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REVIEW ARTICLE

What can CDPK provide as a bioengineering tool?

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Abstract
Plants are the basis of ecology and biodiversity. Plant resources are of paramount importance to humanity. The development of human civilisations has introduced anthropogenic factors that aggravate abiotic and biotic stress effects. Some advanced techniques, such as molecular tools and omics, can provide a comprehensive understanding of the underlying mechanisms of stress tolerance and provide effective tools for artificial adaptation. External negative signals stimulate intracellular calcium fluctuations. Ca^{2+} messengers, such as calcium-dependent protein kinases (CDPKs or CPKs), trigger MAP kinase phosphorylation cascades regulated by certain stress hormones and are triggered by biotic (ET, JA, and SA) and abiotic (ABA) stresses. In addition to activating regulatory mechanisms, CDPKs exert independent effects on transcription factors and enzymes by bypassing the direct pathway. In this review, we focused on the role of CDPKs in the plant defense system and the promise of their use as activators of the defense system. To fully evaluate the promise of CDPKs, we conducted a (1) detailed analysis of their role in the plant defense system, (2) analysis of their responsiveness to stress, (3) analysis of their overexpression effects, and (4) analysis of intradomain modifications. An in-depth analysis of the available information revealed that CDPKs play a pivotal role in the regulation of plant defense; the expression of certain isoforms is stress-specific and essential for resistance. We suggest that intradomain modifications may be effective tools for increasing plant stress tolerance.

Keywords Ca^{2+} messengers · Calcium-dependent protein kinases · Plant defense system · Secondary metabolites · Resistance to biotic or abiotic stress

Abbreviations

ABA	Abscisic acid
CDPKs (CPKs)	Calcium-dependent protein kinases
ET	Ethylene
ETI	Effector-triggered immunity
ISR	Rhizobacterium-induced system resistance
JA	Jasmonic acid
LAR	Local acquired resistance
NPR	Nonexpressor of pathogen-related gene
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
PRRs	Pattern-recognition receptors
PTI	Pattern-triggered immunity
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SMs	Secondary metabolites
TS	Thermal stress

Introduction

External stressors affect the evolutionary development of plants, becoming increasingly important under the influence of human activities that have altered habitats and added many new stressors, including the pollution of ecosystems. Human factors significantly aggravate the effects of natural, biotic and abiotic stress factors on cultivated and wild plants. Biotic factors include plant pathogens, whereas

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abiotic factors include temperature extremes, drought or dampness, and other physical factors. The interaction of two or more stressors exacerbates unfavourable effects on plant growth and development. Advances in molecular biology and plant biotechnology can increase the stress tolerance of crop plants to meet increasing food demands in a cost-effective manner (Cramer et al. 2011). Calcium ion currents play a central role in the transmission of external signals in plant cells. Calcium-dependent enzymes, including calcium-dependent protein kinases (CDPKs or CPKs), are the main transmitters (Resentini et al. 2021). CDPKs are currently the subject of intense study by many global research teams (Yip Delormel and Boudsocq 2019).

Reactive oxygen species (ROS) and calcium are common secondary messengers involved in the signalling of biotic and abiotic stress. The initial phases of plant responses to biotic and abiotic stressors (Fraire-Velazquez et al. 2011). Pathogen exposure, osmotic stress, and temperature stress are the only aggressive impacts and environmental factors that cause the levels of cytosolic Ca^{2+} in plant cells to rise (Takahashi et al. 2011). To explain the independence of signal transmission in the downwards direction and the uniqueness of molecular and adaptive reactions in plant systems, the response is based on mutual regulation, which guarantees the character of the individual "signature" of calcium and ROS (Mazars et al. 2010). The mechanism of transmission of the external signal via fluctuations in $[Ca^{2+}]_{cyt}$ is schematically shown in Fig. 1. Ca^{2+} messengers, including CDPK, trigger cascades of reactions through phosphorylation. The first step involves MAPK (mitogen-activated protein kinase) kinase activity. They regulate many processes, such as gene expression, mitosis, differentiation, proliferation, resistance, and PCD (Wu et al. 2020). Furthermore, according to the Ca^{2+} signature, Ca^{2+} conversion is initiated and regulated by a certain stress hormone, depending on the effect. Generally, phytohormones that control reactions to negative cues fall into one of two categories: regulated responses to abiotic stress (ABA) or regulated responses to biotic stress (ET, JA, and SA). Phytohormone-mediated biotic defence networks rely on the pathogenicity and type

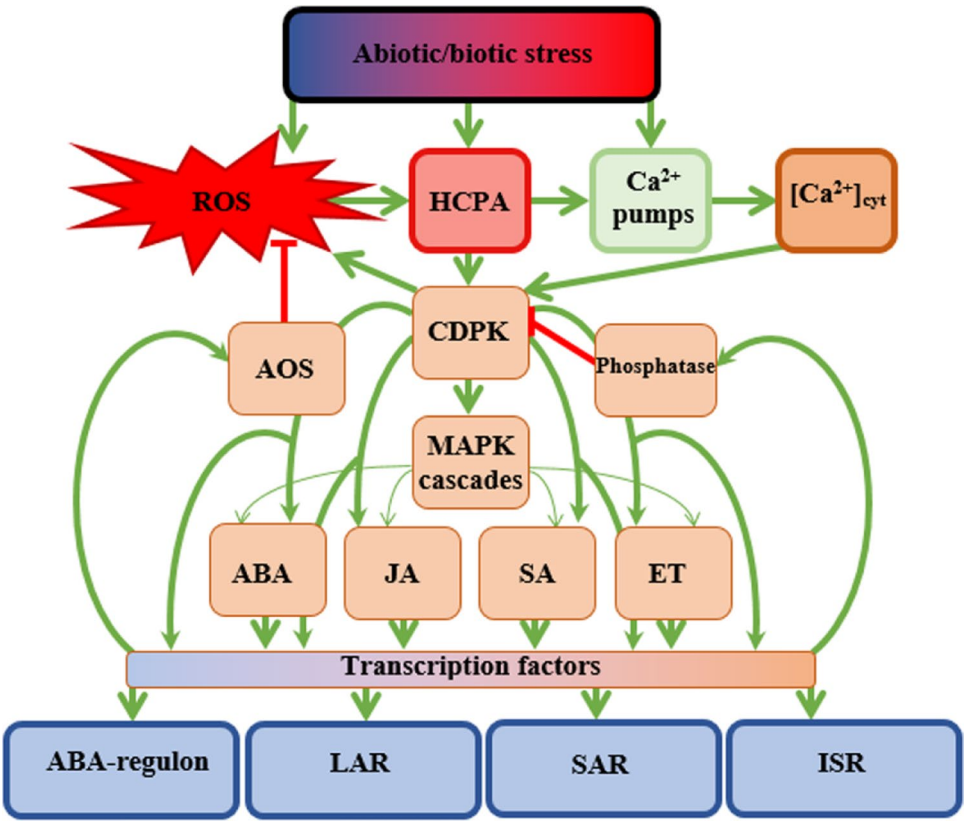

Fig. 1 Simplified scheme of the interaction of plant signal systems under the influence of biotic and abiotic stress factors (according to Fraire-Velazquez et al. 2011; Dvořák et al. 2021). External signals are transmitted via fluctuations in the $[Ca^{2+}]_{cyt}$ concentration. In addition, through the H_2O_2 receptor and HPCA1, ROS regulate Ca^{2+} channels. Ca^{2+} messengers, including CDPK, trigger MAPK kinase phosphorylation cascades. These responses are regulated by certain stress hormones—biotic (ET, JA, and SA) and abiotic (ABA) stresses. The activated pathway regulates the expression or activity of transcription factors (TFs). TFs trigger the corresponding resistance processes (activating pathways are indicated by green arrows). The independent action of CDPK on finite transcription factors bypassing the direct pathway is indicated by additional green arrows. Feedback loops, represented by phosphatases and the antioxidant system, regulate hypersensitive reactions, as indicated by red blocking lines (Erickson et al. 2022)

of the pathogen. SA is essential for both the development of systemic acquired resistance and the initiation of defense responses against biotrophic and hemibiotrophic infections. On the other hand, JA and ET are typically linked to defense against herbivores and necrotrophic infections. The most researched stress response hormone, ABA, has a role in how the body reacts to cold stress, osmotic stress, and drought (Gupta et al. 2017). Furthermore, the antioxidant system and phosphatases are crucial components of feedback loops (Fig. 1, red blocking lines) that control hypersensitivity reactions (Robert-Seilaniantz et al. 2011). Calcium signal decoders, CDPK, play a key role in regulating this complex defense system.

Recently, genome editing technologies have been rapidly developed to solve the problem of stable food supplies. Selecting the optimal genetic target is a critical step in obtaining plants with desired properties. The main requirements for a target gene are (1) a pronounced effect on the given parameters; (2) the presence of several positive effects; (3) the absence of negative effects; (4) the level of complexity of editorial manipulation; and (5) the transparency of the molecular mechanism of action. At first glance, the CDPK appears to be an excellent target for the genetic manipulation of plants. However, most studies have focused on the native forms of genes. In this case, it appears that the observed effects depend on calcium levels and stress factors. Moreover, the response of native CDPK to stress is also dependent on calcium levels, which are modulated by other signalling and feedback systems under different stress conditions. As a result, the overexpression of CDPK did not produce the expected results. In this review, we summarise and schematise the available experimental data on CDPK as a tool for plant bioengineering. As such, CDPKs could solve problems such as regulating the biosynthesis of medicinal secondary metabolites or increasing plant resistance to biotic or abiotic stress. To assess the capabilities of CDPKs, we characterised their role in the regulation of the plant defense system in detail. We also summarise the experimental data on the use of native and subdomain-modified CDPKs.

Calcium signalling system

In plants, the early reaction to stress takes several seconds or minutes. In different cell compartments, this time depends on the intensity of treatment. The first reaction of a plant cell to stress involves rapid changes in the concentrations of Ca^{2+}, H^+, K^+, NO_3, and Cl^- ions, which depend on their movement through membranes, triggering response processes (Costa et al. 2018; Resentini et al. 2021). Among these ions, calcium (Ca^{2+}) plays a key role in many signalling pathways (Kudla et al. 2010). At rest in plant cells, the concentration of free Ca^{2+} in the cytosol ($[Ca^{2+}]_{cyt}$) is within 100–200 nM, but it may increase sharply in response to the perception of various stimuli (Ranf et al. 2008). The induced increase in $[Ca^{2+}]_{cyt}$ is defined as an analogue signal that, depending on the type and intensity of the stimulus, has a peculiar dynamic called the cytosolic "Ca^{2+} signature" (Kudla et al. 2010; Resentini et al. 2021).

