

A head-to-head NLR gene pair from wild emmer confers stripe rust resistance in wheat

Corresponding Author: Dr Lin Huang

Version 0:

Decision Letter:

11th Jun 2024

Dear Dr. Huang,

Thank you very much for your enquiry about submitting a manuscript to Nature Genetics. It sounds interesting and we would like to see it, although I am sure you will appreciate that it can be very difficult to judge a paper without having seen it in its entirety. We cannot guarantee that we'll send it out for review until we have had a chance to look at it.

Please note that supplementary materials must not be included with the main text and figures of your manuscript. Supplementary items should be uploaded as separate files.

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We look forward to receiving it.

Sincerely,
Wei

Wei Li, PhD
Senior Editor
Nature Genetics
New York, NY 10004, USA
www.nature.com/ng

Version 1:

Decision Letter:

24th Jul 2024

Dear Dr Huang,

Your Article, "A head-to-head NLR gene pair from wild emmer confers resistance to wheat stripe rust" has now been seen by 3 referees. You will see from their comments below that while they find your work of interest, some important points are

raised. We are interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

To guide the scope of the revisions, the editors discuss the referee reports in detail within the team with a view to identifying key priorities that should be addressed in revision. In this case, we think all three referees have provided constructive reviews aimed at strengthening the analyses and improving the presentation, and we particularly ask that you address their technical comments as thoroughly as possible with appropriate revisions. We hope that you will find the prioritized set of referee points to be useful when revising your study.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

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*3) Include a revised version of any required Reporting Summary: <https://www.nature.com/documents/nr-reporting-summary.pdf>

It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.

A revised checklist is essential for re-review of the paper.

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We hope to receive your revised manuscript within 3 to 6 months. If you cannot send it within this time, please let us know.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

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We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,
Wei

Wei Li, PhD
Senior Editor
Nature Genetics
www.nature.com/ng

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

The major claim of the paper is that a head-to-head pair of NLRs confers resistance to stripe rust and that this type of resistance is novel.

The resistance as described in this manuscript is novel for the wheat stripe rust pathosystem. The companion paper emphasizes that other head-to-head NLR pairs contain an integrated domain of some sort and that there is not an integrated domain with this NLR pair. I suggest that Hu et al. similarly emphasize this point to highlight the novelty of this NLR pair. A functional NLR pair without an integrated domain would be indeed a significant advance in the field and this paper would be cited going forward as the example of this form of NLR-mediated resistance.

The work described is convincing because the authors performed functional validation of the genes through transgenic complementation, VIG silencing, and CRISPR editing. The data that the authors summarize is valid.

The introduction section is very wheat stripe rust -specific. I would strongly encourage the authors to re-envision the introduction section to emphasize not only wheat stripe rust, but also NLR generally throughout the introduction section besides only the first paragraph.

Statistics were accurately applied throughout the manuscript. Statistics were not applied to the disease phenotypes. This is because the phenotypes were qualitative presence or absence of disease. Even for the VIGS experiment with variation in the amount of disease, the comparison was made to the resistant line with no disease. Quantitative values of disease would make the data stronger, though applying statistics is not necessary since appropriate tests would be comparing the value of "zero" (no pustules) to variations in amount of pustules.

This paper will be of broad influence in plant science because they authors report a novel type of NLR resistance. This will influence thinking on R gene evolution including the in-silico based discovery/prediction of R gene function which is rapidly emerging.

I would indeed recommend publication of this report in Nature Genetics. This paper is a groundbreaking report on a unique form of NLR resistance where a head-to-head pair of NLR genes confers resistance without an integrated domain. The companion paper fully describes the context of this discovery in comparison with other NLR gene pairs. In contrast to the companion paper, this paper is valuable because of (1) the functional validation of the genes through several different assays, (2) the nuances of the transgenic complementation showing that NLR2 is not necessary for resistance in certain backgrounds (Fielder) – (this will result in follow up studies), and (3) the cell death assays provide insight into the role of NLR1.

The finding of NLR2 functional redundancy in Fielder wheat is intriguing result. Since Fielder is hexaploid with a AABBDD subgenome, whereas TD121 is tetraploid AABB, it may be possible that functional redundancy for AABBDD exists on the D genome. Have the authors checked for NLR2 homologs on the D genome?

The authors surveyed several tetraploid accessions for NLR1/2 and reported that some carried NLR1 alone or NLR2 alone? Is it possible for the accessions to possess homologs of NLR1 or NLR2 with polymorphisms that prevented amplification of the sequences used in the survey?

I do not agree with the sentence at the end of the discussion that the authors' gene can be combined with "other slow rusting genes" for two reasons: 1. The authors did not demonstrate that their resistance gene is a slow rusting gene (hence do not use the word "other") and 2. The authors should use a "more durable resistance" instead of "durable resistance".

Reviewer #2:

Remarks to the Author:

The authors used map-based cloning and long-read sequencing to isolate two NLR receptors from wild emmer wheat. Using mutagenesis, VIGS and stable transformation, they confirmed the candidate genes and showed that the two NLRs were in head-to-head orientation and both are required for YrTD12-mediated resistance to wheat stripe rust pathogen. The project was well thought after and the experiments were done nicely. Overall, the manuscript was well written and clear to the point, which I enjoyed reading. The two back-to-back papers not only confirmed each other's findings and also complement each other's discoveries. This is the first time that a pair of NLRs was found to be required for the wheat stripe rust resistance. Once the avirulence gene is also cloned, the mechanism how the two NLRs work together can be revealed. Their discovery will be of great interest to the wheat community working on disease resistance.

A question for the authors to ponder: Is the adjacent head-to-head orientation important or just by chance? When you think about the transformation, unless the two genes were transformed together, their integration positions were random. More often than not, they may not be adjacent to each other or on different chromosomes, or they may not have the head-to-head orientation. Therefore, it seems that this orientation may not be important.

Another question is that if both genes are required to confer the resistance, how they are different from two complementary

genes if they do not have to be adjacent in a head-to-head orientation?

L255-257: As I mentioned in the reviewer' report on the other paper, it is actually very intriguing that TdNLR2 and TdNLR1 are in the same clade with Pik-1 and Pik-2 in rice, respectively. It will be great if the authors could expand a bit more on the implications, from the sequence similarity to function similarity.

Because no specificity difference was found between YrTD121 and Yr84, the authors are correct stating that they are functional haplotypes. For both manuscripts, not too many races have been used so it is difficult to tell whether they are haplotypes or alleles, unless different specificities are found.

L367-369: Better leave this model out because it is purely hypothetical, not evidence based.

L375: They could cite the Canadian work and also the test done in Israele here.

L380: Yr36 has a completely different mode of action compared with Yr18 and Yr46 loci.

L402: For curiosity, why "three-leaf stage"? Normally "two-leaf stage" is sufficient.

Here are some minor changes for the authors to consider when revising the manuscript.

No need to spell out the numbers above ten, and to spell out if the numbers are below ten.

L2: Should be "wheat stripe rust pathogen".

L37: Do not think "genetically linked" is needed here.

L43: Should be "TdNLR1".

L62: Should be "Stripe rust disease".

L77: ASR needs to be spelt out "all-stage resistance".

