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The rice LysM receptor-like kinase *Os*CERK1 is required for the perception of short-chain chitin oligomers in arbuscular mycorrhizal signaling

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Heading for social media: Rice chitin receptor OsCerk1 has a role in the perception of symbiotic fungal signals

Summary

- The rice LysM receptor-like kinase *Os*CERK1 is now known to have a dual role in both pathogenic and symbiotic interactions. Following the recent discovery that the *Oscerk1* mutant is unable to host arbuscular mycorrhizal (AM) fungi, we have examined whether *Os*CERK1 is directly involved in the perception of the short chain chitin oligomers (Myc-COs) identified in AM fungal exudates and shown to activate nuclear Ca^{2+} spiking in the rice root epidermis.
- An *Oscerk1* knock-out mutant expressing the cameleon NLS-YC2.60 was used to monitor nuclear Ca^{2+} signaling following root treatment with either crude fungal exudates or purified Myc-COs.
- Compared to wild type rice, Ca^{2+} spiking responses to AM fungal elicitation were absent in root atrichoblasts of the *Oscerk1* mutant. In contrast, rice lines mutated in *OsCEBiP*, encoding the LysM receptor-like protein which associates with *Os*CERK1 to perceive chitin elicitors of the host immune defense pathway, responded positively to Myc-COs.
- These findings provide direct evidence that the bi-functional *Os*CERK1 plays a central role in perceiving short chain Myc-CO signals and activating the downstream conserved symbiotic signal transduction pathway.

Keywords: Arbuscular mycorrhiza, *Oryza sativa*, Chitin oligomer signaling, LysM RLK receptors, Nuclear calcium spiking Plant-microbe interactions, Root symbiosis

Introduction

Arbuscular mycorrhizal (AM) symbioses with soil borne glomeromycetes are believed to have developed over 400 My ago when the first plant ancestors moved from aquatic to terrestrial environments and are present today in the majority of land plants, including most crops (Fitter et al., 2011; Berruti et al., 2016). This success derives from the ability of obligate mutualistic AM fungi to provide their host plants with privileged access to soil nutrients and water, in return for an ecological niche and host photosynthates (Willis et al., 2013). Metabolite exchange occurs within the root inner cortex, where highly branched hyphal structures known as arbuscules develop within living plant cells (Harrison, 2012). Strong evidence indicates that the establishment of this endosymbiosis requires reciprocal chemical signaling prior to fungal root entry, with plant-exuded strigolactones triggering fungal differentiation and in return fungal signal molecules activating a specific signaling pathway (Delaux et al., 2015) in host epidermal cells (Schmitz and Harrison, 2014).

The activation of a conserved core module of this signaling pathway is required not only during AM, but also during the establishment of symbiotic nitrogen fixation between rhizobia and legumes as well as between *Frankia* and actinorhizal hosts (Barker et al., 2016). For this reason, the core module is known as the common symbiotic signaling pathway, or CSSP, and mutations in key CSSP components display an early block in either fungal or bacterial penetration of the root epidermis (Kistner et al., 2005). Finally, a characteristic feature of the CSSP is the generation of repetitive nuclear-associated Ca^{2+} oscillations known as Ca^{2+} spiking (Oldroyd and Downie, 2006), which means that the activation of the CSSP can be conveniently monitored in outer root tissues using *in vivo* calcium reporters such as cameleons (Miwa et al., 2006).

Studies in legumes have led to the identification of decorated lipo-chitooligosaccharidic (LCO) Nod factors as specific rhizobial signaling molecules recognized by the appropriate host plant. Nod factors are perceived *via* lysin-motif receptor-like kinases (LysM RLKs; Antolın-Llovera et al. 2012), and mutations in these LysM RLKs are defective in nodulation. More recently, chitin-based molecules have also been identified as putative fungal signals perceived by legume host plants during pre-infection stages of the AM association. These include both Nod factor-like Myc-LCOs (Maillet et al., 2011) as well as simpler short-chain chito-oligosaccharides referred to as Myc-COs (Genre et al., 2013). Although both types of molecule are able to trigger CSSP-dependent Ca^{2+} spiking, their respective biological roles still remain to be established. Furthermore, since knock-out mutations in individual legume LysM RLK genes have not yet yielded AM phenotypes with an

efficient block in fungal entry, it is currently difficult to evaluate the relationship between Myc-LCO/CO perception and the establishment of the AM association in these species. Part of the reason for this may be due to functional redundancy between members of the very large family of LysM RLKs present in legume genomes (e.g. Arrighi et al., 2006).

