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EXPERT VIEW

From growth to stress: RAF-like kinases as integrators of hormonal signals in plants

Hye Lin Park and Gyeong Mee Yoon*,

Department of Botany and Plant Pathology and the Center for Plant Biology, Purdue University, West Lafayette, IN 47907, USA

* Correspondence: yoong@purdue.edu

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Abstract

RAF-like kinases, members of the mitogen-activated protein kinase kinase kinase (MAPKKK) family, are central integrators of external and internal signals in plant stress responses and growth regulation. These kinases mediate signaling through multiple hormone pathways, including abscisic acid-dependent and -independent pathways, ethylene signaling, and rapid auxin responses. Unlike typical MAPKKKs that function through kinase cascades, RAF-like kinases primarily employ direct phosphorylation of downstream targets and dynamic subcellular localization to mediate specific physiological responses. Here, we review the emerging roles of RAF-like kinases in *Arabidopsis thaliana*, highlighting their integrative functions in hormone signaling, stress responses, and growth control. The complex interplay between different RAF-like kinase subgroups and their diverse cellular targets underscores the intricate regulatory mechanisms plants have evolved to coordinate environmental responses with development.

Keywords: ABA, Arabidopsis, auxin, CTR1, ethylene, RAF-like MAPKKKs.

Introduction

Due to their sessile nature, plants constantly adapt to diverse environmental stresses, including drought, salinity, nutrient deficiency, and extreme temperatures (Zhu, 2016). To cope with these challenges, plants have evolved complex stress response mechanisms, in which plant hormones serve as crucial signaling molecules (Yoshida *et al.*, 2014; Waadt *et al.*, 2022). These hormonal signals trigger cascades of biochemical and cellular changes, modulating gene expression, metabolic activity, and physiological responses to enable plant stress adaptation. Central to these mechanisms is the integration of hormonal signals into the cellular signal transduction pathway, where protein kinases play a pivotal role in relaying stress signals to downstream effectors through phosphorylation cascades.

Protein kinases, particularly those involved in the mitogen-activated protein kinase (MAPK) cascade, play crucial roles in plant responses to various environmental stresses and developmental signals (Zhang and Zhang, 2022). These evolutionarily conserved cascades in eukaryotes comprise three kinase types: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK, which amplify and transmit signals from receptors to downstream targets (Caffrey *et al.*, 1999; Andrianova *et al.*, 2023). The Arabidopsis genome encodes ~80 MAPKKKs, classified into three subfamilies, namely MAPK/ERK-kinase kinase (MEKK)-like MAPKKKs, the Rapidly Accelerated Fibrosarcoma (RAF)-like MAPKKKs, and Zipper Interacting Protein Kinase (ZIK)-like MAPKKKs. Among these, RAF-like kinases are subdivided into 11 subgroups (B1–B4 and

C1–C7) totaling 48 members (Jonak *et al.*, 2002; Fàbregas *et al.*, 2020). While the roles of MAPKKs and MAPKs in plants are relatively well established, the functions of MAPKKKs have remained obscure. Recent studies, however, have shed light on the crucial roles of MAPKKKs, particularly RAF-like MAPKKKs, in coordinating plant hormone signaling with stress responses and growth regulation, with their dynamic subcellular movement serving as a key regulatory mechanism (Katsuta *et al.*, 2020; Soma *et al.*, 2020; Park *et al.*, 2023; Kuhn *et al.*, 2024). This review discusses recent advances in understanding RAF-like kinases in *Arabidopsis thaliana*, focusing on their integration of hormonal signals in plant stress responses and growth regulation.

RAF-like kinases in osmotic stress responses via ABA-dependent and -independent pathways

