Independent signalling cues underpin arbuscular mycorrhizal symbiosis and large lateral root induction in rice

Chai Hao Chiu[1], Jeongmin Choi[1] and Uta Paszkowski*

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK.

[1] These authors contributed equally to the work.

* Author for correspondence: Tel: +44 01223 748981 Email: up220@cam.ac.uk

Brief Heading: Chiu *et al* identifies the co-receptor required for symbiont-induced root development in rice.

SUMMARY

- **Perception of arbuscular mycorrhizal fungi (AMF) triggers distinct plant signalling responses for parallel establishment of symbiosis and induction of lateral root formation. Rice receptor kinase CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) and alpha-beta hydrolase DWARF14-LIKE (D14L) are involved in pre-symbiotic fungal perception.**
- **After six weeks of inoculation with *Rhizophagus irregularis*, root developmental responses, fungal colonisation and transcriptional responses were monitored in two independent *cerk1* null mutants; a deletion mutant lacking *D14L*, and with *D14L* complemented as well as their respective wild-type cultivars (cv. Nihonmasari and Nipponbare).**
- **Here we show that although essential for symbiosis, D14L is dispensable for AMF-induced root architectural modulation, which conversely relies on CERK1.**
- **Our results demonstrate uncoupling of symbiosis and the symbiotic root developmental signalling during presymbiosis with CERK1 required for AMF-induced root architectural changes.**

Keywords: arbuscular mycorrhizal symbiosis, root system architecture, lateral root, signalling, receptor kinase, D14L, rice (*Oryza sativa*), *Rhizophagus irregularis*

Introduction

Arbuscular mycorrhizal (AM) symbiosis is an evolutionarily ancient mutualistic relationship, representing an important adaptation in the terrestrialisation of plants (Humphreys et al., 2010). Present in more than 80% of land plants today, this symbiosis with Glomeromycotina fungi contributes significantly to global carbon and nutrient cycles. The extraradical mycelium of AM fungi (AMF) acquires minerals beyond the roots nutrient-depletion zone and delivers a proportion of these to the plant in exchange for organic carbon (Smith and Read, 2008). Despite fundamental differences in root system architecture of mono- and dicotyledons (Osmont et al., 2007), in both lateral roots are preferentially colonised by AMF. Remarkably, their formation is induced upon symbiosis establishment, whereby the interface available for symbiotic nutrient exchange is effectively increased (Gutjahr et al., 2009, Olah et al., 2005, Gutjahr and Paszkowski, 2013).

AM symbiosis-induced lateral root formation is regulated at different stages of the interaction, proposed to involve presymbiotic or intraradical signalling cues (Gutjahr and Paszkowski, 2013). It has been well documented that chitinaceous signals from either rhizobia or AMF mediate root

architectural remodelling prior to fungal colonisation (Maillet et al., 2011, Olah et al., 2005, Mukherjee and Ane, 2011, Sun et al., 2015). Microbial chitin-based signals such as lipochitooligosaccharides (LCOs), the nod- and myc-factors from beneficial rhizobia and AMF respectively, and chitin oligomers (COs) released by fungi are recognised by lysin-motifs (LysM) with chitin-binding properties in the extracellular domain of receptor-like kinases (RLK). Legume Nod Factor Receptor 1 (NFR1) and rice Chitin-Elicitor Receptor Kinase 1 (CERK1) are homologous LysM RLKs on the cell membrane that act via association with other receptor-like proteins (RLP, Kouzai et al., 2014). In legumes, perception of both fungal and bacterial chitinaceous signals by nod-factor receptors stimulates nuclear Ca^{2+}-oscillations, and the activation of the Common Symbiosis Signalling Pathway (CSSP), a conserved signal transduction pathway which is necessary for root invasion by AMF and nitrogen-fixing rhizobia (reviewed in Gobbato, 2015). The similar requirement for intact CSSP in rice indicated the taxonomically broad functional conservation (Gutjahr et al., 2008). Interestingly, evidence for the importance of CSSP for lateral root promotion in response to AMF inoculation is equivocal. This response is dependent on CSSP components in *Medicago truncatula* (Olah et al., 2005) but not in rice (Gutjahr et al., 2009, Mukherjee and Ane, 2011), suggesting a fundamental difference in signalling pathways underpinning root system modulations between the two plant species, and possibly more generally between Leguminosae and Poaceae.

