The calcium signaling module CaM–IQM destabilizes IAA–ARF interaction to regulate callus and lateral root formation

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Induction of a pluripotent cell mass, called callus, from detached organs is an initial step in in vitro plant regeneration, during which phytohormone auxin-induced ectopic activation of a root developmental program has been shown to be required for subsequent de novo regeneration of shoots and roots. However, whether other signals are involved in governing callus formation, and thus plant regeneration capability, remains largely unclear. Here, we report that the Arabidopsis calcium (Ca\textsuperscript{2+}) signaling module CALMODULIN IQ-MOTIF CONTAINING PROTEIN (CaM–IQM) interacts with auxin signaling to regulate callus and lateral root formation. We show that disruption of IQMs or CaMs retards auxin-induced callus and lateral root formation by dampening auxin responsiveness, and that CaM–IQM complexes physically interact with the auxin signaling repressors INDOLE-3-ACETIC ACID INDUCIBLE (IAA) proteins in a Ca\textsuperscript{2+}-dependent manner. We further provide evidence that the physical interaction of CaM6 with IAA19 destabilizes the repressive interaction of IAA19 with AUXIN RESPONSE FACTOR 7 (ARF7), and thus regulates auxin-induced callus formation. These findings not only define a critical role of CaM–IQM-mediated Ca\textsuperscript{2+} signaling in callus and lateral root formation, but also provide insight into the interplay of Ca\textsuperscript{2+} signaling and auxin actions during plant regeneration and development.

Significance

Calcium (Ca\textsuperscript{2+}) is a universal signal in eukaryotic cells that regulates multiple cellular and developmental events, and the link between Ca\textsuperscript{2+} signaling and auxin actions in plants has been considered to be missing. Here, we identified the Arabidopsis Ca\textsuperscript{2+} signaling module CALMODULIN IQ-MOTIF CONTAINING PROTEIN (CaM–IQM) as an important regulator of auxin-induced callus and lateral root formation. We further demonstrated that CaM–IQM complexes physically interact with auxin signaling repressors, INDOLE-3-ACETIC ACID INDUCIBLE (IAAs), in a Ca\textsuperscript{2+}-dependent manner to destabilize IAA–AUXIN RESPONSE FACTOR 7 interactions and thus modify auxin responsiveness. These findings reveal a layer of molecular interplay between Ca\textsuperscript{2+} signaling and auxin actions in plant regeneration and development.
phosphatases, metabolic enzymes, transcription factors, and chaperones—to decode specific Ca\(^{2+}\) signals (20, 21). These Ca\(^{2+}/CaM\) signaling networks have been reported to regulate plant development and responses to environmental stimuli, including root hair growth, pollen tube development, hormone response, heat-shock signaling, nitric oxide accumulation, and plant immunity (22–27). As a type of CaMBPs in plants, the IQ-MOTIF CONTAINING PROTEINS (IQMs) have been shown to be involved in regulation of plant stomatal closure, flowering, seed dormancy, and immune response (28–31). Moreover, all the basal media used for in vitro plant regeneration contain a certain level of Ca\(^{2+}\) (3, 32); however, whether Ca\(^{2+}\) signaling participates in plant regeneration programs remains elusive.

Here, we report that the Ca\(^{2+}\) signaling module CaM–IQM is required for auxin-induced callus and lateral root formation in Arabidopsis. We demonstrate that CaM–IQMs physically interact with IAs to antagonize their repressive interaction with ARF7, and thus promote auxin-induced callus formation as well as lateral root formation. Our findings define a layer of molecular interplay between Ca\(^{2+}\) and auxin signaling during plant regeneration and development.

Results

cfc1 Is Defective in Callus and Lateral Root Formation. We previously reported that disruption of the Arabidopsis KCS1 gene, which encodes an enzyme, 3-ketoacyl-CoA synthase 1, that catalyzes a rate-limiting step in the biosynthesis of very long-chain fatty acids, results in enhanced callus formation from multiple organs (15). To further identify the signals or molecules governing auxin-induced callus formation during in vitro regeneration of Arabidopsis, we performed a genetic screen with an ethylmethylsulfone (EMS)-mutagenized population of kcs1-5, which harbors a T-DNA insertion in KCS1, to identify mutants with defective or reduced callus-forming capacity when seedlings were incubated on CIM. One such mutant, initially named callus formation capacity 1 (cfc1), displayed an apparent defect in callus formation from the primary roots of seedlings when compared with kcs1-5 or WT (Fig. 1A). To examine the effect of the cfc1 mutation on the callus-forming capacity of other organs, we incubated hypocotyl and cotyledon explants of WT, kcs1-5, and cfc1 on CIM, and observed that callus formation in the cfc1 explants was also dampened (Fig. 1A), indicating that cfc1 impedes callus-forming capacity of multiple organs.

As auxin-induced callus formation occurs from pericycle or pericycle-like cells through a root development pathway (6), we crossed the pericycle marker line J0121 of Col-0 background (15, 33) with the kcs1-5 and cfc1 mutants and obtained the respective F3 progenies homozygous in both J0121 marker and mutant background, and next compared the fluorescent signals in the pericycle cells and resulting calli in the primary roots of WT, kcs1-5, and cfc1 seedlings. Before seedlings were incubated on CIM, comparable J0121 signals were observed in the

![Image](https://doi.org/10.1073/pnas.2202669119)
pericycle cells of WT, kcs1-5, and cfc1 roots (Fig. 1B). After seedlings were incubated on CIM for 96 h, active callus formation occurred from the pericycle cells of WT and kcs1-5 roots where the J0121 signals almost disappeared; however, callus formation from the cfc1 pericycle was apparently delayed, as the J0121 signal was still detectable (Fig. 1B). Notably, unlike the lateral root primordium-like calli formed from WT pericycle, continuous callus formation was observed from a whole layer of the cfc1 pericycle and from the kcs1-5 pericycle (Fig. 1B), implicating that the effect of cfc1 on callus formation is independent of the kcs1-5 mutation. Moreover, the cfc1 seedlings were also defective in lateral root initiation and developed shorter primary roots than kcs1-5 and WT (Fig. 1C), and growth and development of cfc1 plants were also retarded (SI Appendix, Fig. S1A). These observations demonstrate that the mutation in cfc1 impairs callus and lateral root formation as well as plant development.

