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Single residues in the LRR domain of the wheat PM3A immune receptor can control the strength and the spectrum of the immune response

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Abstract: The development of improved plant nucleotide-binding, leucine-rich repeat (LRR) immune receptors (NLRs) has mostly been based on random mutagenesis or on structural information available for specific receptors complexed with the recognized pathogen effector. Here, we use a targeted mutagenesis approach based on the natural diversity of the Pm3 powdery mildew resistance alleles present in different wheat (*Triticum aestivum*) genotypes. In order to understand the functional importance of the amino acid polymorphisms between the active immune receptor PM3A and the inactive ancestral variant PM3CS, we exchanged polymorphic regions and residues in the LRR domain of PM3A with the corresponding segments of PM3CS. These novel variants were functionally tested for recognition of the corresponding AVRPM3A2/F2 avirulence protein in *Nicotiana benthamiana*. We identified polymorphic residues in four regions of PM3A that enhance the immune response, but also residues that reduce it or result in complete loss of function. We found that the identified critical residues in PM3A modify its activation threshold towards different protein variants of AVRPM3A2/F2. PM3A variants with a lowered threshold gave a stronger overall response and gained an extended recognition spectrum. One of these variant proteins with a single amino acid change was stably transformed into wheat, where it conferred race-specific resistance to mildew. This is a proof of concept that improved PM3A variants with an enlarged recognition spectrum can be engineered based on natural diversity by exchanging single or multiple residues that modulate resistance function.

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**Single residues in the LRR domain of the wheat PM3A immune receptor
can control the strength and the spectrum of the immune response.**

Stefan Lindner¹, Bettina Keller¹, Simrat Pal Singh¹, Zsuzsanna Hasenkamp², Esther Jung¹,
Marion C. Müller¹, Salim Bourras^{1,3*}, Beat Keller^{1*}

¹ Department of Plant and Microbial Biology, University of Zürich, Zürich, Switzerland

² Institute of Pharmacology and Toxicology, University of Zurich, Zürich, Switzerland

³ Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural
Sciences, Uppsala, Sweden

* Corresponding authors (bkeller@botinst.uzh.ch; salim.bourras@slu.se)

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aestivum*, resistance gene, allelic diversity, mutagenesis, genetic engineering

Summary

The development of improved plant nucleotide-binding, leucine-rich repeat immune receptors (NLRs) has mostly been based on random mutagenesis or on structural information available for specific receptors complexed with the recognized pathogen effector. Here, we use a targeted mutagenesis approach based on natural diversity of the *Pm3* powdery mildew resistance alleles present in different wheat genotypes. In order to understand the functional importance of the amino acid polymorphisms between the active immune receptor PM3A and the inactive ancestral variant PM3CS, we exchanged polymorphic regions and residues in the LRR domain of PM3A with the corresponding segments of PM3CS. These novel variants were functionally tested for recognition of the corresponding AVRPM3^{A2/F2} avirulence protein in *Nicotiana benthamiana*. We identified polymorphic residues in four regions of PM3A that enhance the immune response, but also residues that reduce it or result in complete loss of function. We found that the identified critical residues in PM3A modify its activation threshold towards different protein variants of AVRPM3^{A2/F2}. PM3A variants with a lowered threshold gave a stronger overall response and gained an extended recognition spectrum. One of these variant proteins with a single amino acid change was stably transformed into wheat where it conferred race-specific resistance to mildew. This is a proof of concept that improved PM3A variants with an enlarged recognition spectrum can be engineered based on natural diversity by exchanging single or multiple residues that modulate resistance function.

Significance

To engineer plant immune receptors with enhanced and broader recognition specificity, it is essential to identify critical residues for receptor function. Here, we show that natural allelic diversity can be used to determine key residues for specific receptor function. We demonstrate their impact on receptor activity, and use these observations to design an improved immune receptor.

Introduction

Plant pathogenic microorganisms have evolved secreted proteins called effectors that interfere with the host's immune system and allow the pathogen to invade the host's tissue (Bent and Mackey, 2007). In turn, plants have evolved immune receptors that can perceive the presence of a specific effector, which often results in a hypersensitive cell death response (HR). This highly localized reaction deprives the pathogen of its nutrient source and renders the plant immune (Dodds and Rathjen, 2010). A pathogen effector that is recognized by the plant causes the pathogen to be avirulent, and accordingly the effector is considered an avirulence protein (AVR), which is encoded by an avirulence gene (*Avr*). On the plant side, the receptors that recognize the AVR are called resistance or immune receptor proteins, encoded by resistance (*R*) genes. The pairwise *R/Avr* interaction has been described by the gene-for-gene model which was formulated based on genetic studies of race-specific resistance (Flor, 1971). This model has recently been expanded with the addition of suppressors of avirulence (*Svr* genes) (Bourras et al., 2016). *R* genes frequently encode nucleotide-binding, leucine-rich repeat receptor proteins (NLRs) (Jones et al., 2016).

The powdery mildew disease of hexaploid wheat (*Triticum aestivum*) is caused by the obligate biotrophic ascomycete *Blumeria graminis* f.sp. *tritici* (Bgt). In wheat, resistance against Bgt can be encoded by several powdery mildew (*Pm*) resistance genes. The *Pm3* locus provides the largest allelic series of wheat *Pm* genes, with 17 functional alleles that confer race-specific resistance against distinct groups of Bgt isolates. The proteins encoded by the different alleles share at least 97% sequence identity (Bhullar et al., 2009, 2010). The reference allele *Pm3CS* represents the consensus sequence of all known *Pm3* resistance alleles but does not confer resistance and is considered the susceptible, ancestral allele (Yahiaoui et al. 2006, Bhullar et al. 2009). The NLR proteins encoded by the different *Pm3* resistance alleles differ in polymorphisms in the NB (nucleotide binding), ARC (Apaf1, resistance proteins, CED4) and

LRR (leucine-rich repeat) domains, and these polymorphisms determine the signaling activity and recognition spectrum of the respective natural PM3 variants (Brunner et al., 2010, Stirnweis et al. 2014a). For example, PM3E has only two polymorphic residues compared to PM3CS (Fig. 1), and those two residues are sufficient to confer resistance. Similarly, one polymorphic residue shared by PM3B and PM3C is sufficient to convert PM3CS to a functional immune receptor (Brunner et al., 2010). In addition, there are pairs of strong and weak *Pm3* alleles: the strong *Pm3a* confers resistance against the same set of mildew isolates as the weak *Pm3f*, plus an additional set of isolates that *Pm3f* does not recognize (Brunner et al., 2010). The substitution of two amino acids in the ARC2 domain of PM3F was sufficient to expand the recognition spectrum of PM3F (Stirnweis et al., 2014a) (red asterisk in Fig. 1). By now, three *Avr* genes of wheat mildew have been identified for different *Pm3* alleles (*AvrPm3^{a2/f2}* (Bourras et al., 2015), *AvrPm3^{b2/c2}* and *AvrPm3^{d3}* (Bourras et al., 2019)), and additionally, a suppressor of avirulence gene (*SvrPm3^{a1/f1}*) has been described (Parlange et al. 2015; Bourras et al., 2015). Like the *R* genes, *Avr* genes exist in natural variants. For example, there are twelve natural variants of *AvrPm3^{a2/f2}*, and *Pm3a* confers resistance only against variant A, which is the originally described *Avr* gene (McNally et al., 2018). The importance of single residues for NLR function has been described in mutagenesis studies in a variety of pathosystems, such as flax/rust (Ravensdale et al., 2012, Bernoux et al., 2016), potato/*Phytophthora* (Segretin et al., 2014), potato virus X (Farnham and Baulcombe, 2006; Harris et al., 2013), and in wheat/mildew (Brunner et al., 2010; Stirnweis et al., 2014a). There, the mutation of one or two residues can disrupt or enhance resistance. Similarly, single-residue mutations in the AVR can result in gain of virulence by escaping recognition or to the contrary, can lead to a stronger immune response (McNally et al., 2018). In the rice/*Magnaporthe* system of *Pikp*/*AVR-Pik*, similar observations have been reported and could be connected to direct NLR/AVR interaction (Maqbool et al., 2015). In both natural and artificial variants of AVR-Pik polymorphisms of one or two residues

were sufficient to reduce or disrupt both the immune response and the direct interaction with the receptor.

For agricultural applications, resistance genes with a broad resistance spectrum are desirable, and several attempts to improve existing NLRs have been described. Random mutagenesis was used to artificially evolve NLRs to gain a stronger response or a broader spectrum (Farnham and Baulcombe, 2006; Harris et al., 2013; Segretin et al., 2014). These studies generally implied that mutations in the NB and ARC domains influenced overall NLR signaling activity, similar to the observations of Stirnweis et al. (2014a) specifically for PM3, and that mutations in the LRR domain affected AVR recognition. However, random mutagenesis largely generates non-functional mutants. In contrast, mutations based on knowledge of natural diversity possibly allows to study structurally intact proteins. In cases of direct NLR/AVR interaction, and when the interaction site is well characterized, detailed information about the involved residues can be used to optimize the NLR/AVR interface, and expand the recognition spectrum (de la Concepcion et al., 2019).

In this study, we used an approach that exploits natural diversity and exchanged regions as well as individual amino acid residues in the LRR domain of PM3A, based on the polymorphisms with the ancestral, susceptible variant PM3CS. We focused on sequence diversity in the LRR domain, because previous studies have shown that the LRR is responsible for AVR recognition and specificity (Farnham and Baulcombe, 2006; Brunner et al., 2010; Wang et al., 2019). We show that groups of polymorphic residues in different regions in the PM3A LRR domain contribute to NLR function in different ways. While some groups enable the recognition of the AVR, one group is reducing the immune response. We describe similar effects at the single-residue level, with some residue exchanges enhancing the immune response, and some reducing or completely abolishing NLR activity. Furthermore, we show that several point-mutants with

enhanced NLR activity also have a broader recognition spectrum. We conclude that there is a potential for NLR improvement based on the natural diversity of resistance receptors.

