REVIEW ARTICLE

Decoding Cold-Induced Ca^{2+} Signals: the CaM/CML-CBP Sensor Network in Plant Cold Tolerance and Its Functional Dissection by CRISPR-Cas9/Cas12a Genome Editing

Soheila Aghaei Dargiri[1,2] **and Ali Movahedi**[1,*]

[1] State Key Laboratory of Tree Genetics and Breeding, College of Life Sciences, Nanjing Forestry University, Nanjing 210037, China
[2] Department of Biological Control Research, Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

Abstract

Cold stress limits the geographical distribution and the yield of crops, and climate variability is raising the frequency of chilling and freezing events. Within seconds of cold perception, plant cells generate cytosolic Ca^{2+} transients, the so-called Ca^{2+} signatures, whose information content is read by Ca^{2+} sensor proteins. This review synthesises current knowledge of the sensor network formed by calmodulins (CaMs), calmodulin-like proteins (CMLs) and their downstream calmodulin-binding proteins (CBPs), with a focus on cold stress. Because the literature mixes genetically validated interactions with plausible but untested ones, every reported interaction is graded here as validated [V], inferred [I] or hypothetical [H]. We argue that cold-associated Ca^{2+} decoding is better described as a distributed network than as a linear CaM-CML-CBP cascade. The strongest genetic support exists for the CAMTA-CBF module that lies downstream of Ca^{2+}/CaM; by contrast, placing CRLK proteins upstream of the MEKK1-MKK2-MPK4 pathway, and linking that branch to ICE1 turnover, remains a working model assembled from separate studies. Evidence for CBL-CIPK and CML-dependent branches is thinner and context dependent. We then discuss how CRISPR-Cas9, Cas12a and transcriptional-regulation tools can be used to test these gaps, bearing in mind that editing outcomes, pleiotropy and regeneration capacity differ between species and target loci.

Keywords: Calcium signalling, calmodulin, calmodulin-like protein, calmodulin-binding protein, cold stress, CRISPR-Cas9, CRISPR-Cas12a.

1 Introduction

Low temperature is one of the most consequential abiotic constraints on crop production: it damages tissues directly, delays development and lowers yield

Submitted: 03 August 2026
Revised: 16 October 2026
Accepted: 18 October 2026
Published: 18 November 2026

Vol. 2, **No.** 1, 2027.
10.62762/PIJ.2026.351999

***Corresponding author:**
✉ Ali Movahedi
ali_movahedi@njfu.edu.cn

Citation
Aghaei Dargiri, S., & Movahedi, A. (2026). Decoding Cold-Induced Ca^{2+} Signals: the CaM/CML-CBP Sensor Network in Plant Cold Tolerance and Its Functional Dissection by CRISPR-Cas9/Cas12a Genome Editing. *Plant Innovation Journal*, 2(1), 1–12.

© 2026 by the Authors. Published by Institute of Central Computation and Knowledge. This is an open access article under the CC BY license (https://creativecommons.org/licenses/by/4.0/).

in cereals, horticultural crops and vegetables [1, 2]. Plants counter cold through cold acclimation, in which exposure to non-freezing temperatures reprograms gene expression - most prominently through the C-repeat binding factor (CBF) regulon - and thereby raises freezing tolerance [3, 4]. This reprogramming begins with Ca^{2+}. Cold triggers a rapid rise in cytosolic Ca^{2+} that draws on both extracellular and intracellular pools [5], and Ca^{2+} signatures of this kind are read by sensor proteins that convert them into phosphorylation events and transcriptional output, a principle described for abiotic stress in general and for cold in particular [6–8].

Three sensor families carry out this decoding: (i) calmodulins (CaMs) and the extended family of calmodulin-like proteins (CMLs), which relay Ca^{2+} binding to downstream targets [9, 10]; (ii) calcineurin B-like proteins (CBLs) together with their CBL-interacting protein kinases (CIPKs); and (iii) Ca^{2+}-dependent protein kinases (CDPKs/CPKs), which combine sensor and effector in a single polypeptide. All three classes contain EF-hand Ca^{2+}-binding proteins, which have been catalogued in *Arabidopsis* [11]. The downstream effectors of Ca^{2+}/CaM, collectively the calmodulin-binding proteins (CBPs), include transcription factors, receptor-like kinases, metabolic enzymes and ion transporters, and they execute the cellular response [12–14].

Two gaps remain. The field has no evidence-graded synthesis of how these sensor and effector classes cooperate under cold, and - for translational work - no concrete plan for testing the network by genome editing. This review addresses both. We first define the molecular players and introduce an explicit grading scheme, then review the decoding events for which genetic evidence exists, present an integrated network model, compare sensor repertoires across species, set out the contradictions that remain, and finally show how CRISPR-Cas9 and CRISPR-Cas12a, together with base editors, prime editors and dCas9 effectors, can be deployed against this network in a hypothesis-driven editing campaign.

2 The molecular toolbox for Ca^{2+} decoding

2.1 Cold-induced Ca^{2+} signatures

Cold perception begins at the plasma membrane (Figure 1). In rice, the G-protein regulator *COLD1* interacts with the Gα subunit *RGA1* to activate Ca^{2+} channels; *cold1* loss-of-function plants show a reduced cold-induced Ca^{2+} influx and chilling sensitivity [V] [15]. In *Arabidopsis thaliana*, the mechanosensitive channels *MCA1* and *MCA2* mediate cold-induced cytosolic Ca^{2+} increases, and *mca1 mca2* double mutants are impaired both in the Ca^{2+} rise and in freezing tolerance [V] [16]. Cyclic nucleotide-gated channels (CNGCs) and glutamate receptor-like channels (GLRs) are implicated in stress-evoked Ca^{2+} entry and are proposed to contribute to cold-evoked Ca^{2+} signals, whereas the individual contribution of most CNGC members is still unresolved [I] [6]. In tomato, *GLR3.3* and *GLR3.5* are required for cold acclimation-induced chilling tolerance through apoplastic H_2O_2 production and redox homeostasis [V] [17]; whether they act through cold-evoked Ca^{2+} entry remains inferred [I]. The signature itself - its amplitude, duration and frequency - is shaped by efflux systems (autoinhibited Ca^{2+}-ATPases, ACAs; Ca^{2+}/H^+ exchangers, CAXs) and is thought to encode stimulus information [I] [18].

2.2 Calmodulins and calmodulin-like proteins

CaMs are highly conserved proteins of about 148 amino acids carrying four EF-hand Ca^{2+}-binding motifs in two globular lobes; Ca^{2+} binding exposes hydrophobic surfaces that engage target proteins [9, 12]. *A. thaliana* has seven CaM genes encoding four protein isoforms, whereas the CML family has expanded to roughly 50 members [9, 11, 13]. CMLs keep the EF-hand architecture but diverge in sequence, particularly in their terminal domains, and like CaMs they occur only in plants and protists [10, 11]. Phylogenetically, CaMs form a tight clade at the base of the CML radiation, which subdivides into several subgroups [9]. Such an expansion points to functional specialisation alongside partial redundancy, with direct consequences for the design of genome-editing experiments.

