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Short title: Biotrophy drives host adaptation in *Phytophthora infestans*

Abstract

Despite their ability to infect both plant species, natural populations of *Phytophthora infestans*, the pathogen causing late blight on potato and tomato, are usually separated into genetically distinct lineages that are mainly restricted to either host. Laboratory cross-inoculation tests revealed a host-related local adaptation between genotypes, with asymmetric fitness performance between generalist lineages, mainly present on tomato, and specialist lineages confined to potato. To further understand the basis of host-related adaptation in *P. infestans*, we combined experimental evolution and analysis of effectors involved in pathogenicity and cell death modulation. We aimed to check in this way (i) if natural host adaptation of *P. infestans* is reversible during one growing season and (ii) if this process is accompanied by changes in pathogenicity-related gene expression. Two isolates differing substantially by their level of specialization were passaged for nine generations on susceptible potato (cv. Bintje), tomato (cv. Marmande) or alternately on both hosts. Pathogenic fitness and the expression of eight pathogen effectors with known host targets (*AVRblb2, EPIC2B, EPI1, PexRD2, SNE1, PiNPP, INF1 and Pi03192)* and the candidate effector carbonic anhydrase (*CA*) were quantified before and after experimental evolution on these hosts. Fitness and gene expression varied during the experimental evolution experiment, but independently of the subculturing host. However, the level of host-related specialization of both isolates was stable over time and linked to distinct expression patterns of antagonistic host cell death regulator genes, such as *SNE1* and *PiNPP*. Relations between fitness and effector expression proved to be host- and/or isolate-dependent. Altogether, our results demonstrate host adaptation of *P. infestans* to be a rather stable trait that is not prone to fluctuate by transitory host changes. They further suggest that pathogenicity of *P. infestans* strongly depends on its ability to establish a steady biotrophic interaction with its hosts by regulating effector gene expression.

Author Summary

The infamous Irish potato famine pathogen *Phytophthora infestans* causes late blight on potato and tomato, and extensive losses on both crops worldwide. Isolates causing tomato late blight markedly differ in genotype and phenotype from isolates causing potato late blight: under controlled conditions, isolates from tomato perform well on both hosts, while isolates highly pathogenic from potato struggle to produce large lesions on tomato. Mechanisms explaining these differences are unknown, but might provide clues to better understand the fundamental process of host specialization in pathogens. *P. infestans* is known to secrete many effectors, modulating the outcome of the interaction with its hosts. We thus coupled experimental evolution, by subculturing isolates nine times on different hosts, and expression of host cell death regulating effectors to explain pathogenic specialization. We showed that the level of pathogenic specialization depends on the pathogen ability to maintain a biotrophic interaction with its host, and hence to suppress cell death. Host specialization was not altered during serial passages, irrespective of the hosts, although overall pathogenicity increased. These findings show that *P. infestans* is primarily a biotrophic pathogen, feeding on living host tissue, and open ground for new breeding targets for improved resistance to late blight.

Introduction

Parasitism is the relationship between two species in which one benefits at the expense of the other. Relationships of parasites with plants are diverse, and their classification depends largely on physiological/nutritional and ecological criteria [1-3]. According to the nutritional behavior of the parasite, plant-parasite interactions may be biotrophic or necrotrophic. Biotrophy means that the parasite keeps the host alive and derives energy from living cells (e.g. *Blumeria graminis*) [4], whereas necrotrophy refers to relationships where the parasite kills the host cells to feed on dead matter (e.g. *Botrytis cinerea*) [5, 6]. An initially biotrophic relationship may become necrotrophic (e.g. *Colletotricum graminicola*, *Sclerotinia sclerotiorum*) [7, 8]. This situation is commonly referred to as hemi-biotrophy, but there is some ambiguity due to divergent use of this term. For example, interactions of the rice blast fungus *Magnaporthe oryzae* are called hemi-biotrophic [9], but there is no single discrete switch from biotrophy to necrotrophy in infected tissue. Biotrophic invasion of host cells is rather sequential and continues in neighboring rice cells while previously parasitized cells die.