L93: Move "on a 0-9 scale" from L100 to here.

L113: It is clearer to use "Nine KASP markers" to be more specific.

L115: There are a few sub-tables in Supplementary Data 1. It is better to add which one, Supplementary Data 1.X.

L188-189: This is possible. However, this can be checked out easily by testing the TdNLR2 marker in Fielder.

L198: What leave stage for the leaf samples?

L243: Should be "on group 1 chromosomes".

L248: Add an "may", change to "may involve".

L253-254: Capitalise "Clade", also in Fig. 4.

L264: Add "fused to the C-terminus" before included cell death.

L274: Change to "Self-association of the CC domain of NLR proteins".

L278: Should be "in vivo" instead of "in vitro".

L283: "backcross".

L286: "stripe rust pathogen".

L304: Change "necessary" to "both required".

L324: Delete "conferred".

L337: Should be "evolutionary".

L365: Delete "with each other".

L437: Which generation are these RILs?

L441: "SMRTbell".

L494-498: Not clear. Need to rewrite.

L504: "vector combinations".

L507: "the third leaf".

L823: Typo "mutagenesis".

L825: "hexaploid".

Supplementary Fig. 1b: Need to adjust the upper arrow to where it should point to.

Supplementary Fig. 15a: Need to spell out "Un" where it appears the first time.

Supplementary Fig. 19b: The difference between CM42 and Cm42+YrTD121 in this figure is marginal.

Reviewer #3:

Remarks to the Author:

The study performed a map-based cloning and genome sequencing&assembly to identify a genomic region containing several candidate genes for stripe rust resistance. Two genes that are organized head-to-head were demonstrated to be required for the resistance. The study provided solid evidence for validating these two resistance genes: mutagenesis via EMS and/or CRISPR, gene knockdown, and transformation of genomic DNAs and coding sequences. Other related exploration about the allele distribution in the population and contributions of domains to HR responses was conducted. Importantly, the finding added a new pair of head-to-head NLR functioning together to conferring plant disease resistance. The resistance resource may be used to combat stripe rust, an important disease to be controlled in many wheat production areas.

It is still puzzling that the transgene of TdNLR1 only can enhance stripe rust resistance although both TdNLR1 and TdNLR2 are required for the resistance. The explanation that Fielder might contain a TdNLR2-like gene is reasonable. I wonder if the TdNLR2 homologous gene can be identified in the Fielder genome (<https://doi.org/10.1093/dnares/dsab008>).

BSR-seq and its analysis need proper citations. The parameters of Allele frequency difference (AFD) and bulk frequency ratio (BFR) were described but no reference was cited. It is difficult to know what these terms exactly mean. I think it is important to have a citation and briefly explain these terms. In the main text (L110-113), the number of SNP was mentioned. It is not clear these SNPs are from the GATK result or the resistance-associated SNPs passing the filtering criteria.

L43-44: "The CC domain of NLR1 is active in cell-death signaling and self-associates in planta." What does "active" mean?

L323-234: "The deletion of 21 nucleotides at positions 87-107 in the CC domain of TdNLR1_h4 may cause its conferred susceptibility." If a new experiment would be added, this variant of the CC domain could be used to test the cell death induction. If not, the statement would be well supported.

L473-478: Both Bowtie and HiSAT2 were used for RNA-seq read alignments to the reference genome. Please justify why both tools were used.

L588-589: A citation is needed for "The respective sequences of TdNLR1 and TdNLR2 were fused with the yeast two hybrid system vectors pGBKT7 and pGADT7."

What are values on the edges in Fig. 4?

Version 2:

Decision Letter:

Our ref: NG-A65745R1

17th Dec 2024

Dear Dr. Huang,

Thank you for submitting your revised manuscript "A head-to-head NLR gene pair from wild emmer confers stripe rust resistance in wheat" (NG-A65745R1). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Genetics, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements soon. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Genetics Please do not hesitate to contact me if you have any questions.

Sincerely,
Wei

Wei Li, PhD
Senior Editor
Nature Genetics
www.nature.com/ng

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed each of my previous concerns from the initial review. The authors performed the necessary additional studies to alleviate these concerns in a comprehensive manner. The authors also updated the manuscript accordingly. I believe that this manuscript can now be accepted by the journal.

Reviewer #2 (Remarks to the Author):

It is great to see that the authors spent efforts in carrying out the experiments recommended by the reviewers. They have responded nicely to all reviewers' comments and the quality of the manuscript has improved significantly. I think it warrants its publication. A few minor edits are needed. Here are some comments/suggestions.

L246: "starting with ATG".

L297-299: The description here is not accurate. What they implied was the expression of these two with deletion abolished the induction of cell death. From their results, they cannot reach this conclusion. The expression of these two and the intact CC had the same expression as shown in Supplementary Fig. 20c. They need to reword the sentence.

L311: It was my mistake before, which should be "in vitro".

L533-536: Did you inoculate and screen M1 plants? It is quite interesting because we normally only start screening M2 plants.

L548: ...was inoculated "on" the second leaf...

L1048: Need to add a reference or link to MEGA 11.

Reviewer #3 (Remarks to the Author):

Thank the authors for all the efforts to revise the manuscript. The overall quality of the manuscript has been improved and my previous comments have been well addressed. The result from the new experiment testing the 21-nt deletion in the CC domain of TdNLR1_h4 strengthens the conclusion.

Version 3:

Decision Letter:

In reply please quote: NG-A65745R2 Huang

25th Apr 2025

Dear Dr. Huang,

I am delighted to say that your manuscript "A head-to-head NLR gene pair from wild emmer confers stripe rust resistance in wheat" has been accepted for publication in an upcoming issue of Nature Genetics.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Genetics style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

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Sincerely,

Wei Li, PhD
Senior Editor
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NG-A65745R: “A head-to-head NLR gene pair from wild emmer confers stripe rust resistance in wheat”

Response to Reviewers' Comments:

We appreciate the reviewers' valuable comments/suggestions and for highlighting the contributions of our manuscript. Each suggested revision and comment were accurately incorporated and considered. We have revised the manuscript according to these valuable comments/suggestions and provide here our point-by-point responses.

The reviewer's comments are listed below and marked in black, response to the reviewer's comments is marked in purple, while the edited/revised/new text from the paper is marked in blue.

Reviewer #1:

Remarks to the Author:

Comment 1.1 The major claim of the paper is that a head-to-head pair of NLRs confers resistance to stripe rust and that this type of resistance is novel.

The resistance as described in this manuscript is novel for the wheat stripe rust pathosystem. The companion paper emphasizes that other head-to-head NLR pairs contain an integrated domain of some sort and that there is not an integrated domain with this NLR pair. I suggest that Hu et al. similarly emphasize this point to highlight the novelty of this NLR pair. A functional NLR pair without an integrated domain would be indeed a significant advance in the field and this paper would be cited going forward as the example of this form of NLR-mediated resistance.

Response: Thank you for taking the time to review our manuscript. We are grateful for your positive feedback and valuable comments/suggestions for our study. Indeed, the work described in our study and the companion paper represent the first reported case of a functional NLR pair without an integrated domain in the wheat stripe rust pathosystem. We have emphasized this point to highlight the novelty of this type of resistance.