2.3 Calmodulin-binding proteins

CBP here means calmodulin-binding protein in the strict sense: a protein that interacts with Ca^{2+}-loaded CaM (or with CMLs) and consequently changes its activity, localisation or stability. Genome-wide protein microarrays identified 173 candidate CBPs in *A. thaliana*, among them transcription factors, kinases, phosphatases and metabolic enzymes [I] [14]. A few CBP classes are functionally characterised, chiefly the calmodulin-binding transcription activators (CAMTAs) and the Ca^{2+}/CaM-regulated receptor-like kinases (CRLKs). For most predicted CBPs, and for nearly all CML targets, no in planta function under

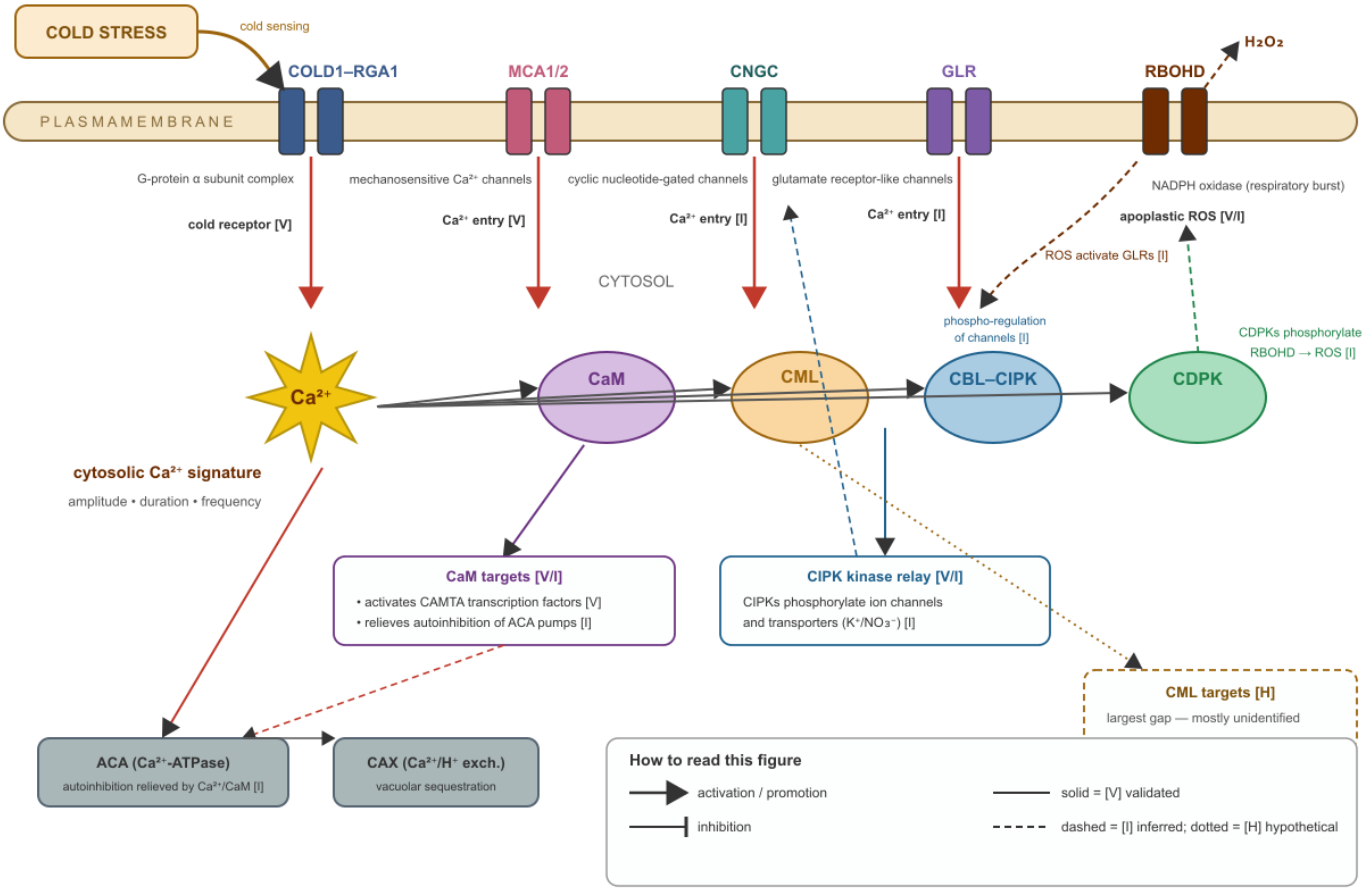


Figure 1. Cold perception at the plasma membrane and generation of the cytosolic Ca^{2+} signature. Cold is sensed by the COLD1-RGA1 G-protein complex [V], which promotes Ca^{2+} influx, and by the mechanosensitive channels MCA1 and MCA2 [V]; CNGC and GLR channels also contribute to cold-evoked Ca^{2+} entry [I]. Cytosolic Ca^{2+} is read in parallel by four sensor systems: (i) CaM, which activates CAMTA transcription factors and relieves the autoinhibition of ACA Ca^{2+}-ATPases [V/I]; (ii) CMLs, whose physiological targets are largely unidentified [H]; (iii) the CBL-CIPK relay, whose CIPKs phosphorylate ion channels and transporters [I]; and (iv) CDPKs, which phosphorylate substrates including RBOHD, promoting apoplastic H_2O_2 accumulation that feeds back onto GLR channels [I]. The signature is terminated by Ca^{2+} efflux through ACA pumps and by vacuolar sequestration through CAX exchangers. Arrows denote activation; solid lines mark validated [V] interactions, dashed lines inferred [I] and dotted lines hypothetical [H] ones.

cold has been demonstrated; this is the largest gap in the field.

2.4 An evidence-grading scheme

To separate direct evidence from interpretation, each interaction discussed below carries one of three grades. V (validated) denotes reproducible genetic perturbation and/or functional evidence in planta under cold or closely related low-temperature conditions. I (inferred) denotes biochemical interaction, reporter, expression, localisation, pharmacological or correlative evidence without direct functional validation in a cold context. H (hypothetical) denotes a plausible connection proposed for testing but without supporting data. Where evidence comes from a species other than the one under discussion, the statement is presented as an orthology-based inference rather than as demonstrated conservation. The grades are applied consistently in Figure 2 and Table 1, and they refer to the specific interaction or phenotype stated, not to the general importance of a protein family.

