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The CBL-CIPK network is involved in the physiological crosstalk between plant growth and stress adaptation

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Abstract

Plants have evolved to deal with different stresses during plant growth, relying on complex interactions or crosstalk between multiple signalling pathways in plant cells. In this sophisticated regulatory network, Ca^{2+} transients in the cytosol ($[Ca^{2+}]_{cyt}$) act as major physiological signals to initiate appropriate responses. The CALCINEURIN B-LIKE PROTEIN (CBL)-CBL-INTERACTING PROTEIN KINASE (CIPK) network relays physiological signals characterised by $[Ca^{2+}]_{cyt}$ transients during plant development and in response to environmental changes. Many studies are aimed at elucidating the role of the CBL-CIPK network in plant growth and stress responses. This review discusses the involvement of the CBL-CIPK pathways in two levels of crosstalk between plant development and stress adaptation: direct crosstalk through interaction with regulatory proteins, and indirect crosstalk through adaptation of correlated physiological processes that affect both plant development and stress responses. This review thus provides novel insights into the physiological roles of the CBL-CIPK network in plant growth and stress adaptation.

KEYWORDS
abiotic stress, biotic stress, CBL, CIPK, crosstalk, plant development, stress adaptation

1 | INTRODUCTION

During their lifecycle, plants are continuously exposed to a plethora of abiotic and biotic stresses. Being sessile, plants need to develop defence mechanisms to adapt to these stresses and not only survive but also grow and reproduce (Shanker & Venkateswarlu, 2011). Stress responses typically come at the cost of growth and yield in crops (Huot et al., 2014; Karasov et al., 2017). More and more studies show that the negative correlations between growth and defence are partially caused by crosstalk between sophisticated molecular regulatory pathways (Karasov et al., 2017). These regulatory networks connect plant growth and stress tolerance, enabling a rapid adaptation of plant metabolism to mitigate the effects of the constantly changing environmental conditions. Campos et al. (2016) have shown that it is in principle possible to uncouple pathways involved in the crosstalk between plant growth and defence, thus considerably reducing the trade-off of growth for defence. Knowledge of the integration of the molecular regulatory networks related

to stress response and plant development may, therefore, be crucial for yield improvement of crops in a changing environment.

Ca^{2+} is a vital secondary messenger for both plant development and early stress responses (Hepler, 2005; Kudla et al., 2010; Reddy et al., 2011; Steinhorst & Kudla, 2014). Specific $[Ca^{2+}]_{cyt}$ transients are triggered in the cytoplasm when plants are exposed to environmental stimuli (Haley et al., 1995; Okazaki et al., 1996; Price et al., 1994). The $[Ca^{2+}]_{cyt}$ transients are recognised by calcium sensors that relay the Ca^{2+} signals to downstream response pathways. These sensors include CALCINEURIN B-LIKE PROTEINs (CBLs) that bind Ca^{2+} through EF-hand motifs (Sánchez-Barrena et al., 2013). The naming "EF-hand" describes its helix-loop-helix structure that resembles a right hand with the thumb (F-helix) and the forefinger (E-helix) extended at 90° (Kretsinger & Nockolds, 1973). Upon binding Ca^{2+}, the molecular surface properties of the CBL proteins are modified (Sánchez-Barrena et al., 2013). This facilitates the interaction of CBL with the NAF domain of a CBL-INTERACTING PROTEIN KINASE (CIPK) (Sánchez-Barrena et al., 2013). The Ca^{2+}-induced interaction of CBL with CIPK uncovers a phosphorylation site located in the activation loop of the CIPK protein (Sánchez-Barrena et al., 2013). The exposed activation loop of the CIPK protein is assumed to be phosphorylated and activated by as yet unknown upstream kinases (Sanyal et al., 2015). The activated CIPK protein typically phosphorylates and thus regulates multiple downstream targets (Sánchez-Barrena et al., 2013), including ion channels and enzymes (Drerup et al., 2013; Nunez-Ramirez et al., 2012). An exception for this general activation mechanism of CBL-CIPK was reported for potassium channel AKT2, which was shown to be activated by the interaction with but not the phosphorylation by the CBL-CIPK complex (Held et al., 2011).

The CBL-CIPK modules, as part of environmental signalling pathways, are well-conserved across the plant kingdom. With the availability of the whole-genome sequence data of many plant species, the evolution of the CBL-CIPK network (from green algae to flowering plants) can be studied (Edel & Kudla, 2015; Kleist et al., 2014; Weinl & Kudla, 2009). A single CBL-CIPK pair is present in green algae *Chlorella spec*., suggesting that the prototype of this signalling module may date back to single-cell plant ancestors (Edel & Kudla, 2015; Tang et al., 2020a; Weinl & Kudla, 2009). The number of CBL and CIPK members expanded from lower plants to higher plants by gene duplication and whole-genome duplication events, indicating that the CBL-CIPK network plays an essential role in plant adaptation to a changing environment (Kleist et al., 2014).

Numerous expression and functional studies have demonstrated the broad involvement of the CBL-CIPK network in plant development and stress response. Recently, more and more studies indicate that the CBL-CIPK network connects plant development and stress adaptation at two levels. At the first level, the CBL-CIPK complexes directly interact with regulatory components involved in plant development and stress response. At the second level, the physiological adaptations regulated by CBL-CIPK pathways in response to stress affect plant growth and development, and vice versa.