Line 42: Both genes lack an integrated domain previously associated with effector perception.

Lines 160-161: Neither TdNLR1 nor TdNLR2 contained integrated domains previously implicated in effector perception.

Lines 396-398: Our findings, together with the companion paper³⁸, represent the first reported example of a functional NLR pair without an integrated domain in the wheat stripe rust pathosystem.

Comment 1.2 The work described is convincing because the authors performed functional validation of the genes through transgenic complementation, VIG silencing, and CRISPR editing. The data that the authors summarize is valid.

Response: Many thanks. In the revised manuscript, we also generated *TdNLR2* knockout plants using CRISPR editing. We demonstrated that knockout *TdNLR2* in HY-45 background also leads to the loss of stripe rust resistance. Therefore, our data have provided strong evidence that the *TdNLR1/TdNLR2* pair is important for the *YrTD121*-mediated resistance.

Lines 189-195: We identified three mutant lines for *TdNLR1* (*TdNLR1KO-1* to *TdNLR1KO-3*), carrying 4-bp, 7-bp, or 13-bp deletions; and two mutant lines for *TdNLR2* (*TdNLR2KO-1* and *TdNLR2KO-2*) with a 1-bp insertion and a 6-bp deletion in the target sites, respectively. These mutants were susceptible to *Pst* race CYR34, whereas non-edited HY-45 plants were highly resistant, indicating that both *TdNLR1* and *TdNLR2* were required for resistance in HY-45.

Comment 1.3 The introduction section is very wheat stripe rust-specific. I would strongly encourage the authors to re-envision the introduction section to emphasize not only wheat stripe rust, but also NLR generally throughout the introduction section besides only the first paragraph.

Response: Thank you for your suggestions. We have expanded the introduction section to emphasize the NLR generally and shortened the wheat stripe rust paragraphs accordingly. There are four paragraphs now in the revised introduction section:

Lines 53-62: Paragraph 1 Typical architecture of NLRs.

Lines 63-72: Paragraph 2 Introduction on singleton, paired NLRs and NLR networks in plant immunity.

Lines 73-82: Paragraph 3 A short introduction about wheat-stripe rust pathosystem and the studied *Yr* genes.

Lines 83-96: Paragraph 4 The objectives of the current study.

Comment 1.4 Statistics were accurately applied throughout the manuscript. Statistics were not applied to the disease phenotypes. This is because the phenotypes were qualitative presence or absence of disease. Even for the VIGS experiment with variation in the amount of disease, the comparison was made to the resistant line with no disease. Quantitative values of disease would make the data stronger, though applying statistics is not necessary since appropriate tests would be comparing the value of “zero” (no pustules) to variations in amount of pustules.

Response: Thank you for your comments and suggestions. We used an infection type of 0-9 scale to record the disease phenotype due to the qualitative presence or absence of disease, while the statistics were generally used for quantitative traits. Following your good advice, we have used the image analysis software, ImageJ, to quantify the percentage of leaf surface covered with sporulating uredinia and applied statistics to estimate *Pst* biomass accumulation on *YrTD121* lines (zero) and gene silencing lines in VIGS experiments (Fig.1R). We have revised the text accordingly.

Lines180-184: *TdNLR1* or *TdNLR2* gene-silenced leaves exhibited reduced stripe rust resistance with average percentage of leaf areas covered by uredinia ranging from 14.02% to 21.14%, whereas the negative control leaves were resistant to stripe rust with no visible uredinia.

Lines 556-559: The digital images (each approximately 4-cm leaf segment) were further analyzed using color thresholds in ImageJ software as described previously to quantify the percentage of leaf surface covered with sporulating uredinia.

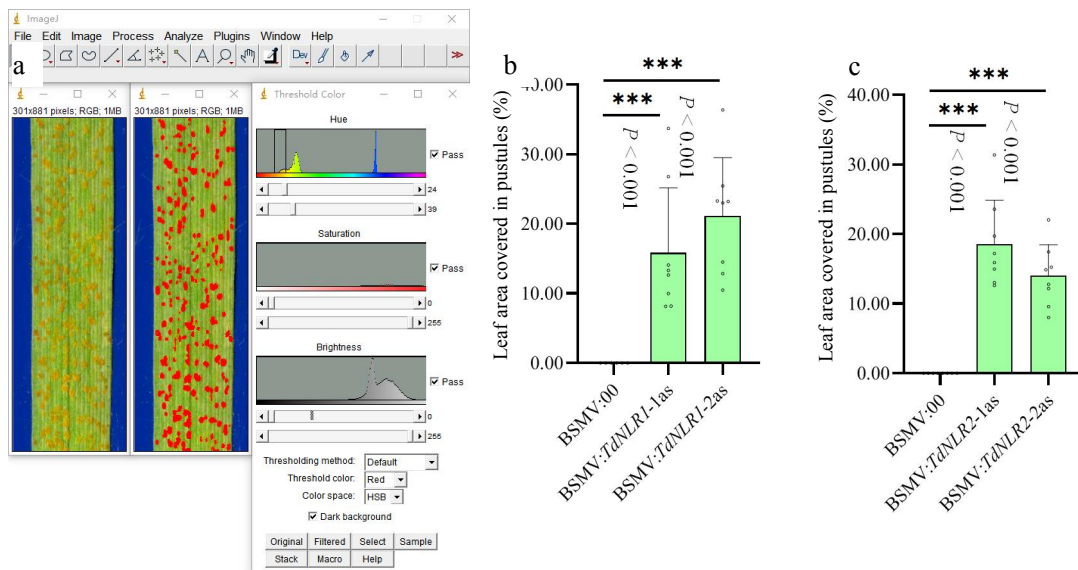


Fig.1R Quantification of the leaf area covered by *Pst* pustules. a Screenshot of the color transformation step in ImageJ software used to identify stripe rust pustules. b, c Mean percentage (%) of leaf area covered with *Pst* pustules on either *TdNLR1* (b) or *TdNLR2* (c) gene silenced leaves inoculated with *Pst* race CYR34.

This new data is added as Supplementary Fig. 5c, d and Supplementary Fig. 6c in the revised manuscript.

Comment 1.5 This paper will be of broad influence in plant science because they authors report a novel type of NLR resistance. This will influence thinking on R gene evolution including the in-silico based discovery/prediction of R gene function which is rapidly emerging.

I would indeed recommend publication of this report in Nature Genetics. This paper is a groundbreaking report on a unique form of NLR resistance where a head-to-head pair of NLR genes confers resistance without an integrated domain. The companion paper fully describes the context of this discovery in comparison with other NLR gene pairs. In contrast to the companion paper, this paper is valuable because of (1) the functional validation of the genes through several different assays, (2) the nuances of the transgenic complementation showing that NLR2 is not necessary for resistance in certain backgrounds (Fielder) – (this will result in follow up studies), and (3) the cell death assays provide insight into the role of NLR1.

Response: Thank you for your recommendation.

Comment 1.6 The finding of NLR2 functional redundancy in Fielder wheat is intriguing result. Since Fielder is hexaploid with a AABBDD subgenome, whereas TD121 is tetraploid AABB, it may be possible that functional redundancy for AABBDD exists on the D genome. Have the authors checked for NLR2 homologs on the D genome?