**IQMs Function Redundantly in Regulating Callus Formation.**

To identify the gene responsible for the cfc1 phenotype, we backcrossed cfc1 with kcs1-5 and examined the callus-forming phenotype in the F2 generation on CIM. We found that the F2 seedlings segregated for the kcs1-5, intermediate, and cfc1 phenotypes in a ratio of 1:2:1 (53:119:49; $\chi^2 = 0.1189$) (SI Appendix, Fig. S1B), demonstrating that the cfc1 phenotype is caused by a semidominant mutation of a single gene. Using a map-based cloning approach with an F2 population obtained from the cross of cfc1 with the Landsberg erecta (Ler) accession (34), we mapped the cfc1 mutation to a 160-kb region on chromosome 5, in which a transition of G-to-A was identified in the coding region of At5g57010, which led to a premature truncation of the IQM5 with 426 amino acids (SI Appendix, Fig. S1C). To further verify that this mutation is responsible for the cfc1 phenotype, we introduced the IQM5 coding sequence driven by the CaMV35S promoter or its native promoter into cfc1, respectively. As expected, constitutive overexpression of IQM5 driven by the CaMV35S promoter fully rescued the callus-forming defect of the cfc1 seedlings (SI Appendix, Fig. S1D), whereas introduction of IQM5 driven by its native promoter led to a partial or full rescue of callus formation among different cfc1 individuals (SI Appendix, Fig. S1E). These findings confirm that the IQM5 mutation in the cfc1 mutant has a dominant effect and is responsible for the callus-formation defect. Next, we crossed cfc1 with WT and obtained a mutant without kcs1-5, designated as iqm5-d. As expected, the iqm5-d seedlings still displayed a reduced callus-forming capacity in their primary roots on CIM and developed short primary roots with fewer lateral roots when compared with WT (Fig. 1D), further supporting the notion that the effect of iqm5-d on callus formation is independent of kcs1-5.

Next, we obtained a loss-of-function mutant of IQM5, iqm5-1 (SALK_134786), in which a T-DNA was inserted in the first exon and disrupted the transcription of IQM5 (31) (SI Appendix, Fig. S1C). Interestingly, the iqm5-1 seedlings incubated on CIM did not have an obvious callus-forming defect when compared with WT (SI Appendix, Fig. S2A). As the Arabidopsis IQM family contains six members—among which the IQM5, IQM1, and IQM4 belong to a phylogenetic clade (35) (SI Appendix, Fig. S2B)—we thus speculated that some IQM members might function redundantly with IQM5 in regulating callus formation. Transcriptional analysis revealed that both IQM5 and IQM1 were highly responsive to CIM (SI Appendix, Fig. S2C), and IQM5 and IQM1 were found to abundantly accumulate in the pericycle cells of primary roots and the resulting calli (SI Appendix, Fig. S2D). We thus obtained the T-DNA insertion mutant iqm1-1 (SALK_127727), in which the transcription of IQM1 was disrupted (29, 36) (SI Appendix, Fig. S2E). As expected, like iqm5-1, the iqm1-1 seedlings on CIM did not show any obvious callus-forming defect, while apparently dampened callus formation was observed in the primary roots of the iqm1-1 iqm5-1 double mutant, and this callus-forming defect could be rescued by introduction of a native promoter-driven IQM1 (SI Appendix, Fig. S2A). Therefore, we conclude that IQM5 and IQM1, possibly together with other IQM members, function redundantly in governing callus formation.

**CaM–IQM-Mediated Calcium Signaling Is Required for Callus Formation.** As IQMs belong to a family of CaMBPs and can interact with CaMs in a Ca2+-independent manner to decode Ca2+ signals (29), we reasoned that the Ca2+ signaling module CaM-IQMs are involved in regulation of callus formation. To test this, we first determined which of the seven Arabidopsis CaM members (37) could physically interact with IQM5 or IQM1. Yeast two-hybrid assays showed that IQM5 could interact with CaM3, CaM5, and CaM6, while IQM1 interacted with CaM5 and CaM6 (SI Appendix, Fig. S3A). Further luciferase complementation imaging (LCI) assays performed in Nicotiana benthamiana leaves revealed that both IQM5 and IQM1 interacted with CaM3, CaM5, and CaM6 in planta (SI Appendix, Fig. S3B). Moreover, using transgenic plants harboring a pCaM3::CaM3-GFP, pCaM5::CaM5-GFP, or pCaM6::CaM6-GFP construct, we showed that CaM3, CaM5, and CaM6 accumulated abundantly in the pericycle or resulting callus (SI Appendix, Fig. S3C), indicating that these CaM members are potential partners of IQM5 and IQM1 during callus formation.

