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論文内容要旨

Studies on co-evolution of an *Arabidopsis thaliana resistance* gene *RCY1* and cucumber mosaic virus

（シロイヌナズナ抵抗性遺伝子 *RCY1* とキュウリモザイクウイルスの

共進化に関する研究）

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General Introduction

Plants are continually threatened by various types of pathogens in the ecosystem where they live. To neutralize the threats, plants have evolved a multitude of defense systems. Unlike animals, adaptive and circulatory immune systems are absent in plants; instead, plants possess a complex immune system to recognize different types of pathogens and induce resistance against them (Chiang and Coaker, 2015). The immune system of plants consists of two interconnected pathways: one induced by recognition of common molecule patterns of pathogens, and the other induced by recognition of specific pathogens (Jones and Dangl, 2006). Plant *resistance* (*R*) genes encode receptor proteins, R proteins, which contribute to the latter pathway in the recognition of specific pathogens. R proteins share a common structure of nucleotide-binding (NB) domain and leucine-reach repeat (LRR) domain. In many cases, the variations in the LRR domain determine the recognition specificity to a pathogen-derived protein. The plant *R* genes have accumulated at high copy numbers in the genomes of land plants. For instance, *Arabidopsis thaliana* and *Oryza sativa* have accumulated 160 and 445 copies of *R* genes in their genomes, respectively (Barragan and Weigel, 2021). Since a plethora of pathogens threatening plants continually, plant genomes might have regularly evolved to accumulate more copy numbers of *R* genes through gene duplication, and to diversify or to change their recognition specificities through nucleotide substitutions. Pathogens have also experienced adaptation to escape recognition by the host *R* genes through gaining mutations in the recognized proteins. In such a way, both the plants and pathogens have been co-evolving.

RCY1 is an *R* gene of *A. thaliana* ecotype C24 that confers resistance to a yellow strain of CMV [CMV(Y)] (Takahashi et al., 1994; 2002). Inoculation of CMV(Y) to *A. thaliana* ecotype C24 results in induction of a hypersensitive response (HR), which is characterized by restriction of systemic infection of the virus and induction of programmed cell death (PCD) within the infected tissues. The gene product of *RCY1* was suggested to recognize capsid protein (CP) of CMV(Y) (Takahashi et al., 2001), while the gene products of allelic *RCY1* orthologs in ecotype Landsberg *erecta* and Dijion-17, *RPP8* and *HRT*, were shown to recognize turnip crinkle virus CP and *Hyaloperonospora parasitica*, respectively (Cooley et al., 2000), suggesting that these allelic genes of *A. thaliana* ecotypes have experienced functional diversification. In the current study, we did both *in vivo* and *in silico* analyses to study the co-evolution of *RCY1* and CMV(Y) as a model pathosystem. Based on the results, we discuss the possible co-evolution trajectories of *R* genes and viruses.

Chapter 1: Characterization of systemic HR (SHR) caused by a single amino-acid substitution in cucumber mosaic virus coat protein

The HR induction in *A. thaliana* C24 upon CMV(Y) infection can be reproduced in an *R*-gene-transformant *N. benthamiana* [hereafter *N. benthamiana* (*R*+)], which carries a chimeric *R* gene consisting of coiled-coil (CC) and NB domain-coding regions from *RPP8* and LRR domain-coding region from *RCY1*: inoculation of CMV(Y) to this *N. benthamiana* (*R*+) plant results in inclusion of the infected regions within the inoculated leaves and PCD induction within the infected regions. However, two weeks after inoculation of several *N. benthamiana* (*R*+) plants with CMV(Y), one plant showed mosaic and necrotic symptoms on the upper non-inoculated leaves (**Fig. 1a**). Sequence analysis of the virus genome in the upper leaves showed that the virus had a single amino-acid substitution (Thr45 to Met: T45M) in its CP (**Fig. 1b**). Inoculation of *N. benthamiana* (*R*+) plants with a mixture of wildtype RNA1, RNA2 labeled with a *yellow fluorescent protein* (*YFP*) gene (RNA2-YFP), and RNA3 with CP-T45M substitution (RNA3-T45M) reproduced the systemic infection and necrosis within 4 days after inoculation (**Fig. 2a**), suggesting that the T45M substitution in CP was responsible for the systemic infection and necrosis. Systemic necrosis was also observed in *A. thaliana* plants which carry the *RCY1* gene (data not shown). On the other hand, inoculation of the T45M variant to wildtype *N. benthamiana* or wildtype *A. thaliana* Col-0 (i.e., without *RCY1* gene) showed systemic infection without necrosis (**Fig. 2b**; data not shown), suggesting that the necrosis occurred in an *R*-gene-dependent manner.

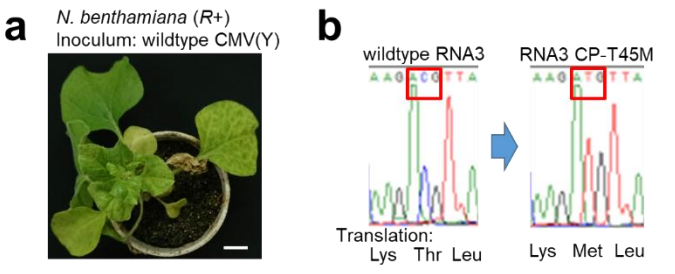


Fig. 1: Identification of a CP-T45M variant in *N. benthamiana*. **a** Mosaic and necrotic symptoms appeared in upper uninoculated leaves of a *N. benthamiana* (*R*+) plant inoculated with wild-type (WT) CMV(Y) at 2 weeks after inoculation. **b** Sequence chromatograms for direct sequencing of reverse-transcription polymerase chain reaction (RT-PCR) product from WT and variant-infected plants. Scale bar = 1 cm.

