Very-long-chain fatty acids restrict regeneration capacity by confining pericycle competence for callus formation in *Arabidopsis*

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Edited by Philip N. Benfey, Duke University, Durham, NC, and approved March 25, 2016 (received for review November 13, 2015)

The already differentiated organs in plants have a remarkable capacity to regenerate new individuals under culture conditions. Plant in vitro regeneration practically starts with the induction of a pluripotent cell mass, the callus, from detached organs on auxin-rich callus-inducing medium (CIM), which is generally required for subsequent regeneration of new bodies. Recent studies show that CIM-induced callus formation occurs from the pericycle or pericycle-like cells through a root developmental pathway, whereas the signals involved in governing callus-forming capacity of pericycle cells remain unknown. Here we report that very-long-chain fatty acids (VLCFAs) play a critical role in confining the pericycle competence for callus formation and thus the regeneration capacity of *Arabidopsis*. By genetic screening, we identified the *callus formation-related 1* (*cfr1*) mutant, which bypasses the inhibition of callus-forming capacity in roots by *solitary-root* (*slr*/*iaa14*). We show that *CFR1* encodes 3-ketoacyl-CoA synthase 1 (KCS1), which catalyzes a rate-limiting step of VLCFA biosynthesis. Our biochemical and genetic analyses demonstrate that VLCFAs restrict the pericycle competence for callus formation, at least in part, by regulating the transcription of *Aberrant Lateral Root Formation 4* (*ALF4*). Moreover, we provide evidence that VLCFAs act as cell layer signals to mediate the pericycle competence for callus formation. Taken together, our results identify VLCFAs or their derivatives as the confining signals for mediating the pericycle competence for callus formation and thus the regeneration capacity of plant organs.

VLCFA | pericycle | callus formation | regeneration

In plants, the already differentiated organs or tissues have a remarkable capability to regenerate new organs or entire individuals under appropriate culture conditions (1, 2). The initial step of a typical plant in vitro regeneration often starts with the induction of a pluripotent cell mass known as a callus from detached organs (explants) on auxin-rich callus-inducing medium (CIM), which is generally required for the subsequent regeneration of new organs or whole plant bodies (3, 4). Thus, callus formation has long been considered a process through which already-differentiated somatic cells acquire regenerating capability (2).

The molecular events of callus formation have begun to be described only recently (2, 5). Studies with *Arabidopsis* explants of multiple organs, including roots, hypocotyls, and petals, have revealed that the CIM-induced callus formation occurs from pericycle or pericycle-like cells, and that the derived calli resemble some characteristics of root meristem by ectopically expressed root meristem genes (6, 7). The recent findings that the four *Arabidopsis* lateral organ boundary domain (LBD) transcription factors play key roles in directing CIM-induced callus formation, and that the root meristem *PLETHORA* (*PLT*) genes are required for subsequent regeneration, supports the idea that CIM-induced callus formation from the pericycle follows a root developmental program (8, 9). In contrast, wound-induced callus formation has been shown to be directed by the AP2 transcription factors WOUND INDUCED DEDIFFERENTIATIONs (WINDs). The wound-induced callus does not exhibit the expression of root meristem genes, and its formation is not blocked in the mutant defective in lateral root formation (10), suggesting that WIND-mediated callus formation likely represents a cell dedifferentiation program in plants (5).

Because CIM-directed callus formation occurs from pericycle or pericycle-like cells, the appropriate competence of pericycle or pericycle-like cells appears to be critical for CIM-induced callus formation and thus the regeneration capacity of various organs. Indeed, specific ablation of the pericycle function in *Arabidopsis* by the pericycle-specific transactivation of a diphtheria toxin chain A effector indeed abolishes both lateral root formation and CIM-induced callus formation (11, 12). The *Arabidopsis Aberrant Lateral Root Formation 4* (*ALF4*), which encodes a nuclear protein expressed in multiple organs and was initially shown to modulate lateral root formation and other developmental processes (13), was recently demonstrated to be involved in the regulation of pericycle competence for CIM-induced callus formation. Disruption of *ALF4* leads to the loss of callus-forming capability in multiple organs, including roots, cotyledons, and petals (7). The protoplasts prepared from *alf4-1* plants fail to reinitiate cell division (14), suggesting that ALF4 may be required for pericycle and possibly other cell types to enter the regeneration programs. However, the signals governing ALF4-mediated pericycle competence remain unclear.

The very-long-chain fatty acids (VLCFAs) generally include fatty acids with an acyl chain length of ≥18 carbons, which are biosynthesized by the fatty acid elongase complex that sequentially adds two carbons into the acyl chain (15). The fatty acid elongase

Significance

Callus induction is an initial step for typical plant in vitro regeneration, and recent studies show that auxin-induced callus formation in multiple organs occurs from the pericycle or pericycle-like cells via a root developmental pathway. We demonstrate here that very-long-chain fatty acids (VLCFAs) or their derivatives act as the critical signal in restricting the callus-forming capacity of the pericycle and thus the regeneration capability in *Arabidopsis*. Our work not only discloses an unidentified role of VLCFAs in defining the regeneration capacity, but also sheds light on the signals that govern the cell states in plant organs. Our findings also may have relevance for investigating the possible role of VLCFAs in the regulation of cell states in animals.