2 | LEVEL 1: THE CBL-CIPK MODULES CONNECT PLANT DEVELOPMENT AND STRESS ADAPTATION BY INTERACTING WITH REGULATORY PROTEINS

The SALT OVERLY SENSITIVE (SOS) pathway is the first CBL-CIPK pathway that was uncovered in *Arabidopsis thaliana* and it is a well-studied example of crosstalk through direct interaction of components involved in plant development and stress adaptation. Under saline conditions, the induced Ca^{2+} transient promotes the phosphorylation activity of the AtCBL4-AtCIPK24 complex which activates the plasma membrane (PM)-localised Na^+/H^+ antiporter AtSOS1, leading to Na^+ extrusion from the roots into the root environment and Na^+ loading into the xylem for long-distance transport (Nunez-Ramirez et al., 2012; Qiu et al., 2002; Shi et al., 2002). The SOS pathway is conserved in other plant species such as rice, poplar, and apple (Mao et al., 2021). Of all AtCIPKs, AtCIPK8 resembles AtCIPK24 the most (60.9% AA pairwise identity) (Kleist et al., 2014), and it has been shown that the AtCBL10-AtCIPK8 complex also functions in the SOS pathway, positively regulating AtSOS1 activity to extrude excess Na^+ from cells in the shoot under saline conditions (Yin et al., 2019).

Under normal conditions, the SOS pathway is suppressed by several proteins including 14-3-3, BRASSINOSTEROID INSENSITIVE 2 (BIN2), and GIGANTEA (GI). These proteins interact with AtCIPK24 and repress its kinase activity (Kim et al., 2013; Li et al., 2020; Yang et al., 2019; Zhou et al., 2014), thus working as molecular switches of the SOS pathway during dynamic growth and floral transition under varying soil salinity (Figure 1) (Kim et al., 2013; Park et al., 2013).

Plant 14-3-3 proteins, like their highly conserved homologs in mammals, function by binding to phosphorylated target proteins and thus modulating their function (Denison et al., 2011). Under normal conditions, AtCIPK11 phosphorylates Ser^{294} of AtCIPK24 and this promotes the interaction of AtCIPK24 with 14-3-3 proteins, which blocks AtCIPK24 activity and shuts down the SOS pathway. Under saline conditions, the interaction between 14-3-3 and AtCIPK11 is stimulated, releasing AtCIPK24 (Yang et al., 2019). The released AtCIPK24 then interacts with Ca^{2+}-binding AtCBL4, thus activating downstream AtSOS1 to regulate cellular Na^+ homeostasis (Nunez-Ramirez et al., 2012; Qiu et al., 2002; Shi et al., 2002).

The SOS pathway is also linked to plant growth through BIN2, a GLYCOGEN SYNTHASE KINASE 3-like kinase. BIN2 phosphorylates and represses the transcription factors BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR (BES1) and thus retards plant growth under salt stress (Li et al., 2020). In the rapid recovery phase after salt stress, however, AtCBL4 and AtCBL10 interact with BIN2 and recruit it to the PM. BIN2 then phosphorylates AtCIPK24 and inhibits the kinase activity of AtCIPK24, effectively switching off the AtSOS1-induced Na^+ efflux. At the same time, the repression of the transcriptional activity of BZR1/BES1 by BIN2 is released, and growth is restored (Li et al., 2020).

AtCIPK24 also interacts with GI, a nuclear protein that functions to promote the elicitation of photoperiod-dependent

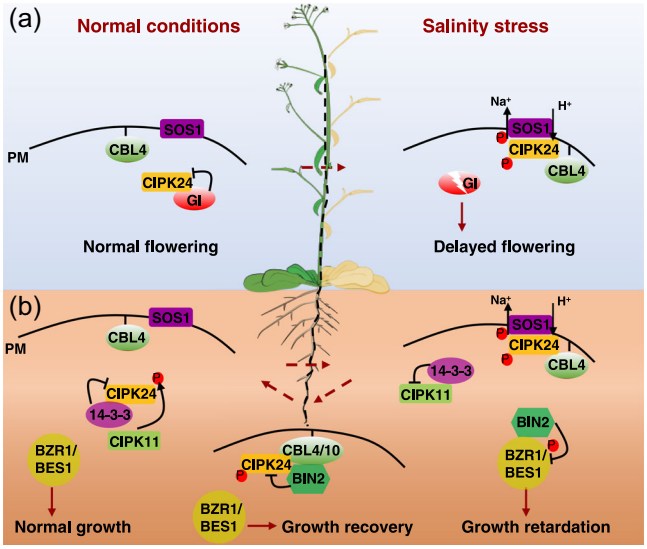

FIGURE 1 SOS pathway is involved in the crosstalk between plant growth and salt stress response. (a) The interaction between GI and SOS pathway (Kim et al., 2013; Park et al., 2013). Under normal conditions (left side of the figure), GI interacts with AtCIPK24 and inhibits its activity, therefore AtCIPK24 does not interact with AtCBL4 and cannot phosphorylate AtSOS1. Salt stress (right side of the figure) induces the degradation of GI, releasing AtCIPK24. AtCIPK24 then interacts with PM-localised Ca^{2+}-binding AtCBL4 and activates AtSOS1. At the same time, salt-dependent degradation of GI retards or abolishes the initiation of flowering. (b) A proposed working model of the role of the SOS pathway in the dynamic growth of plants when switching between normal and salinity conditions (Li et al., 2020). Under normal conditions, AtCIPK11 phosphorylates AtCIPK24 and promotes its interaction with 14-3-3, inhibiting AtCIPK24 activity. BZR1/BES1 stimulates plant growth. Under salt stress, AtCBL4/AtCBL10 recruits AtCIPK24 to the PM to activate AtSOS1. BIN2 is disassociated from the PM to phosphorylate and inhibit BZR1/BES1, thus retarding plant growth. In the recovery phase, AtCBL4/AtCBL10 recruits BIN2 to the PM to inhibit the activity of phosphorylated AtCIPK24. This reduces Na^+ efflux and releases BZR1/BES1 to stimulate plant growth. Black pointed and blunt arrowheads indicate activating and inhibiting interactions, respectively. PM, plasma membrane; SOS, salt overly sensitive [Color figure can be viewed at wileyonlinelibrary.com]