Ca^{2+} signature and its "analogue nature"

Evolution has equipped plants with resistance to basic levels of abiotic and biotic treatments. Plants also have a complex mechanism of protection against strong stress factors, whether individually or in combination. An adequately mitigated effect on plant cells is possibly due to spatiotemporal fluctuations in intracellular ions. As an essential macronutrient, calcium has three functions in the cell: as a structural component of cell walls and membranes, as a counteraction for inorganic and organic anions in the vacuole, and as an intracellular second messenger. The concentration of cytosolic calcium ions ($[Ca^{2+}]_{cyt}$) coordinates responses to numerous developmental processes and environmental stimuli (White and Broadley 2003; Thor 2019). The manner in which Ca^{2+} ions move through membranes determines the analogue nature of the Ca^{2+} signature. In this respect, two things are important. First, how do plant cells generate specific Ca^{2+} signatures? Second, how are they strictly regulated? The formation of the cytosolic signature Ca^{2+} depends on the activity of Ca^{2+}-permeable channels, Ca^{2+} transporters, and Ca^{2+} buffers (Sanders et al. 2002; Resentini et al. 2021). The main source of cytosolic Ca^{2+} is the apoplast; the movement of Ca^{2+} through the plasma membrane plays a key role in the generation of cytosolic Ca^{2+} fluctuations. However, increasing amounts of data show that subcellular compartments are involved in the generation of specific Ca^{2+} signatures (Resentini et al. 2021; Hilleary et al. 2020). Compartments such as chloroplasts and the nucleus can also generate their own Ca^{2+} organellar signature (Resentini et al. 2021; Hilleary et al. 2020).

Thus, calcium is the most important secondary messenger that coordinates the physiological response of plants to external or internal signals. Various signals, including stress, cause a unique combination of intracellular calcium fluctuations (Resentini et al. 2021). In plants, four classes of Ca^{2+} sensor enzymes perform calcium signal decoding: calmodulins (CaM), CaM-like proteins (CML), calcineurin b-like proteins (CBL), and Ca^{2+}-dependent protein kinases (CDPKs or CPKs). Calcium signal decoders cause precise and adaptable responses by modulating gene expression and metabolism (Kudla et al. 2010; Resentini et al. 2021). The uniqueness of calcium-dependent protein kinases lies

in their versatility. CDPK can simultaneously bind directly to calcium, bypass any mediator, and activate target proteins via substrate-specific phosphorylation. The plant genome contains several dozen different isoforms of CDPK genes. CDPKs differ in parameters such as sensitivity to calcium, substrate specificity, and localisation. This variety ensures CDPK involvement in many cell processes (Yip Delormel and Boudsocq 2019). Thus, calcium-dependent protein kinases are the main transducers of the external negative signal, accelerating intracellular calcium and transferring this signal into the cellular response. CDPKs generate phospho-code-defined "logical gates", which dictate transcriptional reprogramming during protection (Erickson et al. 2022).

Synergy of Ca^{2+} with other signalling molecules

Plants have a wide range of adaptation strategies to biotic and abiotic stressors. These mechanisms include physiological, biochemical, and molecular reactions. Defense responses include processes such as the production of stress hormones and the activation of genes associated with stress. The mechanism of resistance to a combination of stressors is not well understood. The impact of multiple factors on plants is more negative than the other effects are (Zandalinas and Mittler 2022). The convergence of extreme environmental events caused by climate change, including temperature increases, unfavourable soil conditions, limited availability and quality of water, and the introduction of numerous anthropogenic pollutants, is a special and difficult task for plants (Lopez-Delacalle et al. 2020). The simultaneous effects of abiotic and biotic stresses modulate complex physiological and biochemical processes, influencing growth and survival through molecular pathways and hormonal regulation, which are crucial for acclimatisation to stress (Zandalinas and Mittler 2022).

In plants, Ca^{2+} and ROS are important and common signalling molecules in the early stages of reactions to abiotic and biotic stresses. Ca^{2+} and ROS levels rise rapidly in local tissue cells soon after attack by a pathogen or environmental stress. The levels of cytosolic Ca^{2+} in plant cells increase in response to various aggressive effects and environmental conditions, including pathogen exposure, osmotic stress, and temperature stress (Takahashi et al. 2011). For example, Ca^{2+} signals in plants are involved in a variety of intracellular signalling pathways after pest infestation: there is a sharp influx of Ca^{2+} followed by the activation of the Ca^{2+}-dependent transduction pathway, which includes interacting lower kinase networks (Arimura and Maffei 2010). Ca^{2+} is a universal transceiver of external signals that acts on the subcellular and spatiotemporal patterns of protein accumulation and interaction. The influx of Ca^{2+} via membrane channels or Ca^{2+} vectors causes specific spatial and temporal oscillations in the concentration $[Ca^{2+}]_{cyt}$. ROS are also important secondary messengers involved in the response to a variety of abiotic and biotic forms of stress. Oxidative stress occurs in response to biotic and abiotic stressors. Plants have a positive feedback mechanism involving NADPH oxidase, ROS, and Ca^{2+}. A decrease in ROS stimulates the flow of Ca^{2+} into the cytoplasm, and Ca^{2+} in turn activates NADPH oxidase and the generation of ROS (Fraire-Velazquez et al. 2011).

The Ca^{2+}-induced generation of ROS and the ROS-mediated regulation of Ca^{2+} channels depend on the pathosystem and the environment. Responses to some forms of stress (biotic or abiotic) may overlap or converge in a common signalling element, such as Ca^{2+} or ROS, or both, leading to similar subsequent events. Calcium and ROS are ubiquitous secondary messengers in abiotic and biotic stress signalling. The response is based on mutual regulation, which ensures the character of the individual "signature" of calcium and ROS (Mazars et al. 2010), which explains the independence of signal transmission in the downwards direction and the uniqueness of molecular and adaptive reactions in plant systems (Fraire-Velazquez et al. 2011). The mechanism is based on the modification of the oxidation–reduction potential of the H_2O_2 receptor on the cell surface, which is capable of transducing a signal from extracellular ROS-produced intracellular signal cascades (Wu et al. 2020). This receptor is the transmembrane enzyme HPCA1 (HYDROGEN-PEROXIDE-INDUCED Ca^{2+} INCREASES 1), which belongs to a family of leucine-rich repeat receptor kinases (LRR-RKs). The oxidation of two pairs of cysteine residues in its extracellular domain results in its autophosphorylation. This process results in an influx of Ca^{2+} via Ca^{2+} channels and subsequent stomatal closure (Wu et al. 2020). Furthermore, Ca^{2+} messengers, including CDPK, trigger cascades of reactions through phosphorylation. The first step involves MAPK kinase activity. Mitogen-activated protein kinases are a widely used family of highly conserved Ser/Thr kinases. They regulate many processes, such as gene expression, mitosis, differentiation, proliferation, resistance, and PCD (Wu et al. 2020).

MAPK cascades accelerate stress signals. Among the first three hierarchically arranged interacting MAPK kinases, which in turn are phosphorylated by MAPKK kinases (Colcombet and Hirt 2008), MAPK acts as the last component of the protein kinase cascade, and one of its main tasks is to transform the extracellular stimulus into a transcriptional response in the nucleus (Wurzinger et al. 2011). Furthermore, according to the Ca^{2+} signature, Ca^{2+} conversion is initiated and regulated by a certain stress hormone, depending on the effect. Typically, phytohormones that regulate the response to adverse signals are grouped into two types: regulated responses to biotic stress (ET, JA, and SA) and

regulated responses to abiotic stress (ABA). Biotic defence signalling networks mediated by phytohormones depend on the nature of the pathogenic agent and its pathogenicity. SA plays a central role in the activation of defensive reactions against biotrophic and hemibiotrophic pathogens, as well as the emergence of systemic acquired resistance. In contrast, JA and ET are usually associated with protection against necrotrophic pathogens and herbivores. ABA is the most studied stress response hormone; it is involved in the response to drought, osmotic stress, and cold stress (Gupta et al. 2017). In addition, phosphatases and the antioxidant system play important roles in feedback loops, which regulate hypersensitivity reactions (Robert-Seilaniantz et al. 2011).

The role of calcium and calcium-dependent protein kinases in stress responses

The role of calcium and CDPK in biotic stress responses

For optimal plant growth, strong regulation of the immune system is needed to prevent the unnecessary diversion of valuable resources. The molecular mechanism of biotic stress reception is still poorly understood. The immunity triggered by PTI and/or ETI is cross-linked with two-level potentials, which involve close interactions between signalling pathways and defense reactions. Two antagonistic processes, phosphorylation and dephosphorylation, regulate these processes. Phosphorylation at the early stage guarantees that the PRR's constituent parts identify the receptor complex. The next level of phosphorylation involves intracellular protein kinases/phosphatases in the "signal relay", namely, calcium-regulated kinases (including CDPKs). In addition to calcium-regulated kinases, mitogen-activated protein kinases (MAPKs) and various protein phosphatases regulate the conversion of hazard signals from surface PRRs to intracellular reactions (Erickson et al. 2022). Most research on the role of CDPK in response to biotic stress has been conducted on the model plant *Arabidopsis thaliana*. To simplify the perception and schematisation of the available information, it is necessary to mention the phylogenetic relationships of CDPK isoforms (Fig. 2). *Arabidopsis* has 34 isoforms of *CDPK* genes, as well as *CRK* genes close to *CDPK*. A phylogenetic tree was constructed with Mega 7 via the neighbor–joining method on the basis of the alignment of the full-size amino acid sequences CDPK and CRK obtained from the MUSCLE program. A phylogenetic tree revealed the reliable division of the CDPK family into four main subgroups (I–IV), and CRK was a separate group (Yip Delormel and Boudsocq 2019). The CDPKs most involved in the biotic response are members of subgroup I (Fig. 2, marked in red). AtCPK5/6/11 are positive regulators of protective reactions against *Pseudomonas syringae* and *Botrytis cinerea* (Dubiella et al. 2013). RBOHD is a

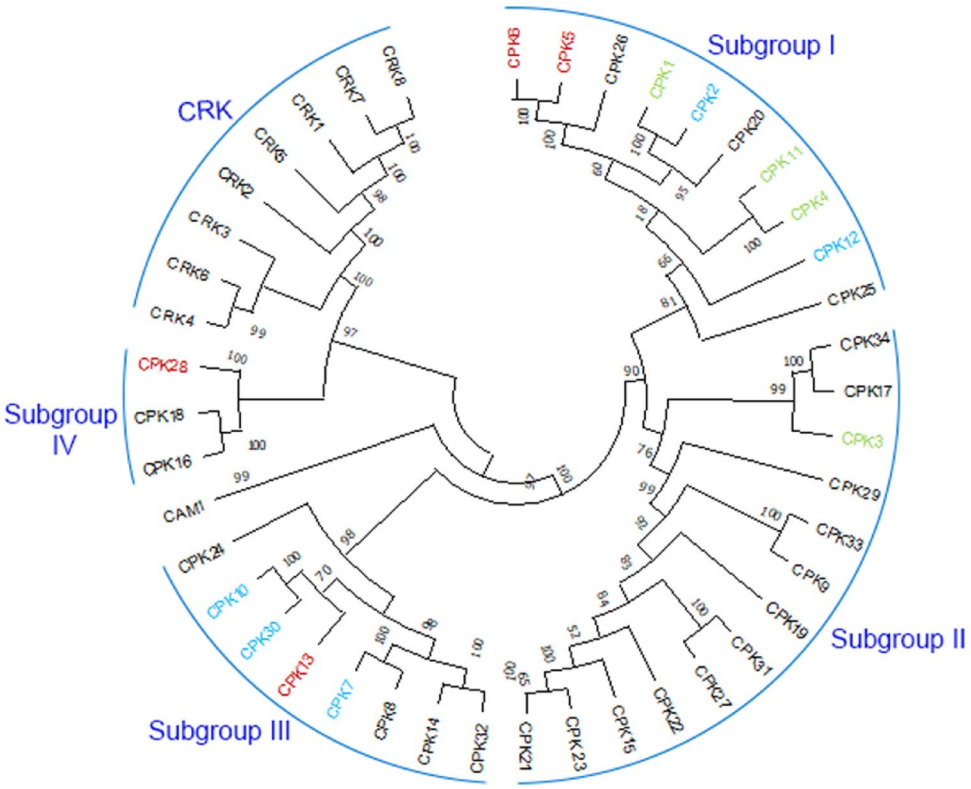

Fig. 2 Dendrogram illustrating *Arabidopsis thaliana* CDPK/CRK phylogenetic relationships. CRKs are a distinct group among the four major subgroups (I–IV) of the CDPK family (Yip Delormel and Boudsocq 2019). Red highlights CDPK isoforms implicated in biotic responses, blue highlights those implicated in abiotic responses, and green highlights those implicated in both biotic and abiotic responses