Next, we obtained a T-DNA insertion mutant of CaM5, cam5-4 (SALK_027181) (37), with disrupted CaM5 transcription (SI Appendix, Fig. S3D). As mutants for CaM6 and CaM3 were publicly unavailable, we generated two allelic mutants in each gene by CRISPR/Cas9 approach, and designated as the cam6-1, cam6-2 and cam3-1, cam3-2, respectively (SI Appendix, Fig. S3E). Interestingly, cam6-1 and cam6-2 seedlings but not the cam5-4, cam3-1, and cam3-2 seedlings on CIM exhibited a callus-forming phenotype distinguishable from that of WT, and this callus-forming defect in cam6-1 could be restored by introduction of a pCaM6::CaM6-GFP construct (SI Appendix, Fig. S3 F and G). Next, we generated the double-mutants cam3-1 cam5-4, cam3-1 cam6-1, and cam5-4 cam6-1, and the triple-mutant cam3-1 cam5-4 cam6-1, and observed that the callus-forming defect of cam6-1 seedlings could be enhanced by cam5-4 but not by cam3-1 (SI Appendix, Fig. S3F), demonstrating that CaM6 and CaM5 govern callus formation in a partially redundant manner.

To further define the involvement of CaM–IQM-mediated Ca2+ signaling in callus and lateral root formation, we compared the callus-forming phenotypes of WT, cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings on CIM with or without Ca2+. As expected, an increase of Ca2+ concentration in CIM promoted callus formation from WT roots in a dose-dependent manner, whereas the effect of Ca2+ was obviously attenuated in the cam6-1, iqm5-d, and cam6-1 iqm5-d roots (Fig. 2A). Similarly, Ca2+ also promoted lateral root formation in WT seedlings in a dose-dependent manner, and this response was largely dampened in the cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings (SI Appendix, Fig. S3H). In addition, the callus- and lateral root-forming phenotypes of cam6-1 iqm5-d resembled those of
CaM–IQM modules are required for auxin-induced callus formation. (A) Callus-forming phenotypes of WT, cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings on CIM supplemented with the indicated concentrations of CaCl₂ for 12 d (n = 12). (Scale bars, 10 mm.) (B) GCaMP6s::GFP fluorescent signals visualized in the primary roots of WT, cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings. The 7-d-old seedlings were incubated in liquid CIM for 0 and 5 min, and the GFP fluorescence was quantified (n = 12). (Scale bars, 50 μm.) (C) Effect of the Ca²⁺ chelator EGTA and CaM antagonist TFP on callus-forming capacity. The 7-d-old WT seedlings were incubated on CIM supplemented with the indicated concentrations of EGTA (n = 14) or TFP (n = 15) for 12 d. (Scale bars, 10 mm.) Data are shown as means ± SD. Different letters indicate significant differences at P < 0.05 determined by one-way or two-way ANOVA with Tukey’s multiple comparison test.

**Disruption of CaM–IQM Dampens Auxin Responsiveness.** Given that auxin plays a key role in directing callus and lateral root formation (3, 4, 13), we investigated whether CaM–IQM modules could impact auxin responsiveness. We first compared lateral root formation among WT, cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings in response to exogenous auxin. As expected, when seedlings were treated with the low concentrations of 1-naphthylacetic acid (NAA), lateral root formation was induced in WT seedlings in a dose-dependent manner; however, the induction of lateral root formation by NAA was obviously dampened in the cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings (SI Appendix, Fig. S4A). Consistent with this, fluorescent signals of the DR5::GFP, a well-used indicator of auxin response (39), were weaker in the cam6-1, iqm5-d, and cam6-1 iqm5-d roots than in WT roots, either with or without treatment with the natural form of auxin IAA (SI Appendix, Fig. S4B). These observations illustrate that disruption of the CaM–IQM module attenuates auxin responsiveness.

To further verify the impact of CaM–IQM modules on auxin responsiveness, we also monitored the transcription of early auxin-responsive genes—namely IAA5, IAA14, IAA19, IAA28, and IAA29 (40), as well as LBD16, LBD17, and LBD29—that act downstream of the auxin signaling module IAA–ARF to direct callus and lateral root formation (13), in the WT, cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings after treatment with IAA. As expected, compared with that in WT, the transcriptional induction of IAA5, IAA14, IAA19, IAA28, and IAA29 by exogenous IAA was obviously attenuated in the cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings (SI Appendix, Fig. S4C). Likewise, the IAA-induced transcription of LBD16, LBD17, and LBD29 was also reduced in these mutant genotypes (SI Appendix, Fig. S4C). These results suggest that CaM–IQM modules are required for proper auxin responsiveness during callus and lateral root formation.

CaMs Physically Interact with IAA5s in a Ca²⁺-Dependent Manner. Because CIM contains a high level of the synthetic auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4-D has been shown to not require auxin efflux carriers for polar transport (4, 41), we thus reasoned that the auxin responsiveness mediated by CaM–IQM during callus induction is likely attributable to the alteration of auxin signaling rather than polar transport or homeostasis. Since auxin signaling is mainly mediated by the SCF{TIR1/AFB} complex via targeting the downstream signaling repressor IAA proteins for proteolytic degradation (42, 43), we thus performed a yeast two-hybrid assay with IQM5 and CaM6 to test whether IQM and CaM physically interact with key auxin signaling components, including TRANSPORT INHIBITOR RESPONSE 1 (TIR1), AUXIN SIGNALING F-BOX 2 (AFB2), AFB3, S PHASE KINASE-ASSOCIATED PROTEIN 1 (SKP1), and IAA members involved in callus and lateral root formation, such as IAA5, IAA14, IAA19, IAA28, and IAA29. We failed to detect any physical interaction between IQM5 and these auxin signaling factors (SI Appendix, Fig. S5A), but physical interactions
between CaM6 and AFB2, AFB3, and IAA19 were detectable in yeast cells (SI Appendix, Fig. S5B). Furthermore, physical interactions between CaM5 and AFB2, AFB3, and IAA19 were also detected in yeast cells (SI Appendix, Fig. S5C). To verify the interactions of CaM5 and CaM6 with AFB2, AFB3, and IAA members in planta, we conducted an LCI assay in N. benthamiana leaves, and found that both CaM6 and CaM5 only interacted with IAA19 and IAA28 (Fig. 3A). The interaction of CaM6 with IAA19 was further validated by a coimmunoprecipitation (co-IP) assay (Fig. 3B).

