Gene editing for accelerated breeding in wheat

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Abstract: Diseases adversely affect grain yield of crop plants. Leaf rust is a major disease of wheat. As race-specific resistance breaks down, introduction of newer sources of resistance often from older accessions is necessary. Linkage drag from the donor accessions adversely impacts grain yield. As a result, CIMMYT breeding effort has shifted to using durable resistance, also referred to as non-host resistance, which allows slow rusting but maintains grain yield. Two of the three known genes for durable resistance have been isolated. One of these two, *Lr67*, is absent in the elite CIMMYT lines. We have initiated efforts to convert the susceptible copy of *Lr67* into its resistant form directly in elite CIMMYT lines using gene editing. This would eliminate backcrossing and thus save time as well as eliminate linkage drag that would accompany the resistant copy of the gene if it were to be introgressed from an older accession. As first steps, we have isolated and sequenced the genomic copies from each of the A, B, and D genome of the *Lr67* gene from three elite CIMMYT lines and an experimental line. Identification of more than 50 single nucleotide polymorphisms (SNPs) in the open reading frames (ORF) among these lines would be useful in designing the guide RNA molecules with precision. Further, we have streamlined genetic transformation of the elite CIMMYT lines, a prerequisite step for gene editing.

Introduction: Wheat is grown on more agricultural area than any other crop in the world followed by maize, which is more productive of the two crops, perhaps because it is a C4 crop but also because it is grown in the high-yielding soils of the USA (Dhugga 2007). Whereas wheat provides approximately 20% of the calories to the world population on average (Hawkesford et al. 2013). Potential grain yield is the ability of the locally adapted crop varieties to produce grain under non-limiting environmental conditions, for example, irrigation and fertilizers, and absence of disease and insect pressure (Wulff and Dhugga 2018). Whereas the potential grain yield of

wheat has been reported to be ~13 t/ha, the actual yield is 3.5 t/ha. The harvested yield is so much lower because of limitation in growth that results from limited water or fertilizer availability plus disease and insect pressure. Competition from weeds and other unpredictable factors, for example, wind-induced lodging, further suppress the yielding potential of crop plants (Oerke 2006).

Genetic engineering has helped boost harvestable yield in maize by allowing selective destruction of the weeds with herbicides, and by conferring resistance against various insects (Wulff and Dhugga 2018). Farmers have benefited substantially from the availability of the genetically-modifies (GM) crops (Klumper and Qaim 2014). Further, bumper crop harvests have helped stabilize grain prices. The GM traits have been available and proven to be safe in maize and soybean for more than 20 years, but none has been approved for commercial planting in wheat (Klumper and Qaim 2014; Wulff and Dhugga 2018). Even for maize, moving GM hybrids outside of the Americas has been a challenge.

Whereas GM crops have encountered resistance from the consumer groups, gene editing offers an alternative avenue to expedite varietal development (Voytas and Gao 2014). Gene editing, which was first accomplished by oligo-mediated homologous recombination but with a very low rate of success, has evolved over time as attempts have focused on increasing the specificity and frequency of edited events (Carroll 2017). Nucleotide editing has been achieved with varying degrees of success using zinc fingers, transcription activator-like effector nucleases (TALENs), and meganucleases (Carroll 2017). Recent introduction of a new technology, clustered regularly interspersed short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (CRISPR-Cas9), has revolutionized the field of gene editing because it is easy to use and returns edited events in high frequency (Carroll 2017).

The conventional method to introduce new traits into cultivated varieties has been through crossing an elite variety to a donor line that contains the desirable trait, followed by repeated backcrossing to the elite line to reduce the genetic contribution of the donor line other than the gene of interest. Once disease resistance breaks down, however, newer sources of resistance must be introduced (Singh et al. 2016). Additional sources of resistance are generally found in wild relatives of the crop plants (Wulff and Dhugga 2018). Linkage drag is a term used to describe the adverse effect of the undesirable genes of the donor parent on the performance of the newly improved variety. It is nearly impossible to eliminate all the undesirable genes of the donor parent through recurrent backcrossing to the elite variety. Linkage drag can manifest in different ways, like increased root or stem lodging, which reduce grain yield.