To confirm that the systemic necrosis occurred in an *R*-gene-dependent manner, we introduced an additional amino-acid substitution N31T (Asn 31 to Thr) to CP. CP-N31T variant was previously found to escape *RCY1*-mediated resistance in our lab. The resultant CP variant with both N31T and T45M substitutions systemically infected *N. benthamiana* (*R*+) plants without inducing necrosis (**Fig. 3**), suggesting that the systemic necrosis observed in *N. benthamiana* (*R*+) plants upon infection by the CP-T45M variant occurred in an *R*-gene-dependent manner, and thus is a systemic HR (SHR), where HR-like response goes systemic.

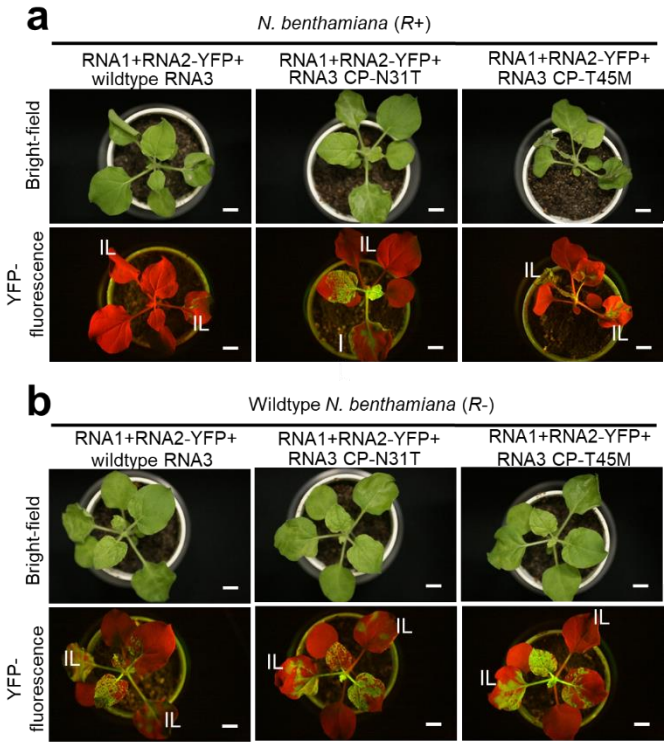


Fig. 2: Characterization of a CP-T45M variant in *N. benthamiana*. Representative images of bright-field and YFP-fluorescence observations of *N. benthamiana* (*R*+) plants (**a**) and wildtype *N. benthamiana* (*R*-) plants (**b**) at 4 days after inoculation of RNA1, RNA2-YFP, and WT RNA3 or CP variants. IL, inoculated leaves. Note that uninfected regions of leaves appear red in YFP-fluorescence observations due to autofluorescence; infected regions appear yellow. Scale bars = 1 cm.

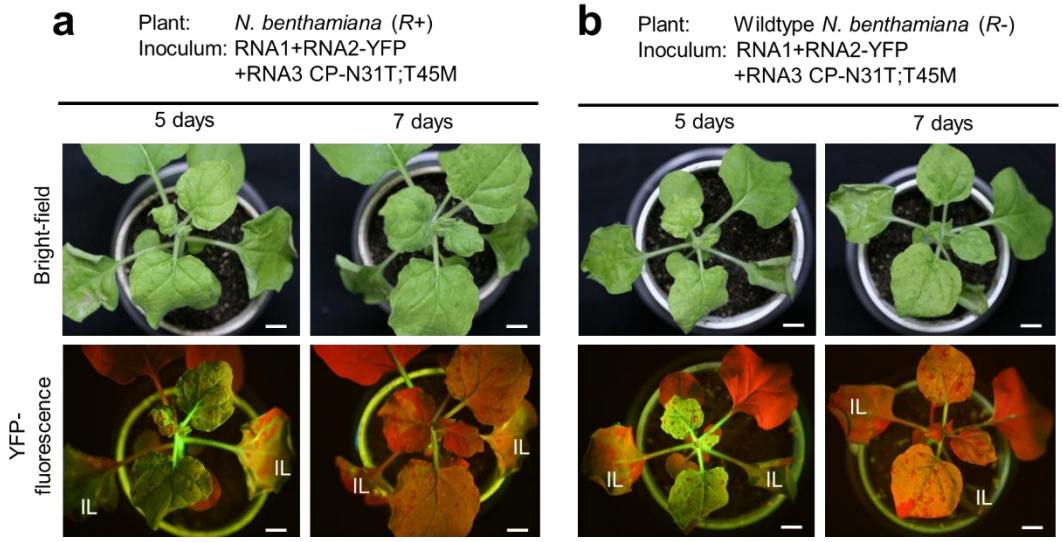


Fig. 3: Inoculation of CP double mutant with N31T and T45M substitutions. Images for bright-field and YFP-fluorescence observations of a *N. benthamiana* (*R*+) plant (**a**) and a wildtype *N. benthamiana* (*R*-) plant (**b**) at 5 and 7 days after inoculation of RNA1, RNA2-YFP, and RNA3 CP-N31T;T45M double mutant. IL indicate inoculated leaves. Scale bars =1 cm.