Author contributions: B.S., C.X., and Y.H. designed research; B.S., C.X., X.Z., H.C., and W.X. performed research; B.S., C.X., and Y.H. analyzed data; and B.S., C.X., and Y.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1522466113/-/DCSupplemental.

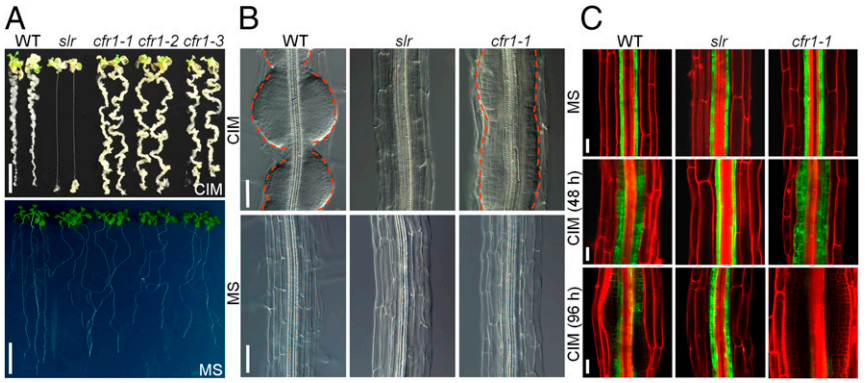

Fig. 1. *cfr1* enhances the callus-forming capacity of pericycle. (*A*) Callus-forming phenotype (*Upper*) and morphology (*Lower*) of WT, *slr*, and three alleles of *cfr1* seedlings. (Scale bars: 1 cm.) (*B*) Cytology of callus formation (*Upper*) and cellular organization (*Lower*) of the mature zone in WT, *slr*, and *cfr1-1* roots. (Scale bars: 50 μm.) (*C*) Expression of the pericycle marker J0121 in the mature zone of WT, *slr*, and *cfr1-1* roots on MS or CIM. (Scale bars: 25 μm.)

complex in plants consists of ketoacyl-CoA synthase (KCS), ketoacyl-CoA reductase (KCR), 3-hydroxy acyl-CoA dehydratase (HCD, also known as PASTICCINO 2, or PAS2), and enoyl-CoA reductase (ECR) (16–19). Recent studies suggest that the VLCFAs or their derivatives, such as cuticular lipids, phospholipids, and sphingolipids, are not only components of protective barriers or cell membranes, but also may act as signaling molecules to mediate various biological processes. In mammals, VLCFAs have been shown to play important roles in cell apoptosis and cell differentiation, as well as in termination of cell proliferation (20–22). In plants, the *Arabidopsis* loss-of-function mutants *pas2* and *kcr1* are embryo-lethal, whereas their leaky alleles exhibit enlarged shoot apical meristems, fused rosette leaves, and altered lateral root branching (17, 18, 23). VLCFAs are also known to regulate programmed cell death during plant–pathogen interactions, to promote cell elongation in cotton fibers by activating ethylene biosynthesis, and to act as a cell layer signal to regulate cell proliferation in the *Arabidopsis* shoot apex by suppressing cytokinin biosynthesis (24–27).

Here we report that VLCFAs play a crucial role in restricting the competence of the pericycle for callus formation and thus the regeneration capacity in *Arabidopsis*. We provide evidence that VLCFAs act as cell layer signals to confine the pericycle competence for callus formation, at least in part, by inhibiting *ALF4* transcription. Our findings indicate that VLCFAs or their derivatives serve as critical signals in mediating CIM-directed callus formation and hence the regeneration capacity in plants.

Results

***cfr1* Bypasses the Inhibition of Callus-Forming Capacity by *solitary-root*.** We previously demonstrated that the four *Arabidopsis* LBD transcription factors act downstream of auxin response factor (ARF) 7 and ARF19 to direct CIM-induced callus formation (8). To further explore the molecular basis of plant regeneration, we performed a genetic screen with ethyl methanesulfonate (EMS)-mutagenized *solitary-root* (*slr*, also known as *iaa14*) plants (28), based on the knowledge that the primary roots of *slr*, with the exception of root apical meristems, are incapable of forming calli on CIM (Fig. 1*A*). This screen allowed us to identify several mutants, termed *callus formation-related* (*cfr*), by their apparently callus-forming phenotype in their primary roots (Fig. 1*A*). Three of the *cfr* mutants displayed a similar phenotype, and genetic analyses showed that they resulted from a recessive mutation in a single gene and were genetically allelic to each other; thus, they were named *cfr1-1*, *cfr1-2*, and *cfr1-3* (Fig. 1*A*).

The *cfr1* seedlings exhibited a strong callus-forming phenotype throughout the primary roots when incubated on CIM, which restored the defect in callus formation of the *slr* roots (Fig. 1*A* and Fig. S1*A*). However, when grown on the medium lacking plant hormones, *cfr1* seedlings were still defective in lateral root initiation and gravitropism, as were the *slr* roots (Fig. 1*A* and Fig. S1 *B* and *C*). Moreover, like *slr*, *cfr1* mutants still displayed hyposensitivity to exogenous auxin in initiating the lateral roots (Fig. S1*B*), suggesting that the overall auxin responses are not altered in *cfr1* plants. In addition, the *cfr1* and *slr* plants grown in soil had a similar morphology, including small rosette leaves, short inflorescence stems, and enhanced apical dominance (Fig. S1 *D*–*F*). These observations demonstrate that the mutation in *CFR1* could bypass the callus-forming capacity inhibited by *slr*.