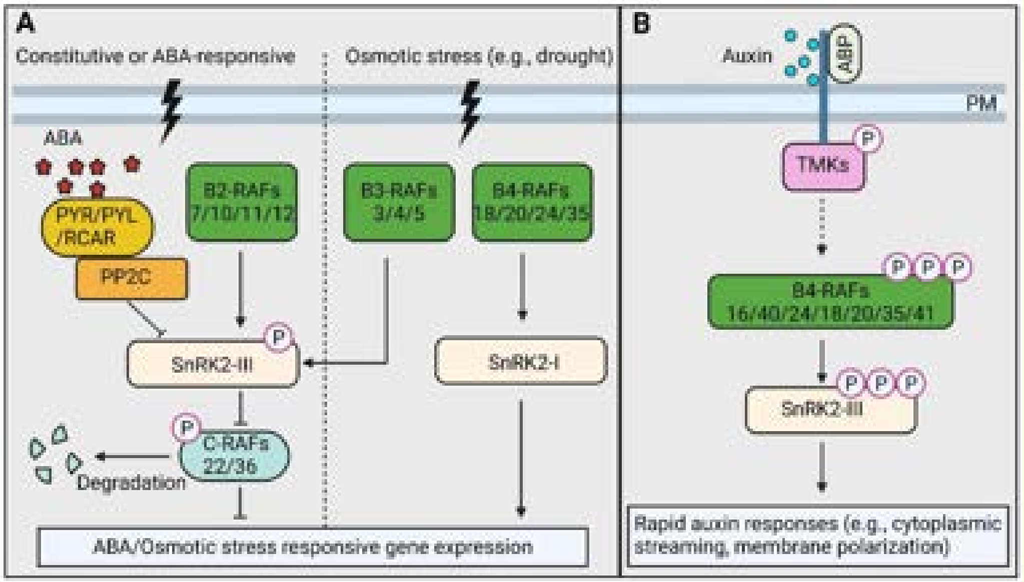
The abscisic acid (ABA) signaling pathway plays a fundamental role in plant stress response. Recent studies have identified that RAF-like MAPKKKs act as essential regulators of sucrose non-fermenting-1-related protein kinase 2 (SnRK2), which mediate both ABA-dependent and ABA-independent pathways (Fàbregas *et al.*, 2020; Lin *et al.*, 2020, 2021; Soma *et al.*, 2020, 2023; Kamiyama *et al.*, 2021a). The ABA signaling pathway comprises three main signaling components: ABA receptors [PYRABACTIN RESISTANCE (PYR)/PYR-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) family proteins], the negative regulator type 2 C protein phosphatase (PP2C), and the positive regulator SnRK2s (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002; Schweighofer *et al.*, 2004; Fujii *et al.*, 2007; Ma *et al.*, 2009; Park *et al.*, 2009). The SnRK2 family, which is classified into three subgroups (I, II, and III), is a key player in mediating osmotic stress responses through both ABA-dependent (subclasses II and III) and ABA-independent (subclass I) signaling pathways (Cutler *et al.*, 2010; Fujii and Zhu, 2012; Kamiyama *et al.*, 2021b). Under osmotic stress, elevated ABA levels trigger ABA–receptor complex formation, inhibiting PP2C activity and releasing SnRK2s to activate downstream gene expression (Box 1A) (Chong *et al.*, 2022).

The subclass III SnRK2s are known to be self-activated after being released from PP2Cs in response to ABA (Ng *et al.*, 2011; Soon *et al.*, 2012). However, autophosphorylation of SnRK2s alone is not sufficient for their full activation, implying the involvement of unidentified upstream kinases (Takahashi *et al.*, 2020; Soma *et al.*, 2023). Supporting this hypothesis, the kinase inhibitor staurosporine blocked SnRK2 activity *in vitro* but failed to prevent its activation by osmotic stress or ABA (Kelner *et al.*, 2004; Boudsocq *et al.*, 2007). Studies in *Physcomitrium patens* identified ABA and abiotic stress-responsive RAF-like kinase (ARK) as an upstream regulator of SnRK2s. This B3 RAF-like kinase is activated in response to drought and hyperosmosis, and regulates SnRK2s (Saruhashi *et al.*, 2015; Islam *et al.*, 2021). B3 RAF MAPKKKs from other land plants, including *A. thaliana* and *Selaginalla moellendorffii* complemented ABA insensitivity in *P. patens ark* mutants (Saruhashi *et al.*, 2015). In Arabidopsis, analysis of artificial miRNA (amiRNA) lines targeting B1 and B3 RAF-like MAPKKKs identified that B3 RAF-like MAPKKKs (RAF3/4/5) regulate the ABA signaling pathway by phosphorylating ABA-responsive subclass III SnRK2s (Takahashi *et al.*, 2020) (Box 1A). Further studies identified B2 RAF-like MAPKKKs (RAF7/10/11/12) as interaction partners and activators of subclade III SnRK2s in response to dehydration and ABA (Soma *et al.*, 2023) (Box 1A). Intriguingly, while both B2 and B3 RAF kinases are involved in SnRK2 signaling, they regulate drought stress tolerance through distinct pathways: B2 RAF–SnRK2s via ABA-dependent signaling and B3 RAF–SnRK2s via ABA-independent mechanisms (Lin *et al.*, 2021; Soma *et al.*, 2023) (Box 1A). B2 RAF kinases (RAF7/10/11/12) maintain constant activity and activate SnRK2s upon their release from PP2C-mediated inhibition by ABA-binding receptors during early drought stress (mild stress). In contrast, B3 RAF kinases (RAF3/4/5) become activated only under sustained or severe drought conditions independently of ABA. This dual system suggests a specialized role for B2 and B3 RAF kinases. B2 RAF kinases serve as housekeepers, activating subclass III SnRK2s in response to ABA during mild osmotic stress, while B3 RAF kinases provide additional reinforcement of SnRK2 activity when stress conditions become severe (Box 1A) (Soma *et al.*, 2023). Such layered activation mechanisms are likely to help plants balance the energy costs of stress responses while ensuring survival under severe conditions. This distinct temporal and mechanistic specialization of B2 and B3 RAFs represents a prime example of subfunctionalization, where evolutionarily related kinases have developed complementary roles in stress response while maintaining their core function in SnRK2 regulation.