Results

Polymorphic regions in the PM3A LRR domain have different roles in NLR function

Polymorphic regions and residues of resistant NLR variants that descend from a common, susceptible ancestor reveal the evolutionary events towards resistance, and should hypothetically be better adapted to modern pathogen strains, either individually or in combination. The susceptible *Pm3* allele *Pm3CS* is the likely ancestor of the whole *Pm3* allelic series in hexaploid wheat (Yahiaoui et al. 2006). Therefore, we hypothesized that any polymorphism in the active PM3 variants compared to the PM3CS protein could have contributed to resistance activity against powdery mildew, either due to increased NLR signaling activity, or improved AVR recognition. Here we focused on the effects of the LRR domain on AVR recognition. With 26 polymorphic residues compared to PM3CS, PM3A is the most divergent variant in the LRR domain (Fig. 1). For a better understanding of the effects of these natural polymorphisms on resistance function we decided to exchange the four polymorphic regions of the LRR domain of PM3A with the corresponding regions of PM3CS (Fig. 1). We anticipated that these exchanges should affect either NLR activation, AVR recognition, or both.

First, we wanted to determine the functional role of individual polymorphic regions on the constitutively activated PM3 receptor. Therefore, we introduced the four exchanges into the autoactive mutant PM3A^{HR}, which carries a D502V mutation in the IHD motif in the C-terminal region of the ARC2 domain (red arrow in Fig. 1). The IHD motif of PM3 receptors is a variant of the highly conserved MHD motif found in many NLR proteins, and mutations in this motif cause a constitutive immune response when transiently expressed in *N. benthamiana* (e.g.

Bendahmane et al., 2002; Williams et al., 2011, Stirnweis et al., 2014a). Based on the assumption that PM3A accumulated mutations that are advantageous for resistance, we expected a reduction or loss of function when we exchanged polymorphic regions from PM3A with the corresponding regions from the inactive PM3CS. We generated four chimeric constructs: PM3A^{HR}- α , with a total of four polymorphic residues, located in LRR repeats 1 and 2, PM3A^{HR}- β , with four polymorphisms in repeats 13 and 14, PM3A^{HR}- γ , with 13 polymorphisms in repeats 19 to 22, and PM3A^{HR}- δ , with five polymorphic residues in repeat 27 (Fig. 1). Region γ is the region of the LRR domain that distinguishes PM3A from the weaker variant PM3F; regions α , β and δ are shared between PM3A and PM3F (Brunner et al., 2010). We transiently expressed these four chimeric constructs in *N. benthamiana* using *Agrobacterium* infiltration, and determined the resulting HR intensity five days after infiltration (5 dpi), using high-sensitivity reading (HSR) imaging. The original PM3A^{HR} was used as a reference. We found that the region exchanges had either no significant effect (PM3A^{HR}- α), or caused a small but significant reduction in relative HR intensity down to 0.85 - 0.90 (PM3A^{HR}- β to δ ; $p < 0.01$) compared to the original PM3A^{HR} (Fig. 2a, b). To test if the observed effects can be attributed to NLR protein abundance, we performed a Western Blot analysis (Fig. 2c). Due to very rapid development of cell-death, samples for protein expression studies were harvested as early as 1 day after leaf infiltration, to avoid that overall estimation of proteins levels is masked by tissue degradation due to HR. While the protein levels of PM3A^{HR}- β , γ and δ were similar to that of the original PM3A^{HR}, PM3A^{HR}- α was less abundant suggesting that the introduced mutation in variant α could possibly influence protein synthesis and stability. From these observations, we can conclude that regions β , γ and δ influence NLR signaling activity and are directly advantageous to resistance, although only to a minor extent, while region α contributes to protein abundance.

Then, we investigated the effect of the four regions on AVR recognition. We introduced the same exchanges into the natural PM3A protein, resulting in the chimeric constructs PM3A- α , - β , - γ and - δ , and transiently co-expressed them with the corresponding AVRPM3^{A2/F2} (Bourras et al., 2015) in *N. benthamiana*. The natural PM3A was used as a reference for immune response quantification, which was performed 5 dpi, using HSR imaging. We performed Western Blot analysis again, and there were no differences in protein abundance between the chimeric constructs and the natural PM3A in the absence of the AVR (Fig. 2f). We found that the exchange of region β had no significant effect on the immune response (Fig. 2d, e), indicating that the polymorphic residues in this region are not involved in AVR recognition. Exchange of region α , however, caused a relative reduction of mean HR intensity to 0.3 ($p < 0.01$) compared to the PM3A reference, and the exchange of region δ led to a complete loss of HR. This suggests that both regions α and δ are crucial for AVR recognition. Most surprisingly, the exchange of region γ caused an increase in mean HR intensity by a factor of 1.4 ($p < 0.01$), which suggests a modulating role of this polymorphic region on immune response. In none of these assays, autoactivity of the novel PM3 chimeric receptor variants was observed.

To summarize, we observed that the four polymorphic regions have different roles in AVR recognition. Region α is involved in AVR recognition and/or subsequent signaling, and – only in the constitutively active PM3A^{HR} – possibly stabilizes the protein. Region β seems to be mostly involved in NLR signaling activity, region γ has a modulating role, and region δ is crucial for AVR recognition.

Single polymorphic residues can control the strength of the immune response

Based on these findings, we expanded the analysis with site-directed mutagenesis to exchange each polymorphic residue of the LRR domain of PM3A individually with the corresponding amino acid of PM3CS. For simplicity, we named them according to their region. For example,

mutant PM3A^{N922S} is based on the exchange of the second polymorphism in region β , and therefore named PM3A- β 2 (Fig. 3a). The full list of mutants is shown in Table S1. We transiently co-expressed all point mutants with AVRPM3^{A2/F2} in *N. benthamiana*, and measured the HR intensity by HSR imaging, 5 dpi, using the natural PM3A as a reference. The relative mean values of HR intensity for each mutant are shown in Table S1. Of the 26 residue exchanges tested, 15 had no significant effect on HR intensity, or the effect was very small (PM3A- γ 9, with 0.94 compared to the reference, $p < 0.01$) (Figs. 3a, S1, S2). PM3A- α 2 showed a reduction of HR intensity to 0.7 ($p < 0.01$), while PM3A- γ 11, - δ 1, - δ 2, - δ 3 and - δ 4 caused a complete loss of HR (Fig. 3a, S1, S2), indicating that these residues are essential for AVR recognition. In contrast, PM3A- β 1, - γ 2, - γ 7, - γ 13 and - δ 5 strongly increased the response intensity to between 1.4 and 1.8 (Fig. 3a, S1, S2; $p < 0.01$), which indicates the role of these residues as modulators of NLR activity in PM3A, similar to that of the complete γ -region. None of the novel receptor variants was autoactive. Western Blot analysis showed that the exchange of neither the resistance-increasing nor the -reducing residues affected protein abundance (Figure S3). Strikingly, region δ of PM3A consists of four polymorphic residues that are crucial for NLR function (δ 1-4) and one that, when mutated, increases the immune response (δ 5), which means that it reduces the immune response in the natural PM3A. In total, the additive effects of the five residues seem to contribute to the overall effect of their region, which was also observed for region α (one HR-decreasing mutant, with the whole region exchange causing decreased HR) and region γ (three enhancing and one decreasing mutations, whole region exchange increasing HR) (Fig. 3, Fig. 2d, e). While the majority of the 26 polymorphic residues had no impact on NLR function, there are almost as many residues that reduce NLR function (five) as there are residues enhancing it (six). This demonstrates that the overall NLR activity level is controlled in a very fine-tuned balance, and any exchange of the involved residues disturbs this state.

We therefore conclude that these assays demonstrate that it is possible to significantly alter the strength of the immune response based on one single point mutation of specific residues in the LRR which can be identified from the natural diversity of the PM3 alleles.

The increased immune response of PM3A- $\delta 5$ is based on increased receptor sensitivity

To further characterize the enhancing effect of PM3A- $\delta 5$, we performed a sensitivity assay by co-expressing it with a dilution series of AVRPM3^{A2/F2} in *N. benthamiana*. Receptor sensitivity describes the ability of the immune receptor to detect the corresponding AVR. The mutant PM3A- $\delta 5$ induced a stronger immune response than the natural PM3A (Fig. S2, S3), which indicates that it might be more sensitive. We stepwise reduced the ratio of bacteria containing the *Avr* gene, from the usual *NLR:Avr* ratio of 1:4 down to 1:0.2, and analyzed the resulting HR intensity by comparing the effect of the dilution between PM3A and PM3A- $\delta 5$ (Fig. 3b, c). We found that with reduced amounts of AVRPM3^{A2/F2}, the natural PM3A quickly lost the ability to trigger HR, which was almost undetectable at an *NLR:Avr* ratio of 1:1 (Fig. 3b), while PM3A- $\delta 5$ lost visible HR at a ratio of only 1:0.4 (Fig. 3c). Also, PM3A induced an HR intensity of 0.8 at a ratio of 1:3, while PM3A- $\delta 5$ did so at a ratio of only 1:2 (Fig. 3b). This shows that PM3A- $\delta 5$ has a lower activation threshold than the natural PM3A. At an *NLR:Avr* ratio of 1:0.8 the natural PM3A would not be considered to induce HR, while PM3A- $\delta 5$ still shows a response (Fig. 3c). Therefore, we conclude that the amount of AVR protein is an important factor in NLR function.

