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Exploring Immune Related Alterations in OPS/BIN2 Interactions and Investigating TCP8 Localization Patterns to Enhance Understanding of the Brassinosteroid Signaling Pathway

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Exploring Immune Related Alterations in OPS/BIN2 Interactions and Investigating TCP8 Localization Patterns to Enhance Understanding of the Brassinosteroid Signaling Pathway

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Maura Adare Donnelly

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Appendix

ARF: Auxin Response Factor, a family of transcription factors that mediate the responses of plant genes to the plant hormone auxin

BAK1: BRI1-Associated-Receptor Kinase 1: Co-receptor kinase that interacts with BRI1, modulating brassinosteroid signaling and defense responses

BIN2: Brassinosteroid-Insensitive 2: Protein kinase involved in the brassinosteroid signaling pathway, regulating the activity of transcription factors such as BZR1 and BZR2

BL: Brassinolide, a type of brassinosteroid hormone

BR: Brassinosteroid: Plant steroid hormones involved in various physiological processes such as growth and development responses

BRZ: Brassinazole: Chemical compound used to inhibit brassinosteroid biosynthesis, allowing for the study of brassinosteroid functions

BRI1: Brassinosteroid-Insensitive 1: Receptor kinase involved in the brassinosteroid signaling pathway, perceiving brassinosteroid signals to initiate downstream responses

BZR1: Brassinazole Resistant 1: Transcription factor involved in the brassinosteroid signaling pathway, regulating gene expression related to growth and development

BZR2 (BES1): Brassinazole Resistant 2/BRI1-EMS-Suppressor 1: Transcription factor involved in the brassinosteroid signaling pathway, regulating gene expression related to growth and development

IDRs: Intrinsically Disordered Regions: Protein regions lacking defined three-dimensional structure, often involved in protein-protein interactions and signaling

LLPS: Liquid-Liquid Phase Separation: Process where certain proteins form membrane-less organelles or structures within cells, influencing various cellular functions

OPS: OCTOPUS: Protein localized in the phloem of plants, implicated in phloem development and possibly immune signaling

PR: Pathogenesis-Related proteins, which are induced in plants in response to pathogen attack or other environmental stresses

SA: Salicylic Acid: Plant hormone involved in defense responses against pathogens

SAR: Systemic Acquired Resistance: A plant defense mechanism where exposure to certain pathogens induces enhanced resistance throughout the plant, preparing it to better defend against future pathogen attacks

TCP8: Teosinte Branched 1/Cycloidea/Proliferating Cell Factor 8: Transcription factor specific to Arabidopsis thaliana, implicated in various developmental processes

TF: Transcription Factor, a protein that binds to specific DNA sequences, thereby controlling the transcription of genetic information from DNA to mRNA

WT: Wildtype: Protein with the normal, unchanged genotype or phenotype typically found in nature

Abstract

The brassinosteroid (BR) signaling pathway is vital in regulating development and stress response in plants. The hormone BR binds a receptor kinase at the plasma membrane forming a receptor complex, causing an intracellular signal transduction carried out through protein interactions within the pathway. These interactions allow for transcription factors to bind to and regulate BR regulated genes within the nuclei; this is important as their expression is important for growth and development. The project's proteins of interest, including *Arabidopsis thaliana* proteins OPS and BIN2, along with transcription factors TCP8 and BZR2, are involved in these pathway interactions. Recent research has identified circular clusters of TCP8 protein, known as condensates, forming in the nuclei of *Nicotiana benthamiana*. Creating truncation mutations that remove regions of interest in TCP8 will allow us to identify sites behind punctae formation. This project utilized a spinning disk confocal microscope and the plant hormones BRZ and SA, which deplete BR, to observe changes in OPS/BIN2 interactions within the brassinosteroid pathway and quantified the presence of truncated TCP8/BZR2 localization via nuclei punctae counts.

Background/introduction

As summarized by Huot et al., 2014, there are complicated interactions between growth and defense mechanisms in the field of plant biology. Plants have limited energy resources, so they must balance their allocation between these two essential aspects of survival at the expense of the other. This phenomenon, commonly known as the 'growth–defense tradeoff,' was first observed in studies conducted by Coley et al., 1985, on plant and insect interactions. The growth defense trade-off implies that plants must invest their finite resources in either growth or defense at a single time. This phenomenon is a fundamental aspect of plant physiology, defining their responses to environmental cues and stressors such as nutrient availability, pathogens, temperature, hormones, pH etc. As they navigate this equilibrium, many factors come into play: hormonal regulations, signaling cascades, and transcription factors and their regulation of gene expression. Hormones are signaling molecules produced by organisms that act as chemical messengers to facilitate complex regulatory pathways by binding receptors and triggering cascades of protein interactions. Hormone crosstalk is a central mechanism for controlling the balance between growth promotion and defense activation. Hormone crosstalk refers to the intricate communication and interaction between different hormones present in the environment within an organism's biological system. This interaction involves hormones influencing each other's synthesis and related signaling pathways, allowing the plant to take cues from the environment and switch pathways to focus on either growth or defense in response. Studying how certain hormones affect protein interactions in signaling pathways, through hormone crosstalk, helps us understand how plants switch from growth to defense.

These findings could potentially reveal novel insights crucial for agriculture, ecology, and beyond.

Many mechanisms are involved to mediate the switch between growth and defense. This thesis will focus on one important hormonal pathway for growth, the brassinosteroid signaling pathway. The brassinosteroid signaling pathway is activated by the presence of the hormone brassinosteroid (BR) which initiates a signaling cascade triggering the sequential activation and interaction of other proteins within the pathway (Clouse, 2011; Anne et. al., 2015). When plants are attacked by pathogens they produce SA, which triggers the systemic acquired resistance (SAR) in the plant, leading to the downstream activation of defense genes (Conrath, 2006). The presence of SA downregulates the BR signaling pathway cueing the plant to direct its energy toward expression of defense genes, halting growth while it fights the pathogen at hand (Khan et al., 2022). We hypothesize that this downregulation occurs through altering the interaction between proteins within the BR signaling pathway.