B4 RAF-like kinases (RAF18/20/24/35) share functional similarity with B2 and B3 RAF-like kinases but specifically target ABA-independent subclass I SnRK2s. Both SnRK2s and B4 RAF-like kinases typically reside in the cytoplasm under normal conditions, but osmotic stress triggers their interaction within processing bodies (P-bodies) (Box 1A) (Lin *et al.*, 2020; Soma *et al.*, 2020). Transcriptome analysis of *raf18/20/24* and *srk2abgh* (subclass I SnRK2 knockout mutant) under dehydration stress revealed that ~60% of genes regulated by B4 RAF kinases overlap with those controlled by subclass I SnRK2, establishing these MAPKKKs as upstream activators in osmotic stress signaling (Soma *et al.*, 2020).

Unlike B2, B3, and B4 RAF-like MAPKKKs that activate stress responses, a subset of C RAF-like MAPKKKs (RAF22/36) negatively regulates ABA signaling (Box 1A) (Kamiyama *et al.*, 2021a). Reverse genetics and biochemical analyses identified RAF36 and its close paralog, RAF22, as direct substrates of subclass III SnRK2s. The RAF36 knockout mutant affects post-germination seedling growth,

Box 1. Key recent developments in RAF-like MAPKKKs integrating ABA and auxin signaling in plant stress responses and growth



- Takahashi *et al.* (2020) identified a group of functionally redundant B3 RAF-like MAPKKKs that activate subclass III SnRK2s in response to ABA and osmotic stress (A).
- Lin *et al.* (2020) reported that B2, B3, and B4 RAF-like kinases play an essential role in early osmotic stress and ABA signaling by activating class I and III SnRK2s in *Arabidopsis thaliana* (A).
- Soma *et al.* (2020) identified three B4 RAF-like MAPKKKs as upstream regulators of subclass I ABA-unresponsive SnRK2 kinases. These RAF kinases interact with and regulate SnRK2s at processing bodies under osmotic stress conditions, thereby modulating the osmotic stress-responsive transcriptome (A).
- Lin *et al* (2021) highlighted the collaborative roles of B2 and B3 RAFs in initiating and amplifying SnRK2 activation within the ABA signaling pathway, underscoring their importance in plant stress responses (A).
- Kamiyama *et al.* (2021a) found that a group C RAF-like kinase in Arabidopsis negatively regulates ABA responses through interaction with SnRK2s. SnRK2s phosphorylate the C RAF kinases, and ABA enhances their degradation. These findings suggest that C RAF kinase suppresses ABA responses under normal conditions while ABA promotes C RAF kinase degradation, enhancing ABA signaling (A).
- Soma *et al.* (2023) discovered that B2 RAFs constantly activate SnRK2s when ABA binds to receptors, while B3 RAFs activate SnRK2s independently of ABA under severe stress (A).
- Kuhn *et al.* (2024) elucidated that B4 RAF-like protein kinases are key mediators of rapid auxin signaling. Phosphoproteomic analyses revealed that this signaling cascade triggers rapid phosphorylation events across a conserved subset of proteins, notably hyperphosphorylation of B4 RAF-like kinases and SnRK2s. Through genetic analyses with B4 RAF-like kinase null mutants, they showed that B4 RAF-like kinases are essential components that transduce auxin perception into rapid cellular responses, revealing an ancient signaling module conserved throughout the green lineage (B). The figure was created in BioRender. Yoon, G. (2025) https://BioRender.com/v08g296.