As the physical interaction of CaM and IQM has been shown to be independent of Ca\(^{2+}\) (29), we thus investigated whether the interaction of CaM6 and IAA19 is Ca\(^{2+}\)-dependent and whether IQMs are required for this interaction. Indeed, co-IP assays performed with transgenic plants coexpressing epitope-tagged IAA19 and CaM6 clearly showed that Ca\(^{2+}\) treatment could induce the physical interaction of CaM6 and IAA19 and that the effect of Ca\(^{2+}\) was dose-dependent (Fig. 3C). In contrast, the physical interaction of CaM6 and IAA19 could be largely disrupted by EGTA treatment and in the iqm5-d background (Fig. 3D), demonstrating that both Ca\(^{2+}\) and IQMs are required for the physical interaction of CaM6 and IAA19.

**CaM6 Destabilizes the Interaction between IAA19 and ARF7.** Since most IAAs function as auxin signaling repressors by interacting with ARFs to repress their transcriptional activities, and IAA19 has been reported to physically interact with ARF7 to inhibit lateral formation (10), we speculated that the interaction of CaMs and IAA19 might modify auxin signaling either by affecting IAA19 stability or by antagonizing its interaction with ARF7. To test this, we first compared IAA19 accumulation among WT, cam6-1, iqm5-d, and cam6-1 iqm5-d seedlings harboring a pIAA19::IAA19-GFP construct, but found that the subcellular localization of IAA19 and its abundance in the primary roots were comparable among these four genotypes (SI Appendix, Fig. S6A). We also generated transgenic plants harboring a pIAA19::mIAA19-GFP construct, which expressed a gain-of-function mutated isofrom of IAA19 (mIAA19) that cannot be degraded by auxin-induced proteolysis in the masugu2-1 (msg2-1) background (10, 44), and monitored the accumulation of IAA19 and mIAA19 in response to Ca\(^{2+}\). Indeed, the abundance of mIAA19 was found to be much higher than that of IAA19 in the transgenic seedlings, while Ca\(^{2+}\) treatment did not affect the abundance of either IAA19 or mIAA19 (SI Appendix, Fig. S6B). These observations exclude the possibility that physical interaction of CaM6 and IAA19 affects IAA19 abundance or stability. Consistent with this, msg2-1 seedlings on CIM still exhibited some degree of callus formation in response to Ca\(^{2+}\) (SI Appendix, Fig. S6C).

Next, we tested whether CaM6 could antagonize ARF7 to interact with IAA19. Both LCI and co-IP assays clearly showed that ARF7 physically interacted with IAA19. Both LCI and co-IP assays clearly showed that ARF7 physically interacted with IAA19 and mIAA19, and...
that the interaction of ARF7 with IAA19 or mIAA19 was remarkably antagonized by the coexpression of CaM6 (Fig. 4 A and B). This might explain why callus formation is still inducible by Ca\(^{2+}\) in nsg2-1 seedlings (SI Appendix, Fig. S6C). Consistent with this, a yeast two-hybrid assay revealed that CaM6 could physically interact with the C terminus of IAA19 (SI Appendix, Fig. S6D), which is required for physical interaction with ARFs (10, 45, 46). Thus, the derepression of ARF7 by CaMs seems to be a possible mechanism behind the interplay of CaM–IQM and auxin signaling. To test this, we first examined whether Ca\(^{2+}\) could elevate the transcription of LBD16 and LBD29, two genes directly targeted by ARF7 (17). As expected, the transcript abundances of LBD16 and LBD19 were obviously increased by Ca\(^{2+}\) treatment (Fig. 4C). Next, we overexpressed ARF7 in the iqm5-d mutant, and observed that overexpression of ARF7 could partially restore callus formation in the iqm5-d seedlings (Fig. 4D), supporting that ARF7 is genetically downstream of the CaM–IQM module in directing callus formation. We thus conclude that the physical interaction of CaM6 with IAA19 releases ARF7 activity and thus promotes callus formation.

**Discussion**

Recent studies in Arabidopsis have revealed that two types of cellular reprogramming occur during plant organ repair and regeneration: auxin-induced callus formation and wound-induced cell dedifferentiation (6, 13, 47, 48). Because CIM contains a high level of auxin, and auxin-induced ectopic activation of root meristematic genes is required for subsequent de novo shoot or root regeneration (5–7, 49), it is likely that auxin-induced callus formation represents a major cellular event in the acquisition of regeneration capability during in vitro plant regeneration. Accumulating evidence indicates that auxin-induced callus formation shares a developmental program with lateral root formation, and that some of the key auxin signaling components involved in lateral root formation are required for callus formation (6, 7, 13). However, it is yet unclear whether Ca\(^{2+}\) signaling plays a role in the callus-forming program. Here, we demonstrate that Ca\(^{2+}\) signaling module CaM–IQM plays an important role in auxin-induced callus and lateral root formation by modifying auxin signaling module IAA–ARFs, which defines a layer of molecular interplay between Ca\(^{2+}\) and auxin signaling during in vitro plant regeneration and development. Obviously, as both Ca\(^{2+}\) and auxin are endogenous signals of plants, the modification of IAA–ARFs by CaM–IQM module wouldn’t be an immediate “all-or-none” but a step-wise process with concurrent activation of ARFs in response to Ca\(^{2+}\).