In rice, CERK1 acts as a bifunctional switch that activates both symbiotic and immune responses (reviewed in Shinya et al., 2015, Zipfel and Oldroyd, 2017), leading to an increased susceptibility to foliar rice blast (*Magnaporthe oryzae*) infection and reduced root colonisation by the AMF *Rhizophagus irregularis* (Miyata et al., 2014, Zhang et al., 2015, Kouzai et al., 2014). Interestingly, *cerk1* mutants failed to exhibit the diagnostic Ca^{2+}-spiking in response to fungal exudates or chitotetratose (CO_4), consistent with a CERK1-dependent pre-symbiotic chitin perception in rice (Carotenuto et al., 2017). Despite the lack of Ca^{2+} oscillations, AMF colonisation of *cerk1* mutants still occurred, yet at lower levels than the wild type (Miyata et al., 2014, Zhang et al., 2015, Kouzai et al., 2014). In contrast, DWARF14-LIKE (D14L) is an intracellular α/β- fold hydrolase receptor that in rice is indispensable for pre-symbiotic AMF perception; although the AM symbiosis-relevant ligand(s) of D14L is unknown, deletion of *D14L* impairs the sensitivity of rice to AMF and abolishes any physical interaction between the plant and the fungus (Gutjahr et al., 2015). *D14L* could either be directly involved in the perception of myc-factors from AMF; or in conditioning root tissue to be competent in perceiving myc-factors.

To address signalling specificity for AMF perception and root architectural changes, we here compared rice lines functionally lacking either of the two receptor proteins, CERK1 or D14L (Gutjahr et al., 2015, Kouzai et al., 2014). Surprisingly, signalling for symbiosis and lateral root induction diverges early on, at the level of presymbiotic perception of AMF-released molecules, with a central involvement of

CERK1 in mediating the transduction of the environmental microbial signal into a developmental response.

Materials and Methods

Plant material, plant growth and growth conditions

Rice (*Oryza sativa* L. ssp. *Japonica*) seeds of *OsCERK1* (*LOC_Os08g42580*) knockouts comprise two independent homozygous knockout lines (*KO#53, #117*) alongside wild-types (Rev#53, #117) from the segregating T2 plants of Nipponbare background, as described previously (Miyata et al., 2014, Zhang et al., 2015). For mutants with compromised pre-symbiotic responses, *hebiba*^{AOC} mutants which arose in the Nihonmasari background were used. *hebiba* mutants have a 170kb, 26 gene deletion, and complementation with *ALLENE OXIDE CYCLASE (AOC)* restored jasmonate deficiency and male sterility but not the defective AM colonisation response. *hebiba*^{AOC} mutants complemented with the *D14L* gene, *hebiba*$^{AOC/D14L}$ had restored AMF colonisation (Gutjahr et al, 2015).

Seeds were surface-sterilised briefly in 70% (v/v) ethanol, then for 20 minutes in 3% (v/v) sodium hypochlorite. Imbibed seeds were germinated on 0.9% (w/v) bactoagar at 30^{o}C for 7 days. Plantlets were then transferred into cones containing sterile quartz sand in walk-in growth chambers at 12-hour/12-hour light/dark cycle at 28 ^{O}C/20 ^{O}C and 60% relative humidity. Plants were inoculated with 300 spores of *Rhizophagus irregularis* per plant, as described previously (Gutjahr et al., 2008). AM fungal inoculum was sub-cultured and extracted from hairy carrot (*Daucus carota* L.) root cultures as described in (Gutjahr et al., 2008, Bécard and Fortin, 1988). Plants were watered three times weekly for the first 2 weeks post inoculation (wpi), thereafter fertilised twice a week with half Hoagland solution (25 µM Pi) and 0.01% (w/v) Sequestren Rapid (Syngenta). These growth conditions were demonstrated previously to promote efficient mycorrhizal colonisation (Gutjahr et al., 2008).