***cfr1* Enhances Pericycle Competence for Callus Formation.** We then used differential interference contrast (DIC) microscopy to compare the cytological characteristics of mature region of primary roots in WT, *slr*, and *cfr1-1* plants before and after incubation on CIM. Before being transferred to CIM, the *cfr1-1*, *slr*, and WT roots had the same arrangement of cell layers with a normally organized structure (Fig. 1*B*); however, after seedlings were incubated on CIM, the callus formation in WT occurred at regular intervals from pericycle cells with a structure of lateral root-like initials, whereas the pericycle cells of *cfr1-1* proliferated along the entire roots, leading to formation of a continuous callus layer without an apparent interval structure (Fig. 1*B*). We next visualized the expression of J0121, a widely used pericycle identity marker (11), in WT, *slr*, and *cfr1-1* roots before and after incubation on CIM. Similarly, the expression of J0121 in *cfr1-1* roots was similar to that in both WT and *slr* roots before incubation on CIM (Fig. 1*C*). After seedlings were incubated on CIM, J0121 expression was exclusively maintained in pericycle cells of *slr* primary roots but gradually decreased in the WT pericycle cells in which the initial structures developed (Fig. 1*C*). In contrast, J0121 signals became disappeared in the entire *cfr1-1* pericycle where the extensive cell proliferation occurred (Fig. 1*C*). These observations indicate that the callus in *cfr1* originates from pericycle cells, and that the pericycle cells of *cfr1* have a high competence to enter the callus-forming program.

To determine whether the calli derived from *cfr1* have root meristem characteristics, we monitored the expression of *pWOX5::GFP-ER* and *pPLT1::PLT1:YFP*, two markers expressed in the root meristem and recently shown to be characteristic markers of CIM-induced calli derived from multiple organs (7, 29, 30). The fluorescent signals were detected in both WT and *cfr1-1* calli, but not in *slr* pericycle cells (Fig. S2 *A* and *B*), indicating that the callus derived from *cfr1* has the property of root meristems. We incubated *cfr1-1* seedlings on CIM for 12 d and then transferred them to shoot-inducing medium (SIM), and observed that adventitious shoots regenerated efficiently from *cfr1-1* calli (Fig. S2 *C* and *D*).

To test whether *cfr1* has an effect on the shoot-regenerating capability of the callus, we compared the expression of *PLT* genes, which is considered to reflect the shoot-regenerating competence of calli (9), in WT, *slr*, and *cfr1-1* root-derived calli. We observed that expression levels of these *PLT* genes were comparable among the three genotypes (Fig. S2*E*), suggesting that *cfr1* might affect mainly the callus-forming capacity of the pericycle rather than the regenerating capability of derived calli.

***CFR1* Encodes the 3-Ketoacyl-CoA Synthase 1 (KCS1).** Using an F2 population of *cfr1-1* crossed with the *Arabidopsis* Landsberg *erecta* (L*er*) ecotype, we finely mapped *CFR1* to a region of ∼110 kb on