Loss-of-function PM3A mutants can be rescued by overabundant AVR variants

Given that the stronger immune response triggered by PM3A- $\delta 5$ is caused by a lower activation threshold, it seems likely that some of the mutants that did not cause HR, such as PM3A- $\delta 1$ or - $\delta 4$, have reduced sensitivity, and thus a higher activation threshold, and that the amounts of AVR present in the previous infiltration assay were not sufficient to trigger the immune

response. For simplicity, we will use the term “loss-of-function” mutants for these variants. We tested this hypothesis by utilizing a selection of artificial AVR variants that were described by McNally et al. (2018). These constructs correspond to single-residue mutants of AVRPM3^{A2/F2} (AVRPM3^{A2/F2}-G71N, -L83Y, -Q112N, -L116Y and -R124E) and give a strongly increased HR when co-expressed with the natural PM3A. By Western Blotting, using a custom-made antibody for detection of AVRPM3^{A2/F2} (Fig. S6a), we found that these artificial protein variants were more abundant compared to the natural AVR (Fig. S6b), with the L116Y variant having the highest protein level. We hypothesized that by presenting higher levels of AVR protein to the loss-of-function PM3A mutants, their activation threshold might be reached, and therefore, they may trigger HR. We co-expressed some of these PM3A mutants (PM3A- γ 11, - δ 1 and - δ 4, and the δ -region mutant) with the five AVR variants, and tested for immune response. We found that while PM3A- δ and PM3A- γ 11 did not develop HR with any of the artificial AVR mutants, PM3A- δ 4 showed HR when combined with AVRPM3^{A2/F2}-L116Y, and PM3A- δ 1 showed HR with all five AVR variants, with the most intense HR occurring with the L116Y variant (Fig. 4a). Here, we cannot exclude the possibility that this gain of function by PM3A- δ 1 and especially δ 4 might be due to the mutations introduced to the AVR variants, and not caused by AVR protein abundance. However, AVRPM3^{A2/F2}-L116Y was the variant with the highest abundance (Fig. S6b), it was the only one that triggered HR with PM3A- δ 4 (Fig. 4a), and from all five AVRPM3^{A2/F2} variants it gave the strongest HR with PM3A- δ 1 (Fig. 4a). In addition, PM3A- δ 1 recognized all five of the artificial AVR variants, all of which are characterized by a different mutation in different parts of the AVR proteins. Since the shared property of the five AVRPM3^{A2/F2} variants is their overabundance, and we get the strongest effect with the most abundant one, we assume that this is the cause for the observed results.

These assays demonstrate that a seemingly inactive PM3A mutant can gain recognition when the AVR is sufficiently abundant, and that each PM3A mutant has a different threshold in that

regard. This further supports our previous observation that NLR function depends on AVR abundance and receptor sensitivity which can be controlled by single residue changes. This leads to the question if sufficiently sensitive NLR mutants can detect additional natural AVR variants which are not recognized by the natural PM3A.

Over-sensitive NLR mutants exhibit a broader recognition spectrum

Beside NLR sensitivity, another component of NLR function is the recognition spectrum. Testing 272 isolates from a global mildew collection, McNally et al. (2018) described a total of 12 haplotypes of *AvrPm3^{a2/f2}* that encode for the natural variants AVRPM3^{A2/F2}-varA to -varM, of which only variant A, the originally described AVR, is recognized by PM3A. Some of these variants are polymorphic to variant A in only one or two residues, and this is already sufficient to disrupt recognition by PM3A. We speculated that a sensitizing mutation of PM3A might confer a broader recognition spectrum. Therefore, we cross-tested our enhanced PM3A mutants PM3A-β1, -γ2, -γ7, -γ13 and -δ5 with the 12 AVR variants by co-expression in *N. benthamiana* as described earlier. We found that all five PM3A mutants, but not the natural PM3A, triggered HR at different levels of intensity in the presence of AVRPM3^{A2/F2}-varF (N66K, R69S, G86E) (Fig. 4b, 4c, S5). PM3A-δ5 could additionally trigger detectable HR when co-expressed with AVRPM3^{A2/F2}-varC (F95L), although with a very low intensity (0.2 compared to PM3A with AVRPM3^{A2/F2}-varA, $p < 0.01$).

To conclude, all five PM3A mutants that caused stronger HR with AVRPM3^{A2/F2}-varA were able to detect one (PM3A-β1, -γ2, -γ7, -γ13) or two (PM3A-δ5) additional natural variants, although with a lower intensity of subsequent HR induction. This indicates that like the PM3A mutants that have different thresholds of AVR recognition, the stronger PM3A mutants also have different thresholds for detection of AVR variants. Altogether, we propose that both the

response-enhancing and -reducing residues in the LRR domain contribute to the overall NLR function of PM3A and act as gradual modulators.

A stable transgenic *Pm3a-δ5* wheat line expresses a functional receptor

A PM3A variant that is able to recognize more than one natural variant of the AVR would be extremely valuable in agriculture granted it is also functional in wheat. Therefore, we tested the enhanced *Pm3a-δ5* in the natural wheat/powdery mildew system. We generated stable transgenic wheat lines overexpressing *Pm3a-δ5* in the susceptible Bobwhite (BW S26) cultivar. The gene was introduced under the control of the maize ubiquitin promoter and transformed into BW S26 by particle bombardment as previously described (Pellegrineschi et al., 2002, Brunner et al., 2011 and 2012). Together with non-transgenic Bobwhite, and a transgenic BW-*Pm3a* line generated earlier (Brunner et al., 2011), we subjected three independent BW-*Pm3a-δ5* lines (T2 plants, lines #4, #23B and #32) to a powdery mildew infection test with the natural isolates Bgt_96224 (encoding the original AVRPM3^{A2/F2}-varA, avirulent on *Pm3a*) and Bgt_C3-1, the only isolate in our collection encoding for AVRPM3^{A2/F2}-varC (recognized only by PM3A-δ5, but not the natural PM3A in *N. benthamiana*). Some wheat leaf tissue of each line was sampled for protein extraction (Fig. S7a). As expected, all four transgenic lines (BW-*Pm3a* and BW-*Pm3a-δ5*) were resistant against isolate Bgt_96224, although the transgene of lines BW-PM3A-δ5 #23B and #32 still segregated, which is also visible on the Western Blot, where the protein is not present in one of the tested samples for both lines (Fig. S7a). Untransformed BW was susceptible towards Bgt_96224 (Fig. 5). All tested transgenic lines and untransformed BW were susceptible towards isolate Bgt_C3-1, with no visible difference between the *Pm3a* and the *Pm3a-δ5* lines (Fig. 5).

Thus, the *Pm3a-δ5* lines appear to be functional and confer resistance against Bgt_96224. However, the weak recognition of AVRPM3^{A2/F2}-varC we observed in the *N. benthamiana*

overexpression system (Fig. 4b, c) did not result in resistance in wheat when challenged with Bgt_C3-1. First, it is possible that the weak HR from *N. benthamiana* (80% weaker than with AVRPM3^{A2/F2}-varA) is simply not strong enough to confer resistance in wheat. In addition, we found that isolate Bgt_C3-1 encodes the active allele of the *SvrPm3*^{a1/f1} gene which can suppress the resistance response of several alleles of *Pm3* including *Pm3a* (Bourras et al., 2015, 2019). We therefore performed RT-qPCR to determine its expression level, as well as that of *AvrPm3*^{a2/f2}-varC. We found that both genes are expressed in Bgt_C3-1 (Fig. S7b), with *SvrPm3*^{a1/f1} at a level that is comparable to that of isolate Bgt_JIW2 as described by Bourras et al. (2015), which is at least sufficient to suppress the weaker allele *Pm3f*. Therefore, consistent with previous observation that the response of PM3A-δ5 to AVRPM3^{A2/F2}-varC is visibly weaker than that of PM3A to AVRPM3^{A2/F2}-varA (ca. 0.2; Fig. 4c), we conclude that the expression of SVRPM3^{A1/F1} by isolate Bgt_C3-1 might be sufficient to suppress the weak recognition of variant C by PM3A-δ5. Consistent with this observation, we observed no further gain of resistance when the *Pm3a*-δ5 lines were challenged with additional mildew isolates encoding and expressing AVRPM3^{A2/F2}-varA and SVRPM3^{A1/F1} (specifically Bgt_07004 (SVR higher than AVR) and Bgt_Chikara (SVR lower than AVR) (Fig. S8).

Overall, these results are in line with previous observations by Bourras et al. (2015) demonstrating that *Avr* and *Svr* gene expression levels are an important component of *Avr* recognition in the *AvrPm3*-*Pm3* interaction, and congruent with our protein expression assays demonstrating there is a threshold for PM3 activation based on AVR protein abundance.

We conclude that the artificially generated PM3A-δ5 NLR can be fully functional in wheat and that the observed enhanced spectrum in the *N. benthamiana* overexpression system is likely restricted in field conditions by the availability of mildew races encoding the active

SVRPM3^{A1/F1} suppressor and/or expressing AVRPM3^{A2/F2}-varC below the recognition threshold.

