Brief Communication

CRISPR/Cas9-mediated generation of fls2 mutant in Nicotiana benthamiana for investigating the flagellin recognition spectrum of diverse FLS2 receptors

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Plant cell surface pattern-recognition receptors (PRRs) mount pattern-triggered immunity (PTI) by recognizing the typical molecular structures of pathogen-associated molecular patterns (PAMPs), providing the first line of defence against various phytopathogens. Flagellin-sensing 2 (FLS2) of Arabidopsis thaliana, which perceives conserved epitopes (flg22), is an important pattern-triggered immunity (PTI) by recognizing the typical motif of wild-type and KO lines. After flg22Psy (Pseudomonas syringae) treatments, wild type and KO1 generated reactive oxygen species (ROS) bursts (Figure 1f), accumulated activated MPK3/6 (Figure 1g), and exhibited significant growth inhibition (Figure 1h, i). In contrast, there were no obvious responses by KO1&2 and KO2. In addition, transient expression with 35S::gNbFLS2 and 35S::gNbFLS2:GFP (gNbFLS2, the full-length genomic DNA sequences of NbFLS2; GFP, coding sequence of green fluorescent protein) revealed that 35S::gNbFLS2-2 and 35S::gNbFLS2-2:GFP can recover the ability to generate ROS bursts in KO1&2 after flg22Psy treatment, but 35S::gNbFLS2-1 and 35S::gNbFLS2-1:GFP cannot (Figure 1j). Moreover, immunoblotting detected the accumulation of NbFLS2-2-GFP (~210 kDa) but did not detect NbFLS2-1-GFP (Figure 1k). RT-PCR and qRT-PCR results demonstrated the expression of two gNbFLS2s in transient assay (Figure 1la–c). Furthermore, no accumulation of target protein was observed in transient expression of the coding sequence of NbFLS2-1 (Figure 1k). Therefore, the lack of function of NbFLS2-1 may be due to translational level regulation.

Flagellin-induced ROS burst assays using N. benthamiana leaves that transiently express heterologous FLS2s represent a robust and convenient experimental method for identifying the function of FLS2s, but the presence of functional endogenous FLS2s, which can recognize a range of flagellin epitopes and/or may interact with downstream elements, limits the method’s application. The NbFLS2 double-mutant generated here can help overcome this limitation. We cloned the genomic DNA sequences of FLS2 homologues from multiple plants and generated binary vectors with the 35S::gFLS2:GFP construct. Their transient expression in KO1&2 revealed that 29 GFP-fused FLS2s (GenBank accession No. ON556647–ON556668, MH079052, MH079054, MH079055, MH079056 and MH079058) with molecular weights of approximately 200 to 210 kDa were successfully accumulated (Figure 1l). The leaf discs of KO1&2 expressing heterologous FLS2s were challenged with three flagellin epitopes (flg22Psy, flg15Eco, and flg22Agro) in ROS burst assays. Four FLS2 homologues failed to confer KO1&2 the ability to respond to flg22Psy, among which FLS2 from Nelumbo nucifera, Kalanchoe laxiflora and Ginkgo biloba lacked the 14–17th, 4–6th, and 26 & 28th LRR motifs, respectively, whereas Morus alba FLS2, lacking the 15th LRR motif and Populus euphratica FLS2, lacking the 26th LRR motif, still recognized flg22Psy (Figure 1m). In addition, there was suggesting their complete loss of function (Figure 1a–d). Furthermore, qRT-PCR results showed that the expression levels of mutated FLS2 genes were lower than that of wild type (Figure 1e).
Figure 1. Using CRISPR/Cas9 to knockout two FLS2 genes in N. benthamiana and verify the functions of FLS2s from multiple plants. (a–c) Alignment of nucleotide sequences targeted by sgRNA1 (a), sgRNA2 (b), and sgRNA3 (c). Red letters and hyphens: insertions and deletions caused by Cas9/sgRNAs, respectively. DNA sequencing chromatograms of sgRNA target regions are provided for KO lines. The sequences of sgRNA1 and sgRNA3 are overlined, and the sequence of sgRNA2 is indicated by the red rectangle. (d) Amplification of the Cas9 fragment with genomic DNAs of T0 and T1 lines. (e) The expression levels of NbFLS2s in wild type and KO lines as determined by qRT-PCR. Asterisks (*P < 0.05 and **P < 0.01) denote significant differences from the NbFLS2 expression level of wild type (one-way ANOVA and Tukey’s test, with three independent experiments). (f) ROS burst assay with leaf discs after treatment with flg22Psy (50 nM) and H2O (mock). The error bars represent the means ± SDs (n = 8). (g) MAPK activation of leaf discs by flg22Psy (1 μM) using a phospho-p44/42 MAPK antibody. (h, i) Fresh weight (h) and root length (i) of seedlings growing in liquid medium with and without flg22Psy (5 μM) for 2 weeks. Asterisks (*P < 0.05 and **P < 0.01) denote significant differences from the fresh weight of flg22Psy-free seedlings of each line (one-way ANOVA and Tukey’s test, n > 10). (j) ROS burst produced by KO1&2 leaves transiently expressing NbFLS2-GFPs and NbFLS2s after treatment with 50 μM flg22Psy. (k) Immunoblot of transiently expressing NbFLS2-GFPs in KO1&2 using an anti-GFP antibody. Transiently expressing AtFLS2-GFP served as control for molecular weight. (l) Immunoblot of 29 transiently expressing FLS2-GFPs in KO1&2 using an anti-GFP antibody. (m) ROS burst produced by KO1&2 leaves transiently expressing QvFLS2-GFP, TjFLS2-GFP, and SbFLS2–GFP after treatment with 1 μM flg22Agro. Transiently expressing AtFLS2-GFP served as a negative control of the flg22Agro response. (o) Phylogeny of FLS2 homologues. The phylogenic tree was inferred using the maximum-likelihood method. Numbers at each node indicate the bootstrap percentage (n = 1000). Missing LRRs, based on alignments with AtFLS2; RLU, relative light units; ++, RLU more than 50 000; +, RLU more than 10 000; −, RLU less than 10 000. ROS burst assays were performed using the luminol-based method with a GloMax™ 96 Microplate Luminometer. The full-length genomic sequences of FLS2s were used for all binary vector construction.
a difference in flg15\textsuperscript{Eco} recognition among poplar FLS2s, i.e., FLS2 from \textit{P. trichocarpa} and \textit{P. euphratica} recognized flg15\textsuperscript{Eco}, but FLS2s from five other poplar species did not (Figure 1n). Furthermore, FLS2 from \textit{Quercus variabilis} and \textit{Trachelospermum jasminoides} are highly sensitive to flg22\textsuperscript{Agro} (Figure 1o).

Here, we used CRISPR/Cas9 technology to knock out two FLS2 genes in \textit{N. benthamiana} both separately and together, and we found that only NbFLS2-2 contributed to the recognition of flg22\textsuperscript{Agro}. In addition, we combined transient expression and ROS burst assays to rapidly validate the FLS2 flagellin epitope recognition spectrum from 29 plant species in an \textit{N. benthamiana} FLS2 double-mutant. This convenient approach, combined with a large number of FLS2 homologues currently revealed by plant genome sequencing, will facilitate screening of the FLS2s that can trigger broad-spectrum resistance or resistance targeting specific pathogens, and investigating co-evolutionary dynamics of plant FLS2 and bacterial flagellin in a given environment.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

LW, HX, LZ, and QC performed research and analysed data, LW and QC wrote the paper. All the authors read and approved the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Detecting the expression of NbFLS2s in transient assay.

Appendix S1 Supplementary materials and methods.