SHR is sometimes observed in crop fields and in laboratories, and is often considered to be a failure of resistance. To characterize SHR in a quantitative manner, we estimated viral multiplicity of infection (MOI) of wildtype CMV and its CP variants. Viral MOI is an average number of viral genomes that establish cell infections; previous studies in our group established

the estimation procedures for MOI in cell-to-cell movements of plant viruses (Miyashita and Kishino 2010; Miyashita et al., 2015). We utilized the procedure for MOI estimation in the first and second cell-to-cell movements of CMV at 14~16 hours post inoculation (hpi), when PCD is not induced yet. The obtained MOI estimates are summarized in **Table 1**. Inoculation of wildtype RNA3 to wildtype *N. benthamiana*, a susceptible combination, resulted in MOI of 5.72 ± 0.24 (mean ± standard error). On the other hand, inoculation of wildtype RNA3 to *N. benthamiana* (*R*+) plants, an HR-inducing combination, resulted in MOI of 4.08 ± 0.22, showing that *R*-gene-mediated decrease in MOI was 28.7% in HR induction. Inoculation of the RNA3-T45M variant exhibited a modest decrease in MOI upon resistance induction: 5.73 ± 0.35 in wildtype *N. benthamiana*, a susceptible interaction; 5.00 ± 0.26 in *N. benthamiana* (*R*+), an SHR-inducing combination. Thus, *R*-gene-mediated decrease in MOI was 12.7% in SHR induction. Such a decrease in MOI was not detected for the inoculation of RNA3-N31T variant that completely escaped *R*-gene-mediated resistance.

Table 1. Multiplicity of infection (MOI) estimates in wild-type (WT) and *RPRPCY*-transformant *Nicotiana benthamiana* plants inoculated with WT and variant RNA3s at 14-16 hpi. Mean ± standard error for each MOI estimate is shown.

Inoculated RNA3	MOI in wildtype *N. benthamiana* (*R*–) [A]	MOI in *N. benthamiana* (*R*+) [B]	*R*-gene-dependent MOI change [(B – A)/A]
WT	λ_{12} = 5.72 ± 0.24	λ_{12} = 4.08 ± 0.22	–28.7%
RNA3-N31T	λ_{12} = 5.75 ± 0.36	λ_{12} = 5.67 ± 0.28	–1.4%
RNA3-T45M	λ_{12} = 5.73 ± 0.35	λ_{12} = 5.00 ± 0.26	–12.7%

The above results provided the first direct evidences that MOI, the number of viral genomes to infect a cell, decreases upon induction of *R*-gene-mediated resistance, and that the level of MOI reduction in SHR is lower than in HR. This notion well explains how HR and SHR occur: efficient decrease in MOI will stop the expansion of infected regions to restrict systemic infection, while inefficient decrease in MOI cannot stop the expansion of infected regions and thus allows systemic infection; and post-hoc PCD in the infected tissues cause HR and SHR, respectively. The fates of infected plants drastically differ by HR or SHR inductions, whether they survive or die; however, the current study showed that such a change can be brought by a comparably small difference in the level of resistance induction. We also showed that such a change can be caused by a single spontaneous nucleotide substitution in the viral genome.

Chapter 2: Simulation studies on the conditions that make SHR-inducing genotype adaptive

In the previous chapter, we showed inefficient resistance induction results in SHR that leads to the death of the infected plant. SHR that kills the infected plant cannot be beneficial to the plant individual even when compared with susceptibility in the absence of the *R* gene, because susceptible virus infections rarely kill the infected plant. The above thoughts favor loss of *R* genes from plant genomes in the evolution history of plants, especially because even a single spontaneous nucleotide substitution in the viral genome can change HR to SHR. However, for a plant population that consists of individuals sharing close genetic information, the immediate death of the infected plants by SHR induction could eliminate the source of infection for the neighboring kin plants, leading to population-level resistance (**Fig. 4a**). To discuss on what conditions such suicidal population-level resistance functions, we performed some simulations in this chapter.