3 Decoding events in cold stress: validated and inferred links

3.1 The Ca^{2+}/CaM-CAMTA-CBF module

CAMTAs are Ca^{2+}/CaM-regulated transcription factors that bind the CM2 motif (CCGCGT) in CBF promoters [19]. The *camta1 camta3* double mutant shows reduced CBF induction and impaired freezing

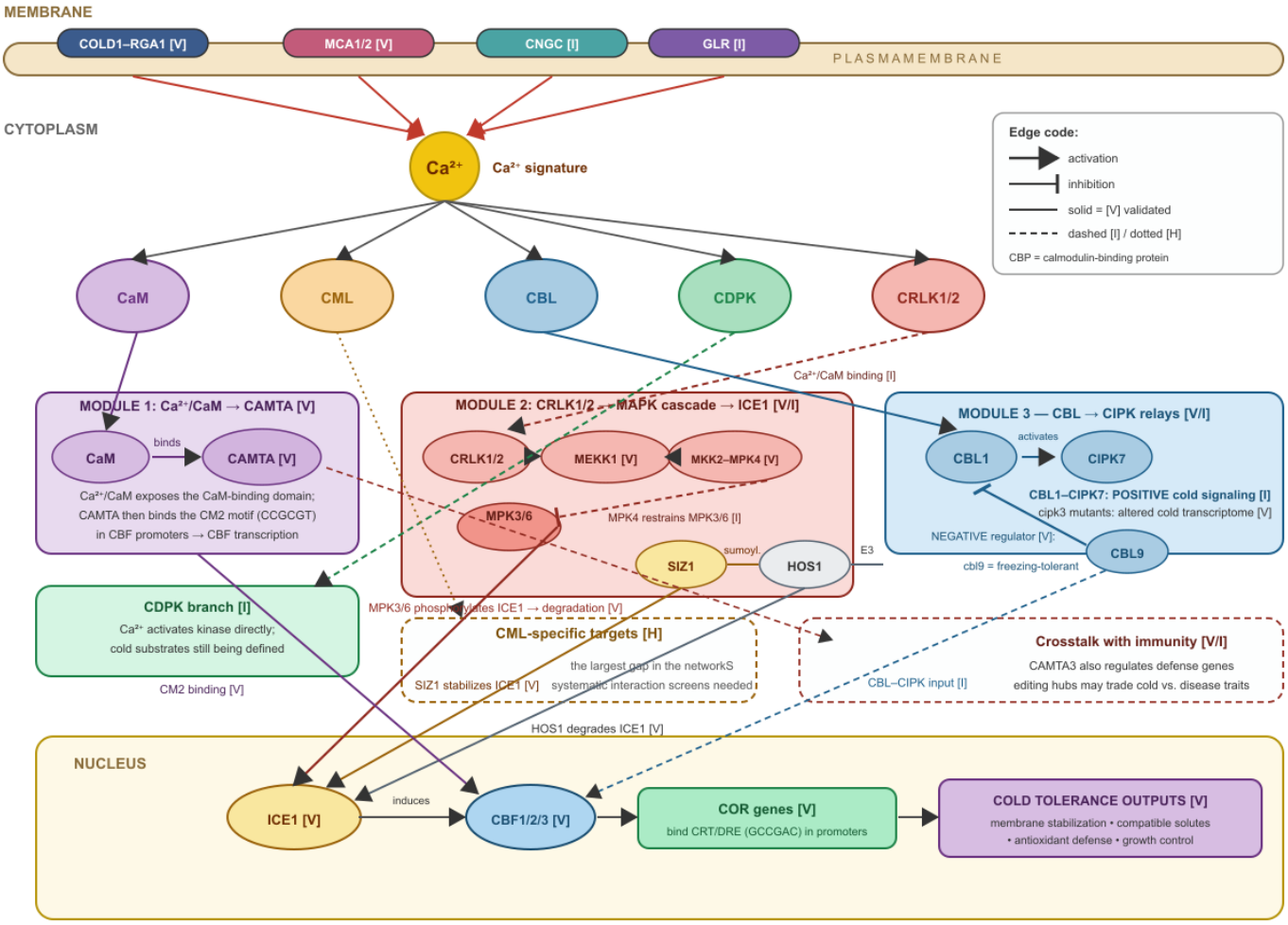


Figure 2. Interaction map of the CaM/CML-CBP decoding network under cold stress. Three modules integrate the Ca^{2+} signal onto the CBF regulon. Module 1 [V]: Ca^{2+}-loaded CaM binds the CaM-binding domain of CAMTA transcription factors, which then bind the CM2 motif (CCGCGT) in the *CBF1/2/3* promoters. Module 2 [I]: the proposed route from CRLK1 (CRLK2 untested) through MEKK1-MKK2-MPK4 to MPK3/MPK6-dependent ICE1 turnover (Section 3.2); ICE1 stability is additionally set by SIZ1-mediated SUMOylation, which stabilises it, and HOS1-mediated ubiquitination, which promotes its degradation. Module 3 [V/I]: CBL1-CIPK7 [I] is associated with the cold response, whereas CBL9 [V] acts negatively and its knockout enhances freezing tolerance; the CDPK branch [I] and CML-specific targets [H] remain undefined. CAMTA3 also regulates immune-response genes (dashed box), so perturbing CaM-CAMTA hubs may entail cold-immunity trade-offs. In the nucleus, ICE1 and CAMTAs promote *CBF1*, *CBF2* and *CBF3* expression; CBF proteins bind CRT/DRE elements and activate cold-responsive genes acting in membrane stabilisation, compatible-solute accumulation and antioxidant defence. Edge code: arrows, activation; T-bars, inhibition; solid, validated [V]; dashed, inferred [I]; dotted, hypothetical [H]. CBP, calmodulin-binding protein.

tolerance [19], and CAMTA1 and CAMTA2 act together with CAMTA3 in the cold induction of *CBF1*, *CBF2* and *CBF3* [20]; together these results make CAMTA activity a genetically validated link to the central cold regulon [V]. That this link depends on Ca^{2+}/CaM in the cold is inferred from CaM regulation of CAMTAs [I] [7]. CAMTA3 additionally acts in Ca^{2+}/CaM-regulated, salicylic acid-mediated immunity [21], which shows how a single CaM-effector hub can serve more than one signalling context.

3.2 The CRLK1-MAPK module and control of ICE1

CRLK1 is a plasma-membrane receptor-like kinase with two CaM-binding sites, and CRLK2 is its closest paralogue. The studies cited here address CRLK1 only, so any role of CRLK2 in this module is hypothetical [H]. In *A. thaliana*, genetic and biochemical data indicate that CRLK1 contributes to cold-responsive gene expression and freezing tolerance and can interact with MEKK1 [22, 23]. The MEKK1-MKK2-MPK4 pathway has also been implicated in cold signalling and in the

Table 1. Master evidence table of Ca^{2+}-decoding components under cold stress.