including cotyledon greening, suggesting that RAF36 normally suppresses ABA responses. However, ABA-activated SnRK2s phosphorylate RAF36, and presumably RFA22, triggering their degradation in response to ABA. This degradation enhances ABA responses, allowing plants to better adapt to adverse environments (Kamiyama *et al.*, 2021a).

The interplay between B RAF and C RAF kinases in SnRK2s regulation exemplifies evolutionary subfunctionalization in hormone signaling networks. While maintaining overlapping functions in the control of SnRK2s, these RAFs have evolved distinct regulatory roles, enabling responses to different hormonal cues such as ABA and auxin and environmental cues. Their preservation across plant genomes suggests essential, non-redundant roles in cellular signaling networks, allowing integration of diverse hormonal signals through specific RAF–SnRK2 modules based on environmental and developmental context. Key questions remain about how cells integrate competing signals from different RAF–SnRK2 modules, what determines their regulatory hierarchy, and how hormone signaling dynamics influence this regulation, the answers to which will illuminate plant stress responses and adaptation mechanisms.

RAF-like kinases in salinity and drought stress responses via the ethylene-dependent pathway

The gaseous plant hormone ethylene is one of the primary regulators that modulate plant stress responses, in addition to its diverse roles in growth and development (Bleecker and Kende, 2000; Alonso and Ecker, 2001). Plants perceive ethylene signals via endoplasmic reticulum (ER)-localized receptors, which relay signals downstream by directly interacting with Constitutive Triple Response 1 (CTR1), a RAF-like MAPKKK tethered at the ER. Originally identified in Arabidopsis as a recessive mutant displaying constitutive ethylene responses, CTR1 (also known as RAF1) belongs to the B3 RAF-like MAPKKK family and shares high sequence homology with five paralogs (RAF2/3/4/5/6), including Enhanced Disease Resistance 1 (EDR1) (RAF2) involved in disease resistance (Kieber *et al.*, 1993; Jonak *et al.*, 2002; Wawrzynska *et al.*, 2008). The ancestral CTR1-like protein found in the bryophyte *P. patens* regulates both ethylene and ABA signaling, but modern CTR1 proteins have specialized in ethylene response regulation through direct interaction with ethylene receptors (Yasumura *et al.*, 2015).

In the absence of ethylene, ethylene receptors activate CTR1, which then phosphorylates Ethylene-Insensitive 2 (EIN2), a positive regulator of the ethylene signaling pathway (Kieber *et al.*, 1993; Alonso *et al.*, 1999; Binder, 2020). This phosphorylation triggers EIN2 degradation via the 26S proteasome, suppressing ethylene signaling. In contrast, when ethylene binds to its receptors, the receptors and CTR1 are inactivated, releasing EIN2 from inhibition. This leads to proteolytic cleavage of EIN2 at the C-terminus via unknown proteases (Ju *et al.*, 2012; Qiao *et al.*, 2012; Wen *et al.*, 2012). The cleaved EIN2 C-terminal fragment then moves to the nucleus or P-bodies, where it indirectly activates the transcription factor EIN3 and its paralog EIN3-like (EIL) to regulate stress-responsive gene expression (Fig. 1) (Li *et al.*, 2015; Merchante *et al.*, 2015).