Response: We thank the reviewer for this question. We have checked carefully the Fielder genome and identified three *TdNLR2-like* homologous sequences with relatively low sequence identity to *TdNLR2*. The 1B copy was truncated due to a premature stop codon. Based on their similar three-dimensional (3D) structures, we hypothesize that the paralogs copies of *TdNLR2* from chromosomes 2B and 2D of Fielder genome may contribute redundantly to *TdNLR1*-mediated immunity. We have revised the manuscript accordingly. This new data is added as Supplementary Fig. 8 in the revised manuscript. We will validate the potential redundant function of *TdNLR2-like* homologs in the future.

Lines 205-212: Three *TdNLR2-like* homologs were identified in Fielder chromosomes 1B (TraesFLD1B01G018900.1), 2B (TraesFLD2B01G357400.1), and 2D (TraesFLD2D01G344800.1), respectively. The 1B copy was truncated due to a premature stop codon; the 2B and 2D copies shared relatively low amino acid sequence similarity (approximately 44.0%) with *TdNLR2*, although their predicted protein structures were similar, indicating a potentially redundant function of *TdNLR2* in conferring resistance.

Lines 388-390: Given that expressing *TdNLR1* alone conferred resistance, we hypothesize that the paralogous copies of *TdNLR2* from chromosomes 2B and 2D in Fielder may contribute redundantly to *TdNLR1*-mediated immunity.

Comment 1.7 The authors surveyed several tetraploid accessions for NLR1/2 and reported that some carried NLR1 alone or NLR2 alone? Is it possible for the accessions to possess homologs of NLR1 or NLR2 with polymorphisms that prevented amplification of the sequences used in the survey?

Response: We thank the reviewer for this comment. We have conducted additional work to detect the wild emmer accessions that carry either *TdNLR1* or *TdNLR2* alone using homolog primers or whole genome re-sequencing. Indeed, we found two accessions carried *TdNLR1* and *nlr2* allelic sequences, and three had *nlr1* allelic sequences and *TdNLR2*. We found one accession (WEW110843) previously detected to carry *TdNLR1* alone, also had a truncated *TdNLR2* with a 649-nucleotide deletion spanning 465 bp of the 5'-UTR) and 184 bp of coding sequence that starts in the ATG. We have updated these findings in the revised manuscript. This new data is also added as Supplementary Figs.10,11 and Supplementary Figs.14,15 in the revised manuscript.

Lines 232-235: A survey using additional sets of homolog primers and re-sequencing identified two accessions carrying *TdNLR1* and *nlr2* and three with *TdNLR2* and *nlr1*.

Lines 244-246: *TdNLR2_h11* had a 649-nucleotide deletion spanning 465 base pairs (bp) of the 5'-UTR and 184 bp of the coding sequence, starting with the ATG.

Lines 360-362: We found two wild emmer accessions harboring *TdNLR1* and *nlr2* allelic sequences and three accessions carrying *TdNLR2* and *nlr1* allelic sequences.

Comment 1.8 I do not agree with the sentence at the end of the discussion that the authors' gene can be combined with "other slow rusting genes" for two reasons: 1. The authors did not demonstrate that their resistance gene is a slow rusting gene (hence do not use the word "other") and 2. The authors should use a "more durable resistance" instead of "durable resistance".

Response: We have used "slow rusting genes" instead of "other slow rusting genes" and "more durable resistance" instead of "durable resistance" as suggested.

Reviewer #2:

Remarks to the Author:

Comment 2.1 The authors used map-based cloning and long-read sequencing to isolate two NLR receptors from wild emmer wheat. Using mutagenesis, VIGS and stable transformation, they confirmed the candidate genes and showed that the two NLRs were in head-to-head orientation and both are required for YrTD12-mediated resistance to wheat stripe rust pathogen. The project was well thought after and the experiments were done nicely. Overall, the manuscript was well written and clear to the point, which I enjoyed reading. The two back-to-back papers not only confirmed each other's findings and also complement each other's discoveries. This is the first time that a pair of NLRs was found to be required for the wheat stripe rust resistance. Once the avirulence gene is also cloned, the mechanism how the two NLRs work together can be revealed. Their discovery will be of great interest to the wheat community working on disease resistance.

Response: We are grateful for your careful reading and valuable comments/suggestions, which have significantly improved the presentation of our manuscript. We fully agree that the discovery of the first paired NLRs for wheat stripe rust disease will offer potential novel avenues to investigate molecular mechanisms of paired-NLR function and the corresponding avirulence gene(s).

Comment 2.2 A question for the authors to ponder: Is the adjacent head-to-head orientation important or just by chance? When you think about the transformation, unless the two genes were transformed together, their integration positions were random. More often than not, they may not be adjacent to each other or on different chromosomes, or they may not have the head-to-head orientation. Therefore, it seems that this orientation may not be important.

Response: Thank you for your inspiring question. Although the TdNLR1/TdNLR2 pair are closely linked in a head-to-head orientation at *YRTD121* locus, their integration positions in Fielder transformants was random, and may be integrated on different chromosomes, not in a head-to-head orientation. Therefore, we fully agree with your comments that the head-to-head orientation of TdNLR1 and TdNLR2 genes in our study may not be so important for the function. However, the genetic linkage mechanism of TdNLR1 and TdNLR2 may have an evolutionary advantage by facilitating co-segregation, co-evolution of the TdNLR1/TdNLR2 gene pair to cope with pathogens (Contreras et al. 2023). We have used this comment to improve our manuscript.

Lines 377-381: Random introduction of *TdNLR1* and *TdNLR2* into Fielder conferred resistance and indicated that the head-to-head orientation of the

gene pair might not be critical for resistance function, whereas the genetic linkage mechanism of *TdNLR1/TdNLR2* may facilitate adaptive co-evolution and genetic co-segregation of the gene pair to cope with pathogen infection.

Comment 2.3 Another question is that if both genes are required to confer the resistance, how they are different from two complementary genes if they do not have to be adjacent in a head-to-head orientation?

Response: Thank you for your challenging question. To our best knowledge, gene complementation is the interaction between two genes in a genotype to produce a particular phenotypic trait, while they do not have individual effects in separate genotypes. In addition, two genes act in a complementarily way often positioned on different chromosomes. For example, complementary genes *Yr73* on chromosomes 3DL and *Yr74* on 5BL for stripe rust resistance (Dracatos et al. 2016); *Lr27* (3BS) and *Lr31* (4BS) for leaf rust resistance (Singh and McIntosh 1984); *Ne1* (5BL) and *Ne2* (2BS) for hybrid necrosis (Nishikawa et al. 1974; Hewitt et al. 2021). Furthermore, few studies have showed that some complementary genes encoded proteins with different architecture. For complementary dominant leaf rust APR genes *LrN3B* and *LrN4B*, a transducing family protein/WD-40 repeat family protein and a tetratricopeptide repeat protein were considered as the most likely candidates for *LrN3B* (Wang et al. 2024). The *Ne1* encodes protein with ABH domain (Prof. Zhiyong Liu personal communication), while *Ne2* encodes NLR protein (Hewitt et al. 2021). To date, the description of the molecular mechanism underlying complementary genes in wheat is still largely unknown. In contrast, the NLR pairs typically require two NB-LRR genes for function. They work as specialized sensor-helper pairs in which a sensor NLR (sNLR) recognizes pathogen effectors and a helper NLR (hNLR) induces the downstream immune responses (Adachi et al. 2019). In some cases, sNLRs function together with multiple hNLRs at different loci to form an intricate NLR network (Wu et al. 2017).