Root counting, staining and mycorrhizal colonization quantification

Roots were harvested and preserved in 50% (v/v) ethanol for scoring. Number of crown roots (CRs), large lateral roots (LLRs) and fine lateral roots (FLRs) were counted under a stereomicroscope (Wild Heerbrugg, Switzerland) and CR lengths were measured manually. Trypan blue (Sigma-Aldrich, St. Louis, MO, USA) staining and mycorrhizal colonisation of the different genotypes were quantified as described previously (Gutjahr et al., 2008). Representative images were taken using Keyence VHX-5000 Digital Microscope (Keyence, Milton Keynes, UK).

RNA Extraction, cDNA Synthesis, and Gene Expression Analysis

Roots were harvested and frozen in liquid nitrogen for gene expression analysis. Root tissues were homogenised using metal beads using TissueLyserII (Qiagen) at 30 Hz for 2 minutes. RNA was extracted from ground tissue, assessed for their integrity and purity before conversion into cDNA as described in (Gutjahr et al., 2008). Absence of contaminating genomic DNA was confirmed by performing PCR with primers on two exons flanking a spliced intron in *GAPDH* to yield a lighter product **(Table S1)** following gel electrophoresis on a 0.8% (w/v) agarose gel. gDNA sample was used as a positive control.

Quantitative PCR (qPCR) was performed as described previously, using SYBR Green Fluorophore on C1000 Thermal Cycler with CFX96 real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA) (Gutjahr et al., 2008). Specific primers were used for *CYCLOPHILIN2*, *ACTIN, GAPDH* and *UBIQUITIN* as constitutive reference genes, for *AM1*, *AM3*, *PT11*, *AM14* as AM marker genes specifically induced during symbiosis, for *RiEF1α (R. irregularis ELONGATION FACTOR1α)* as a fungal marker gene and for genotyping *CERK1*, *D14L* **(Table S1)**. Gene expression values were normalised to the geometric mean of the three reference genes, and displayed as a function of *CYCLOPHILIN2* mRNA levels.

Statistical analyses

For root architecture analysis and root colonisation, five entire root systems were analysed for each genotype and treatment. Differences between mock- and AM fungi-inoculated plants were assessed by the non-parametric Mann-Whitney test at 5% significance level using RStudio (http://www.Rproject.org/). For gene expression analysis, 3-6 root systems were analysed. To ensure equal variance, gene expression values were $\log_{10}$ transformed before analysis by One-way ANOVA followed by post hoc Tukey HSD as described (Pimprikar et al., 2016).

Results

To monitor the relevance of CERk1 and D14L on AMF-induced root system architecture, *cerk1* and *hebiba*^{AOC} mutants and corresponding wild-types were co-cultivated with *R. irregularis* to reproduce the natural rhizosphere interactions during AM symbiosis, achieving physiological concentrations and possible gradients of signalling molecules between plants and AMF. All plants were examined for their post-embryonic root system architectural responses at the stage of a fully established symbiosis at 6 wpi. Rice root systems consist of crown (CR), large (LLR) and fine lateral roots (FLR, Rebouillat et al., 2009) with CRs modestly and LLRs extensively colonised but FLRs immune to AMF (Gutjahr et al., 2009). While CRs of the wild type rice cultivars Nipponbare and Nihonmasari did not increase in number or length upon co-cultivation with *R. irregularis* (**Fig. S1a-b**), the total number and density of LLR was

higher on colonised as opposed to non-colonised roots of both cultivars (**Fig. 1a-b**). However, promotion of FLR formation by co-cultivation of plant and fungus was not observed **(Fig. S1c-d)**, arguing against a general activation of lateral root (LR) development (Gutjahr et al., 2009) but for the specific induction of the preferred tissue (LLR) available for colonisation.