Biotrophic hyphae growing ahead of dead tissue have also been reported in the late blight pathogen *Phytophthora infestans* (Mont.) De Bary [10, 11], an oomycete [2] with outstanding scientific and economic importance [12, 13]. Under natural conditions, *P. infestans* depends on living host tissue to complete its life cycle and has low or no saprotrophic survival ability outside its hosts [see 14 and literature cited therein]. However, as tissue damage (necrosis) occurs during later states of colonization, its lifestyle has also been classified as hemi-biotrophic or even necrotrophic. As schematized in Figure 1, the duration of biotrophy varies among *P. infestans* isolates and has been related with the level of pathogenic fitness on tomato in laboratory conditions [15-18]. Biotrophy has also been associated with distinct gene expression patterns on potato [19].

Plant pathogens face in their hosts a two-branched innate immune system [20]: pathogen-associated molecular pattern triggered immunity (PTI) and effector triggered immunity (ETI). As outlined by the

79 authors of this seminal review, successful pathogens secrete effectors proteins to suppress PTI and
80 lose/evolve effectors to overcome ETI. These principles also apply to oomycete-plant interactions
81 [21-23]. *P. infestans* is known to employ various effectors during the interaction with its hosts [24,
82 25] and gene expression polymorphisms among isolates have been reported on potato [19].
83 Interestingly, these polymorphisms can be associated with the duration of biotrophy [19, figure 7]. *P.*
84 *infestans* has a repeat-bloated 240 megabases (Mb) genome containing hundreds of fast-evolving
85 RxLR, CRN and other effector-coding genes that are expressed in a coordinated way during its
86 interaction with potato and tomato [24-26]. Secreted into the host apoplast or cytoplasm, they
87 disable host defense components and facilitate colonization: *P. infestans* effectors such as *EPI1* [27],
88 *EPI1*0 [28], *EPIC1*/*EPIC2B* [29, 30] and *AVRblb2* [31] are implicated in counter-defense against
89 apoplast-localized host proteases. Other effector genes manipulate host gene expression by
90 downregulating defense related genes [e.g. effector PI03192, 32] or upregulating susceptibility
91 factors [e.g. effector Pi04089, 33]. *P. infestans* also induces/suppresses host cell-death through the
92 coordinated action of cell death antagonistic effectors. As illustrated by Zuluaga et al. [24],
93 biotrophy-related suppressors of cell death such as *ipiO1* [34, 35] and *SNE1* [36] are secreted in the
94 early phases of the interaction, followed by the later secretion of synergistically interacting necrosis-
95 inducing effectors (NIE) such as *PiNPP*1.1 and *INF1* [37]. As the timing of expression and biological
96 activity fit trophic phases of the interaction, effectors countering host cell-death may serve *P.*
97 *infestans* to fine tune the duration of biotrophy by taking control over the host plant cell death
98 machinery [e.g. SNE1/PINPP1.1, 38]. Therefore, *P. infestans* effectors are thought to be involved in
99 host specialization [39]. This view is supported by the fact that *P. infestans* isolates are genetically
100 diverse and may carry different variants of an effector (e.g. ipiO family) [40].
101
102 Ecological specialization is a main concept in ecology, but its definitions are highly context-
103 dependent (see [41]). Historically developed as a species attribute (generalist and specialist species),
104 the concept of specialization is now largely extended in the ecological literature to any ecological

level (individual, population, species, community). Within-species variation of ecological specialization is indeed widespread [42]: a species considered as ecological generalist may actually be a heterogeneous collection of specialized individuals. Here, we will refer to ecological specialization at the individual level, as the aim of our work is to explore concomitantly ecological and mechanistic aspects of previously documented within-species variation of specialization in *P. infestans* [18, 43, 44]. Considering the important distinction between fundamental and realized specialization [45], it should also be clarified that ecological specialization in the present study refers to Grinnellian specialization assessed in controlled cross-inoculation experiments. Inferred here from direct measures of species performance (i.e. pathogenic fitness) in multiple host environments (i.e. potato, tomato) [46], it analyses the variance in performance across these hosts according to the Levins' metric [47]. The terms 'generalist' and 'specialist' refer here to previously identified isolate types (potato isolate, tomato isolate; [15]) showing distinct level of pathogenic specialization.

