PLAAC	http://plaac.wi.mit.edu	PrLDs prediction	Lancaster et al., 2014
CIDER	http://157.245.85.131:8000/CIDER/analysis/	amino acid analysis	Kyte and Doolittle, 1982; Uversky, 2002; Campen et al., 2008; Das and Pappu, 2013; Martin et al., 2016
ProtScale	https://web.expasy.org/protscale/	amino acid analysis	Wilkins et al., 1999
SGnn	http://sgnn.ppmclab.com	heat-stress-induced SG prediction	Iglesias et al., 2021

Table 1. Websites suitable for analyzing proteins with potential LLPS in plants.

there any network relationships among the different biomolecular condensates derived in the stress response mechanism? These questions deserve further investigation.

Crops grown in natural environments are far more complex than model plants, and more precise work units are required to efficiently coordinate the work of intracellular biomolecules. The complex genomes, allopolyploidy, cross-pollination, and growth cycles of crops make the study of LLPS in crops quite rudimentary. To fully explore the molecular mechanisms that regulate growth and stress resistance in plants and to apply these mechanisms to production practices are two fundamental challenges for plant researchers. Is there any natural variation that affects LLPS in plants? In *A. thaliana*, there are two isoforms, full-length FLOE1.1 and FLOE1.2, that lack most of the DS region. Absence of the DS region results in larger condensates and higher seed germination rates. Expression of FLOE1.2 is much lower in environments with unpredictable rainfall. Natural variation in the DS region between FLOE1 isoforms can fine-tune seed germination rate for local environmental adaptation. More importantly, FLOE homologs with different phase-separation behaviors have been found in all green plants, suggesting that this seed germination fine-tuning mechanism is widespread (Dorone et al., 2021). The thermosensor ELF3 also exhibits climate-adapted variation in phase-separation behavior, and the polyQ repeat embedded in the PrLD of *A. thaliana* ELF3 is positively correlated with temperature sensing. By contrast, only a small PrLD has been found in *Solanum tuberosum* growing in temperate climates, and no PrLD has been found in *Brachypodium distachyon* growing in warmer climates (Jung et al., 2020). We can combine the properties of natural variations in DS and polyQ for targeted breeding of crops with high germination rates or heat tolerance adapted to local environments. More natural variations in crops that regulate traits, yield, and resistance by altering phase-separation behavior await discovery.

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Q.L. and J.D. designed and wrote the manuscript. W.L., Y.N., and T.W. revised the manuscript. The authors have read and agreed to the published version of the review.

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