Therefore, we think the NLR pair in our study (if it does not have to be adjacent in a head-to-head orientation) may differ from the two complementary genes by the gene architecture (NLR pair vs. potential different gene structure) and the mode of action ('sensor-helper' pair or network vs. two genes complementation). We apologize for not putting our assumption in the revised manuscript. The picture will be clear when additional complementary genes were cloned in the future.

Comment 2.4 L255-257: As I mentioned in the reviewer' report on the other paper, it is actually very intriguing that TdNLR2 and TdNLR1 are in the same clade with Pik-1 and Pik-2 in rice, respectively. It will be great if the authors could expand a bit more on the implications, from the sequence similarity to function similarity.

Response: We thank the reviewer for this suggestion. We compared the amino acid sequences between TdNLR2/TdNLR1 and Pik-1/Pik-2 (Figs. 2R, 3R), and revealed relatively low sequence identities (less than 44%), while both function in plant defense responses. Based on this finding, we therefore added sentences (Lines 284-287) to expand a bit on the implications, from the sequence similarity to function similarity of these two NLR pairs.

Lines 284-287: TdNLR1 shared 43.3% amino acid identity with Pik-2, whereas the similarity between TdNLR2 and Pik-1 was 25.8%, suggesting sequence divergence between these paired NLRs, whereas both retain critical roles in regulation of plant immunity.

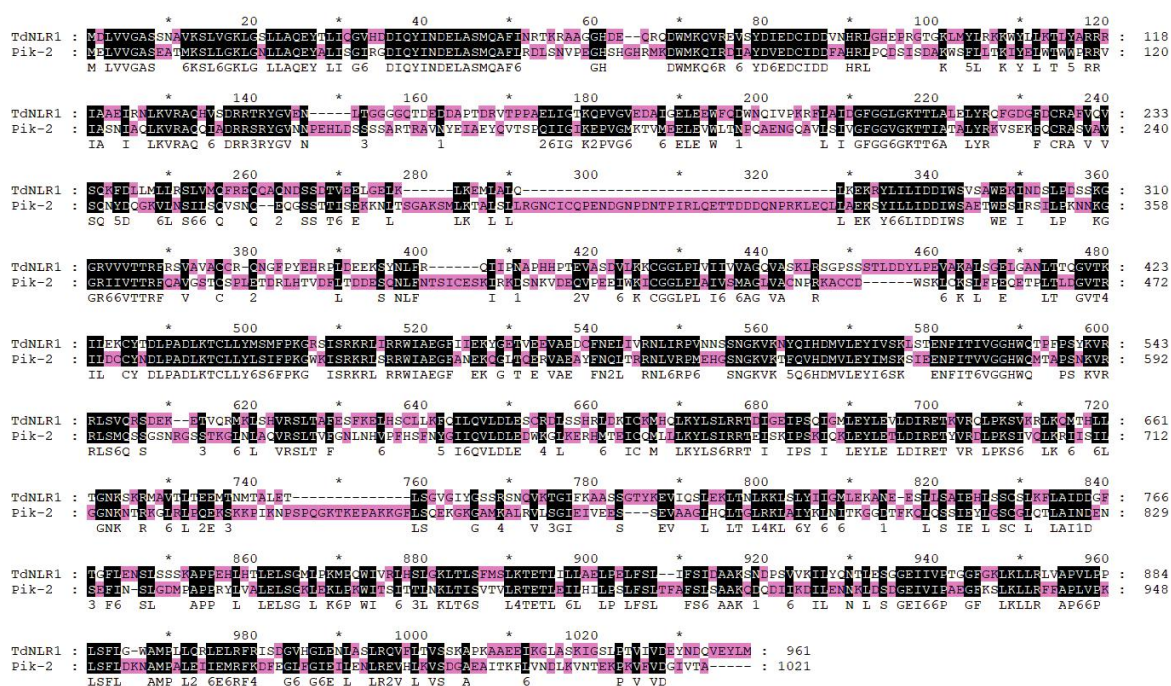


Fig. 2R Sequence alignments of TdNLR1 and Pik-2 proteins. The identical sequences were highlighted in dark shading.

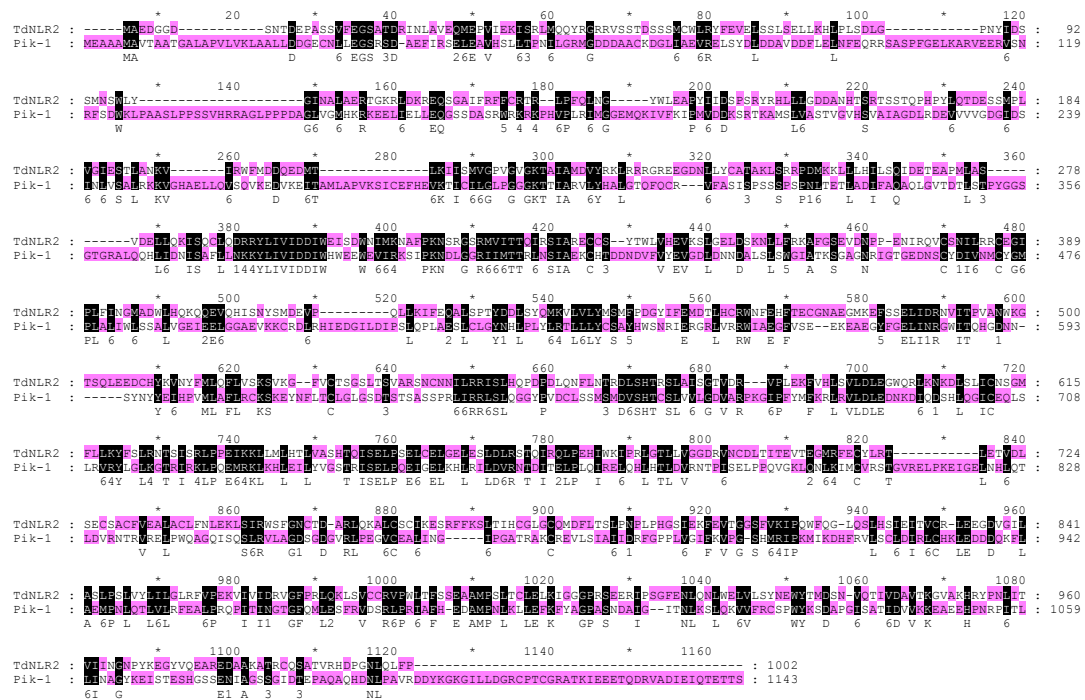


Fig.3R Sequence alignments of TdNLR21 and Pik-1 proteins. The identical regions were highlighted in dark shading.

Comment 2.5 Because no specificity difference was found between YrTD121 and Yr84, the authors are correct stating that they are functional haplotypes. For both manuscripts, not too many races have been used so it is difficult to tell whether they are haplotypes or alleles, unless different specificities are found.