Residues critical for PM3A-triggered immune response share common characteristics

In the work presented here we have specifically studied the residues that are naturally polymorphic between PM3A and PM3CS. However, it is likely that there are more residues with a similar enhancing or reducing function in both PM3 proteins, which are simply not polymorphic. To support future research for their identification, we analyzed if the residues that we found to enhance or reduce PM3A resistance function had common features. We separated the total 26 single-residue exchange mutants of PM3A into two groups: 12 that caused a significant effect in NLR function (functional mutants), and 14 that did not (neutral mutants), without differentiating between those having an enhancing or reducing effect. We then classified them on the basis of six parameters and checked if any of these are distinctly different between the two groups (Fig. 6). The parameters were (a) solvent exposure, (b) location on a 3D-model of the PM3A LRR domain (Sela et al, 2014), (c) location in the LxxLxLxx motif of the LRR repeat motif, (d) how conservative the polymorphism between PM3A and PM3CS was regarding size or (e) charge, and finally (f) residue position on the primary sequence. A detailed description of the parameters is given in Supplementary Note 1, and the scoring in Table S2. We found that a mutation is more likely to have a significant effect on the PM3A immune response when it is solvent exposed (Fig. 6a), located on the inner arc of the model, or close to it (Fig. 6e), within the LRR core motif (Fig. 6b), not conservative in both size and charge (Fig. 6c, d), and finally, located towards the C-terminus of the protein (Fig. 6e), which can be observed with exchanges $\gamma 7$, $\delta 2$ and $\delta 5$. Furthermore, on the 3D-model, the majority of the resistance-enhancing and -reducing residues cluster on an area on the inner arc surface towards the C-terminus of the LRR domain, which might indicate a possible AVR recognition interface that can be specifically targeted for NLR improvement (Fig. 6e).

Discussion

In this project, we wanted to achieve a deeper understanding of the functional role of naturally occurring polymorphisms in the LRR domain of the PM3 receptor family. We hypothesized that if we understand how the polymorphic regions and residues contribute to NLR function, especially AVR recognition, this would support future attempts to design improved NLRs. We found that polymorphic regions in the LRR domain mostly contribute to AVR recognition (Fig. 2), and that there are polymorphic residues in PM3A that enhance the immune response, but also residues that reduce the response (Fig. 3a, S1, S2). Exchanging response-reducing residues with their ancestral counterparts from an inactive (susceptible) protein resulted in PM3A variants with a reduced activation threshold (Fig. 3b, c), a stronger immune response in general (Fig. S1, S2), and a broader recognition spectrum (Fig. 4b, c, S5). A similar observation has been reported for randomly generated mutants of the potato NLR R3a, which confers resistance against *Phytophthora*, where a single residue mutant in the LRR domain increased HR intensity and expanded the recognition spectrum (Segretin et al., 2014). Similar results have also been obtained with structure-guided engineering of the rice NLR *Pikp* (de la Concepcion et al., 2019) where the introduced mutations were based on detailed knowledge on the NLR/AVR interaction site, and caused increased response and a broader recognition spectrum.

The lower recognition threshold and the resulting increased sensitivity and broader recognition spectrum we observed in the PM3A- $\delta 5$ mutant would make it more difficult for the pathogen to rapidly evolve to avoid recognition, e.g. by expressing lower levels of the AVR, or – provided there is a direct interaction – slightly changing its amino acid composition. Therefore, we stably transformed PM3A- $\delta 5$ into wheat, and we found that the receptor was expressed and conferred resistance towards the isolate that expressed variant A of AVRPM3^{A2/F2} (Fig. 5), but not to the isolate expressing variant C. This shows that in principle, the enhanced PM3A mutants act as functional immune receptors in wheat, but we could not show that the lower activation threshold

of PM3A- $\delta 5$ that we observed in *N. benthamiana* also resulted in a stronger response or an extended recognition spectrum in wheat, at least in the leaf segment infection test. Lack of resistance to isolate Bgt_C3-1 can be attributed to a combination of the already weak response in the *N. benthamiana* overexpression system with the presence of the active suppressor gene *SvrPm3^{a1/f1}* in this isolate. Since our *Pm3a- $\delta 5$* lines are principally functional, we suggest further testing of these transgenic lines, specifically with isolates that express the AVR variant C, or AVR variant A at very low levels, but do not have the active *SvrPm3^{a1/f1}* gene. The *Pm3a- $\delta 5$* lines should also be tested in field trials, to determine if there is an effect of the generally stronger response on mildew infection, and if there is any adverse effects of the transgene. Finally, *Pm3a- $\delta 5$* might also be further improved by combining with another response-increasing mutation, such as $\gamma 2$, $\gamma 7$ or $\gamma 13$, or by random mutagenesis, where alleles with stronger interaction with other AVRPM3^{A2/F2} variants could be selected.

In the studies of de la Concepcion et al. (2019) on the rice *Pikp* resistance gene, it was demonstrated that the observed stronger response and expanded spectrum, which is very similar to our observations, resulted from an increased NLR/AVR binding affinity. Furthermore, our results resemble observations from the flax/rust system, where recognition specificity and resistance response strength of the L5, L6 and L7 receptor variants towards natural AVRL567 variants is determined by polymorphisms in both the NLRs and the AVRs, and recognition is based on direct interaction (Dodds et al., 2006). In addition, our observations that the N- and C-terminal repeats of the LRR domain are the main contributors to receptor specificity are also in accordance to the findings of Ravensdale et al. (2012) in flax. These findings are highly similar to ours, and combined with our observation that the PM3A-triggered immune response depends directly on AVR abundance (Fig. 3b, c), and the hypothetical interaction interface we identified (Fig. 6e), a direct interaction between PM3A and AVRPM3^{A2/F2} is one hypothetical explanation. This hypothesis is further substantiated by the similarities between the *Pm3* allelic

series from wheat and *Mla* from barley. In both genes, polymorphic sites in the sequences encoding the LRR domain are under positive selection (Seeholzer et al., 2010), and the distinct allelic variants recognize mildew AVR_s that are sequence-unrelated (*Mla*: Lu et al., 2016; *Pm3*: Bourras et al., 2019). Considering that direct interaction has been demonstrated between MLA receptors and their corresponding AVR_s (Saur et al., 2019), the same could be assumed for the *Pm3* allelic series. That would imply that the critical polymorphic residues between PM3A and PM3CS were directly involved in NLR/AVR interaction, and modulated the binding affinity, i.e. the equilibrium between receptor-bound and -unbound AVR. This could be integrated into the equilibrium-based switch model suggested for the flax rust receptors L6 and L7 by Bernoux et al. (2016). There, intramolecular interactions keep the NLR in an equilibrium between an inactive and a transient active state. The active state is stabilized when the AVR binds to the receptor, and HR is triggered. Our findings would add a second equilibrium to this model that is defined by the binding affinity between NLR and AVR. However, since an experimental proof of a direct PM3A/AVRPM3^{A2/F2} interaction has yet to be provided, other models of interactions should not be excluded.

Our observations of the residue characteristics that make a significant impact on NLR-triggered immune response are very much in line with earlier observations. It has been shown for the tomato receptor-like protein Cf-9 that specificity is determined by residues that are located in the LxxLxLxx motif of the leucine-rich repeats (Wulff et al., 2009). From LRR crystal structures (Enkhbayar et al., 2003; Wei et al., 2008), we know that the x residues of the LxxLxLxx motif are solvent exposed and that they form the inner arc surface of the LRR domain. Sela et al. (2014) showed that residues towards the C-terminus (repeats 21 to 28) of the wheat PM3 immune receptor are highly diverse and positively selected. Accordingly, Brunner et al. (2010) demonstrated that the predicted solvent-exposed residues located in the LRR motif of more C-terminal repeats are heavily involved in PM3 resistance and specificity.

Considering this, the exceptions we found that do not follow the observed patterns may be of interest. The mutant PM3A- β 1 gives a significantly stronger immune response than the natural PM3A, but the respective residue it is not predicted to be part of the LRR motif and it is located on the outer surface (Fig. 6e), which indicates that it might not be directly involved in AVR recognition. Instead it could modulate intramolecular interactions, and be involved in the inactive/active state equilibrium suggested by Bernoux et al. (2016). Furthermore, assuming that upon activation, the PM3 receptors form circular structures similar to mammalian NLRs (Hu et al., 2015; Zhang et al., 2015) or ZAR1 from *Arabidopsis* (Wang et al., 2019), it may be involved in intermolecular interactions and modulate the formation of the polymeric complex. The other exception we found based on the predicted three dimensional structure, was mutant PM3A- γ 12. The residue is located in the LxxLxLxx motif and therefore exposed in the inner arc surface. There, while the exchange is radical in both size and charge, it didn't affect the NLR response significantly (Fig. S1, S2, Tables S1, S2). Most likely it is simply not involved in AVR recognition and therefore does not affect the activation threshold.

We have shown that within the natural diversity of the PM3 immune receptor family, there are polymorphic residues that allow for artificial NLR improvement. Surprisingly, these critical amino acids are not already present in the functional PM3A, but instead in the ancestral, inactive PM3CS. There are several possible explanations for this observation. First, PM3A is evolutionary young and does probably not represent an 'optimal' receptor, but a product of still ongoing evolution, explaining the unexploited potential for improved resistance. The sequences encoding polymorphic amino acid regions in PM3A were possibly introgressed into the *Pm3* gene by gene conversion from related genes (Yahiaoui et al. 2006). Thus, they represent protein regions removed from their original evolutionary context, resulting in the observed, diverse functional consequences in the wheat PM3A immune receptor. Second, there can be tradeoffs between a functionally optimized receptor and other physiological or agronomically important

traits. For example, a potato *Rx* mutant that conferred resistance against additional strains of PVX, was more susceptible to the distantly related PoMV than the natural *Rx* gene (Farnham and Baulcombe, 2006), thus correlating improved resistance with adverse effects. Considering this, it is possible that over-sensitive PM3 variants have been selected against in evolution, and that the response-enhancing and -reducing residues tightly regulate the immune response to a certain level which in total results in an active, functional receptor.