It is likely that, under normal growth conditions, a low Ca\(^{2+}\) gradient in the pericycle is perceived by a CaM–IQM complex for interacting with IAA proteins to relieve the ARF activity, and such low Ca\(^{2+}\) signature coordinates with endogenous auxin signaling to allow proper lateral root initiation. Upon CIM treatment, high levels of auxin and Ca\(^{2+}\) lead to a high Ca\(^{2+}\) gradient in the pericycle or pericycle-like cells, and this high Ca\(^{2+}\) signature is perceived by CaM–IQMs to derepress IAA-inhibited ARFs, which derepresses the ARF activity, and this derepression of ARF7 by CaMs seems to be a possible mechanism behind the interplay of CaM–IQM and auxin signaling. To test this, we first examined whether Ca\(^{2+}\) could elevate the transcription of LBD16 and LBD29, two genes directly targeted by ARF7 (17). As expected, the transcript abundances of LBD16 and LBD19 were obviously increased by Ca\(^{2+}\) treatment (Fig. 4C). Next, we overexpressed ARF7 in the iqm5-d mutant, and observed that overexpression of ARF7 could partially restore callus formation in the iqm5-d seedlings (Fig. 4D), supporting that ARF7 is genetically downstream of the CaM–IQM module in directing callus formation. We thus conclude that the physical interaction of CaM6 with IAA19 releases ARF7 activity and thus promotes callus formation.

**Fig. 4.** CaM6 destabilizes the ARF7-IAA19 interaction. (A) CaM6 dampens ARF7-IAA19 interaction. LCI assay was performed with N. benthamiana leaves transiently coexpressing Cluc-ARF7 and IAA19/mIAA19-NLuc with GFP (empty vector) or CaM6-GFP. Representative images of an infiltrated leaf, the coexpressed proteins, the quantified fluorescent signals (n = 15), and the immunoblotted protein abundances are shown from left to right. (B) CaM6 destabilizes the physical interaction of ARF7 and IAA19. Co-IP assay was performed with N. benthamiana leaves transiently expressing ARF7-GFP and IAA19-MYC or mIAA19-MYC with Cluc-CaM6 or Cluc (empty vector) using an agarose-conjugated anti-GFP matrix and immunoblotting with anti-GFP and anti-MYC antibodies. The experiments in A and B were performed for at least two biological replicates. (C) Transcriptional induction of LBD16 and LBD29 by Ca\(^{2+}\), qRT-PCR was performed with 7-d-old WT seedlings incubated on B5 medium containing 10^{-3} M CaCl\(_2\) for 0, 6, and 12 h (n = 3 biological replicates). (D) Overexpression of ARF7 partially rescued the callus-forming defect of the iqm5-d roots. The 7-d-old seedlings of p35S:ARF7, iqm5-d, and three independent p35S:ARF7 iqm5-d lines were incubated on CIM for 12 d, and the area of callus formed in primary roots (n = 14) and the transcript abundances of ARF7 (n = 3 biological replicates) were determined. (Scale bar, 10 mm.) Data are presented as means ± SD. Different letters indicate significant differences at P < 0.05 determined by one-way ANOVA with Tukey’s multiple comparison test.
changes in Ca$^{2+}$ in the plant stem cell niche alters the polarity of PIN-FORMED1 (54). The Arabidopsis CALCIURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE 6 participate in lateral root formation, as well as callus formation, by affecting auxin transport (55, 56). Although a few lines of evidence have suggested that Ca$^{2+}$ or CaM signaling affects auxin response (27, 57, 58), little is yet known about the interplay of Ca$^{2+}$ and auxin at the signaling level. Therefore, the link of Ca$^{2+}$ signaling and auxin actions has been considered to be still missing (59). Here, we provide substantial evidence that the Ca$^{2+}$ signaling module CaM–IQM interacts with the key auxin signaling component IAA–ARF by antagonistically interacting with IAAAs, and thus regulating callus and lateral root formation. Interestingly, although IQM forms a complex with CaM via its N-terminal IQ-motif (29), our finding that the truncated IQM5 protein in iqm5-5 has had a dominant effect strongly suggests that the C-terminal regions of IQMs are essential for the CaM–IQM module to decode Ca$^{2+}$ signals. Moreover, as both IAAAs and ARFs act largely redundantly in auxin signaling (43), and we only identified IAA19–ARF7 as a physical target of the CaM–IQM module, it is possible that other IAAAs, including IAA28, or ARFs are also the potential targets of CaM–IQM modules during auxin-regulated regeneration and development. On the other hand, since CaMs decode specific Ca$^{2+}$ signals by forming multiple complexes with various types of CaMBPs, it is also plausible that similar CaM–IAA interactions might exist in these Ca$^{2+}$ signaling modules to modify auxin actions during plant development and environmental responses. Therefore, further work on these interactions will shed light on the molecular links between Ca$^{2+}$ signaling and auxin actions.

