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3. The evolution of post-zygotic isolation barriers by immune-triggered hybrid incompatibilities in *Arabidopsis thaliana*

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Abstract. A long-standing question in evolutionary biology is what defines a species. The biological species concept considers a species as a population of individuals that interbreeds freely and produces viable offspring. Therefore, reproductive isolation is the essence of species. Hybrid necrosis is one form of post-zygotic reproductive isolation. In this chapter, we summarize what is known to date about this phenomenon and highlight progress made in the understanding of these immune-triggered hybrid incompatibilities through our research in the plant model *Arabidopsis thaliana*.

1. Introduction

1.1. Reproductive isolation: The essence of speciation

According to the biological species concept (BSC) two populations are considered as separate species if they have departed from an interbreeding

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situation [1]. Therefore, reproductive isolation (RI) leading to restriction and ultimately disruption of gene flow between populations [2] is, by definition, the essence of species.

Isolating barriers impeding gene flow can act before (pre-zygotic) or after (post-zygotic) fertilization. In plants, pre-zygotic RI barriers comprise pre-pollination and pollen recognition barriers. Pre-pollination barriers include factors such as ecological or geographical isolation, flower morphology and color with influence on pollinator-visitation, as well as (substantial) differences in flowering time. Pollen recognition barriers lead to the abortion and inhibition of pollen tube growth on stigma of other plant species. These processes and their implications in contributing to RI have been subject of recent reviews [2].

If post-zygotic RI barriers are at play, fertilization is possible but hybrid offspring may display a range of physiological and morphological defects. These defects range from embryo lethality, sterility or hybrid necrosis affecting growth and reproduction. Since some genetically characterized hybrid necrosis phenotypes have been associated to the immune system, the term ‘immune-triggered hybrid incompatibilities’ is being generalized, although there might be other cases where the immune system is not the cause of cell death.

The underlying genetic causes for post-zygotic RI barriers in plants can be polyploidy, large chromosome-rearrangements and lethal epistatic interactions between parental alleles among others [2,3]. A number of recent reviews summarize the major findings for the first two causes of post-zygotic RI barriers in plants [2,4,5]. In this chapter we will rather focus on the latter, which is the aim of our research: Epistatic interactions between parental alleles, which conform to the Dobzhansky-Muller (DM) model of HI and leading to an autoimmune-like response.

The DM model explains, in a relatively simple way, how incompatibilities between closely related species arise without either one having to go through a fitness valley (Fig. 1) [6,7]. It proposes that incompatible genes may arise in divergent lineages, which are neutral in a non-hybrid genetic background. However, it is agnostic about the reasons that drive divergence at incompatible loci. The major advance made by Dobzhansky and Muller was the conclusion that incompatibilities are due to the epistatic interaction between parental alleles [8]. This explains why, in the first place, incompatibilities can arise without going through an adaptive valley [8]. This would likely be the case, if incompatibility results from allelic divergence at a single locus (e.g. alleles ‘A’ and ‘a’ of one gene), because individuals with genotype ‘Aa’ would suffer from reduced fitness. Despite the theory, single-locus incompatibilities, like heterozygous disadvantage, have been described elsewhere. However, evidence tells us that not all mutations supposedly lethal in the laboratories are

deleterious in nature and can be somehow tolerated for some generations. Anyhow, the focus of this chapter are DM incompatibilities owing to epistasis between two or more loci.

Typically, post-zygotic RI barriers are thought to contribute less to total RI than early-acting pre-zygotic RI barriers [2]. However, pre-zygotic RI such as geographical isolation or occupation of ecological niches (ecological speciation) might drive the evolution of post-zygotic RI, in particular those conforming to the DM-model of hybrid incompatibilities [2]. Furthermore, plant speciation, involving post-zygotic RI barriers often requires the accumulation of multiple independent DM-incompatibilities to achieve and maintain a sufficient cessation of gene-flow between separating species [2,5]. Studying speciation is possible in two different approaches: one is the “spyglass” – looking at individual species and identifying processes that might have lead to speciation [9]. The other is the “magnifying glass” – studying within-species incompatibilities between ecotypes or races that may be indicative of incipient speciation [9,10].

For evolutionary biologists and geneticists, studying DM incompatibilities within species offers the unique opportunity to examine possible incipient speciation and the underlying molecular mechanisms [9].

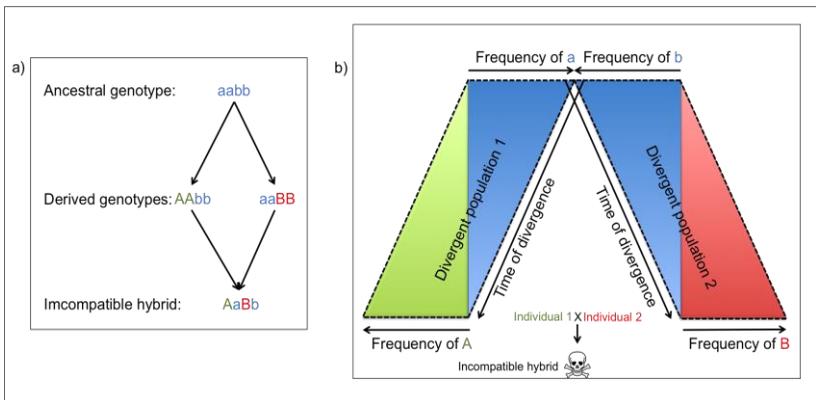


Figure 1. The Dobzhansky–Muller (DM) model of genetic incompatibilities. (a) In the DM model, starting from an ancestral genotype (aabb) different incompatible alleles (A and B, depicted in green and red, respectively) arise in divergent populations which are neutral in their own non-hybrid genetic backgrounds (AAbb and aaBB). Hybridization brings together both incompatible alleles that have not met before together (AaBb) and triggers incompatibility. (b) In divergent populations genetic drift and/or selection may lead to an increase of incompatible alleles. This is illustrated by an increase of green (A) and red (B) area.