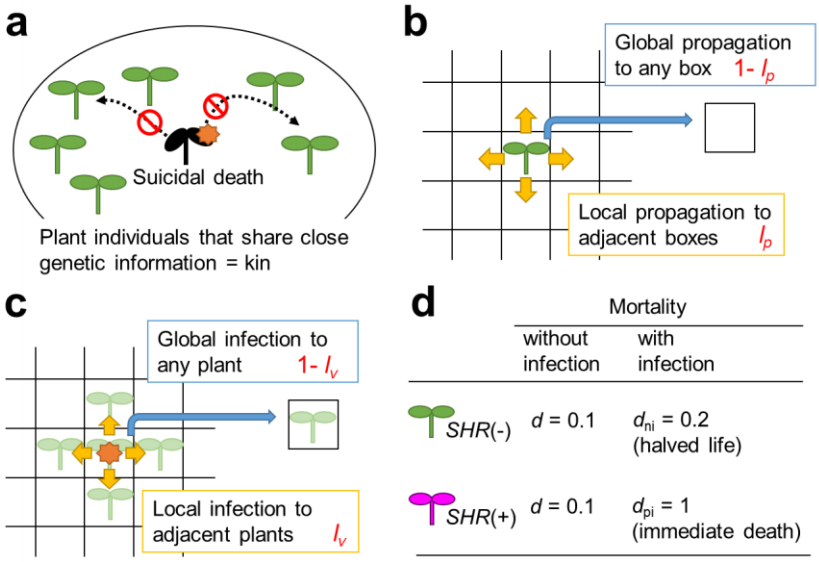


Fig. 4: Conceptual model for a suicidal population resistance via SHR that may protect next of kin. a Protection of adjacent kin plants by suicidal death. **b** Local and global propagation of plants. **c** Local and global propagation of virus. **d** Mortality of *SHR*(–) and *SHR*(+) plants with and without virus infection. Parameter values were set to answer the question: whether it is more fit to suffer lower reproduction due to viral infection, or to induce inefficient resistance against the virus, and, by opposing, end them.

Land plants often propagate locally through their seeds or via vegetative reproduction. Plant viruses also propagate locally because their vectors usually travel only short distances. These situations might help population-level resistance. To assess if such localities in plant and virus reproduction contribute to population resistance, we introduced a spatial structure to the simulation model. A two-dimensional 100 × 100-lattice were assumed as a field model, where the population dynamics of plants and viruses were simulated. Dependency of the host plant on local propagation was parameterized as l_p (**Fig. 4b**) and dependency of the virus on local transmission was parameterized as l_v (**Fig. 4c**). Two plant genotypes, plants with an SHR-

inducing *R* gene [*SHR*(+) plants] and without the SHR-inducing gene [*SHR*(–) plants] were assumed. They have the same reproduction rate and mortality rate when they are not infected by the virus but differ in mortality when they get infected (**Fig. 4d**).

The simulations were performed by assuming equal (i.e., 50% and 50%) initial subpopulations of *SHR*(+) and *SHR*(-) plants (**Fig. 5**, t=-200). After a short running-in period before virus introduction (**Fig. 5**, t=0), different sizes of plant subpopulation patches were formed at different l_p values: smaller l_p resulted in smaller patches and larger l_p resulted in larger patches, though the ratio of *SHR*(+) plants to *SHR*(-) plants were not different. This suggested that difference in l_p changes the size of subpopulations formed by genetically close kin. After starting constant introduction of the virus to the field at t=0, the occupancy of *SHR*(+) plants increased at l_p=1.00, while it decreased at l_p=0.95 or 0.90 (**Fig. 5**, t=5000). These simulation results indicated that local propagation of the host is required for suicidal population-level resistance to be functional.

