Swapping nematode and virus resistance in potato

Sequence exchange between homologous NB-LRR genes converts virus resistance into nematode resistance, and *vice versa*.

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One sentence summary

Domain swapping between the NB-LRR immune receptors Rx1 and Gpa2 converts extreme resistance to potato virus X in the shoots into potato cyst nematode resistance in the roots, and *vice versa*. 
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Plants have evolved a limited repertoire of NB-LRR disease resistance (R) genes to protect themselves against a myriad of pathogens. This limitation is thought to be counterbalanced by the rapid evolution of NB-LRR proteins, as only a few sequence changes have been shown to be sufficient to alter resistance specificities towards novel strains of a pathogen. However, little is known about the flexibility of NB-LRR R genes to switch resistance specificities between phylogenetically unrelated pathogens. To investigate this, we created domain swaps between the close homologs Gpa2 and Rx1, which confer resistance in potato to the cyst nematode Globodera pallida and Potato virus X (PVX), respectively. The genetic fusion of the CC-NB-ARC of Gpa2 with the LRR of Rx1 (Gpa2\text{CN}/Rx1\text{L}) results in autoactivity, but lowering the protein levels restored its specific activation response including extreme resistance to PVX in potato shoots. The reciprocal chimera (Rx1\text{CN}/Gpa2\text{L}) shows a loss-of-function phenotype, but exchange of the first 3 LRRs of Gpa2 by the corresponding region of Rx1 was sufficient to regain a wild type resistance response to G. pallida in the roots. These data demonstrate that exchanging the recognition moiety in the LRR is sufficient to convert extreme virus resistance in the leaves into mild nematode resistance in the roots, and \textit{vice versa}. In addition, we show that the CC-NB-ARC can operate independently of the recognition specificities defined by the LRR domain, either above or belowground. These data show the versatility of NB-LRR genes to generate resistances to unrelated pathogens with completely different lifestyles and routes of invasion.
INTRODUCTION

Plants are constantly exposed to a diverse array of pathogens and parasites that attempt to invade leaves, stems, or roots by various mechanisms. To sense foreign invaders, plants have evolved a cell autonomous multilayered immune system consisting of extra- and intracellular immune receptors (Jones and Dangl, 2006; Cook et al., 2015). Immune receptors encoded by so called dominant resistance \((R)\) genes operate in a gene-for-gene specific manner, in which the \(R\) protein limits the growth of viruses, bacteria, oomycete, fungi or invertebrate pests by triggering a host specific defense response upon recognition of pathogen-derived elicitors. This recognition may involve a direct interaction between the \(R\) protein and its cognate elicitor, or an indirect interaction by sensing elicitor-dependent modifications of host proteins (Asai and Shirashu 2015). The subsequent host defense response may include the production of anti-microbial compounds, the induction of a reactive oxygen burst, and a local programmed cell death, also called hypersensitive response (HR) (Feechan et al., 2015).

Most known \(R\) genes encode intracellular NB-LRR immune receptors consisting of a nucleotide-binding site (NB), a domain homologous to Apaf1 and CED4 (ARC) and a leucine-rich repeat domain (LRR) responsible for the direct or indirect recognition of the pathogen. Within the NB-LRR class of disease resistance genes two large families are distinguished: CC-NB-LRR proteins that have a N-terminal coiled-coil (CC) domain and TIR-NB-LRR proteins with a N-terminal Toll/ Interleukin-1 receptor (TIR) domain. These multidomain proteins function as a molecular switch and their activity is tightly controlled by inter- and intradomain interactions. The protein is kept in an autoinhibited “off” state and switches “on” upon recognition of its cognate pathogen elicitor by a multistep activation process (reviewed by Takken and Tameling 2009; Takken and Goverse 2012; Sukarta et al
Plant resistance genes of the NB-LRR class are highly polymorphic and are among the most rapidly evolving genes in the genome of plants (Mondragon-Palomino, Meyers et al. 2002; Cork and Purugganan 2005; Meyers, Kaushik et al. 2005; Ballvora et al., 2007). Of all presently known NB-LRR sequences, e.g. 149 in the Arabidopsis genome (Meyers et al. 2003), about 400 in poplar (Kohler et al. 2008), over 500 in rice (Zhou et al. 2004), and more than 700 in diploid potato (Jupe et al., 2012), only a few have been linked to resistance to a specific pathogen. Even in clusters containing R genes of known specificities, the functions of adjacent paralogs are often unknown (Bakker et al., 2011).

Although mutations are a major source of variation, much of the diversity within resistance gene families arises from intra- and intergenic sequence exchanges that shuffle polymorphic sites between individual genes. While several simple R gene loci do exist, most R genes belong to gene families located at complex loci harbouring several tandemly repeated NB-LRR gene homologs (Michelmore and Meyers 1998; Gebhardt and Valkonen, 2001). The occurrence of R gene homologs in clusters is thought to promote sequence exchange by gene conversion and unequal crossing-over. For example, a detailed study of the Rp1 rust resistance complex of maize showed that reshuffling of sequences played a central role in the creation of genetic diversity and even led to new specificities (Hulbert 1997; Sun, Collins et al. 2001; Smith and Hulbert 2005).

Various clusters of highly similar R genes are found that recognize series of variants of a single pathogen species, which are the result of a co-evolutionary arms race between host and pathogen (Hayes, Jeong et al. 2004). However, the mechanisms underlying the ability of NB-LRR R genes to switch resistance specificities between taxonomically unrelated pathogens are largely unknown. An indication that altering resistance specificities towards widely different
pathogens involves relatively few molecular changes, comes from the observation that for example resistance genes for downy mildew (RPP8) and for Turnip Crinkle virus (HRT) in Arabidopsis are highly homologous and are found at the same genomic position in different accessions (Cooley, Pathirana et al. 2000).

Gpa2 and Rx1 are two highly similar CC-NB-LRR genes located in the same R gene cluster of potato (Solanum tuberosum ssp. andigena), but confer resistance to two completely different types of pathogens, the potato cyst nematode Globodera pallida and Potato virus X (PVX), respectively (Bendahmane, Kanyuka et al. 1999; van der Vossen, van der Voort et al. 2000). Potato cyst nematodes penetrate the roots and fuse plant cells into multinucleate feeding cells, on which they depend for development and reproduction. In Gpa2 resistant plants, the nematode-induced feeding cells degenerate in a late stage of infection and finally block the development of fertile adult females. PVX is a single stranded RNA virus that is transmitted above ground by insects and by mechanical injury, resulting in systemic infection of the aerial parts of the plant. A striking feature of Rx1-mediated resistance is the rapid arrest of PVX accumulation in the initial infected cells, resulting in symptomless resistance, so-called extreme resistance (Kohm, Goulden et al. 1993; Bendahmane, Kanyuka et al. 1999). However, recognition of the PVX coat protein (CP) by the LRR domain of Rx1 activates a specific cell death response in agroinfiltration assays on leaves of Nicotiana benthamiana (Bendahmane, Kanyuka et al. 1999). Similarly, co-expression of the nematode effector RBP-1 with Gpa2 results in the induction of a specific cell death response (Sacco et al., 2009). Thus, Gpa2 and Rx1 provide an excellent test system to investigate the flexibility of NB-LRR genes in the plant genome to evolve novel functional R genes, which recognize two distinct elicitors and confer resistance to two taxonomically unrelated pathogens with different life strategies.