1.2. Hybrid Necrosis: Immune-triggered HI

Frequently, plant breeders have observed a common phenomenon of post-zygotic RI, known as hybrid necrosis, which occurred in F_1 and F_2 -hybrids as well as in later back cross generations. Thus, the causal genes can be either dominant or recessive [11]. This phenomenon is accompanied by a set of recurring characteristics: growth inhibition, cell death and necrosis of leaf tissue. Therefore, it is commonly referred to as hybrid necrosis or hybrid weakness [12].

It occurs in crosses within and between plant species, monocots and dicots, inbreeding and outcrossing, annual and perennial as well as wild plants species and many crops [11]. Most hybrid necrosis cases known to date have been reported for crop plants. Perhaps the most well-known hybrid necrosis in a crop plant comes from wheat and involves the interaction between the two “necrosis alleles” Ne_1 and Ne_2 [12]. Modifiers in different genetic backgrounds were suggested as a reason for the range of severity [13].

Plant breeders have been particularly systematic in describing and analyzing the phenotypes of hybrid necrosis. However, as plant breeders are interested in hybrid vigor, hybrid necrosis was rather treated as an irritating problem when trying to introgress desirable traits into elite cultivars. In this regard, the wide-spread occurrence of Ne_1 and Ne_2 alleles in many wheat cultivars was recognized as a problem for wheat breeders [14]. Conversely, this is a fascinating case for evolutionary biologists.

Extensive (out-) crossing by plant breeders has been suggested as a potential reason for this “crop bias” in the occurrence of HI [11]. Interestingly, a milder form of hybrid necrosis, leaf-tip necrosis, has been successfully used as a morphological marker that is associated to increased resistance to spot blotch disease in wheat e.g. [15], thus already pointing to an involvement of the plant immune-system components in hybrid necrosis. Many of these cases have been compiled recently [11] and the authors concluded that, at least in some cases, autoimmunity might be the underlying cause for hybrid necrosis.

Although it is astonishing that hybrid necrosis has so far never been reported for maize, mutational studies in this crop identified a vast collection of lesion mimic mutants (LMM), that exhibited programmed cell death (PCD) and necrosis, which in many cases is accompanied by increased or altered disease resistance [15]. Emerson [16] conducted perhaps one of the earliest studies in one of these maize mutants. He described a recessive mutation that causes blotched leaves, a necrosis that is reminiscent to the plants’ response to infection, but lacked pathogen attack in this mutant.

Another LMM, *Les1* has been studied by Hoisington *et al.* [15]. The *Les1* mutant-phenotype was found to depend on the genetic background and initiation of necrotic lesions could be suppressed by elevated ('permissive') temperature, features which are now well known for many autoimmune mutants.

Similarly, a large number of autoimmune mutants exist in *Arabidopsis thaliana*, currently the most popular and best-studied model organism in plant sciences. These mutants display hallmarks of autoimmunity due to deregulated immune responses. These include development of spontaneous necrotic lesions, temperature- and humidity-dependent dwarfing and increased disease resistance owing to a constitutively active immune system in the absence of pathogen attack. Recent studies on hybrid necrosis in *Arabidopsis thaliana* reinforced the notion that autoimmunity underlies hybrid necrosis and may contribute to the establishment of post-zygotic RI barriers [17–19].

Thus, immune-triggered hybrid incompatibilities offer interesting possibilities for both evolutionary biologists aiming to elucidate the forces that drive incompatibilities between plant lineages, and plant breeders seeking to increase resistance of their cultivars without detriment to it.

2. The plant immune system

2.1. A very brief introduction to plant immunity

In nature, plants interact with a large amount of beneficial, non-detrimental and detrimental microbes, known as the plant microbiota [20]. In this context it is intriguing how little susceptibility we observe in nature, at least with the naked eye. To detect and effectively defend themselves against pathogenic microbes, plants have evolved a two-layered innate immune system. Specific epitopes of conserved microbial features - so-called pathogen-associated molecular patterns (PAMPs) – are detected by pattern recognition receptors (PRRs). This comprises the first layer of plant innate immunity. For example the PAMPs flagellin and EF-tu are recognized by the PRRs FLAGELLIN SENSING 2 (FLS2) and EF-tu RECEPTOR (EFR), respectively, which belong to a class of trans-membrane receptor-like kinases (RLKs) [21].

Indeed, most PRRs and their interaction partners are RLKs, belonging to the leucine-rich repeat (LRR) class. Recognition of PAMPs triggers PAMP-triggered immunity (PTI). This involves the induction of mitogen-activated protein kinase (MAPK) cascades, deposition of callose (an hemicellulose) at plasmodesmata to inhibit molecular flux between cells [21–24], generation of

reactive oxygen species (ROS) and gene expression changes to restrict growth of microbial pathogens [25]. To subvert PTI, adapted pathogens have evolved numerous effectors. These are deployed into plant cells to manipulate host targets in order to facilitate successful invasion of the host. To counteract these attacks, plants have evolved intracellular NUCLEOTIDE-BINDING LEUCINE-RICH REPEAT (NB-LRR) proteins. Based on their N-terminal motif they are classified into COILED-COIL (CC)- or TOLL INTERLEUKIN 1 RECEPTOR (TIR) – NB-LRRs. These R-proteins act as molecular switches and sense pathogen effectors directly or indirectly through the effectors' action on a virulence target [26]. This layer of plant innate immunity is called effector-triggered immunity (ETI). Upon recognition, NB-LRRs undergo a conformational change and trigger a strong and effective reaction that leads to a hypersensitive response (HR) at the site of infection, which is often accompanied by cell death to deprive (hemi-) biotrophic pathogens of nutrients [27]. In this context it becomes clear that ETI-responses must be tightly regulated in order to prevent aberrant signaling with the associated effects. The strong negative effect on plant fitness can be seen in several *Arabidopsis* autoimmune-mutants, which display strong temperature- and humidity-dependent growth retardation and necrosis [28]. Autoimmunity in these mutants may result from gain-of function in *NB-LRR* genes or mutations in genes, which are thought to negatively regulate *NB-LRR* gene expression [29].