common substrate for these four CDPKs. AtCPK5/6 also regulate ethylene production in response to *B. cinerea* infection by modulating ACC synthase at the transcriptional level or by stabilising proteins (Li et al. 2018). Overexpression of the *AtCPK1* or *AtCPK5* gene triggers SA accumulation by activating SA biosynthesis gene expression, which provides resistance to a wide range of pathogens (Coca and San Segundo 2010; Dubiella et al. 2013; Bredow and Monaghan 2019). The accumulation of phytoalexins mediated by AtCPK1 through the phosphorylation of L-phenylalanine ammonia lyase also positively regulates ETI (Gao and He 2013). In parallel, AtCPK4/5/6/11 synergistically induced *WRKY46* expression in combination with *WRKY8/28/48*. AtCPK4/5/11 phosphorylate WRKYs in vitro, contributing to increased DNA binding (Gao and He 2013).

Additionally, some CDPKs from subgroups II and III contribute to plant immunity. In particular, AtCPK3 is involved in various responses to *F. moniliforme* (Lachaud et al. 2013). Genetic screening of 17 single *cpk* mutants revealed that *cpk3* and *cpk13* specifically impaired *PDF1.2* gene expression. Both kinases phosphorylate the transcription factor HSFB2a in vitro, which activates the expression of the gene *PDF1.2* (Kanchiswamy et al. 2010). Moreover, for the first time, it was recently shown that CDPK may be involved in protection against viral infection. AtCPK3 can limit the spread of *Potato virus X* from cell to cell (Perraki et al. 2018). AtCPK28 of subgroup IV negatively regulates PAMP-induced oxidative stress by reducing the stability of the kinase RBOHD-stimulating enzyme BIK1 induced by *B. cinerea* (Monaghan et al. 2014). AtCPK28 phosphorylates plant E3 ubiquitin ligases, resulting in the degradation of BIK1 (Wang et al. 2018). In this way, RBOHD undergoes fine-tuned CDPK-dependent regulation to ensure local PCD without spreading throughout the entire plant. In this context, AtCPK5 can play an important role in system signalling (Dubiella et al. 2013). Thus, several CDPKs (AtCPK1–6, 11, and 28) act as key nodes regulating multiple cellular responses in plants under various pathogenic conditions.

The role of calcium and CDPK in osmotic stress responses

Osmotic stress often refers to salinity and drought. The ABA signalling system plays a key role in the regulation of osmotic stress. To a lesser extent, ABA regulates temperature and other abiotic stresses. Osmotic stress causes transcriptional changes and reprogramming, which are partially mediated by CDPK. CDPKs regulate at least two types of transcription factors. One type is a group of ABA-sensitive (ABRE) ABF binding factors (Chinnusamy et al. 2010). AtCPK32 interacts with ABF4, providing ABA-induced activity for ABF4 (Choi et al. 2005). Similarly, AtCPK4 phosphorylates ABF2 in vitro and stimulates its activity (Lu et al. 2013). In addition, AtCPK4/11 can phosphorylate ABF1/4. The overexpression of AtCPK4/11 provided tolerance to drought associated with the activation of the expression of genes such as RAB18 and KIN1/2, whereas *cpk4/11* mutants presented opposite phenotypes (Zhu et al. 2007). Isoforms AtCPK7/10/12/30 are also capable of phosphorylating ABF1, ABF2, ABF3, and/or ABF4. CPK and ABF probably constitute a complex signalling network that regulates gene expression in response to osmotic stress (Yip Delormel and Boudsocq 2019). As repeatedly shown, some CDPK isoforms are involved in osmotic tolerance regulation (Dong et al. 2020; Erickson et al. 2022; Hettenhausen et al. 2016; Huang et al. 2018). This explains the extensive evidence for CDPK-mediated increases in resistance to osmotic stress. ABA-related phosphatases have been shown to physically interact with AtCPK1, dephosphorylating and reducing, but not completely inhibiting, its activity (Sheen 1996; Erickson et al. 2022; Durian et al. 2020a). There is currently no evidence of autophosphorylation of AtCPK1. Without a reverse regulatory system, CDPK-dependent activation of the ABA system could lead to a hypersensitivity reaction, as one target is the ABA-dependent senescence-associated protease (SAG12) and its transcription factors (Yip Delormel and Boudsocq 2019).

Calcium plays an important role in salt tolerance (Manishankar et al. 2018). Soil salinity can significantly reduce crop yields (Sun et al. 2020). When the electrical conductivity of the soil increased to 4 dS m^{-1} (40 mM NaCl), the soil was considered saline. Most crops can survive and fruit in soils with no more than 100 mM NaCl. However, the soil salinity can be greater than 2300 mM NaCl (Manishankar et al. 2018). Exogenous calcium concentrations ranging from 2 to 10 mM increase the salt tolerance of plants (Maeda et al. 2005; Manishankar et al. 2018; Tahjib-Ul-Arif et al. 2018; Salahshoor and Kazemi 2016; Larbi et al. 2020). These findings suggest that, under salinity treatment, calcium is essential for activating the SOS pathway. The molecular mechanisms of salt resistance involve calcium-dependent proteins such as Ca^{2+} sensors, protein kinases, and the CBL-CIPK pathway (Manishankar et al. 2018). However, high concentrations of calcium are toxic to plants (Bassett 1980; White and Broadley 2003).

Drought is one of the most significant abiotic stresses for agriculture (Sharma et al. 2023). Many studies have focused specifically on plant resistance to drought. In addition to the numerous experimental scientific articles published in the past year alone, more than 400 review articles on plant drought resistance were found in PubMed. This number of review articles is justified by a truly large array of experimental data that need regular systematisation. As

noted above, ABA is the pivotal phytohormone involved in the regulation of the response to osmotic and drought stress (Yoshida and Fernie 2024), and CDPKs play important roles in these processes (Chen et al. 2021; Kim et al. 2024). Since the discovery of CDPKs in the early 1990s and over the next 20 years, many studies have been conducted on the effects of *CDPK* overexpression on plant drought tolerance. This may be the most striking effect of the overexpression of various forms of *CDPK* genes in different plants. Research in this area can be divided into 3 categories: (1) tolerance to drought of *CDPK*-overexpressing plants; (2) differential expression analysis; and (3) genome-wide analysis of the *CDPKs* of various plant species. An increase in the drought tolerance of CDPK-overexpressing Arabidopsis plants was first shown in 1994 (Urao et al. 1994). CDPK was subsequently shown to be involved in the molecular mechanism of osmotic stress responses (Xu et al. 2010). This was followed by a series of works to identify isoforms of CDPK genes that regulate drought resistance in crops: rice (Saijo et al. 2000; Wei et al. 2014), bread bean (Liu et al. 2006), maize (Jiang et al. 2013), and wheat (Hou et al. 2024).

The role of calcium and CDPK in cold stress responses

The role of calcium and calcium-dependent protein kinases in thermotolerance is not yet clear. The overexpression of various isoforms of *CDPK* genes increases resistance to short-term intense cold stress (Almadanim et al. 2018; Chen et al. 2013; Dubrovina et al. 2015; Liu et al. 2018; Lv et al. 2018). The role of CDPK in tolerance to long-term cold stress and cultivation under low temperatures is not known (Dekomah et al. 2022). However, the positive role of intracellular calcium in the regulation of cold resistance has been sufficiently studied. Cold shock causes an immediate increase in the cytosolic calcium concentration in both cold-tolerant *Arabidopsis* and cold-sensitive tobacco plants. In *Arabidopsis*, calcium channel blockers inhibited the cold-induced increase in the cytosolic calcium concentration and the expression of cold-induced genes. These findings suggest that calcium influx plays an important role in the cold response. Pretreatment of *Arabidopsis* with *E. coli* or hydrogen peroxide altered the calcium signature in response to cold shock. Differences were also observed in the responses of the plants to repeated cold stimulation. These findings suggest that acclimation involves the modification of calcium signalling to promote cold memory (Knight et al. 1996; Song et al. 2008; Gao and Zhang 2019). Calcium fluctuations depend on the intensity and timing of cold treatments. Moreover, cold-induced calcium influx is reduced by repeated cold treatments, which ensures the development of "cold memory" (Plieth et al. 1999). Exogenous Ca^{2+} induces the expression of Ca^{2+} signalling pathway genes and genes involved in plant responses to cold stress. Increased expression of calcium signalling genes (CAMTA, CBL6, CIPK31, and CIPK2) and cold sensitivity genes (WCOR413, WCOR410, WCOR14, and Wrab17) maintains cellular redox homeostasis, increases photosynthetic rates, and ultimately increases stress tolerance (Malko et al. 2023). Thus, the overexpression of CDPK is expected to significantly increase resistance to long-term cold stress. However, we did not find such data. It can be assumed that antagonistic interactions between calcium and other signalling systems block the effect of calcium during prolonged cold stress, which reduces the efficiency of transformation by calcium-dependent protein kinases.

The role of calcium and CDPK in heat stress responses

There are many reports on the involvement of CDPKs in heat tolerance. Certain isoforms have been shown to respond to short-term, intense heat stress (Ray et al. 2007; Romeis 2001; Dubrovina et al. 2013; Dong et al. 2020; Veremeichik et al. 2022). The expression of 4 of the 19 isoforms of the *CDPK* genes of the wild Chinese grape *V. pseudoreticulata* has been shown to increase in response to short-term (2–48 h) intense (42 °C) thermal stress (Zhang et al. 2015). The increase in the expression of *GmCDPK5* and *GmCDPK10* under intense short-term stress was significantly greater in wild soy than in cultivated soybeans (Veremeichik et al. 2022). The activation of protective mechanisms in CDPK-overexpressing plants in response to short-term heat treatment has also been shown (Tan et al. 2011; Wang and Song 2014). In particular, under heat stress, AtCPK28 directly phosphorylates the stress marker enzyme APX2, which should lead to increased resistance to heat (Hu et al. 2021). Moreover, a decrease in heat tolerance in the *cdpk* mutant has also been shown (Wei et al. 2023). However, there is no evidence of a direct increase in heat tolerance in CDPK-overexpressing plants (Dekomah et al. 2022). There are many conflicting data concerning the role of calcium in thermotolerance. The inability of CDPK to increase tolerance to prolonged heat stress can be explained by the ambiguous role of calcium in the response to heat stress.

