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Short title: NRC2/3/4 are not essential for flg22 responses

NRC4 gene cluster is not essential for bacterial flagellin-triggered immunity[1]

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One-Sentence Summary: CRISPR/Cas9-mediated mutation of NRC2/3/4 genes did not affect bacterial flagellin-triggered immunity.

Plants utilise cell surface pattern recognition receptors (PRRs) and intracellular nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) to fend off invading pathogens (Dodds and Rathjen, 2010; Win et al., 2012). Both types of immune receptors detect pathogen molecules directly or indirectly to activate complex immune responses and disease resistance (Kourelis and van der Hoorn, 2018). Although PRR- and NLR-triggered immunity are generally thought to activate distinct pathways, they can induce similar outputs such as production of reactive oxygen species (ROS) and hypersensitive cell death (Peng et al., 2018). Both PRR- and NLR-activated pathways involve calcium-dependent protein kinases, mitogen-activate protein kinases (MAPKs), phytohormone signalling, and transcriptional reprogramming (Peng et al., 2018). However, whether these two pathways converge at some point to potentiate and strengthen the immune response remains unclear. A recent study suggested that the tomato NLR helper NRC4 positively regulates the ROS burst induced by the bacterial flagellin peptide flg22 (Leibman-Markus et al. 2018b). We took advantage of the CRISPR/Cas9 system to knock out multiple *NRC* genes in tomato and *Nicotiana benthamiana*. Although these mutants failed to respond to the NRC-dependent NLRs, they remained unaltered in flg22-induced responses. We conclude that the *NRC* genes are not essential for flg22-induced responses in tomato and *N. benthamiana*.

Throughout evolution, a subset of NLR proteins have functionally diversified into sensors that detect pathogen molecules and helpers (also known as executors) that operate genetically downstream of sensor NLRs in mediating the hypersensitive response (HR) and disease resistance (Cesari, 2018; Adachi et al., 2019). The emerging view is that although some singleton NLRs carry both activities, sensor and helper NLRs form receptor complexes that range from pairs to networks (Wu et al., 2018; Adachi et al., 2019). One example of an NLR network is formed by the NRCs (NLR-required for cell death) in asterid plants (Gabriels et al. 2007; Wu et al., 2017). Over the last ~100 million years, the NRC network has dramatically expanded from a pair of sensor and helper genes to form a complex network of phylogenetically related sensor and helper NLRs. In *N. benthamiana,* the NLR helpers *NRC2, NRC3*, and *NRC4* are partially redundant but display varying degrees of specificity towards sensor NLRs that confer resistance to oomycete, bacterial, and viral pathogens (Wu et al., 2017). Interestingly, a recent study linked the tomato NRC *SlNRC4a* to PRR-triggered immunity (Leibman-

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C.-H.W., H.A., J.C.D.C., R.C.-G., and V.N. performed experiments. All authors designed the research and analysed data. C.-H.W., V.N. and S.K. wrote the letter with contributions from all the authors.

Markus et al., 2018b). Leibman-Markus et al. (2018) reported that overexpression of *SlNRC4a* in *N. benthamiana* enhances ROS production elicited by the bacterial flagellin peptide flg22 and the fungal protein ethylene-inducing xylanase (EIX). Furthermore, SlNRC4a associates with the PRRs AtFLS2 and LeEIX in co-immunoprecipitation experiments. These results led Leibman-Markus et al. (2018a and 2018b) to conclude that SlNRC4a is a positive regulator of the immune response mediated by PRRs, notably the extensively studied FLS2 receptor.

Whereas *Agrobacterium*-mediated transient expression of *SlNRC4a* (Solyc04g007070, hereafter referred to as *NRC4a*) in *N. benthamiana* can enhance flg22-induced ROS burst (Leibman-Markus et al., 2018b), it remains unclear whether knocking out *NRC4a* affects flg22-induced responses in tomato. *NRC4a* occurs in the tomato genome as a gene cluster together with two closely related paralogous genes (*SlNRC4b*, Solyc04g007060, *NRC4b*; *SlNRC4c*, Solyc04g007030, *NRC4c*) (Supplemental Fig. S1A). This gene cluster also contains another gene Solyc04g007050, that we named *SlNRC5* (*NRC5*), which is phylogenetically related to the NRCs (Wu et al., 2017). In this study, we decided to take advantage of the CRISPR/Cas9 system to generate loss-of-function mutants in the clustered NRC genes. We reasoned that the contribution of *NRC4* paralogs in FLS2-mediated responses can be addressed by deleting the entire *NRC4/5* gene cluster.