2.2. Predispositions for generating immune-triggered HIs

Several features predispose the plant immune system for generating hybrid incompatibilities.

Firstly, *NB-LRR* genes belong to the most variable class of plant genes and are highly polymorphic between *Arabidopsis* accessions [30]. As well, *R*-genes show a general tendency towards clustering. High quality whole-genome sequencing of the *Arabidopsis thaliana* reference accession Col-0 found 159 *NB-LRR* genes, including those family members that do not have both, *NB* and *LRR* domains. Of these genes, 113 were found in 38 clusters with a varying cluster size of two to nine *NB-LRR* genes, while 46 *NB-LRR* genes were designated as singletons [31]. Clustering offers the opportunity to generate variation in resistance and new recognition specificities by interallelic recombination and gene conversion, thereby rapidly creating divergent haplotypes [30,32–34]. The need to respond to rapidly evolving microbial pathogen populations that are spatially and temporally heterogenous might reflect a major force for the observed plant immune system diversification. Some (but not all) *R*-genes with different recognition

strengths and capacities to launch effective immune responses are maintained within plant populations by transient balancing selection [32]. A valid explanation for this might be the associated fitness-costs of increased disease resistance in the absence of pathogen pressure [32,35], although this is still a hypothesis.

Secondly, the guard-guardee model promotes indirect recognition of pathogen effectors [36,37]. In this model a guard – mostly an NB-LRR protein – monitors the status of the guardee – an effector target, which might be a central hub [26,38,39]– and induces ETI upon detection of effector manipulations on the guardee. Indirect recognition, rather than direct recognition might maximize recognition capacities and may be the main driver for *R*-gene diversification [28]. Perhaps the best-studied example of a guardee in *Arabidopsis thaliana* is RIN4 (RPM1-interacting protein 4). *Pseudomonas syringae* type III effectors phosphorylate (AvrRpm1 and AvrB) or cleave (AvrRpt2) RIN4. The NB-LRR receptors RPM1 (RESISTANCE TO PSEUDOMONAS SYRINGAE PV. MACULICOLA 1) and RPS2 (RESISTANCE TO PSUDOMONAS SYRINGAE 2) guard RIN4 against these effector modifications, respectively [40–42]. Indeed, *rin4* null mutants are embryo lethal, due to constitutive RPM1 and RPS2-triggered immune responses [43]. *rin4 rps2* double knock-out mutants rescue the embryo-lethal phenotype but retain some constitutive activation of ETI-responses, dependent on *RPM1*. This was shown by increased expression of the marker gene *PR1* and enhanced resistance to *Pseudomonas syringae* strain DC3000, which does not deliver AvrRpm1 effectors in *rin4 rps2*, but not *rin4 rps2 rpm1* [43]. Perhaps not surprisingly, RIN4 underlies one of the interacting loci conditioning immune-triggered hybrid incompatibility in offspring derived from an interspecific cross between the domesticated *Lactuca sativa* and its wild relative *Lactuca saligna* [44].

As mentioned before, induction of MAPK signaling cascades upon PAMP recognition is a hallmark of PTI [24]. One MAPK cascade that functions in PTI is the MEKK1-MKK1/2-MPK4 cascade [45]. The type III effector HopA1 is known to inactivate MAPK signaling to successfully suppress this defense response [46]. In a recent study it was shown that autoimmunity in *Arabidopsis mek1*, *mkk1/2* fully and *mpk4* largely depends on activation of the CC-NB-LRR receptor SUMM2 (*suppressor of mek1*, *mkk2*, 2) [47]. Furthermore, the study showed direct interaction of HopA1 and MPK4 in co-immunoprecipitation (Co-IP) experiments. However, the absence of a direct interaction of SUMM2 with MPK4 suggests that SUMM2 indirectly guards the above MAPK cascade against effector perturbations by monitoring MPK4 activity, rather than directly [47].

Thirdly, ligand-induced heterooligomeric complexes of PRRs with other RLKs or signaling partners initiate MAPK cascades in PTI [21]. For example, FLS2 and EFR associate in complexes with their signaling partner BAK1 (BRI1-ASSOCIATED KINASE 1) upon recognition of flagellin and EF-Tu, respectively [48,49]. These complexes may also contain additional RLKs [21]. It is conceivable that PRRs, PRR-containing complexes and individual RLKs in these complexes are well-suited targets for pathogen effectors aiming to evade PTI. For example, BAK1-containing complexes are targeted by bacterial effectors, to suppress downstream signaling [50]. However, *bak1* knock-out mutants do not display obvious autoimmunity. But a double knock-out of *BAK1* and *BKK1* (*BAK1-LIKE1*) shows severe dwarfing and constitutive activation of immune responses, indicating some functional redundancy between BAK1 and BKK1 [51]. Another receptor-like kinase, which interacts with BAK1 is BIR1 (BAK1-INTERACING RECEPTOR-LIKE KINASE 1) and temperature-dependent dwarfing and autoimmunity is observed in *bir1* mutants [52]. In the latter case, the temperature-dependent phenotype can be partially rescued by loss-of function of the *TIR-NB-LRR* gene *SNC1* (*SUPPRESSOR OF NPR1-CONSTITUTIVE 1*) [52]. Interestingly, RLKs - many of which have a function in plant immunity - are numerous and among the more variable proteins between accessions in the *Arabidopsis* genome with a capacity to form divergent clusters and chimeric genes [30,53].