Initially, $[Ca^{2+}]_{cyt}$ increased in heat-treated plants (Klein and Ferguson 1987; Biyaseheva et al. 1993). Lenzoni and Knight (2019) reported that sudden increases in absolute temperature led to increased calcium concentrations in the chloroplast but not in the cytosol (Lenzoni and Knight 2019). The increase in the calcium concentration in chloroplasts is dynamic, dose dependent, and dependent on the absolute temperature rather than the rate of heating. Notably,

differences in threshold temperature have been shown for different plant species. Long-term heat exposure leads to a decrease in antioxidant activity (Jiang and Huang 2001; Lopez-Delgado et al. 1998; Dat et al. 1998). During prolonged heat exposure, exogenous calcium, as well as blocking calcium channels, does not have a significant effect on heat-induced parameters such as ROS, MDA content, or membrane permeability (Zhao and Ji-Fang 2005; Shen et al. 2016). Moreover, after short-term heat treatment, exogenous calcium resulted in an increase in these heat-induced parameters (Tan et al. 2011). These results were subsequently confirmed by other plant authors (Rikhvanov et al. 2014; Wang and Li 2006). However, microdoses of calcium have been shown to protect chlorophyll under long-term heat stress (Wang et al. 2022).

HSPs provide protection from death during heat treatments. Mild heat stress induces HSP expression. Calcium was previously thought to be unimportant for HSP (Gong et al. 1997). The appearance of denatured proteins in the cell is assumed to trigger the expression of HSPs (Voellmy and and Boellmann 2007). Recent findings, however, suggest that this mechanism does not require protein denaturation (Rikhvanov et al. 2014). Calcium is transported from the cytosol to intracellular compartments, including the mitochondria. The entry of Ca^{2+} into mitochondria is accompanied by hyperpolarisation of the inner mitochondrial membrane and an increase in the production of reactive oxygen species (ROS). Increased ROS production promotes the activation of HSP expression under mild heat stress (Volkov et al. 2006; Miller and Mittler 2006; Pucciariello et al. 2012) but leads to plant death under severe heat shock. Thus, mitochondria and possibly other organelles play crucial roles in determining the life or death of heat-treated plant cells. The cytosolic Ca^{2+} content and ROS production control these key processes (Rikhvanov et al. 2014). An increase in $[Ca^{2+}]_{cyt}$ plays an important role in the activation of HSP expression (Saidi et al. 2011). The Ca^{2+} signature depends not only on the type of stress treatment but also on the plant organ. The amplitude, timing, duration, and number of $[Ca^{2+}]_{cyt}$ peaks are critical for the expression of HSP genes. In the early stages of heat stress, an increase in cytoplasmic calcium leads to the activation of the expression of HSPs. The inhibition of Ca^{2+} transport during the first 25 min of heat stress caused no HSP expression. Although $[Ca^{2+}]_{cyt}$ and HSP expression are linked (Saidi et al. 2009), increasing $[Ca^{2+}]_{cyt}$ above a certain level no longer activates HSP expression. Heat-induced calcium influx was subsequently shown to have a cytotoxic effect (Wang et al. 2009, 2004; Yan et al. 2002). Although an increase in $[Ca^{2+}]_{cyt}$ plays an important role in defense responses, an excess of $[Ca^{2+}]_{cyt}$ can have adverse consequences (Medvedev 2005). The main cause of cell death under intense heat treatment is usually associated with increased ROS production and the aggregation of cellular proteins (Saidi et al. 2011). An excessive increase in $[Ca^{2+}]_{cyt}$ is another obvious cause of cell death. The toxicity of calcium is likely determined by the formation of Ca^{2+} complexes with phosphates (Medvedev 2005). In addition, calcium may regulate the development of programmed cell death (PCD). Thus, Ca^{2+} clearly plays a dual role in plant responses to elevated temperatures. On the one hand, it activates the expression of HSPs and may protect the plant from death; on the other hand, it can stimulate plant death (Rikhvanov et al. 2014). It can be assumed that the possible positive effect of the CDPK under heat stress can be blocked by feedback loops. Calcium ions may be blocked through currents or the activation of phosphatases. This process may involve antagonism of ABA signalling systems and SAR (Yasuda et al. 2008).

We have described in detail how signalling molecules interact during stress treatments (key information from "The role of calcium and calcium-dependent protein kinases in stress responses" section is summarised in Table 1). Recent data have shown that CDPKs can independently act on finite transcription factors and enzymes by bypassing the direct pathway. The important role of CDPKs in the regulation of plant stress tolerance suggests that CDPKs are prospective subjects of plant genetic engineering (Erickson et al. 2022).

Calcium-independent and engineered CDPKS

Structure of the CDPK

When plants are exposed to a stress stimulus, CDPK modulates the activity of key enzymes in plant cell signalling systems. The fine-tuned organisation of the elements of the protective system ensures the necessary and sufficient level of response to a certain treatment. The CDPK structure plays an important role in defense mechanisms. Plants have several dozen structurally different isoforms of the CDPK gene. These differences indicate that the specific multifunctionality of the CDPK family is necessary for the regulation of many reactions. The main functional characteristics of CDPKs are substrate specificity, activator ability, calcium binding ability, and autoinhibition. Substrate specificity and calcium dependence represent the most important intrafamilial differences in CDPK. Typically, CDPK proteins consist of four domains: N-terminal variable, C-terminal Ca^{2+}-binding, kinase, and autoinhibitory domains.

N-terminal variable domains contain specific amino acid motifs that recognise different substrates. In addition, the N-terminal variable domains contain motifs that determine the intracellular localisation of CDPK. Thus, the N-terminal

Table 1 Involvement of CDPKs in biotic and abiotic stress responses

CDPK isoforms	Plant species	Comments	References
Biotic stress			
AtCPK5/6/11	*A. thaliana*	Protective reactions against *Pseudomonas syringae* and *Botrytis cinerea*	Dubiella et al. (2013) and Li et al. (2018)
AtCPK1/5	*A. thaliana*	Triggers SA and phytoalexins accumulation	Coca and San Segundo (2010), Dubiella et al. (2013) and Bredow and Monaghan (2019)
AtCPK4/5/6/11	*A. thaliana*	Synergistically induced *WRKY46*	Gao and He (2013)
AtCPK3	*A. thaliana*	Involved in responses to *F. moniliforme* and the *Potato virus X*	Lachaud et al. (2013) and Perraki et al. (2018)
AtCPK28	*A. thaliana*	Negatively regulates oxidative stress induced by *B. cinerea*	Monaghan et al. (2014) and Wang et al. (2018)
Osmotic stress			
AtCPK4	*A. thaliana*	Phosphorylates ABF2 in vitro and stimulates its activity	Lu et al. (2013)
AtCPK1	*A. thaliana*	Positively regulates salt/drought tolerance	Huang et al. (2018)
AtCPK4/11	*A. thaliana*	Provided tolerance to drought via activation of the expression of RAB18 and KIN1/2	Zhu et al. (2007)
AtCPK7/10/12/30	*A. thaliana*	Phosphorylate ABF1, ABF2, ABF3, and/or ABF4	Yip Delormel and Boudsocq (2019)
AtCPK32	*A. thaliana*	Interact with ABF4, providing ABA-induced activity for ABF4	Choi et al. (2005)
Heat stress			
AtCPK28	*A. thaliana*	Directly phosphorylates the APX2, which should lead to increased resistance to heat	Hu et al. (2021)
OsCPK13	*Oryza sativa*	Expressed due to heat stress	Ray et al. (2007)
VaCPK9/20/21/29	*Vitis amurensis*	Expressed due to high heat stress	Dubrovina et al. (2013)
GmCDPK5	*Glycine max*	Expressed due to short-term heat stress	Veremeichik et al. (2022)
VpCDPK4/6/9/10/19	*Vitis pseudoreticulata*	Expressed due to short-term heat stress	Zhang et al. (2015)
Cold stress			
OsCPK17	*Oryza sativa*	phosphorylates in vivo: aquaporin OsPIP2;1/6 and sucrose-phosphate synthases	Almadanim et al. (2018)
PeCPK10	*Populus euphratica*	Enhance freezing tolerance	Chen et al. (2013)
OsCPK24	*Oryza sativa*	Enhance tolerance to short-term cold stress	Liu et al. (2018)
VaCPK20	*Vitis amurensis*	Enhance tolerance to short-term cold stress	Dubrovina et al. (2015)
SlCPK27	*Solanum lycopersicum*	Positive regulator of ABA generation in cold adaptation	Lv et al. (2018)
VpCDPK6/9/ 16	*Vitis pseudoreticulata*	Expressed in response to short-term cold stress	Zhang et al. (2015)
MdCPK1a	*Malus domestica*	Enhance tolerance to short-term cold stress due to scavenging ROS accumulation	Dong et al. (2020)
GmCDPK5/10	*Glycine max*	Expressed in response to short-term cold stress	Veremeichik et al. (2022)

domain allows CDPK to control different processes in different cellular compartments, determining the functional diversity of CDPK. The topic of CDPK localisation is covered in detail in the review by Simeunovic et al. (2016). In brief, using the example of the CDPK of *Arabidopsis*, it can be summarised that most *Arabidopsis* CDPKs are either exclusively membrane localised or are both soluble and membrane associated (Simeunovic et al. 2016). This membrane association is mediated by N-terminal acylation provided by N-myristoylation and palmitoylation (Martín and Busconi 2000). Notably, the reversibility of palmitoylation allows a few CDPKs to translocate to the cytosol, nucleus or endoplasmic reticulum (ER) in response to a certain stimulus (Chehab et al. 2004; Simeunovic et al. 2016). Moreover, tissue-specific expression ensures the localisation of certain forms of CDPK in certain organs, tissues and even compartments (Simeunovic et al. 2016).

The C-terminal Ca^{2+}-binding domain (CaM-like domain, CLD) contains four EF-hand domains. The number and sequence of EF hands determine the level of sensitivity to calcium. CLD activates the PK kinase domain (Harper et al. 2004). CLD is bound to PK via an autoinhibitory domain represented by a 35 a.a. junction, J (Hrabak et al. 2003). The J and CLD domains do not function independently (Chandran et al. 2006) and form a single functional CDPK activation domain (CAD). The CAD splice fragment blocks

kinase activity when $[Ca^{2+}]_{cyt}$ is released. $[Ca^{2+}]_{cyt}$, in response to external influences, binds Ca^{2+} with CAD, and conformational changes remove the J-terminal fragment from the active protein kinase center. Free PK domains provide access to substrates (Bender et al. 2018). The functional diversity of CDPKs, provided by the variability of the C- and N-terminal domains, was described in detail in a previous review (Yip Delormel and Boudsocq 2019).