Importantly, AMF-induced LLR promotion is lost in both independent knockout lines of *cerk1* mutants where LLR numbers, density and proportion of colonised plants remained at equivalent levels to mock inoculated plants **(*p*>0.4; Fig. 1a-b).** On the contrary, deletion of *D14L* in *hebiba*^{AOC} did not compromise AMF-induced LLR formation, and mirrored the wild-type enhancement (**Fig. 1a-b**). Thus LLR promotion is dependent on *CERK1*, but independent on *D14L*. To verify the development of symbiosis on the same plants, roots were microscopically and molecularly examined for the extent of fungal colonisation. Both *cerk1* null alleles displayed significantly reduced intraradical fungal structures relative to the wild-types (***p*<0.001, Fig. 2a and S2a**), which was also reflected by the limited induction of AM-specific rice marker genes *AM1, AM3, PT11* and *AM14* (**Fig. S3a**, Gutjahr et al., 2008) thereby confirming earlier reports (Miyata et al., 2014, Zhang et al., 2015). Interestingly, the effect of *cerk1* mutation on extraradical fungal structures was less pronounced, matching the statistically equivalent abundance of the fungal housekeeping gene *R. irregularis ELONGATION FACTOR 1α* (*RiEF1α*, ***p*>0.05**; **Fig S3a**), together suggesting considerable fungal growth and thus adequate nourishment. Also consistent with our earlier observations, AMF colonisation was absent from *hebiba*^{AOC} lines and restored to wild-type levels when *D14L* was reintroduced under its native promoter **(Fig. 2b, S2b, Gutjahr et al., 2015)**. Consistently, there was no detectable expression of marker genes including *RiEF1α* in *hebiba*^{AOC} but wild-type levels were restored in genetically complemented *hebiba*$^{AOC, D14L}$ **(Fig. S3b)**.

To establish whether transcriptional cross-talk occurs between CERK1 and D14L signalling, we examined the transcript levels of *CERK1* and *D14L*, and found that both were constitutively expressed independent of the presence or absence of AMF or the perturbation of gene function of the respective other receptor **(Fig. S3a-b)**. In summary, both the abundance of fungal structures and marker gene transcript levels documented that *cerk1* mutants establish AM symbiosis, albeit at lower levels but are compromised in LLR promotion; and that the loss of *D14L* abolishes all AM symbiosis signalling but retains enhanced LLR formation.

Discussion

We conclude that perception of AMF activates at least two independent signalling pathways in rice with D14L and CERK1 as central components with distinct outcomes (**Fig. 3**). Compelling evidence for the uncoupling of symbiotic root developmental and AM symbiosis signalling is provided by the wild type-like LLR induction in the AMF-insensitive *hebiba*^{AOC} mutant. Lack of *D14L* rendered *hebiba*^{AOC} unresponsive to AMF as reflected by the absence of diagnostic transcriptional responses within the first 24 hours post exposure to germinated spore exudates (GSEs, Gutjahr et al., 2015) and also in this study with the lack of induced *AM1* expression. However, the increased LLR production in AMF-inoculated *hebiba*^{AOC} conclusively demonstrated the activation of a developmental signalling pathway mediated by CERK1, but independent of symbiotic signalling that establishes AMF colonisation. On the contrary, despite displaying fungal colonisation, *cerk1* mutants failed to induce the LLR promotion response, lending further support for separate signalling pathways leading either to symbiosis establishment or LLR promotion.