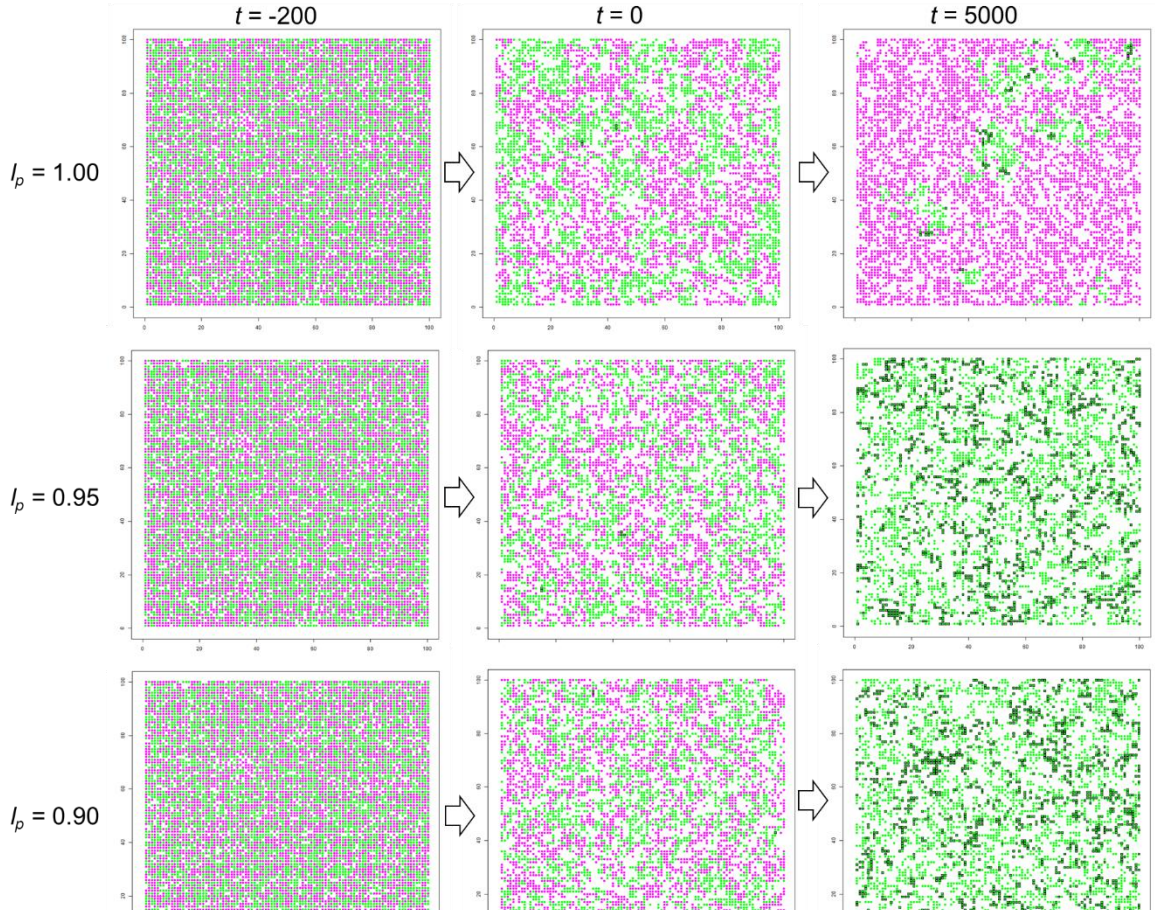


Fig. 5: Simulation results for a suicidal resistance mechanism at different levels of dependency on local propagation (l_p). Time course presentation of simulation results at different l_p values under condition (R_{SHR+} = 0.5). The results of single trials for each value of l_p are shown. Green and magenta dots indicate *SHR*(–) and *SHR*(+) plants, respectively. The dependency on local infection of the virus was set to l_v = 0.90. After a short running-in period (t = –200 to t = 0), constant virus influx was started. Infected plants are shown as green or magenta dots surrounded by black squares.

Fig. 6 schematically explains the possible benefit of population resistance by SHR and the possible co-evolution trajectories of host plant and the virus. Though the fitness of SHR-inducing genotypes is low at the individual level even compared with full susceptibility, benefit of suicidal population resistance may increase the fitness of SHR-inducing genotype over full susceptibility (**Fig. 6a**). As a result, SHR-inducing genotypes are not excluded from the population; this provides the time and opportunities for the SHR-inducing genotypes to gain additional nucleotide substitution(s) to increase the level of resistance induction toward HR- or extreme resistance (ER)-inducing genotypes (**Fig. 6b**). This "top-up" of fitness by population resistance in SHR have critical importance for evolution of antiviral *R* genes, because newly generated *R* genes are not fully adapted and thus likely to induce SHR. In addition, viral spontaneous mutations may frequently decrease the level of resistance induction to SHR; however, the plant *R* gene can again start adaptation (**Fig. 6b**). Interestingly, drastic increase in the copy number of *R* genes occurred in the history of land plants evolution (Gao et al., 2018). This may reflect the need for local propagation for the suicidal population resistance to be beneficial.

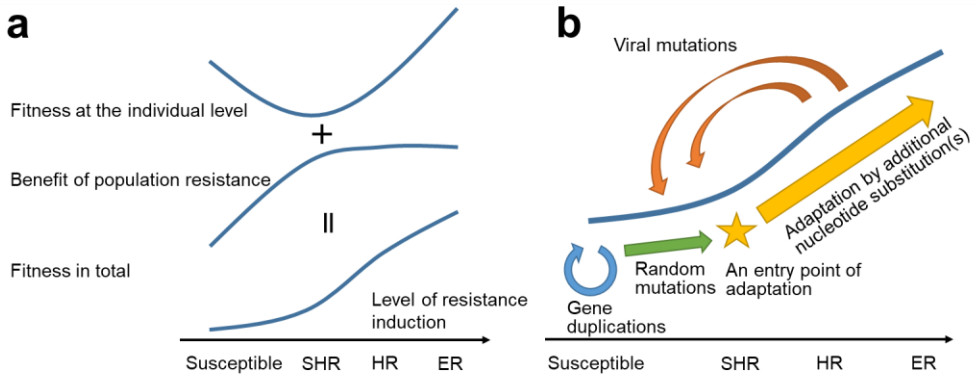


Fig. 6: Schematic representation of fitness curves and proposed evolutionary trajectory of antiviral *R* genes. a Fitness at the individual and population levels for full susceptibility and the induction of SHR, HR and extreme resistance (ER). **b** Proposed trajectory of antiviral *R* gene evolution.

Chapter 3: Artificial evolution of *RPRPCY* gene to screen for gain-of-function mutants that recognize a resistance-breaking CMV variant

To study the acquisition process of new recognition specificities by *R* genes, we performed artificial evolution of an *R* gene by error-prone PCR, and screened for gain-of-function mutants that recognize a new target protein (**Fig. 7**). Specifically, we introduced random mutations to the LRR domain-coding region of the chimeric *RPRPCY* cDNA which was cloned into a binary vector pRI201. We obtained a mutant library consisting of more than 2000 clones. Sequence analysis of randomly selected 7 clones revealed that in average 5.6 mutations were introduced into the region, suggesting that less than 1% ($e^{-5.6}$=0.0037) of the clones stay free from mutations. The binary vector library was introduced into *Agrobacterium tumefaciens* LBA4404, and each transformant clone was used for transient expression of the mutated *R* gene in *N. benthamiana* by infiltration method. As a recognition target, we transiently co-expressed a CMV *CP* variant with N31A (Asn 31 to Ala) substitution, which escapes recognition by the original *RPRPCY*. Cell death induction was assessed till 7 days after co-infiltration.