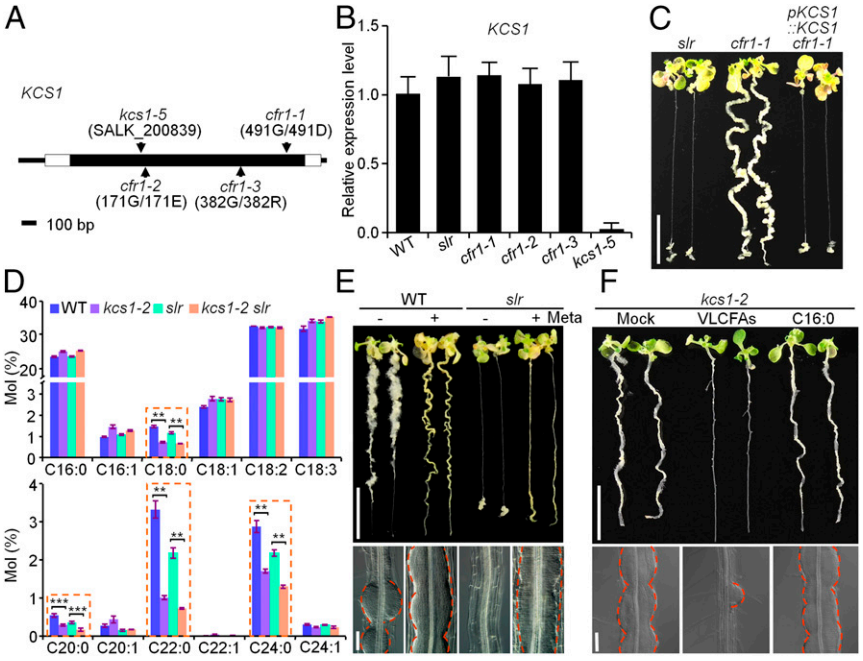

Fig. 2. VLCFAs restrict the pericycle competence for callus formation. (*A*) Map-based cloning and sequencing showing the mutation sites of *cfr1* in the coding region of *KCS1*. The T-DNA insertion site of *kcs1-5* is also indicated. (*B*) The expression level of *KCS1* in WT, *slr*, the *cfr1* alleles, and *kcs1-5*. $n = 3$ biological replicates. Error bars are SD. (*C*) Callus-forming phenotype of *slr*, *cfr1-1*, and transgenic *cfr1-1* seedlings carrying *pKCS1::KCS1*. (Scale bar: 1 cm.) (*D*) Total fatty acid composition of WT, *kcs1-2*, *slr*, and *kcs1-2 slr* roots. $n = 3$ biological replicates. Error bars are SD. Significance was determined by Student's *t* test. $^{**}P < 0.01$; $^{***}P < 0.001$. (*E*) Effect of metazachlor (Meta) on pericycle competence for callus formation. WT and *slr* seedlings were incubated on CIM supplemented with (+) or without (–) exogenous application of 5 μM metazachlor. (Scale bars: 1 cm in *Upper*, 50 μm in *Lower*.) (*F*) Exogenous VLCFAs inhibit callus-forming capacity in the *kcs1-2* pericycle. *kcs1-2* seedlings were incubated on CIM supplemented with VLCFAs (a mixture of C18:0, C20:0, C22:0, and C24:0) or C16:0 fatty acids; the *tert*-butyl methyl ether for dissolving VLCFAs served as a control (Mock). (Scale bars: 1 cm in *Upper*, 50 μm in *Lower*.)

chromosome 1. Sequencing of genes in this region in the *cfr1-1* genome enabled the identification of a G-to-A transition at a position +1,472 bp from the start codon of *KCS1* that resulted in an amino acid substitution of 491Gly to 491Asp in KCS1 (Fig. 2*A*). Further sequencing of *KCS1* in *cfr1-2* and *cfr1-3* validated that the coding regions of *KCS1* contained allelic mutations (Fig. 2*A*). Expression analysis showed that the *KCS1* transcript levels in the three *cfr1* alleles were comparable to those in WT and *slr* plants (Fig. 2*B*). We then introduced the WT *KCS1* genomic DNA (~4.5 kb, including a 2.7-kb promoter region) into *cfr1-1*, and found that the callus-forming phenotype in *cfr1-1* roots was fully blocked in these transgenic plants (Fig. 2*C*), indicating that the *KCS1* mutation confers the enhanced callus-forming phenotype observed in the *cfr1* plants.

We then crossed *cfr1* mutants with WT and obtained *kcs1* mutants that lacked the *slr* mutation. Because the *kcs1-1* mutant has been previously characterized in *Arabidopsis* (31), we designated the newly identified alleles as *kcs1-2*, *kcs1-3*, and *kcs1-4* and the *cfr1* allele as *kcs1 slr*. We also obtained a T-DNA insertion mutant (SALK_200839), *kcs1-5*, from the *Arabidopsis* Biological Resource Center (ABRC), in which a T-DNA sequence was inserted in the *KCS1* coding region and *KCS1* mRNA was undetectable (Fig. 2 *A* and *B*). The *kcs1-2* and *kcs1-5* seedlings incubated on CIM still exhibited the enhanced callus-forming phenotype in their primary roots, with an additive morphology of WT and *kcs1 slr* roots by flattened initial structures (Fig. S3 *A* and *B*). Further introduction of WT *KCS1* genomic DNA into *kcs1-2* fully restored the callus-forming phenotype of *kcs1-2* to the morphology observed in WT (Fig. S3*C*). Moreover, lateral root formation in *kcs1-2* and *kcs1-5* was not altered (Fig. S3*D*), and KCS1 accumulation in *slr* roots was similar to that in WT (Fig. S3*E*). These results support the idea that the KCS1-mediated pericycle competence for callus formation is independent of SLR-modulated lateral root formation.

VLCFAs Play an Inhibitory Role in Confining Pericycle Competence for Callus Formation. KCS1 is a part of the fatty acid elongase complex and catalyzes a rate-limiting step in VLCFA biosynthesis (Fig. S4*A*) (15, 31). To test whether the VLCFAs are responsible for altered pericycle competence for callus formation in *kcs1* and *kcs1 slr*, we first compared the total fatty acid levels in roots of WT, *kcs1-2*, *slr*, and *kcs1-2 slr*. In agreement with the known function of KCS1 in VLCFA biosynthesis, the saturated VLCFA levels for C18:0, C20:0, C22:0, and C24:0 in *kcs1-2* and *kcs1-2 slr* were only approximately 30–60% of those in WT and *slr* plants (Fig. 2*D*).