Gene editing offers an alternative to backcrossing if a disease-susceptible elite lines can be directly transformed. Editing of the susceptible copy of a gene to its resistant version directly in elite lines not only eliminates linkage drag but also provides an elite disease-resistant variety that could then be used as a donor of resistance for the other disease-susceptible elite varieties that might be recalcitrant to transformation. Plant transformation, thus, is the first step in successful gene editing. We have overcome this hurdle by successfully transforming three elite wheat varieties from CIMMYT. In this report, we elaborate on our success in wheat and briefly discuss our work in wheat.

Materials and methods: DNA isolation and PCR: Wheat genotypes were grown in the greenhouse with proper irrigation and photoperiod at 16/8 h with an ambient temperature at 26ºC/16ºC day/night temperature. Three-week-old seedlings were harvested, and genomic DNA extracted using the DNeasy Plant Mini Kit (Qiagen). Genomic PCR was performed using Q5 High

Fidelity DNA polymerase (New England Biolabs) with gene specific oligonucleotide primers. Oligonucleotide primers were synthesized by T4 Oligo (Irapuato, Mexico). PCR products were gel purified using the QIAquick gel extraction kit (Qiagen). PCR products were sequenced directly by Langebio-CINVESTAV Genomic Services (Irapuato, Mexico). Nucleic and amino acid sequences were aligned using Vector NTI.

Donor materials for explant preparation: At 15 DAF, spikes were collected and immature seeds were extracted for each variety. The isolated seeds were sequentially surface sterilized in 70% (v/v) ethanol for 1 min, and 10% (v/v) Clorolex solution containing 100 µl of the surfactant Tween 80 for 10 minutes, successively three times rinsed with autoclaved distilled water to remove surface adhered sterilizing reagents. The embryos were excised under a light microscope.

Transformation was performed using *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) using the protocol of Ishida et al. (Ishida et al. 2015) with some modifications as described. The AGL1 strain harbored a combinations of pAL154 and pAL156 vectors system derived from pSoup/pGreen constructs (Hellens et al. 2000), which were kindly provided by BBSRC, UK. The vector pAL154 possess 15kb Komari fragment, which transacts to induce the proliferation of pAL156 vector. The pGreen based plasmid PAL156 carries T-DNA integrating *Bar* gene (phosphinothrocin-resistant gene), *gusA* gene (modified β-glucuronidase gene) and maize RPOL intron inserted at 385th nucleotide to prevent the gene expression in *Agrobacterium* strain (Bourdon et al., 2001; Ke et al., 2002). Both genes were driven by ubiquitin promoter and ubiquitin intron derived from *Zea mays* (Christensen and Quail 1996). Bar and *gusA* genes were adjacent to left and right borders respectively. Purity of the *A. tumefaciens* AGL1 strain was maintained by adding 100 mg/l of kanamycin and 200mg/l of carbenicillin.

A. tumefaciens stock culture (430 µl) was inoculated in 10 ml of MG/L medium containing 20 µl of 100 mg/ml carbenicillin and 20 µl of 50 mg/ml kanamycin and incubated overnight at 37°C in a shaker at 250 rpm. Bacteria were quantified by measuring turbidity (OD at 0.4 at A600) after 16 h and the culture tubes were centrifuged at 3500 rpm (2000 g) in a centrifuge for 10 min.

Embryos were extracted from the immature seeds under a light microscope and the radicle excised with the help of scalpel and forceps. *A. tumefaciens* pellet obtained from centrifugation was briefly mixed with 4.8 ml 1X liquid media containing appropriate amount of silwet, acetosyringone and phloroglucinol. Isolated embryos (about 30 embryos for each microfuge tube) were immersed in 600 µl of the 1X inoculation medium containing *A. tumefaciens*. The tubes were mildly vortexed three times for 15-20 sec at 10 min intervals and incubated in a water bath for 3 min at 46°C. The treated embryos were transferred to the inoculation/co-cultivation solid medium. Subsequently, the induced embryos were transferred to the callus regeneration medium containing the antibiotic timentin.