In contrast, promising advances in this direction have recently come from studies on the monocot rice, where it has been shown that either knock-out (Miyata et al., 2014) or silencing (Zhang et al., 2015) of the *OsCERK1* gene results in a clear defect in AM fungal penetration of the root epidermis. *Os*CERK1 was originally identified as a LysM RLK that associates with *Os*CEBiP, a LysM RLP (receptor-like protein) lacking a kinase domain to perceive long-chain chitin oligomers (such as chito-octaose, CO8) as part of a host immune defense signaling pathway responding to fungal pathogen elicitors (Shimizu et al., 2010). Thus, mutations in either *OsCERK1* or *OsCEBiP* fail to activate the chitin-triggered immune defense response (Kouzai et al., 2014; Miyata et al., 2014). In contrast to *Oscerk1*, *Oscebip* mutants establish AM symbiosis normally, suggesting that this particular LysM RLP is probably not essential for the perception of chitin-based AM symbiotic signals (Miyata et al., 2014). In addition to these findings, a recent study has shown that the short-chain Myc-CO chito-tetraose (CO4) is an active elicitor of nuclear Ca^{2+} spiking in rice atrichoblasts (Sun et al., 2015), the non-root hair epidermal cells that are targeted for infection by AM hyphae. In contrast, the same study showed that even high concentrations of Myc-LCOs were unable to trigger Ca^{2+} spiking in rice atrichoblasts.

In this paper we have made use of the *Oscerk1* knock-out mutant to directly investigate the role of this rice LysM RLK in perceiving symbiotic AM signals. Experiments using transgenic rice lines expressing a nuclear Ca^{2+}-sensing cameleon probe have revealed that, by comparison with wild type (WT) plants, the *Oscerk1* mutant is unable to respond to crude germinated AM fungal spore exudates. Furthermore, the fact that purified CO4 also fails to trigger Ca^{2+} spiking in the *Oscerk1* background provides additional evidence that Myc-COs present in the fungal exudate are important signals during the initial stages of fungal/host communication.

Materials and Methods

Plant material and cameleon constructs

The nuclear-localized yellow cameleon NLS-YC2.60 (Nagai et al 2004; Suzaki et al 2013) was introduced into the pUB-GW-Hyg vector (Maekawa et al 2008) and used to transform wild type rice (*Oryza sativa* L. *japonica* cv. Nipponbare BL no. 2) using *Agrobacterium*-mediated

transformation (Ozawa and Takaiwa, 2010). The expression of NLS-YC2.60 was confirmed in rice seedling primary roots by fluorescence microscopy. NLS-YC2.60 was also introduced into *Oscerk1*, *Osnfr5* and *Oscebip* mutants by crossing with the WT/NLS-YC2.60 line. Genotyping of the F2 progenies from these crosses were performed and mutant lines expressing NLS-YC2.60 fluorescence were selected.

Rice seeds were surface-sterilized as described in Campos-Soriano et al. (2011) and placed on water-agar (0.8% Plant Agar, Duchefa) in 12 cm-square Petri dishes. Dishes were kept in the dark for 3 days to induce germination, and then exposed to a light period of 16h at a constant temperature of 23°C with an aluminium foil wrap to limit light illumination of the root system. Since the cameleon fluorescence appeared to be strongly reduced in older roots, 2 cm-long apical segments of primary roots from 7-10 day old plantlets were used for the various treatments and subsequent FRET-based imagery.