It seems evident that under these circumstances guard-guardee pairs must evolve under the above molecular constraints in order to prevent aberrant signaling and constitutive activation of ETI. This might be due to recognition of a divergent guardee as modified-self, which can be altered protein function, folding or altered downstream response. Thus, genes encoding guard and guardee in divergent populations might provide excellent epistatic pairs for the evolution of immune-triggered hybrid incompatibilities.

3. Immune-triggered hybrid incompatibilities

Despite the numerous examples of hybrid necrosis among various plant taxa and hints that plant immunity might be involved in some of these cases as early as 1926 [54], only recently have the causal genes in this epistatic interactions been identified [17–19,44,55]. The picture has emerged, that the model plant species *Arabidopsis thaliana* provides an excellent system for the study of genetic incompatibilities, such as immune-triggered hybrid incompatibilities, indicative of incipient speciation [10,56].

3.1. Immune-triggered HI between *Landsberg erecta* and Central Asian accessions

In a recent study by Alcázar *et al.* [18] we have examined a set of *Arabidopsis thaliana* recombinant inbred line (RIL) populations for growth at 14 °C (referred to as low), a temperature that plants frequently encounter in nature, compared to 20 °C (standard laboratory conditions, referred to as moderate).

In two RIL populations derived from a cross of the North European (NE) accession *Landsberg erecta* (*Ler*) to the Central Asian (CA) accessions Kashmir (Kas-2) or Kondara (Kond), plants which displayed extreme dwarfism, delay in flowering time, reduced number of seeds and necrosis (referred to as incompatible lines) at 14 °C were identified (Fig. 2).

The same genotypes resembled their parents when grown at moderate temperature. Quantitative trait locus (QTL) analysis revealed epistatic interactions involving 3 loci in *Ler* x Kas-2 and 2 loci in *Ler* x Kond incompatible lines to be causative of the above genotype by environment (GxE) interaction. In both cases incompatibility segregates as a recessive trait and commonly shares homozygous *Ler* alleles on QTL 3 (chromosome 3) and homozygous Kas-2- or Kond-alleles on QTL 4 (chromosome 4). All F₁ plants derived from crosses of incompatible *Ler* x Kas-2 and incompatible *Ler* x Kond RILs displayed dwarfism. This, and the recessive nature of QTL4 suggests that incompatibility in both RIL populations is controlled by the same loci. The study additionally identified another locus, QTL 5 on chromosome 5 in *Ler* x Kas-2 RILs, which is dominant for incompatible Kas-2 alleles and acts as a modulator for growth. Fine-mapping of QTL 3 to an



Figure 2. Incompatible phenotype of the *Ler* x Kas-2 near-isogenic line (NIL) compared to its parental lines (Kas-2 and *Ler*). Plants were grown for 5 weeks at 14-16 °C.

interval of 71,4 kb was performed. This interval includes 2 *RPP1*-like genes of the *TIR-NB-LRR* class in the reference accession Colombia (Col). However, absence of dwarfism in F_2 progenies of Col x Kas-2 crosses with Col alleles on QTL 3 and Kas-2 alleles on QTL4 and 5 suggested that polymorphisms between *Ler* and Col in this interval are the likely determinants for mediating incompatibility. Sequencing of a *Ler* bacterial artificial chromosome (BAC) spanning this interval revealed a cluster of 8 *RPP1*-like genes in *Ler*, instead of 2 in Col. The other genes in this interval were mostly conserved between *Ler* and Col, thus indicating that interactions of one or more *RPP1*-like genes from *Ler* (*RPP1*-like^{*Ler*}) with Kas-2 or Kond alleles on QTL 4 induce incompatibility. This *RPP1*-like locus was also identified to underlie DANGEROUS MIX 2 (DM2) of Uk-1 in F_1 hybrid necrosis of two accessions from Umkirch, Germany, namely Uk-1/Uk-3 hybrids [17]. However, the *RPP1*-like^{Uk-1} cluster is fully compatible with Kas-2 alleles on QTL 4 and QTL 5 and represents a different allele of this locus [18]. Regarding the fact that 38 gene clusters of the *NB-LRR* class exist in *Arabidopsis thaliana* [31] it is intriguing that the first two published cases of immune-triggered hybrid incompatibility in this species involve polymorphic alleles at this locus. Particular “trigger-happiness” could be a potential reason for this. Indeed, this polymorphic *RPP1*-like cluster locus might underlie additional other epistases leading to immune-triggered hybrid incompatibilities. We observed variation in the degree of incompatibility in *Ler* x Kas-2 incompatible lines. A Near Isogenic Line (NIL) with an introgression spanning the *RPP1*-like^{*Ler*} cluster in a Kas-2 genetic background was developed. These plants are severely stunted in growth and show strong leaf lesions, exacerbating the effects observed in incompatible RILs. This, and the observed variation among incompatible RILs points to additional modifiers in Kas-2 genetic background [18]. The involvement the *RPP1*-like^{*Ler*} *TIR-NB-LRR* gene cluster underlying the effects of QTL 3 in this epistatic interaction and the strong temperature-modulation of the above phenotypes resemble phenotypes seen in *Arabidopsis* autoimmune-mutants, displaying aberrant ETI signaling involving *TIR-NB-LRR* genes. Investigation of cell death by trypan blue (TB) staining of incompatible *Ler* x Kas-2 RILs, the NIL and both parental accessions at low and moderate temperature revealed a correlation between induction of cell death and temperature dependent dwarfing. However, parental Kas-2 lines displayed some necrotic spots at 14 °C which was not seen in *Ler*, thus pointing to different thresholds for the activation of cell death programs at low temperature in both parents. QTL analysis for cell death at 14 °C revealed four main effect QTL in *Ler* x Kas-2 RILs of which 2 co-located with the previously identified growth-related QTL 3 and 4. We concluded that growth

is suppressed at the expense of cell death program initiation. Growth retardation might be associated with the metabolic cost of constitutive immune responses [35,57].