Strategies for creating calcium-independent CDPKs (mutations, truncations)

Thus, the manipulation of CAD and J fragments may be more promising for genetic engineering than the overexpression of native CDPK genes. The genetic removal of CAD from AtCPK1 or CDPKα in soy led to the constitutive activation of the enzyme (Harper et al. 1994; Yoo and Harmon 1996). Moreover, the absence of CAD in another CDPK, CPK17, in rice (the AtCPK1 orthologue) led to a loss of catalytic activity (Almadanim et al. 2018). Furthermore, the inhibition of AtCPK10 by EGTA suggests that a CAD associated with Ca^{2+} may be important for CDPK function (Boudsocq et al. 2012). Harper et al. (1994) and Huang et al. (1996) are the first and perhaps the most interesting scientific works in this field. These two studies analysed several types of mutations in the AtCPK1 J domain and truncated mutants. The object of research in those works was the isoform AtCPK1 (GenBank accession number, AT5G04870; http://ww.uniprot.org/UniProt/Q06850). This isoform is one of the most studied (Bender et al. 2018). Among the other mutants, the mutant KJM23 (with the substitution of AV-424 with PD and QFSA-430 with PEDL) was the most active in the presence or absence of Ca^{2+}. Another type of mutation in the J-domain (KJM4, LRV-I444 in DLPG) was described by Huang et al. (1996). This mutation disrupted Ca^{2+}-induced activation, leading to latent KJM4 activity (Huang et al. 1996). The native form in the presence of Ca^{2+} ions showed 100% activity; in the absence of Ca^{2+} ions, the activity decreased to 1.5%. The mutant isoform KJM23 showed 97% and 86% activity in the presence and absence of Ca^{2+} ions, respectively (Harper et al. 1994). The KJM4 mutation completely abolished the binding of CaMLD. The mutant form of KJM4 showed latent activity in the presence and absence of Ca^{2+} ions (Huang et al. 1996). Interestingly, both types of substitutions change the highly conserved residues of the J-domain of CDPK. The J-domain contains pseudosubstrate autoinhibitory (17–26 a.a.) and CaMLD binding (23–35 a.a.) subdomains (Raichaudhuri et al. 2006). The KJM23 mutation replaces the most constitutive residues of the pseudosubstrate autoinhibitory subdomain (QFSA to PEDL). The KJM4 mutation replaces the most constitutive residues of the CaMLD binding subdomain (LRVI to DLPG). Thus, the application of mutagenesis to the J-domain is molecularly justified. Further work in this direction is justified by the need to fully understand the molecular mechanism of these physiological effects. The key information from "Calcium-independent and engineered CDPKS" section is summarised in Table 2.

Table 2 Structure and functional domains of CDPKs and their modifications

	CDPK structure (domains)			
	N-terminal variable	Kinase (PK)	CDPK activation domain (CAD)	
			Autoinhibitory domain, 35 a.a. junction (J)	C-terminal Ca^{2+}-binding domain (CaM-like domain, CLD)
Function	Recognition substrates; determination of the intracellular localisation	Phosphorylation of the substrates	CLD is bound to PK via J when $[Ca^{2+}]_{cyt}$ is released; binds Ca^{2+} with CAD, led to removal of the J-terminal fragment from the kinase centre	Ca^{2+}-binding
Structural features	N-terminal acylation via N-myristoylation and palmitoylation	Active protein kinase centre	Pseudosubstrate autoinhibitory (17–26 a.a.) and CaMLD binding (23–35 a.a.) subdomains	Number and sequence of EF hands determine the level of sensitivity to calcium
Modifications	–	–	The genetic removal of CAD from AtCPK1 or CDPKα in soy led to the constitutive activation (Harper et al. 1994; Yoo and Harmon 1996) The absence of CAD in CPK17 in rice led to a loss of catalytic activity (Almadanim et al. 2018) KJM23 (AV-424 with PD and QFSA-430 with PEDL) mutation leading to constitutive activity (Harper et al. 1994) KJM4 (LRV-I444 in DLPG) mutation leading to latent KJM4 activity	

Functional advantages over native forms

The development of human civilisation has introduced anthropogenic factors that significantly aggravate the negative impacts of natural stresses on plant ecosystems. Genome editing technologies may be promising approaches for solving the problem of producing stress-resistant plants. To obtain plants with desired properties, the most important consideration is to choose the optimal genetic target. The basic requirements for the target gene are (1) a pronounced effect according to the specified parameters; (2) the presence of several positive effects; (3) the absence of negative effects; (4) an accessible level of complexity in editorial manipulation; and (5) transparency of the molecular mechanism of action. According to numerous studies, plant calcium-dependent protein kinases (CDPKs) appear to be among the most suitable candidates for genetic manipulation. These unique plant regulatory proteins are involved in many routine and stress-mediated processes, including responses to pathogen attack and osmotic and temperature stress. Certain isoforms of CDPK phosphorylate key enzymes involved in the metabolism of reactive oxygen species, ethylene, abscisic acid and salicylic acid. However, it has been repeatedly shown that the overexpression of promising isoforms of CDPK genes leads to the formation of plants that are highly resistant only to osmotic stress. The increase in the expression of CDPK, a modulator of plant stress resistance, is not as important as conformational modifications. Studies have shown that overexpression of the modified *AtCPK1* gene has a more powerful effect than the native form of the gene on (1) resistance not only to osmotic stress but also to temperature stress; (2) biosynthesis of phytoalexins; and (3) metabolism and homeostasis of reactive oxygen species (ROS) and other signalling molecules. The main idea was to determine whether it is possible to improve the results of plant transformation with the CDPK gene by modifying the autoinhibitory domain. The following questions must be answered: (1) Does transformation with a calcium-independent form of the *AtCPK1* gene have a more powerful effect on secondary metabolism than does transformation with the native form of the gene? (2) Will the effect of secondary metabolism activation be universal, that is, applicable to various classes of phytoalexins? (3) Is it possible to improve AtCPK1-induced resistance to osmotic stress through modification? (4) Will the modification provide resistance to temperature stress?

Applications of engineered CDPKS in plant biotechnology

CDPK as an activator of secondary metabolite productivity

There are few publications on the application of CDPK for the activation of secondary metabolism. For example, the expression of the native form of *VaCDPK20* in grape cell culture positively regulates the biosynthesis of stilbenes (Aleynova-Shumakova et al. 2014). How can one explain such weak interest in this direction? We can assume that, in most cases, researchers encounter a stable effect on secondary metabolism when the gene is transformed with its native form. The reason may be that a stably growing plant cell culture is not exposed to stress, and the level of intracellular calcium remains basic, which does not allow CDPK to show its capabilities due to autoinhibition. However, few studies have compared the effects of *CDPK* gene expression on transgenic plants. In most cases, in the absence of plant stress factors, plants in which CDPK genes are overexpressed or knocked out do not differ significantly from control plants (Huang et al. 2018; Durian et al. 2020b).

In a previous study, the effects of the overexpression of native and mutant constitutively active forms of the *AtCPK1* gene on the biosynthesis of anthraquinones were compared in transgenic cell cultures of *Rubia cordifolia* L. Analysis of the productivity of *R. cordifolia* callus culture revealed that the overexpression of the native form *AtCPK1* led to a fivefold increase in productivity, and the overexpression of the mutant constitutively active form of *AtCPK1* increased productivity by more than tenfold (Shkryl et al. 2011, 2016). However, these data suggest that AtCPK1 at the basal intracellular calcium level is also involved in the routine life-support processes of plant cells. The overexpression of a mutant nonactive form led to a smoothing of the activatory effect. The expression of a constitutively active calcium-independent mutant multiplied the positive effect on secondary metabolism. Therefore, replacing 6 of the 35 amino acid residues of the CDPK autoinhibitory domain multiplies the gene expression effect on secondary metabolism, whereas replacing the 4 amino acid residues reverses any effect (Shkryl et al. 2016). The effectiveness of applying a mutant constitutively active form of the *AtCPK1* gene for secondary metabolism activation was subsequently confirmed. The overexpression of the constitutively active form of the *AtCPK1* gene resulted in a 70-fold increase in productivity (mg/l) and a 40-fold increase in content (% dry weight) compared with those of the control line for *V. amurensis* cell cultures (Veremeichik et al. 2017). The *G. max* cell line produces a total of approximately 40 mg/L isoflavonoids, of which 2 mg/L and approximately 0.3 mg/L

are prenylated. The overexpression of the constitutively active form of *AtCPK1* in the *G. max* cell line led to an increase in the yield of isoflavones to 202 mg/L, of which the yield of the corresponding aglycones was 6.7 mg/L and that of the prenylated forms was 4 mg/L (Veremeichik et al. 2019). Moreover, native soybean plants contain mainly flavonoids. Compared with that in field crops, the amount of isoflavonoids in the cell culture transformed with a mutant form of the gene was fivefold greater. Moreover, soybean cell cultures are monoproducers of isoflavonoids, whereas in plants, isoflavonoids are minor components (Akitha Devi and Giridhar 2014; Veremeichik et al. 2019, 2021c). These works revealed that replacing 6 of the 35 amino acid residues of the CDPK autoinhibitory domain multiplies the effect of AtCPK1 on secondary metabolism, whereas replacing the other 4 amino acid residues abolishes any effect. It can be assumed that this result will be important in the future due to the active development of genomic editing technologies. In terms of the application of this approach to the activation of biosynthesis in cell cultures of medicinal plants, an important observation is the activation of biosynthetic minor phytoalexins, which allows the production of monoproducer cultures of the most bioactive compounds. Studies have been conducted on plant cell cultures via various biosynthetic pathways for the biosynthesis of specific compounds. Both the shikimic pathway of anthraquinone biosynthesis and several branches (isoflavonoid and stilbene biosynthesis) of the phenylpropanoid pathway were reinforced by the overexpression of modified AtCPK1 in plant callus culture without growth inhibition.

CDPK as a modulator of abiotic stress tolerance

The biosynthesis of secondary metabolites accompanies the response of plant cells to stresses of both biotic and abiotic natures. It has previously been shown that AtCPK1 positively regulates a plant cell's response to pathogen attack, which is consistent with the role of CDPK in secondary metabolism regulation (Coca and san Segundo 2010). The expression of several CDPK gene isoforms has been shown to respond to various abiotic stresses (Ray et al. 2007; Romeis 2001; Dubrovina et al. 2013; Dong et al. 2020). The overexpression of the native *AtCPK1* gene has been shown to increase the tolerance of tobacco plants to salinity (Huang et al. 2018; Veremeichik et al. 2021b). Germination of WT tobacco was inhibited at 120 mM NaCl, whereas AtCPK1-OE plants were tolerant to 180 mM NaCl (Veremeichik et al. 2021b). The same results were obtained in the studies of Huang et al. *A. thaliana* plants transformed with *AtCPK1* were resistant to 140 mM NaCl (Huang et al. 2018). Moreover, the accelerating effect of a calcium-independent mutant form of AtCPK1 was demonstrated. Salt tolerance can be increased by up to 80% by inactivating the autoinhibitory domain (Veremeichik et al. 2021b).

In contrast to plants transformed with the native form of the *AtCPK1* gene, we demonstrated in this study that plants transformed with a constitutively active form of the gene were substantially more resistant to long-term cold stress. The growth of the control plants decreased fourfold after 30 days of low-temperature treatment. Resistance increased marginally but considerably when the native form of the *AtCPK1* gene was overexpressed by no more than 20%. Furthermore, the effect was evident during the long-term cultivation of plants as well as during seed germination. In addition, compared with the control plants, the plants expressing the mutant constitutively active variant of the *AtCPK1* gene presented twofold greater resistance to cold. During seed germination, weak but significant resistance to prolonged cold (12 °C) was caused by the overexpression of the native form of the *AtCPK1* gene. Moreover, the germination of the KJM23-OE plants was not inhibited by low temperatures. We can therefore conclude that cold tolerance is conferred by the native form of *AtCPK1* and that this effect is amplified by autoinhibitory domain inactivation. Notably, however, in this instance, there is virtually no correlation between cold resistance and the expression of the gene's native form. Thus, CDPK is an effective tool for creating cold-tolerant plants through direct inactivation of the autoinhibitory domain (Veremeichik et al. 2025).