The interaction of rice *cerk1* with either rice blast or AMF led to quantitative phenotypes, reflecting that CERK1 is required but not essential for the respective interactions (Kouzai et al., 2014, Miyata et al., 2014, Zhang et al., 2015). In contrast, we describe here that unexpectedly, CERK1 is vital for the developmental LLR response to fungal inoculation. As AM fungi produce a cocktail of chitinaceous compounds, including short chain chitin oligomers such as CO_4 (Genre et al., 2013) which in rice elicit CERK1-dependent Ca^{2+}-spiking (Carotenuto et al., 2017), the perception of such chitin oligomers by CERK1 may be key to LLR induction. However, chitin binding assays had previously revealed that whereas Arabidopsis CERK1 effectively bound chitin oligomers, rice CERK1 did not (Kouzai et al., 2014). This further suggests that CERK1 interacts with a chitin-binding competent receptor protein to perceive short chain chitin oligomers in GSEs and together transduce signals that result in enhanced LLR development. However, the identity of the ligand(s) that activate this developmental signalling response remains at present elusive (**Fig. 3**).

Furthermore, simulating a more natural condition with fungal inoculum or GSEs instead of the uniform application of high concentrations of chitin signals to rice roots (Sun et al., 2015) repeatedly revealed LLR promotion to be independent of CSSP (Gutjahr et al., 2009, Mukherjee and Ane, 2011), indicating that in rice, other signalling components operate downstream of CERK1 to integrate the rhizosphere signal with the developmental read-out.

In summary, we hypothesise that perception of chitin signals within GSEs involves CERK1 and other high-affinity, ligand-binding RLKs and RLPs at the cell surface, while intracellular D14L could be involved in either direct perception or indirectly via constitution of unknown receptor complex for AM symbiosis. Ligand-binding potentially initiates several independent signalling cascades that mediate

different responses via CERK1, including immunity, symbiosis and LLR promotion. Because AM symbiosis pre-dates the evolution of roots in land plants (Humphreys et al., 2010), and because the toolkits for mycorrhizal symbiosis were already present in the algal ancestors of land plants (Delaux et al., 2015), it is unsurprising for receptor complexes to reprogramme plant development to optimally respond to AMF; or that their roles in regulating development of multicellular plants in response to environmental signals pre-disposed them for symbiosis signalling. Here we identify CERK1, with known roles in immune and symbiosis signalling, to have additional developmental roles, offering a crucial molecular lead for elucidating the signalling pathways for AM symbiosis and LLR promotion.

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Author Contributions

C.H.C. and J.C. performed experiments; J.C. and U.P. designed the experiments; all authors wrote the manuscript.

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Supporting Information:

Fig. S1 Characterisation of crown and fine lateral root properties.
Fig. S2 Representative images of AM colonisation phenotypes of wild-type and mutant plants.
Fig. S3 AM marker gene expression
Table S1 List PCR primers used in this study.