Fungal signals and root treatments

The AM fungus used in this study was *Gigaspora margarita* isolate BEG 34 (International Bank for Glomeromycota, University of Kent, UK). Germinated spore exudates (GSE) were produced as described in Chabaud et al. (2011). Briefly, batches of 100 surface-sterilized *G. margarita* spores were germinated in 1 ml of sterile distilled water over a 7-day period. The resulting fungal germination medium was then concentrated 10-fold using a Lio5P lyophilizer (Cinquepascal, Milan, Italy) and stored at -20°C. All experiments were performed using the 10-fold concentrated *G. margarita* spore exudate.

Purified CO4 was purchased from Megazyme (Libios, France). A CO4 stock solution was prepared in sterile distilled water at 10^{-3} M and stored at -20°C. Preliminary tests performed on the rice seedling primary roots expressing adequate levels of cameleon fluorescence revealed that a concentration of 10^{-5} M CO4 was required for the efficient induction of Ca^{2+} spiking in root atrichoblasts. (Fig. S1).

The protocol for root treatment was modified from that described in Genre et al. (2013). Root segments were placed in a 2 mm-thick microchamber containing sterile distilled water on a microscope slide. The water in the microchamber was rapidly (< 30 s) substituted by 100µl of either GSE, 10^{-5} M CO4 or sterile distilled water (as control) before initiating confocal image acquisition. To prevent a cold-shock response, all solutions were warmed to 25°C before use.

Imaging was carried out on atrichoblast-rich areas of the root epidermis located on the part of the root in contact with the agar medium and between 1-2 cm from the root tip.

Confocal microscopy and data analysis

FRET-based imaging for detecting and plotting relative changes in nuclear Ca^{2+} concentrations corresponding to changes in the ratio of yellow fluorescent protein (YFP) to cyan fluorescent protein (CFP) emission intensity over time was performed using a slightly modified version of the protocol described by Genre et al. (2013). A Leica TCS SP2 AOBS confocal laser-scanning microsope was equipped with a long-distance HCX Apo L NA 40X 0.80 water-immersion objective or a HCX PL APO 40X 0.85 dry objective (Leica Microsystems GmbH, Wetzlar, Germany). Fluorescence intensities corresponding to both the CFP and YFP moieties of the NLS-YC2.60 nuclear cameleon were measured after exciting the probe at 458 nm (80% Ar laser) and recording the emitted fluorescence at 470–500 and 530–570 nm respectively. In order to optimize fluorescence excitation and acquisition, the beam expander was set at 1 and the pinhole diameter at 4-6 Airy units. Transmitted light images were acquired simultaneously to confirm cell identity. Images were scanned at a resolution of 512 x 512 pixels and collected every 5 s over a period of 30 min, starting 20 min after the treatment. The reason for this is that, as originally observed by Sun et al. (2015), spiking in rice atrichoblasts generally initiates only after a delay of 20 to 30 min following root treatment.

Previous studies using the legume *Medicago truncatula* had shown highly variable Ca^{2+} spiking profiles in root atrichoblasts in response to both AM fungal GSEs and short-chain COs such as CO4 (Genre et al., 2013; Russo et al., 2013). Since this also appears to be the case in rice (Sun et al., 2015; this manuscript) comparisons between spiking responses to different AM elicitors and in different host mutant backgrounds have been performed on the basis of the percentage of responding atrichoblasts. As previously (Russo et al., 2013), we have considered two Ca^{2+} peaks within a 30 min period as the minimum threshold for defining a positive spiking response, and for each elicitor condition we present representative profiles for two cells. The total numbers of cells and independent roots analyzed for each experimental condition are presented in Table S1 and statistical tests were carried out using non-parametric analysis of variance (Kruskal-Wallis) with a probability level of $p < 0.05$.