For future attempts of PM3 NLR improvement, we suggest to utilize the identified promising residues and combine them in an artificial immune receptor. Additional residues could be identified either by random mutagenesis, based on existing knowledge of natural diversity, or a resolved crystal structure. To improve NLRs without a known 3D-structure and limited knowledge of the natural diversity, we suggest to make use of the established knowledge on critical residues, and select amino acids in the LxxLxLxx motif (which are solvent exposed and located in the inner arc) and mutate them radically in both size and charge. Combining two or three mutations that enhance the immune response could, however, produce oversensitive receptors. This could be mitigated by integrating additional, response-reducing residues. We propose that mutations that modulate the intramolecular interactions between the receptor domains (receptivity), as well as mutations that change the intermolecular interactions between receptor and AVR (sensitivity), can lead to an increased immune response, and could be targeted for NLR engineering. Effects of combining such mutations, and possibly fine-tuning high-receptivity with low-sensitivity mutations, or vice versa, may be highly interesting, especially in the light of the tightly regulated response level of natural PM3A that we have observed. As a proof of concept, this has been demonstrated by Harris et al. (2013). There, a previously generated potato NLR *Rx* mutant (in the LRR domain) that had a broader spectrum, but also showed severe adverse effects in transgenic potato (Farnham and Baulcombe, 2006), was randomly mutated further in the NB-ARC domains, and indeed, the resulting secondary

mutants kept the enhanced properties, but lost the negative effects. If this can be generalized, balancing the equilibrium of receptivity and sensitivity might produce synthetic PM3 variants with a broader spectrum, but without the debilitating effects of oversensitivity.

To conclude, we have demonstrated that the diversity within the PM3 alleles can be utilized to generate immune receptors with a stronger response and a broader recognition spectrum that remain functional in wheat. We therefore consider diversity-based mutagenesis a highly relevant, complementary approach for generating improved NLRs together with structure-guided mutagenesis, random mutagenesis, or artificial evolution.

Experimental Procedures

LRR region exchange

The regions α , δ , γ and δ and the remaining plasmid backbone were amplified by PCR from vectors containing *Pm3a* and *Pm3CS*, using Phusion High Fidelity DNA Polymerase (NEB, #M0503L). The respective primers are listed in Table S3. The template plasmids were pENTR221 vectors that had been generated by TOPO cloning and contained the respective genes with a C-terminal HA-tag (Stirnweis et al., 2014b). The primers were designed so that the products would overlap to allow for recombination with the Gibson-Assembly Cloning Kit (NEB, #E2611). For example, to generate the *Pm3a- α* construct, the *Pm3a* backbone (including the vector), and the *Pm3a* fragments β , γ , and δ were combined with the *Pm3CS* fragment α . The recombined constructs were then transformed into DH5 α *E. coli* cells.

Site-directed mutagenesis

Site-directed mutagenesis was performed on a pENTR221 plasmid containing the *Pm3a* gene with a C-terminal HA-tag that had been generated by TOPO cloning (Stirnweis et al., 2014b). An inverse PCR was performed to introduce the desired mutation using Phusion High Fidelity

DNA Polymerase (NEB, #M0503L). The respective primers are listed in Table S3. The template was digested with *DpnI* (NEB, #R0176) and the linearized product then purified. The product was phosphorylated using T4 polynucleotide kinase (NEB, #M0201) in T4 ligase buffer with 10 mM ATP (NEB, #B0202), then ligated by T4 ligase (NEB, #M0202), and afterwards transformed into DH5 α *E. coli* cells.

Generation of *Agrobacterium* strains

The transformed DH5 α cells were selected on kanamycin medium. Colony-PCR was performed to further select clones, and the pENTR221 vectors encoding the gene cassette were purified, verified by Sanger sequencing, then the cassette was recombined into the pIPKb004 expression vector (Himmelbach et al., 2007) using the LR clonase II enzyme mix (Invitrogen, #11791) and transformed into DH5 α *E. coli* cells, which were then selected for spectinomycin resistance. Sequence verified pIPKb004 expression vectors were transformed to GV3101 *Agrobacterium tumefaciens* cells as previously described (Bourras et al., 2015), which were selected on medium containing rifampicin, gentamycin and spectinomycin. Primers used for colony-PCR in both the pENTR221 and the pIPKb004 vectors are given in Table S2.

Agro-infiltration for transient expression studies in *N. benthamiana*

Transient expression of NLR and AVR proteins in *N. benthamiana* was performed as previously described (Bourras et al., 2019). Briefly, GV3101 *Agrobacterium* cultures were grown overnight at 28°C, 200 rpm in selective medium containing Rifampicin, Gentamycin and Spectinomycin. The cultures were centrifuged for 5 min at 3300 g, and the cells re-suspended in LB medium and incubated for 30 min at 28°C, 200 rpm. The cells were then centrifuged again for 5 min at 3300 g and the OD₆₀₀ was adjusted to 1.2 in induction buffer (10 mM MES-KOH pH 5.6, 10 mM MgCl₂, 0.2 mM Acetosyringone). The bacterial suspensions were

incubated again at 28°C at 200 rpm for 2-3 hours for induction. Prior to infiltration suspensions were mixed as indicated. If not stated otherwise, *NLR/Avr* ratio was 1:4.

Imaging of the hypersensitive response

The intensity of the hypersensitive response was determined 5 days after infiltration as previously described (Bourras et al., 2019). Briefly, leaves were imaged using high sensitivity reading (HSR) with the Vilber Lourmat Fusion FX system, with the following settings: fluorescence sample; excitation: blue-light epi-illumination; emission: filter F535 Y2; aperture 0.84-open; sensitivity: full resolution. HR was quantified as the mean gray value of the infiltrated area as determined by ImageJ, and normalized against the uninfiltrated background and the reference area (PM3A and AVRPM3^{A2/F2}, ratio 1:4, or PM3A^{HR}) that was present on each separate leaf.

SDS-PAGE and Western Blotting

To determine protein abundance, the respective constructs were transiently expressed in *N. benthamiana*, as described above. For direct comparison, the reference and sample constructs were expressed in the same tobacco leaf, and 2 days (1 day for PM3A^{HR} in order to avoid protein degradation resulting from cell death) after infiltration leaf disks (diameter 0.7 cm) were collected from 6 independent leaves. The leaf disks were frozen in liquid nitrogen, ground to a powder, suspended in 400 µl 2x protein extraction buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 0.04% Bromophenol blue, 20% Glycerol, 2% SDS) and boiled for 10 min at 95°C. For protein extraction from wheat leaves, a detached leaf segment (ca. 3 cm long) was frozen, ground, suspended in 400 µl 2x protein extraction buffer and boiled for 10 min at 95°C. 15 µl of the extracts were loaded onto SDS gels (8% gels for NLRs and 4-20% gradient gels for AVR), and then transferred to a nitrocellulose membrane (Amersham, #10600001) by semi-dry Western blotting (Bio-Rad Trans-Blot Semi-Dry Transfer Cell). The membrane was then

stained with Ponceau-S, destained in 1xTBS-T (10 mM Tris-HCl pH 7.5, 0.1% Tween-20) and blocked for 1 h in 10 ml blocking buffer (5% skim milk powder in 1xTBS-T) at room temperature. The HA-tagged NLR proteins were detected by an HRP-conjugated anti-HA antibody (monoclonal from rat, Roche, clone 3F10, 1:2000). The untagged AVR proteins were detected by a custom-made antibody against the LNDQIEKAYREALEAGT peptide (AVRPM3^{A2/F2} res. 22 to 38; rabbit, polyclonal; Pineda Antikörper-Service (Berlin), 1:500). The anti-HA and anti-AVR antibodies were added to the 10 ml blocking buffer and incubated with shaking at room temperature for 2 h. The membranes were then washed 3 times in 1xTBS-T. The anti-AVR membranes were then decorated with the HRP-conjugated secondary antibody (mouse-anti-rabbit, LabForce, 1:15'000) in 15 ml blocking buffer at 4°C over night. For peroxidase activity detection, the Vilber Lourmat Fusion FX system (chemiluminescence setting) and ECL HRP substrate (Advansta, #K-12045-D20) were used.

PM3A and PM3A- $\delta 5$ sensitivity assay

For the sensitivity assay of PM3A and PM3A- $\delta 5$, the *Agrobacteria* were prepared as described above. Before infiltration, the different mixes were prepared. Each mix was 1 ml in total, of which 200 μ l were always the NLR-expressing bacteria. The remaining 800 μ l were a mix of AVRPM3^{A2/F2}- and GUS-expressing bacteria: 800 μ l AVR and no GUS for the NLR/AVR ratio of 1:4, 600 μ l AVR and 200 μ l GUS for 1:3, 400 μ l AVR and 400 μ l GUS for 1:2, 200 μ l AVR and 600 μ l GUS for 1:1, 160 μ l AVR and 640 μ l GUS for 1:0.8, 120 μ l AVR and 680 μ l GUS for 1:0.6, 80 μ l AVR and 720 μ l GUS for 1:0.4, 40 μ l AVR and 760 μ l GUS for 1:0.2, and no AVR and 800 μ l GUS for 1:0.

Generation of transgenic wheat lines

The *Pm3a- $\delta 5$ -HA* gene was cloned into the pAHC17 plasmid (Christensen and Quail, 1996), under the control of the maize ubiquitin promoter and the NOS terminator, using Gibson-

Assembly (#NEB, E2611), which was verified by Sanger sequencing. The primers used to generate the fragments are listed in Table S2. The whole *pUbi-Pm3a-δ5-HA-NOS* cassette was then transformed by particle bombardment into the hexaploid spring wheat cultivar Bobwhite SH 98 26 (BW S26), that does not have an endogenous *Pm3* gene (Brunner et al., 2011), as described by Pellegrineschi et al., 2002, and by Brunner et al., 2011 and 2012. T₀ plants were selected by Western Blot for the presence of the HA epitope tagged transgene, and allowed to self. Positively selected lines were propagated into the T2 generation.