We next incubated WT and *slr* seedlings on CIM supplemented with metazachlor, a known inhibitor of VLCFA biosynthesis that acts by inhibiting the activities of KCS1 and other KCS enzymes (32). As expected, the WT and *slr* roots incubated on CIM with metazachlor recapitulated the callus-forming morphology observed in the pericycle of *kcs1* and *kcs1 slr* roots, respectively (Fig. 2*E*). We then incubated the *kcs1-2* seedlings on CIM supplemented with a mixture of VLCFAs (C18:0, C20:0, C22:0, and C24:0) or their precursor C16:0 fatty acids, and observed that the exogenous application of VLCFAs, but not of C16:0 fatty acids, almost fully blocked the callus-forming capacity of *kcs1-2* roots (Fig. 2*F*). We also obtained a T-DNA insertion mutant (SALK_051324), *pas1-4*, from the ABRC, in which the *PASTICCINO 1* (*PAS1*) that encodes a scaffold protein of the fatty acid elongase complex was disrupted (Fig. S4 *A* and *B*) (33). Consistently, *pas1-4* seedlings grown on CIM also displayed an enhanced callus-forming phenotype, as did *kcs1*, and introduction of *pas1-4* into *slr* resulted in bypassed callus formation in *pas1-4 slr* roots (Fig. S4*C*). These findings demonstrate that VLCFAs play an inhibitory role in confining pericycle competence for callus formation.

We next tested whether VLCFA deficiency affects the callus-forming capacity of aerial organs by incubating hypocotyls and cotyledons of *kcs1-2* and *pas1-4* on CIM. As shown in Fig. S4 *D* and *E*, although callus formation in the *kcs1-2* cotyledon appeared to be slightly enhanced compared with that in WT, the strong callus-forming phenotype was observed in the *kcs1-2* and *pas1-4* hypocotyls and the *pas1-4* cotyledon, implicating that VLCFAs also have an effect on the callus-forming capacity of aerial organs.

Because auxin plays an essential role in directing callus formation and VLCFAs have been suggested to regulate polar auxin transport on lateral root formation (3, 8, 33), we also explored whether the VLCFA-mediated pericycle competence for callus formation is related to endogenous auxin hemostasis or spatial accumulation. Careful comparison of DR5::GFP and PIN1:GFP in the roots of WT, *kcs1-2*, *slr*, and *kcs1-2 slr* before and after incubation on CIM revealed that the overall auxin accumulation in *kcs1-2* or *kcs1-2 slr* roots was similar to that in WT or *slr* roots, respectively (Fig. S5 *A* and *B*). Likewise, the expression levels of auxin-induced *LBD16*, *LBD17*, *LBD18*, and *LBD29*, which are targets of SLR-ARF7/ARF19 (8, 34), were comparable in the *kcs1-2*

and WT roots but were reduced to a similar level in the *slr* and *kcs1-2 slr* roots (Fig. S5*C*). These observations suggest that VLCFA-mediated pericycle competence for callus formation is not attributable to endogenous auxin homeostasis or spatial accumulation.

***ALF4* Acts Downstream of VLCFAs.** Because *Arabidopsis* ALF4 has been reported to be necessary for CIM-induced callus formation in multiple organs (7, 14), we speculated that *ALF4* may be involved in VLCFA-mediated pericycle competence for callus formation. To test this, we first compared the expression of *ALF4* in WT, *kcs1-2*, *slr*, and *kcs1-2 slr* seedlings. Our real-time quantitative RT-PCR (qRT-PCR) analysis showed that the *ALF4* expression was indeed elevated by approximately twofold in *kcs1-2* and *kcs1-2 slr* compared with that in WT and *slr* (Fig. 3*A*). This elevation was further validated by GUS staining assayed with the primary roots of transgenic plants harboring a *pALF4::β-glucuronidase* (*GUS*) construct (Fig. 3*B*). In contrast, *ALF4* expression was found to be comparable in the WT and *slr* seedlings on either MS or CIM (Fig. 3 *A* and *B* and Fig. S6 *A* and *B*), implicating that the alteration of *ALF4* transcription caused by *kcs1* is independent of the *slr* mutation. Moreover, treatment with metazachlor resulted in an elevation of *ALF4* expression in WT roots (Fig. 3 *C* and *D*), whereas exogenous application of the VLCFA mixture, but not the C16:0 fatty acids, caused decreased *ALF4* transcription in *kcs1-2* roots (Fig. 3 *E* and *F*). These results demonstrate that VLCFAs could suppress *ALF4* transcription.

To further examine the possibility that *ALF4* acts downstream of VLCFAs, we generated *alf4-1 kcs1-2* double-mutant plants by crossing *kcs1-2* with *alf4-1*$^{-/+}$ plants, and examined the callus-forming capacity of these plants when incubated on CIM. As shown in Fig. 3*G*, disruption of *ALF4* completely blocked pericycle cells from forming callus in *kcs1-2*. Furthermore, the transgenic plants overexpressing *ALF4* on CIM recapitulated the enhanced callus-forming phenotype observed in *kcs1-2* (Fig. 3*H*), and the overexpression of *ALF4* partially rescued the callus-forming defect in *slr* roots (Fig. S6*C*). These findings suggest that the inhibition of pericycle competence for callus formation by VLCFAs occurs, at least in part, through the regulation of *ALF4* transcription.