Results

AM fungal exudates fail to trigger symbiotic Ca^{2+} spiking in the *Oscerk1* mutant background

When concentrated germinated spore exudate (GSE) prepared from the AM fungus *Gigaspora margarita* was applied to the roots of the WT rice transgenic line expressing the nuclear-localized NLS-YC2.60 cameleon, nuclear Ca^{2+} spiking was observed in approximately one third of root atrichoblasts (Fig. 1 and 2). These spiking responses are reminiscent of those previously observed on roots of both *Medicago* and *Daucus* (Genre et al. 2013) and the two representative profiles shown in Fig. 1 underline the considerable irregularity and variability of the spiking responses. In contrast, spiking was not observed in control experiments when the GSE was replaced by distilled water (Fig. 1).

The NLS-YC2.60 cameleon was then introduced into the *Oscerk1* background, and roots of the transgenic mutant line were treated with the crude fungal exudate. In contrast with WT, atrichoblasts of the *Oscerk1* mutant failed to respond to the *G. margarita* GSE (Fig. 1). This was confirmed statistically by quantifying the percentage of responding atrichoblasts based on the combined results from a total of over 100 atrichoblasts and up to ten independent roots for each line (Fig. 2; Table S1). In conclusion, in the absence of functional *Os*CERK1, rice roots are no longer able to perceive the fungal signal molecules present in the AM GSE that normally trigger Ca^{2+} spiking in WT atrichoblasts.

The *Oscerk1* mutant is also defective in responding to Myc-COs

As stated earlier, short-chain chitin oligomers such as chito-tetraose (CO4) are biologically active components of the AM GSE, capable of triggering epidermal Ca^{2+} spiking responses in both *Medicago* and *Daucus* (Genre *et al*., 2013) as well as in rice (Sun *et al*., 2015). Furthermore, the fact that strigolactones can boost Myc-CO levels in AM GSEs provides direct evidence for a pre-infection molecular dialogue between symbiotic partners (Genre et al. 2013). CO4 activity was therefore evaluated for both the WT and *Oscerk1* transgenic rice lines. Figure 1 shows that, as expected, 10^{-5} M CO4 is able to initiate calcium spiking in root atrichoblasts of WT rice. However, in line with the GSE treatment, *Oscerk1* mutants did not display the sustained Ca^{2+} spiking response observed in the WT line (Fig. 1), and this was again confirmed by quantitative analysis (Fig. 3). These results are therefore consistent with an essential role for *Os*CERK1 in a receptor

complex capable of activating the rice CSSP following the perception of Myc-COs as fungal symbiotic factors.

Both GSE and CO4 can elicit Ca^{2+} spiking in *Oscebip* and *Osnfr5* mutants

*Os*CERK1 is known to form a receptor complex with the LysM RLP *Os*CEBIP during defense-related perception of long-chain chitin oligomers such as CO8 (Shimizu et al., 2010) and that a knock-out mutation of the *OsCEBiP* gene is only defective in the host immunity response (Miyata et al., 2014). For this reason it was important to examine whether the *Oscebip* mutant is still able to respond to AM fungal signals. Results presented in Fig. 2 and Fig. S2 show that, in contrast to *Oscerk1,* exogenous GSE treatment can trigger Ca^{2+} spiking responses in *Oscebip* root atrichoblasts. Equally, the application of purified 10^{-5} M CO4 to roots of the *Oscebip* mutant elicited Ca^{2+} spiking responses which could not be distinguished quantitatively from those observed with the wild type line (Fig. 3; Fig. S2). These findings are therefore coherent with the lack of an AM phenotype for *Oscebip,* and provide additional evidence that, in rice, distinct receptor complexes are required for perceiving the appropriate chitin oligomers which activate either symbiotic or defense-related downstream signaling pathways (Miyata et al. 2014; Shinya et al., 2015).