The callus was then transferred to the induction medium and placed in a light chamber at 26°C for 4 to 5 weeks. Subsequent selection was done on medium containing phosphinothricin and timentin for 4 to 5 weeks and hardening of the plants was done in soil-filled mini pots for 4 to 5 weeks. GUS assays were performed as described (Wu et al. 2008).

Finale Bayer's PPT, stock concentration 13.5 % was taken. Required of Ammonium Glufosinate concentration for PPT leaf painting assay was 2%, thus Finale Bayers PPT solution was diluted to obtain 2%, additionally 0.1 % Tween-20 was added in order to make an adhesive PPT leaf painting solution. Working solution preparation formulae: (13.5%/100) x 2%=0.27ml or 270 µl of PPT was added to make 100 ml of aqueous solution, in which 0.1% or 100 µl of Tween-20 was also

included. Matured leaves of both putative transgenic and non-transgenic Fielder varieties were painted using PPT solution upon dipping the cotton swab and painted over the selected area of the leaf tissues. Approximately after 3 to 4 days, the painted leaves of both transgenic and non-transgenic genotypes were observed for the leaf necrosis.

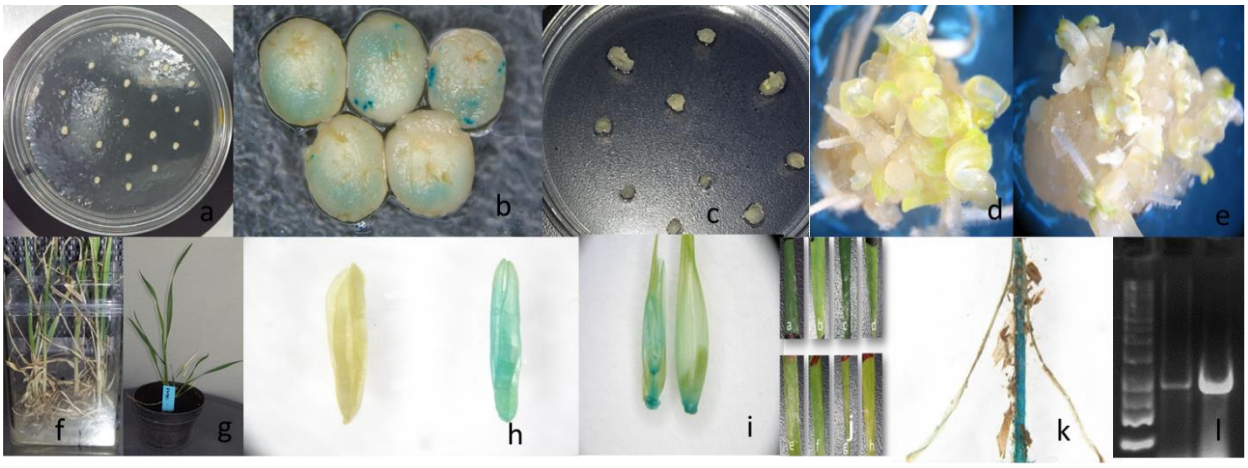
The wheat elite line Reedling was also transformed with a pCAMBIA vector, pSG19, where the expression of the DS-RED protein fused to a nuclear targeting sequence, N7, on the C-terminus, was driven by the CaMV 35S promoter and its transcription terminated by a NOS-polyA signal (Gillmor et al., 2010). Selection was carried out on bialaphos, the resistance gene the expression of which was also driven by the CaMV 35S promoter and terminated by the NOS-polyA signal.
PCR volume was fixed with 2µl of dNTP (20mM each), 0.1 µl of Phusion Polymerase (2U/ µl) (Thermo Fisher Scientific Co.), 0.2 µl of 2 pM Forward primer, 0.2µl of 2 pM Reverse primer R, 0.6 µl of DMSO (100%), 4 µl of HF5X buffer, 0.5 µl of MgCl2 (50mM), 15.4 µl of Sigma H2O. PCR was performed using Mastercycler® pro (Eppendorf) using the following cycling program: Initial denaturation at 98°C for 40 seconds, 35 cycles consists of 12 seconds denaturation at 98°C, 45 seconds annealing at 66°C, 23 seconds extension at 72°C and final extension at 72°C for 7 minutes. Primers used for gusA gene, forward: AGT GTA CGT ATC ACC GTT TGT GTG AAC (Tm: 67.5°C), reverse: ATC GCC GCT TTG GAC ATA CCA TCC GTA (76.5°C). Primers used for gusA gene, forward: GGT CTG CAC CAT CGT CAA CC (58.6 oC), reverse: GTC ATG CCA GTT CCC GTG CT (Tm 60.4oC). Samples were stored at 4°C. Subsequently, PCR amplified fragments along with O' Generuler 1kb ladder (Promega) were resolved using 1.2% of 10X diluted GelRed™ (Biotium Inc.) pre-stained agarose gel at 100 V for 2 h. The expected band was visualized using UV illumination-based documentation unit.