flowering (Kim et al., 2013; Oliverio et al., 2007; Park et al., 1999). Under normal conditions, GI interacts with AtCIPK24 and prevents AtCIPK24 from phosphorylating AtSOS1. Under saline conditions, however, the AtCIPK24-GI complex disintegrates and GI is then degraded, leading to delayed flowering (Figure 1). At the same time, the SOS pathway is activated to regulate Na^+ homeostasis and Na^+ extrusion (Kim et al., 2013; Park et al., 2013). This is consistent with the finding that *GI*-overexpressing *Arabidopsis* plants exhibited a salt-sensitive phenotype and *gi* mutants had increased salt tolerance (Kim et al., 2013).

One CIPK can be regulated by different proteins (AtCIPK24 by AtCIPK11, GI, 14-3-3, and BIN2). These interactions of CIPKs with other proteins provide the molecular basis for the first level of direct crosstalk between plant growth and stress response.

3 | LEVEL 2: THE CBL-CIPK MODULES CONNECT PLANT DEVELOPMENT AND STRESS ADAPTATION BY MODULATING CORRELATED PHYSIOLOGICAL PROCESSES

To adapt to stress conditions, new biochemical pathways are activated and others that are characteristic of the non-stressed state are repressed in plants (Bohnert & Sheveleva, 1998). Protective metabolic adaptation of plants includes changes in phytohormones, ion homeostasis, and the concentration of metabolites such as sugars, sugar alcohols, low-complexity carbohydrates, tertiary amines, sulfonium compounds, and amino acids (Bohnert & Sheveleva, 1998; Wolters & Jurgens, 2009). These metabolic adaptations of plants to stress alter their physiology (Bohnert & Sheveleva, 1998), and affect plant development at the same time. Therefore, the CBL-CIPK network connects plant growth and stress adaptations by modulating correlated physiological processes, including mediating phytohormone signals (Guo et al., 2002; Pandey, 2005, Pandey et al., 2008; Song et al., 2005) and regulating the homeostasis of sugar and various ions (Dong et al., 2021; Ma et al., 2019a, 2019b).

3.1 | Phytohormone-related plant development and stress adaptation connected by CBL-CIPK network

Phytohormones are central regulators of plant growth and stress response. ABSCISIC ACID (ABA) stimulates dormancy during seed maturation (Sano & Marion-Poll, 2021), and also accumulates under osmotic stress to promote stomatal closure and reduce water loss (Luan, 2002). It has been reported that the CBL-CIPK network affects ABA-regulated plant development and abiotic stress response.

ABSCISIC ACID REPRESSOR 1 (ABR1) is a repressor of the ABA response because disruption of *ABR1* leads to a hypersensitive response to ABA in seed germination (Pandey, 2005). The AtCBL9-AtCIPK3 complex was found to activate ABR1 in the nucleus, suppressing ABA-dependent dormancy and stimulating seed germination (Figure 2a) (Pandey, 2005; Pandey et al., 2008; Sanyal et al., 2017b). When treated with ABA or under osmotic stress, *At-cbl9*, *At-cipk3*, *At-cbl9cipk3*, and *At-abr1* mutants all showed reduced germination frequency but also impaired early seedling development (Pandey, 2005; Pandey et al., 2008). The germination impairment of these mutants under osmotic stress could be caused by the osmotic stress-induced increase in ABA levels. In addition, *At-abr1* adult seedlings exhibited smaller stomatal aperture after ABA treatment, which could be the reason why they had less water loss and showed a tolerant phenotype under drought stress (Sanyal et al., 2017b).

ETHYLENE RESPONSE FACTOR 7 (AtERF7) also represses the ABA response of plants. The RNAi-mediated suppression of *AtERF7* led to increased ABA sensitivity during seed germination, while the *AtERF7*-overexpressing lines had less stomatal closure and higher sensitivity to drought stress than wild-type seedlings (Song et al., 2005). AtCIPK15 has been shown to phosphorylate AtERF7 and this may further repress

