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

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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/la4). 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) gene family is 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.
complex in plants consists of ketocarotenoid synthase (KCS), ketocarotenoid 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 embryonal, 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 (sr, also known as aux1-14) plants (28), based on the knowledge that the primary roots of sr, with the exception of root apical meristems, are incapable of forming calli on CIM (Fig. L4). This screen allowed us to identify several mutants, termed callus formation-related (cfr), by their apparently callus-forming phenotype in their primary roots (Fig. L4). 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. L4).

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 sr roots (Fig. L4 and Fig. S1A). However, when grown on the medium lacking plant hormones, cfr1 seedlings were defective in lateral root initiation and gravitropism, as were the sr roots (Fig. L4 and Fig. S1 B and C). Moreover, like sr, cfr1 mutants still displayed hypersensitivity to exogenous auxin in initiating the lateral roots (Fig. S1B), suggesting that the overall auxin responses are not altered in cfr1 plants. In addition, the cfr1 and sr 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 sr.

**cfr1 Enhances Pericycle Competence for Callus Formation.** We then used differential interference contrast (DIC) microscopy to compare the cytochemical characteristics of mature region of primary roots in WT, sr, and cfr1-1 plants before and after incubation on CIM. Before being transferred to CIM, the cfr1-1, sr, and WT roots had the same arrangement of cell layers with a normally organized structure (Fig. 1B); 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. 1B). We next visualized the expression of J0121, a widely used pericycle identity marker (11), in WT, sr, 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 sr roots before incubation on CIM (Fig. 1C). After seedlings were incubated on CIM, J0121 expression was exclusively maintained in pericycle cells of sr primary roots but gradually decreased in the WT pericycle cells in which the initial structures developed (Fig. 1C). In contrast, J0121 signals became disappeared in the entire cfr1-1 pericycle where the extensive cell proliferation occurred (Fig. 1C). 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 sr 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, sr, and cfr1-1 root-derived calli. We observed that expression levels of these *PLT* genes were comparable among the three genotypes (Fig. S2E), 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 (Ler) ecotype, we finely mapped *CFR1* to a region of ~110 kb on
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. 2A). Further sequencing of KCS1 in cfr1-2 and cfr1-3 validated that the coding regions of KCS1 contained allelic mutations (Fig. 2A). Expression analysis showed that the KCS1 transcript levels in the three cfr1 alleles were comparable to those in WT and slr plants (Fig. 2B). 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. 2C), 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_083893), 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. 2A 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. S3A 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. S3C). Moreover, lateral root formation in kcs1-2 and kcs1-5 was not altered (Fig. S3D), and KCS1 accumulation in slr roots was similar to that in WT (Fig. S3E). 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. S4A) (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. 2D).

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. 2E). We then introduced 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. 2F). 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. S4C). 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 auxin biosynthesis 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

Fig. 2. VLCFAs restrict the pericycle competence for callus formation. (A) Map-based cloning and sequencingshowing 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 levels 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.)
and WT roots but were reduced to a similar level in the slr and kcs1-2 slr roots (Fig. S5C). 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. S4). This elevation was further validated by GUS staining assayed with the primary roots of WT and in the emerged lateral root; however, GFP signals were detected in the WT and slr seedlings on either MS or CIM (Fig. 3 A and B and Fig. S6 A and B), implying 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 slr mutants, and examined the callus-forming capacity of these plants when incubated on CIM. As shown in Fig. 3G, 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. 3H), and the overexpression of ALF4 partially rescued the callus-forming defect in slr roots (Fig. S6C). 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 AgNO3 (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 (CRO1), which results in a cytokinin deficiency in transgenic plants (37), did not block or attenuate the callus-forming phenotype of kcs1-2 (Fig. 7 C and D). Similarly, application of AgNO3 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-mediated 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. 4A, 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 undetectable in the pericycle cells of mature zones and in the meristem region (Fig. 4 B). 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
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 gently maintained the differentiated states of the cells under development (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 pluriotent 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, pWOXS::GFP-ER, pPTLT::PTL::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_00839) and pas1-4 (SALK_05124) were obtained from the ABRC. p35S::LBD16-SRDX, p35S::LBD16::SRDX, 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 Valkenbos et al. (4).

Cytochemical Analyses. For histological analysis, roots were fixed and cleared according to a previously described method for DIC microscopy (51). Thin sections were cut as described by Wang et al. (52) and confocalized microcopy was performed using a Leica SPS 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 >505 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 the SI Materials and Methods. The primers used in this study are listed in Table S1.


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