In addition to its established role at the ER as a negative regulator of the ethylene signaling pathway, recent studies have revealed the more complex role of CTR1 through diverse subcellular localizations. This spatial distribution proves critical for plant responses to stresses such as drought and salinity (Chien *et al.*, 2023; Li *et al.*, 2023; Park *et al.*, 2023). Park *et al.* (2023) demonstrated that along with the C-terminus of EIN2, CTR1 also translocates into the nucleus upon perception of ethylene at the ER (Fig. 1). This ER-to-nucleus movement occurs independently of its kinase activity and probably involves displacement of its N-terminus, which normally anchors CTR1 to the ER through receptor interaction. Once in the nucleus, CTR1 directly interacts with EIN3-binding F-Box proteins (EBFs), the RING-type E3 ligases responsible for EIN3 degradation. This CTR1–EBF interaction enhances EIN3 protein stability, leading to amplified ethylene responses in plants (Fig. 1). The nuclear role of CTR1 in promoting ethylene responses contrasts with its established negative regulatory function at the ER, where it modulates EIN2 activity. This implies that nuclear CTR1 plays a direct role in fine-tuning ethylene responses, distinct from its cytoplasmic role where its inactivation upon ethylene perception promotes EIN2 nuclear translocation. The dual functionality of CTR1—acting as a negative regulator at the ER and a positive modulator in the nucleus—points to a sophisticated regulatory mechanism that allows plants to precisely control their ethylene responses under stress conditions, highlighting the spatiotemporal control of plant hormone signaling components.

Further evidence for the role of the RAF-like kinase CTR1 in osmotic stress came from a recent study demonstrating direct interaction between CTR1 and Salt Overly Sensitive 2 (SOS2), a Ser/Thr kinase playing a key role in the SOS pathway (Li *et al.*, 2023) (Fig. 1). The SOS pathway is central to plant salinity tolerance, involving a calcium-binding protein SOS3, SOS2, and a plasma membrane Na^+/H^+ antiporter SOS1. Under high salinity, SOS3 senses elevated cytosolic calcium levels and activates SOS2, forming a complex that then stimulates SOS1 to promote Na^+ efflux and enhance salt tolerance (Ali *et al.*, 2023). Li *et al.* (2023) demonstrated that SOS2 phosphorylates and inactivates CTR1, thereby enhancing ethylene responses under salt stress. This finding aligns with the positive role of nuclear CTR1 in managing salinity stress independent of its kinase activity, suggesting complex regulation of CTR1 function under salt stress (Li *et al.*, 2023; Park *et al.*, 2023). Supporting this, Quan *et al.* (2017), showed synergistic effects of SOS2 and EIN3 on Arabidopsis salt tolerance. SOS2 phosphorylates EIN3 at Ser325 *in vitro* and that mutation of this site reduces EIN3 transcriptional activity and fails to rescue the salt hypersensitivity of *ein3* mutants. Collectively, these findings suggest that CTR1 functions as a regulatory hub connecting the salt response and ethylene signaling pathways.

The discovery of the subcellular localization-dependent functions of CTR1/RAF1 reveals a novel paradigm in RAF-like kinase signaling. Unlike canonical MAPK cascades, CTR1 operates through direct substrate phosphorylation and interactions in distinct cellular compartments, as shown by its dual role in phosphorylating EIN2 at the ER and modulating EIN3 stability in the nucleus through