Because heat stress upsets cellular homeostasis, which eventually leads to stunted growth and even death, it poses a serious threat to crop productivity globally (Hall 2000). Research has shown that certain *CDPK* gene isoforms respond to short intense heat stress (Ray et al. 2007; Romeis 2001; Dubrovina et al. 2013; Dong et al. 2020; Veremeichik et al. 2022). Four of the 19 isoforms of the *CDPK* genes of the wild Chinese grape *V. pseudoreticulata* are expressed at relatively high levels in response to short-term (2–48 h) acute (42 °C) heat stress (Zhang et al. 2015). Under intense short-term heat stress, wild soy presented significantly greater increases in *GmCDPK5* and *GmCDPK10* expression than cultivated soybeans did (Veremeichik et al. 2022). It has also been demonstrated that short-term heat treatment activates defensive mechanisms in plants overexpressing CDPK (Tan et al. 2011; Wang and Song 2014). AtCPK28 directly phosphorylates the stress marker enzyme APX2, leading to increased heat resistance (Hu et al. 2021). Furthermore, the *cdpk* mutant is relatively insensitive to heat (Wei et al. 2023). However, there is no evidence that plants with higher levels of *CDPK* overexpression are more heat tolerant (Dekomah et al. 2022). The role of Ca^{2+} in thermotolerance has been the subject of numerous conflicting studies. The inability of *CDPKs* to build tolerance to prolonged heat stress in plants overexpressing *CDPK* may be explained

by the unknown involvement of Ca^{2+} in the reactions of these cells to heat stress. It was later demonstrated that heat-induced Ca^{2+} influx has a deleterious effect (Wang et al. 2009). Furthermore, Ca^{2+} may control how programmed cell death (PCD) develops. Therefore, Ca^{2+} clearly has two functions in how plants react to high temperatures. The triggering of the formation of heat shock proteins (HSPs) may prevent plants from dying, but it may also result in plant death. Feedback loops are thought to prevent the potential beneficial effects of CDPK. Currents or phosphatase activation can impede phosphorylation-regulated Ca^{2+} channels or Ca^{2+}-induced phosphorylation-dependent processes. The ABA and SAR signalling systems may be antagonistic during heat treatment (Yasuda et al. 2008). The resistance or sensitivity of tobacco to heat stress is unaffected by *AtCPK1* overexpression. Furthermore, resistance to extended heat exposure is markedly increased by the development of the modified AtCPK1 form, which is unaffected by changes in intracellular Ca^{2+} levels. Nevertheless, nothing is known about how intracellular Ca^{2+} regulates heat tolerance both in vitro and in soil (Veremeichik et al. 2025; Tikhonova et al. 2025). On the basis of the information gathered, inactivating the CDPK autoinhibitory domain appears to be a viable method for increasing a plant's resistance to temperature stress.

A significant increase in endogenous ABA was detected in plants expressed constitutively active form of the *AtCPK1* gene (Veremeichik et al. 2025). An important indicator of elevated endogenous ABA levels (Ali et al. 2022; Schopfer et al. 1979) is a significant delay in the germination of plants expressed constitutively active form of the *AtCPK1* gene (Veremeichik et al. 2025). Increased sensitivity to exogenous ABA is another significant sign of elevated endogenous ABA. Both exogenous and endogenous ABA have favourable effects on stress-induced senescence and normal aging processes. This is demonstrated by the more severe chlorophyll degradation linked to senescence (Ren et al. 2018). It has been demonstrated that plants transformed with the native and constitutively active versions of the *AtCPK1* gene exhibit significantly more severe ABA-induced senescence. According to Durian et al. (2020b), these results are in line with the previously documented CPK1-mediated activation of ORE1, one of the most significant master regulators of ABA-dependent PCD and senescence. This finding also indicates the need for a detailed analysis of the hormonal status of CDPK-overexpressing plants since ABA and SA are antagonistic agents (Cao et al. 2011; Nahar et al. 2012). However, under long-term heat treatment, heat-induced senescence was less intense in heat-treated plants expressing constitutively active versions of the *AtCPK1* gene, whereas WT plants and plants transformed with the native *AtCPK1* gene were damaged to the same extent. Moreover, heat-induced increases in ABA levels were blocked in heat-treated plants, which expressed constitutively active versions of the *AtCPK1* gene as well as genes involved in ABA biosynthesis (Veremeichik et al. 2025).

Early heat exposure causes an increase in the intracellular Ca^{2+} concentration, which initiates CDPK-regulated cascades. Later, however, Ca^{2+} levels decrease, which prevents CDPK from functioning. Chlorophyll catabolism is then triggered by heat-induced excess ABA (Kane and McAdam 2023), which lowers the physiological load during brief exposure but causes plant death during prolonged and severe heat stress (Song et al. 2008). ABA production is generally activated by the constitutively active version of AtCPK1, but heat exposure results in a decrease in ABA content. Consequently, a reduction in SAG12 expression was noted. As a result, under heat stress, the leaves of KJM23-OE plants were significantly less yellow than those of WT plants. We can presume that the CDPK-dependent stimulation of SA signalling is responsible for this result. According to Cao et al. (2011) and Nahar et al. (2012), SA and ABA are known to be antagonistic, and ABA signalling is inhibited when SAR is activated. Data on CDPK-dependent activation of SA signalling, SA biosynthesis (Coca and San Segundo 2010), and activation of the expression of proteins linked to SAR (Veremeichik et al. 2023) corroborate this theory. However, the information gathered for this study supports this theory. The SA-dependent protease ACD6 expression was shown to be downregulated. ACD6 mediates the hypersensitive response and PCD and is implicated in the feedback regulation of SA signalling (Rate et al. 1999).

We propose the following hypothesis concerning the function of CDPK during long-term abiotic stress exposure on the basis of the acquired molecular and biochemical data (Fig. 3). Therefore, a thorough examination of the biosynthesis and signalling of ABA and SA under prolonged heat stress, as well as the functions of the native and constitutively active versions of AtCPK1 in these processes, is necessary for future research in this field. Several problems were addressed by using a modified calcium-independent version of AtCPK1. First, why is heat resistance not as great as it is for salt, drought, and cold when native forms of CDPK genes are overexpressed? The priority of ABA signalling activation is determined by the reliance on heat-induced calcium ion currents. Therefore, an inadequate response from BR and ET signalling may not result from CDPK-dependent activation of ABA signalling (Fig. 3). Modified CPK1 stimulates BR signalling in the absence of heat-induced ion currents, improving the ability of transgenic plants to withstand high temperatures. BR may be blocked under heat stress, leading to the buildup and activation of BIN2, which leads to the activation of ERF49 (DREB2D) expression (Chen et al. 2022). In another paper, the authors claimed that BR is

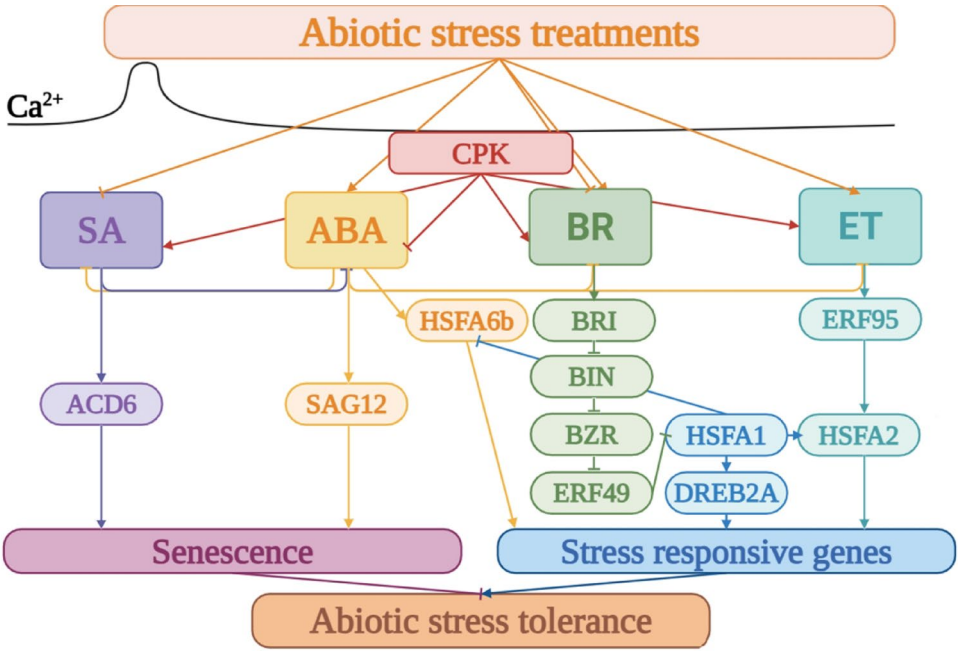

Fig. 3 Proposed model of the role of CDPK under abiotic stress. During the early stages of heat/cold/osmotic stress exposure, the intracellular calcium concentration increases, which triggers cascades regulated by CDPK. Later, calcium levels decrease, which blocks CDPK; stress-induced excess ABA triggers chlorophyll catabolism (Kane and McAdam 2023). SA and ABA are known to be antagonistic (Cao et al. 2011; Nahar et al. 2012). ACD6 is involved in the feedback regulation of SA signalling and mediates the hypersensitivity response and PCD (Rate et al. 1999). BR may be blocked under heat stress, leading to the buildup and activation of BIN2, which leads to the activation of ERF49 (DREB2D) expression. Arabidopsis HSFA1s function as "master regulators" and are induced under heat stress to directly regulate the expression of downstream HSR genes or indirectly by activating the AP2/ERF TF DREB2A. In Arabidopsis, baseline thermotolerance is increased by ERF95 or ERF97 expression, providing a detailed description of this phenomenon. In contrast to the calcium-independent version KJM23, overexpression of the native form of the AtCPK1 gene resulted in a heat-induced increase in the expression of genes involved in ABA production, as we previously described. The calcium-independent version KJM23 provides CDPK-dependent activation of ROS- and/or SA-dependent and ABA-independent responses to abiotic stress treatment to mitigate ABA-dependent negative effects. As a result, KJM23-plants were more tolerant to long-term heat/cold/osmotic stress than were plants overexpressing the native form of AtCPK1

produced by heat stress, leading to the activation of BZR1, which represses ERF49 expression. As "master regulators", Arabidopsis HSFA1s are activated in response to heat stress and either directly or indirectly control the expression of downstream HEAT STRESS RESPONSIVE (HSR) genes by activating the AP2/ERF TF DREB2A (Finka et al. 2012). In Arabidopsis, baseline thermotolerance is increased by ERF95 or ERF97 expression (Huang et al. 2023). Wang et al. (2024) provided a detailed description of this paradigm. In contrast to the calcium-independent version KJM23, overexpression of the native form of the AtCPK1 gene resulted in a heat-induced increase in the expression of genes involved in ABA production, as we previously described. The calcium-independent version KJM23 provides CDPK-dependent activation of ROS- and/or SA-dependent and ABA-independent responses to abiotic stress treatment to mitigate ABA-dependent negative effects. As a result, KJM23-overexpressing plants were more tolerant to long-term heat/cold/osmotic stress than were plants overexpressing the native form of AtCPK1.