Protein (gene)	Species	Interaction/target	Cold phenotype	Level	Reference
COLD1-RGA1	*Oryza sativa*	G-protein-coupled Ca^{2+} channel activation	Reduced cold-induced Ca^{2+} influx; chilling sensitivity (*cold1*)	[V]	[15]
MCA1/MCA2	*A. thaliana*	Mechanosensitive Ca^{2+} channels	Reduced cold-induced $[Ca^{2+}]_{cyt}$; freezing sensitivity (*mca1 mca2*)	[V]	[16]
Ca^{2+}/CaM	*A. thaliana*	CAMTA transcription factors	CAMTA activity is required for normal *CBF* induction; the CaM-dependence of this step in the cold is inferred	[I]	[7, 19]
CAMTA1/2/3	*A. thaliana*	CM2 (CCGCGT) motif of *CBF* promoters	Impaired cold acclimation and freezing sensitivity (*camta1 camta3*); CAMTA1/2 act with CAMTA3 in *CBF* induction	[V]	[19, 20]
CRLK1	*A. thaliana*	Ca^{2+}/CaM-regulated receptor-like kinase with two CaM-binding sites	*crlk1* shows impaired cold-responsive gene induction and increased chilling/freezing sensitivity	[V]	[22, 23]
CRLK1-MAPK-*ICE1* module (*CRLK2* untested)	*A. thaliana*	CRLK1 interacts with MEKK1 (biochemical evidence); proposed route from CRLK signalling through MEKK1-MKK2-MPK4 to MPK3/MPK6-dependent ICE1 turnover	Integrated pathway not established in a single genetic framework	[I]	[23, 24]
CBL1-CIPK7	*A. thaliana*	CIPK kinase relay	CBL1 interacts with CIPK7; cold induction of *CIPK7* is influenced by CBL1; role in the cold response proposed	[I]	[25]
CBL9	*A. thaliana*	Target unknown	Enhanced freezing tolerance (*cbl9*); negative regulator	[V]	[26]
CIPK3	*A. thaliana*	Cold/ABA transcriptome regulation	Altered cold-responsive gene expression (*cipk3*)	[V]	[27]
CML24 (*TCH2*)	*A. thaliana*	Targets unknown	Touch-/cold-induced; pleiotropic ABA, daylength and ion-homeostasis phenotypes	[I]	[28]
CML12 (*TCH3*)	*A. thaliana*	Targets unknown	Touch-responsive stress-related CML; cold-specific role untested	[I]	[29]
CDPK family	*A. thaliana*/*O. sativa*	Direct phosphorylation of targets	Cold-responsive; contribution to cold tolerance; substrates largely undefined	[I]	[7]
GLR3.3/GLR3.5	*Solanum lycopersicum*	Apoplastic H_2O_2; redox homeostasis	Required for cold acclimation-induced chilling tolerance	[V]	[17]
SlCaM6	*Solanum lycopersicum*	Binds ICE1 (Ca^{2+}-independent interaction) and attenuates its transcriptional activity	*CaM6* overexpression reduces, and knockdown increases, chilling tolerance; negative regulator	[V]	[30]

control of MPK3/MPK6 activity, and MPK3/MPK6 phosphorylate ICE1 and promote its degradation [24]. What has not been resolved in a single genetic framework is the complete causal chain running from cold-induced Ca^{2+}/CaM perception through CRLK1 to MAPK activity, ICE1 stability and CBF

transcription. We therefore treat the CRLK1-MEKK1 interaction as biochemical evidence only [I] and the MPK3/MPK6-ICE1 relationship as validated [V], while the integrated CRLK1-MAPK-ICE1 route is graded inferred [I] in Figure 2 and Table 1. CRLK paralogue specificity, pathway directionality and the quantitative contribution of CRLK-dependent signalling to the cold regulon all remain open.

3.3 CBL-CIPK relays and CDPKs

CBL-CIPK modules and CDPK/CPK proteins offer further routes for reading cold-associated Ca^{2+} signals. In *A. thaliana*, CIPK3 affects ABA- and cold-responsive transcriptional output, which places it at a cross-talk point between the two pathways [27]. CBL1 interacts with CIPK7, and the cold induction of *CIPK7* is influenced by CBL1, which implicates the module in the cold response, although the relevant substrates and the position of the module relative to the CBF pathway are unresolved [25]. CBL9, in contrast, has been reported as a negative regulator of freezing tolerance, so the sign of the response is a property of the individual module rather than of the CBL-CIPK system as a whole [26]. Several *CDPK/CPK* genes are cold responsive and have been linked to cold tolerance, but cold-specific substrates and causal relationships with the CBF regulon are still poorly defined [7]. Figure 2 and Table 1 therefore grade the CIPK3 and CBL9 phenotypes as validated, and the broader CBL1-CIPK7 branch and the CDPK/CPK-to-CBF connections as inferred [I].

4 The CaM/CML-CBP system is a decoding network, not a linear cascade

The evidence does not support a linear cascade in which CaM activates CMLs, which in turn activate CBPs. CaMs and CMLs are parallel sensor families with largely distinct target preferences, and CBPs are direct targets of Ca^{2+}/CaM - and, where tested, of CMLs. A decentralised decoding network fits the data better (Figure 2): several sensors (CaM, CML, CBL, CDPK) read the same Ca^{2+} signature at the same time; effector modules of differing validation status (CAMTA-CBF [V]; CRLK-MAPK-ICE1 [I]; CBL-CIPK [V/I]) integrate these inputs onto the CBF regulon; and the resulting transcriptional output (*COR* genes) confers tolerance. Several edges are inferred or hypothetical, most conspicuously the identity of CML-specific targets [H], and are annotated as such. The distinction has practical consequences: a linear cascade would predict clean, interpretable single-gene knockouts, whereas a network predicts redundancy and partial phenotypes.

5 Comparative view of Ca^{2+}-decoding repertoires

To identify candidates for functional validation, we compare the information available for *A. thaliana*, *Oryza sativa* and *Solanum lycopersicum* (Figure 3). The depth of evidence is uneven. Most genetically resolved decoding modules come from *A. thaliana*; the COLD1-RGA1 perception module is supported in rice [15]; and in tomato the evidence covers GLR3.3 and GLR3.5 [17] and the CaM6-ICE1 module, in which CaM6 binds ICE1 in a Ca^{2+}-independent interaction and attenuates *COR* gene expression, so that CaM6 acts as a negative regulator of chilling tolerance [30]. Similarity in gene-family size, sequence or expression response is a basis for prioritising candidates, not evidence of conserved function. Each crop annotation in Figure 3 therefore states whether it rests on direct functional evidence, on transcript-level responsiveness, or on orthology. Rice-specific nodes such as COLD1 need to be tested in rice, and modules transferred from *A. thaliana* to tomato or other crops require species-specific validation before being proposed as breeding or editing targets.