Previously, Rx1 and Gpa2 have been used as model system to investigate the role of intra-
and interdomain interactions in the functioning of CC-NB-LRR proteins as molecular switch in plant immunity (refs). Sequence exchange between different domains of Rx1 and Gpa2 showed that genetic fusions of the CC-NB-ARC of Rx1 with the LRR of Gpa2 (referred to as Rx1_{CN}/Gpa2_{L} in our study) and the reciprocal domain swap (referred to as Gpa2_{CN}/Rx1_{L} in our study) are not functional despite their high sequence homology. Whereas the chimera Gpa2_{CN}/Rx1_{L} results in a constitutive cell death response, a loss-of-function phenotype was observed for Rx1_{CN}/Gpa2_{L} (Rairdan and Moffett, 2006). Exchanging additional polymorphic regions between Rx1 and Gpa2 followed by targeted mutagenesis of specific residues revealed that the N-terminal end of the LRR domain cooperates with the NB-ARC domain to control the molecular switch function of the R protein (Slootweg et al., 2013). So, sequence exchange between homologous R genes like Rx1 and Gpa2 may result in aberrant CC-NB-LRR protein functioning both \emph{in vitro} and \emph{in vivo} due to the incompatibility between domains.

Here, we used this knowledge to provide experimental support for the hypothesis that NB-LRR proteins are composed of flexible modules that allow plants to generate novel functional R proteins against distinct pathogens by sequence exchange between homologous genes at R gene loci. Exchanging the CC-NB-ARC domains of the paralogs Gpa2 and Rx1 showed that activation of a resistance response is independent of the recognition specificity module of resistance proteins. Using the non-functional chimera Rx1_{CN}/Gpa2_{L} as a template, an elicitor-dependent cell death response was regained upon agroinfiltration of \emph{N. benthamiana} leaves by introducing different N-terminal segments of the LRR domain of Rx1 in the Rx1_{CN}/Gpa2_{L} background, probably by restoring the compatibility between the NB-ARC and LRR domain. Moreover, these data reveal that the recognition specificity of Gpa2 is determined by the C-terminal end of the LRR domain as observed for Rx1 (Farnham and Baulcombe et al 2006). To test the functionality of the chimera’s in disease resistance, transgenic potato plants were
generated. Indeed, a resistance response comparable to Gpa2-mediated resistance was observed against the potato cyst nematode G. pallida in plants harboring a chimera in which the first three N-terminal LRR repeats were introduced in the Rx1<sub>CN</sub>/Gpa2<sub>L</sub> background. These data support a model in which novel R genes can be created by sequence exchange of the C-terminal LRR sensor domain while keeping the molecular switch function intact.

For the reciprocal chimera Gpa2<sub>CN</sub>/Rx1<sub>L</sub>, we could demonstrate that artificially reducing the protein expression levels - by modulating the translational efficiency - results in the loss of autoactivity and the regain of an elicitor-dependent Rx1 wild type response in leaves of N. benthamiana. Moreover, also extreme resistance against PVX was restored in transgenic potato plants, which was indistinguishable from wild type Rx1 plants. Together, these findings show that not only the amino acid sequence and the protein structure, but also the expression levels of NB-LRR proteins, can play a role in the evolution of novel R genes in plants. In addition, the conversion of nematode resistance into virus resistance and vice versa demonstrates the potential to engineer novel functional R genes against distinct pathogens in major food crops like potato.
RESULTS

The CC-NB-ARC domain of Gpa2 is able to activate extreme resistance to PVX

To test the versatility of the various domains of Gpa2 and Rx1 in triggering defense responses to PVX and potato cyst nematodes, a chimera encoding the CC-NB-ARC domain of Gpa2 and the LRR domain of Rx1 was used (Gpa2_{CN}/Rx1_{L}, Fig. 1A). Under control of the strong double enhanced CaMV 35S promoter, the Gpa2_{CN}/Rx1_{L} domain fusion results in a constitutive and elicitor independent cell death response (Rairdan and Moffett 2006; Slootweg et al., 2013). To investigate whether overexpression of this recombinant R protein contributed to its autoactivity, the 35S promoter and Tnos terminator sequences were replaced by the endogenous promoter and terminator regions of Rx1. However, under the control of the Rx1 promoter the chimera Gpa2_{CN}/Rx1_{L} still induced a constitutive cell death response in leaves of N. benthamiana, whereas a specific response was observed for wild type Rx1 with both the 35S and Rx1 promoter (Fig. 1B). We concluded that the expression level of this chimera Gpa2_{CN}/Rx1_{L} was still above the threshold for autoactivity. This probably explains why we were unable to generate stable transgenic potato plants for this construct for further testing (data not shown).

To test whether reduced R protein levels would abolish autoactivity of the Gpa2_{CN}/Rx1_{L} fusion, we introduced an out of frame start codon directly upstream of the original translation initiation site (Fig. 2A). The translation efficiency of the R gene reading frame thus became dependent on the leaky scanning (LS) by the ribosome (Kozak 1995; Kozak 1999). The leaky scanning (35S_{LS}) promoter results in a substantial reduction in Rx1
protein levels *in planta* (Slootweg et al., 2010). Expression of the recombinant protein Gpa2<sub>CN</sub>/Rx1<sub>L</sub> under control of the 35S<sub>LS</sub> promoter no longer triggered an autoactive hypersensitive response compared to the use of the 35S promoter in an agroinfiltration assay (Fig. 2B). Interestingly, co-expression of the elicitor of Rx1, the PVX coat protein CP106, and the 35S<sub>LS</sub>:Gpa2<sub>CN</sub>/Rx1<sub>L</sub> construct induced a specific HR, which was determined by visual scoring of cell death on the leaves of *N. benthamiana* and the quantification of cell death by measuring the chlorophyll contents of cells after Harris et al., 2013 (Fig. 2B and 2C). This response was absent when the construct was co-infiltrated with either the virulent PVX coat protein CP105 or the GFP control. We therefore conclude that the chimeric Gpa2<sub>CN</sub>/Rx1<sub>L</sub> protein can still specifically recognize the PVX elicitor by the Rx1 LRR domain and activate a cell death response through the CC-NB-ARC domains of Gpa2. Furthermore, our results demonstrate that elicitor-dependent activity and autoactivity can be uncoupled by attenuating the protein levels of recombinant R proteins like Gpa2<sub>CN</sub>/Rx1<sub>L</sub>, resulting in a functional NB-LRR immune receptor.

The lack of autoactivity allowed us to generate transgenic potato plants harboring the 35S<sub>LS</sub>::Gpa2<sub>CN</sub>/Rx1<sub>L</sub> construct and to test them for PVX resistance. Plants were inoculated with either the avirulent strain PVX<sub>UK3</sub> or the virulent strain PVX<sub>HB</sub> and three weeks after inoculation the compound leaves near the shoot apex were harvested for virus detection using ELISA. No detectable amounts of PVX<sub>UK3</sub> could be observed in the transgenic plants harboring 35S<sub>LS</sub>::Gpa2<sub>CN</sub>/Rx1<sub>L</sub> (Fig. 2D). Similar results were obtained for the potato clone SH containing the endogenous *Rx1* gene and transgenic lines containing the *Rx1* gene under control of the 35S promoter, which were included as resistant controls. In transgenic plants harboring the empty vector high levels of PVX<sub>UK3</sub> could be detected, indicating that the inoculation with the avirulent strain was successful. Infection of the plants with the *Rx1*-
resistance breaking strain resulted in systemic spreading of the virus in all plants irrespective of the genotype, although reduced levels were observed in the plants expressing \textit{Rx1} from the 35S promoter. These data support earlier findings that the recognition specificity of \textit{Rx1} is determined by the LRR domain (Rairdan and Moffett, 2006), but more interestingly, that the CC-NB-ARC domain of the nematode resistance protein Gpa2 is able to activate an extreme resistance response against potato virus X in the aboveground parts of the plant.