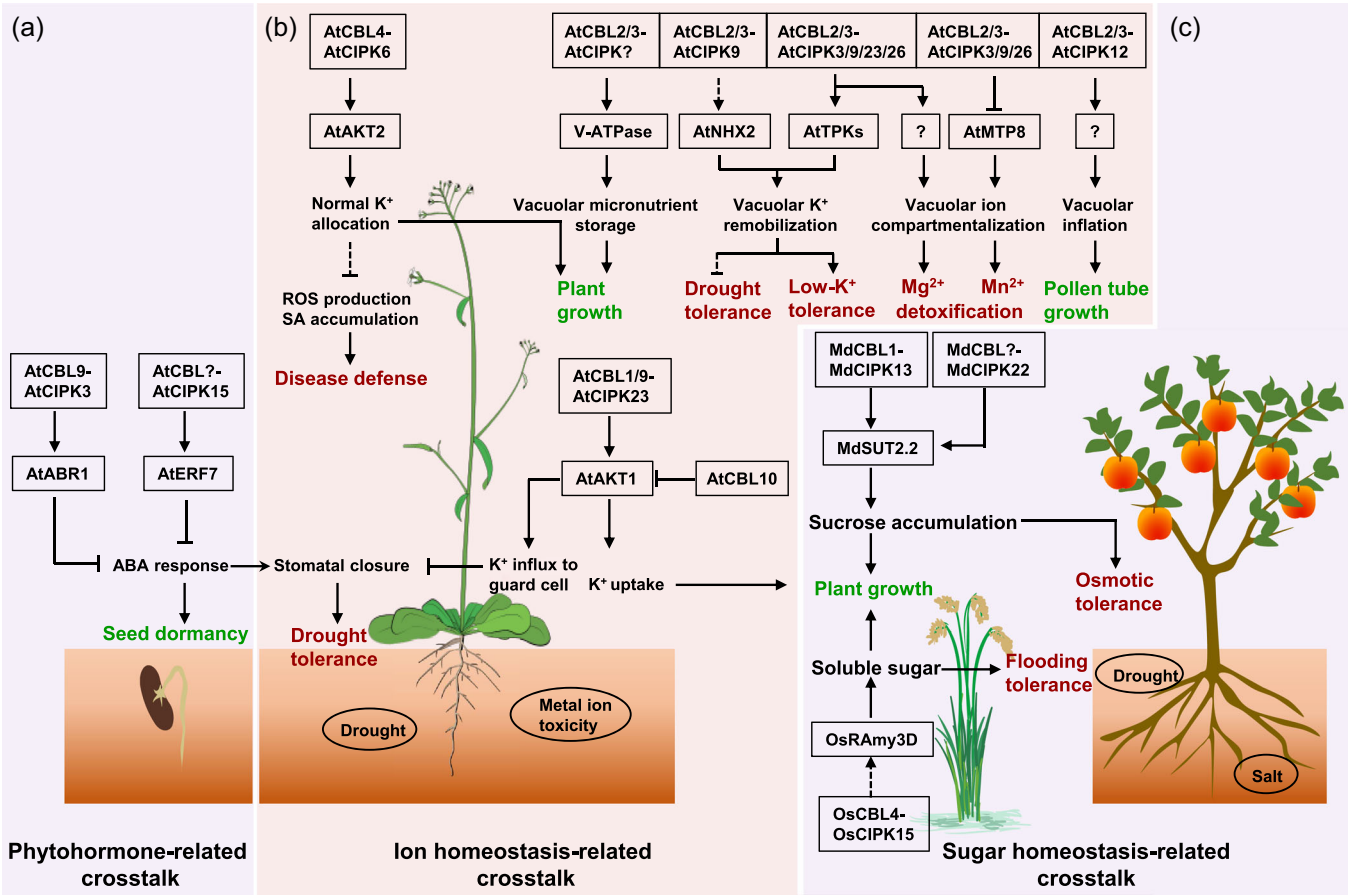

FIGURE 2 Representative CBL-CIPK pathways involved in the crosstalk between plant development and stress adaptation. (a–c) CBL-CIPK pathways involved in phytohormone-related (a), ion homeostasis-related (b), and sucrose homeostasis-related (c) crosstalk between plant development and stress adaptation. Black pointed and blunt arrowheads indicate activating and inhibitory processes, respectively. The dotted lines indicate proposed processes. The green colour in the text indicates plant development processes, while the red colour in the text indicates plant stress responses. CBL, calcineurin B-like protein; CIPK, CBL-interacting protein kinase [Color figure can be viewed at wileyonlinelibrary.com]

the ABA response (Figure 2a) (Song et al., 2005). *At-cipk15* RNAi lines exhibited a series of ABA hypersensitivity phenotypes, including delayed seed germination, arrested early seedling development, and dramatically reduced stomatal pores and leaf transpiration (Guo et al., 2002; Song et al., 2005). The reduced water loss of the *At-cipk15* RNAi lines may also enhance its drought tolerance.

3.2 | Ion homeostasis-related plant development and stress adaptation connected by CBL-CIPK network

Bioinformatic analysis showed that the algal CIPKs are likely to be the closest orthologs of AtCIPK24, a key regulator of Na^+ homeostasis when interacting with AtCBL4 (Edel & Kudla, 2015; Kleist et al., 2014). All CIPKs from *Physcomitrium patens* and *Selaginella moellendorffii* are orthologs of AtCIPK24 and AtCIPK23 (Weinl & Kudla, 2009). AtCIPK23 is a key regulator of K^+ homeostasis when interacting with AtCBL1 and AtCBL9 (Xu et al., 2006). In addition, algal CBLs feature the dual-lipid modification motif (or relicts of this motif) MGCXXS/T that presents in *Arabidopsis* AtCBL1/AtCBL9/AtCBL4 for PM localisation (Kleist et al., 2014). This indicates that the ancestral role of the CBL-CIPK network is linked to the regulation of ion transport across the PM (Edel & Kudla, 2015; Kleist et al., 2014; Weinl & Kudla, 2009).

From lower to higher plants, the number and complexity of CBLs and CIPKs increase (Kleist et al., 2014; Weinl & Kudla, 2009), but the physiological functions of most CBL-CIPK pathways are still linked to ion homeostasis regulation (Dong et al., 2021). As the regulation of ion transport and homeostasis is essential in growth, development, as well as adaptation to stress conditions, it is not unexpected that the adaptation of ion transport regulated by CBL-CIPK pathways in stress response affects plant growth and development, and vice versa.

3.2.1 | Ion homeostasis regulated by the CBL1/CBL9-CIPK23 pathway

Potassium (K^+) is a vital nutrient that affects many biochemical and physiological processes in plants. K^+ not only regulates plant growth and metabolism, but also contributes greatly to plant defence against various stresses (Wang et al., 2013).