The CML family remains the largest gap, because most members have neither a validated target nor a demonstrated cold-specific function. In *A. thaliana*, *CML24/TCH2* transcripts are induced by touch and cold [28], and *CML12/TCH3* is a touch-responsive CML discussed among stress-related Ca^{2+} sensor hubs [29]; yet responsiveness alone neither establishes a role in cold tolerance nor identifies downstream effectors. They are therefore well-suited candidates for loss-of-function, combinatorial and complementation studies rather than established cold-tolerance determinants. Table 1 lists the strongest protein-phenotype links, with the evidence level for each assignment.

5.1 Contradictions and open questions

Four observations complicate any simple picture. First, CBL-CIPK members may carry different signs, as set out in Section 3.3: CBL9 acts as a negative regulator, whereas CBL1-CIPK7 is associated with the cold response, so the family cannot be treated as a uniform input and editing different members may produce different or opposite phenotypes. Second, CAMTA3 acts both in cold-responsive transcription [19, 20] and in Ca^{2+}/CaM-regulated immunity [21, 31], which

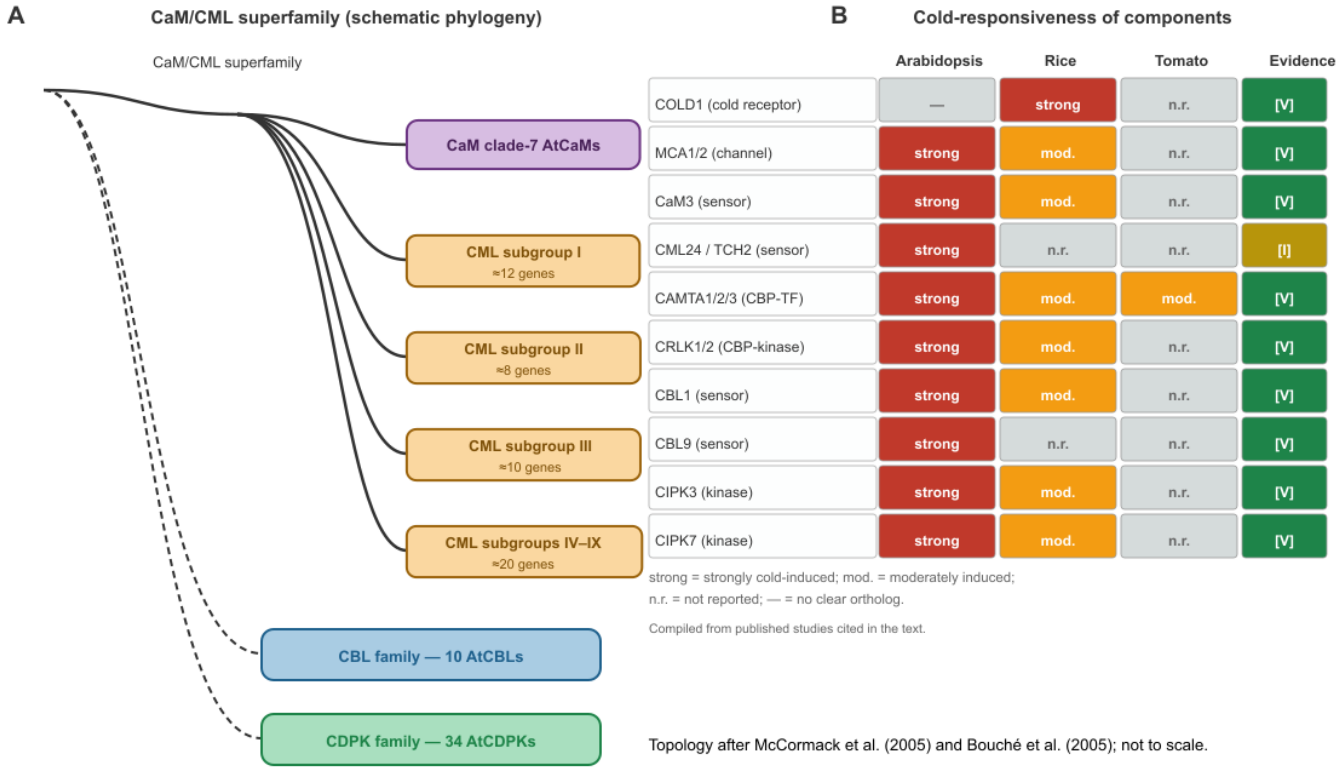


Figure 3. Comparative view of Ca^{2+}-decoding components. (A) Schematic phylogeny of the CaM/CML superfamily: the seven canonical *A. thaliana* CaMs form a tight clade basal to the CML radiation, which subdivides into several subgroups; the CBL and CDPK families are shown for comparison (topology after [9, 13]; branch lengths not to scale). (B) Cold-responsiveness of representative components in *A. thaliana*, rice and tomato, compiled from published transcriptomic and functional studies: strong, strongly cold-induced; mod., moderately induced; n.r., cold-responsiveness not reported; -, no clear orthologue. The right-hand column gives the evidence grade ([V]/[I]) for the component's role in cold signalling.

raises the agronomically relevant question of whether editing CaM-effector hubs trades cold tolerance against disease resistance. Third, the specificity problem is unsolved: it is not known whether different cold regimes produce distinct Ca^{2+} signatures read by distinct sensor sets, or whether specificity arises mainly downstream [18]. Fourth, the size of the CML family relative to its handful of characterised members implies either extensive redundancy or highly specialised roles; either way, single-gene knockouts are unlikely to suffice and multiplex editing is indicated.

6 CRISPR-Cas9/Cas12a-based functional dissection

6.1 Editing platforms and derivative tools

CRISPR-Cas9 and CRISPR-Cas12a are established plant genome-editing platforms, but their performance depends on the target locus, guide design, delivery method, regeneration system and crop genotype. SpCas9 recognises an NGG PAM and underpins targeted mutagenesis as well as base editing and programmable transcriptional regulation [32]; prime editing builds on the same Cas9 scaffold. Cas12a recognises T-rich PAM sequences and generates staggered double-strand breaks [33]. It also processes its own crRNA arrays, which makes it attractive for multiplexed editing wherever compatible target sites exist. Neither platform guarantees efficient editing, predictable repair outcomes or uniform performance across species. Off-target edits and other technical bottlenecks are recognised limitations of CRISPR/Cas editing in plants [34]. On-target structural variation, chimerism, genotype-specific transformation and regeneration limits, and editing efficiencies likewise require empirical validation in each campaign. Ribonucleoprotein (RNP) delivery can reduce persistent nuclease expression and transgene integration, yet it does not remove the need for thorough molecular characterisation. Catalytically inactive Cas proteins fused to activator or repressor

Table 2. Prioritised CRISPR-Cas9/Cas12a editing campaign for the CaM/CML-CBP network.