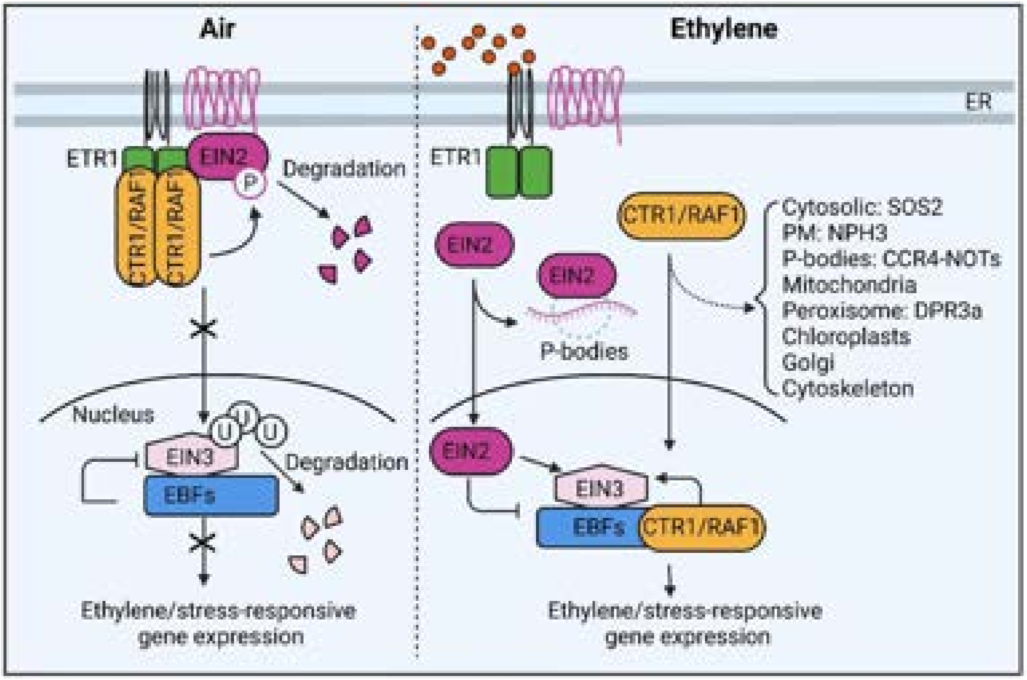


Fig. 1. Model of ethylene signaling and CTR1/RAF1 functions in the pathway. In the absence of ethylene, ETR1-activated CTR1 phosphorylates EIN2 directly, triggering its degradation and suppressing ethylene signaling. Simultaneously in the nucleus, EIN3 undergoes degradation through EBF1/2-mediated ubiquitination. Upon ethylene perception, both receptor function and CTR1 kinase activity are inhibited, enabling EIN2 cleavage at its C-terminus. This cleaved C-terminal fragment localizes to either processing bodies (P-bodies) or the nucleus, where it leads to EIN3 activation. At the ER, CTR1, like EIN2, translocates into the nucleus where it stabilizes EIN3 via direct interaction with EBFs. Beyond its role in ethylene signaling, CTR1 can also associate with various cellular compartments via protein–protein interactions, participating in diverse signaling pathways. Created in BioRender. Yoon, G. (2025) https://BioRender.com/v08g296.

kinase-independent interactions with EBFs. This spatial segregation of functions demonstrates how plants achieve signaling specificity through localization-dependent regulation beyond classical MAPK cascades, suggesting that similar mechanisms may exist for other RAF-like kinases in stress responses.

RAF-like kinases in auxin-mediated fast, conserved responses in plants

RAF-like MAPKKs regulate not only stress responses through ABA and ethylene signaling but also rapid auxin responses across diverse plant species (Kuhn *et al.*, 2024). Auxin influences a wide range of plant growth and developmental processes through both slow and rapid auxin response pathways (Mockaitis and Estelle, 2008; Yu *et al.*, 2022). The slow auxin responses, which regulate cellular growth, division, and differentiation, are primarily mediated by Transport Inhibitor Response 1 and Auxin-Signaling F-box (TIR1/AFB1) receptors (Prigge *et al.*, 2020; Qi *et al.*, 2022; Chen *et al.*, 2023; Dubey *et al.*, 2023). However, the mechanisms underlying fast auxin responses mediated by ABP1–Transmembrane Kinase 1 (TMK1) remain poorly understood. These rapid responses including membrane polarization, cytoplasmic streaming, and ion flux remain largely enigmatic (Etherton, 1970; Barbez *et al.*, 2017; Narasimhan *et al.*, 2021; Serre *et al.*, 2021; Friml *et al.*, 2022).

A recent study identified B4 RAF-like protein kinases as key mediators of rapid auxin responses across the green lineage, from land plants to algae (Kuhn *et al.*, 2024) (Box 1B). Phosphoproteomic analyses across five diverse species revealed conserved auxin-induced changes in phosphorylation and GTPase activity, suggesting an ancient origin of this signaling module. In Arabidopsis, auxin treatment triggered hyperphosphorylation of 26 kinases, including the blue light receptor PHOT1, two B RAFs (B3 RAF5 and B4 RAF20), and SnRK2s (subclass III), known substrates of B RAF MAPKKKs (Box 1B) (Kuhn *et al.*, 2024). Experiments with OK^{130} (B4 RAF nulls; RAF16/40/24/18/20/35/42) in Arabidopsis and a *Marchantia* ortholog null mutant (Mppraf^{KO}) showed that RAF dysfunction impaired auxin sensitivity in root growth inhibition and cytoplasmic streaming (Koide *et al.*, 2019; Lin *et al.*, 2020; Kuhn *et al.*, 2024). Notably, the auxin-induced hyperphosphorylation of B4 RAFs depends mainly on the ABP1–TMK1 pathway rather than the AFB1 receptor (Box 1B) (Kuhn *et al.*, 2024). These findings highlight RAF kinases as central, evolutionarily conserved mediators of rapid auxin responses in the green lineage.