Transcription factors regulate gene expression, controlling activity by binding to DNA sequences and enabling cells to respond to environmental stimuli. The transcription factor TCP8 has known roles in controlling the expression of genes related to growth and defense (Kim et al., 2014). Within the nucleus TCP8 interacts with Brassinazole Resistant 2 (BZR2), a transcription in the BR signaling pathway, to promote expression of genes important for growth (Spears et al., 2022). In the nucleus transcription factors have been found to localize into circular clusters called condensates or punctae, and TCP8 has exhibited this behavior (Spears el al., 2022). This interesting phenotypic behavior may explain TCP8's ability to promote gene expression of genes in pathways related to

growth and defense, which cannot occur simultaneously. Exploring the importance of specific regions within TCP8's genome may answer how TCP8 forms these punctae and if this observed localization pattern is important to TCP8's interaction with BZR2 within the BR signaling pathway.

The rest of this thesis will go into more detail about the brassinosteroid pathway, hormones influencing its activation, and protein interactions involved. As needed please refer to the appendix for definitions of the key terms mentioned.

Brassinosteroid Signaling Pathway

The brassinosteroid signaling pathway is pivotal in orchestrating plant growth and development (Huot et al., 2014). Brassinosteroids (BRs) are a class of plant hormones that play crucial roles in various aspects of plant growth and development (Clouse, 2011). The brassinosteroid signaling pathway is a complex signal transduction pathway that mediates the effects of brassinosteroids on plant physiology through regulating over a thousand genes (Clouse, 2011). BRs exert their effects by promoting cell elongation, division, and differentiation, crucial processes in seed germination, stem elongation, leaf expansion, and fruit development (Huot et al., 2014). Upon perception by cell surface receptors, brassinosteroids initiate a cascade of molecular events that traverse intracellular components, culminating in alterations in gene expression and physiological responses driving plant growth and development. Plants deficient in or insensitive to BR signaling exhibit severe growth stunting and male infertility, underscoring the pathway's significance. Conversely, external application of BR enhances both the quality and quantity of crop yield, highlighting the potential agricultural implications of understanding and manipulating this signaling pathway (Huot et al., 2014). The research

surrounding the BR signaling pathway and the plant proteins involved remains relatively limited, with the current understanding having emerged within the last 15 years (Clouse et al., 2011). The following paragraphs will divide protein interactions involved in BR signal transduction into known and unknowns, highlighting gaps within the literature on the regulation of the pathway.

The pathway begins with the binding of BR to brassinosteroid-insensitive 1 (BRI1) receptor kinase on the surface of the cell membrane. After BRI1 is activated, it phosphorylates the negative regulator BKI1 allowing BRI1to form a receptor complex with BRI 1-associated-receptor kinase 1 (BAK1) (Clouse, 2011*)*. BAK1 phosphorylates downstream signaling components, including brassinosteroid-insensitive 2 (BIN2) , a glycogen-synthase-kinase-3-like kinase, inactivating it. As shown in the model in Fig. 1, when BIN2 is inactivated, transcription factors BZR1 and BZR2 are able to translocate to the nucleus and bind to specific DNA sequences in the promoters of target genes related to plant growth and development (He et al., 2002). In contrast, in the absence of BR, when BIN2 is activated, it phosphorylates BZR1 and BZR2 (He et al., 2002). Phosphorylation of these transcription factors by BIN2 leads to their inactivation and degradation, preventing them from translocating into the nucleus and activating the transcription of target genes, preventing growth and development of the plant (Huot et al., 2014; Anne et al., 2015). While it is known that BR signaling inhibits BIN2 activity, less is known about the mechanisms involved behind BIN2's deactivation and proteins involved (He at el., 2002). Researchers have proposed that there could be additional proteins interacting with BRI1, BAK1, and BIN2 within the pathway and that finding such proteins would be important for understanding the pathway (Clouse, 2011).

Since the inactivation of BIN2 is vital for the expression of BR regulated genes for growth and development, the protein which inactivates BIN2 is important in determining whether the plant invests its energy into growth or defense. Transgenic lines of *Arabidopsis* overexpressing OPS showed characteristics similar to lines overacted BR mutants, indicating OPS has a role in BR signal transduction (Anne et al., 2015). The protein OCTOPUS (OPS) which exists in the phloem of *Arabidopsis*, is a polar protein which localizes at the plasma membrane and plays a role in phloem development and differentiation (Truernit et. al 2012). The phloem, a carbon-rich tissue in plants that transports sugars from the leaves to the roots and flowers, is also highly susceptible to pathogens due to its rich carbon content (Jiang et al., 2019). Following the indication that OPS may be involved in BR signal transduction it was found that OPS recruits BIN2 to the plasma membrane where they interact, inactivating BIN2 (Anne et al., 2015). These findings suggest that OPS acts as a positive regulator of the BR signaling pathway by inhibiting BIN2 activity, as illustrated in Fig. 1. This inhibition prevents BIN2 from phosphorylating TCP8-interacting transcription factors BZR1 and BZR2, thereby facilitating their translocation into the nucleus (Anne et al., 2015). Once in the nucleus these transcription factors bind to the promoter region of BR regulated genes, allowing for their expression, contributing to the plant's growth and development. Previous research has shown that OPS plays a role in phloem development but its role in immune signaling has not been studied (Jiang et al., 2019). Through continued exploration of OPS and its involvement in the brassinosteroid signaling pathway, particularly in relation to what influences the interaction between OPS and BIN2, we can gain deeper insights into how plants use the brassinosteroid signaling pathway to balance growth and defense.