Target (gene set)	Tool and strategy	Network rationale	Primary readout
CML12 + *CML24* (single → double → with paralogues)	Cas9 multiplex sgRNAs; Cas12a crRNA array	Tests sensor necessity and redundancy (H1)	*CBF*/*COR* induction; freezing tolerance (electrolyte leakage, F_v/F_m)
CAMTA3	Cas9 knockout + dCas9-VPR activation + dCas9-SRDX repression	Tests hub sufficiency and pleiotropy (H2)	*COR* expression; freezing tolerance; parallel pathogen phenotyping
CBL9	Cas9 knockout	Tests whether loss of *CBL9* raises freezing tolerance in the chosen species and background; contrasts with the *CBL1-CIPK7* branch	Freezing survival; cold-induced Ca^{2+} dynamics; *CBF*-regulon expression
CBL1 + *CIPK7*	Cas12a array multiplex knockout	Tests epistasis with *cbl9*	*CBF* reporter output; kinase activity
CIPK3	Cas9 knockout	Tests the contribution of CIPK3 to cold- and ABA-responsive transcription	Cold-responsive transcript profiling; ABA-response assays; freezing tolerance
CRLK1, *CRLK2*	Cas12a multiplex knockout, with independently validated single and combined mutants	Tests the contribution of each *CRLK* paralogue to cold-induced MAPK activity and ICE1 stability, without presuming a shared pathway	ICE1 abundance; MPK3/MPK6 and MPK4 activity; *CBF*/*COR* expression; freezing tolerance
Cold-responsive *CDPK*/*CPK* candidates	Cas9 knockout panel	Tests whether individual members contribute to cold-associated Ca^{2+} decoding and identifies candidate substrates	Cold phenotyping; phosphoproteomics under cold; *CBF*/*COR* expression
CaM isoforms (seven loci in *A. thaliana*)	Staged multiplex editing, or base/regulatory editing of selected loci	Tests whether partial changes in *CaM* dosage alter cold-associated Ca^{2+} decoding while limiting the pleiotropy of broad *CaM* disruption	Ca^{2+} signature dynamics; *CBF*/*COR* expression; growth; fertility; freezing tolerance
ICE1	Cas9 knockout or targeted base editing	Provides a reference perturbation against which Ca^{2+}-decoding modules can be judged on the *ICE1-CBF* axis	ICE1 abundance; *CBF*/*COR* expression; freezing tolerance

domains offer a way to perturb expression without double-strand breaks, again subject to validation in the chosen species and tissue.

6.2 Matching editing strategy to network architecture

Network architecture should guide strategy, and each approach is best framed as a test rather than as a predicted outcome. For closely related CaM and CML members, multiplex editing can expose redundancy, though the extent of redundancy, the developmental pleiotropy and the editability of each locus have to be determined empirically. At dosage-sensitive loci, regulatory or splice-site editing offers an alternative to complete knockout, provided the edited alleles and their expression consequences are verified. dCas9-based activation or repression complements knockouts by asking whether altered expression of a hub such as CAMTA3 changes cold-response output. Tissue-specific or inducible systems can limit pleiotropy; cold-inducible editing deserves particular caution, because editing kinetics need not match the timescale of acute cold signalling. The strategy matrix in Table 2 is accordingly a framework for hypothesis-driven work, not a universal workflow.

6.3 A prioritised editing campaign

Table 2 sets out candidate perturbations derived from the evidence map in Table 1 and Figure 3. They constitute a proposed agenda, not validated targets. Before deployment in any crop, each target

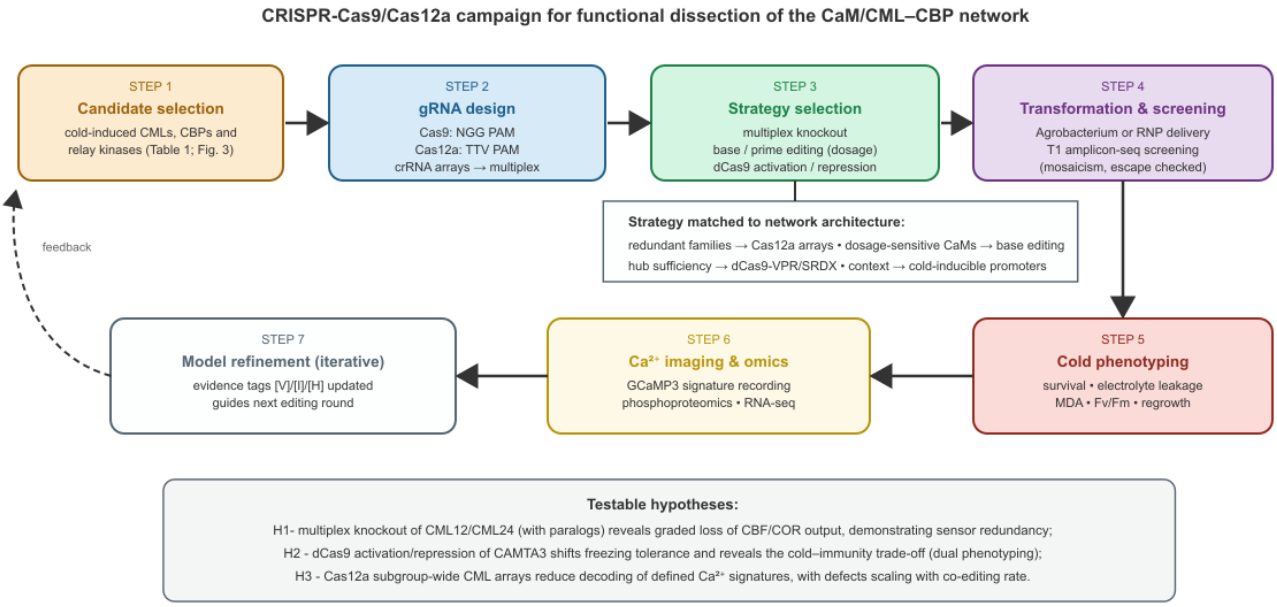


Figure 4. Proposed CRISPR-Cas9/Cas12a campaign for functional dissection of the CaM/CML-CBP network. Step 1, target prioritisation from the evidence map (Table 1; Figure 3). Step 2, guide-RNA design matched to network architecture: Cas9 targets compatible NGG PAM sites, Cas12a targets T-rich PAM sites and processes crRNA arrays for multiplexing. Step 3, strategy selection, including multiplex knockout of candidate redundant CMLs, base or prime editing at dosage-sensitive *CaM* loci, and dCas9-VPR activation or dCas9-SRDX repression at transcriptional hubs. Step 4, *Agrobacterium*-mediated or RNP delivery, followed by molecular validation of edited alleles, assessment of mosaicism and screening for unintended on-target outcomes. Steps 5-7, cold phenotyping: survival, electrolyte leakage, malondialdehyde accumulation, F_v/F_m, GCaMP-based Ca^{2+}-signature recording where available, and phosphoproteomic or RNA-seq readouts. Results iteratively refine the network model and its evidence grades.

needs confirmation of gene identity, expression under the relevant cold regime, paralogue relationships, accessible PAM sites, guide specificity, and the feasibility of transformation or RNP delivery. Primary phenotyping should pair molecular verification of edited alleles with standardised cold-response assays: survival, electrolyte leakage, chlorophyll fluorescence, *CBF*/*COR* expression and, where feasible, dynamic Ca^{2+} imaging. Where growth, fertility or immunity trade-offs are plausible, performance should be assessed across developmental stages and under both controlled and field-relevant conditions. Figure 4 shows the resulting iterative workflow, in which genotype and phenotype data feed back to refine the network model.