Response: Thanks for your comments. Although the *YrTD121* and *Yr84* have two amino acid changes in TdNLR1 and one amino change in TdNLR2 at protein level, we did not find specificity differences between *YrTD121* and *Yr84* based on limited *Pst* races tested in our study. To be on the safe side, we claimed in our study and together in the companion paper that *YrTD121* and *Yr84* are different functional haplotypes. We fully agree with the reviewer's comment. Once different resistance specificities are found between *YrTD121* and *Yr84*, then we can call them different alleles, while this relies on the testing of numerous *Pst* races worldwide. We apologize for the inability to test many races (we also do not have them) at the moment to detect potential specificity differences. In addition, *YrTD121* and *Yr84* are currently present in different wheat genetic backgrounds that may affect resistance specificity tests. In the future, we plan to express different haplotypes of *YrTD121*, including *Yr84*, in the same transgenic wheat background and then compare the potential specificity differences using different *Pst* races. This will be done in collaboration with other groups that have access to various *Pst* races and may take time.

Comment 2.6 L367-369: Better leave this model out because it is purely hypothetical, not evidence based.

Response: Thanks for your suggestion. We have removed this hypothesis model and revised the text based on the LRR domain interaction data present in the companion paper.

Lines 400-403: In the companion study, a ligand-independent interaction through the LRR domains of the TdNLR1/TdNLR2 pair was detected, a feature that is distinct from the NLR pairs that form hetero-complexes and interact through their CC or TIR domains.

Comment 2.7 They could cite the Canadian work and also the test done in Israel here.

Response: Done.

Comment 2.8 L380: *Yr36* has a completely different mode of action compared with *Yr18* and *Yr46* loci.

Response: We thank the reviewer for this suggestion. We have revised the text as suggested.

Lines 413-415: Therefore, the *YrTD121* should be pyramided with slow-rusting resistance genes, such as *Yr18/Lr34/Sr57*, *Yr36*, and *Yr46/Lr67/Sr55*, with different modes of action to achieve more durable resistance.

Comment 2.9 L402: For curiosity, why “three-leaf stage”? Normally “two-leaf stage” is sufficient.

Response: We apologize for this mistake. Indeed, two-leaf stage is sufficient. In our study, we have inoculated wheat plants at the two-leaf to four-leaf stages. We have revised this description. The plants inoculated at the older leaf stage were used for the VIGS experiment since the HY-45 plants at the younger leaf stage had easily died upon virus inoculation.

Line437: Plants at the two- to four-leaf stage...

Comment 2.10 Here are some minor changes for the authors to consider when revising the manuscript.

Response: Thanks for your careful review and comments. As you suggested, we have revised the manuscript accordingly.

Comment 2.11 No need to spell out the numbers above ten, and to spell out if the numbers are below ten.

Response: Many thanks. We have revised this issue throughout the manuscript.

Comment 2.12 L2: Should be “wheat stripe rust pathogen”.

Response: We changed the title to “A head-to-head NLR gene pair from wild emmer confers stripe rust resistance in wheat” (14 words) instead of as suggested in previous title “A head-to-head NLR gene pair from wild emmer confers resistance to wheat stripe rust pathogen” (15 words).

Comment 2.13 L37: Do not think “genetically linked” is needed here.

Response: We have removed the “genetically linked” from the sentence.

Comment 2.14 L43: Should be “TdNLR1”.

Response: Revised.

Comment 2.15 L62: Should be “Stripe rust disease”.

Response: Revised (Line75).

Comment 2.16 L77: ASR needs to be spelt out “all-stage resistance”.

Response: We have revised this paragraph.

Comment 2.17 L93: Move “on a 0-9 scale’ from L100 to here.

Response: Done.

Comment 2.18 L113: It is clearer to use “Nine KASP markers” to be more specific.

Response: Thank you. We have revised the text as “Six kompetitive allele-specific PCR (KASP) and three PCR markers” (Line122).

Comment 2.19 L115: There are a few sub-tables in Supplementary Data 1. It is better to add which one, Supplementary Data 1.X.

Response: Thanks, we have revised the supplementary Data and combined sub-tables (Supplementary Data 2 in the revised manuscript).

Comment 2.20 L188-189: This is possible. However, this can be checked out easily by testing the TdNLR2 marker in Fielder.

Response: Thank you for your suggestion. We have checked carefully the Fielder genome and identified three *TdNLR2-like* homologous sequences (on chromosomes 1B, 2B, and 2D). The 1B copy was truncated due to a premature stop codon. Based on their similar three-dimensional (3D) structures, we hypothesize that the paralogous copies of *TdNLR2* from chromosomes 2B and 2D of Fielder may contribute redundantly to TdNLR1-mediated immunity. More details can be found in response to reviewer#1 comment 1.6.

Comment 2.21 L198: What leave stage for the leaf samples?

Response: Two-leaf stage. Revised.(Line221)

Comment 2.22 L243: Should be “on group 1 chromosomes”.

Response: Revised.

Comment 2.23 L248: Add an “may”, change to “may involve”.

Response: Revised as “may have evolved”(Line274)

Comment 2.24. L253-254: Capitalise “Clade”, also in Fig. 4.

Response: Done.

Comment 2.25 L264: Add “fused to the C-terminus” before included cell death.

Response: Revised.

Comment 2.26 L274: Change to “Self-association of the CC domain of NLR proteins”.

Response: Done.

Comment 2.27 L278: Should be “in vivo” instead of “in vitro”.

Response: Revised.

Comment 2.28 L283: “backcross”.

Response: Revised.

Comment 2.29 L286: “stripe rust pathogen”.

Response: Revised.

Comment 2.30 L304: Change “necessary” to “both required”.

Response: Revised.

Comment 2.31 L324: Delete “conferred”.

Response: We have revised this sentence.

Comment 2.32 L337: Should be “evolutionary”.

Response: Revised.

Comment 2.33 L365: Delete “with each other”.

Response: We have rewrote this sentence.

Comment 2.34 L437: Which generation are these RILs?

Response: F₄₋₆ generations. We have revised as F₄₋₆ RILs(Line475).

Comment 2.35 L441: “SMRTbell”.

Response: We have corrected this typo error.

Comment 2.36 L494-498: Not clear. Need to rewrite.

Response: Thank you. We have rewritten these sentences as:

Lines 533-541: Taking advantage of the incompletely dominant inheritance of *YrTD121*, spikes were harvested from M₁ plants with reduced stripe rust resistance, assuming they were heterozygous for *YrTD121* mutations. Then, 10–15 plants of each M₂ family were inoculated with a mixture of *Pst* races CYR32, CYR33, CYR34, Zhong 4, and HY46-8 in the field. The M₃ seeds collected from susceptible M₂ plants were re-evaluated with race CYR34 in a growth chamber. Finally, all the susceptible mutants identified were confirmed using *YrTD121*-flanking markers *Sicauw26* and *Sicauw76* to rule out the possibility of seed contamination.

Comment 2.37 L504: “vector combinations”.

Response: Revised.

Comment 2.38 L507: “the third leaf”.

Response: Revised.

Comment 2.39 L823: Typo “mutagenesis”.

Response: Corrected already.

Comment 2.40 L825: “hexaploid”.