To knockout the *NRC4* gene cluster in tomato, we designed four guide RNAs based on the conserved sequences in the NRC4 paralogs (Supplemental Fig. S1B). We transformed these guide RNAs together with Cas9 and a kanamycin selection marker into tomato (*Solanum lycopersicum*) GCR758 (Balint-Kurti et al., 1995). We recovered 13 independent transformants that are kanamycin resistant. To determine whether these transformants are mutated in the *NRC4* gene cluster, we used gene specific primers to amplify fragments of *NRC4a*, *NRC4b*, *NRC4c*, and *NRC5* (Supplemental Fig. S1C, Supplemental Table S1). These primers amplified fragments with expected sizes when genomic DNA from wild-type plants was used as a template in the PCR reaction, but failed to amplify some of the *NRCs* (such as *NRC4c* and *NRC4a*) with genomic DNA from the line T0-1 (Supplemental Fig. S1C). Interestingly, we could not amplify any of the *NRC4* and *NRC5* fragments from the genomic DNA of the line T0-7, suggesting that this line contained multiple deletions or a large deletion in the locus of *NRC4* gene cluster (Supplemental Fig. S1C). To further confirm the genotype of the T0-7 plant, we designed four additional primers based on the sequences adjacent to *NRC4c* and *NRC4a*. Due to the distance between the primers (over 50 kb based on the reference sequence), these primer pairs LR1F x 7075F and 7020R x 7075F could not amplify any fragments when the genomic DNA from the wild type plant was used as a template (Supplemental Fig. S2). However, we successfully amplified fragments of 1.3 kb and 3.8 kb with the primer pairs LR1F x 7075F and 7020R x 7075F, respectively, using DNA from T0-7 (Fig. 1; Supplemental Fig. S2). Thus, we sequenced the 1.3 kb fragment amplified using the primer pair LR1F x 7075F by Sanger sequencing and confirmed that this plant contains a 53 kb deletion in the *NRC4* locus, connecting the open reading frame (ORF) of *NRC4c* to the ORF of Solyc04g007075 (Fig. 1; Supplemental Fig. S3). In addition to the 53 kb deletion, we also found a 290 bp deletion in *NRC4c* (Fig. 1C). The remaining sequence resulted in a fusion of ORFs from Solyc04g007075 and NRC4c with multiple frameshift mutations leading to premature stop codons in *NRC4c* (Supplemental Fig. S3). We further obtained a homozygous T2 line (*nrc4*_7.4) and used this line for further experiments (Supplemental Fig. S4).