In the search for a potential LysM RLK/RLP partner for *Os*CERK1 in perceiving AM fungal signals, Miyata et al (2016) identified *Os*NFR5 (previously known as *Os*RLK2) as the closest rice ortholog of the Nod factor receptor component NFR5 from *Lotus japonicus.* The reasoning behind this was based on the fact that NFR5 associates with a second LysM RLK, NFR1, for which *Os*CERK1 is a close ortholog. However, despite the induction of *OsNFR5* expression in rice roots following AM fungal inoculation, knock-out mutants of this gene were colonized normally by AM fungi (Miyata et al., 2016). To examine this further, we also evaluated the capacity of the *Osnfr5* mutant to respond to the exogenous application of GSE/CO4 by introducing the nuclear Ca^{2+} cameleon construct into the mutant background (Methods). The Ca^{2+} spiking data presented in Fig. 2, Fig. 3 and Fig. S2 show that, as for *Oscebip*, the percentage of *Osnfr5* root atrichoblasts capable of responding positively to the application of either GSE or 10^{-5} M CO4 is statistically indistinguishable from the WT line. In conclusion, the capacities of the three rice LysM RLK/RLP mutants to respond to GSE/CO4 are fully in line with their corresponding AM phenotypes, thus further underlining the pertinence of the Ca^{2+} spiking assay as a reliable indicator of host perception of the symbiotic fungal signals required for initial root colonization.

Discussion

Since the breakthrough discovery of Nod factor LCOs as the key rhizobial signal molecules involved in the initial molecular dialogue leading to successful legume nodulation, it has become a priority to identify the equivalent "Myc factors" produced by endosymbiotic glomeromycota AM fungi. Although several chitin-based molecules (Myc-COs and Myc-LCOs) secreted by AM fungi have emerged as potential Myc factors from research in legumes (Maillet et al. 2011; Genre et al. 2013), the evaluation of their biological significance as signaling molecules has been compromised both by the absence of AM fungal genetic approaches as well as difficulties in identifying legume LysM RLK/RLP receptors essential for initial fungal entry. To circumvent this, attention has turned to the monocot rice, where recent findings have unexpectedly revealed that the *Oscerk1* mutant is not only defective in immune defense responses, but also refractory to AM fungal colonization (Miyata et al. 2014; Zhang et al. 2015). By introducing a cameleon Ca^{2+} reporter into the AM-defective *Oscerk1* background, we demonstrate here that this mutant is no longer able to perceive the symbiotic signal molecules present in AM fungal exudates, as revealed by the failure to initiate Ca^{2+} spiking in root atrichoblasts (Figs. 1&2). The triggering of these characteristic nuclear-associated Ca^{2+} oscillations is considered a hallmark for the activation of the conserved CSSP endosymbiotic signaling pathway (Oldroyd and Downie, 2006). Furthermore, these experiments have also revealed that short chain Myc-COs such as CO4, whose concentrations are preferentially enhanced in AM fungal exudates in the presence of host strigolactones (Genre et al. 2013), are no longer able to elicit Ca^{2+} spiking when applied to roots of the *Oscerk1* mutant (Figs. 1&3). Together, these findings argue firstly that the *Os*CERK1 LysM RLK is necessary for the successful perception/transduction of AM fungal signals in rice, and secondly provide direct evidence for the role of short-chain Myc-COs during this critical stage of host-fungal communication. In the light of these results it will now be important to confirm the significance of Myc-COs during initial AM fungal-host signaling in other plants, including both legumes and dicot non-legumes such as tomato (Buendia et al., 2015) and *Parasponia andersonii* (Op den Camp et al., 2011) for which LysM RLK RNAi knock-down experiments have revealed defective AM phenotypes.

What can we infer about the likely role of *Os*CERK1 in perceiving Myc-COs based on studies of the immune defense receptor complex? Firstly, affinity labeling experiments have shown that *Os*CEBiP is the major receptor for long-chain chitin oligomers such as CO8 in rice, whereas *Os*CERK1 does not appear to directly bind chitin oligosaccharides (Kaku et al., 2006; Shinya et al., 2012; Kouzai et al., 2014). Secondly, structural studies have revealed that long-chain chitin oligomers bind to two

molecules of *Os*CEBiP *via* the central LysM domains, and that this leads to receptor dimerization (Hayafune et al., 2014; Liu et al., 2016), a requisite for the activation of defense signaling. The current model therefore proposes that *Os*CEBiP homo-dimerization recruits *Os*CERK1 to form the active receptor complex (Shinya et al., 2015). This model also explains why CO4/CO5 are unable to trigger defense signaling, since these short-chain oligosaccharides cannot simultaneously bind to two LysM domains. However, they can act as antagonists to CO8-induced receptor dimerization (Liu et al., 2012).