Results and discussion:

Agrobacterium-mediated transformation and transgenic screening

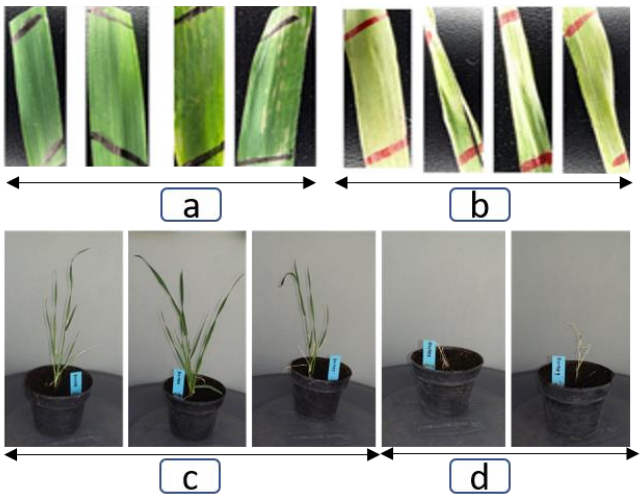
Editing native genes in the host genome is a promising approach to improve specific traits in crop plants. However, gene editing requires transformation of the host plant through biolistic or *Agrobacterium*-mediated methods. *Agrobacterium*-mediated transformation is the preferred strategy to edit genes in T0 generation as it results in simple, low copy integration of the T-DNA as compared to the biolistic method (Zhang et al, 2019). We have optimized the existing *Agrobacterium* transformation procedures and report here successful generation of transgenic events. (Fig.1).

Figure 1: Schematic representation of wheat transformation. a) Infected embryos on growth induction media, b) transformed embryos expressing GUS, c) callus induction after 2 weeks, d) and e) regeneration of shoots after 3-4 weeks from pro-embryos of each callus in light chamber, f) PPT selection of young plantlets in light chamber, g) a PPT-selected young seedling, h) GUS assay showing difference between transgenic and non-transgenic anthers, i) GUS assay staining of young caryopsis, j) PPT leaf painting assay differentiating transgenic leaves without necrosis with non-transgenic leaves with necrosis, k) GUS staining of rootlet from transgenic plant, l) Transgenic confirmation with *Bar* gene fragment PCR amplification.