Application of calcium-independent CDPK for the investigation of CDPK-mediated processes

To study the functions of certain proteins, two main approaches are used: overexpression and knockout. However, for enzymes that depend on something, these two approaches do not always provide a complete picture. The ability to obtain an independent, constantly active mutant allows one to unlock the full potential of the enzyme under study. A good example is the study of the function of the MPK4 key relay of MPK4 in the plant defense system. The acquisition of a constantly active form made it possible to clarify functions (Colcombet et al. 2013) that remained in the shadows when standard approaches were used (Petersen et al. 2000). According to the literature, two controversial aspects prevent a full understanding of AtCPK1-associated biochemical processes. First, despite numerous studies over the last 20 years, the effect of AtCPK1 on the metabolism of ROS in cells has not been reliably established. On the one hand, AtCPK1 has been shown to activate the generation

of ROS directly when it interacts with NADPH oxidases (Xing et al. 2001). On the other hand, when the *AtCPK1* gene was overexpressed in *A. thaliana* plants, a stress-induced decrease in intracellular ROS was detected, but the basal and stress-induced states of the ROS metabolism systems were not investigated (Huang et al. 2018). Second, transcriptomic data (Coca and San Segundo 2010) indicate that AtCPK1 stimulates salicylic acid signalling but not systemic acquired resistance. Investigating the role of AtCPK1 in regulating the antagonism of systemic or local acquired resistance and establishing a priority direction are of paramount importance for the interpretation of AtCPK1-mediated physiological and biochemical effects. The molecular mechanism of AtCPK1 integration in response to abiotic stress has been studied much less extensively. AtCPK1 activates the ABA-dependent promoter (Sheen 1996). In addition to the "calcium signature", "phosphocodes" and autophosphorylation modulate AtCPK1 activity (Erickson et al. 2022). This, in turn, determines the location of the CDPK in the feedback loop of ABA signalling. Thus, the presence of feedback loops complicates the study of the role of CDPK in response reactions. Using modified AtCPK1, it was possible to shed light on controversial aspects, such as CDPK-mediated ROS metabolism and the regulation of PR gene expression. Wide investigations of modified CDPKs may allow for the study of other related mechanisms.

ROS metabolism

In a calcium-independent mutant, AtCPK1 was shown to activate the expression of genes encoding key enzymes involved in ROS metabolism, which led to an increase in basal ROS levels in both undifferentiated and plant cells. The overexpression of the native form of the gene had little effect on the intracellular ROS content, which is also consistent with the data of Huang et al. (2018). The overexpression of the modified *AtCPK1* gene led to a significant increase in ROS. The increase in the ROS content in the exponential phase was consistent with the increase in the expression of the key genes encoding anthraquinone biosynthesis enzymes. An increase in ROS in both mesophyll cells and stomatal cells was observed in tobacco plants expressing the modified AtCPK1 gene. Among other ROS scavengers, an increase in the expression of the *Apx2* gene accompanied an AtCPK1-induced increase in the expression of NADPH oxidase genes. *Apx2* is one of the core target genes of the stress-inducible heat stress transcription factor HSF (Guo et al. 2016). The expression of *AtHSFA1* is regulated through ROS-induced Ca^{2+} signalling systems, and AtHSFA1 activates the expression of *AtHSFA2* and AtHSFA3 and suppresses the expression of ABA-inducible AtHSFA6b (Huang et al. 2016). The overexpression of the native and modified forms of the *AtCPK1* gene led to a significant increase in the expression of ROS-regulated *NtHSFA1*, *NtHSFA2*, and *NtHSFA3* and a decrease in the expression of the ABA-regulated form *NtHSFA6b* (Veremeichik et al. 2021b). The ROS signalling system is stimulated at the level of ROS generation, ROS detoxification, and the regulation of the expression of ROS-dependent stressed HSFs.

SAR/LAR antagonism

A working hypothesis on the role of CDPK was proposed on the basis of a proteomic study of *V. amurensis* cell lines transformed with mutated AtCPK1 (Veremeichik et al. 2023). PR-2 and PR-5 protein expression increased, but PR-3 protein expression decreased in the transgenic cells. While PR-3 is linked to local acquired resistance, PR-2 and PR-5 are proteins linked to systemic acquired resistance. Alterations in primary metabolism signify the initiation of polyphenol and salicylic acid precursor production. Additionally, fewer enzymes have been implicated in the production of precursors of jasmonate and fatty acids (Fig. 4). These findings suggest that LAR-associated JA signalling is inhibited, whereas SAR-associated SA signalling is induced by CDPK (Wang et al. 1999). Proteomic analysis revealed that the calcium-independent form of AtCPK1 blocks local acquired resistance and activates systemic resistance at the level of pathogen-associated proteins and primary metabolism. The results of biochemical, molecular, and proteomic analyses, as well as confocal microscopy, expand the current understanding of the role of CDPK in the regulation of ROS metabolism and the antagonism of systemic and local acquired resistance. These results are important not only for understanding the molecular mechanism of action of calcium-independent CDPK but also as a basis for further studies of the physiological functions of CDPK, which cannot be revealed when working with native forms of the CDPK genes.

Future prospects and challenges

Potential of CDPKs in gene editing and synthetic biology

Plants have historically been the primary source of phytochemicals that humans use for food, medicine, culture, and cosmetics (Jamshidi-Kia et al. 2018). Currently, global climate change, limited access to fresh water, limited food supplies, achievements in sustainable development, and growing energy demands are among the most important global challenges facing humanity. The search for approaches for the efficient cultivation of crops is the

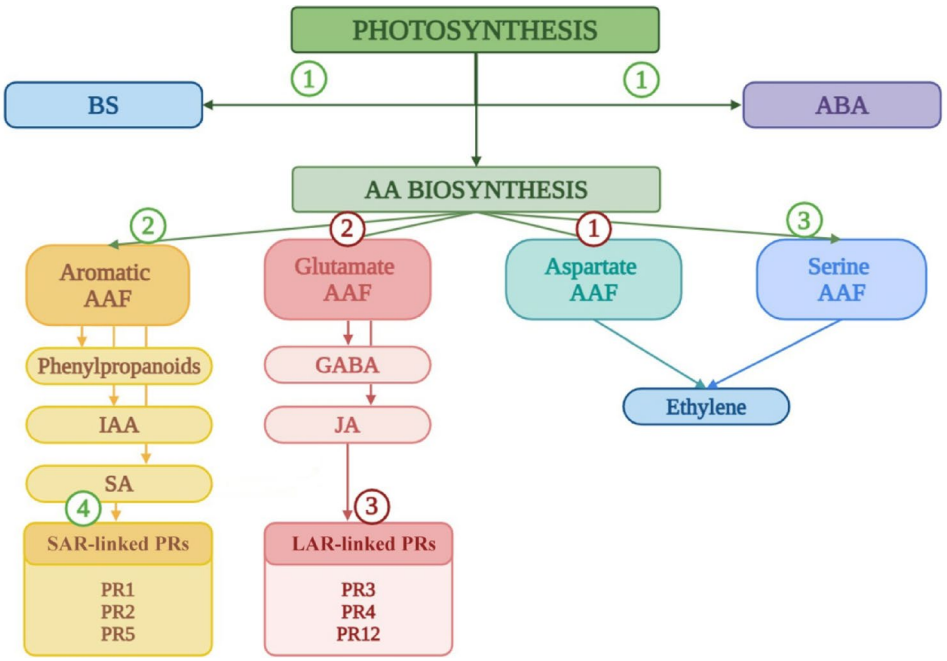

Fig. 4 A simplified model suggesting the mechanisms of CPK action on primary metabolism, signalling molecules, and PR proteins. This model is based on a study by Trovato et al. (2021). Green boxes, photosynthesis as a precursor of the biosynthesis of BS and ABA and the amino acid families (AAF): blue boxes, aspartate and serine AAF as precursors of ethylene biosynthesis; red boxes, glutamate AAF as precursors of GABA and ABA; yellow boxes, aromatic AAF as precursors of phenylpropanoids, IAA and SA. The scheme shows crosstalk between salicylic acid (SA) and jasmonic acid. The presented model is based on a review by Enoki and Suzuki (2016). As a result of AtCPK1-Ca action, the activation of systemic acquired resistance and the inhibition of localised acquired resistance through PR proteins can be proposed. The green numbers in the circles represent AtCPK1-Ca-induced increases in enzyme abundance: (1 and 2) biosynthesis of pentose phosphate intermediates (with the participation of glyceraldehyde-3-phosphoglycerate kinase and phosphoglycerate kinase); (3) cysteine biosynthesis catalysed by 3PG (cysteine synthase); and (4) increased abundance of SAR-associated PR1, 2 and 5. The red numbers in the circles represent *AtCPK1-Ca*-induced decreases in the abundance of enzymes: (1) biosynthesis of the aspartate pool from oxaloacetate (by malate dehydrogenases and phosphoserine aminotransferase); (2) biosynthesis of glutamate from citrate (by isocitrate dehydrogenases, aconitate hydratases, glutamine synthetase, and glutamate dehydrogenase); and (3) decreased abundance of LAR-associated PR3, 4 and 12

main and primary task of fundamental research. Cytosolic calcium ions are essential for controlling both routine and life-threatening conditions. A "calcium signature" that is suited for the circumstances at hand is produced by multi-level modulation of calcium ion fluctuations. The "calcium signature" causes calcium messengers to be released, which in turn activates proper signalling pathways and reactions. Calcium-dependent protein kinases are unique among calcium decoders in that they can modulate the response by activating critical defense system components on the basis of calcium levels. The unique structure of CDPK ensures the involvement of family members in the regulation of the main physiological processes of plant cells. Recent studies have shown that many specific CDPK isoforms play key roles in regulating stress resistance, which is of primary importance for the cost-effective cultivation of crops. The overexpression of some native *CDPK* isoforms resulted in improved tolerance to osmotic stress. In this review, we report that site-directed mutagenesis of the autoinhibitory domain enhances the effect of CDPK overexpression on the cellular defense system.

It has been repeatedly shown that the overexpression of promising CDPK gene isoforms allows for the production of plants resistant only to osmotic stress. To unlock the potential of CDPK as a modulator of plant stress resistance, increased expression, but not conformational modifications, is needed. Compared with the native form of the gene, site-directed mutagenesis of the pseudosubstrate autoinhibitory subdomain of AtCPK1 has a more powerful effect on (1) resistance not only to osmotic stress but also to temperature stress; (2) biosynthesis of phytoalexins; and (3) metabolism and homeostasis of ROS and other signalling molecules. Even less important is the possibility of a more detailed study of the functions of CDPK by analogy with MAPK4. The acquisition of a constitutively active form made it possible to clarify functions (Colcombet et al. 2013) that remained in the shadows when standard approaches were used (Petersen et al. 2000).