As a consequence, transcript levels of defense marker genes were analyzed by real time-PCR in a set of *Ler* x *Kas-2* RILs grown at low and moderate temperature. Enhanced expression of marker genes for the plant defense hormone salicylic acid (SA; *EDS1* and *PRI*) and for oxidative stress (*GST-1*) broadly correlated with cell death induction in incompatible lines at low temperature. Moreover, measuring free and total SA, it was established that the degree of incompatibility in these lines depended on the accumulation of SA. This was most evident in the highly incompatible NIL that accumulated 2 to 3-fold more free SA than *Kas-2* at low and moderate temperature. Indeed, SA-pathway activation drives hybrid incompatibility in these lines, as dwarfism and cell death at low temperature were fully suppressed by the dominant transgene *NahG*, which converts SA to catechol, and by mutations in genes involved in SA-biosynthesis (*SID2*) and regulation (*EDS1*) [18].

In addition to this, and in line with the above observations, incompatible plants displayed increased resistance towards the virulent pathogen *Hyaloperonospora arabidopsidis* (*Hpa*), which is able to infect both parental accessions (*Ler* and *Kas-2*) even at moderate temperatures. Incompatible dwarf plants readily developed pathogen-induced cell death upon infection, suggesting a lowered threshold for defense activation. Thus, the positive trait of enhanced resistance in incompatible hybrids at moderate temperature may become detrimental in the absence of pathogen attack and at low temperature [18]. Because even the highly incompatible NIL was ultimately able to flower and set viable seeds even at low temperature, the authors conclude that the characterized immune-triggered hybrid incompatibilities have incomplete penetrance and additional DM incompatibilities may be required to achieve complete hybrid breakdown [18].

3.2. A natural variant of a receptor-like kinase underlies QTL 4

In a follow-up study, the causal gene underlying the effects of QTL 4 was identified as the Receptor-Like Kinase (RLK) *STRUBBELIG RECEPTOR FAMILY 3* (*SRF3*), which was not predicted to be involved in plant immunity [19]. Sequencing of incompatible *SRF3*^{*Kas-2*} and *SRF3*^{*Kond*} genes revealed different alleles at this locus inducing similar responses by incompatible interactions with the *RPPI*-like^{*Ler*} cluster. Further analysis by genotyping 603 accessions covering the natural growth range of *Arabidopsis thaliana* found the presence of these two incompatible allelic forms only in Central Asian accessions, but not in European accessions (Fig. 3).

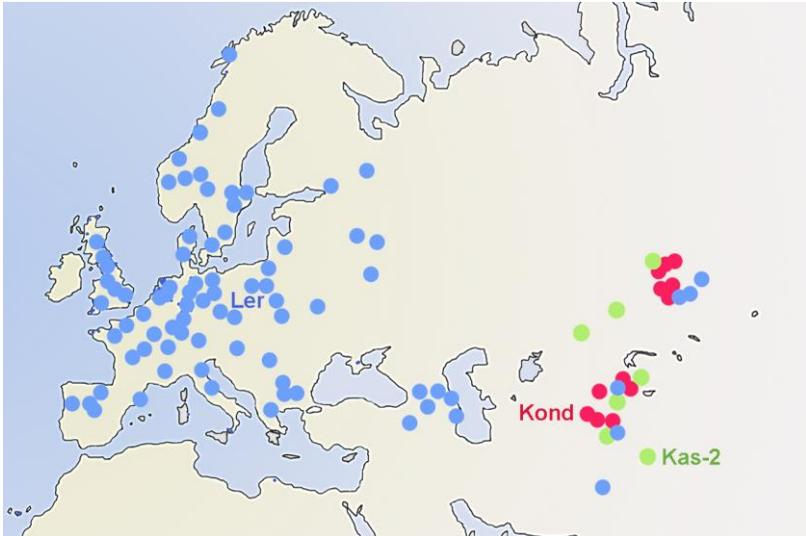


Figure 3. Distribution of SRF3 alleles in Eurasia. The origin of accessions carrying *Ler*-like alleles (blue), *Kas-2* alleles (green) and *Kond* alleles (red) is indicated. *Kas-2* and *Kond* alleles are only found in Central Asia.