6.4 Testable hypotheses

H1: sensor redundancy in CML-mediated cold responses. Multiplex disruption of *CML12*, *CML24* and selected close paralogues may uncover cold-response phenotypes invisible in single mutants. If these CMLs act redundantly, progressively edited genotypes should show altered *CBF*/*COR* induction and freezing tolerance relative to matched single mutants and the wild type. The experiment presupposes verified expression of the selected paralogues in the relevant tissues and cold regime.

H2: CAMTA3 dosage, cold tolerance and immunity. Changing *CAMTA3* expression by CRISPR-based activation or repression may alter cold-responsive transcription and pathogen-response traits. Neither the direction nor the magnitude of the effect can be assumed, since CAMTA3 operates in several signalling contexts. Parallel cold and pathogen phenotyping with transcript analysis would test for a trade-off.

H3: network-level decoding capacity. Editing a defined CML subgroup may change the relationship between cold-evoked Ca^{2+} dynamics and CBF reporter output. Robust Ca^{2+} imaging and reporter systems must first be established in the chosen genotype, and any reduction in reporter output must then be distinguished from pleiotropic developmental or physiological defects.

7 Conclusions

Cold-induced Ca^{2+} signals are read by several sensor and effector modules, a number of which converge

on cold-responsive transcriptional programmes. The evidence favours a network model over a single linear CaM/CML/CBP cascade, and the functions and direct targets of most CMLs are still unknown. CRISPR-Cas9, Cas12a, base editing and transcriptional-regulation platforms provide complementary ways to close these gaps, provided they are applied with species-specific validation, rigorous genotyping and phenotyping for pleiotropy. The framework in Table 2 and Figure 4 is offered as a falsifiable roadmap rather than a prediction of uniform editing outcomes. Causal links between defined Ca^{2+} signatures, sensor combinations and cold-tolerance output will have to be established before these pathways can be translated reliably into crop improvement.

Abbreviations

ABA, abscisic acid; ACA, autoinhibited Ca^{2+}-ATPase; CAX, Ca^{2+}/H^{+} exchanger; CBF, C-repeat binding factor; CBL, calcineurin B-like protein; CBP, calmodulin-binding protein; CDPK/CPK, Ca^{2+}-dependent protein kinase; CIPK, CBL-interacting protein kinase; CML, calmodulin-like protein; CNGC, cyclic nucleotide-gated channel; COR, cold-responsive gene; CRLK, Ca^{2+}/CaM-regulated receptor-like kinase; dCas9, catalytically dead Cas9; GLR, glutamate receptor-like channel; ICE1, inducer of CBF expression 1; MDA, malondialdehyde; MCA, MID1-complementing activity channel; PAM, protospacer-adjacent motif; RGA1, G-protein α subunit; RNP, ribonucleoprotein.

Data Availability Statement

No new data were generated in this review. All data discussed are available in the cited publications.

Funding

The authors thank colleagues at the State Key Laboratory of Tree Genetics and Breeding for discussion. This work received no specific funding.

Conflicts of Interest

Ali Movahedi served as an Editor-in-Chief of the *Plant Innovation Journal* at the time of manuscript submission. To ensure the integrity of the peer-review process, Ali Movahedi was not involved in the editorial handling, peer review, or decision-making process for this manuscript, which was handled independently by another editor. The remaining authors declare no conflicts of interest.

AI Use Statement

The authors declare that no generative AI was used in the preparation of this manuscript.

Ethical Approval and Consent to Participate

Not applicable. This review involved no experiments on humans or animals, and no human participants, human data or animal subjects were used.

References

[1] Yadav, S. K. (2010). Cold stress tolerance mechanisms in plants. A review. *Agronomy for Sustainable Development, 30*(3), 515-527. [CrossRef]

[2] Ding, Y., Shi, Y., & Yang, S. (2019). Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. *New Phytologist, 222*(4), 1690-1704. [CrossRef]

[3] Hannah, M. A., Heyer, A. G., & Hincha, D. K. (2005). A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. *PLoS Genetics, 1*(2), e26. [CrossRef]

[4] Zhu, J. K. (2016). Abiotic stress signaling and responses in plants. *Cell, 167*(2), 313-324. [CrossRef]

[5] Knight, H., Trewavas, A. J., & Knight, M. R. (1996). Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. *The Plant Cell, 8*(3), 489-503. [CrossRef]

[6] Wilkins, K. A., Matthus, E., Swarbreck, S. M., & Davies, J. M. (2016). Calcium-mediated abiotic stress signaling in roots. *Frontiers in Plant Science, 7*, 1296. [CrossRef]

[7] Yuan, P., Yang, T., & Poovaiah, B. W. (2018). Calcium signaling-mediated plant response to cold stress. *International Journal of Molecular Sciences, 19*(12), 3896. [CrossRef]

[8] Li, Y., Liu, Y., Jin, L., & Peng, R. (2022). Crosstalk between Ca^{2+} and other regulators assists plants in responding to abiotic stress. *Plants, 11*(10), 1351. [CrossRef]

[9] McCormack, E., Tsai, Y. C., & Braam, J. (2005). Handling calcium signaling: arabidopsis CaMs and CMLs. *Trends in plant science, 10*(8), 383-389. [CrossRef]

[10] Bender, K. W., & Snedden, W. A. (2013). Calmodulin-related proteins step out from the shadow of their namesake. *Plant physiology, 163*(2), 486-495. [CrossRef]

[11] Day, I. S., Reddy, V. S., Shad Ali, G., & Reddy, A. S. N. (2002). Analysis of EF-hand-containing proteins in *Arabidopsis*. *Genome Biology, 3*(10), research0056-1. [CrossRef]

[12] Zielinski, R. E. (1998). Calmodulin and calmodulin-binding proteins in plants. *Annual*

Review of Plant Physiology and Plant Molecular Biology, 49(1), 697-725. [CrossRef]