Because previous work has also shown that VLCFAs can repress cytokinin biosynthesis but activate ethylene biosynthesis (26, 27), we investigated whether VLCFA-mediated pericycle competence for callus formation is associated with cytokinin or ethylene homeostasis. Both qRT-PCR and GUS staining assays showed that *ALF4* expression was not affected by the exogenous application of either kinetin or the widely used ethylene signaling inhibitor $AgNO_3$ (35), demonstrating that *ALF4* does not transcriptionally respond to cytokinin or ethylene (Fig. S7 *A* and *B*). The transgenic plants overexpressing *ISOPENTENYLTRANSFERASE 3* (*IPT3*), a gene that encodes an enzyme that catalyzes a rate-limiting step in cytokinin biosynthesis (36), did not recapitulate the callus-forming phenotype observed in *kcs1-2*, and ectopic expression of *Cytokinin Oxidase 1* (*CKX1*), which results in a cytokinin deficiency in transgenic plants (37), did not block or attenuate the callus-forming phenotype of *kcs1-2* (Fig. S7 *C* and *D*). Similarly, application of $AgNO_3$ to WT plants or the treatment of *kcs1-2* with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) had no obvious effect on callus formation (Fig. S7 *E* and *F*). These observations suggest that VLCFA-modulated pericycle competence for callus formation does not rely on the alteration of cytokinin or ethylene homeostasis.

VLCFAs as Cell Layer Signals in Confining Pericycle Competence for Callus Formation. *Arabidopsis KCS1* is expressed in almost all organs, including roots, stems, leaves, and flowers (16, 31). To examine whether KCS1 accumulates in the pericycle cells, we visualized KCS1 accumulation in the roots of transgenic *kcs1-2* plants harboring a *pKCS1::KCS1:GFP* construct in which the enhanced callus-forming phenotype was blocked. As shown in Fig. 4*A*, abundant GFP signals were observed in the endodermis of primary roots, in the proliferating cells of lateral root primordium, and in the emerged lateral root; however, GFP signals were

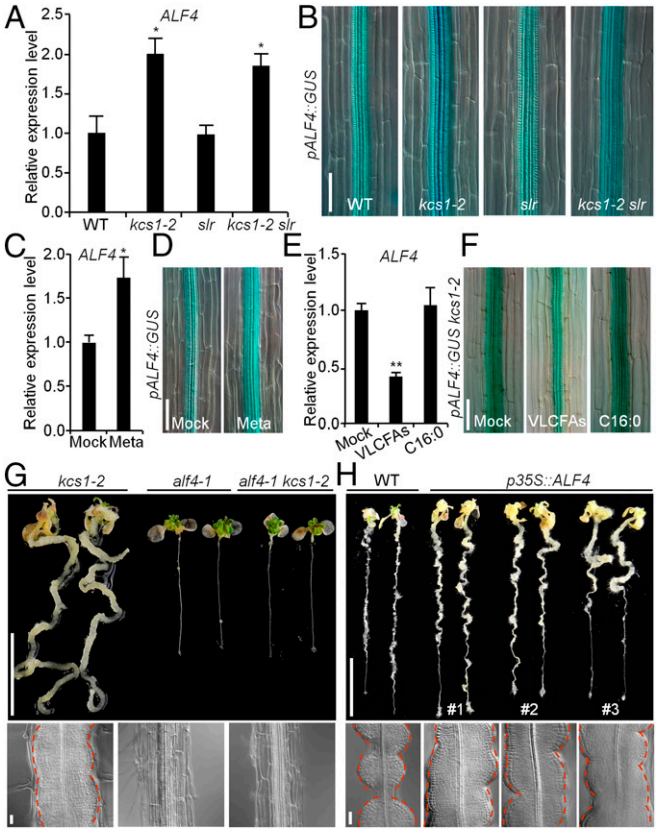

Fig. 3. *ALF4* acts downstream of VLCFAs. (*A* and *B*) qRT-PCR and GUS staining analyses of *ALF4* expression in WT, *kcs1-2*, *slr*, and *kcs1-2 slr*. $n = 3$ biological replicates. Error bars are SD. Significance was determined by Student's *t* test. $^*P < 0.05$. (Scale bar: 50 μm.) (*C* and *D*) *ALF4* expression in WT or *pALF4::GUS* seedlings treated with or without (Mock) 5 μM metazachlor (Meta). $n = 3$ biological replicates. Error bars are SD. Significance was determined by Student's *t* test. $^*P < 0.05$. (Scale bar: 50 μm.) (*E* and *F*) *ALF4* expression in *kcs1-2* or *pALF4::GUS kcs1-2* seedlings treated with VLCFAs or C16:0 fatty acids. $n = 3$ biological replicates. Significance was determined by Student's *t* test. $^{**}P < 0.01$. (Scale bar: 50 μm.) (*G* and *H*) Callus-forming phenotype (*Upper*) and cytological morphology (*Lower*) of *kcs1-2 alf4-1* plants (*G*) and transgenic *p35S::ALF4* plants (*H*). (Scale bars: 1 cm in *Upper*, 20 μm in *Lower*.)

undetectable in the pericycle cells of mature zones and in the meristem region (Fig. 4*A*). This finding suggests that VLCFAs synthesized in the endodermis may act as cell layer signals to affect *ALF4* expression in the pericycle and thus restrict pericycle competence for callus formation.