The CBL1/CBL9-CIPK23-AKT1 pathway connects K^+ homeostasis to plant growth, which is conserved in several plant species. CBL1 and CBL9 are two CBL proteins that have strong sequence similarity and have functional redundancy in ion uptake and transport. In *Arabidopsis*, AtCBL1/AtCBL9-AtCIPK23 complexes are important positive regulators of root K^+ uptake through phosphorylation of K^+ transport systems HAK5 or AKT1 under different external K^+ concentrations (Aleman et al., 2011; Ragel et al., 2015; Xu et al., 2006). Leaf development and growth of *At-cbl1cbl9* double mutant, *At-cipk23* mutant, and *At-akt1* mutant were extremely impaired under K^+ deficiency (Figure 2b) (Xu et al., 2006). Rice (*Oryza sativa*) *OsCIPK23* RNAi lines and *Os-akt1* mutant showed similar K^+-deficient symptoms under low K^+ conditions, including reduced K^+ content, leaf brown spots, and growth inhibition (Li et al., 2014). In addition, *OsCIPK23* RNAi lines had irregularly shaped pollen grains without any starch granules, which is typical for rice sterile pollen and may be related to impaired K^+ loading ability into the pollen (Yang et al., 2008). In grapevine (*Vitis vinifera*), the VvCBL1-VvCIPK4 complex (homologs of AtCBL1-AtCIPK23 complex) can activate the AKT1 ortholog VvK1.2, potentially mediating whole plant K^+ transport and translocation in fleshy fruit development (Cuellar et al., 2010, 2013). A dysfunctional SlCBL1/SlCBL9-SlCIPK23-LKT1 (AKT1 ortholog) pathway in tomato (*Solanum lycopersicum*) did not affect growth, but the tomato *lkt1* mutant displayed sensitivity to excessive Mg^{2+} with chlorosis at leaf margins (Amo et al., 2021). This may be linked to impaired K^+ accumulation in the cytosol (Amo et al., 2021): Mg^{2+} is preferentially stored in vacuoles (Shaul, 2002), and impaired K^+ loading may affect the osmotic adjustment that compensates for the increased osmotic pressure in the vacuole caused by the accumulation of metal ions (Wang et al., 2013).

The K^+ nutritional status of plants affects their drought tolerance by regulating stomatal aperture, osmotic adjustment, and reactive oxygen species (ROS) scavenging (Wang et al., 2013). AtCBL1/AtCBL9-AtCIPK23 complexes activate AKT1 and increase K^+ influx from leaf apoplast to guard cells, leading to stomatal opening and increased leaf transpiration (Figure 2b) (Cheong et al., 2007; Nieves-Cordones et al., 2012). Mutants with a disabled AtCBL1/AtCBL9-AtCIPK23-AKT1 pathway had decreased stomatal opening, which contributes to drought tolerance (Cheong et al., 2007; Nieves-Cordones et al., 2012). AtCBL10 was reported to be an inhibitor of the AtCBL1/AtCBL9-AtCIPK23-AKT1 pathway, by competing with AtCIPK23 for binding to AKT1 (Ren et al., 2013). The AKT1-mediated inward K^+ currents are inhibited in the *At-cbl10* mutant (Ren et al., 2013), which could be a reason why the *At-cbl10* mutant displayed a drought-tolerant phenotype (Kang & Nam, 2016). In addition, the NO_3^- transporter CHLORATE RESISTANT 1 (CHL1) is also activated by the AtCBL1/AtCBL9-AtCIPK23 complexes, switching on high-affinity nitrate transport under low external NO_3^- concentrations (Ho et al., 2009; Leran et al., 2015; Liu & Tsay, 2003). The decreased nitrate accumulation in the guard cells may also contribute to stomatal closure and reduced transpiration rates in the mutants with a disabled AtCBL1/AtCBL9-AtCIPK23-AKT1 pathway. Indeed, there is evidence for enhanced drought tolerance in *At-chl1* mutants, with reduced stomatal opening and transpiration rates (Guo et al., 2003). It should be noted that the behaviour of mutants with an impaired CBL1/CBL9-CIPK23 pathway under drought stress may vary between plant species. For instance, the *OsCIPK23* RNAi lines with reduced *OsCIPK23* expression were more sensitive to drought stress compared to wild-type plants rather than more tolerant, which is different from the phenotype of *At-cipk23* (Cheong et al., 2007; Yang et al., 2008). Possibly, the K^+ deficiency of *OsCIPK23* RNAi lines negatively affected their ability for osmotic adjustment or ROS detoxification.

3.2.2 | Ion homeostasis regulated by the CBL4-CIPK6 pathway

Regulation of K^+ homeostasis also affects disease infection by inducing the synthesis of molecules like ROS and phytohormones (Amtmann et al., 2008; Ashley et al., 2006). AKT2 is a phloem-expressed weakly rectifying K^+ channel that is active in a wide membrane potential range and can mediate both influx and efflux of K^+ (Lacombe et al., 2000). The AtCBL4-AtCIPK6 complex regulates K^+ allocation by modulating the activity of AKT2 for both K^+ loading in the source and unloading in the sink (Figure 2b) (Held et al., 2011; Lacombe et al., 2000). *At-cipk6* mutants had enhanced disease resistance with increased ROS production and SA accumulation compared to wild-type plants after the infection of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Sardar et al., 2017). Increased ROS production and SA accumulation in *At-cipk6* mutants may be caused by disruption of the AtCBL4-AtCIPK6 regulated K^+ transport and allocation.