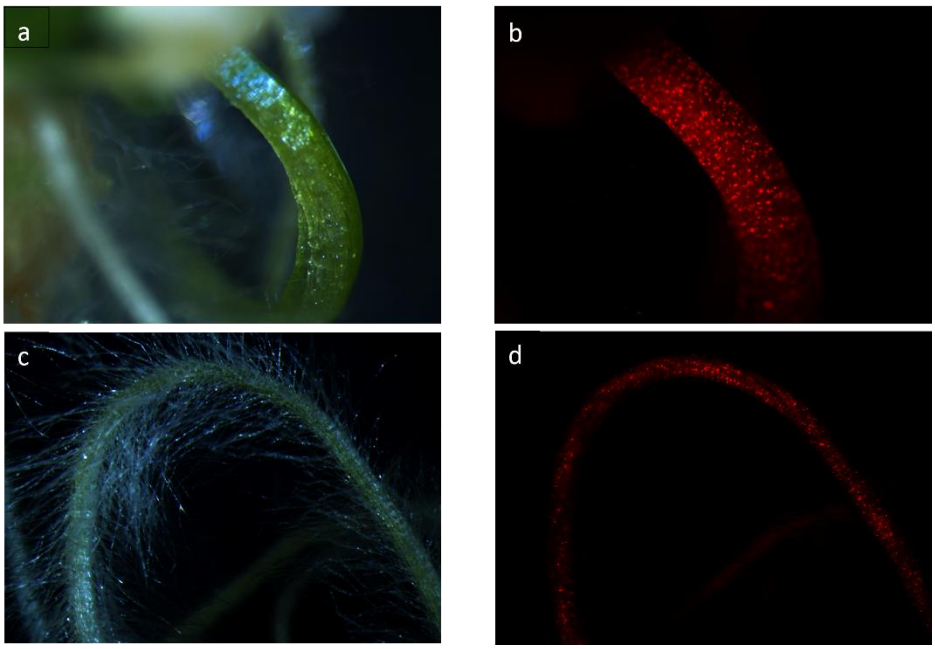
Callus bearing multiple shoots and roots from the regeneration medium was transferred to solid selection medium containing PPT. PPT inhibits glutamine synthetase in non-transgenic plantlets, which lack the *Pat*/*Bar* gene, causing senescence. Selected putative PPT-resistant seedlings were transferred to pots for hardening in a light chamber, where only the transgenic plants with apparently higher relative expression of the PAT or BAR gene survived. We observed that 2.5 mg/ml PPT stunted the plantlet growth along with root inhibition, and caused yellowing as previously described by Abdeen and Miki, 2009. Such senescence-prone plantlets with vestigial root formation along with retarded growth didn't survive further in the light chamber, while healthy plantlets with resistance to PPT survived. PPT leaf painting assay showed that the transgenic genotypes exhibited a lack of necrosis or partial necrosis, apparently because of the presence of phosphinothricin acetyltransferase (PAT) or bialaphos resistance (BAR) genes (Fig.2).

Figure 2: a) PPT painted leaves appeared as normal in putative transgenics, b) PPT painted leaves exhibited necrosis in non-transgenics, c) From left, the first three pots with healthy transgenic lines selected from PPT amalgamated media, d) Absence of apparent survival of last two genotypes with retarded growth from PPT selection media (experimental line Baj).