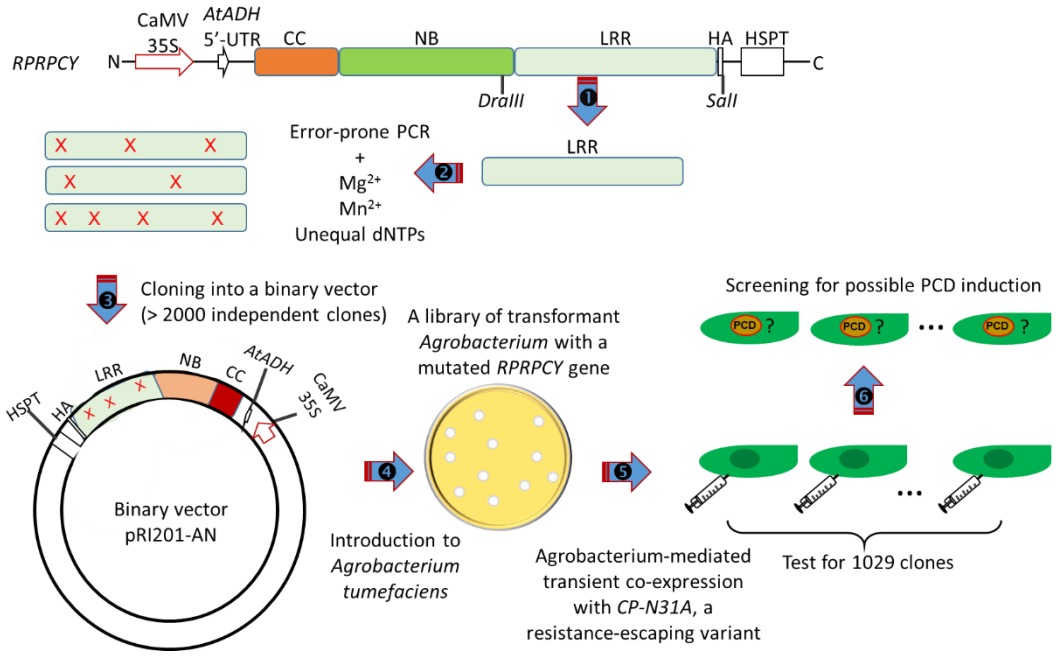


Fig. 7: Schematic representations of *RPRPCY* LRR-domain mutants construction and screening for gain-of-function resistance-breaking CMV CP-variant in *N. benthamiana*. Mutations to *RPRPCY* LRR domain were introduced through error-prone (EP) polymerase-chain reaction (EP-PCR) in the presence of Mn^{2+} and under unequal concentration of dNTPs. The EP-PCR product was then cloned into a binary vector pRI201-AN. *Agrobacterium tumefaciens* LBA4404 strain was transformed with the mutant library of *RPRPCY*. The transformants were used to screen LRR-domain mutants for gain-of-function to induce PCD in the presence of CMV(Y)-CP-N31A that escapes recognition by the original *RPRPCY*. PCD development was followed up till 7 days post-infiltration (dpi).

Our pilot tests for 176 clones showed that 37 % (65 clones) of the LRR-domain mutants lost recognition ability even for the wildtype CMV CP. This loss of function could be due to generation of premature termination codons and amino-acid substitutions that affected the stability and/or recognition function. The other 63 % of the clones maintained recognition ability for the wildtype CP, suggesting that ~60% of the clones could be appropriately screened for our purpose. However, screening of 1,029 clones did not give any clones that can recognize CP-N31A. This indicates that we couldn't introduce gain-of-function mutations by artificial evolution. It is possible that we may find such mutations by increasing the screening scale and/or by employing different methods for random mutagenesis. However, for the current study, we concluded that gain-of-function mutations rarely occur, although there are some examples reported for other pathosystems (Harris et al., 2013; Huang et al., 2021).

Chapter 4: Detection of selection pressure on *RCY1* and its allelic genes of *A. thaliana* ecotypes at the 1-aa resolution

As acquisition of new recognition specificity by an *R* gene was suggested to be a rare event, we next tried to learn from the evolution history of the *RCY1*/*RPP8*/*HRT* allele. To obtain the allelic gene sequences, we first obtained public RNA-seq data sets for ~700 ecotypes of *A. thaliana*, and mapped the short reads to known *RCY1*/*RPP8*/*HRT* cDNA sequences with BWA-mem software. Mapped reads were extracted, and subjected to *de novo* assemble with Trinity software. After the curation steps (clustering and ORF search with CD-Hit and transdecoder, respectively), we obtained 667 coding sequences of the allelic gene candidates in different ecotypes (**Fig. 8**). The obtained sequences shared at maximum 86% to 100% nucleotide identities with either of *RCY1*, *RPP8*, or *HRT*.