While these findings establish RAF kinases as evolutionarily conserved mediators of rapid auxin signaling, their role extends beyond single hormone pathways to integrate multiple signaling networks. The convergence of auxin and ABA signaling through SnRK2s reveals an additional layer of hormone response integration in plants. Beyond the ABP1–TMK1 pathway discussed above, RAF kinases also phosphorylate SnRK2s, suggesting a direct molecular link between rapid auxin responses and ABA signaling networks. The hyperphosphorylation of SnRK2s in response to both hormones positions these kinases as critical integration hubs for environmental and developmental signals. During stress conditions, this dual regulation enables precise adjustment of growth patterns through coordinated hormone responses. Such sophisticated integration through RAF–SnRK2 modules represents an evolutionary innovation that helps plants maintain growth plasticity under varying environmental conditions. Deciphering the specific phosphorylation patterns and downstream targets of this signaling node could reveal new strategies for enhancing crop resilience without sacrificing productivity.

Spatiotemporal integration of RAF-like kinases in stress and hormonal signaling

The subcellular localization of signaling components is crucial for their function in growth and stress responses. Most MAPKKKs are primarily cytoplasmic with some associated with membranes or translocating to various organelles. The B3 RAF-like MAPKKK CTR1/RAF1 was traditionally viewed as an ER-bound inhibitor of ethylene signaling through complex formation with ethylene receptors (Clark *et al.*, 1998; Gao *et al.*, 2003). However, upon ethylene signaling activation, CTR1 translocates to the nucleus, where it positively regulates ethylene responses by stabilizing EIN3 transcription factors, enhancing tolerance to osmotic stresses such as drought and salinity (Park *et al.*, 2023). Beyond the ER and nucleus, proteomic studies using proximity labeling revealed that CTR1 potentially localizes to multiple subcellular locations, including the cytoskeleton, mitochondria, chloroplasts, Golgi apparatus, and P-bodies, through proximal interactions with proteins in these organelles (Fig. 1) (Chien *et al.*, 2023). The stress-induced translocation of RAF-like MAPKKKs is not only limited to CTR1. For instance, the Arabidopsis B4 RAF kinase RAF18 moves from the cytoplasm to P-bodies under osmotic stress, regulating stress-responsive mRNAs through interaction with subclass I SnRK2s (Soma *et al.*, 2020). Similarly, two other B4 RAF-like MAPKKKs (RAF20/24) and a *P. paten*s B RAF involved in rapid auxin responses exhibit broad distribution between the cytoplasm and distinct cytoplasmic puncta (Kuhn *et al.*, 2024). These spatial and temporal patterns of RAF kinases establish discrete cellular domains for integrating environmental and developmental signals, enabling tissue-specific adjustment of growth patterns through coordinated hormone responses. This dynamic distribution pattern suggests that RAF-like MAPKKKs may employ diverse mechanisms, including translocation, oligomerization, or phase separation, to facilitate diverse subcellular localizations.