To test this, we attempted to express *KCS1* in the cortex of *kcs1-2* roots by generating transgenic *kcs1-2* plants expressing *KCS1* driven by the promoter of *Plastid Endopeptidase* (*PEP*), which is expressed exclusively in the cortex of elongation and mature zones of roots (38). We observed that the cortex-expressed *KCS1* could also suppress the enhanced callus-forming phenotype but did not affect the cell layer organization and callus origin in the *kcs1-2* roots (Fig. 4 *B*–*D*). These findings support the VLCFAs or their derivatives as cell layer signals in confining the pericycle competence for callus formation and thus the regeneration capacity in plants.

Discussion

The maintenance of varied cell competences in an organ is critical for body construction in both animals and plants, and the properly maintained states of pericycle or pericycle-like cells within plant organs also greatly contribute to their remarkable regeneration capabilities (2, 7). Recent studies have suggested that ALF4 is critical for the pericycle competence for CIM-induced callus

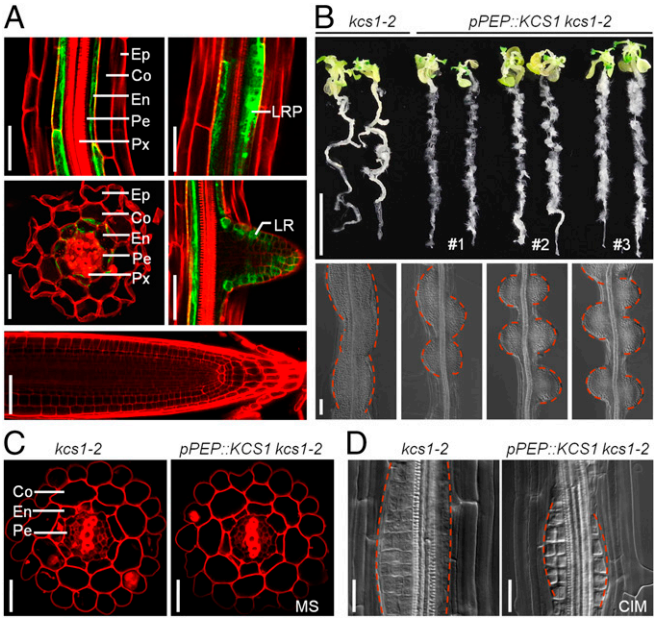

Fig. 4. VLCFAs as cell layer signals in confining pericycle competence for callus formation. (*A*) KCS1 accumulation in roots of *kcs1-2* plants harboring a *pKCS1::KCS1:GFP* construct. (Scale bars: 50 μm.) (*B*) Callus-forming phenotype (*Upper*) and cytological characteristics (*Lower*) in *kcs1-2* seedlings carrying a *pPEP::KCS1* construct. (Scale bars: 1 cm in *Upper*, 50 μm in *Lower*.) (*C* and *D*) Cell layer organization and callus origin in the mature zones of *kcs1-2* and *pPEP::KCS1 kcs1-2* roots. Sectioning and a clearing assay were performed with the roots on MS (*C*) and on CIM (*D*), respectively. (Scale bars: 20 μm.) Ep, epidermis; Co, cortex; En, endodermis; Pe, pericycle; Px, protoxylem; LRP, lateral root primordium; LR, lateral root.

formation, whereas the signals and molecular basis that govern the pericycle competence for regeneration are unclear. Here we have demonstrated that a deficiency of VLCFAs in *Arabidopsis* enhances the callus-forming capacity of pericycle cells, whereas exogenous VLCFAs inhibit pericycle cells from forming the callus. We also provide evidence that VLCFAs act as cell layer signals to restrict the pericycle competence for callus formation partially through regulation of *ALF4* transcription. These findings thus identify the VLCFAs or their derivatives as important signal molecules for mediating pericycle competence for the regeneration capacity of plant organs. More importantly, the signals that stringently maintain the differentiated states of the cells under developmental progression remain unclear (39), and thus it is likely that VLCFA-mediated ALF4 signaling is also necessary to maintain the optimal states of pericycle or pericycle-like cells and thereby prevent excess callus formation in response to external cues. Our findings may shed light on how plant cell states are stringently maintained during normal growth and development.

VLCFAs are components of the cellular membrane in animal and plant cells, and are present mainly in the form of sphingolipids and phospholipids (15). Increasing evidence suggests that VLCFAs or their derivatives are likely bioactive signals that mediate a variety of developmental processes and environmental responses. In plants, VLCFAs have been shown to mediate development as well as biotic and abiotic responses, including hypersensitive cell death and defense (15, 24–27, 40). In yeast and animals, VLCFAs also serve as precursors of bioactive lipid signaling molecules that regulate cell proliferation and apoptosis (20, 41, 42). Recent studies also suggest that VLCFAs or their derivatives participate in the regulation of animal cell differentiation or organ regeneration processes, such as activation of quiescent muscle stem cells known as satellite cells to proliferate in the process of skeletal muscle regeneration after injury (43, 44). Given that a large number of diverse metabolites are derived from VLCFAs (15, 21, 45), which VLCFA-derived molecule(s) act as signals and what signaling components are involved in regulation of specific biological events in both plants and animals remain unknown. Therefore, it is of interest to further define the molecules and signaling components involved in the regulation of pericycle competence for callus formation and to explore whether they have a similar role in animal cells. Any new knowledge gained through such work also would benefit the manipulation of cell pluripotency in both kingdoms.