Different from the general activation mechanism of CBL-CIPK, the activation of AKT2 is dependent on the interaction but not the phosphorylation by the AtCBL4-AtCIPK6 complex. AKT2 is activated when it is translocated from the endoplasmic reticulum to PM and this translocation depends on AtCBL4 (Held et al., 2011). Aberrant K^+ transport and allocation in the *At-cbl4*, *At-cipk6*, and *At-akt2* mutants were accompanied by reduced rosette size and delayed flowering phenotypes (Held et al., 2011). The *At-cipk6* mutants also had a remarkably decreased shoot-to-root and root basipetal auxin transport, resulting in fused cotyledons, swollen hypocotyls, and compromised lateral root growth (Tripathi et al., 2009).

3.2.3 | Ion homeostasis regulated by CBL2/CBL3-CIPK3/CIPK9/CIPK23/CIPK26 pathway

Vacuolar ion homeostasis affects ion compartmentalisation, vacuole inflation, and polar growth, which are required for multiple physiological processes in plant development and stress adaptation, including metal ionic tolerance, stomatal movement, pollen germination, and polarised pollen tube growth.

Tonoplast-localised AtCBL2 and AtCBL3, another two partly functionally redundant CBLs, work as crucial regulators of vacuolar

ion homeostasis. Ion compartmentalisation of *At-cbl2cbl3* double mutant was affected, thus its abilities of ionic tolerance (to excessive Ca^{2+}, Fe^{3+}, Cu^{2+}, K^+, and Zn^{3+}) and micronutrient storage (of Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}) were compromised (Tang et al., 2012). It was suggested that AtCBL2 and AtCBL3 positively regulate the activity of vacuolar H^+-ATPase (V-ATPase) (Figure 2b) (Tang et al., 2012), which is the major proton pump in establishing and maintaining an electrochemical proton gradient across the tonoplast to drive ion transport (Lüttge et al., 2001). However, a more recent study showed that AtCBL2 and AtCBL3 regulate Mg^{2+} compartmentalisation together with multiple AtCIPKs (AtCIPK3/AtCIPK9/AtCIPK23/AtCIPK26) through a V-ATPase-independent pathway (Figure 2b) (Tang et al., 2015), indicating that AtCBL2 and AtCBL3 regulate ion homeostasis through several different downstream pathways. Recently, more pathways under the control of AtCBL2 and AtCBL3 were discovered: AtCBL2/AtCBL3-AtCIPK3/AtCIPK9/AtCIPK26 negatively regulates the transport activity of vacuolar Mn^{2+} transporter AtMTP8, inhibiting Mn^{2+} transport from the cytoplasm to the vacuole (Ju et al., 2022).

In addition, AtCBL2/AtCBL3-AtCIPK3/AtCIPK9/AtCIPK23/AtCIPK26 complexes were found to activate the vacuolar two-pore K^+ channels AtTPKs for K^+ remobilisation to the cytoplasm under low-K^+ stress (Tang et al., 2020b), explaining why the *At-cbl2cbl3* double mutants retain more K^+ even though they showed more sensitive to K^+ deficiency than wild-type (Tang et al., 2012, 2020b). The mutation of *AtCIPK9* alone leads to a discernible vacuolar K^+ conductance reduction and sensitivity to K^+ deficiency, suggesting that *AtCIPK9* might be a dominating *AtCIPK* in the regulation of vacuolar K^+ homeostasis (Tang et al., 2020b). The importance of *AtCIPK9* for vacuolar K^+ homeostasis is exemplified by the study of Song et al. (2018), in which the *At-cipk9* mutant had reduced vacuolar K^+ influx through K^+/H^+ antiporter AtNHX1 and AtNHX2 but not defective vacuolar K^+ release through AtTPK1, resulting in ABA-hypersensitive stomatal closure and thus enhanced drought tolerance (Song et al., 2018).

Interestingly, Tang et al., (2012; 2020b) reported on contrasting phenotypes of the *At-cbl2cbl3* double mutant under low-K^+ stress. In their earlier study, they reported *At-cbl2cbl3* double mutant was more tolerant than WT under low-K^+ (50, 100, and 200 μM) conditions (Tang et al., 2012), while they recently observed the *At-cbl2cbl3* double mutant was more sensitive than WT under low-K^+ (10, 100 μM) conditions (Tang et al., 2020b). The different NH_4^+ concentrations used in root media (20 mM in the previous study and 1 mM in the recent study) were suggested as the cause for the contradicting phenotypes (Tang et al., 2020b), as NH_4^+ toxicity might mask the true phenotype of mutants under low-K^+ stress (Shi et al., 2020). It is well known that NH_4^+ toxicity enhances the plant sensitivity to low-K^+ stress while K^+ relieves plant sensitivity to NH_4^+ toxicity (Shi et al., 2020), while the *At-cbl2cbl3* double mutant exhibited a more tolerant phenotype than the wild-type under the more severe combined stress conditions (low-K^+ and high NH_4^+), suggesting that AtCBL2 and AtCBL3 might also be involved in the regulation of NH_4^+ homeostasis. For another study, however, *At-cbl3* and *At-cipk9* mutant were reported to be more tolerant than wild-type under K^+ deficiency even when NH_4^+ concentration in root media was as low as 1.25 mM (Liu et al., 2013), indicating the possible role of CBL-CIPK network in regulating K^+ and NH_4^+ homeostasis is worth exploring in more detail.