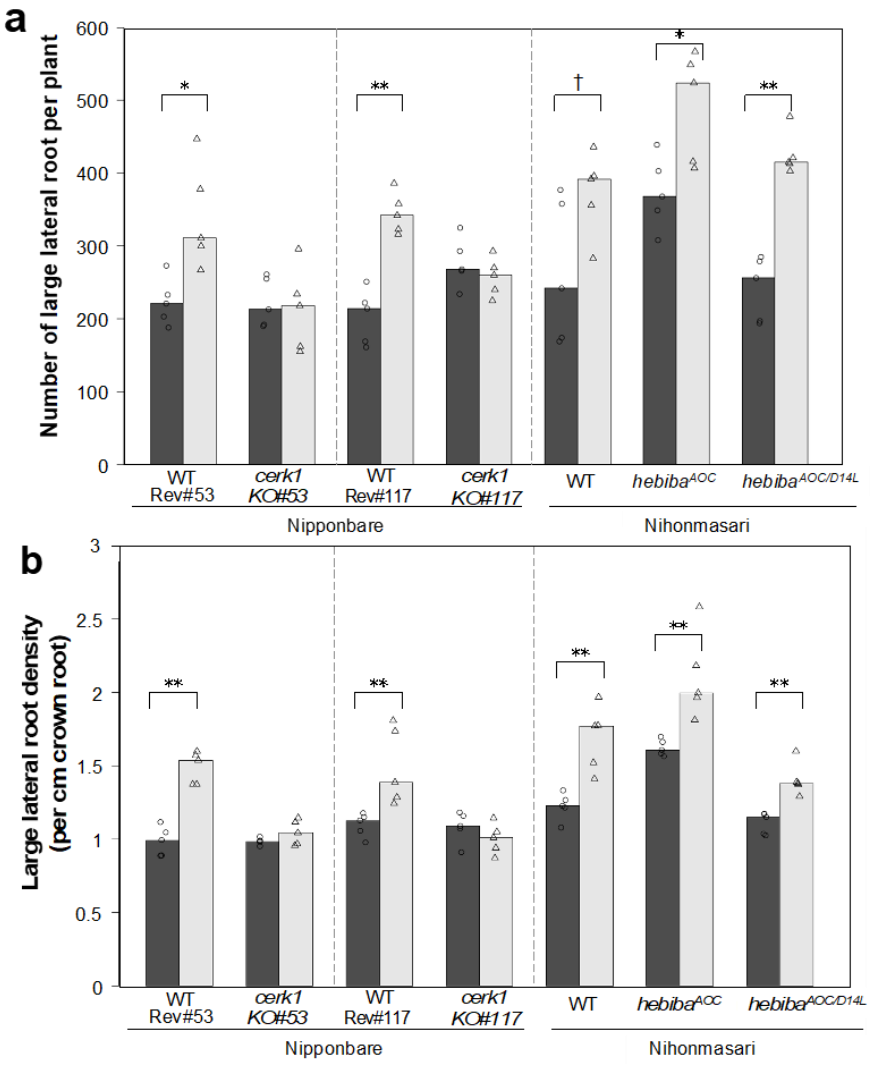

Figure 1: *Rhizophagus irregularis*-induced large lateral root changes are dependent on rice *CERK1* but not *D14L*.

(a) Absolute number of large lateral roots (LLRs) and (b) LLR density expressed as number of LLR per cm of crown roots. The number and lengths were determined at 6 weeks post inoculation (wpi) for both mock treated plants (dark bars, mock) and *R. irregularis*-inoculated plants (light bars, myc). In each graph, the median and individual data points are shown (circles, mock; triangles, myc). Five biological replicates were used for every treatment and genotype. *KO#53* and *KO#117* denote *cerk1* homozygous knockouts lines; Rev#53 and Rev#117 denote the corresponding wild-types derived from segregating T2 generation (cv. Nipponbare); *hebiba*^{AOC} refers to the *hebiba* mutant genetically complemented with the *ALLENE OXIDE CYCLASE* (*AOC*) gene (Riemann et al., 2013); and *hebiba*$^{AOC/D14L}$ with reintroduced *D14L* (Gutjahr et al., 2015). Root architectural changes were compared between mock control and inoculated plants for individual genotypes using the non-parametric Kruskal-Wallis test. † $P < 0.10$, * $P < 0.05$, ** $P < 0.01$.