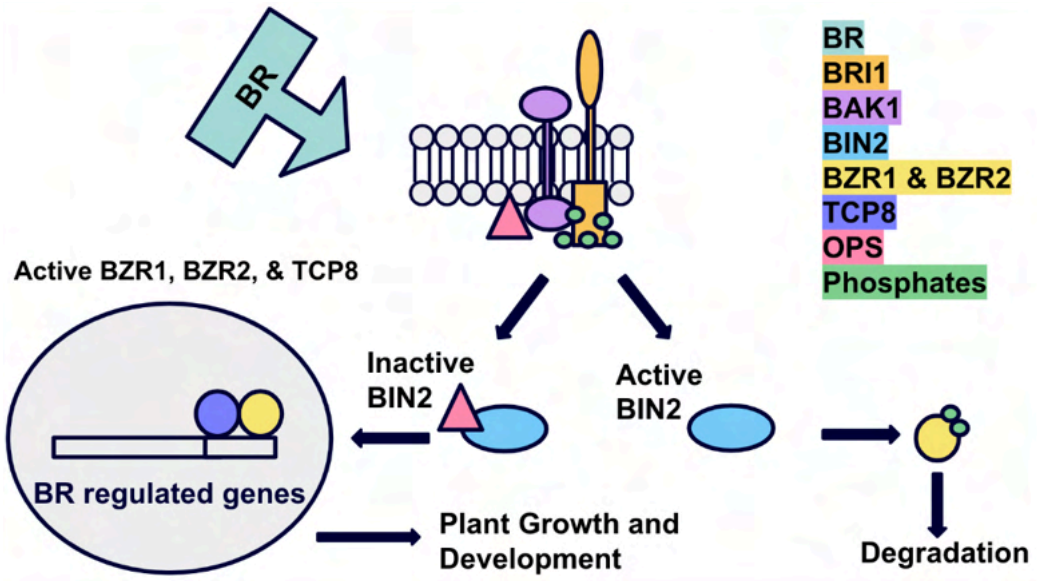

Figure 1. Brassinosteroid (BR) signaling pathway based on Clouse (2011). This model simplifies the protein interactions involved in BR signal transduction. BR binds BRI1, a receptor kinase at the membrane, forming a receptor complex with BAK1. This model proposes that the protein OPS located in the plasma membrane with the receptor complex negatively regulates BIN2 in the presence of BR, inactivating it. When BIN2 is inactive it does not tag transcription factors BZR1 and BZR2 for degradation with phosphates. The active, unphosphorylated BZR1 and BZR2 enter the nucleus, interact with transcription factor TCP8, and bind to the promoter regions of BR regulated genes, allowing for BR regulated genes to be expressed. The expression of BR regulated genes promotes plant growth and development. If BR is not present in the system OPS does not bind and inactivate BIN2 preventing translocation of the transcription factors into the nucleus, decreasing expression of BR regulated genes, thus allowing the plant more energy to devote to expression of defense related genes.

Hormone Crosstalk

As plants allocate limited resources between growth and defense mechanisms, hormonal signaling pathways act as key regulators in this balance. For instance, brassinosteroids (BR) promote growth processes, such as cell elongation and differentiation, while simultaneously inhibiting the plant's defense response against pathogens (Huot et al., 2014). Conversely, salicylic acid (SA) is known for its role in activating plant defense mechanisms but can also suppress growth when present in elevated levels. This delicate interplay between hormones allows plants to adjust their allocation of resources based on environmental cues and the level of threat encountered. Moreover, the intricate crosstalk between different hormonal pathways further modulates the trade-off between growth and defense, highlighting the complexity of plant adaptation strategies in response to changing environmental conditions (Huot et al., 2014).

The hormone salicylic acid (SA) is a plant hormone important in regulating plant survival and immunity (Asami et. al 2000). Systemic acquired resistance (SAR) is a defense mechanism in plants where prior exposure to necrotizing pathogens induces enhanced resistance to future pathogen attacks throughout the plant (Conrath, 2006). Salicylic acid (SA) plays a central role in SAR by serving as a signaling molecule that activates defense genes and primes the plant for quicker and more effective defense responses upon subsequent pathogen encounters (Conrath, 2006). SA influences the translation of Pathogenesis-Related (PR) proteins which allow plants to respond to pathogens and environmental stressors (Khan et al., 2022). In *Arabidopsis*, SA has been demonstrated to stimulate the activation of Brassinosteroid-Insensitive 2 (BIN2) kinase, which then activates the PR gene expression (Han et al., 2022). When BIN2, a negative

regulator of BR signaling, is active, expression of BR genes is downregulated, indicating a shift from growth and development responses to stress and immune responses (Khan et al., 2022). These findings highlight the role of SA in orchestrating plant immunity by downregulating growth responses through activating BIN2.

The BR hormone biosynthesis inhibitor brassinazole (BRZ) is one that directly depletes BR, through preventing its biosynthesis (Asami et al., 2000). The study found that BRZ treatment caused deficiency of a type of BR called brassinolide (BL) in *Arabidopsis*, leading to morphological changes resembling BR-deficient mutants, including dwarfism due to a failure of cell elongation (Asami et al., 2000). BRZ (brassinazole) inhibits brassinosteroid (BR) biosynthesis by interfering with the enzymatic steps involved in the synthesis pathway (Rozhon et al., 2019). Specifically, BRZ is known to inhibit the conversion of cathasterone to teasterone, a critical step in the BR biosynthesis pathway (Rozhon et al., 2019). This novel BR biosynthesis inhibitor could serve as a valuable tool for investigating the function of BRs in various plants and biochemical processes and may have potential applications as a commercial plant growth regulator. (Asami et al., 2000).

SA and BRZ work in different ways to downregulate the brassinosteroid signaling pathway. SA, known for its role in activating plant defense mechanisms, activates Brassinosteroid-Insensitive 2 (BIN2) kinase, which subsequently downregulates BR gene expression (Han et al., 2022). While BRZ directly inhibits BR biosynthesis, preventing the signaling cascade from being initiated and down regulates expression of BR genes (Rozhon et al., 2019). The presence of BR leads to the degradation of BIN2, a negative regulator of the BR signaling pathway (Peng et al., 2008). Thus in the absence of BRs,

BIN2 is active and tags the transcription factors for degradation (Rozhon et al., 2019). Further manipulation of BR and the brassinosteroid signaling pathway through the introduction of salicylic acid (SA) and brassinazole (BRZ) will allow for results that offer more insight into the specific interactions behind BIN2's regulation and whether the plant expresses genes for growth and development or is able to direct its energy into stress and immune responses.

Transcription Factors

The brassinosteroid signaling pathway utilizes transcription factors BZR1 and BZR2 to regulate the expression of genes involved in regulating growth and defense (He et al., 2002). Transcription factors (TFs) play a crucial role in controlling both growth and defense pathways in plants allowing plants to adapt and respond to environmental stimuli by binding at the promoter region of genes (Kim et al., 2014). Many TFs contain intrinsically disordered regions (IDRs), which are regions that lack a stable three-dimensional structure making them highly flexible and dynamic, often adopting multiple conformations or remaining extended (Brodsky, Jana, & Barkai, 2021). IDRs can interact directly with DNA, likely playing a role in TF binding specificity (Brodsky, Jana, & Barkai, 2021). Previous research discusses the role of IDRs in transcription factors contributing to protein interactions and the formation of transcriptional condensates (Brodsky, Jana, & Barkai, 2021). The formation of biomolecular condensates refers to the localization of proteins via liquid-liquid phase separation into round clusters within cells, visible via microscopy (Emenecker et al., 2020). IDRs ability to form protein interactions drives phase separation into condensates, allowing TFs to activate gene expression (Boija, et al., 2018). Condensates provide plants with a means of

organization and concentration of molecules involved in responses to environmental stimuli (Emenecker et al., 2020). Condensates have been found to play a role in growth and defense pathway responses through the involvement of transcription factors (TFs) in the formation of plant-specific condensates, which regulate gene expression in the nucleus (Shigenaga et al., 2017). However, TF IDRs, the mechanisms behind condensate formation, and binding specificity to DNA are poorly characterized and require further research (Brodsky, Jana, & Barkai, 2021).