Since the *Oscebip* mutant is defective for chitin-triggered immunity, but unaffected for either AM fungal colonization (Miyata et al., 2014) or the capacity to respond to both exogenous AM fungal GSE and 10^{-5} M CO4 (Figs. 2&3), it appears unlikely that *Os*CEBiP has a role in perceiving/transducing the symbiotic AM fungal signals. Bearing in mind that there is currently no evidence for direct binding of the *Os*CERK1 co-receptor to chitin oligomers and that all known plant LysM RLP-mediated receptors comprise at least two partners, the most likely scenario at this stage is that *Os*CERK1 associates with a second LysM-containing membrane protein. Following this reasoning, the role of one possible LysM RLK partner, *Os*NFR5, the rice ortholog of LjNFR5/MtNFP, was examined by creating an *Osnfr5* knock-out mutant. However, this mutant can be successfully colonized by AM fungi (Miyata et al., 2016), and also responds with nuclear Ca^{2+} spiking to both exogenous AM GSE and CO4 (Figs. 2&3). Furthermore, no heterodimerization could be observed between *Os*CERK1 and *Os*NFR5 in BiFC assays (Miyata et al. 2016). Future research will now need to focus on additional candidate rice LysM-based receptors by evaluating both the AM phenotypes and the GSE/CO4-responsiveness of the respective mutant lines.

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Author Contribution

G.C., M.Ch. and M.C. performed experiments and data analysis. N.T., H.K. and K.M. developed the transgenic plant lines. A.G., D.B., M.Ch. and T.N. wrote the manuscript. A.G., D.B., M.Ch., N.S. and T.N. designed the experiments.

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Figure legends

Figure 1. Nuclear Ca^{2+} spiking in response to AM fungal signals in both wild type and *Oscerk1* mutant lines. The figure shows representative profiles from independent atrichoblasts of wild type and *Oscerk1* mutant roots treated with sterile water (control), 10-fold concentrated *G. margarita* germinated spore exudate (GSE) or 10^{-5} M chito-tetraose (CO4). Both GSE and CO4 trigger a sustained response in the wild type, but not in *Oscerk1* plants, indicating a role for *Os*CERK1 in the perception of AM fungal signals in *Oryza sativa*. Values on the Y axis represent the ratio between YFP and CFP fluorescence.

Figure 2. Percentage of responding atrichoblasts in wild type, *Oscerk1, Oscebip and Osnfr5* mutant roots of rice in response to *G. margarita* GSE. GSE (10-fold concentrated) treatment (grey) elicited a spiking response in 33% of wild type, 40% of *Osnfr5* and 59% of *Oscebip* atrichoblasts, but failed to trigger spiking in the *Oscerk1* background (asterisk indicates a statistically significant difference). Bars on each histogram indicate standard deviations. Differences between water controls (white) were not statistically significant.

Figure 3. Percentage of responding atrichoblasts in wild type, *Oscerk1*, *Oscebip* and *Osnfr5* mutant rice roots in response to 10^{-5} M CO4. Nuclear Ca^{2+} spiking was elicited in a significant proportion of root atrichoblasts from wild type (51%), *Oscebip* (31%) and *Osnfr5* (31%) lines treated with 10^{-5} M CO4 (grey), but not in the *Oscerk1* mutant (asterisk indicates a statistically significant difference). Bars on each histogram indicate standard deviation. Differences between water controls (white) were not statistically significant.