Finally, although auxin-induced callus formation is an initial step in in vitro plant regeneration and largely determines the regeneration capacity of the plant, we still know little about how the varied regeneration capacities are determined among different plant species. In Arabidopsis, ABERRANT LATERAL ROOT FORMATION (ALF4), which encodes a nuclear protein initially identified to be required for lateral root formation, has been shown to be essential for callus formation (6, 60), as disruption of ALF4 leads to the loss of callus-forming capability in multiple organs, including roots, cotyledons, and petals (6). A recent study showed that, although ALF4 is not responsive to auxin, the ALF4 protein could bind to RING BOX 1 (RBX1), a subunit of the SCFTIR1 complex, to inhibit the activity of SCFTIR1 and thus affect auxin sensitivity (61). Recently, very long-chain fatty acids or their derivatives have been defined as restrictive signals limiting callus-forming capability, at least in part, by modulating transcription of ALF4 (15). Interestingly, we demonstrate here that the Ca$^{2+}$ signaling module CaM–IQM regulates callus-forming capacity by modifying the interaction of IAA–ARFs and thus auxin response. It seems that auxin response or sensitivity might be one of the molecular mechanisms behind callus-forming capacity. Thus, further identification of signals or factors governing callus formation will be necessary to clarify how regeneration capability is determined in plants.

**Materials and Methods**

**Plant Materials and Growth Conditions.** The Arabidopsis thaliana Columbia-0 accession was used in this study. The T-DNA insertion mutants iqm5-1 (SALK_134786), iqm1-1 (SALK_127727), and cam5-4 (SALK_027181) were obtained from the Arabidopsis Biological Resource Center (ABRC) and verified by PCR analyses, as described previously (30, 31, 37). The Arabidopsis J0121, DR5::GFP, and GCAmp6s::GFP marker lines and msg2-1 mutant were described previously (10, 33, 38, 39). The Arabidopsis seeds were surface-sterilized in ∼1% sodium hypochlorite, rinsed three times with sterile water, and germinated on half-strength Murashige and Skoog medium (3) (1/2 MS medium; Coolabber), 1% sucrose, 0.5% plant agar (pH 5.7) after stratification at 4°C for 2 d. The seedlings and plants were grown in a culture room or growth chamber at 22 ± 2°C with a 16/8 h light/dark photoperiod and an illumination intensity of 80 to 90 μmol m$^{-2}$s$^{-1}$.

**EMS Mutagenesis and CRISPR/Cas9 Editing.** EMS mutagenesis was carried out according to the method described previously (62). The kcs1-5 seeds were immersed in 100 mM phosphate buffer (pH 7.5) for 3 d at 4°C and dried on filter paper for 1 d. The seeds were subsequently mutagenized in 100 mM phosphate buffer containing 0.4% (vol/vol) EMS (Sigma) at room temperature for 8 h, and then washed three times with sterilized water. The mutagenized seeds (M1 generation) were grown in soil and allowed to self-pollinate. The seeds of M1 plants (M2 generation) were collected and germinated in 1/2 MS medium. The 7-d-old seedlings were transferred on CIM, and callus formation in the primary paper was examined at 7 d. The candidate cfr mutants were transferred onto 1/2 MS medium for recovery and then grown in soil, and their callus-forming phenotypes were further validated in the M3 generation.

To generate the cam3 and cam6 allelic mutants, an Arabidopsis egg cell-specific promoter-controlled CRISPR/Cas9 gene-editing system was used as previously described (63). Briefly, the 23-bp specific target sequences with PAM sites (5’-N20NGG-3’) were manually identified within the exons of CAM3 and CAM6, and their specificities were evaluated using the BLAST tool on the TAIR website (https://www.arabidopsis.org/BLAST/index.jsp). Primers corresponding to these gRNAs were designed (SI Appendix, Table S1), and PCR was performed using the pCWC-DT1T2 plasmid as a template. The PCR products were cloned into the pHEE401 vector and transformed into Arabidopsis. Mutations within the target genes were identified in the transgenic T1 plants, and the homozygous T3 plants without the construct were identified by sequencing and used for further characterization.

**Callus Induction and Lateral Root Formation.** For characterization of the callus-forming phenotype, 7-d-old seedlings or their explants were incubated on CIM (B5 medium [Coolabber], 2% glucose, 0.5% MES, 0.25% phytagel supplemented with 2.26 μM 2,4-D, and 0.23 μM kinetin, pH 5.7) (7) for 12 or 20 d. To examine the effect of Ca$^{2+}$ on callus formation, the 7-d-old seedlings were cultured on CIM without or with different concentrations of CaCl$_2$ (Sigma) or on CIM supplemented various concentrations of EGTA (Sigma) or TFP (Sigma) for 12 d. The formed callus was photographed and the area of callus was quantified with ImageJ software (15). To characterize lateral root formation, the seedlings were grown on 1/2 MS medium for 10 d, or the 5-d-old seedlings were transferred to 1/2 MS supplemented with various concentrations of NAA for 12 h or to 1/2 MS with or without CaCl$_2$ for 5 d, and the numbers of lateral root initiates were counted under a stereo microscope. All experiments above were repeated for at least 3 independent biological replicates with more than 12 independent plants each time.

**Plasmid Construction and Arabidopsis Transformation.** For generation of the transgenic plants, a genomic IQM5 fragment containing a 1,464-bp promoter and a 1,997-bp coding region, an IQM1 fragment containing a 2,103-bp promoter and a 2,130-bp coding region, and a genomic CAM6 fragment containing a 1,518-bp promoter and a 1,208-bp coding region were fused with a GFP sequence and cloned into the pCAMBIA1300 plasmid (Cambia) to generate the pIOMS::IQM5-GFP, pIOM1::IQM1-GFP, and pCAM6::CAM6-GFP constructs, respectively. The cDNA fragments of IQMS and ARF7 were cloned into the pSuper1300 vector to generate the p35S::IQM5 and p35S::ARF7 constructs, respectively. For determination of the tissue-specific expression of CaMs and IAA19, a DNA fragment of CAM3 containing a 1,072-bp promoter and a 940-bp coding sequence, CaMS containing a 1,851-bp promoter and a 2,108-bp coding sequence, and the DNA fragments of IAA9 from W1 or msg2-1 containing a 2,038-bp promoter and a 594-bp coding sequence were fused with a GFP sequence and cloned into the pCAMBIA1300 plasmid for generation of pCaM3::CAM3-GFP, pCaM5::CaM5-GFP, pIAA19::IAA19-GFP, and pIAA19::IAA19-GFP, respectively. All the plasmids were verified by sequencing and introduced into the Agrobacterium tumefaciens strain EHA105 or ABI and transformed into Arabidopsis by the standard floral-dipping method (64). At least eight independent transgenic
lines with a single T-DNA insertion were generated for each construct, and at least three independent T3 homozygous lines were used for subsequent characterization. All primers used for the generation of the constructs are listed in SI Appendix, Table S1.