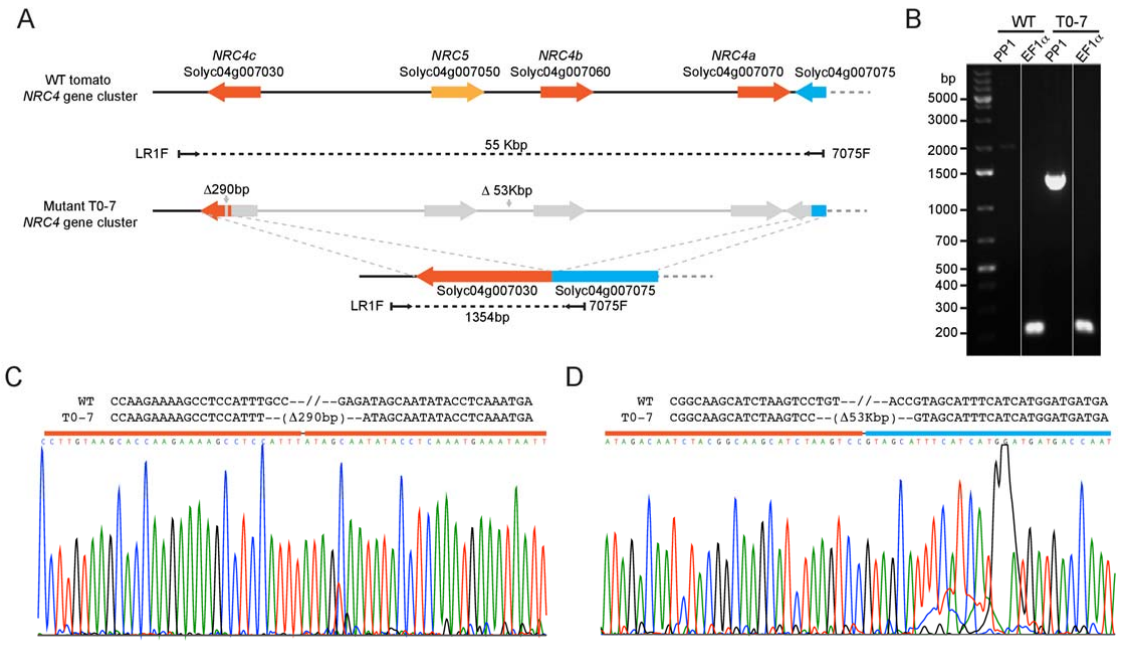

Figure 1. The T0-7 transformant carries a large (>53 kb) deletion spanning across the *NRC4* gene cluster. A. Schematic view of the tomato *NRC4* gene cluster in wild-type (WT) and mutant T0-7. Orange, *NRC4* paralogs; yellow, *NRC5*; blue, Solyc04g007075, which contains incomplete sequence information due to a sequencing gap in the reference genome. The deleted regions in the mutant T0-7 are marked in grey. B. PCR-genotyping for the large deletion. PP1, amplification with primer pair 1:LR1F x 7075F indicated in A; *EF1*α amplification control with *EF1*α primers. The uncropped image is provided in Supplemental Fig. S2B. C. Sequence alignment and chromatograms of Sanger DNA sequencing results. In this region, the mutant T0-7 contains a 290bp deletion based on reference genome and the results of sequencing. D. Sequence alignment and chromatograms of Sanger DNA sequencing results. In this region, the mutant T0-7 contains a 53kbp deletion based on the reference genome and the results of sequencing.

We previously reported that the sensor NLR Rpi-blb2, which confers resistance to potato late blight pathogen *Phytophthora infestans*, depends on NRC4 when expressed in *N. benthamiana*. To test whether Rpi-blb2 signals through NRC4 in tomato, we expressed Rpi-blb2/AVRblb2, Rpi-vnt1/AVRvnt1 (NRC-independent), or GFP in wild type and the *NRC4* knockout tomato line using agroinfiltration (see Supplemental methods). Rpi-blb2-mediated cell death was compromised in the *NRC4* knockout plants, whereas Rpi-vnt1 triggered strong cell death in both wild type and *NRC4* knockout plants, consistent with the earlier finding from the *N. benthamiana* experimental system (Supplemental Fig. S4).

Leibman-Markus et al. (2018) proposed that *NRC4a* participates in immunity mediated by FLS2 because overexpression of *NRC4a* in *N. benthamiana* enhances ROS production after flg22 treatment. Leibman-Markus et al. (2018) obtained a CRISPR/Cas9 mutagenized tomato line that expresses a truncated variant of SlNRC4a. However, the effect of this mutation on flg22-induced responses was not reported. As *NRC4a* exists in a gene cluster with the highly homologous *NRC4b* and *NRC4c* that are potentially functionally redundant, we reasoned that our *NRC4/5* gene cluster deletion would be ideal to test whether the *NRC4* genes are required for FLS2-mediated responses. To test the hypothesis, we monitored apoplastic ROS production in response to flg22 peptides. We observed a transient flg22-induced ROS burst with the leaf discs from wild-type tomato plant GCR758. However, we did not observe a notable difference in terms of flg22-induced ROS burst between the wild type and the *NRC4* deletion line *nrc4*_7.4 (Fig. 2A and B). As mitogen-activated