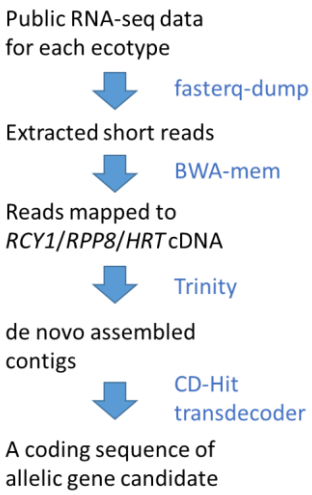
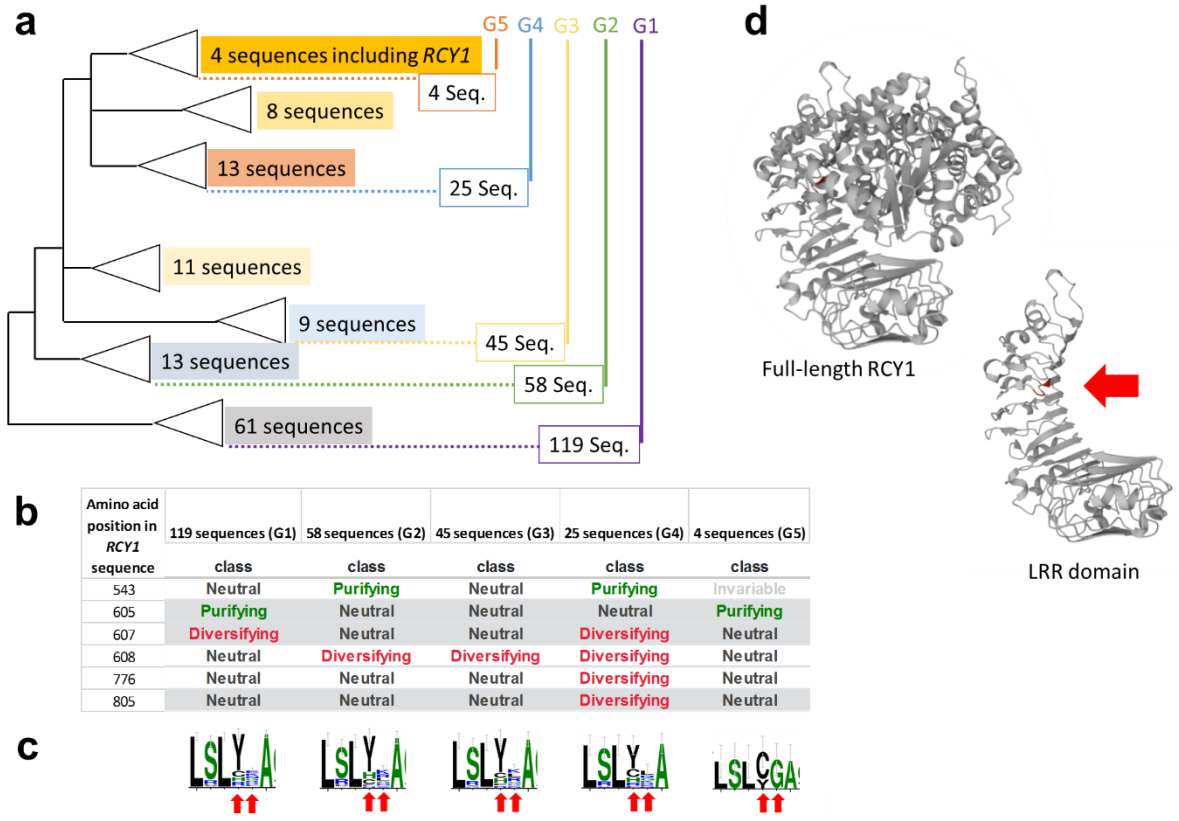


Fig. 8: Analysis flow for obtaining coding sequences of *RCY1* allelic gene candidates

The 667 sequences were analyzed as follows: We aligned their LRR domain sequences with that of RCY1 using Muscle software, and drew phylogenetic tree with maximum-likelihood method using MEGA11 software. We expected that critical amino-acid substitution(s) at certain position(s) conferred recognition specificity to CMV, thus

diversification occurred at the position(s) during such evolution period, but the same position(s) might have experienced purifying selection after that, because of its/their importance in CMV recognition. To analyze such change in selection pressures at the 1-aa resolution, we employed dN/dS analysis with FEL software to detect both diversifying and purifying selections within different sizes of clades (from G1 to G5), which contain the LRR sequence of RCY1 (**Fig. 9a**). The analysis suggested 6 amino-acid positions that may have experienced the evolution trajectory explained above (**Fig. 9b**). Further comparison of amino-acid sequences showed that Cys607 and Gly608 are specific to *RCY1* and its genetically close genes, thus might be critical for CMV recognition (**Fig. 9c**). We mapped these positions to the RCY1 protein structure expected by AlphaFold2 software. The two positions were found in the "inside" of the LRR domain, which is believed to be important for target recognition by R proteins, supporting the above results (**Fig. 9d**). Further studies are required to test this finding.



Amino acid position in *RCY1* sequence	119 sequences (G1)	58 sequences (G2)	45 sequences (G3)	25 sequences (G4)	4 sequences (G5)
	class	class	class	class	class
543	Neutral	Purifying	Neutral	Purifying	Invariable
605	Purifying	Neutral	Neutral	Neutral	Purifying
607	Diversifying	Neutral	Neutral	Diversifying	Neutral
608	Neutral	Diversifying	Diversifying	Diversifying	Neutral
776	Neutral	Neutral	Neutral	Diversifying	Neutral
805	Neutral	Neutral	Neutral	Diversifying	Neutral

Fig. 9: Detection of selection pressures in the evolution history of RCY1 and its allelic genes. a schematic representation of different sizes of clades G1 to G5 that include the LRR domain coding sequence of *RCY1*. **b** Positions that might have experienced purifying selection after diversifying selection. Note that detection of selection pressure becomes difficult in smaller clades, due to the stronger limitation in detection power of statistic analyses. **c** Weblogo representation of possible critical positions for CMV recognition. **d** Possible critical positions in the expected structure of RCY1.