Studies on *Arabidopsis thaliana* transcription factors auxin response factors (ARFs) have found that these TFs form hormonal regulated condensates (Powers et al., 2019). ARF7 and ARF19 (ARFs) in the presence of the phytohormone auxin exhibit nuclear localization via the formation of condensates (Powers et al., 2019). ARF's IDRs could contribute to the formation of these condensates contributing to transcriptional regulation of auxin genes (Powers et al., 2019).

TCP8 has been observed forming condenates in *Nicotiana benthamiana,* in response to the presence of BR, potentially modulating BR-responsive gene expression dynamically (Spears el al., 2022). The Spears' lab works with *Arabidopsis thaliana* TF teosinte branched 1/cycloidea/proliferating cell factors (TCPs), specifically TCP8 (Spears et al., 2022). TCP transcription factors 8, 14, and 15 play a role in regulating defense genes through protein interactions (Kim et al., 2014). These three TCPs are positive regulators of defense and immunity gene expression, binding to DNA and promoting transcription of genes necessary for stress-induced responses to pathogens (Kim et al., 2014). TCPs also regulate signaling pathways of plant hormones such as salicylic acid (SA), and brassinosteroids (BRs) (Schommer et al., 2008; Guo et al., 2010;

Mukhopadhyay and Tyagi, 2015; Wang et al., 2015; Gonzalez-Grandio et al., 2017). Figure 2 demonstrates the experimental design and observed results depicting the relationship between BR and nuclear condensates, also referred to as punctate (Spears et al., 2022). BR causes TCP8 to move into punctae while introducing BRZ, which prevents biosynthesis of BR causes TCP8 to move out of the punctae. TCP8 has been found to interact with TFs BZR1 and BZR2 which regulate the expression of BR genes important for growth and development, and the movement of TCP8 in and out of punctae may affect these interactions (Spears el al., 2022). To this date little is known about TCP8 condensates and their presence has not been identified in systems besides N. *Benthamiana.* Further research is required to determine TCP8's precise mechanisms utilized in punctae formation and the role of these punctae in the BR signaling pathway (Spears at el., 2022).

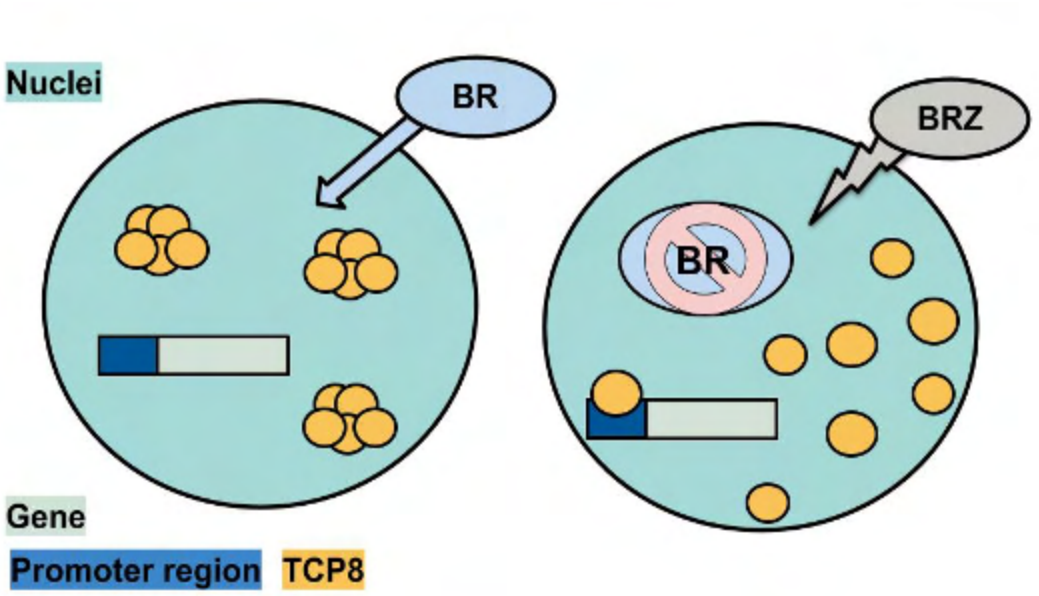

Figure 2. A depiction of the dynamic response of TCP8 within the nucleus of N. *benthamiana* cells when BRZ, an inhibitor of BR, is synthetically introduced into the system through the spraying of the leaves. BR is a naturally occurring plant hormone important for growth and development. The introduction of BRZ depletes BR, causing the TCP8 to activate gene expression of genes associated with growth, regulating the BR hormone pathway.

Similar to ARF, TCP8 has prion-like domains or IDRs (Powers et al., 2019 & Valsecchi et al., 2013). It has been found that TCPs are part of the intrinsically disordered protein family, and TCP8 is one of the most disordered of all the TCPs (Valsecchi et al., 2013). This study identified three regions of disorder within TCP8, as identified in Fig. 3., the first is the N-terminal IDR, then the middle IDR, and lastly the C-terminal IDR. These regions are enriched with specific amino acids which may indicate specific

structural or functional properties of each IDR. The enrichment of certain amino acids within these regions might suggest a propensity for phosphorylation, a post-translational modification crucial for regulating protein function. The N-terminal IDR is enriched in threonine (Thr) and arginine (Arg), the middle IDR contains a higher abundance of histidine (His), glutamine (Gln), and asparagine (Asn), and the C-terminal IDR is enriched in serine (Ser), asparagine (Asn), and glutamine (Gln). Phosphorylation sites are often characterized by the presence of specific amino acids such as serine, threonine, and tyrosine, which are frequently targeted by kinases. These regions of TCP8 are likely to undergo phosphorylation and may be involved in mediating interactions with other proteins or in modulating TCP8's activity in response to cellular signals or environmental cues (Valsecchi et al., 2013). Other studies have confirmed that post-translational modifications do exist in the IDRs of TCP8 (Xu et al., 2017). It has been hypothesized by the molecular genetics plant community that the IDRs in TCP8 are targets of post-translational modifications that determine whether the plant is actively regulating growth or defense (Xu et al., 2017). Additional research needs to be conducted on the significance of these IDRs on the function of TCP8 and may reveal whether these regions, similar to other TFs, play a role in punctae formation.