Modified CDPKs activate the biosynthesis of secondary metabolites of various groups, mainly polyphenols. On the one hand, this effect is important as an indicator of the plant's enhanced defense status. Polyphenols play crucial roles in

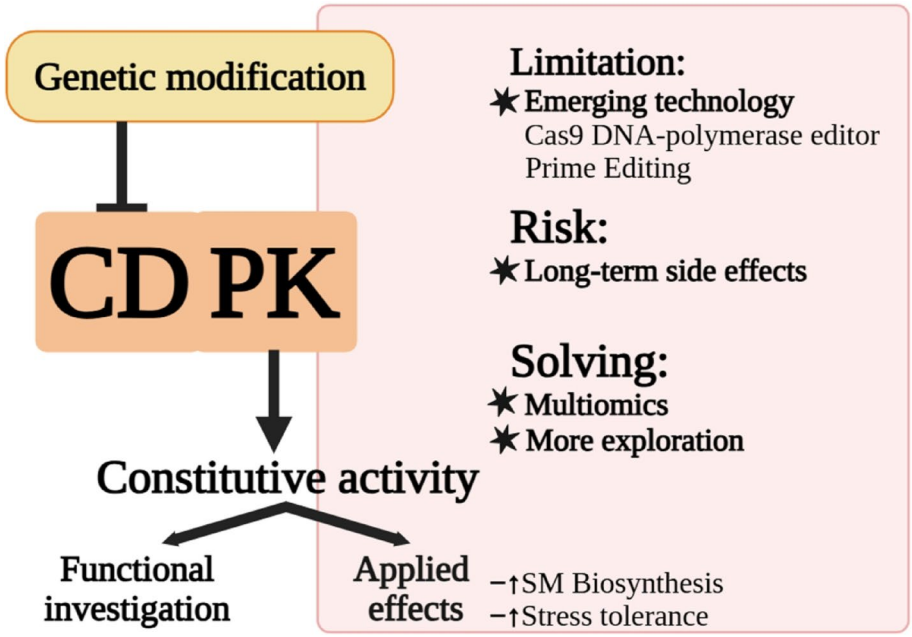

Fig. 5 Future prospects and challenges of CDPKs in gene editing and synthetic biology. Shutdown of the calcium dependence via genetic modification provides the constitutive kinase activity of modified CDPK. As a result, we have the opportunity to study the functions of the enzyme in more detail by analogy with the study of the function of MPK4 in the plant defense system. The acquisition of a constantly active form made it possible to clarify functions (Colcombet et al. 2013) that remained in the shadows when standard approaches were used (Petersen et al. 2000). This approach allows for stable activation of the plant's protective status: an increase in the biosynthesis of secondary metabolites and an increase in tolerance to biotic and abiotic stresses. The main limitation for using this approach for crop plants is the lack of a suitable method of genetic modification. However, the active development of Cas9-DNA polymerase editor technology suggests the possibility of using this approach for cultivated plants. The risks associated with the widespread use of modified CDPKs are related primarily to the lack of research on long-term side effects. Integration with multiomics, precision breeding strategies, and active research in this area will help determine the safety level of the proposed approach

adaptation and resistance to abiotic and biotic stress. On the other hand, this approach is highly important for plant cell culture biotechnology. The production of secondary metabolites, including pharmacologically active compounds, in native plants is low (usually less than 1% of dry weight) and mainly depends on the physiological state and developmental stage of the plant (Dixon 2001). An increase in the production and accumulation of secondary metabolites occurs in plants when they are exposed to various stresses. The plant's hormonal system perceives signals and triggers the plant's adaptation to the environment, including the biosynthesis of a certain group of secondary metabolites. Plant cell culture technology can provide efficient and sustainable sources of phytochemicals with reduced energy and carbon footprints (Krasteva et al. 2021). Research is actively underway on new approaches to increase the productivity of cell cultures via genetic engineering methods. Efforts have led to the development of transgenic plant crops, which are viable and suitable systems for the production of compounds of pharmaceutical importance, giving rise to the terms "biopharmaceuticals" and "molecular farming" (Ma et al. 2003). As shown and described above, modified, constantly active CDPK has a powerful effect on the biosynthesis of various groups of secondary metabolites without inhibiting the growth of cultures. The overexpression of the native form had a significantly weaker effect.

Shutdown of the calcium dependence via genetic modification provides the constitutive kinase activity of the modified CDPK (Fig. 5). As a result, we have the opportunity to study the functions of the enzyme in more detail by analogy with the study of the function of MPK4 in the plant defense system. The acquisition of a constantly active form made it possible to clarify functions (Colcombet et al. 2013) that remained in the shadows when standard approaches were used (Petersen et al. 2000). Thus, using a modified form, it was possible to shed light on ROS-dependent regulation, SAR-LAR antagonism, and regulation of the response to temperature stress. However, there remains a wide field for research into the ability of CDPK to be independent of calcium fluctuations. Modulation of the defense system by modifying CDPK may become a universal approach for obtaining highly resistant and productive varieties of agricultural plants.

Limitations in the use of constitutively active CDPKs

Shutdown of the calcium dependence via genetic modification provides the constitutive kinase activity of modified CDPK, a promising tool for agrobiotechnology. Multiplex genome editing via CRISPR has transformed areas of biotechnology, such as plant engineering for agricultural applications and metabolic pathway engineering for industrial biomanufacturing (Pacesa et al. 2024). Genetically edited organisms, including crop plants, are not classified as GMOs. Moreover, various approaches for genetic editing are currently being actively studied to obtain not only gene knockout effects but also the introduction of specific point mutations (Chen et al. 2019). However, the state of the art of such genetic editing techniques is still far from large-scale use. Thus, the main limitation of using this approach for crop plants is the lack of a suitable method of genetic modification.

Two prominent examples of emerging techniques can potentially enable successful editing of CDPK to disable the dependence on calcium ion fluctuations (Fig. 5). One of them, ‘‘search-and-replace’’ genome editing technology or prime editing (PE), was developed in human cells. This approach can mediate 12 possible base-to-base conversions via reverse transcriptase (RT) paired with the CRISPR–Cas9 nickase and a prime editing guide RNA (Anzalone et al. 2019). On the basis of this approach, a method for editing plants was developed. A wide variety of single or multiple nucleotide edits at several target sites were successfully introduced by prime editing in rice (Xu et al. 2020). In contrast to PEs, which use a Cas9 nickase and an RT fused to the RT to introduce alterations at the target site RNA template utilising a portion of the guide RNA as a template, novel strategies that use DNA polymerases to introduce specific mutations into the genome are being investigated. A designed error-prone DNA polymerase linked to a Cas9 nickase variant was utilised in an early attempt to diversify nucleotides continuously within a tunable window of up to 350 nucleotides from a target site. A more recent study showed that a tethered linear DNA template can be used to induce modifications at a Cas9-nicked spot utilising a phage-derived DNA polymerase. Unlike RT-based prime editing, this method allows for longer insertions of more than 100 nucleotides and circumvents autoinhibitory intramolecular base pairing inside the guide RNA. Another new method, called “click editing”, uses Cas9 in conjunction with HUH endonucleases and DNA-dependent DNA polymerases (DDPs) to introduce a variety of genome edits, such as small insertions and deletions and all single-nucleotide changes. By utilising the bioconjugation activity of HUH endonucleases, the procedure covalently affixes “click DNA” templates to a HUH-nCas9-DDP protein fusion. This method prevents unwanted insertions while enabling accurate genome editing with few indels. The ability of DNA polymerase-based editing methods to produce a broad range of genetic changes, providing a high degree of control and a variety of results, makes them unique (Pacesa et al. 2024).

Integration with multiomics and precision breeding strategies

The uniqueness of CDPKs lies in their self-sufficiency: CDPKs are able to bind directly to calcium ions, bypass intermediaries, and activate specific target proteins through substrate-specific phosphorylation. Modification of the pseudosubstrate autoinhibition subdomain blocks the enzyme's dependence on stress-induced calcium fluctuations, rendering it constitutive. This mimicry of stable stress exposure leads to an increased sensitivity threshold to adverse external stimuli. To recommend a modified form of AtCPK1 as a bioengineering tool or to use such a modification as an editing platform, the molecular mechanism of action must be transparent. However, we understand that there is currently insufficient information about the positive and negative effects of this approach. In our opinion, the use of modified constitutively active forms of CDPK genes is of high scientific and applied interest. With this review, we would like to draw attention to research in this direction. However, the active development of Cas9-DNA polymerase editor technology suggests the possibility of using this approach for cultivated plants.

Large portions of the genomes of key crops are set, and genetic variability has significantly decreased owing to thousands of years of guided evolution through breeding, which limits the possibility of enhancing numerous qualities. The main methods of crop improvement in precision breeding strategies of modern agriculture are currently crossing, mutation breeding and transgenic breeding. Mutation breeding has expanded genetic variation by introducing random mutations via chemical mutagens or physical irradiation. However, these procedures are restricted by their stochastic nature, and generating and screening large numbers of mutants are challenging. Such time-consuming, laborious, untargeted breeding programs cannot keep pace with the demands for increased crop production, even if marker-assisted breeding approaches are adopted to increase selection efficiency. Transgenic breeding, which generates desired traits through the transfer of exogenous genes into elite background varieties, can overcome the bottleneck of reproductive isolation. However, the commercialisation of genetically modified crops is limited by long and costly regulatory evaluation processes as well as by public concerns (Chen et al. 2019).

The risks associated with the widespread use of modified CDPKs are related primarily to the lack of research on long-term side effects. Integration with multiomics, precision breeding strategies, and active research in this area will help determine the safety level of the proposed approach. However, calcium dependence prevents the effective use of CDPKs in plant bioengineering and prevents comprehensive investigations of their functions. Moreover, the multiplicity of feedback control loops makes it difficult to analyse individual components of calcium signalling as well as the system as a whole. In terms of the calcium signalling components used for bioengineering, CDPKs are distinct and exciting. We emphasise the function of CDPK in the plant defense system in this review. To scale the involvement of calcium and calcium decoders, we attempted to schematise the multilevel plant defense mechanism against biotic and abiotic stress factors. Additionally, we attempted to highlight the potential use of CDPK intradomain alterations as a bioengineering tool as well as for a thorough examination of the underlying features. Over the past decade, genome editing technologies have developed rapidly. As a result, gene knockout via CRISPR/Cas9 technology has become a simple and accessible way to modulate plant properties. However, edited plants do not fall under the definition of GMOs. We hope that site-directed gene modification via a similar approach will soon become readily available. This approach makes inactivation of the autoinhibitory domain of CDPK at the level of genome editing possible.

Conclusion

Calcium-dependent protein kinases are unique among calcium decoders in that they can modulate the response by activating critical defense system components on the basis of calcium levels. However, calcium dependence prevents the effective use of CDPKs in plant bioengineering and prevents comprehensive investigations of their functions. We emphasise the function of CDPK in the plant defense system in this review. To scale the involvement of calcium and calcium decoders, we attempted to schematise the multilevel plant defense mechanism against biotic and abiotic stress factors. Additionally, we attempted to highlight the potential use of CDPK intradomain alterations as a bioengineering tool as well as for a thorough examination of the underlying features. Over the past decade, genome editing technologies have developed rapidly. As a result, gene knockout via CRISPR/Cas9 technology has become a simple and accessible way to modulate plant properties. However, edited plants do not fall under the definition of GMOs. We hope that site-directed gene modification via a similar approach will soon become readily available. This approach makes inactivation of the autoinhibitory domain of CDPK at the level of genome editing possible. However, we understand that there is currently insufficient information about the positive and negative effects of this approach. In our opinion, the use of modified constitutively active forms of CDPK genes is of high scientific and applied interest. With this review, we would like to draw attention to research in this direction.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Consent to participate Not applicable.

Ethics approval Not applicable.

Consent for publication The authors of this work agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Conflict of interest The authors declare that they have no competing interests.

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