33 Central Asian and 30 North and South European accessions were selected and crossed to *Ler*. Consistently, segregation of dwarfism in F_2 populations at low temperature was observed only in crosses derived from *Ler* with Central Asian accessions. Importantly, the identified incompatible alleles fall into distinctive genotypic clades within Central Asian accessions, as suggested by STRUCTURE analysis based on genotyping with 139 genome-wide SNP markers [58]. Transiently expressed in *Nicotiana benthamiana* SRF3 localizes to the plasma membrane. In addition to this, SRF3 is differentially phosphorylated in response to flg 22 [59]. Flg22 is a conserved epitope of the bacterial MAMP flagellin, which is sensed by FLS2 [19]. This prompted the authors to investigate the involvement of different SRF3 allelic forms in plant immunity. Strikingly, incompatibility in the highly incompatible NIL (*Kas-2* genetic background with *RPP1*-like^{*Ler*} introgression) could be suppressed by transformation with compatible and dominant *SRF3*^{*Ler*} (referred to as cNIL). Immune responses dependent on compatible (*Ler*) and incompatible (*Kas-2*, *Kond*) *SRF3* alleles in compatible and incompatible genetic interactions with the *RPP1*-like^{*Ler*} cluster were further examined. As already mentioned, initiation of MAPK cascades upon pathogen recognition is an early event of

PTI signaling. Activation of MAPKs by treatment with flg22 was analyzed in these lines based on activities of MPK3, MPK4 and MPK6 and concluded that MPK4 activity (suppressor of immune signaling) and MPK6 activity kinetics (activator of immune signaling) correlated with the extent of immune activation in isogenic backgrounds. In addition, plants with different allelic combinations were infected with the oomycete *Hpa* and the virulent bacterial strain *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and avirulent *Pst* DC3000 *hrcC*. The *hrcC* mutant mainly induces PTI responses, because it fails to deliver effectors into host cells. Infection phenotypes (measured as bacterial growth) displayed enhanced susceptibility towards *Pst* DC3000 as well as *Pst* DC3000 *hrcC* compared to the parental accessions when Kas-2 backgrounds were transformed with *SRF3*^{Ler} alleles [19]. This suggests that Central Asian alleles of *SRF3* contribute positively to basal resistance, and the opposite holds true for *Ler* *SRF3* alleles. Since *srf3* mutant lines in Col were neither more susceptible to *Hpa* isolates Noco2 and Cala2 nor towards *Pst* DC3000 or its mutant version *hrcC* [19], the effect of enhanced early immune responses observed for Central Asian alleles is background dependent or it requires specific point mutations rather than loss-of-function alleles. The study of different genetic backgrounds is the basis for the genetics of natural variation that allows the identification of weak alleles that do not show any phenotype in common laboratory accessions. Different crosses between Col, Kas-2 and *Ler* are underway to identify this complex background dependence. In line with activation of ETI responses in incompatible lines, the NIL showed enhanced resistance towards *Pst* DC3000 which deploys effectors into host cells compared to its parents. As mentioned in the introduction, new alleles such as those involved in DM incompatibilities can arise and may increase in frequency owing to genetic drift or natural selection in isolated or divergent populations. By applying an appropriate statistical test (Fay and Wu's *H*) across the genomic region of *SRF3* we looked for molecular signatures of selection. An excess of derived alleles as indicated by high negative values for Fay and Wu's *H*, suggested a recent selective sweep in Central Asian populations. Thus, HI between *Ler* and Central Asian accessions likely evolved as a by-product of natural selection for incompatible *SRF3* alleles in Central Asian populations to the biotic environment [19]. A current hypothesis for incompatible hybrids displaying autoimmunity is that polymorphisms in *SRF3*^{Kas-2} mimic effector modifications that are sensed by one or more RPP1-like receptors from *Ler* (Fig. 4). Thus, RPP1-like receptors and the LRR-RLK *SRF3* would normally be in a guard-guardee relationship (Fig. 4).

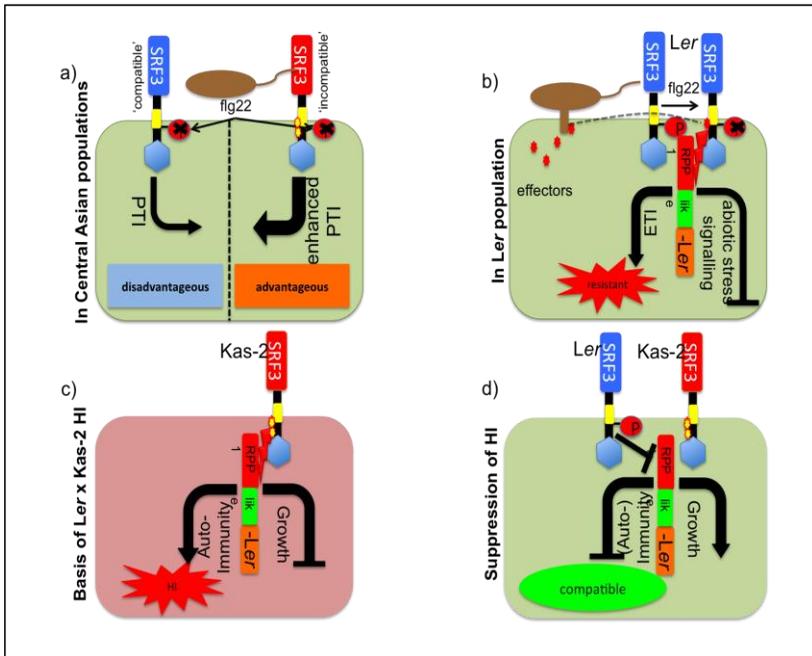


Figure 4. Simplified model of different SRF3 allelic forms and RPP1-like^{Ler} receptors in plant immunity and immune-triggered HI. a) In Central Asian populations “incompatible” SRF3 forms may contribute to early PTI responses. This may confer a relative advantage under high pathogen pressure (indicated by weight of the arrows). b) In the original *Ler* population pathogens may have evolved effectors to subvert PTI responses mediated by SRF3. In turn, RPP1-like^{Ler} immune receptors arose to guard SRF3 against these perturbations and trigger effective local immune responses (ETI). c) Both incompatible alleles evolved independently. In a hybrid context incompatible SRF3 forms mimic effector modifications sensed by RPP1-like^{Ler} TIR-NB-LRRs. This leads to autoimmunity at the expense of growth. d) Dominance of compatible SRF3^{Ler} may be achieved, because a normal guard-guardee relationship is established and suppresses autoimmunity, thus promoting growth.