Figure 3. A representation of the intrinsically-disordered regions within AtTCP8. Based on the three regions of disordered identified in Valsecchi et al. (2013). IDR 1 is the N-terminal IDR, 2 is the middle IDR, and 3 represents the C-terminal IDR.

Transcription factors play an important role in the regulation of gene expression, so further research on IDRs and their importance in the formation of punctae may help further the understanding of how these TFs regulate signaling pathways. Specifically, mutating TCP8 to remove entire IDRs may help us identify sites important for TCP8's function and help to better understand punctae formation. Additionally, mutating TCP8 and testing the effect on TCP8's interactions with transcription factors BZR1 and BZR2 can provide more insight into TCP8's role in BR signaling pathway.

Significance

From hormone signaling pathways to transcriptional regulation, plants meticulously balance resource allocation to respond to environmental cues they encounter. Through hormonal crosstalk, the formation of biomolecular condensates, and transcription factors, plants are able to respond dynamically, balancing gene expression and signaling pathways vital to growth and defense. Through the balance between these molecular pathways, plants optimize their fitness and resilience, ultimately shaping their adaptation strategies crucial for agriculture and ecology.

This research aims to enhance the understanding of how plants respond to environmental stressors, including hormones, through the BR signaling pathway and the formation of TCP8 condensates in nuclei via LLPS. The role of the protein OPS in BR signaling, and its impact on growth and immunity, remains understudied. By artificially manipulating hormones, we can observe and gain insights into OPS' interactions with BIN2. This will allow for a better understanding of OPS' role in growth and defense and highlight the novel role this phloem localized protein has in these processes. Furthermore,

TCP8, a transcription factor that interacts with BZR2, a critical regulator of BR gene expression for growth and development, has been observed forming punctae in the nucleus. TCP8's IDRs are suspected to influence this phenotype, so broad mutation studies, which remove entire IDRs will be used to pinpoint specific IDRs responsible for TCP8 punctae formation. Understanding how plant responses to the environment rely on condensate formation through liquid-liquid phase separation can significantly advance our understanding of this mechanism's importance across different systems. This project aimed to address several of the knowledge gaps mentioned earlier in this introduction across the BIN2/OPS and TCP8/BZR1/BZR2 signaling continuity.

Methodology

Samples/Plant Maintenance

N. benthamiana plants used in interaction and localization assays were grown under an 8-h light and 16-h dark cycle at 22°C and 55% relative humidity.

Experiment 1

In the context of the BR signaling model, the interaction between OPS and BIN2 at the plasma membrane is significant (Greenwood et al., 2023). The disruption of their protein complex may lead to the activation of downstream signaling responses triggered by brassinosteroids (BRs). These responses are dependent on the presence of hormones such as BR and SA, which activate specific cellular responses. Therefore, the interaction between OPS and BIN2 at the plasma membrane represents a mechanism by which the BR signaling pathway can be altered in response to environmental cues, ultimately affecting plant growth and development. This experiment was designed to confirm that OPS and BIN2 do interact and then synthetically introduce SA, a hormone which initiates

immune response, to determine the effect on this interaction. The purpose of this experiment is to identify the role of OPS in the BR signaling pathway and to investigate if a change in interaction between OPS and BIN2 could be important in the plant's defense response.

Molecular cloning

Full-length cDNAs of Ops and Bin2, lacking stop codons, were amplified from *Arabidopsis* Col-0 genomic DNA and cloned into the Gateway‑compatible donor vector pDONR221 (Invitrogen). The coding regions were transferred via LR reaction into the split luciferase vectors pCAMBIA-NLuc and pCAMBIA-CLuc, as described by Chen et al. (2008). Detailed primer information is provided in Table S1 of (Greenwood et al., 2023). Once expression of cloned genes was validated via sequencing they were transformed from *E. coli* into plant pathogen *Agrobacterium*.

Split Luciferase Assay

The split luciferase assays were performed following protocols established in Spears et al. (2022). The coding sequences of BIN2 and OPS were inserted into split-luciferase expression constructs and inserted into leaf epidermal cells of *Nicotiana benthamiana.* A negative control GUS was used to standardize the date in order to compare interactions between OPS and BIN2 (Anne et al., 2015). Constructs of OPS-nLUC, BIN2-cLUC, and GUS-cLUC were transformed into *Agrobacterium tumefaciens* strain C58C1. Overnight cultures were generated for each construct at 30°C, then pelleted and resuspended in 10-mM MgCl2 buffered with 1-mM MES (pH 5.6) and 100-nM acetosyringone (3',5'-dimethyoxy-4'-hydroxyacetophenone). Suspensions were incubated for 4–5 h. Bacterial inocula were mixed to an optical density at 600 nm of 0.2

for each strain and syringe infiltrated into mature *Nicotiana benthamiana* leaves. Infiltrated plants were placed back in the growth chamber and 24 hours before sampling, leaves were sprayed with either 1 μM salicylic acid (SA), 1 μM brassinazole (Brz), or a mock solution containing 0.01% Tween-20 which acted as a control. After 72 hours of incubation, leaf discs were obtained using a sharp 0.5 cm diameter bore and placed abaxial side down on 100 μl of infiltration solution (50 mM MES pH 5.6, 10 mM MgCl2, 0.5% DMSO), which helps break down the cell wall of the samples, in a white 96-well plate. The plate was then wrapped in foil and incubated in the growth chamber for approximately 20 minutes. Following incubation the infiltration solution was removed and replaced with 100 μl of reaction solution (1× infiltration solution, 1 mM luciferin). Luminescence was measured at 10-minute intervals for a period of 2 hours in a BioTek Synergy HTX plate reader. The expression of luminescence indicates interaction between the OPS and BIN2 as the N and C terminus of luciferase unite and express light in the presence of luciferin if the proteins interact.