Conclusion

In the present study, we discovered and analyzed a CMV(Y) mutant that induces SHR in the presence of *RCY1* or an equivalent *R* gene. The analysis showed that SHR is a result of inefficient induction of resistance. Simulations indicated that SHR-inducing genotype can be more fit than susceptible genotype due to suicidal population resistance that protect genetically close individuals. The dependency of suicidal population resistance on local propagation of the host implied that this resistance mechanism is specific for land plants. Based on the findings we proposed common co-evolution trajectories of antiviral *R* genes and the viruses. Our failure in screening for gain-of-function variants after artificial evolution of an *R* gene implied that a gain-of function mutation is a rare event, further emphasizing the importance of SHR-mediated suicidal population resistance that help new *R* genes stay in the ecosystem. The plenty of public RNA-seq data for *A. thaliana* ecotypes allowed us to analyze the history of selection pressure on *RCY1* and the ancestors at the 1-aa resolution. The analysis suggested candidates for amino-acid positions in RCY1 that are critical for CMV recognition. We believe that this study provides an important basis for understanding co-evolution of antiviral *R* genes and the recognized viruses.

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論文審査の結果の要旨及び担当者

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学位論文題目	Studies on co-evolution of an *Arabidopsis thaliana* resistance gene *RCY1* and cucumber mosaic virus（シロイヌナズナ抵抗性遺伝子 *RCY1* とキュウリモザイクウイルスの共進化に関する研究）

論 文 審 査 の 結 果 の 要 旨

植物は様々な病原体の脅威にさらされており、それに対抗する抵抗性機構を複数有する。その一つに、*R* 遺伝子と総称される数十～数百コピーの遺伝子群による抵抗性機構がある。*R* 遺伝子の産物である R タンパク質はレセプターとして機能し、それぞれ病原体由来タンパク質を特異的に認識して強い抵抗性を誘導する。R タンパク質がウイルス感染を認識した場合に起こる現象としてよく知られるのは過敏感反応(HR, hypersensitive reaction)であり、ウイルス感染域の拡大停止と感染域のプログラム細胞死により特徴づけられる。しかし特定の *R* 遺伝子・ウイルスの組み合わせでは細胞死を伴わずに 1 細胞レベルでウイルス増殖を抑制する高度抵抗性(ER, extreme resistance)になる場合や、HR 様の反応が全身で起きて植物体が全身壊死する全身 HR (SHR, systemic HR)になる場合がある。ER はより強い抵抗性が誘導された結果であるとされるが、SHR については抵抗性が不十分な場合に起こるという考えと、より強い抵抗性応答の結果であるという考えがあり、はっきりしていなかった。また細胞死自体が感染植物におけるウイルスの感染拡大を止めないことから、細胞死が起きる意義は明らかでなかった

審査論文の第一章では、ウイルスの MOI、すなわちウイルスが細胞感染する際に感染を成立させるウイルスゲノム数の平均値に着目し、*R* 遺伝子による抵抗性誘導時の MOI の低下を示すことで、抵抗性誘導強度を数値化して理解する試みがなされた。具体的には、*R* 遺伝子をもたない *N. benthamiana* ではキュウリモザイクウイルス(CMV)の MOI は 5.7 程度であったが、CMV 外被タンパク質(CP)を認識する *RCY1* 由来 *R* 遺伝子の存在下で MOI は 4.1 程度に低下することが示された。SHR を引き起こす CMV の CP 変異体 T45M では MOI は 5.0 程度までしか低下しないことが示された。これらの結果からは *R* 遺伝子によるウイルス抵抗性誘導強度が MOI の低下で数値化できることと、SHR で誘導される抵抗性は HR の場合よりも弱いことが明らかとなった。第二章では、細胞死の意義について議論をする目的でシミュレーションによる研究が行われた。SHR による全身壊死は個体レベルでは不利だが周囲の個体への感染源を環境中から除去する効果があるため、陸上植物のように宿主の遺伝的血縁度が高い集団では自らを犠牲にして血縁個体を生き残らせる自殺型の集団適応性機構として機能する可能性が示された。この結果から、細胞死は集団レベルの抵抗性のために誘導される可能性を指摘した。また *R* 遺伝子が認識特異性を獲得する進化についての知見を得る目的で第三章は人為的にランダム変異を導入した *R* 遺伝子のスクリーニング、第四章では *RCY1* 遺伝子座の進化過程を配列解析から検討する試みがなされた。第一章、第二章で得られた知見は独自の手法やアイデアで従来からの疑問を解明するものであり、科学的価値が特に高い。また第三章と第四章で行われた試みは今後継続の必要があるものの、研究の過程で確立した解析手法には普遍性があり、今後の発展が十分に期待できる。よって審査員一同は、この博士論文は博士（農学)の学位を授与するに値するものであると判断した。