4. Concluding remarks and future perspectives

Hybrid necrosis has been long observed and characterized by plant breeders and evolutionary biologists alike. Studying hybrid necrosis between different accessions of the model plant species *Arabidopsis thaliana* has contributed significantly to the understanding of the molecular bases of this

phenomenon [17–19] offering the opportunity to examine possible incipient speciation processes [9]. With the cloning of the first causal genes previous notions could be validated that, indeed in many cases autoimmunity underlies this form of DM incompatibility. As presented in the introduction, the DM model is agnostic with regard to the reason of allele divergence. Our studies [18,19] have identified immune-triggered hybrid incompatibility between a North European accession and numerous Central Asian accessions. The observed effects are caused by the negative epistatic interaction between a highly polymorphic cluster of *TIR-NB-LRR* genes (*RPP1*-like^{Ler}) and incompatible alleles of the receptor-like kinase *SRF3* in Central Asian accessions. We have established that incompatible *SRF3* alleles in Central Asian populations have undergone a recent selective sweep and enhance early immune responses, potentially conferring selective advantage for these alleles in Central Asian populations. Thus, our studies provide an important example where DM incompatibilities displaying autoimmunity may arise as a by-product of adaptation to the biotic environment that is strongly modulated by temperature.

However, our studies also offer interesting future perspectives.

R-gene clusters are highly polymorphic and show signatures of transient balancing selection in local populations rather than world-wide selective sweeps [32]. Since the *RPP1*-like^{Ler} haplotype of this cluster is only found in *Ler*, it would be interesting to see its detailed geographical distribution on a large screen of accessions. Furthermore, incompatible hybrids offer the unique opportunity to identify additional modifiers of the described epistasis. With regard to *SRF3*, several interesting perspectives exist. Why are Central Asian alleles of this RLK incompatible? *SRF3* was not identified in a plant immune context before our publication [19]. We know that RLKs often function in heterooligomeric complexes. Also *SRF3* is dephosphorylated upon *flg22* treatment. This immediately poses the questions, which are the molecular interaction partners of this RLK, and how *flg22* signaling interferes with *SRF3* phosphorylation.

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References

1. Mayr, E.: *Systematics and the Origin of Species*. Columbia University Press, New York; 1942.
2. Rieseberg, L.H., Willis, J.H. 2007, *Science*, 317, 910.
3. Mallet, J. 2007, *Nature*, 446, 279.
4. Orr, H. A. 1995, *Genetics*, 139, 1805.
5. Orr, H. A., Turelli, M. 2001, *Evolution*, 55, 1085.
6. Muller, H. J., 1942, *Biol. Symp.*, 6, 71.
7. Dobzhansky, T.: *Genetics and the Origin of Species*. Columbia University Press, New York; 1937.
8. Orr, H. A. 1996, *Genetics*, 144, 1331.
9. Via, S. 2009, *Proc. Natl. Acad. Sci. U.S.A.*, 106, 9939.
10. Bomblies, K., Weigel, D. 2010, *Philos. Trans. R. Soc. B Biol. Sci.*, 365, 1815.
11. Bomblies, K., Weigel, D. 2007, *Nature Rev. Genet.*, 8, 382.
12. Hermesen, J. G. T. *Hybrid necrosis in wheat*. In: Proefschrift Landbouwhogeschool Wageningen, 1962, pp.129.
13. Hermesen, J. G. T. 1963, *Genetica*, 33, 245.
14. Hermesen, J. G. T. 1963, *Euphytica*, 12, 1.
15. Hoisington, D. A., Neuffer, M. G., Walbot, V. 1982, *Dev. Biol.*, 93, 381.
16. Emerson, R. A. 1926, *Cornell Mem.*, 70, 3.
17. Bomblies, K., Lempe, J., Epple, P., Warthmann, N., Lanz, C., Dangl, J. L., Weigel, D. 2007, *PLoS Biol*, 5, e236.
18. Alcázar, R., García, A. V., Parker, J. E., Reymond, M. 2009, *Proc. Natl. Acad. Sci. U.S.A.*, 106, 334.
19. Alcázar, R., García, A. V., Kronholm, I., de Meaux, J., Koornneef, M., Parker, J. E., Reymond, M. 2010, *Nature Genet.*, 42, 1135.
20. Bulgarelli, D., Schlaeppli, K., Spaepen, S., van Themaat, E. V. L., Schulze-Lefert, P. 2013, *Annu. Rev. Plant Biol.*, 64, 807.
21. Zipfel, C. 2008, *Curr. Opin. Immunol.*, 20, 10.
22. Zipfel, C., Robatzek, S. 2010, *Plant Physiol.*, 154, 551.
23. Faulkner, C., Petutschnig, E., Benitez-Alfonso, Y., Beck, M., Robatzek, S., Lipka, V., Maule, A. J. 2013, *Proc. Natl. Acad. Sci. U.S.A.*, 110, 9166.
24. Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W.-L., Gomez-Gomez L., Boller, T., Ausubel, F. M., Sheen, J. 2002, *Nature*, 415, 977.
25. Nürnberger, T., Brunner, F., Kemmerling, B., Piater, L. 2004, *Immunol. Rev.*, 198, 249.
26. Jones, J. D. G., Dangl, J. L. 2006, *Nature*, 444, 323.
27. Mehdy, M. C. 1994, *Plant Physiol.*, 105, 467.
28. Alcázar, R., Parker, J. E. 2011, *Trends Plant Sci.*, 16, 666.
29. Hua, J. 2013 *Curr. Opin. Plant Biol.*, 16, 406.