Western Blot

Samples obtained from the same leaves used in the split luciferase assay were utilized to verify equivalent levels of protein in leaves infiltrated with each of the 3 treatment groups (Mock, SA, and BRZ). This procedure was previously used in Spears et al., 2022 to verify the levels of TCP8 in the split luciferase assay. Western blot analysis was conducted to investigate protein levels of OPS in each sample. For this analysis, one gram of total protein was extracted from the leaf samples using 1 mL of 2× sodium dodecyl sulfate (SDS) buffer containing 100-mM Tris–HCl (pH 6.8), 4% w/v SDS, 20% v/v glycerol, and 250 mM dithiothreitol. Following extraction, the protein samples were

cleared by centrifugation at 16,000 × g and subsequently loaded onto an 8% bis–acrylamide SDS–polyacrylamide gel electrophoresis gel. Detection of the protein of interest was achieved using a 1:2,000 dilution of horseradish peroxidase-conjugated anti-HA antibody (Roche, Basel, Switzerland).

Experiment 2

Truncation Design

In order to more broadly determine which IDRs contain regions of importance for TCP8's functions, truncation mutations removing entire IDRs were designed (Fig. 4). Using Benchling, a versatile molecular biology software platform, we designed truncation mutations aimed at removing entire intrinsically disordered regions (IDRs) of TCP8. Starting with the retrieval of the TCP8 amino acid sequence, we analyze the sequence to pinpoint IDRs, as their exact base numbers have been previously identified. We designed truncation mutations primers, ensuring the removal of IDRs while preserving essential functional domains or motifs. Once the primers were ordered and arrived PCR was used to amplify the mutated TCP8 sequences within a pDONR vector and they were transformed into C58C1 for experimentation. We successfully generated truncation 1 and 2 mutant constructs to be used in experimentation. Previous experiments revealed that regions of PTMs were located in IDRs 1 and 2 so this experiment focused on Truncation mutations 1 and 2 shown in Fig. 4. Thus any differences in phenotypes between truncations 1 and 2 as compared to the wildtype will allow us to pinpoint which IDR is vital for TCP8's function.

Figure 4. A representation of the truncation mutations targeting intrinsically-disordered regions within AtTCP8. Truncation mutations were made to broadly target IDR regions of TCP8; IDRs 1, 2, and 3 are labeled. One mutation will eliminate IDR 3; one will eliminate IDR 2 and IDR 3, and therefore affect Cluster 2; the last will eliminate IDR 1. IDRs 1 and 2 contain areas enriched in post-translational modification sites that may affect TCP8 activities.

Microscopy/colocalization analysis

Following TCP8 infiltration, leaf tissue from each treatment group was cut to obtain a sample to observe localization of GFP-TCP8 within the cells. A spinning disk confocal microscope was used to observe and quantify the presence of GFP-TCP8 punctae in the wildtype (WT), truncation mutation 1, and truncation mutation 2 samples. As described in Spears et al., 2022, GFP was excited using an argon laser with peak excitation at 488 nm at 2% intensity. Emission from GFP was collected between 500 and 550 nm with gain of 400. Single Z-plane images from both laser channels were captured and then colocalization was analyzed using Fiji (ImageJ). Punctae in each image were

assessed independently by myself and my lab partner. Each nuclei per image was accounted for and the number of punctae per nucleus was recorded. Only the punctae with the brightest and most clearly defined edges were considered for counting. In an effort to remain consistent the nucleolus was also counted. The best images from each sample were also noted to be compared qualitatively.

Results

Experiment 1

The split luciferase assay showed no interaction between OPS and our negative control GUS so interactions between OPS and BIN2 were normalized to this control as shown in Fig. 5. An interaction between OPS-cLUC and BIN2-nLUC was observed which confirmed previously reported data (Anne et al., 2015). Fig. 5 highlights that the samples treated with SA and BRZ had lower relative signals indicating a decrease in interaction between OPS-cLUC and BIN2-nLUC. Less interaction between OPS and BIN2 results in the two ends of the split luciferase enzyme not connecting, leading to reduced reconstitution of the active luciferase enzyme. Luciferase catalyzes the oxidation of luciferin, producing bioluminescence, so less activated luciferase causes a decrease in luminescence, corresponding to a lower signal detected by the plate reader. This decrease in signal intensity reflects the diminished interaction between OPS and BIN2 which is prompted by the introduction of the hormones SA and BRZ into the system. Following this experiment a western blot was performed using 2 samples from each treatment: mock, BRZ, and SA. The western blot was performed to ensure equal levels of OPS protein in each sample to verify that decreased interaction was a result of the manipulated

conditions and not less protein expression in the leaf samples. The western blot showed equal bands at the kDa that corresponds to GFP-OPS as indicated by the arrows in Fig. 6.

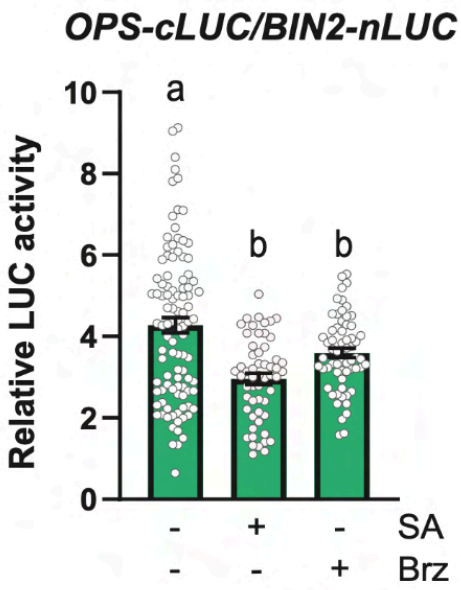

Figure 5. OPS interaction with BIN2 is regulated by SA and BRZ introduction. Interactions between OPS and BIN2 were quantified using a split-luciferase assay in *N. benthamiana* leaf epidermal cells. The column labeled (a) depicts tissue sprayed with a mock solution acting as a control. The columns labeled (b) were sprayed with 1 μM solutions of either SA or Brz 24 hours prior to sampling (+ indicates the treatment used). Relative luciferase (LUC) activity was normalized to the negative control (GUS-nLUC) levels as relative LUC activity and data were combined from 3 independent experiments, n=60-100. A significance in the relative LUC activity between (a) and (b) was determined by ANOVA with Tukey MCS, $p< 0.05$. Error bars represent 1 S.E.