**Gene Expression and Western Blot Analyses.** Total RNAs were isolated using the E.Z.N.A. Plant RNA Kit (OMEGA BioTek), and the reverse-transcription reaction was performed with a reverse transcription kit (Takara) according to the manufacturer’s instructions. RT-PCR was carried out using a standard method, and transcripts of the Glyceraldehyde-3-Phosphate Dehydrogenase C Subunit 1 (GAPC) were used as an internal control. qRT-PCR was conducted as previously described (65), and the relative expression level of each gene was calculated using the $\Delta\Delta CT$ (cycle threshold) method. The transcript abundance of ACTIN2 was used as an internal control. All primers used for RT-PCR and qRT-PCR are listed in SI Appendix, Table S1. To monitor protein abundance, the total proteins were extracted from infiltrated N. benthamiana leaves or homozygous transgenic Arabidopsis plants with a Plant Total Protein Extraction Kit (Cwbio), according to the manufacturer’s instructions, and quantified by colloidal Coomassie brilliant blue. The proteins were separated by polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, which were probed with anti-GFP (MBL) (1:5,000), anti-MYC (MBL) (1:5,000), or anti-LUC (Sigma) (1:3,000) primary antibodies, followed by HRP-labeled secondary antibody (Bioeasy; 1:10,000). Detection was performed using the ECL Super Sensitive Kit (DinIng), and the signals were captured with a Tanon 5200 imaging system. Rubisco stained with Ponceau S was used as a loading control.

**Confocal Microscopy.** To examine the tissue-specific or cellular accumulation of proteins, the primary roots of Arabidopsis seedlings harboring different GFP-tagged constructs or markers were visualized and photographed under an Olympus FV1000-MPE laser scanning microscope after being mounted in 10 mg l$^{-1}$ propidium iodide (Sigma). A GFP excitation/emission filter (488 nm/525 nm) was used to visualize the protein-specific fluorescence. The propidium iodide signal was visualized by excitation with an argon laser at 488 nm and detected with a spectral detector set at >585 nm for emission. The GFP fluorescent signals were quantified with ImageJ software.

**Yeast Two-Hybrid Assay.** The yeast two-hybrid assay was performed using the Matchmaker GAL4 two-hybrid system (Clontech). The coding sequences of IOM5, CaM5, and ARF7 fused with a GFP or MYC sequence were cloned into the pSuper1300 vector (67), and I4A19 was cloned into the pVPMYC binary vector (68). About 2 g of the N. benthamiana leaves transiently expressing proteins of 1 g of transgenic Arabidopsis seedlings were collected for extraction of protein. The total proteins were incubated with agarose-conjugated anti-MYC (MBL) or agarose-conjugated anti-Myc (MBL) matrix for 3 h with rotation at 4°C. The agarose beads were washed five times with 1 ml IP buffer, and then denatured in 50 μl of SDS loading buffer. Immunoprecipitated proteins were detected with anti-GFP antibody (MBL) (1:5,000), anti-MYC antibody (MBL) (1:5,000), and anti-LUC antibody (Sigma) (1:3,000). The experiments were repeated at least two times. All the primers used for the generation of constructs are listed in SI Appendix, Table S1.

**Phylogenetic Analysis.** Amino acid sequences of the IQM family members were obtained from TAIR (https://www.arabidopsis.org/), and the phylogenetic analysis was performed using the Mega X software (maximum-likelihood method, bootstrapping with 1,000 iterations, Jones-Taylor-Thornton model, uniform rates among sites, complete deletion of gaps/missing data, nearest-neighbor-interchange method).

**Data Availability.** All study data are included in the main text and SI Appendix.

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**LCI Assay.** The coding sequences of IQM1, IQM5, IAAS, IAAS4, IAAS42, IAAS49, ARF2, and ARF3 were fused in-frame with the N-terminal half of the luciferase gene (NLuc) in the pCAMBIA-NLuc vector, and those of ARF7 and CaMs were fused downstream of the C-terminal half of the luciferase gene (Cluc) in the pCAMBIA-Cluc vector (66). The NLuc- and Cluc-tagged plasmids were transformed into EHA105 followed by cotransfection into 4-wk-old N. benthamiana leaves via Agrobacterium p19 strain-mediated infiltration. After 2 d, the leaves were immersed in a fluorogenic solution of 1 mM luciferin and kept in the dark for 5 min, and images were captured by the Tanon 5200 imaging system with a 10-min exposition. The proteins from the corresponding leaves were immunoblotted with the anti-LUC antibody (Sigma) (1:3,000) to determine the abundance of expressed fusion proteins. The fluorescent signals from the LCI assay were quantified by ImageJ software, and the experiments were repeated three times. The primers used for the constructs in the LCI assay are listed in SI Appendix, Table S1.