Response: Corrected.

Comment 2.41 Supplementary Fig. 1b: Need to adjust the upper arrow to where it should point to.

Response: Revised.

Comment 2.42 Supplementary Fig. 15a: Need to spell out “Un” where it appears the first time.

Response: We have used “unknown function” instead of “UN” when it appears the first time in Supplementary Fig. 8 in the revised manuscript.

Comment 2.43 Supplementary Fig. 19b: The difference between CM42 and Cm42+YrTD121 in this figure is marginal.

Response: We have revised the text as CM42 (left) and CM42+*YrTD121* (right) plants showing similar phenotypes (Supplementary Fig. 23 in the revised manuscript).

Reviewer #3:

Remarks to the Author:

Comment 3.1 The study performed a map-based cloning and genome sequencing&assembly to identify a genomic region containing several candidate genes for stripe rust resistance. Two genes that are organized head-to-head were demonstrated to be required for the resistance. The study provided solid evidence for validating these two resistance genes: mutagenesis via EMS and/or CRISPR, gene knockdown, and transformation of genomic DNAs and coding sequences. Other related exploration about the allele distribution in the population and contributions of domains to HR responses was conducted. Importantly, the finding added a new pair of head-to-head NLR functioning together to conferring plant disease resistance. The resistance resource may be used to combat stripe rust, an important disease to be controlled in many wheat production areas.

Response: We are grateful for your careful review and valuable comments/suggestions.

Comment 3.2. It is still puzzling that the transgene of TdNLR1 only can enhance stripe rust resistance although both TdNLR1 and TdNLR2 are required for the resistance. The explanation that Fielder might contain a TdNLR2-like gene is reasonable. I wonder if the TdNLR2 homologous gene can be identified in the Fielder genome (<https://doi.org/10.1093/dnares/dsab008>).

Response: We thank reviewer for this comment and suggestion. We have checked carefully the Fielder genome and found three *TdNLR2-like* homologous sequences (on chromosomes 1B, 2B, and 2D, respectively) with relatively low sequence identity to *TdNLR2*. The 1B copy was truncated due to a premature stop codon. Based on their similar three-dimensional (3D) structures, we hypothesize that the paralogous copies of *TdNLR2* from chromosomes 2B and 2D of Fielder may contribute redundantly to *TdNLR1*-mediated immunity in Fielder background. We have revised the manuscript accordingly. This new data is added as Supplementary Fig. 8 in the revised manuscript. More details can be found in response to reviewer#1 comment 1.6.

Comment 3.3 BSR-seq and its analysis need proper citations. The parameters of Allele frequency difference (AFD) and bulk frequency ratio (BFR) were described but no reference was cited. It is difficult to know what these terms exactly mean. I think it is important to have a citation and briefly explain these terms. In the main text (L110-113), the number of SNP was mentioned. It is not clear these SNPs are from the GATK result or the resistance-associated SNPs passing the filtering criteria.

Response: We have provided references for the BSR-seq analysis (Trick et al. 2012) and for the parameter of Allele frequency difference (AFD) (Wang et al. 2017) mentioned in the manuscript. We used both parameters of Allele frequency difference (AFD) and bulk frequency ratio (BFR) for SNP calling, and the results were similar (Table R1). However, the SNP results present in the manuscript are exactly based on the AFD parameter. Therefore, we showed only the AFD results in the revised manuscript.

Table R1 Distribution of resistance-associated SNPs on wheat chromosomes

Chromosome	BSR-Seq (AFD $\geq$ 0.7)	BFR $\geq$ 6
chr1A	8	7
chr1B	130	116
chr2A	3	2
chr2B	2	2
chr3A	5	6
chr3B	36	36
chr4A	2	2
chr4B	2	1
chr5A	4	3
chr5B	5	3
chr6A	1	0
chr6B	11	4
chr7A	3	3
chr7B	5	3
chrUn	3	5
Total	220	193

For the meanings of AFD and BFR terms:

Allele frequency difference (AFD) is defined as the absolute value after subtraction of the allele frequency of the resistant bulk from that of the susceptible bulk.

Bulk frequency ratio (BFR) is defined as the allele frequency ratio of the resistant allele (resistant parent allele) in the resistant bulk compared to the resistant allele frequency in the susceptible bulk.

We have provided citations and brief explanation for the AFD in the revised manuscript:

Lines 462-466: The allele frequency for each variant and allele frequency difference (AFD) between resistant and susceptible bulks were calculated to identify SNP variants. AFD was defined as the absolute value after subtracting the allele frequency of the resistant bulk from that of the susceptible bulk.

We apologize for the inaccurate description of the sentences in the main text. The SNPs are resistance-associated SNPs passing the filtering criteria. We have revised the text as follows:

Lines 117-119: With a filtration criterion of allele frequency difference (AFD) ≥ 0.7 , we identified 220 single-nucleotide polymorphisms (SNPs) potentially associated with the resistant phenotype.

Comment 3.4 L43-44: “The CC domain of NLR1 is active in cell-death signaling and self-associates in planta.” What does “active” mean?

Response: We apologize for the inaccurate description. We have revised this sentence as: The coiled-coil domain of TdNLR1 triggers cell-death and self-associates *in planta*.(Line44)

Comment 3.5 L323-234: “The deletion of 21 nucleotides at positions 87-107 in the CC domain of TdNLR1_h4 may cause its conferred susceptibility.” If a new experiment would be added, this variant of the CC domain could be used to test the cell death induction. If not, the statement would be well supported.

Response: We thank the reviewer for this suggestion. We have performed additional experiments to confirm that the expression of the CC domain and artificial chimeric CC variant of TdNLR1_h4 indeed abolished the CC activity of cell death induction (Fig. 4R), supporting the statement that the deletion of 21 nucleotides at positions 87-107 of TdNLR1_h4 may cause its susceptibility. We have revised the text accordingly.

Lines 297-299: Intriguingly, expression of the CC domain of TdNLR1_h4 and an artificial chimeric CC variant of TdNLR1_h4 with a seven amino acid (GVHDDIQ) deletion abolished the induction of cell death.

Lines 350-352: seven amino acid (GVHDDIQ) deletion in the CC domain of TdNLR1_h4 impaired cell death-inducing activity likely causing a susceptible host response to stripe rust.