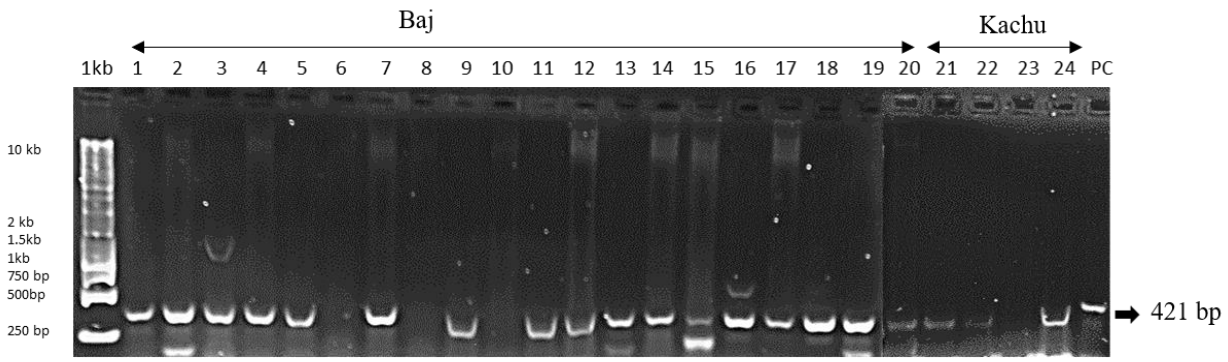
Successful transformation of Reedling, an elite CIMMYT variety, was carried out as described in M&M using a pCAMBIA vector containing the gene for the DS-RED protein fused at its C-terminus with a nuclear-targeting signal, N7, according to Gillmor et al. (2010). Targeting of the DS-RED fluorescent protein to nucleus is shown in Fig. 3, which demonstrates that we successfully transformed an elite CIMMYT variety.

Fig. 3: Transformation of the wheat variety Reedling with Agrobacterium. A vector expressing DS-RED driven by a 35S promoter was used to transform Reedling immature embryos. A regenerating seedling shoot (a) and root (c) visualized under visible light, and the same seedling shoot (b) and root (d) visualized under ultraviolet light using a DS-RED filter. DS-RED can be clearly seen in the nuclei, where it is targeted (right).



Other results are displayed in Fig. 4. Transformation efficiency ranged from 40-80 %. The presence of the transgene was confirmed by PCR of the *Bar* gene fragment size about 421 bp (Fig. 4). We have designed gRNA molecules for *Lr67* gene that are common to all four genotypes and are currently in the process of transforming the lines in collaboration with Corteva Agriscience (Lowe et al. 2018; Lowe et al. 2016). The regenerated events will be screened for gene edits as described previously (Savitashev et al. 2015).

Figure 4. Amplification of bar gene in varieties Baj and Kachu/saul. Gel picture top: Wheat variety Baj Genotypes with lab codes: 1.1B, 2. 2B, 3.3B, 4.4B, 5.4B2. 6. 4B3, 7.5B, 8. 6B, 9.7B1, 10. 7B2, 118B1, 12.8B2, 13. 9B1, 14. 9B2, 15. 10B1, 16. 10B2, 17. 10B3, 18. 11B1, 19. 11B2, 20. 12B. Kachu/saul genotypes: 21. KHA1, 22. KHB2, 23. KHB1, 24. KHC, 25. PC: Plasmid control.



Transgene frequency:~80%

Sequence characterization of genomic copies of the *Lr67* gene for gene editing

Diseases reduce grain yield in wheat by approximately 13% on average (Singh et al. 2016). Leaf rust is the most widely adapted disease that causes varying levels of damage to the crop (Singh et al. 2016). We have specifically focused on *Lr67*, a gene that confers durable resistance against leaf rust but is only available in older accessions (Moore et al. 2015).

In order to edit the susceptible copy of *Lr67* directly in elite wheat lines to its resistant form, we must first sequences each of the alleles corresponding to the A, B, and D genomes in the target genotypes. This step is necessary in order to avoid failure of the guide RNA to target the CRISPR-Cas complex to the gene of interest as even a single nucleotide mismatch could significantly weaken, if not eliminate, targeting. The genomic copies of *Lr67* were approximately 5 kb in length so we amplified allele-specific copies in parts. High quality Sanger sequence was provided by the DNA facility for approximately 700 nt for each run, requiring 7-10 sequence runs both for the forward and the reverse strands.

Two unique polymorphisms distinguish the resistant form of *Lr67* from its susceptible form (Moore et al. 2015). The first one is a G to C transversion at position 430 of the open reading frame (ORF), which causes a non-conservative change in the corresponding amino acid from Gly to Arg (Table 1, Fig. 5).