**Co-IP Assay.** For co-IP assays in N. benthamiana leaves and transgenic Arabidopsis plants, the coding sequences of IOM5, CaM5, CaM6, and ARF7 fused with a GFP or MYC sequence were cloned into the pSuper1300 vector (67), and I4A19 was cloned into the pVPMYC binary vector (68). About 2 g of the N. benthamiana leaves transiently expressing proteins of 1 g of transgenic Arabidopsis seedlings were collected for extraction of protein. The total proteins were incubated with agarose-conjugated anti-MYC (MBL) or agarose-conjugated anti-Myc (MBL) matrix for 3 h with rotation at 4°C. The agarose beads were washed five times with 1 ml IP buffer, and then denatured in 50 μl of SDS loading buffer. Immunoprecipitated proteins were detected with anti-GFP antibody (MBL) (1:5,000), anti-MYC antibody (MBL) (1:5,000), and anti-LUC antibody (Sigma) (1:3,000). The experiments were repeated at least two times. All the primers used for the generation of constructs are listed in SI Appendix, Table S1.
24. Y. Pan et al., Dynamic interactions of plant CNGC subunits and calmodulin drive oscillatory Ca2+ channel activities. Dev. Cell 48, 710–725 e5 (2019).

25. W. Zhang et al., Molecular and genetic evidence for the key role of AtCaM3 in heat-shock signal transduction in Arabidopsis. Plant Physiol. 149, 1772–1784 (2009).

26. Y. Xuan, S. Zhou, L. Wang, Y. Cheng, L. Zhao, Nitric oxide functions as a signal and acts upstream of AtCaM3 in thermotolerance in Arabidopsis seedlings. Plant Physiol. 153, 1895–1906 (2010).

27. J. Yang et al., The CaM1-associated CCMX/MMK1/6 cascade positively affects lateral root growth via auxin signaling under salt stress in rice. J. Exp. Bot. 72, 6611–6627 (2021).

28. Y. P. Zhou et al., Arabidopsis IOMG, a novel calmodulin-binding protein, is involved with seed dormancy and germination in Arabidopsis. Front Plant Sci 9, 721 (2018).

29. Y. P. Zhou, J. Duan, T. Fujibe, K. T. Yamamoto, C. E. Tian, AOM1, a novel calmodulin-binding protein, is involved in stomatal movement in Arabidopsis. Plant Mol. Biol. 79, 333–346 (2012).

30. T. Lu et al., The calmodulin-binding protein IOM1 interacts with CASKLASE2 to affect pathogen defense. Plant Physiol. 181, 1314–1327 (2019).

31. L. P. Gong, J. Z. Cheng, Y. P. Zhou, X. L. Huang, C. E. Tian, Disruption of IOMs delays flowering possibly through modulating the juvenile-to-adult transition. Acta Physiol. Plant. 39, 21 (2017).

32. T. Thorpe, History of plant tissue culture. Methods Mol. Biol. 877, 9–27 (2012).

33. L. Laplaze et al., GAL4-GFP enhancer trap lines for genetic manipulation of lateral root development in Arabidopsis thaliana. J. Exp. Bot. 56, 2433–2442 (2005).

34. V. Arondel et al., Map-based cloning of a gene controlling omega-3 fatty acid desaturation in Arabidopsis. Science 258, 1353–1355 (1992).

35. Y. Zhou, Y. Chen, K. T. Yamamoto, J. Duan, C. E. Tian, Sequence and expression analysis of the Arabidopsis IOM family. Acta Physiol. Plant. 32, 1233–1235 (2010).

36. Y. Zhou et al., Initial characterization of Arabidopsis T-DNA insertion mutants of the IOM1 gene that encodes an IQ Motif containing protein. Plant Cell Physiol. 48, 5197–5197 (2007).

37. N. A. Al-Quraan, R. D. Locy, N. K. Singh, Expression of calmodulin genes in wild type and Arabidopsis IQM family. Annu. Rev. Genet. 43, 265–289 (2009).

38. A. Delbarre, P. Muller, V. Imhoff, J. Guern, Comparison of mechanisms controlling uptake and transduction in Arabidopsis thaliana. J. Exp. Bot. 56, 2433–2442 (2005).

39. J. Friml, Calcium: The missing link in auxin action. Annu. Rev. Plant Biol. 57, 4059–4070 (2006).

40. R. J. DiDonato et al., Arabidopsis ALCF4 encodes a nuclear-localized protein required for lateral root formation. Plant J. 37, 340–353 (2004).

41. R. Baghi et al., The Arabidopsis ALCF4 protein is a regulator of SCI F3 ligases. EMBO J. 37, 255–268 (2018).

42. M. Ruggier et al., Reduced naphthylphthalamic acid binding in the tir3 mutant of Arabidopsis is associated with a reduction in polar auxin transport and diverse morphological defects. Plant Cell 21, 745–757 (1999).

43. Z. P. Wang et al., Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. Genome Biol. 16, 144 (2015).

44. S. J. Elgg, A. F. Bent. Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743 (1998).

45. K. J. Jukic, T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔC T method. Methods 25, 402–408 (2001).

46. S. Li et al., Two Arabidopsis receptor-like cytoplasmic kinases SRK1 and SRK2 associate with the ZAR1-ZED1 complex and are required for effector-triggered immunity. Mol. Plant 12, 967–983 (2019).

47. V. Chinnasamy et al., ICE1: A regulator of cold-induced transcription and freezing tolerance in Arabidopsis. Genes Dev. 17, 1043–1054 (2003).

48. Y. Hu, Q. Xie, H. N. Chua, The Arabidopsis auxin-inducible gene ARGO controls lateral organ size. Plant Cell 15, 1951–1961 (2003).