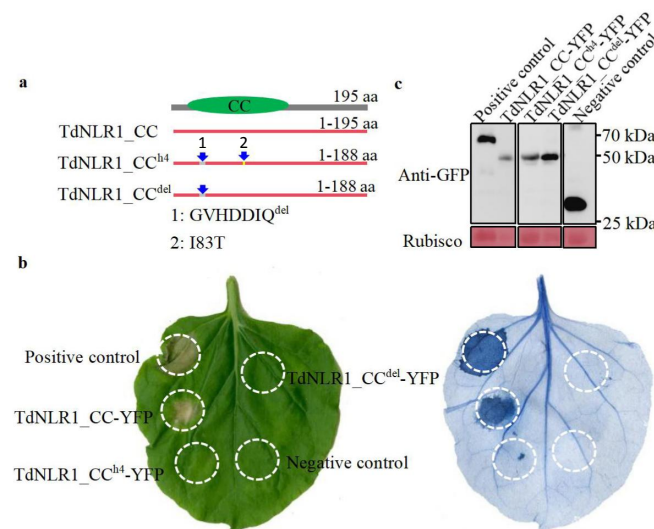


Fig.4R Transient expression of the CC domain and artificial chimeric CC variant of TdNLR1_h4 in *N. benthamiana* leaves.

a Schematic showing different CC variants of TdNLR1. TdNLR1_CC, CC domain of TdNLR1_h1; TdNLR1_CC^{h4}, CC domain of TdNLR1_h4; TdNLR1_CC^{del}, artificial chimeric CC variant of TdNLR1_h4 with the seven amino acid (GVHDDIQ) deletion. **b** A YFP tag was fused to the C terminus of TdNLR1_CC, TdNLR1_CC^{h4}, and TdNLR1_CC^{del}. Infiltrated leaves (left panel) stained with trypan blue (right panel) to observe cell death at 72 hpi. **c** Western blotting to detect the expression of constructs in *N. benthamiana* leaves. Positive control, wheat TaZFP5B protein. Negative control, YFP.

This data was added as Supplementary Fig. 20 in the revised manuscript.

Comment 3.6 L473-478: Both Bowtie and HiSAT2 were used for RNA-seq read alignments to the reference genome. Please justify why both tools were used.

Response: We thank the reviewer for this comment. During the fine mapping stage, the RNA-seq reads were initially aligned to the Zavitan reference genome using Bowtie tool to detect expressed genes in the fine mapping region of *YrTD121*. When we had the TD121 PacBio sequence assembly, RNA-Seq reads were aligned to the 1.18 Mb TD121 PacBio contig sequence of the *YrTD121* region. These two alignments were conducted at different times and by different persons who used different tools. We have revised the manuscript to show that the alignments were carried out at different time stages with different tools (Lines 511-517).

Comment 3.7 L588-589: A citation is needed for “The respective sequences of TdNLR1 and TdNLR2 were fused with the yeast two hybrid system vectors pGBKT7 and pGADT7.”

Response: The citation was provided (Lin et al. 2017, Methods Mol. Biol. 1615:177–187).

Comment 3.8 What are values on the edges in Fig. 4?

Response: We thank the reviewer for pointing this issue out. The values on the edges in Fig.4 represent ‘branch length’. We have revised the text accordingly.

References cited (point-by-point responses):

- Adachi, H. et al. NLR singletons, pairs, and networks: evolution, assembly, and regulation of the intracellular immunoreceptor circuitry of plants. *Curr. Opin. Plant Biol.* **50**, 121-131 (2019).
- Contreras, M.P. et al. NLR receptors in plant immunity: making sense of the alphabet soup. *EMBO Rep.* **24**, e57495 (2023).
- Dracatos, P.M. et al. Complementary resistance genes in wheat selection 'Avocet R' confer resistance to stripe rust. *Theor. Appl. Genet.* **129**, 65-76 (2016).
- Hewitt, T. et al. Wheat leaf rust resistance gene *Lr13* is a specific *Ne2* allele for hybrid necrosis. *Mol. Plant* **14**, 1025-1028 (2021).
- Lin J. S. et al. Protein-protein interactions: yeast two-hybrid system. *Methods Mol. Biol.* **1615**, 177-187 (2017).
- Nishikawa, K. et al. Mapping of progressive necrosis genes, *Ne1* and *Ne2* of common wheat by the telocentric method. *Jpn. J. Breed.* **24**, 277-281 (1974).
- Singh, R.P. & McIntosh, R.A. Complementary genes for reaction to *Puccinia recondita tritici* in *Triticum aestivum*. II. Cytogenetic Studies. *Can. J. Genet. Cytol.* **26**, 736-742 (1984).
- Trick, M. et al. Combining SNP discovery from next-generation sequencing data with bulked segregant analysis (BSA) to fine-map genes in polyploid wheat. *BMC Plant Biol.* **12**, 14 (2012).
- Wang, W. et al. Fine-mapping of *LrN3B* on wheat chromosome arm 3BS, one of the two complementary genes for adult-plant leaf rust resistance. *Theor. Appl. Genet.* **137**, 203 (2024).
- Wang Y. et al. Mapping stripe rust resistance gene *YrZH22* in Chinese wheat cultivar Zhoumai 22 by bulked segregant RNA-Seq (BSR-Seq) and comparative genomics analyses. *Theor. Appl. Genet.* **130**, 2191-2201 (2017).
- Wu, C.H. et al. NLR network mediates immunity to diverse plant pathogens. *Proc. Natl. Acad. Sci. USA.* **114**, 8113-8118 (2017).

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed each of my previous concerns from the initial review. The authors performed the necessary additional studies to alleviate these concerns in a comprehensive manner. The authors also updated the manuscript accordingly. I believe that this manuscript can now be accepted by the journal.

Response: Thank you.

Reviewer #2 (Remarks to the Author):

Comment 2.1 It is great to see that the authors spent efforts in carrying out the experiments recommended by the reviewers. They have responded nicely to all reviewers' comments and the quality of the manuscript has improved significantly. I think it warrants its publication. A few minor edits are needed. Here are some comments/suggestions.

Response: Thank you.

Comment 2.2 L246: "starting with ATG".

Response: Revised.

Comment 2.3 L297-299: The description here is not accurate. What they implied was the expression of these two with deletion abolished the induction of cell death. From their results, they cannot reach this conclusion. The expression of these two and the intact CC had the same expression as shown in Supplementary Fig. 20c. They need to reword the sentence.

Response: We have reworded this sentence as (L296-299):

Intriguingly, compared to TdNLR1_CC, expression of the CC domain of TdNLR1_h4 (TdNLR1_CC^{h4}) or an artificial chimeric CC variant TdNLR1_CC^{del} with a seven amino acid (GVHDDIQ) deletion impaired the ability of CC to trigger cell death in *Nicotiana benthamiana* leaves.

Comment 2.4 L311: It was my mistake before, which should be "in vitro".

Response: We have revised it as "in vitro" (L311).

Comment 2.5 L533-536: Did you inoculate and screen M1 plants? It is quite interesting because we normally only start screening M2 plants.

Response: Indeed, we normally screening M₂ plants when the gene is dominant inherited. The *YrTD121* confers incompletely dominant resistance and heterozygous *YRTD121* locus will show intermediate resistance. Theoretically, if the EMS-treated M₁ plants have mutations at *YrTD121* (caused heterozygous *YRTD121* locus), they will show

intermediate resistance. Therefore, we start screening M₁ plants with reduced stripe rust resistance to simplify the work. The corresponding M₂ and M₃ seeds were also re-evaluated. It works well in our study.

Comment 2.6 L548: ...was inoculated “on” the second leaf...

Response: Thank you, we have corrected ‘to’ to ‘on’ (L783).

Comment 2.7 L1048: Need to add a reference or link to MEGA 11.

Response: We have provided a link to MEGA 11 (L855).

Reviewer #3 (Remarks to the Author):

Thank the authors for all the efforts to revise the manuscript. The overall quality of the manuscript has been improved and my previous comments have been well addressed. The result from the new experiment testing the 21-nt deletion in the CC domain of TdNLR1_h4 strengthens the conclusion.

Response: Thank you.