The CC-NB-ARC domain of \textit{Rx1} is able to activate nematode resistance to \textit{G. pallida}

To test whether the CC-NB-ARC domain of \textit{Rx1} is also able to activate nematode resistance to the potato cyst nematode \textit{G. pallida}, a reciprocal protein was used consisting of the CC-NB-ARC domains of \textit{Rx1} and the LRR domain of Gpa2 (Rx1\textsubscript{CN}/Gpa2\textsubscript{L}; Suppl. Fig. S1A). Members of the RBP-1 effector family of \textit{G. pallida} were shown to induce an HR in an agroinfiltration assay when co-expressed with Gpa2 on leaves of \textit{N. benthamiana} (Sacco, Koropacka et al. 2009). Therefore, we used RBP-1 effectors to test the functionality of the chimera Rx1\textsubscript{CN}/Gpa2\textsubscript{L} first in transient expression assays under control of both the 35S and the endogenous promoter. However, co-expression of Rx1\textsubscript{CN}/Gpa2\textsubscript{L} and the eliciting RBP-1 variant Rook6 did not trigger a clear visible HR, neither when expressed by its native nor the CaMV 35S promoter (Suppl. Data and Suppl. Fig. S1B) despite proper expression of the chimera (Suppl. Fig. S1 C and D). In addition, transgenic potato plants expressing Rx1\textsubscript{CN}/Gpa2\textsubscript{L} did not provide resistance to nematodes irrespective of the promoter used (Suppl. Data and Suppl. Fig. S1E & Fig. S2), consistent with a loss-of-function phenotype as reported (Slootweg et al., 2013).
Previously, it was shown that an autoactive construct similar to $Gpa2_{CN}/Rx1_L$ acquired the regulated functionality of Rx1 by including the N-terminal end of the LRR domain of Gpa2 (Rairdan and Moffett 2006). Because intramolecular interactions between the CC-NB-ARC domain and the N-terminal part of the LRR domain control the R protein’s activity (Slootweg et al., 2013), we reasoned that the replacement of the N-terminal region of the LRR domain of Rx1 might also be required to restore the functionality of the reverse domain swap construct $Rx1_{CN}/Gpa2_L$. Therefore, a set of additional constructs was designed in which parts of the LRR domains of Rx1 and Gpa2 were exchanged (Fig. 3A). As expected, no clear cell death response was observed for $Rx1_{CN}/Gpa2_L$ (hereafter referred to as R13G4G5 based on the nomenclature used for domain swap constructs by Slootweg et al., 2013) when expressed under control of the 35S promoter, whereas a normal cell death response was observed for the wild type Gpa2 protein after co-expression with the activating RBP-1 variant Rook6 in an agroinfiltration assay in *N. benthamiana* leaves (Fig. 3B). Interestingly, this cell death response was partially restored by the replacement of the first three N-terminal repeats of the LRR domain of Rx1 (r) by the corresponding regions of Gpa2 (g) in the construct R13rggG5 (Fig. 3B). A similar mild cell death response was observed for the LRR subdomain swap constructs R13rrgG5 and R13grgG5 (Fig. 3B), in which other LRR segments were exchanged between Rx1 and Gpa2. No cell death response was observed in the presence of the non-activating RBP-1 variant Rook4, which shows that the cell death response obtained by these constructs is the result of specific recognition of the nematode effector RBP-1 and not the activation of an autoimmune response. These data support the notion that different N-terminal repeats of the LRR domain of Rx1 contribute to the controlled activation of the CC-NB-ARC domains of Rx1 by restoring the compatibility between these domains (Slootweg et al., 2013).

To investigate the role of the C-terminal LRR region of Gpa2 in RBP-1-induced
activation, two additional domain swap constructs were used in this study. A complete loss of function was observed for the domain swap construct R13ggrG5 compared to the residual activation response observed for R13G45 in the presence of the RBP-1 effector (Fig. 3B), which suggests that the repeats in the C-terminal end of the Gpa2 LRR domain (G4) are involved in RBP-1 recognition. A similar complete loss of function was observed for R13gggR5, which results in the genetic fusion of the acidic tail of Rx1 (R5) to the LRR domain of Gpa2 (G4). Due to a premature stop codon in the corresponding sequence this acidic tail is not present in the wild type Gpa2 protein (Bendahmane et al., 1999; van der Vossen et al., 2000). So apparently, fusion of the Rx1 acidic tail at the distal end of the Gpa2 LRR also compromises the recognition of the RBP-1 effector. Chlorophyll measurements were used to quantify the specific cell death responses (Fig. 3C), supporting the observed differences in the activity of the domain swap constructs. In addition, Western blot analysis revealed that the chimeric proteins with a loss-of-function phenotype were properly expressed in planta (Fig. 3D). From these data, it seems that the C-terminal region of Gpa2 is indispensable for RBP-1 recognition and the induction of effector-triggered cell death.

To further investigate whether the controlled activity of the chimeric R proteins in cell death assays also translates into nematode resistance, transgenic potato lines were generated for the chimeras R13grgG5, R13ggrG5, R13G4R5 and R13rggG5 in a susceptible potato background. Independent transgenic potato lines expressing the constructs were selected by PCR and challenged with the avirulent G. pallida population D383. Remarkably, all four R13rggG5 lines revealed similar levels of nematode resistance as the transgenic line carrying the Gpa2 gene and the Gpa2 resistant potato clone SH, whereas the empty vector (EV) control plants were fully susceptible as expected (Fig. 3E). The chimera’s R13ggrG5 and R13G4R5 showed also a fully susceptible phenotype, whereas R13G45 and R13rggG5 result
in an intermediate susceptible phenotype. This seems consistent with the response of these chimera’s in the cell death assays shown in Fig. 3B and C. These data demonstrate that the compatibility between the N-terminal repeats of the LRR domain of Rx1 with the CC-NB-ARC domain determines the functionality of the chimera R13rggG5, both when transiently expressed in leaves of *N. benthamiana* as well as in transgenic potato plants. Furthermore, we conclude that the C-terminal end of the LRR region is indispensable for nematode-induced *Gpa2* resistance.

Both the endogenous promoters of *Rx1* and *Gpa2* are interchangeable for driving virus and nematode resistance

We extended our analysis to the flanking regions of *Rx1* and *Gpa2* involved in the transcriptional regulation of the genes, to investigate the flexibility of *R* gene loci to regulate proper expression of novel *R* genes during infection of plants by pathogens with completely different lifestyles. The 2.6 kb DNA upstream of the start codon revealed two extra TA-rich regions in the *Gpa2* sequence compared to *Rx1* at -2458bp (TA<sub>7</sub>) and at -1329 (TA<sub>15</sub>), which are predicted to function as enhancers, and two small indels (T<sub>5</sub> and T<sub>2</sub>) just upstream of the start codons at position -207 bp and -6 bp, respectively. Various single base pair substitutions are distributed over the promoter region, resulting in the prediction of several additional *cis* acting regulatory elements for either the *Rx1* or *Gpa2* promoter. For example, an AT-rich element (binding site for AT rich DNA binding protein ATBP-1), an AT-rich sequence (for maximal elicitor-mediated activation), and ethylene (ERE), auxin (TGA) and wound (WUN) responsive elements were detected (*Suppl. Fig. S3 & Suppl. Table S2*). Analysing the genomic sequence +298 bp downstream of the stop codon revealed that the 3’UTR regions of
Rx1 and Gpa2 (Vossen et al., 2000) were identical until position +160 followed by a more variable region containing 8 single base pair substitutions and two small indels of 1 and 2 nucleotides in case of Gpa2 and one indel of 3 nucleotides for Rx1. These results show that the regulatory sequences of Rx1 and Gpa2 are highly homologous to each other, suggesting potential overlap in the expression regulation involved in Gpa2 and Rx1 activity.