Supporting Information

Figure S1. Representative Ca^{2+} spiking profiles and percentage of responding atrichoblasts in wild type rice roots treated with increasing concentrations of CO4. Based on these results, the concentration of 10^{-5}M CO4 was chosen as the most appropriate for the study of nuclear Ca^{2+} spiking responses in rice plantlet primary root (see Materials and Methods). Bars represent standard deviation; asterisks indicate statistically significant differences compared to the water control. A minimum of 15 atrichoblasts from two independent roots was used for each condition.

Figure S2. Representative Ca^{2+} spiking profiles observed for the *Oscebip* and *Osnfr5* mutants treated with either *G. margarita* GSE or 10^{-5} M CO4. As compared to *Oscerk1* (Fig. 1) spiking responses were detected for both rice mutants treated with either fungal elicitor.

Table S1. Number of atrichoblasts per experimental condition used for the various statistical analyses. Numbers in brackets indicate the number of independent roots used for each condition.

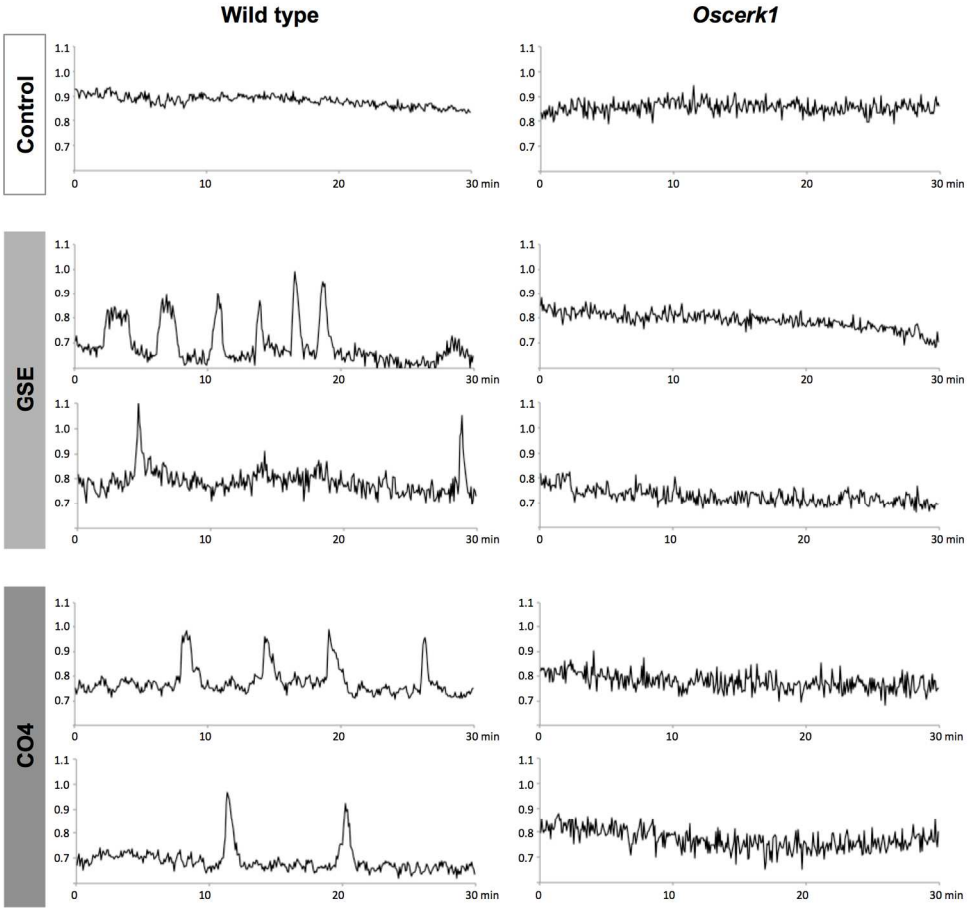

Figure 1. Nuclear Ca2+ spiking in response to AM fungal signals in both wild type and Oscerk1 mutant lines. The figure shows representative profiles from independent atrichoblasts of wild type and Oscerk1 mutant roots treated with sterile water (control), 10-fold concentrated G. margarita germinated spore exudate (GSE) or 10-5 M chito-tetraose (CO4). Both GSE and CO4 trigger a sustained response in the wild type, but not in Oscerk1 plants, indicating a role for OsCERK1 in the perception of AM fungal signals. Values on the Y axis represent the ratio between YFP and CFP fluorescence.