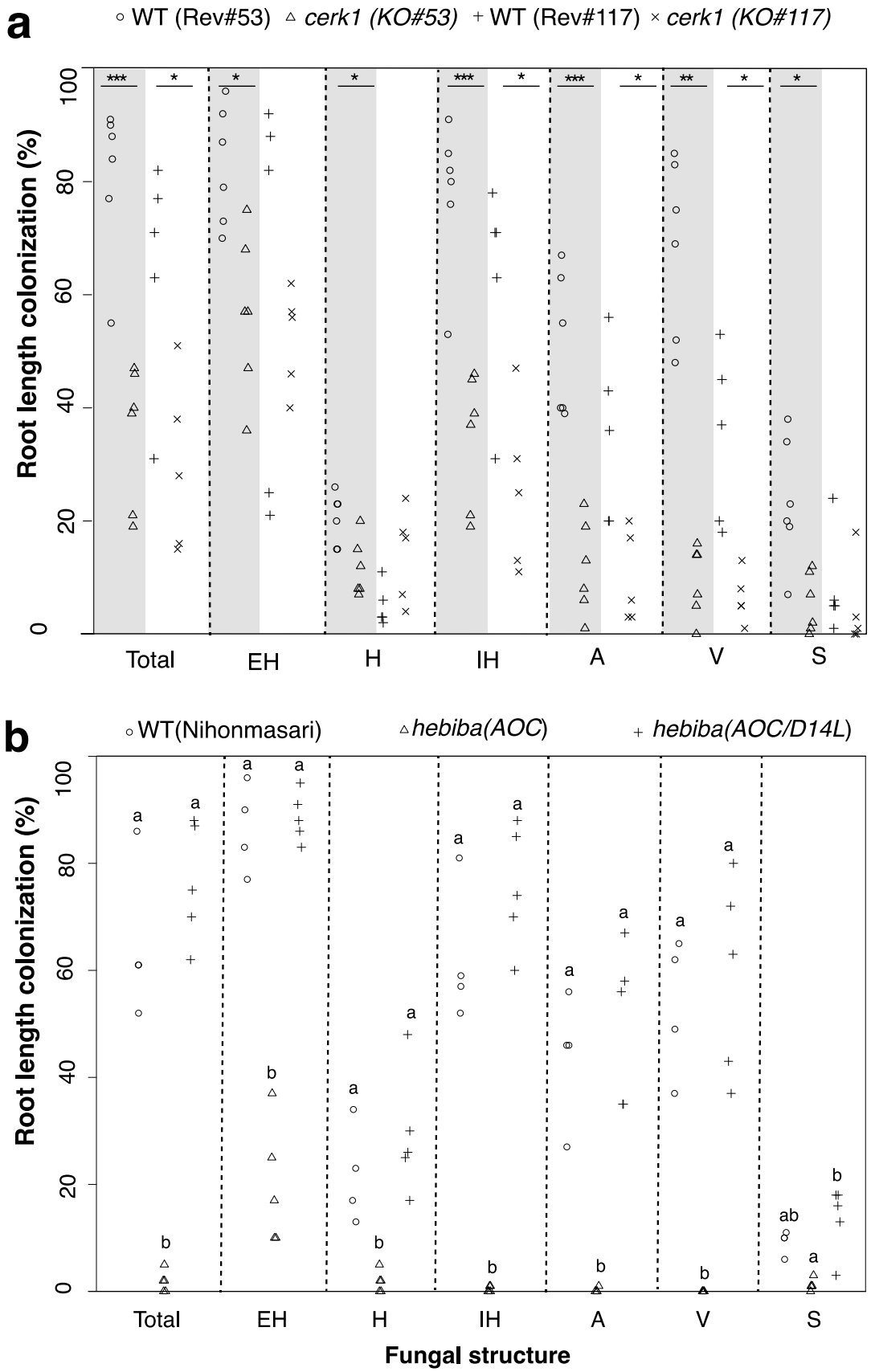

Figure 2. Quantification of colonisation of rice *cerk1* and *hebiba*^{AOC} mutants by *Rhizophagus irregularis*. Percentage root length colonisation in (a) two independent alleles of *cerk1* knockouts