To test whether the highly similar (~97%) regulatory sequences of Rx1 and Gpa2 are also exchangeable, Rx1 expression was placed under control of the Gpa2 promoter (pGPAII) and terminator region (T_{GPA2}) and *vice versa*, Gpa2 expression was driven by the Rx1 promoter (pRXI) and terminator (T_{Rx1}) sequences. Both constructs, pGPAII::Rx1::T_{Gpa2} and pRXI::Gpa2::T_{Rx1}, triggered an HR in the presence of CP106 and RBP-1 variant Rook6, respectively. No cell death response was observed either in the presence of CP105 and non-activating RBP-1 variant Rook4 nor the controls YFP and GUS, showing the specificity of the response. The responses conditioned by these constructs were indistinguishable from the responses obtained with the genes using their own native promoters (Fig. 4A). Next, we investigated whether pGPAII::Rx1::T_{Gpa2} and pRXI::Gpa2::T_{Rx1} were able to confer disease resistance. Transgenic potato plants harbouring pGPAII::Rx1::T_{Gpa2} showed no systemic spreading of the avirulent strain PVX_{UK3}, whereas an accumulation of the virulent strain PVX_{HB} was observed with ELISA (Fig. 4B). Similarly, transgenic potato plants harbouring pRXI::Gpa2::T_{Rx1} showed resistance to the avirulent *G. pallida* population D383, while the plants were fully susceptible to the Gpa2 resistance-breaking population Rookmaker (Fig. 4C). These results demonstrate that the Gpa2 and Rx1 promoter are exchangeable despite the presence of small polymorphisms, and that they are able to drive disease resistance in both roots and shoots to two unrelated pathogens with completely different lifestyles.
Here, we provide experimental evidence for the hypothesis that intragenic sequence exchange at complex $R$ gene loci can result in the generation of novel $R$ genes with resistance specificities to entirely different attackers. Exchanging the recognition moieties in the LRR regions of the two closely related $R$ genes $Gpa2$ and $Rx1$ showed that the corresponding CC-NB-ARC domains could both mediate extreme virus resistance in the shoots and a mild nematode resistance response in the roots of potato. To our knowledge, this is the first example of the formation of functional bidirectional chimeric $R$ proteins between two members of a single $R$ gene cluster that confer resistance to two completely unrelated pathogens with entirely different modes of parasitism and routes of invasion. Our results demonstrate that complex $R$ gene loci provide a genetic framework in which intragenic sequence exchange between homologous genes is allowed. However, it also point to functional constraints that act on the generation of effective novel $R$ genes, as sequence exchange often results in gain or loss of function phenotypes due to incompatibility between functional domains involved in regulating the molecular switch function (Rairdan and Moffett, 2006; Qi et al., 2012; Takken and Goverse 2012; Slootweg et al., 2013, Wang et al., 2015). In addition, we show that the resistance specificity can be retained by reducing the expression levels of autoactive recombinant proteins, resulting in a normal functional $R$ protein. Potentially, this provides the plant with an additional regulatory mechanism to maintain or generate novel $R$ gene specificities during evolution, which would otherwise be lost due to negative selection by an autonomous cell death response.

Resistance assays on transgenic potato plants showed that the endogenous promoters of $Gpa2$ and $Rx1$ are interchangeable and that the resistance phenotypes were indistinguishable
from their wild types. Thus, the flexibility of complex R gene loci extend beyond the coding regions. This implies that the upstream regulatory sequences of both genes allow for proper expression in root and shoot tissues, and pose no limitation to the formation of new recognition specificities against pathogens with diverse lifestyles and routes of invasion. Exchangeability of regulatory sequences of R gene homologs in one cluster as shown in this study provides additional versatility to adopt quickly to a wide range of pathogens irrespective their lifestyle. This could also explain why homologous R genes located in synthenic regions of a genome confer resistance to distinct pathogens in different plant species (Gebhardt & Valkonen, 2009; Bakker et al. 2011). For example, R3a and I2 which are located at a complex R gene locus in a colinear region on chromosome 11 in potato and tomato, confer resistance to the oomycete Phytophtora infestans and the soilborn fungal pathogen Fusarium oxysporum, respectively (Huang et al., 2005). Interestingly, I2 is also able to show a weak recognition response to the cognate R3a elicitor Avr3a, which could be enhanced by a single mutation in the CC domain and even result in partial late blight resistance (Segretin et al., 2014; Giannakopoulou et al., 2015). Apparently, the R3a and I2 recognition specificity has evolved over time by sequence diversification, which enables the detection of different pathogen species. In addition, our results can explain why most R genes are constitutively expressed at low levels throughout the plant, even in tissues that are normally not invaded by the pathogens. An alternative explanation is that R proteins display multiple recognition specificities to (yet unknown) pathogens with different lifestyles. For example, the R gene Mi-1.2 is expressed constitutively at low levels in all plant parts and confers resistance against root-knot nematodes, aphids and whitefly (Milligan et al., 1998; Rossi et al., 1998; Nombela et al., 2003). Our data indicate that no pathogen-specific barriers may exist within R gene clusters and that R gene clusters may generate resistances to novel
pathogens on relatively short evolutionary time scales.

The high sequence homology observed for the promoter and terminator regions of \(Rx1\) and \(Gpa2\) suggest that these genes are co-regulated in potato. The coordinated expression of \(R\) genes was recently shown for specific pairs of immune receptors like \(RRS1/RPS4\) from Arabidopsis (Sinapidou et al., 2004; Narusaka et al., 2009; Williams et al., 2014) and \(RGA4/RGA5\) from rice, which cooperate in plant immunity by forming a functional complex consisting of a sensor and executor (Okuyama et al., 2011; Cesari et al., 2013 and 2014). They are located in the same chromosomal region in a head-to-head orientation and their expression is driven by \(cis\) regulatory elements present in the intergenic regions separating the two genes. The coordinated expression and cooperation of \(R\) genes may be a common theme in plant immunity to increase the repertoire of recognition specificities with a limited set of \(R\) genes (Griebel et al., 2014). Therefore, \(Rx1\) and \(Gpa2\) may be part of a co-regulatory network that activates multiple homologous genes located at complex \(R\) gene loci to allow the coordinated expression and functioning of \(R\) proteins in fine-tuning immune responses to pathogen attack. This hypothesis is supported by the sequence conservation of the flanking regulatory sequences of the other close \(R\) gene homologs present in the \(Rx1/Gpa2\) cluster (Bakker et al., 2003), which suggests that they are under the same transcriptional control as \(Rx1\) and \(Gpa2\). The presence of so called nuclear Matrix Attachment Regions (MARs) in a 115 kb genomic region harbouring the \(Rx1/Gpa2\) cluster (Suppl. Data and Suppl. Fig. S4) also point to the coordinated expression of \(Rx1\) and \(Gpa2\). MARs are wide spread sequence elements in eukaryotic genomes which bind to the nuclear matrix to regulate the higher-order organization of chromatin and affect the transcriptional activity of their flanking genes to orchestrate the spatial and temporal expression of genes during development (Ji et al., 2013; Xu et al., 2013; Iarovaia et al., 2005). This implies that similar regulatory mechanisms may be involved in the
coordinated expression and transcriptional dynamics of $R$ genes at complex loci like $Rx1/Gpa2$.

Our observation that the CC-NB-ARC domains of Gpa2 and Rx1 can mediate resistance to widely different pathogens demonstrates that similar or overlapping defence responses are activated. This indicates that Rx1 and Gpa2 share common downstream signalling components involved in both nematode and virus induced defence responses, which allow the exchange of response domains between homologous $R$ genes as shown in this study. The N-terminal domains of $R$ proteins are thought to function as a scaffold for downstream interacting proteins involved in the regulation of defense responses. The CC domains of Rx1 and Gpa2 are highly conserved and bind to the WPP domain of RanGAP2, a host protein required for both Rx1 and Gpa2 functioning (Sacco, Mansoor et al. 2007; Sacco, Koropacka et al. 2009), (Tameling and Baulcombe 2007)). This supports a role for RanGAP2 as a common accessory protein in Rx1 and Gpa2 mediated resistance and the CC domain as a platform for downstream interacting proteins involved in plant defense. Whether successful sequence exchange at complex $R$ gene loci is restricted to homologous $R$ genes like $Rx1$ and $Gpa2$ which reside in the same $R$ gene cluster and share common downstream signaling components or whether sequence exchange can also occur between homologs from different $R$ gene clusters which involve distinct accessory proteins needs further investigation.