Table 1: Nonsynonymous polymorphisms in the open reading frame of wheat genotypes. Polymorphisms in the open reading frames of three elite CIMMYT lines, Baj, Kachu, and Reedling, an experimental line, Fielder, and the resistant (Res) and susceptible (Sus) open reading frames as reported in Moore et al. (2015) are presented.

Codon (number)	Nucleotide (position in ORF)	Variant (amino acid)	Genotype
144	430	**CGT (Arg)**	**4D Res**
	430	GGT (Gly)	4D Sus, 4D
	430, 432	GGC (Gly)	4A, 4B
151	453	AAC (Asn)	4D, 4B
	453	AAG (Lys)	4A
238	713	AAG (Lys)	4D, 4B
	713	AGG (Arg)	4A
358	1072	TTC (Phe)	4D, 4A, 4B Baj, 4B Fielder, 4B Kachu
	1072	GTC (Val)	4B Reedling
375	1125	AAG (Lys)	4D, 4B
	1124	AGG (Arg)	4A
376	1128	TCG (Ser)	4D, 4B Baj, 4B Fielder, 4B Kachu
	1128	TCC (Ser)	4A
	1127	TTG (Leu)	4B Reedling
387	**1161**	**TTG (Leu)**	**4D Res**
	1159	GTG (Val)	All others
408	1224	CTC (Leu)	4D Res, 4D Fielder, 4D Kachu, 4A, 4B
	1222	GTC (Val)	4D Sus, 4D Baj, 4D Reedling
496	1488	TTC (Phe)	4D, 4B
	1487	TAC (Tyr)	4A
502	1506	CAC (His)	4D, 4A
	1506	CAA (Gln)	4B

504-505	1513-1515	CAC insertion	4A

Fig. 5: Sequence alignment of the *Lr67* predicted protein for each of the genomes from four genotypes of wheat. The reference resistant (Res) and susceptible (Sus) alleles are included from Moore et al. (2015). Only the relevant portions of the protein containing the two polymorphisms (G144R and V387L) associated with rust resistance are shown.

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                        101                                                150
     Lr67-4D-Res  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLRCGVGFA
     Lr67-4D-Sus  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
     Lr67-4D-Baj  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
 Lr67-4D-Fielder  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
   Lr67-4D-Kachu  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
Lr67-4D-Reedling  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
     Lr67-4A-Baj  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
 Lr67-4A-Fielder  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
   Lr67-4A-Kachu  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
Lr67-4A-Reedling  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
     Lr67-4B-Baj  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
 Lr67-4B-Fielder  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
   Lr67-4B-Kachu  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA
Lr67-4B-Reedling  (101) SYTTRRLGRRLTMLIAGVFFIIGVIFNGAAQNLAMLIIGRILLGCGVGFA


                        351                                                400
     Lr67-4D-Res  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILLVVMVCTYVASFAW
     Lr67-4D-Sus  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILVVVMVCTYVASFAW
     Lr67-4D-Baj  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILVVVMVCTYVASFAW
 Lr67-4D-Fielder  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILVVVMVCTYVASFAW
   Lr67-4D-Kachu  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILVVVMVCTYVASFAW
Lr67-4D-Reedling  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILVVVMVCTYVASFAW
     Lr67-4A-Baj  (351) LEAGVQMFLSQVVIAVVLGIKVTDRSDNLGHGWAILVVVMVCTYVASFAW
 Lr67-4A-Fielder  (351) LEAGVQMFLSQVVIAVVLGIKVTDRSDNLGHGWAILVVVMVCTYVASFAW
   Lr67-4A-Kachu  (351) LEAGVQMFLSQVVIAVVLGIKVTDRSDNLGHGWAILVVVMVCTYVASFAW
Lr67-4A-Reedling  (351) LEAGVQMFLSQVVIAVVLGIKVTDRSDNLGHGWAILVVVMVCTYVASFAW
     Lr67-4B-Baj  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILVVVMVCTYVASFAW
 Lr67-4B-Fielder  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILVVVMVCTYVASFAW
   Lr67-4B-Kachu  (351) LEAGVQMFLSQVVIAVVLGIKVTDKSDNLGHGWAILVVVMVCTYVASFAW
Lr67-4B-Reedling  (351) LEAGVQMVLSQVVIAVVLGIKVTDKLDNLGHGWAILVVVMVCTYVASFAW
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The second is at nucleotide position 1161, G to T of the first nucleotide of the codon, which changes the corresponding amino acid from Val to Leu. This is a conservative change, however.