(*KO#53, KO#117*) and their corresponding wild-types (Rev#53, Rev#117); (b) *hebiba*^{AOC} denotes *hebiba* mutant genetically complemented with the *ALLENE OXIDE CYCLASE* (*AOC*) gene (Riemann et al., 2013); and *hebiba*$^{AOC/D14L}$ with reintroduced *D14L* (Gutjahr et al., 2015) and Nihonmasari wild-type cultivar.
Percentage of root length colonisation (RLC) by *R. irregularis* were determined by the grid-line intersect method at 6wpi in plants used for root architecture analysis in **Figure 1**. Data points of 5-6 biological replicates are shown. EH, extra-radical hyphae; H, hyphopodia; IH, intra-radical hyphae; A, arbuscules; V, vesicles; S, spores. For total RLC and individual fungal structures, separate Kruskal-Wallis tests were performed, using the Benjamini-Hochberg adjustment for the post hoc tests in (b).
In (a) the *P* values are denoted by *, $P < 0.1$; **, $P < 0.05$; *** $P < 0.01$ for statistically significant differences between Mock and Myc treatments of the same genotype. Symbols: ○, WT (Rev#53); Δ, *cerk1 (KO#53)*; +, WT (Rev#117); ×, *cerk1 (KO#117)*.
In (b) $P \leq 0.01$ for every fungal structure and different letters above each bar indicate statistically different groups in the post hoc pairwise comparisons. Comparisons are limited to each fungal structure. Degrees of freedom = 2, Total: $\chi^2 = 11.2$, $p = 0$; EH: $\chi^2 = 9.73$, $p = 0.01$; H: $\chi^2 = 10.4$, $p = 0.1$; IH: $\chi^2 = 11.3$, $p = 0$; A: $\chi^2 = 10.3$, $p = 0.01$; V: $\chi^2 = 10.26$, $p = 0.01$; S: $\chi^2 = 9.28$, $p = 0.01$. Symbols: ○, WT (Nihonmasari); Δ, *hebiba*^{AOC} ; +, *hebiba*$^{AOC/D14L}$

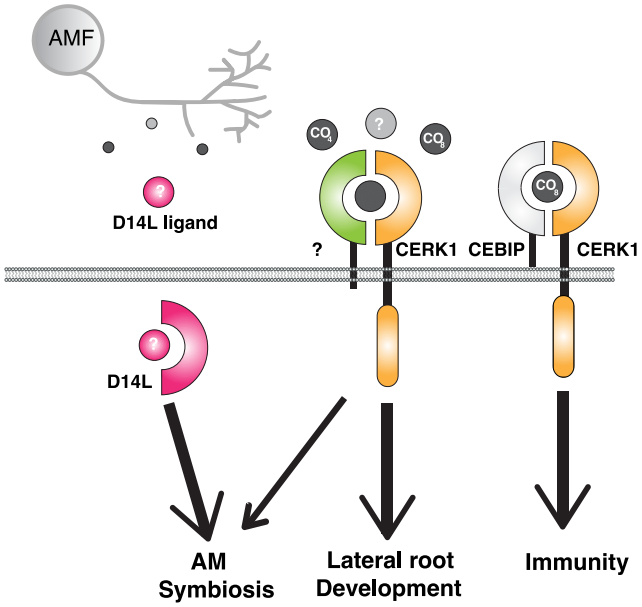

Figure 3: Model summarising the roles of D14L and CERK1 in the independent promotion of AM symbiosis and LLR induction in rice.

Germinating spore exudates (GSE) of arbuscular mycorrhizal fungi (AMF) contain a complex mixture of molecules which includes various Lipo/Chitooligosaccharides (L/COs). In rice, a lysin-motif (LysM) receptor kinase, CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) is required as a co-receptor for the perception of long- (CO_{7-8}) and short-chain (CO_4) chitin oligomers to activate defense and AM symbiosis signalling, respectively (reviewed in Shinya et al., 2015, Zipfel and Oldroyd, 2017). On the other hand, DWARF 14-LIKE (D14L) is an intracellular alpha-beta fold hydrolase responsible for the perception of AMF. Deletion of *D14L* in *hebiba*^{AOC} results in complete absence of fungal colonization and symbiosis signalling, but did not abolish AMF-induced large lateral root (LLR) development. Loss of *CERK1* impairs but does not eradicate AM symbiosis and immunity signalling whereas LLR promotion is abolished. The thickness of the arrows indicate relative importance for the indicated read-outs immunity, AM symbiosis and LLR development.