Despite the capacity of the CC-NB-ARC of Gpa2 to activate a rapid cell death response in agroinfiltration assays or even extreme resistance to PVX when fused to the LRR domain of Rx1, a sluggish nematode resistance develops over two weeks when expressed in potato. Vice versa, a similar mild resistance response to nematodes is obtained when the CC-NB-ARC domain of Rx1 is fused to the LRR of Gpa2 and expressed in plants, suggesting that the
pathogen contributes to the resistance phenotype. Pathogens like bacteria, fungi, oomycetes and nematodes secrete an impressive array of proteins of which many are thought to be involved in suppressing plant defenses (Gurlebeck, Thieme et al. 2005; Ridout, Skamnioti et al. 2006; Thomas 2006; Truman, de Zabala et al. 2006; da Cunha, Sreerekha et al. 2007; He, Shan et al. 2007; Goverse and Smant 2014). The sluggish resistance response associated with Gpa2 may be explained by suppressors of plant innate immunity secreted by the nematode into plant tissues like SPRYSEC19 from the sibling nematode species Globodera rostochiensis, which can modulate Gpa2 and Rx1 mediated activity in N. benthamiana (Postma et al., 2012). However, not all resistance responses to feeding cell-inducing nematodes are mild. The resistance proteins Hero and Mi-1.2 respond with a fast HR upon nematode infection (Sobczak, Avrova et al. 2005; Williamson and Kumar 2006). Other explanations for the slow Gpa2 response are possible differences in the efficiency of effector recognition – either directly or indirectly - by the LRR domain in nematode feeding sites or root specific factors which determine the efficiency of the Gpa2 disease resistance response.

It is likely that the phenomena we observed in this study play a prominent role during the evolution of R proteins. The constitutively active phenotypes obtained for several chimeric constructs show that sequence divergence and coevolution between domains constrain the possibilities for reshuffling sequences within and between R gene clusters. This autoactivity presents a strong selection factor as illustrated by the inability to regenerate transgenic potato plants with the constitutively active R gene chimera. However, fine-tuned regulation of transcript levels, translation efficiency or protein stability may assuage the effects of domain incompatibility in newly formed chimeras as demonstrated in this study. Together, this shows the flexibility of NB-LRR genes to generate novel recognition specificities or to maintain
recognition specificities in natural gene pools.

Our results provide a functional framework for engineering novel functional $R$ genes based on sequence exchange between homologous genes or targeted mutagenesis. It appears that novel functional $R$ genes can be obtained by exchanging or modifying the recognition moiety encoded by the C-terminal end of the LRR region, while keeping the NB-ARC/LRR$_{N-term}$ switch module intact to avoid loss or gain-of-function mutants. However, if perturbations of the NB-ARC/LRR$_{N-term}$ switch module lead to R proteins with autoimmune or trigger-happy responses, normal functionality of the R protein can be restored by down-tuning their expression levels. Alternatively, additional mutations can be introduced in the switch module of these sensitized R proteins to restore full functionality as demonstrated for Rx1 (Harris et al., 2013). Together, these results show the potential of artificial evolution of $R$ genes based on a rational design to obtain durable and broadspectrum resistance to major pathogens in important food crops like potato.
MATERIALS AND METHODS

DNA constructs

For expression under the control of the double enhanced CaMV 35S promoter and Tnos terminator, Rx1 was amplified from the binary plasmid pBINRx1 (van der Vossen, van der Voort et al. 2000) using the primers 5GpRxbn and Rxrev (see Suppl. Table 1 for an overview of the primers used in this study) and cloned into the NcoI-SalI sites of pUCAP (van Engelen, Molthoff et al. 1995), resulting in pUCAPRx1. For Gpa2, the proximal end was amplified from pBINRGC2 (van der Vossen, van der Voort et al. 2000) with the primers 5GpRxbn and GpRxSturev to generate a NcoI-AvrII fragment, which was cloned together with an AvrII-PstI fragment from pBINRGC2 into the NcoI-PstI digested pUCAPRx1.

The Rx1 3'UTR (transcription termination) region was amplified from pBINRx1 using the primers 5UTRkp and 3UTRrev and cloned into the KpnI-PacI sites of the reporter plasmid pUCAPYFP, replacing Tnos. Next, the promoter region of Rx1 (2805 bp between the XbaI site and ATG start codon) was cloned in two steps. First, the region between the DraIII site (-1429 bp) and the start codon was amplified from pBINRx1 using the primers bRxAdeIf and RxbnREV and second, the DraIII-NcoI fragment was cloned together with the 1431 bp AscI-DraIII fragment of pBINRx1, fused to a PacI-AscI adapter consisting of AD1 and AD2, into the AscI-NcoI sites of pUCAPYFP, replacing Tnos. The Gpa2 3'UTR region was amplified from pBINRGC2 using the primers 5UTRkp and 3UTRrev for cloning in the KpnI-PacI sites of pUCAPYFP, replacing Tnos. The Gpa2 promoter region was constructed in two steps. First, the region between the BstZ17I (SnaI) site (-2744 bp) and the start codon was amplified from pBINRGC2 using the primers bGpaSnaIf and GPbnREV. This BstZ17I-NcoI fragment was cloned alongside the 720 bp PacI-BstZ17I fragment of pBINRx1, fused to a PacI-AscI adapter consisting of AD1 and AD2, into the AscI-NcoI
digested pUCAPYFP with Gpa2 3’UTR after digestion with AscI-NcoI. Thereafter, the YFP sequence was subsequently replaced by the coding sequence of Rx1 and Gpa2 via the NcoI and KpnI restriction sites.

The domain swap constructs Gpa2<sub>CN</sub>/Rx1<sub>L</sub> and Rx1<sub>CN</sub>/Gpa2<sub>L</sub> were made by exchanging the LRR fragments of Gpa2 and Rx1 using the unique ApaLI and PstI site, which are conserved and situated in the beginning and the end of the LRR encoding region of the genes, respectively. The LRR subdomain swap constructs R13rggG5, R13rrgG5, R13grgG5, R13ggrG5, R13gggR5 were created after Slootweg et al. (2013) by exchanging sequence fragments between Rx1 and Gpa2 using the restriction sites indicated in Figure 3A at amino acid positions 489 (ApaLI), 593 (ClaI), Gpa2 761/Rx1 756 (BspEI), and Gpa2 879/Rx1 874 (EcoRI).

The N-terminal GFP fusion constructs were created by first providing GFP with NcoI and SstI–KpnI sites and cloning of this fragment in pUCAP. Then the AscI-SstI (35S::GFP) was cloned with a 12 amino acids encoding linker (−GGGSGGGSGGGS−) into the pGPAII driven R gene constructs. A subset of the full-length chimeric Rx1/Gpa2 constructs showing loss-of-function (R13ggrG5 and R13gggR5) was tagged with a single c-Myc tag at their N terminus.

The leaky scan construct 35S<sub>LS</sub>::Gpa2<sub>CN</sub>/Rx1<sub>L</sub> was created following the same procedure as for 35S::Gpa2<sub>CN</sub>/Rx1<sub>L</sub>, but in this case the Gpa2 sequence was amplified with Gpa2LSFor instead of 5GpRxbn as forward primer. For the leaky scan GFPmyc6 construct 35S<sub>LS</sub>::GFPmyc6, GFP was amplified with the primer pair 5nGFP and 3CFP. The PCR fragments were transferred as NcoI-SstI fragments into pRAPmyc6, pGPAIImyc6 and pRXImyc6. The 6 fold myc-tag, present in these vectors was built from 3 tandem repeats generated by triple fusion of the NheI-SpeI fragments of the annealed oligos mMYC1 and...
mMYC2.