Wheat infection tests

The stable transgenic lines BW S26-Pm3a #1, -Pm3a-δ5 #4, -Pm3a-δ5 #23B and -Pm3a-δ5 #32 and the untransformed BW S26 were subjected to infection with the *Blumeria graminis* f.sp. *tritici* isolates Bgt_96224 (AVRPM3^{A2/F2}-varA) and Bgt_C3-1 (AVRPM3^{A2/F2}-varC), as well as with isolates Bgt_07004 and Bgt_Chikara (characterized in McNally et al., 2018). The wheat plants were vernalized for 3 days and grown for 10 days before infection. Detached leaf segments were bedded on 0.37% Agar plates supplemented with 24 ppm benzimidazole and infected with the respective mildew isolates. The isolates were obtained from the in-house powdery mildew storage, and propagated twice for 10 days on detached leaves of the susceptible wheat cultivar Kanzler before infection. Virulence was determined 10 days after infection.

RNA isolation and RT-qPCR

To isolate mRNA from the powdery mildew isolates Bgt_96224 and Bgt_C3-1, detached Kanzler leaf segments were infected as described for the infection tests. Two days after infection, the leaf segments were collected in 2 ml Eppendorf tubes, together with two 4.5 mm glass beads, and flash-frozen in liquid nitrogen. They were then ground to a powder with the GenoGrinder tissue homogenizer (twice at 1500 rpm for 30 seconds). The powder was stored

at -80°C. For mRNA extraction the SV Total RNA Extraction System (Promega, #Z3101) was used, and RNA integrity was tested on a 1.2% agarose gel, with a pH 6 SB buffer. The mRNA samples were stored at -80°C. The RT-qPCR was performed as previously described (Praz et al., 2018). cDNA was prepared with the iScript Advanced cDNA Synthesis Kit (BioRad, #1725038) and then stored at -20°C. As an internal control for gene expression normalization *GAPDH* was chosen. Primers for amplification of *GAPDH*, *AvrPm3^{a2/f2}* and *SvrPm3* are shown in Table S3.

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Supporting Information

Figure S1: Effects of single residue exchanges in the LRR domain of PM3A on HR intensity

Figure S2: Quantitative comparison of HR intensity induced by the single residue exchange PM3A mutants

Figure S3: Abundance of HA-tagged PM3A proteins and the functional single residue exchange constructs

Figure S4: High-Sensitivity Reading (HSR) images of *N. benthamiana* co-expressing PM3A or PM3A- $\delta 5$, respectively, with different amounts of AVRPM3^{A2/F2}

Figure S5: Quantitative comparison of HR intensity of the five tested stronger PM3A mutants when co-expressed with the natural AVRPM3^{A2/F2} variants C and F in *N. benthamiana*.

Figure S6: Detection of AVRPM3^{A2/F2} and its artificial variants by an AVR-specific antibody

Figure S7: Expression of PM3A proteins from transgenic wheat and *AvrPm3*^{a2/f2} and *SvrPm3* mRNA from powdery mildew

Figure S8: Additional mildew infection assays with transgenic *Pm3a- $\delta 5$* wheat lines

Figure S9: Depiction of the topological surfaces of the PM3A LRR model

Table S1: PM3A mutants used in this study and their effect on immune response

Table S2: Characterization of single-residue exchanges used in this study

Table S3: Primers used in this study

Supplementary Note 1: Characteristics of the critical residues affecting the PM3A NLR function

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Figure Legends

Figure 1: Schematic depiction of the domain architecture of the PM3 proteins.

Amino acid polymorphisms occurring in the natural variants PM3A, PM3B, PM3C, PM3D, PM3E and PM3F, and the natural susceptible variant PM3CS are indicated (colored vertical lines; colors relate to the protein domains indicated in PM3) (Brunner et al., 2010). The PM3A polymorphic regions α , β , γ and δ are indicated by brackets. The red asterisk indicates the pair of polymorphic residues from PM3A that enhance PM3F activity (Stirnweis et al. 2014), and the red arrow indicates the location of the D502V mutation that causes constitutive autoactivity of PM3A.

Figure 2: Effects of LRR region exchanges in PM3A^{HR} and PM3A on HR intensity and protein abundance.

(a) High-Sensitivity Reading (HSR) image showing the change in HR intensity at 5 dpi as a result of LRR region exchanges in PM3A^{HR} when expressed in *N. benthamiana*. (b) Quantitative comparison of HR intensity induced by the LRR region exchange constructs of PM3A^{HR}, based on HSR imaging of 40 replicates from four independent experiments. (c) Abundance of HA-tagged PM3A^{HR} proteins and the LRR exchange constructs detected by anti-HA antibody in Western Blot, and corresponding Ponceau staining, representative of three independent experiments with similar results. The extracts were obtained from six identical-sized leaf disks of infiltrated *N. benthamiana* at 1 dpi. (d) HSR imaging showing the change in HR intensity at 5 dpi as a result of LRR region exchanges in PM3A when co-expressed with AVRPM3^{A2/F2} in *N. benthamiana*. (e) Quantitative comparison of HR intensity induced by the LRR region exchange constructs of PM3A, based on HSR imaging of 30 sample pairs from three independent experiments. (f) Abundance of HA-tagged PM3A proteins and the LRR exchange constructs, in the absence of AVRPM3^{A2/F2}, detected by anti-HA antibody in a Western Blot, and corresponding Ponceau staining. The extracts were obtained from six

identical-sized leaf disks of infiltrated *N. benthamiana* at 2 dpi. In the plots, the red dot represents the mean. Statistical significance was determined with the paired wilcox.exact function in R (n.s.: $p>0.05$, *: $p<0.05$, **: $p<0.01$).

Figure 3: Effects of single residue exchanges in the LRR domain on the PM3A immune response.

(a) Schematic depiction of the single residues exchanged between PM3A and PM3CS in the respective regions α , β , γ and δ and their functional consequences. Neutral residues are represented by black bars, residues enhancing the PM3A immune response are depicted in red, and response-reducing residues in blue. PM3A- $\gamma 9$, which has a small but significant enhancing effect, is shown in orange. HSR images, statistical analysis and protein expression are shown in Fig. S1, S2 and S3, respectively. The mean relative HR intensities for each mutant are shown in Table S1. (b, c) Quantitative comparison of the HR triggered by PM3A and PM3A- $\delta 5$, with reduced amounts of AVRPM3^{A2/F2}. Infiltrated *NLR:Avr* ratios range from (b) 1:4 to 1:1, and (c) 1:0.8 to 1:0.2. Data is based on epifluorescence imaging of at least 15 samples from two independent experiments. In the plots, the red dot represents the mean. Statistical significance was determined with the wilcox.exact function in R (n.s.: $p>0.05$, *: $p<0.05$, **: $p<0.01$). Representative HSR images can be found in Fig. S4.

Figure 4: Recognition of artificial and natural AVRPM3^{A2/F2} variants by the PM3A loss-of-function and high-sensitivity mutants.

(a) HSR imaging at 5 dpi of loss-of-function mutants of PM3A when coexpressed with highly abundant artificial AVRPM3^{A2/F2} variants in *N. benthamiana*. (b) HSR imaging at 5 dpi of the high-sensitivity PM3A mutants $\gamma 2$ and $\delta 5$ when co-expressed with the natural AVRPM3^{A2/F2} variants C and F in *N. benthamiana*. (c) Quantitative comparison of HR intensity for the combinations tested in Fig. 4b, based on HSR imaging of 14 paired samples from 2 independent

experiments. In the plots, the red dot represents the mean. Statistical significance was determined with the paired wilcox.exact function in R (n.s.: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$).

Figure 5: Functionality of the PM3A- $\delta 5$ mutant in stable transgenic wheat.

Infection test with isolates Bgt_96224 (AVRPM3^{A2/F2}-varA) or Bgt_C3-1 (AVRPM3^{A2/F2}-varC), respectively, on non-transgenic Bobwhite S26 (BW), transgenic BW-*Pm3a*, and three independent transgenic BW-*Pm3a*- $\delta 5$ lines (#4, #23B and #32). The leaf segments were 3 cm long. Additional infection tests are shown in Fig. S8.

Figure 6: Common properties of the amino acid changes altering PM3A function.

Bar plots are based on the data in Table S2, and detailed descriptions on each parameter is given there and in Supplementary Note 1. Bar plots show the distribution of the possible values of each parameter between the neutral and the functional mutations. (a) Solvent exposure of the residues in PM3A, based on the 3D-model. (b) Localization of the residue within the LxxLxLxx core motif. (c) Conservation of residue charge when polymorphic between PM3A and PM3CS. (d) Conservation of residue size when polymorphic between PM3A and PM3CS. (e) Localization of the solvent-exposed residues on the 3D-model of the PM3A LRR domain. NLR-response-enhancing residues are depicted in red, and response-reducing residues are depicted in yellow. The small-effect $\gamma 9$ residue is colored orange, and the neutral residues in blue. The LRR repeats that host the polymorphic residues are indicated by the dark grey color.

Figure 1: Schematic depiction of the domain architecture of the PM3 proteins.

Figure 2: Effects of LRR region exchanges in $PM3A^{HR}$ and $PM3A$ on HR intensity and protein abundance.

(a)

(b)

(c)

Figure 3: Effects of single residue exchanges in the LRR domain on the PM3A immune response.

Figure 4: Recognition of artificial and natural AVRPM3^{A2/F2} variants by the PM3A loss-of-function and high-sensitivity mutants.