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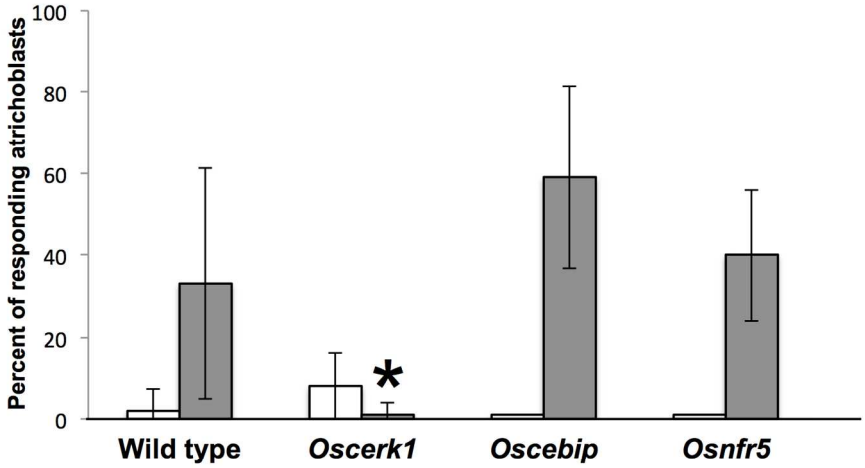

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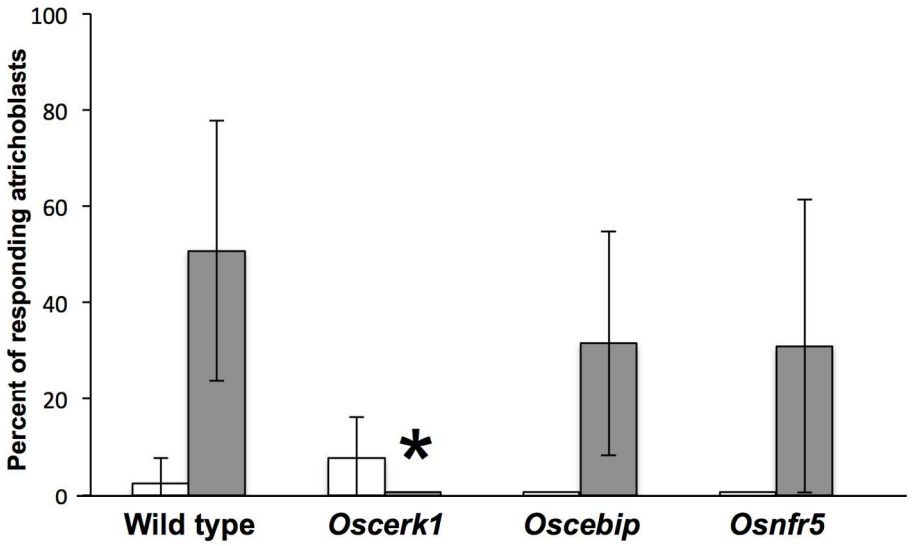

Figure 3. Percentage of responding atrichoblasts in wild type, Oscerk1, Oscebip and Osnfr5 mutant roots in response to 10-5 M CO4. Nuclear Ca^{2+} spiking was elicited in a significant proportion of root atrichoblasts from wild type (51%), Oscebip (31%) and Osnfr5 (31%) lines treated with 10-5 M CO4 (grey), but not in the Oscerk1 mutant (asterisk indicates a statistically significant difference). Bars on each histogram indicate standard deviation. Differences between water controls (white) were not statistically significant.

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