The PVX coat proteins CP106 and CP105 were amplified from the PVX amplicons pGR106 (Jones, Hamilton et al. 1999) containing cDNA of the Rx1-avirulent PVX strain UK3 and pGR105 containing cDNA of the Rx1-resistance breaking strain HB (Goulden, Kohm et al. 1993), respectively, using the primers 5UK3cp and 3UK3CP for CP106 and 5HBcp and 3HBCP for CP105. The products were cloned into the NcoI-KpnI sites of pUCAP between the CaMV 35S promoter and the Tnos terminator. Constructs harboring the Gpa2 elicitors RBP-1 Rook6 (eliciting), and RBP-1 Rook4 (noneliciting) are described elsewhere (Sacco et al., 2009).

For agro-infiltration assays and Agrobacterium tumefaciens – mediated plant transformation, the expression cassettes containing the constructs were cloned into the AscI and PacI sites of the binary vector pBINPLUS (van Engelen, Molthoff et al. 1995) and transformed to A. tumefaciens (pMOG101).

**Agroinfiltration assays**

For agroinfiltration experiments, Agrobacterium tumefaciens strain pMOG101 was cultured in 1 L YEB medium (5 g beef extract, 1 g yeast [Saccharomyces cerevisiae] extract, 5 g bactopeptone, 5 g Suc, and 2 mL MgSO4) with the appropriate antibiotics as described earlier (Van der Hoorn et al., 2000). After growing overnight at 28°C, the bacteria were pelleted by centrifugation, resuspended in 1 L infiltration medium (5 g Murashige and Skoog salts, 1.95 g MES, and 20 g Suc, pH 5.6, with NaOH and 200 mM acetylsyringone), and incubated at room temperature for 2 h. For agroinfiltration, the bacterial suspensions were diluted to final concentrations between optical density at 600 nm (OD600) of 0.2 and 1.0. Leaves were infiltrated of 6 weeks old Nicotiana benthamiana plants grown in the greenhouse at 20°C and
16 hours of light. Each combination was tested at least \textit{in duplo} on two different plants in at least two independent experiments. The strength of observed hypersensitive responses was ranked at a scale of 0 to 5, from no visible signs of hypersensitive response to full necrosis as described (Slootweg et al., 2013).

**Chlorophyll assay**

As a measure for the strengths of the cell-death response, chlorophyll was extracted from the leaf area transiently expressing the tested constructs (modified from (Harris, Slootweg et al. 2013)). Per agroinfiltration spot a 13 mm diameter leaf disc was collected at 3 to 5 days after infiltration and incubated overnight in 500 ul DMSO. 150 ul Chlorophyll extract in DMSO was transferred to a clear 96-well plate and the absorption was measured at a wavelength of 655 nm in a plate reader.

**Plant transformation**

The susceptible diploid potato line V was used for \textit{Agrobacterium}–mediated plant transformation as described (van Engelen, Schouten et al. 1994). Genomic DNA was extracted using the Dneasy plant mini kit (Qiagen) for PCR to analyse the incorporation of the transgene in the plant genome. RNA was extracted using Trizol LS Reagent (Life Technologies) for RT-PCR using the Superscript TM First strand synthesis system (Life Technologies) to test expression of the transgene with gene specific primers.

**Virus resistance test**

To obtain infectious virus particles, leaves of \textit{Nicotiana benthamiana} were agroinfiltrated with the PVX amplicons pGR106 and pGR105 (kindly provided by David Baulcombe,
Cambridge, UK). Systemically infected leaf material was homogenized in 10 ml of 50 mM NaPO4 buffer pH 7 and 20 µl was used for inoculation by rubbing four leaves per plant of 4 weeks old transgenic potato plants with carborundum powder. At least 3 plants per construct were used. As a control for each construct one plant was mock inoculated. Infected plants were grown in the greenhouse at 23°C and 16 hours of light. Three weeks after infection 10 leaf discs were taken from compound leaves of the apex and homogenized as described above. The relative virus concentration was determined using DAS-ELISA (Maki-Valkama, Valkonen et al. 2000). ELISA plates were coated with a 1:1000 dilution of a polyclonal antibody against PVX to bind the antigen and an alkaline phosphatase conjugated version of this antibody against PVX conjugated with alkaline phosphatase was used for detection (a kind gift of J. Saaijer).

Nematode resistance test

For the nematode resistant tests, the avirulent *Globodera pallida* population D383 and the virulent population Pa3-Rookmaker were used for infection of transgenic potato lines. The resistant diploid potato clone SH harboring the *Gpa2* gene (van der Vossen, van der Voort et al. 2000) was used as a control. Stem cuttings of *in vitro* potato plants were grown on agar plates and after three weeks, roots were infected with approximately 300 surface sterilized second stage juveniles per plate as described (Goverse, Overmars et al. 2000). For each construct three independent transformed lines were used. After 21 days and 8 weeks nematode development was monitored by microscopic inspection. For the resistance test in soil, transgenic potato plants were transferred from *in vitro* cultures and grown under greenhouse condition for two months and then inoculated with 10,000 eggs per pot of *G. pallida* Rookmaker or D383. Three and a half month after inoculation cysts were isolated.
from the roots and counted.

**Real time RT-PCR**

Leaves of *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* (pMOG101) carrying constructs of interest. At 48 hours after inoculation leaves were collected and frozen in liquid nitrogen. For RNA extraction, 60 mg of leaf tissue was used for the isolation of total RNA with the RNeasy Plant Mini Kit from Qiagen, including extra DNAse treatment. The total RNA concentration was measured using a NanoDrop spectrophotometer (Isogen) and all samples were adjusted to the same concentration. For cDNA synthesis, Super Script III (Invitrogen) and random hexamer primers were used. For real-time PCR reactions, primers were designed for Gpa2 and Rx1 using the Beacon 4.0 software. Actin was used as a reference gene. The following primers were used: Nb.actinF, Nb.actinR, Gpa.LRR-F A, Gpa.LRR-R B, Rx.C-F, and Rx.D-R (*Suppl. Table 1*). The iQ SYBR Green Supermix (Bio-Rad) was used in a reaction volume of 25 μl (7.5 μl water, 2x 1 μl primer (5 mM), 3 μl template, 12.5 μl Supermix). The annealing temperature for the actin and Gpa.LRR primers was 64 °C and for the Rx primers 63 °C. The applied PCR program was 98 °C for 3 minutes followed by 50 cycles of 95 °C for 10 sec and 63°C for 20 sec and 70 °C for 30 sec.

**Protein analysis**

Total protein extract of *N. benthamiana* leaves transiently expressing the constructs was made by grinding leaf material in protein extraction buffer (50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, 1 mM EDTA, 2% (w/v) polyvinylpolypyrrolidone, 0.4 mg mL–1 Pefabloc SC plus [Roche], and 5 mM dithiothreitol) on ice. The soluble fraction was analyzed by SDS-PAGE and subsequent visualisation by Coomassie Brilliant Blue staining or Western
blotting and protein detection. For immunodetection, the following antibodies were used: 9E10 anti-Myc (Sigma) and ab290 anti-GFP (Abcam). Peroxidase activity was visualized using Thermo Scientific SuperSignal West Femto or Dura substrate and imaging the luminescence with G:BOX gel documentation system (Syngene).

SUPPLEMENTAL DATA

Figure supplement S1. Loss-of-function phenotypes obtained for the chimera Rx1CN/Gpa2L
Figure supplement S2. Gpa2 resistance phenotypes in transgenic and wild type potato roots
Figure supplement S3. Prediction of cis acting regulatory present in the Rx1 or Gpa2 promoter
Figure supplement S4. Prediction of MAR elements present at the Rx1/Gpa2 locus in potato
Suppl. Table 1 Primer, adapter and linker sequences
Suppl. Table 2 PlantCARE prediction of several cis acting regulatory elements involved in plant defense and stress in the Rx and Gpa2 promoter regions.
Figure 1. The chimera $Gpa_{2CN}/Rx_{1L}$ confers a constitutive cell death response

A. The domain swap construct $Gpa_{2CN}/Rx_{1L}$ was obtained by exchanging the LRR domain of $Gpa_{2}$ with the corresponding domain of $Rx_{1}$ using the ApaII restriction site (after Slootweg et al., 2013).