Both *At-cbl2cbl3* double mutants and *At-cipk3/9/23/26* quadruple mutants were severely inhibited in growth (Tang et al., 2012; Tang et al., 2020b), and the *At-cbl2cbl3* double mutant also displayed leaf tip necrosis, underdeveloped roots, shorter siliques, fewer seeds, and defective embryonic development (Eckert et al., 2014; Tang et al., 2012), which are symptoms that may be related to micronutrient deficiency (e.g., Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}) or abnormal vacuolar K^+ homeostasis (Song et al., 2018; Tang et al., 2020b). In addition, *At-cbl2*, *At-cbl3*, *At-cbl2cbl3*, and *At-cipk12* mutant lines all showed dramatically reduced pollen germination rates and altered tonoplast morphologies in pollen tubes. This suggests that AtCBL2, AtCBL3, and AtCIPK12 play a role in maintaining vacuole inflation and polar growth, which is required for essential processes for reproduction like pollen germination, polarised tube growth, and vacuolar morphology (Steinhorst et al., 2015). This reproduction barrier in *At-cbl2*, *At-cbl3*, *At-cbl2cbl3*, and *At-cipk12* mutants therefore may also be resulting from abnormal vacuolar ion homeostasis in pollen.

3.3 | Sugar homeostasis-related plant development and stress adaptation connected by CBL-CIPK network

Sugars are the main carbon- and energy-containing molecules needed for plant growth and development, and they are substrates for cell wall construction as well as cellular solutes for osmotic balance (Griffiths et al., 2016). Several CBL-CIPK complexes were shown to play roles in the phosphorylation of sugar transporters and regulation of sugar homeostasis. For example, cotton (*Gossypium hirsutum*) GhCBL2-GhCIPK6 was reported to promote sugar (glucose) accumulation via interaction with the tonoplast sugar transporter TONOPLAST SUGAR TRANSPORTER 2 (TST2) (Deng et al., 2020). The *GhCIPK6*-overexpressing lines had more energy for growth under starvation treatment (a 10-day dark treatment followed by a 3-day recovery period) compared to the wild-type that had a withering phenotype (Deng et al., 2020).

In apple (*Malus domestica*), the overexpression of *SUCROSE TRANSPORTER 2.2* (*MdSUT2.2*) improves sucrose accumulation and tolerance to salt stress and drought stress (Ma et al., 2019a, 2019b). The enhanced salt and drought tolerance of *MdSUT2.2*-overexpressing apple seedlings were shown to be dependent on the phosphorylation of MdSUT2.2 by MdCIPK13 and MdCIPK22, respectively (Figure 2c) (Ma et al., 2019a, 2019b). In addition, the overexpression of *MdCIPK13* and *MdCIPK22* improved sugar accumulation, as well as the salt stress and drought stress tolerance of transgenic apple seedlings, respectively (Ma et al., 2019a, 2019b). This may be related to osmotic adjustment through increased sucrose accumulation in these

MdCIPK13- and *MdCIPK22*- overexpressing apple seedlings in response to osmotic stress (Figure 2c) (Ma et al., 2019a, 2019b). Sugar content also influences fruit flavour, colour, and other quality traits in apple. Transient overexpression of *MdCBL1* and *MdCIPK13* increased the sugar content of apple fruit, whereas transient suppression of these genes decreased the sugar content of apple fruit (Jiang et al., 2021). These are clear examples of the CBL-CIPK pathway regulating the response to salt and drought stress (through osmotic adjustment) while also affecting apple growth and fruit quality.

Sugar accumulation during cold acclimation may contribute to the stabilisation of membranes, protecting plants from cold-induced cellular damage (Thomashow, 1999). In *Arabidopsis*, AtCBL1 was predicted to regulate sugar metabolism by interacting with AtCIPK7 to target sucrose synthase (Chikano et al., 2001; Huang et al., 2011). The *At-cbl1* mutant was sensitive to low temperature and exhibited much more severe wilting symptoms than the wild-type (Huang et al., 2011). The wilting symptoms of *At-cbl1* under cold stress may be related to cold-induced cellular damage caused by deficient sugar accumulation (Huang et al., 2011). More research needs to be done to confirm the involvement of AtCBL1-AtCIPK7 in sugar metabolism during cold stress.

Soluble sugars are substrates for glycolysis, producing pyruvate that enables alcohol dehydrogenase (Adh) to reoxidize NADH to produce ATP under hypoxia conditions like flooding stress (Zabalza et al., 2009). Rice *OsCIPK15* is necessary for the expression of α-amylase genes (*αAmy3*, *αAmy7*, and *αAmy8*) and *Adh2* and the production of α-amylase and Adh under flooding stress, which are required for sugar production and fermentation (Lee et al., 2009). Energy deficiency of the *Os-cipk15* mutant leads to sensitivity to flooding stress at both the germination stage and seedling growth stage (Lee et al., 2009). Another further study showed that OsCBL4 is the partner of OsCIPK15 in regulating the expression of *αAmy3* (also named *RAmy3D*) (Ho et al., 2017) (Figure 2c). Knowledge about the role of the CBL-CIPK network in sugar homeostasis is as yet limited, but it deserves more attention considering the important physiological roles of sugars in plant development and stress adaptation.

4 | PROSPECTS: BREEDING STRATEGIES TARGETING COMPONENTS OF THE CBL-CIPK NETWORK

Developing crops with improved stress tolerance is a highly sustainable strategy to meet the food demand of an increasing human population. Genome-editing based on the CRISPR-Cas9 technique is regarded as a new plant breeding technology that can help achieve this goal. However, few genes have been successfully used for improving stress tolerance without yield penalties under normal conditions in field trials. One of the reasons is that the molecular regulatory networks of plant growth and stress tolerance are complicated and their crosstalk is poorly understood. The CBL-CIPK network is involved in both stress response and plant development, and components of this network can be a target for plant breeding. More detailed knowledge about how the CBL-CIPK network connects with other pathways can help us to better target and modulate the components in the CBL-CIPK network for breeding varieties with optimised stress tolerance and a minimal yield penalty.