30. Clark, R. M., Schweikert, G., Toomajian, C., Ossowski, S., Zeller, G., Shinn, P., Warthmann, N., Hu, T. T., Fu, G., Hinds, D. A., Chen, H., Frazer, K. A., Huson, D. H., Schölkopf, B., Nordborg, M., Rättsch, G., Ecker, J. R., Weigel, D. 2007, *Science*, 317, 338.
31. Guo, Y.-L., Fitz, J., Schneeberger, K., Ossowski, S., Cao, J., Weigel, D. 2011, *Plant Physiol.*, 157, 757.
32. Bakker, E. G., Toomajian, C., Kreitman, M., Bergelson, J. 2006, *Plant Cell*, 18, 1803.
33. Noel, L., Moores, T. L., van Der Biezen, E. A., Parniske, M., Daniels, M. J., Parker, J. E., Jones, J. D. 1999, *Plant Cell*, 11, 2099.
34. Michelmore, R. W., Meyers, B. C. 1998, *Genome Res.*, 8, 1113.
35. Tian, D., Traw, M. B., Chen, J. Q., Kreitman, M., Bergelson, J. 1998, *Nature*, 423, 74.
36. Dangl, J. L., Jones, J. D. G. 2001, *Nature*, 411, 826.
37. Van Der Biezen, E. A., Jones, J. D. G. 1998, *Trends Biochem. Sci.*, 23, 454.
38. Mukhtar, M. S., Carvunis, A.-R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., Tasan, M., Galli, M., Hao, T., Nishimura, M. T., Pevzner, S. J., Donovan, S. E., Ghamsari, L., Santhanam, B., Romero, V., Poulin, M. M., Gebreab, F., Gutierrez, B. J., Tam, S., Monachello, D., Boxem, M., Harbort, C. J., McDonald, N., Gai, L., Chen, H., He, Y., European Union Effectoromics Consortium, Vandenhaute, J., Roth, F. P., Hill, D. E., Ecker, J. R., Vidal, M., Beynon, J., Braun, P., Dangl, J.L. 2011, *Science*, 333, 596.
39. Chisholm, S. T., Coaker, G., Day, B., Staskawicz, B. J. 2006, *Cell*, 124, 803.
40. Mackey, D., Belkhadir, Y., Alonso, J. M., Ecker, J. R., Dangl, J. L. 2003, *Cell*, 112, 379.
41. Mackey, D., Holt III, B. F., Wiig, A., Dangl, J. L. 2002, *Cell*, 108, 743.
42. Axtell, M. J., Staskawicz, B. J. 2003, *Cell*, 112, 369.
43. Belkhadir, Y., Nimchuk, Z., Hubert, D. A., Mackey, D., Dangl, J. L. 2004, *Plant Cell*, 16, 2822.
44. Jeuken, M. J. W., Zhang, N. W., McHale, L. K., Pelgrom, K., Boer, E. den, Lindhout, P., Michelmore, R. W., Visser, R. G. F., Niks, R. E. 2009, *Plant Cell*, 21, 3368.
45. Gao, M., Liu, J., Bi, D., Zhang, Z., Cheng, F., Chen, S., Zhang, Y. 2008, *Cell Res.*, 18, 1190.
46. Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., Zhou, J. M. 2007, *Cell Host Microbe*, 1, 175.
47. Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., Ba, H., Zhou, J., Zhang, Y. 2012, *Cell Host Microbe*, 11, 253.
48. Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D. G., Felix, G., Boller, T. 2007, *Nature*, 448, 497.
49. Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F. G., Tör, M., Vries, S. de, Zipfel, C. 2011, *Plant Cell*, 23, 2440.
50. Shan, L., He, P., Li, J., Heese, A., Peck, S. C., Nürnberger, T., Martin, G. B., Sheen, J. 2008, *Cell Host Microbe*, 4, 17.

51. He, K., Gou, X., Yuan, T., Lin, H., Asami, T., Yoshida, S., Russell, S. D., Li, J. 2007, *Curr. Biol.*, 17, 1109.
52. Wang, Z., Meng, P., Zhang, X., Ren, D., Yang, S. 2011, *Plant J.*, 67, 1081.
53. Bomblies, K. 2009, *Botany*, 87, 1013.
54. Burkholder, W. H., Muller, A. S. 1926, *Phytopathology*, 16, 631.
55. Yamamoto, E., Takashi, T., Morinaka, Y., Lin, S., Wu, J., Matsumoto, T., Kitano, H., Matsuoka, M., Ashikari, M. 2010, *Mol. Genet. Genomics*, 283, 305.
56. Alcázar, R., Pecinka, A., Aarts, M. G. M., Fransz, P. F., Koornneef, M. 2012, *Curr. Opin. Plant Biol.*, 15, 205.
57. Hulten, M. van, Pelser, M., Loon, L. C. van, Pieterse, C. M. J., Ton, J. 2006, *Proc. Natl. Acad. Sci. U.S.A.*, 103, 5602.
58. Platt, A., Horton, M., Huang, Y. S., Li, Y., Anastasio, A. E., Mulyati, N. W., Ågren, J., Bosssdorf, O., Byers, D., Donohue, K., Dunning, M., Holub, E. B., Hudson, A., Le Corre, V., Loudet, O., Roux, F., Warthmann, N., Weigel, D., Rivero, L., Scholl, R., Nordborg, M., Bergelson, J., Borevitz, J. O. 2010, *PLoS Genet.*, 6, e1000843.
59. Benschop, J. J., Mohammed, S., O'Flaherty, M., Heck, A. J. R., Slijper, M., Menke, F. L. H. 2007, *Mol. Cell. Proteomics*, 6, 1198.