B. Agroinfiltration of the chimera $Gpa_{2CN}/Rx_{1L}$ in leaves of $N. benthamiana$ results in a constitutive cell death response when expressed under control of the native RXI promoter. For the wild type $Rx_{1}$ gene, a specific cell death response was obtained in the presence of the avirulent PVX elicitor CP106 when expression was driven by either the CaMV35S promoter or its native promoter. The virulent PVX elicitor CP105 and YFP were used as controls.

Figure 2. Restoring PVX resistance in potato by reducing $Gpa_{2CN}/Rx_{1L}$ expression levels

A. A second translation initiation site was introduced in the construct 35S$_{LS}$::$Gpa_{2CN}/Rx_{1L}$ to obtain leaky scanning of ribosomes (Kozak 1995; Kozak 1999) and a subsequent reduction of the protein expression levels (Slootweg et al., 2010).

B. Agroinfiltration of $N. benthamiana$ leaves with 35S::$Gpa_{2CN}/Rx_{1L}$ results in a constitutive cell death response in the absence of the PVX elicitor, whereas no such autoactivation response was observed for 35S$_{LS}$::$Gpa_{2CN}/Rx_{1L}$. The activation of a specific cell death response was detected for 35S$_{LS}$::$Gpa_{2CN}/Rx_{1L}$ in the presence of the avirulent PVX elicitor CP106, similar to the wild type $Rx_{1}$ gene under control of the CaMV 35S promoter. The virulent PVX elicitor CP105 and GFP were used as controls.

C. Quantification of cell death by measuring the chlorophyll contents of cells upon agroinfiltration of the constructs in $N. benthamiana$ (after Harris et al., 2013) to confirm
the differences observed by visual scoring. Cell death results in lower absorption values at A655 nm due to chlorophyll loss (dpi = 2; n = 8; mean ± standard error).

**D.** A greenhouse virus resistance assay was performed on transgenic potato plants expressing the wild type *Rx1* gene under control of the CaMV 35S promoter and the domain swap construct *Gpa2* 

/ *Rx1* 

under control of the leaky scanning 35S LS promoter. The diploid potato clone SH, which contains the endogenous *Rx1* gene, was used as the resistant control and the diploid potato clone lineV, which was used for the transformation of the constructs, was used as susceptible control. Leaf material was collected from secondary infected leaves of the plant apex three weeks after infection with the avirulent strain PVXUK3 or the virulent strain PVXHB and systemic spreading of the virus in the plants was detected by DAS-ELISA (mean ± SD).

**Figure 3. Restoring nematode resistance to Globodera pallida in potato by the chimera R13rggG5**

**A.** Schematic overview of the domain swap constructs used in this study to reconstruct a functional chimera by combining *Gpa2* LRR segments required for RBP-1 recognition and *Rx1* LRR segments compatible with the *Rx1* CC-NB-ARC response domains. The top row shows sequence fragments in the LRR domain between *Gpa2* (G4) and *Rx1* (R4) with the positions of the breakpoints in the amino acid sequence given above. If the numbering of the positions differ between *Rx1* and *Gpa2* both numbers are given. The second row shows the domain architecture with the coiled coil (CC=1), nucleotide-binding (NB=2), ARC1 and ARC2 subdomains (=3) and the LRR domain (=4) followed by an acidic tail (=5). In the third row the amino acid positions polymorphic between *Rx1* and *Gpa2* are indicated. The *Rx1* sequence is depicted in black and the *Gpa2* sequence in
white in the chimeric constructs. Specific combinations of LRR segments derived from Gpa2 or Rx1 are indicated in lowercase letters in the construct names, whereas complete domains are indicated in uppercase letters (after Slootweg et al., 2013).

B. Functional analysis of the six domain swap constructs in the presence of the activating RBP-1 variant Rook6 and the non-activating RBP-1 variant Rook4 upon agroinfiltration in leaves of *N. benthamiana* (after Sacco et al., 2009). The *Gpa2* gene was used as control. Expression of the constructs was driven by the CaMV 35S promoter.

C. Quantification of the cell death response induced by the six domain swap constructs in the presence of the nematode effector RBP-1 variant Rook6 based on a reduction in chlorophyll content (after Harris et al., 2013). GFP was used as negative control. Multiple infiltration spots were collected per constructs and pooled for two different plants (*n* = 6; mean ± standard error).

D. *In planta* protein expression of the two chimeric proteins R13ggrG5 and R13gggR5, which showed a loss-of-function phenotype, was confirmed by agroinfiltration of the constructs in leaves of *N. benthamiana* followed by Western blotting using an anti-myc antibody. A diluted sample of GFP-myc was included as control and Commassie Brilliant Blue (CBB) staining of the gel was used to check the loading of equal amounts of protein.

E. Greenhouse nematode resistance assay on transgenic potato lines harboring the chimeric constructs *R13G45* (also known as *Rx1CN/Gpa2L*, see Figure Supplement S1), *R13grgG5*, *R13ggrG5*, *R13G4G5* and *R13rggG5* under control of the CaMV 35S promoter. Transgenic plants harboring the full length *Gpa2* gene were used as resistant controls (line 9.2) like the resistant potato clone SH, which harbours the *Gpa2* gene. A transgenic line containing the empty vector (EV) was used as susceptible control. Roots were inoculated with the avirulent Pa2-D383 population of the potato cyst nematode *Globodera pallida*. 
Two to four independent transgenic lines were assayed in multiple replicates for each transgene. Cysts were counted on these plants at 16 weeks post inoculation and the average number ± SD are shown.

**Figure 4. The Rx1 and Gpa2 promoter are exchangeable driving both nematode resistance in potato roots as well as PVX resistance in potato shoots**

**A.** A specific cell death phenotype was obtained for *Rx1* on leaves of *N. benthamiana* when coexpressed with the avirulent elicitor CP106. The virulent elicitor CP105 and YFP were used as negative controls. *Rx1* was expressed either from the endogenous RXI or the GPAII promoter. Similar results were obtained for *Gpa2* when expressed under controle of the RXI promoter and the GPAII promoter in the presence of the activating RBP-1 variant Rook6. The non-activating RBP-1 variant Rook4 and GUS were used as negative controls.

**B.** Greenhouse virus resistance assay. The mean absorbance values (A405) are shown of homogenate derived from secondary compound leaves in ELISA of transgenic potato plants. *Rx1* was expressed from its own promoter (RXI) or the *Gpa2* promoter (GPAII). Transgenic lines harboring the genomic *Rx1* BAC sequence was used as positive control. Leaves were harvested three weeks after primary leaf inoculation with the avirulent strain PVX<sub>UK3</sub> or the virulent strain PVX<sub>HB</sub>. Between 4 to 12 plants from 2 to 4 independent lines were assayed per construct (mean ± SD).

**C.** Greenhouse nematode resistance assay on transgenic potato plants harboring the *Gpa2* gene under control of either the endogenous RXI or GPAII promoter and terminator region. Plants were tested with the avirulent Pa<sub>2</sub>-D383 population and the virulent
population Pa3-Rookmaker of the potato cyst nematode *Globodera pallida*. Three independent transgenic lines were assayed in multiple replicates for each transgene. Cysts were counted on these plants at 16 weeks post inoculation and the average number ± SD are shown.


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FIGURES AND LEGENDS

A

B

Figure 1. The chimera $Gpa2_{CN}/Rx1_L$ confers a constitutive cell death response

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A. A second translation initiation site was introduced in the construct $35S_{LS}::Gpa2_{CN}/Rx1_L$ to obtain leaky scanning of ribosomes (Kozak 1995; Kozak 1999) and a subsequent reduction of the protein expression levels (Slootweg et al., 2010).