Figure 5: Functionality of the PM3A- $\delta 5$ mutant in stable transgenic wheat.

Figure 6: Common properties of the amino acid changes altering PM3A function.

Supplementary material

Single residues in the LRR domain of the wheat PM3A immune receptor can control the strength and the spectrum of the immune response.

Stefan Lindner¹, Bettina Keller¹, Simrat Pal Singh¹, Zsuzsanna Hasenkamp², Esther Jung¹,
Marion C. Müller¹, Salim Bourras^{1,3*}, Beat Keller^{1*}

¹ Department of Plant and Microbial Biology, University of Zürich, Zürich, Switzerland

² Institute of Pharmacology and Toxicology, University of Zurich, Zürich, Switzerland

³ Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural
Sciences, Uppsala, Sweden

* Corresponding authors (bkeller@botinst.uzh.ch; salim.bourras@slu.se)

Figure S1: Effects of single residue exchanges in the LRR domain of PM3A on HR intensity. Representative HSR images of the respective PM3A single residue mutants co-expressed with AVRPM3^{A2/F2} in *N. benthamiana*. Leaves were scored at 5 dpi. Results were consistent over at least 2 independent assays of 21 to 72 replicates in total.

Figure S2: Quantitative comparison of HR intensity induced by the single residue exchange PM3A mutants, based on HSR imaging of at least 21 sample pairs from at least 2 independent experiments. In the plots, the red dot represents the mean. Statistical significance was determined with the paired wilcox.exact function in R (n.s.: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$).

Figure S3: Abundance of HA-tagged PM3A proteins and the functional single residue exchange constructs highlighted in Fig. 3a, detected by anti-HA antibody in Western Blot, and corresponding Ponceau staining. The extracts were obtained from 6 leaf disks of identical size harvested from agro-infiltrated *N. benthamiana* plants at 2 dpi. (a) Protein abundance of loss-of-function PM3A mutants. (b) Protein abundance of PM3A mutants with a stronger immune response.

Figure S4: High-Sensitivity Reading (HSR) images of *N. benthamiana* co-expressing PM3A or PM3A- $\delta 5$, respectively, with different amounts of AVRPM3^{A2/F2}. HR was scored at 5 dpi. Results were consistent over at least 2 independent assays of 17 replicates in total.

Figure S5: Quantitative comparison of HR intensity of the five tested stronger PM3A mutants when co-expressed with the natural AVRPM3^{A2/F2} variants C and F in *N. benthamiana*. HR was quantified at 5 dpi, based on HSR imaging of 6 paired samples each. In the plots, the red dot represents the mean.

Figure S6: Detection of AVRPM3^{A2/F2} and its artificial variants by an AVR-specific antibody. (a) Confirmation of the specificity of the rabbit polyclonal antibody against AVRPM3^{A2/F2} by Western Blotting, and the corresponding Ponceau staining. “+” and “-” indicate if the AVR has been infiltrated to the tobacco leaf tissue that was used for the respective extracts. The red arrow indicates the position of AVRPM3^{A2/F2} on the 4-20% gradient gel. (b) Abundance of artificial AVRPM3^{A2/F2} variants compared to natural AVRPM3^{A2/F2} detected by anti-AVRPM3^{A2/F2} antibody in Western Blot, and corresponding Ponceau staining. The extracts were taken from equal amount of leaf disks of identical size harvested from agro-infiltrated *N. benthamiana* plants at 2 dpi

Figure S7: Expression of PM3A proteins from transgenic wheat and *AvrPm3*^{a2/f2} and *SvrPm3* mRNA from powdery mildew. (a) Abundance of HA-tagged PM3A and PM3A-δ5 proteins extracted from transgenic wheat, detected by anti-HA antibody in Western Blot, and corresponding Ponceau staining. For each line, samples were taken from two individual plants, at the same day the leaves were subjected to the infection test. (b) Expression levels of *AvrPm3*^{a2/f2} and *SvrPm3*^{a1/f1} in isolates Bgt_96224 and Bgt_C3-1, based on RT-qPCR from samples taken 2 dpi. Isolate Bgt_C3-1 (*Avr* variant C, active *Svr*) was compared to isolate Bgt_96224 (*Avr* variant A, inactive *Svr*). The single data points were normalized with their respective mean values. In the graphs, the red dot represents the mean.

Figure S8: Infection of transgenic *Pm3a-δ5* wheat lines with mildew isolates Bgt_07004 and Bgt_Chikara. Here, mildew isolates Bgt_07004 (a) and Bgt_Chikara (b) were tested against the reference isolate Bgt_96224 on non-transgenic Bobwhite and on transgenic lines BW-*Pm3a*, BW-*Pm3a-δ5* #4, #23B and #32. The results shown are representative for two independent repetitions.

Figure S9: Depiction of the topological surfaces of the PM3A LRR model, as used in Fig 6e and Table S2; Green: inner arc, purple: upper surface, blue: outer arc, not visible: lower surface.

Table S1: PM3A mutants used in this study, and their effect on immune response

Name	Mutations	Relative HR	p-value
Pm3a	none	1.00	-
Pm3a-HR	D502V	1.00	-
Pm3a-α	I587L, R588del, R612S, M613I	0.34	3.00E-06
Pm3a-HR-α	D502V, I587L, R588del, R612S, M613I	1.00	1.95E-01
Pm3a-α1	I587L	1.01	8.15E-01
Pm3a-α2	R588del	0.73	7.92E-07
Pm3a-α3	R612S	1.11	7.90E-01
Pm3a-α4	M613I	1.18	2.10E-01
Pm3a-β	R917P, N922S, E946G, I955V	1.18	7.32E-02
Pm3a-HR-β	D502V, R917P, N922S, E946G, I955V	0.86	2.13E-04
Pm3a-β1	R917P	1.65	2.12E-03
Pm3a-β2	N922S	1.28	1.79E-01
Pm3a-β3	E946G	1.10	6.55E-01
Pm3a-β4	I955V	1.23	1.45E-01
Pm3a-γ	K1087N, R1097T, V1099L, E1104K, E1123Q, L1125P, R1132C, I1133L, E1134R, D1153T, L1155G, E1156G, D1158I	1.43	9.09E-05
Pm3a-HR-γ	D502V, K1087N, R1097T, V1099L, E1104K, E1123Q, L1125P, R1132C, I1133L, E1134R, D1153T, L1155G, E1156G, D1158I	0.91	1.05E-02
Pm3a-γ1	K1087N	1.08	2.66E-01
Pm3a-γ2	R1097T	1.81	2.50E-05
Pm3a-γ3	V1099L	0.92	4.26E-01
Pm3a-γ4	E1104K	1.08	9.79E-01
Pm3a-γ5	E1123Q	1.09	5.22E-01
Pm3a-γ6	L1125P	0.96	2.40E-01
Pm3a-γ7	R1132C	1.45	3.38E-03
Pm3a-γ8	I1133L	1.23	1.78E-01
Pm3a-γ9	E1134R	0.94	6.87E-03
Pm3a-γ10	D1153T	1.08	3.81E-01
Pm3a-γ11	L1155G	0.07	4.16E-05
Pm3a-γ12	E1156G	1.17	2.51E-01
Pm3a-γ13	D1158I	1.43	1.49E-02
Pm3a-δ	K1329E, I1330S, D1332W, R1334E, S1335R	0.06	2.71E-05
Pm3a-HR-δ	D502V, K1329E, I1330S, D1332W, R1334E, S1335R	0.85	1.12E-03
Pm3a-δ1	K1329E	0.14	3.93E-06
Pm3a-δ2	I1330S	0.08	3.89E-06
Pm3a-δ3	D1332W	0.08	3.79E-06
Pm3a-δ4	R1334E	0.07	1.76E-06
Pm3a-δ5	S1335R	1.81	3.79E-06

List of PM3A mutants generated in this work, and their effect on HR intensity. The notations indicate the residue change from PM3A to the corresponding residue of PM3CS in the respective construct or mutant. Relative HR values are the mean, based on the same data as Fig. S1 and S2. The p-value indicates significant difference towards the reference, based on the paired wilcox.exact function in R.

Table S2: Characterization of single-residue exchanges used in this study

Name	Mut	Effect	Ssignif	Region	Solv exp	Topology	LRR motif	Cons size	Cons chrg
Pm3a- α 1	I587L	1.01	0	1	1	1	1	0	0
Pm3a- α 2	R588del	0.73	3	1	1	1	0	2	2
Pm3a- α 3	R612S	1.11	0	1	1	1.5	0	2	1
Pm3a- α 4	M613I	1.18	0	1	0	0	0	0	0
Pm3a- β 1	R917P	1.65	3	3	1	3	0	1	2
Pm3a- β 2	N922S	1.28	0	3	0	0	0	1	0
Pm3a- β 3	E946G	1.10	0	3	0.5	2	0	2	2
Pm3a- β 4	I955V	1.23	0	3	0	0	0	1	0
Pm3a- γ 1	K1087N	1.08	0	4	1	3	0	1	1
Pm3a- γ 2	R1097T	1.81	3	4	1	1	1	1	1
Pm3a- γ 3	V1099L	0.92	0	4	0.5	1	1	1	0
Pm3a- γ 4	E1104K	1.08	0	4	1	2	0	0	2
Pm3a- γ 5	E1123Q	1.09	0	4	0.5	4	0	0	1
Pm3a- γ 6	L1125P	0.96	0	4	0.5	0	0	1	0
Pm3a- γ 7	R1132C	1.45	3	4	1	1	1	2	1
Pm3a- γ 8	I1133L	1.23	0	4	0	0	0	0	0
Pm3a- γ 9	E1134R	0.94	3	4	0.5	1	1	0	2
Pm3a- γ 10	D1153T	1.08	0	4	0.5	1	1	0	1
Pm3a- γ 11	L1155G	0.07	3	4	1	1	1	1	0
Pm3a- γ 12	E1156G	1.17	0	4	1	1.5	1	2	2
Pm3a- γ 13	D1158I	1.43	3	4	1	2	0	1	2
Pm3a- δ 1	K1329E	0.14	3	5	1	1	1	0	2
Pm3a- δ 2	I1330S	0.08	3	5	1	1	1	2	1
Pm3a- δ 3	D1332W	0.08	3	5	0.5	1	1	1	1
Pm3a- δ 4	R1334E	0.07	3	5	1	1.5	1	0	2
Pm3a- δ 5	S1335R	1.81	3	5	1	1.5	1	2	1