The information presented in this review indicates that constitutive overexpression or complete knockout of *CBL* and *CIPK* genes is likely to interrupt other signalling pathways and thus may lead to unwanted side effects. For example, the *At-cipk6* mutant exhibits enhanced disease resistance (Sardar et al., 2017), but the growth and development of the *At-cipk6* mutant are disrupted, showing reduced rosette size and later flowering (Held et al., 2011). In this case, an alternative approach would be to modify the gene expression or protein function rather than knocking out or constitutively over-expressing the gene.

4.1 | Modify gene expression through promoter-editing

Gene promoter provides a rich source of targets for genome-editing, contributing to dissecting tradeoff effects in crop breeding (Song et al., 2022). The DNA double-strand break in the target locus generated through CRISPR-Cas9 genome editing can be repaired through the high-fidelity homology-directed repair, stimulating precise sequence alteration. Based on this, promoter fragment insertion and swap can be generated when a DNA repair template is exogenously supplied (Shi et al., 2017). This would enable fine-tuning the gene expression to a specific tissue, under a specific stress condition, or at a specific developmental stage to reduce the trade-off between disease defence and growth.

The promoter of a gene can be replaced with a tissue-specific driving promoter. For instance, with a phloem-specific expression, *AtCIPK6* is still able to regulate the activity of phloem-expressed AKT2 to ensure the K^+ nutrition for plant growth, and the loss of *AtCIPK6* in leaves will increase resistance to the pathogen infection. This effectively uncouples the tradeoff between plant growth and disease response connected by AtCIPK6. In some cases, *cis*-regulatory elements in a promoter can be deleted through CRISPR-Cas9 to alter the tissue-specific gene expression by avoiding the inhibition of tissue-specific expressed transcription factors, thus uncoupling linkage traits (Song et al., 2022).

In addition, stress-inducible promoters can also be used so that the gene is highly expressed only under specific stress without yield loss under normal conditions (Pino et al., 2007). For example, AtCBL2 and AtCBL3 positively regulate plant tolerance to Mg^{2+} toxicity (Tang et al., 2015), but their overexpression interferes with pollen germination and tube growth (Steinhorst et al., 2015). In this case, an Mg^{2+} toxicity-inducible promoter with a higher driving activity under Mg^{2+} toxicity stress will help to resolve this tradeoff. Another alternative approach would be to insert Mg^{2+} toxicity-responsive *cis*-regulatory elements in the native promoter of *AtCBL2* and *AtCBL3*.

4.2 | Fine-tune protein function through gene-editing

Generating single-nucleotide polymorphism (SNP) is able to modify a protein function, which could be another way to reduce the growth-defence tradeoff. A reference case is about RESISTANCE OF RICE TO DISEASES1 (ROD1), which is a Ca^{2+} sensor that can scavenge ROS by stimulating catalase activity and thus suppress rice immunity (Gao et al., 2021). ROD1 inhibits ROS production under normal conditions to avoid the growth penalty, while it was degraded upon pathogen infection to ensure proper plant defence. Therefore, constitutively activated immunity in *rod1* mutants increased the pathogen resistance but compromised plant growth, leading to reduced grain yield (Gao et al., 2021). The researchers found that a natural SNP mutation of *ROD1* attenuated its ROS scavenging ability, contributing to improved basal defence without yield penalty (Gao et al., 2021). In this theory, inducing targeted mutations can potentially attenuate the protein kinase activity of AtCIPK6, retaining basal defence and plant growth at the same time.

4.3 | Modify the expression of splice variants

Alternative splicing (AS) of genes is a key regulatory mechanism for plants to adapt to the environment (Rigo et al., 2019). The analysis of genome sequencing results has shown that AS-induced splice variants widely exist in *CBL* and *CIPK* families (Hu et al., 2015; Kanwar et al., 2014; Kolukisaoglu et al., 2004). It was reported that splice variants of *AtCIPK3* showed transcript and interaction preference (Sanyal et al., 2017a), which may lead to different expression patterns, protein kinase activity, and downstream targets. The splice variants of some *CBL*s (e.g., *AtCBL4*) may have different protein localisation and interactors because of the difference in the N-terminus and EF domain of their encoded proteins (Figure S1). AS of CBL-CIPK components could play roles in the crosstalk between growth and defence, thus overexpressing or knocking out a specific splice variant could be another possibility to tune the gene function and relieve the growth-defence tradeoff.

In summary, to take optimal advantage of these powerful strategies to target the CBL-CIPK network, advanced knowledge about the roles of the CBL-CIPK network and its crosstalk with other pathways is essential. With more research and deeper insight into the physiological roles of splice variants of these genes, the potential for the moderation of CBL-CIPK gene functions may be exploited in the future for breeding climate-proof crop varieties.

AUTHOR CONTRIBUTIONS

Jingjing Mao: conceived the original writing plans. **Jingjing Mao, C. Gerard van der Linden, and Qian Wang**: completed the writing. **Haobao Liu, Qian Wang, and C. Gerard van der Linden**: co-supervised the writing. **Zhijie Mo, Guang Yuan, and Haiying Xiang**: provided ideas and joined discussion. **Richard G. F. Visser and Yuling Bai**: helped with manuscript revision. **Haobao Liu and Qian Wang**: agree to serve as the authors responsible for contact and ensure communication. All authors contributed to the article and approved the submitted version.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings will be available in [repository name] at [DOI/URL] following an embargo from the date of publication to allow for commercialisation of research findings.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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