B. Agroinfiltration of *N. benthamiana* leafs with $35S::Gpa2_{CN}/Rx1_L$ results in a constitutive cell death response in the absence of the PVX elicitor, whereas no such autoactivation response was observed for $35S_{LS}::Gpa2_{CN}/Rx1_L$. The activation of a specific cell death response was detected for $35S_{LS}::Gpa2_{CN}/Rx1_L$ in the presence of the avirulent PVX elicitor CP106, similar to the wild type Rx1 gene under control of the CaMV 35S promoter. The virulent PVX elicitor CP105 and GFP were used as controls.

C. Quantification of cell death by measuring the chlorophyll contents of cells upon agroinfiltration of the constructs in *N. benthamiana* (after Harris et al., 2013) to confirm the differences observed by visual scoring. Cell death results in lower absorption values at A655 nm due to chlorophyll loss (dpi = 2; n = 8; mean ± standard error).

D. A greenhouse virus resistance assay was performed on transgenic potato plants expressing the wild type Rx1 gene under control of the CaMV 35S promoter and the domain swap construct $Gpa2_{CN}/Rx1_L$ under control of the leaky scanning $35S_{LS}$ promoter. The diploid potato clone SH, which contains the endogenous Rx1 gene, was used as the resistant control and the diploid potato clone line V, which was used for the transformation of the constructs, was used as susceptible control. Leaf material was collected from secondary infected leafs of the plant apex three weeks after infection with the avirulent strain PVX$_{UK3}$ or the virulent strain PVX$_{HB}$ and systemic spreading of the virus in the plants was detected by DAS-ELISA (mean ± SD).
Figure 3. Restoring nematode resistance to *Globodera pallida* in potato by the chimera R13rggG5

A. Schematic overview of the domain swap constructs used in this study to reconstruct a functional chimera by combining Gpa2 LRR segments required for RBP-1 recognition and Rx1 LRR segments compatible with the Rx1 CC-NB-ARC response domains. The top row shows sequence fragments in the LRR domain between Gpa2 (G4) and Rx1 (R4) with the positions of the breakpoints in the amino acid sequence given above. If the numbering of the positions differ between Rx1 and Gpa2 both numbers are given. The second row shows the domain architecture with the coiled coil (CC=1), nucleotide-binding (NB=2), ARC1 and ARC2 subdomains (=3) and the LRR domain (=4) followed by an acidic tail (=5). In the third row the amino acid positions polymorphic between Rx1 and Gpa2 are indicated. The Rx1 sequence is depicted in black and the Gpa2 sequence in white in the chimeric constructs. Specific combinations of LRR segments derived from Gpa2 or Rx1 are indicated in lowercase letters in the construct names, whereas complete domains are indicated in uppercase letters (after Slootweg et al., 2013).

B. Functional analysis of the six domain swap constructs in the presence of the activating RBP-1 variant Rook6 and the non-activating RBP-1 variant Rook4 upon agroinfiltration in leaves of *N. benthamiana* (after Sacco et al., 2009). The Gpa2 gene was used as control. Expression of the constructs was driven by the CaMV 35S promoter.

C. Quantification of the cell death response induced by the six domain swap constructs in the presence of the nematode effector RBP-1 variant Rook6 based on a reduction in chlorophyll content (after Harris et al., 2013). GFP was used as negative control. Multiple infiltration spots were collected per constructs and pooled for two different plants (n = 6; mean ± standard error).

D. *In planta* protein expression of the two chimeric proteins R13ggrG5 and R13gggR5, which showed a loss-of-function phenotype, was confirmed by agroinfiltration of the constructs in leaves of *N. benthamiana* followed by Western blotting using an anti-myc antibody. A diluted sample of GFP-myc was included as control and Comassie Brilliant Blue (CBB) staining of the gel was used to check the loading of equal amounts of protein.

E. Greenhouse nematode resistance assay on transgenic potato lines harboring the chimeric constructs R13G45 (also known as Rx1_C/N/Gpa2_L see Figure Supplement S1), R13ggrG5, R13gggR5, R13G4G5 and R13rggG5 under control of the CaMV 35S promoter. Transgenic plants harboring the full length Gpa2 gene were used as resistant controls (line 9.2) like the resistant potato clone SH, which harbours the Gpa2 gene. A transgenic line containing the empty vector (EV) was used as susceptible control. Roots were inoculated with the avirulent P2-D383 population of the potato cyst nematode *Globodera pallida*. Two to four independent transgenic lines were assayed in multiple replicates for each transgene. Cysts were counted on these plants at 16 weeks post inoculation and the average number ± SD are shown.
A specific cell death phenotype was obtained for Rx1 on leaves of *N. benthamiana* when coexpressed with the avirulent elicitor CP106. The virulent elicitor CP105 and YFP were used as negative controls. Rx1 was expressed either from the endogenous RXI or the GPAII promoter. Similar results were obtained for Gpa2 when expressed under control of the RXI promoter and the GPAII promoter in the presence of the activating RBP-1 variant Rook6. The non-activating RBP-1 variant Rook4 and GUS were used as negative controls.

**B.** Greenhouse virus resistance assay. The mean absorbance values (A405) are shown of homogenate derived from secondary compound leaves in ELISA of transgenic potato plants. Rx1 was expressed from its own promoter (RXI) or the Gpa2 promoter (GPAII). Transgenic lines harboring the genomic Rx1 BAC sequence was used as positive control. Leaves were harvested three weeks after primary leaf inoculation with the avirulent strain PVX UK3 or the virulent strain PVX HB. Between 4 to 12 plants from 2 to 4 independent lines were assayed per construct (mean ± SD).

**C.** Greenhouse nematode resistance assay on transgenic potato plants harboring the Gpa2 gene under control of either the endogenous RXI or GPAII promoter and terminator region. Plants were tested with the avirulent *P. arabidopsis* D383 population and the virulent population *P. a.-Rookmaker of the potato cyst nematode Globodera pallida*. Three independent transgenic lines were assayed in multiple replicates for each transgene. Cysts were counted on these plants at 16 weeks post inoculation and the average number ± SD are shown.

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**Figure 4. The Rx1 and Gpa2 promoter are exchangeable driving both nematode resistance in potato roots as well as PVX resistance in potato shoots**

### Table: PVX resistance

<table>
<thead>
<tr>
<th>Construct</th>
<th>PVX&lt;sub&gt;UK3&lt;/sub&gt; value A&lt;sub&gt;405&lt;/sub&gt; ± SD</th>
<th>PVX&lt;sub&gt;HB&lt;/sub&gt; value A&lt;sub&gt;405&lt;/sub&gt; ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rx1&quot; (BAC)</td>
<td>0.041 ± 0.045</td>
<td>1.7 ± 0.57</td>
</tr>
<tr>
<td>pRXI::Rx1</td>
<td>0.052 ± 0.035</td>
<td>1.3 ± 0.39</td>
</tr>
<tr>
<td>pGPAII::Rx1</td>
<td>0.025 ± 0.024</td>
<td>0.46 ± 0.32</td>
</tr>
</tbody>
</table>

### Table: Nematode resistance

<table>
<thead>
<tr>
<th>Construct</th>
<th>D383 No. cysts ± SD</th>
<th>Rookmaker No. cysts ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGPAII::Gpa2</td>
<td>2 ± 1.9</td>
<td>439 ± 235</td>
</tr>
<tr>
<td>pRXI::Gpa2</td>
<td>0.2 ± 0.4</td>
<td>435 ± 302</td>
</tr>
</tbody>
</table>


Jones JD, Dangl JL. The plant immune system. Nature 2006;444:323-9


Milligan SB, Bodeau J, Yaghoobi J, Kaloshian I, Zabel P, Williamson V (1998) The root knot nematode resistance gene Mi from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. The Plant Cell 10:1307-1319