“effect” shows the mean HR intensity, based on the same data as Fig. S1 and S2. “signif” indicates the significance of the “effect” value, with 0: $p > 0.1$, 1: $p < 0.1$, 2: $p < 0.05$ and 3: $p < 0.01$. “region” indicates where in the primary sequence of the LRR domain the mutation is located, with 1: 516-695, 2: 696-875, 3: 876-1055, 4: 1056-1235 and 5: 1236-1415. “solv exp” indicates if the residue is solvent exposed in the 3D model of the PM3A LRR domain, with 0: not exposed, 0.5: partially and 1: prominently exposed. “topology” indicates where on the surface the residue is positioned, with 0: not exposed to the surface, 1: inner arc, 2: upper surface, 3: outer arc and 4: lower surface; x.5 meaning roughly between two surfaces; see Fig. S8 for visualization of the surfaces. “LRR motif” indicates if the residue is located within the core

LxxLxLxx motif of the repeat, with 0: no and 1: yes. “consSIZE” indicates how conservative the mutation is regarding the size of the residue, based on the Taylor classification (Betts and Russel, 2003), with the number indicating the steps on the scale of tiny – small – large; note that bigger values mean low conservation. Similarly, “consCHRG” indicates how conservative the mutation is regarding charge, with the scales being either pos. charged – polar – neg. charged, or charged – polar – uncharged.

Table S3: Primers used in this study

Primers for Gibson-Assembly fragments for Pm3a-α, -β, -γ, -δ region exchange		
Pm3- α	AAGATCCCTGCTATTCAAACA	AGCAATGGTGCTTCAGGTAATGCTA
Pm3- β	ACAGAGCTTCAAGGCGTGCA	CTCTTGCTTGCCATCTTC
Pm3- γ	AGATGGCAAGCAAGAGGTGT	GATAGGACTTCTGCTTCTGGAGGTAG
Pm3- δ	ACCTCCAGAAGCAGAAGTCCTATC	TTTGATGCTGCCCAGTTG
Pm3 backbone	AACTGGGCAGCATCAAACG	ACAGAGCTTCAAGGCGTGCA
Primers for site-directed mutagenesis of the Pm3a-LRR domain		
Pm3a- α 1	GGCACAGAATCATTTCTACTCA	TCTCAGACAGAGCTTCAAGGC
Pm3a- α 2	GGCACAGAATCATTTCTACTCA	AATACAGAGCTTCAAGGCGTG
Pm3a- α 3	AAAGCACTTCCTGAGGATATAAGTAT	CATAGAACTTTCTGAGAGATCAAGGT
Pm3a- α 4	GCACTTCCTGAGGATATAAGTATTCT	TTTGATACGACTTCTGAGAGATCAA
Pm3a- β 1	CATTGGTTCATGAAAATTGTAG	GTGCTTCACGCAATGG
Pm3a- β 2	CATGAAAGTTGTAGTGGAGGT	AACCAACCTTGCTTCACG
Pm3a- β 3	GACTTGGGGAGTTTTCAGA	TTCCAATGCAAGTACCTTTAG
Pm3a- β 4	GCAGCTGTCGAAGGAGAA	ATCCCATTTCTGAAAACCTC
Pm3a- γ 1	CCAGAGAATGTGTTCCAAA	CCAGTGGACGAGCACA
Pm3a- γ 2	TCGTTGAGGACATTAGTGATT	TACCAAGCTTTGGAACACTT
Pm3a- γ 3	AGATTACTGATTAGAACTGCGA	CCTCAACGATACCAAGCTT
Pm3a- γ 4	AGAAACTGCAAAAATCTGACT	AATCACTAATCTCCTCAACGATA
Pm3a- γ 5	GAAAGGAGTCAGCACCTG	GGATGCCAATGGCTC
Pm3a- γ 6	GAACACCCGAGAGGTCT	ACTCCTTTCGGATGCC
Pm3a- γ 7	GAGTCTCTTTGCATAGAAAACCTG	CAGACCTCTCAGGTGTTCA
Pm3a- γ 8	TCTCTTCGCTTAGAAAACCTGC	CTCCAGACCTCTCAGGTGT
Pm3a- γ 9	CTTCGCATAAGAAACCTGCC	AGACTCCAGACCTCTCAGGT
Pm3a- γ 10	GAAAATGACTATTCTTGAGTGCG	TTGAGAGATGCCGGG
Pm3a- γ 11	AATGGATATTGGTGAGTGCG	TTCTTGAGAGATGCCGG
Pm3a- γ 12	GATATTCTTGCGTGCGATAAG	CATTTTCTTGAGAGATGCCG
Pm3a- γ 13	CTTGAGTGCAATTAAGCTTGA	AATATCCATTTTCTTGAGAGATG
Pm3a- δ 1	TAGAAGCTGCAGTACCCTGGC	AGATCAAGGATTTCCAGCGATG
Pm3a- δ 2	TGCAGTACCCTGGCATCC	GCTTCTAAGATCAAGGATTTCAAG
Pm3a- δ 3	TACCCTGGCATCCCTGCC	CTGCAGCTTCTAAGCCAAAGGATTT
Pm3a- δ 4	ACCCTGGCATCCCTGC	ACTGCAGCTTTCAAGATCAAGG
Pm3a- δ 5	CTGGCATCCCTGCCGA	GGTACTGCATCTTCTAAGATCAAGGATTTT
Primers for colony PCR of Pm3a and related genes in the respective vectors		
in pENTR	TCAAGAGACTGTTCAATTATGG	TTTAATCGCGGCTCGAC
in pIPKb004	TCAAGAGACTGTTCAATTATGG	CAAGACCGGCAACAGGATTC
Primers for Gibson-Assembly for PM3A-δ5-HA into pAHC17 for stable wheat transformation		
Pm3a- δ 5-HA	GGTCGACTCTATGGCAGAGCGGGTGG	CGGATCCTCTTCAAGCATAATCTGGAACATCGTAT
pAHC17 backbone	TTATGCTTGAAGAGGATCCGTCGACCT	GCTCTGCCATAGAGTCGACCTGCAGAAGTAAC
Primers for RT-qPCR		
GAPDH	TATAGCCGCATTGTCTTCC	CCGTCTCGACTGCTTGAT
AvrPm3a2/f2	GCACTTCACCTAACGGACTAAGGAAAGG	GCAATACGCTCTGGACATAAGCCTGTAG
SvrPm3	TTCTTGAGTAGTTTTCTACCA	TGTAAGCCGTTGGGTAAAG

Primer sequences used to generate the region and single residue exchanges in this study, and for colony PCR in the two different vectors. The primer sequences to generate the region exchange fragments from Pm3a, Pm3CS and Pm3a^{HR} are identical.

Supplementary Note 1 – Characteristics of critical residues

The parameters used to characterize the properties of the critical amino-acid changes were selected and scored as follows: (a) solvent exposure (based on the 3D-model of the LRR domain of PM3A, published by Sela et al. (2014); exposed, partially exposed, or not exposed), (b) position on the 3D-model of the LRR domain (not exposed, inner arc, upper surface, outer arc, lower surface; see Fig. S7), (c) location within the LxxLxLxx repeat motif (yes or no), how much the exchange affected (d) size and (e) charge of the residue (here named conservatism; high, medium or low, as defined in the Table S2 legend), and (f) the position on the primary sequence of the LRR domain (C-terminal 900 amino acids of the protein, divided into five regions of 180 residues each). The results are summarized in Table S2. All of the functional mutations are either partially (17%) or prominently (83%) exposed, while the neutral mutations do not show a particular preference, and 29% of them are not exposed to the surface (Fig. 6a). 67% of the functional mutations are located on the inner arc of the horseshoe-shape model of the LRR domain, with additional 17% close to that surface (Fig. 6e). In comparison (with the unexposed residues not included), the neutral mutations again do not seem to show a preference here, with 30% being located on the inner arc, 20% on the upper surface, 20% in between the latter two, and another 20% on the outer arc and lower surface (Fig. 6e). When it comes to the repeat motif, 75% of the functional mutations are located within the LxxLxLxx motif, compared to only 29% of the neutral ones (Fig. 6b). Regarding residue conservatism, the neutral mutations are generally more conservative than the functional mutations: In size, the neutral mutations are mostly highly (43%) or medium (36%) conserved, compared to the functional mutations, which are mainly lowly (42%) or medium (42%) conserved (Fig. 6d). Similarly, regarding charge, the neutral mutations are mostly highly (50%) or medium (29%) conserved, while the functional mutations are mainly lowly (50%) or medium (42%) conserved (Fig. 6c). Finally, while the majority of all introduced exchanges is generally more in the C-terminal half of the LRR domain, the functional mutations are located more towards the C-terminus (42% in region

4 and 42% in region 5) compared to the neutral mutations (21% in region 3, 57% in region 4, and none in region 5) (Fig. 6e).