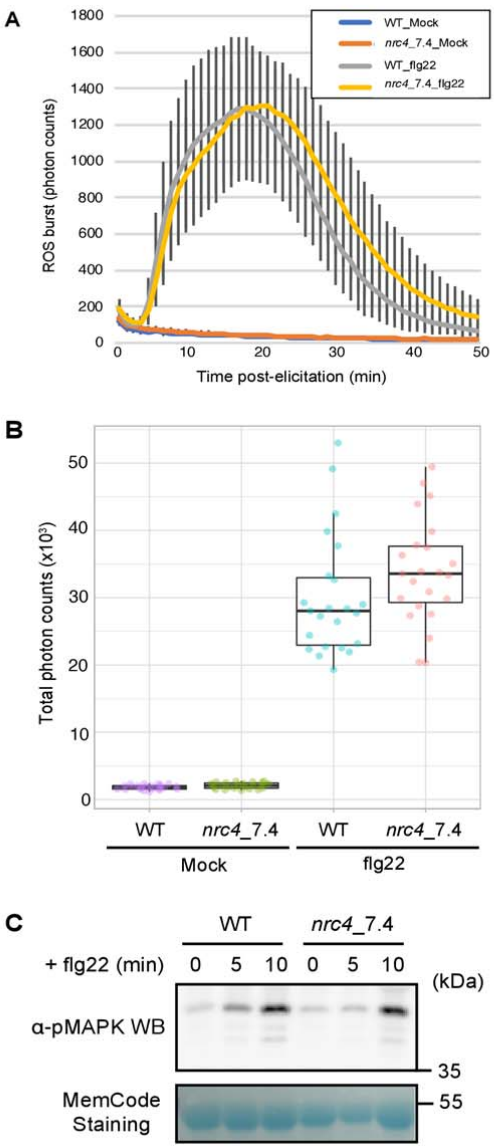

Figure 2. *NRC4* knockout tomato plants are not impaired in flg22-induced defence responses. A. Flg22-triggered ROS bursts were measured for 50 min using leaf discs of the WT and T2 line *nrc4*_7.4. Data are presented as means ± SD. B. Scatter plot and box plot of total photon counts of each treatment in A. Data C. Flg22-triggered MAPK activation was analysed by immunoblots with α-pMAPK. Proteins were extracted from tomato leaf tissues of the WT and T2 line *nrc4*_7.4, 0, 5 or 10 min after treatment with flg22.

protein kinase (MAPK) activation represents another typical output in FLS2-mediated responses, we tested whether MAPK phosphorylation was impaired in the *NRC4* knockout plants. We detected increased phosphorylation of MAPKs in the wild-type plants by immunoblot analysis with p-42/44 antibody after flg22 treatment. We also detected increased phosphorylation of MAPKs in the *NRC4* knockout mutant and, here too, we did not observe a significant difference between the wild type and the *NRC4* deletion mutant (Fig. 2C). Our results indicate that the *NRC4* genes are not essential for flg22-induced responses in tomato.

Previous studies have suggested that the NRC proteins are involved in immune responses mediated by both intracellular NLR and cell surface PRR immune receptors (Gabriels et al., 2007; Wu et al., 2016; Brendolise et al., 2017; Wu et al., 2017). Silencing of *NRC2* and *NRC3* by virus-induced gene silencing (VIGS) and RNAi reduces Cf4- and Prf-mediated cell death in *N. benthamiana*, indicating that NRC2 and NRC3 are involved in cell death responses activated in both pathways (Brendolise et al., 2017; Wu et al., 2016). Furthermore, silencing of *NRC2*, *NRC3*, and *NRC4* together, but not individually, compromises cell death mediated by Rx, Bs2, and some other NLRs in *N. benthamiana*, and this phenotype can be complemented by individual NRCs (Wu et al., 2017). Given that *NRC2*, *NRC3*, and *NRC4* display degrees of genetic redundancy in NLR- and PRR-mediated cell death in *N. benthamiana*, we sought to test whether knocking out *NRC2/3/4* affects flg22-induced responses. We transformed *N. benthamiana* with 2-4 guide RNAs targeting *NRC2*, *NRC3*, or *NRC4* together with Cas9 and a phosphinothricin selection marker and obtained loss-of-function mutants (Supplemental Fig. S5A and Fig S6A). We selected two independent T2 *NRC4* knockout lines and two independent T2 *NRC2/3/4* knockout lines for further characterisation. Due to the complexity of the *N. benthamiana* genome and duplications of each *NRC* gene, these selected lines may express variants of NRC2, NRC3, or NRC4 proteins, ranging from 33 to 123 amino acid truncations to full-length NRCs with a 3 amino acid indel in the coiled-coil domain (Supplemental Fig. S5A and Fig S6A). Consistent with our previous reports with VIGS assays, the two *NRC4* knockout lines (*nrc4*_9.1.3 and *nrc4*_1.2.1) were found to be defective in Rpi-blb2-mediated cell death, and the two *NRC2/3/4* knockout lines (*nrc234*_4.3.1 and *nrc234*_5.5.1) were defective in Rpi-blb2-, Prf(Pto)-, and Rx-mediated cell death (Supplemental Fig. S5 and Fig S6). These results confirmed that the selected *NRC4* and *NRC2/3/4* knockout lines do not contain any NRC2, NRC3, or NRC4 variants that are still functional for the tested sensor NLR genes. Next, we tested the degree to which flg22-induced ROS burst and phosphorylation of MAPKs were affected in these *NRC* knockout *N. benthamiana* lines. Both the wild-type plants and the *NRC* knocked out plants yielded similar results for flg22-induced ROS burst and MAPK phosphorylation assays (Supplemental Fig. S7). In conclusion, our results indicate that *NRC2, NRC3*, and *NRC4* are not essential for flg22-induced responses in *N. benthamiana*.