Each of the polymorphisms corresponds to amino acids in a transmembrane domain (TMD). *LR67* protein is a hexose transporter and possesses 12 TMD (Moore et al. 2015). A non-conservative change like the one from Gly to Arg could cause a disruption in the TMD assembly and inactivate the protein (*LR67i*). Whereas Gly has only a hydrogen as a side chain and is neutral, Arg has a bulky, positively charged side chain and is an extremely basic amino acid with a pKa of 12.5, which would explain inactivation of the transporter protein. The second change, from Val to Leu, where both the amino acids are hydrophobic, is unlikely to disrupt the TMD hence is likely to have coevolved with the Gly to Arg mutation but without perhaps any role in the inactivation of the transporter protein. These observations were actually proved to be true using hexose uptake assays in yeast cells where the reversion of Arg to Gly in the encoded protein restored hexose uptake but Leu to Val did not when Arg (instead of the wildtype Gly) was present at the other site (Moore et al, 2015).

It is also likely that the transporter protein encoded by the *Lr67* gene, although annotated as a hexose transporter, is perhaps involved in the transport of toxin molecules of the pathogenic fungi into the plant cells. When it is inactivated, the remaining copies of the transporter encoded by the A and B genomes are unable to compensate for its loss. Our view is that it might require simple disruption of the *Lr67* ORF, which could be accomplished by employing the non-homologous end joining (NHEJ) after causing a cut with CRISPR-Cas, also referred to as the side-directed nuclease scenario 1 (SDN1) (Podevin et al. 2013). Further, by designing the gRNA molecules in the conserved regions of the ORF, it might be possible to disrupt the A and B copies as well, which will provide us additional tools to determine whether the inactivated *LR67i-A* and *LR67i-B* proteins phenocopy the *LR67i-D* protein, and if stacking them with the *LR67i-D* protein further augments rust resistance.

As to our objective of sequencing each of the *Lr67* copies from each of the genotypes to avoid failure of the gRNA to bind the target sequence, we identified 56 single nucleotide polymorphisms (SNPs) just in the ORF that is ~1.5 kb in length (only partial data shown in Table 1 and Fig. 5). Eleven of these polymorphisms led to nonsynonymous changes in the corresponding amino acids, eight of which are either non-conservative or semi-conservative in nature (Table 1). As discussed previously, the *LR67* protein from the resistant line is distinguishable from the susceptible line as well all the CIMMYT lines by two specific changes, a Gly144Arg and a Val387Leu where Gly and Val apparently correspond to susceptibility (Table 1 and Fig. 5). A unique change is the addition of a complete codon in the A genome near the end of the ORF (Table 1). Whether it has any significance is not clear.

We have initiated editing of the Lr67 gene in Reedling using the tools developed in our laboratory. Successful identification of the edited events where the Lr67 gene is inactivated would allow to test whether it confers additional resistance against a broad spectrum of fungi, something durable resistance genes are known to do (Moore et al., 2015).

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Author contributions: KT: Amplified, sequenced and assembled the genomic copies of Lr67 gene, PCR transgenic confirmation, assisted and co-guided the tissue culture, helping to prepare the manuscript. **LN**: Tissue culture and Agrobacterium mediated transformation of Baj, Kachu, Fielder and Reedling wheat varieties. **MP**: Transformation of wheat line Reedling with a

pCAMBIA vector pSG19 with the expression of the DS-RED protein. **GV** and **PV** provided critical suggestions and helped to improve the manuscript.

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