Our finding that the SLR-mediated lateral root initiation is not necessarily required for CIM-induced callus formation in *kcs1 slr* mutant also raises a question regarding the extent to which the lateral root formation and CIM-induced callus formation programs overlap. In *Arabidopsis*, the root pericycle is responsible for lateral root initiation and CIM-induced callus formation (6, 7, 11, 12), and the CIM-induced callus formation follows a root developmental pathway (6, 7). Indeed, several mutants defective in lateral root initiation, including *slr*, *arf7 arf19*, *p35S::LBD16-SRDX*, and *alf4-1*, display a compromised or blocked callus-forming phenotype on CIM (7, 8, 13, 28, 34). Surprisingly, we found that the enhanced callus-forming capacity in *kcs1* is independent of *slr* mutation. Moreover, the severe mutant or transgenic plants deficient in VLCFA biosynthesis, such as *pas1* or *KCR1-RNAi* plants, have been reported to exhibit retarded lateral root formation (17, 33). Thus, the VLCFA-mediated pericycle competence for callus formation is through a pathway independent of SLR-modulated lateral root formation (Fig. S8). Because the enhanced callus-forming phenotype of *kcs1 slr* and *kcs1* is observed only on CIM, and the resulting calli still have root meristem characteristics, it is likely that the molecular events of the induction of pluripotent cells with root meristem characteristics by auxin are shared for both callus formation and lateral root initiation, whereas the other differentiation programs directed by SLR are still necessary for lateral root patterning (Fig. S8).

Finally, because auxin is a key phytohormone in directing pericycle-derived lateral root initiation and callus formation (3, 8, 46), and because previous studies have suggested that a deficiency of VLCFAs in the *pas1* mutant results in an alteration of polar auxin distribution (33), the extent to which the VLCFA-mediated pericycle competence for callus formation is associated with auxin responses remains unclear. Although we observed that the overall auxin distribution and response in *kcs1* and *kcs1 slr* plants are not obviously altered and previous work has also shown that *ALF4* expression and subcellular localization of ALF4 are not regulated by auxin (13), the enhanced callus-forming phenotype in *kcs1* and *kcs1 slr* is observed only on CIM, which contains excess amounts of the nontransportable auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D) (47). Moreover, a recent study has suggested that the perturbed graft formation in *alf4-1* occurs along with the decreased auxin responsiveness (48). Therefore, we could not exclude the possibility that VLCFA-mediated pericycle competence for callus formation is related to the alteration of auxin response or sensitivity of pericycle cells. Further work is still needed to clarify whether the pluripotent states of cells are closely associated with their auxin responsiveness or sensitivity in plants.

Materials and Methods

Plant Materials and Growth Conditions. The *cfr1* mutant was identified from an EMS-mutagenic population of the *slr* mutant. The *slr* and *alf4-1* mutants, as well as the J0121, *pWOX5::GFP-ER*, *pPLT1::PLT1:YFP*, *DR5::GFP*, and *pPIN1::PIN1:GFP* marker lines, have been described previously (11, 13, 28–30, 49, 50). T-DNA insertion mutants *kcs1-5* (SALK_200839) and *pas1-4* (SALK_051324) were obtained from the ABRC. *pKCS1::KCS1*, *pKCS1::KCS1:GFP*, *pALF4::GUS*, *pPEP::KCS1*, and OE lines of *ALF4*, *IPT3*, and *CKX1* were generated in this experiment. Regeneration assays were performed on CIM and SIM as described by Valvekens et al. (4).

Cytological Analyses. For histological analysis, roots were fixed and cleared according to a previously described method for DIC microscopy (51). Thin sections were created as described by Wang et al. (52), and confocal microscopy was performed using a Leica SP5 confocal microscope. GFP and YFP signals were detected by excitation with an argon laser at 488 nm and a spectral detector set at 505–550 nm for the emission. The propidium iodide

(PI) signal was visualized by excitation with an argon laser at 488 nm and a spectral detector set at >585 nm for the emission.

Analysis of Fatty Acids. Total fatty acids of the roots were methylated and extracted for lipid analysis according to the method described by Browse et al. (53).

More detailed information on the experimental methods is provided in *SI Materials and Methods*. The primers used in this study are listed in Table S1.

ACKNOWLEDGMENTS. We thank Dr. Masao Tasaka for the *slr* mutant, Dr. John L. Celenza for the *alf4-1* mutant, Dr. Ben Scheres for *pWOX5::GFP-ER* and *pPLT1::PLT1:YFP*, Dr. Gerd Jürgens for *DR5::GFP*, Dr. Jiří Friml for *pPIN1::PIN1:GFP*, Dr. Jim Haseloff for the J0121 marker lines, and the *Arabidopsis* Biological Resource Center for the seeds of SALK_200839 and SALK_051324. We also thank Zhen Xue for the total fatty acid measurements. This work was funded by the Ministry of Science and Technology of China (Grant 2013CB967300) and the National Natural Science Foundation of China (Grants 31230009 and 31371447).

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