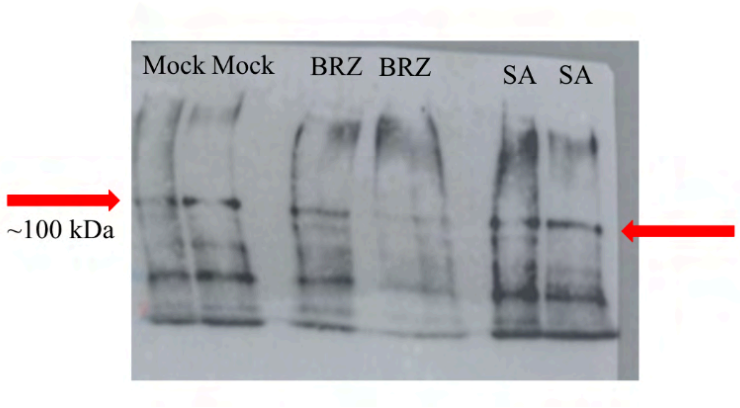

Figure 6. Western blot of OPS-HA-cLUC/BIN2-nLUC samples sprayed with mock, BRZ, and SA. Anti-HA was used as the primary antibody to detect the protein level of OPS-HA-cLUC in the samples. The western blot depicts equal amounts of OPS protein in each sample indicating that the results of the split luciferase assay are not due to unequal levels of protein.

Experiment 2

After observing each treatment group under the microscope, initial qualitative differences were assessed and images of nuclei were captured. As seen in Figure 7 there are distinct differences in the GFP-TCP8 localization between WT and truncation mutation 1 compared to truncation mutation 2. We classified this difference categorically as 'nuclear localization' versus 'non nuclear localization'. Figure 7 shows the best representative of this observed pattern among the captured images for each treatment group. The WT and Trunc 1 images have similar bright green round dots within the nucleus of the cell, while Trunc 2's nuclei is solid green indicating localization of GFP-TCP8 throughout. Trunc 2's GFP-TCP8 localization presents differently as seen in

figure 8. The green fluorescence is throughout the plasma membrane of the cell and along the edges of the cell. All images in Trunc 2 looked similar to this and all looked distinct from the WT and Trunc 1 captured images. To sort the images in order to quantify these punctae and their localization pattern we decided to use the terminology “nuclear vs non-nuclear localization”. Since Trunc 2 has no punctae in the nucleus we identified Trunc 2 as having non-nuclear localization and since GFP-TCP8 was not present in circular groups in the nucleus we characterized its localization pattern as “diffuse”. After observing these qualitative differences between the treatments, we counted the occurrence of nuclear localized punctae to assess quantitative differences between the groups. We downloaded the captured images into Fiji in order to quantify and record the number of punctae within each nucleus. There were 11 cells within the WT and Trunc 1 treatment groups and 9 cells within the Trunc 2 treatment group. Figure 9 (A) shows the distribution of the frequency of counts of punctae within each group. All cells within the WT treatment group had 2 or more punctae and figure 9 (B) shows that the average number of punctae per cell for this group is 5.45. The average number of punctae in the Trunc 1 treatment group is 4.73. Figure 9 (A) and (B) depict that the Trunc 2 treatment group had no cells with nuclear punctae. Figure 9 (C) was created to visualize the proportion of punctae and diffuse nuclear localization. WT and Trunc 1 had 100% punctae and Trunc 2 had 100% diffuse nuclear localization.

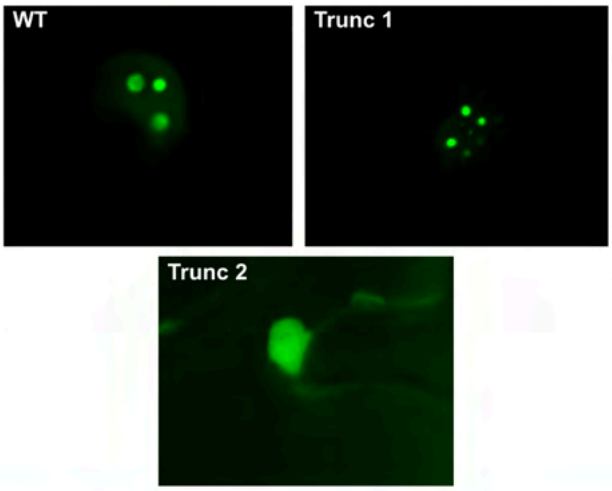

Figure 7. Images taken on a spinning disk confocal microscope of GFP-TCP8 localization within N. *benthaliana*. Each image is centered on one nucleus. Samples expressing TCP8 WT and TCP8 truncation mutation 1 show a similar pattern of GFP-TCP8 localization, as their nuclei contain bright well defined spheres. The nuclei shown for theTCP8 truncation mutation 2 cell has diffuse TCP8-GFP localization throughout the entire nuclei making it solid green and there are no punctae within.

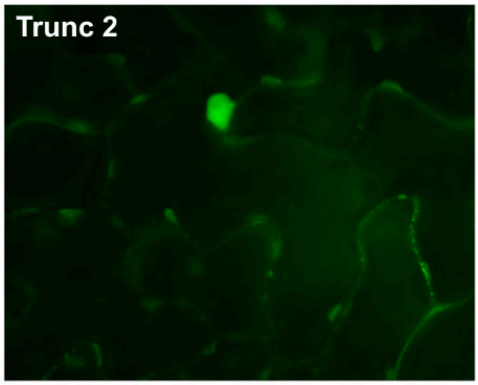

Figure 8. Image of a N. *benthaliana* leaf epidermal cell infiltrated with GFP-Trunc 2 taken on a spinning disk confocal microscope. This is the same image as figure 7 but zoomed out to show the entire cell. TCP8 truncation 2 displays a unique phenotype with GFP localization throughout the plasma membrane, not within the nuclei.