Beyond their dynamic subcellular distribution patterns, RAF-like MAPKKKs also exhibit unique functional characteristics that distinguish them from conventional MAPK cascades**.** The established relationships between RAF-like MAPKKKs and SnRK2s suggest a unique evolutionary path, distinct from their traditional role in MAPKK–MAPK cascades. Unlike MEKK-like MAPKKKs, RAF-like MAPKKKs lack direct biochemical evidence for activating downstream MAPKK–MAPK modules. Instead, they appear to regulate cellular processes through alternative downstream targets. This non-canonical function is well demonstrated by the RAF–SnRK2 modules and two well-characterized B3 RAF kinases, CTR1 and EDR1. CTR1 modulates ethylene responses by interacting with EIN2 and EBFs at the ER and the nucleus, respectively (Ju *et al.*, 2012; Park *et al.*, 2023) (Box 1B), while also interacting with SOS2 in the cytoplasm during osmotic stress conditions (Li *et al.*, 2023) (Box 1B). Recent proximal labeling studies have revealed additional CTR1 targets involved in blue light signaling, organelle division, and mRNA metabolism, including Non-Phototropic Hypocotyl 3, Dynamin-Related Protein 3A-like, and the Carbon Catabolite Repression 4–Negative On TATA-less (CCR4–NOT) complex (Fig. 1) (Chien *et al.*, 2023). Similarly, EDR1/RAF2 regulates plant immune and stress responses through direct interaction with KEEP ON GOING, ENHANCED DISEASE SUSCEPTIBILITY 1, and PHYTOALEXIN DEFICIENT 4 (Gu and Innes, 2011; Serrano *et al.*, 2014; Neubauer *et al.*, 2020). Similar to RAF-like MAPKKKs, ZIK-like MAPKKKs have not been confirmed to phosphorylate MAPKKs (Chardin *et al.*, 2017). Together, these findings reveal RAF-like MAPKKKs as versatile regulators that directly phosphorylate substrates, act as scaffold proteins, and influence protein stability, challenging the conventional view of linear MAPK cascades.

These diverse functions of RAF kinases are likely to be orchestrated through multiple regulatory mechanisms, including their spatiotemporal expression and structural features. A key determinant of their functional specificity is protein–protein interactions, which are mediated by specific motifs including intrinsically disordered regions (IDRs). Intriguingly, many RAF-like kinases contain IDRs that enable diverse protein interactions and phase separation events (Wang, 2024). These IDRs enhance functional specificity through multiple mechanisms: they undergo induced folding upon substrate binding to increase interaction specificity, serve as targets of post-translation modifications to modulate interaction dynamics, and promote liquid–liquid phase separation to form biomolecular condensates such as stress granules or P-bodies (Kurotani *et al.*, 2014; Chakrabarti and Chakravarty, 2022; Holehouse and Kragelund, 2024). Subcellular compartmentalization adds another layer of regulation to RAF kinase substrate specificity, as illustrated by CTR1/RAF1. This spatial organization, together with compartment-specific interaction partners, defines the biochemical behavior and substrate specificity of individual RAF kinases.

Conclusions and future prospects

The diversity of RAF-like kinase signaling mechanisms reveals a complex evolutionary picture. MAPK cascades are a common feature throughout eukaryotes, yet RAF-like kinases exhibit more sophisticated variations in their distribution and function. Mammalian RAF kinases demonstrate this complexity by operating through two distinct mechanisms: the classical MAPK cascade (RAF→MEK→ERK) and direct phosphorylation of targets such as ROKα, MST2, and ASK1 (Niault *et al.*, 2009; Romano *et al.*, 2014; Huang *et al.*, 2024). In contrast, their plant counterparts appear to favor a simpler approach, primarily acting through direct substrate phosphorylation, raising intriguing questions about their evolutionary paths.

The functional diversification of RAF-like kinases is evident in their specialized regulatory roles. For example, B2 and B3 RAF members primarily mediate ABA-dependent osmotic stress responses, whereas B4 RAFs coordinate ABA-independent stress pathways while retaining sensitivity to osmotic stresses. Further specialization is seen in CTR1/RAF1, which modulates ethylene signaling, and EDR1/RAF2, which coordinates pathogen defense pathways. The C-group RAFs function as negative ABA regulators, while B4 RAFs remain to be fully characterized. Although B2 RAFs are well-established ABA regulators, the primary upstream activators of B3 and B4 variants during stress responses are still unclear. Current evidence suggests that both RAFs integrate diverse environmental signals and hormones rather than depending on a single hormone pathway.

The functional distribution of RAF-like MAPKKKs probably emerged through gene duplication events, where duplicated RAF-like kinase genes acquired specialized roles while preserving certain ancestral functions. This balance between specialized and shared functions enables plants to fine-tune stress response networks through maintained functional redundancy. Elucidating the molecular mechanisms driving this specialization requires comprehensive phylogenetic analyses and structural studies across diverse plant species, with particular attention to early diverging groups. Such research would illuminate both the origins and ancestral signaling mechanisms of RAF-like kinases in plants, providing crucial insights into their evolutionary trajectory across eukaryotic lineages.

Author contributions

Both HLP and GMY contributed to the writing of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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