[13] Bouché, N., Yellin, A., Snedden, W. A., & Fromm, H. (2005). Plant-specific calmodulin-binding proteins. *Annual Review of Plant Biology, 56*(1), 435-466. [CrossRef]

[14] Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Seay, M., Gerstein, M., Snyder, M., & Dinesh-Kumar, S. P. (2007). Differential binding of calmodulin-related proteins to their targets revealed through high-density Arabidopsis protein microarrays. *Proceedings of the National Academy of Sciences, 104*(11), 4730-4735. [CrossRef]

[15] Ma, Y., Dai, X., Xu, Y., Luo, W., Zheng, X., Zeng, D., Pan, Y., Lin, X., Liu, H., Zhang, D., Xiao, J., Guo, X., Xu, S., Niu, Y., Jin, J., Zhang, H., Xu, X., Li, L., Wang, W., ... & Chong, K. (2015). COLD1 confers chilling tolerance in rice. *Cell, 160*(6), 1209-1221. [CrossRef]

[16] Mori, K., Renhu, N., Naito, M., Nakamura, A., Shiba, H., Yamamoto, T., Suzaki, T., Iida, H., & Miura, K. (2018). Ca^{2+}-permeable mechanosensitive channels MCA1 and MCA2 mediate cold-induced cytosolic Ca^{2+} increase and cold tolerance in *Arabidopsis*. *Scientific Reports, 8*(1), 550. [CrossRef]

[17] Li, H., Jiang, X., Lv, X., Ahammed, G. J., Guo, Z., Qi, Z., Yu, J., & Zhou, Y. (2019). Tomato GLR3.3 and GLR3.5 mediate cold acclimation-induced chilling tolerance by regulating apoplastic H_2O_2 production and redox homeostasis. *Plant, Cell & Environment, 42*(12), 3326-3339. [CrossRef]

[18] Kudla, J., Becker, D., Grill, E., Hedrich, R., Hippler, M., Kummer, U., Parniske, M., Romeis, T., & Schumacher, K. (2018). Advances and current challenges in calcium signaling. *New Phytologist, 218*(2), 414-431. [CrossRef]

[19] Doherty, C. J., Van Buskirk, H. A., Myers, S. J., & Thomashow, M. F. (2009). Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *The Plant Cell, 21*(3), 972-984. [CrossRef]

[20] Kim, Y., Park, S., Gilmour, S. J., & Thomashow, M. F. (2013). Roles of CAMTA transcription factors and salicylic acid in configuring the low-temperature transcriptome and freezing tolerance of *Arabidopsis*. *The Plant Journal, 75*(3), 364-376. [CrossRef]

[21] Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A. S. N., & Poovaiah, B. W. (2009). Ca^{2+}/calmodulin regulates salicylic-acid-mediated plant immunity. *Nature, 457*(7233), 1154-1158. [CrossRef]

[22] Yang, T., Chaudhuri, S., Yang, L., Du, L., & Poovaiah, B. W. (2010). A calcium/calmodulin-regulated member of the receptor-like kinase family confers cold tolerance in plants. *Journal of Biological Chemistry, 285*(10), 7119-7126. [CrossRef]

[23] Yang, T., Shad Ali, G., Yang, L., Du, L., Reddy, A. S. N., & Poovaiah, B. W. (2010). Calcium/calmodulin-regulated receptor-like kinase CRLK1 interacts with MEKK1 in plants. *Plant Signaling & Behavior, 5*(8), 991-994. [CrossRef]

[24] Zhao, C., Wang, P., Si, T., Hsu, C. C., Wang, L., Zayed, O., Yu, Z., Zhu, Y., Dong, J., Tao, W. A., & Zhu, J. K. (2017). MAP kinase cascades regulate the cold response by modulating ICE1 protein stability. *Developmental cell, 43*(5), 618-629. [CrossRef]

[25] Huang, C., Ding, S., Zhang, H., Du, H., & An, L. (2011). CIPK7 is involved in cold response by interacting with CBL1 in *Arabidopsis thaliana*. *Plant Science, 181*(1), 57-64. [CrossRef]

[26] Gao, Y., & Zhang, G. (2019). A calcium sensor calcineurin B-like 9 negatively regulates cold tolerance via calcium signaling in *Arabidopsis thaliana*. *Plant Signaling & Behavior, 14*(3), e1573099. [CrossRef]

[27] Kim, K. N., Cheong, Y. H., Grant, J. J., Pandey, G. K., & Luan, S. (2003). CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. *The Plant Cell, 15*(2), 411-423. [CrossRef]

[28] Delk, N. A., Johnson, K. A., Chowdhury, N. I., & Braam, J. (2005). CML24, regulated in expression by diverse stimuli, encodes a potential Ca^{2+} sensor that functions in responses to abscisic acid, daylength, and ion stress. *Plant Physiology, 139*(1), 240-253. [CrossRef]

[29] Ranty, B., Aldon, D., Cotelle, V., Galaud, J. P., Thuleau, P., & Mazars, C. (2016). Calcium sensors as key hubs in plant responses to biotic and abiotic stresses. *Frontiers in Plant Science, 7*, 327. [CrossRef]

[30] Lin, R., Song, J., Tang, M., Wang, L., Yu, J., & Zhou, Y. (2023). CALMODULIN6 negatively regulates cold tolerance by attenuating ICE1-dependent stress responses in tomato. *Plant Physiology, 193*(3), 2105-2121. [CrossRef]

[31] Aldon, D., Mbengue, M., Mazars, C., & Galaud, J. P. (2018). Calcium signalling in plant biotic interactions. *International journal of molecular sciences, 19*(3), 665. [CrossRef]

[32] Vats, S., Kumawat, S., Kumar, V., Patil, G. B., Joshi, T., Sonah, H., Sharma, T. R., & Deshmukh, R. (2019). Genome editing in plants: exploration of technological advancements and challenges. *Cells, 8*(11), 1386. [CrossRef]

[33] Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., Volz, S. E., Joung, J., van der Oost, J., Regev, A., Koonin, E. V., & Zhang, F. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *cell, 163*(3), 759-771. [CrossRef]

[34] Hwarari, D., Radani, Y., Ke, Y., Chen, J., & Yang, L. (2024). CRISPR/Cas genome editing in plants: mechanisms, applications, and overcoming bottlenecks. *Functional & Integrative Genomics, 24*(2), 50. [CrossRef]



Soheila Aghaei Dargiri received her postdoctoral training in molecular genetics and biotechnology at the Department of Biological Control Research, Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran. Her research addresses plant-microbe interactions and abiotic stress tolerance. (Email: s.aghaei@areeo.ac.ir)



Ali Movahedi is a Professor at the College of Life Sciences, Nanjing Forestry University, China. His research focuses on genome editing, genetic engineering, regulation of gene expression, and plant responses to abiotic stress, with contributions to plant biotechnology and molecular biology. (Email: ali_movahedi@njfu.edu.cn)