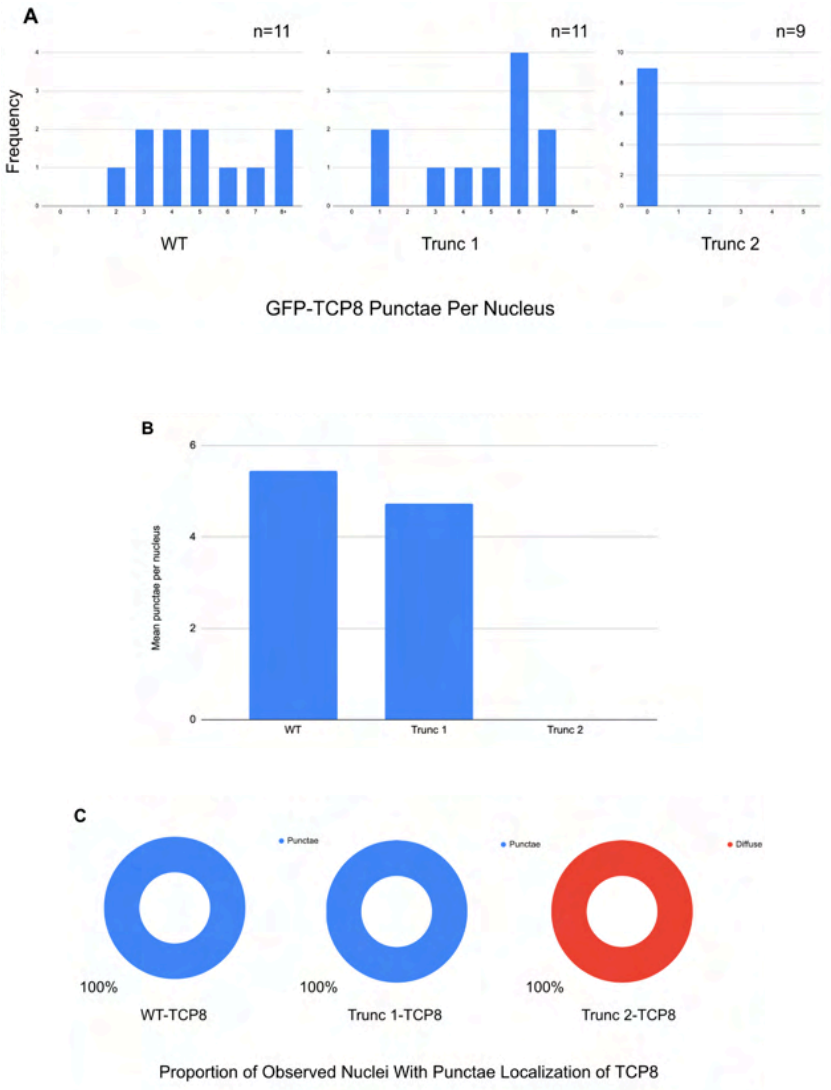

Figure 9. (A) Histograms assessing the frequency of GFP-TCP8 punctae per nucleus of n = the number of observed cells. No cells in the Trunc 2 sample have punctae within their nucleus. **(B)** A bar graph highlighting the average number of punctae per nucleus. The mean number of punctae per nucleus for WT = 5.45 and the mean number of punctae per nucleus for Trunc 1 = 4.73. There is no significant difference between these values. **(C)** Pie charts depicting the proportion of punctae vs diffuse localization within the nuclei. All of the cells in WT and Trunc 1 samples had nuclear punctae while no cells in Trunc 2 had punctae within the nuclei.

Discussion

The results from these experiments shed light on the connections between hormone signaling pathways, transcription factors, and biomolecular condensates in plants. Our split luciferase assay not only confirmed the interaction between OPS and BIN2 but also identified that this interaction is important in the context of immunity, as well as growth. In response to treatment with SA we observed the plant downregulate the brassinosteroid signaling pathway through decreasing the interaction between OPS and BIN2, allowing the plant to allocate its energy and resources to initiating immune responses. These observed shifts in these interactions allows us to better understand the mechanisms plants use to balance growth and defense. The decrease in interaction between OPS and BIN2, also highlights the hormone-dependent modulation of this interaction, suggesting a regulatory role for hormones in the BR signaling pathway. Understanding how OPS, a protein localized in the phloem, negatively regulates BIN2, a key kinase in the pathway, provides novel insights into the role of OPS in the initiation of immune responses. These findings offer new perspectives on the role of known BR signaling proteins in governing the growth-defense tradeoff as well as the role of hormone crosstalk in initiating these changes in protein interaction. This research also opens up future avenues for continued research to understand how plants optimize their responses to environmental cues and stressors, with implications for agriculture and ecological resilience.

The localization patterns of transcription factor TCP8 are important within the brassinosteroid signaling pathway because TCP8 interacts with transcription factor BZR2 to promote transcription of BR regulated genes. Regions of PTMs located in IDRs 1 and

2 have been identified as sites of interest that may be important for TCP8's function and interactions with other proteins. Truncation mutation 1 removed IDR 3 and truncation mutation 2 removed IDR 2 to determine the significance of IDR 1 and 2 on localization patterns within the cell. Truncation mutation 2 loses the nuclear localization pattern present in wildtype and truncation mutation 1 indicating that IDR 2 is important for this phenotype. These qualitative and quantitative differences underscore the importance of specific regions within TCP8 in mediating its behavior and interactions, further emphasizing the significance of IDRs in regulating transcription factor function. This experiment's findings highlight the importance of sites within IDR 2 for future testing. Future experiments could use Truncation mutation 2 and introduce BRZ into the system to track and quantify the dynamic movements of TCP8 into and out of punctae in response to environmental stimuli, providing more insight into the ways that plants use molecular condensates in their pathways. Another future experiment could successfully grow *Arabidopsis* GFP expressing lines to observe these TCP8 punctae in their naturally occurring system for the first time.

Despite observing a decrease in interaction between OPS and BIN2 upon hormone treatment, the exact molecular mechanisms underlying this phenomenon remain unclear. Additionally, the absence of nuclear punctae in Truncation 2 mutants prompts further investigation into the precise regions within TCP8 responsible for condensate formation. The captured images represent only a small portion of the samples and samples of larger than 11 cells could provide more data on the occurrence of nuclear vs non nuclear localization. A better method for quantifying and classifying the punctae could also be determined to eliminate human error.

Overall this study has provided valuable insights into the distinct roles of OPS and TCP8 and their respective protein interactions within the brassinosteroid signaling pathway. The finding that the interaction between OPS and BIN2 is altered in the context of immunity opens up avenues for further research into the ways in which plants alter protein interactions to switch between growth and defense. Our initial efforts to categorize TCP8 punctae and the identification that IDR 2 is important for punctae formation lay important groundwork for future studies to determine how the formation of condensates affects interactions with transcription factors BZR1 and BZR2 in the nucleus. By addressing these unanswered questions, we can continue to unravel the complexities of plant hormone signaling pathways and transcriptional regulation, ultimately contributing to a more comprehensive understanding of plant responses to environmental stressors. This research underscores the importance of hormonal crosstalk and biomolecular condensates in shaping plant adaptation strategies, aligning with the broader goal of advancing agricultural and ecological practices.

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