The NRC network is phylogenetically restricted to asterids and caryophyllales, but is missing in Arabidopsis and other rosid species (Wu et al., 2017). Therefore, our results may not be that surprising given that FLS2 belongs to an ancient receptor-like kinase subfamily XII that broadly occurs in angiosperms (Dufayard et al., 2017; Liu et al., 2017). In contrast, NRCs may be involved in Cf-4- and LeEIX-mediated immunoresponses considering that these cell surface receptor-like proteins are phylogenetically restricted to some asterid clades (Kang and Yeom, 2018) and, unlike flg22, trigger hypersensitive cell death in plant tissues (Gabriels et al., 2007; Brendolise et al., 2017; Wu et al., 2016). Future work will need to address how cell surface receptors mechanistically engage NLR proteins to induce cell death and other immune responses.

Supplemental Data

The following supplemental materials are available.

Supplemental Methods

Supplemental Figure S1. Targeting the *NRC4* gene cluster with CRISPR/Cas9 in tomato.

Supplemental Figure S2. Primer design and characterisation of the large deletion in mutant T0-7.

Supplemental Figure S3. Sanger sequencing result of the *NRC4* deletion allele from T0-7.

Supplemental Figure S4. The *NRC4* knockout homozygous T2 line (*nrc4*_7.4) is impaired in Rpi-blb2-mediated cell death.

Supplemental Figure S5. Genotypes and phenotypes of *NRC4* knockout *N. benthamiana*.

Supplemental Figure S6. Genotypes and phenotypes of *NRC2/3/4* knockout *N. benthamiana*.

Supplemental Figure S7. Knocking out of *NRCs* in *N. benthamiana* did not affect flg22-induced defence responses.

Supplemental Table S1. Primers used in this study.

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Figure Legends

Figure 1. The T0-7 transformant carries a large (>53 kb) deletion spanning across the *NRC4* gene cluster. A. Schematic view of the tomato *NRC4* gene cluster in wild-type (WT) and mutant T0-7. Orange, *NRC4* paralogs; yellow, *NRC5*; blue, Solyc04g007075, which contains incomplete sequence information due to a sequencing gap in the reference genome. The deleted regions in the mutant T0-7 are marked in grey. B. PCR-genotyping for the large deletion. PP1, amplification with primer pair 1:LR1F x 7075F indicated in A; *EF1*□□amplification control with *EF1*□□primers. The uncropped image is provided in Supplemental Fig. S2B. C. Sequence alignment and chromatograms of Sanger DNA sequencing results. In this region, the mutant T0-7 contains a 290bp deletion based on reference genome and the results of sequencing. D. Sequence alignment and chromatograms of Sanger DNA sequencing results. In this region, the mutant T0-7 contains a 53kbp deletion based on the reference genome and the results of sequencing.

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