Overall, what determines the level of host specificity of *P. infestans* is not well understood. Besides the potential effect of cultural practices [48], quantitative differences in pathogenic fitness of isolates may be a particular strong explanatory factor [49-51]. The evolutionary process of pathogenic specialization in *P. infestans* populations on potato and tomato is just as little understood as its underlying determinants. Experimental evolution experiments are thus often used to explore those evolutionary dynamics in real-time. There is evidence, from Mexico and Israel, that tomato isolates could evolve from a local potato population and acquire specialization to tomato [43, 52]. In other words, aggressiveness on tomato would have evolved in isolates already pathogenic on potato. This could explain the lower host specificity of tomato isolates with respect to potato isolates. As documented for various parasite-host systems [53], the observed increase of fitness/virulence/growth on the new host on which the parasite has been passed is often associated with attenuation of these traits on the original host. In the case of highly adaptive pathogens, as little as one [54] or only several passages [55, 56] on the alternative hosts may be sufficient to alter or

131 revert adaptation patterns. *P. infestans* may be considered as a highly adaptive pathogen, as it has
132 been reported to be locally adapted to the host species potato and tomato [15, 18] and generally
133 adapted to particular potato cultivars as well [57-59]. However, to the best of our knowledge,
134 ongoing specific adaptation of *P. infestans* to a particular host cultivar or host species has not yet
135 been established in controlled conditions by experimental evolution experiments. Some evidence
136 arises from an early study of *P. infestans* maintained prior to experiments on chickpea agar [60]. The
137 authors suggest that *P. infestans* could recover initial virulence, but only on its original potato cultivar
138 after serial passages on that specific cultivar.
139
140 The present work investigates the lability of specialization in *P. infetans* interactions with
141 potato and tomato using experimental evolution, and explores molecular mecanisms potentially
142 involved in host specialisation. For this purpose, expression of nine effector genes – selected for their
143 established or presumed functions in pathogenicity – were assessed concomittantly to the level of
144 host specialization. Our underlying hypotheses were that i) the original adapation patterns
145 (generalist or potato specialist) could be altered by serial passages through alternate hosts, ii)
146 alterations of pathogenicity towards less host specificity would lower the pathogenicity level on the
147 original host, and iii) each adaptation pattern involves specific combinations or kinetics in the
148 expression of pathogenicity-related effector genes. Our results proved these hypotheses to be
149 wrong, but highlighted the importance of the biotrophic stage in the determination of host specificity
150 in *P. infestans*. More specifically, they showed that the ability for the pathogen to maintain the
151 biotrophic stage for a long enough period is crucial in the sucessful exploitation of the host.
152
153
154
155

Results

Pathogenic fitness of *Phytophthora infestans*

***P. infestans* isolates differ in their level of host specialization.** Before experimental evolution, both *P. infestans* isolates were equally fit on potato but their level of pathogenic fitness differed markedly on tomato, the isolate collected on tomato (15.TII.02) being fitter than the isolate collected on potato (15.P50.09) (Fig 2A). Each isolate appeared to be fitter on its host of origin than on the other host (Fig 2A). This was most noticeable and statically significant for the potato isolate ($P = 0.005$), but less pronounced and not statistically significant for the tomato isolate ($P = 0.08$). Considering potato and tomato as different ecological niches, the Levin's measure of standardized niche breath B_A (scale 0-1) differed significantly between both isolates ($P = 0.03$), confirming isolate 15.TII.02 ($B_A = 0.88$; se = 0.06) to be more generalist than isolate 15.P50.09 ($B_A = 0.61$; se = 0.04).

Subculturing hosts do not differentially impact pathogenic fitness. Pathogenic fitness of replicate lineages subcultured nine times on either potato, tomato or on alternating hosts did not significantly differ (Fig 2B). This was true for both isolates and whatever the tested host (potato and tomato), showing that subculturing habitats had no differential effect on the pathogenic fitness of *P. infestans* isolates. However, there was a slight general increase of pathogenic fitness during experimental evolution, that was statistically significant only for the tomato isolate.

Host specialization is a stable trait (at least over a short-term). After experimental evolution, the pathogenic fitness of both isolates was still similar on potato and still highly different on tomato (Fig 2B, compare to Fig 2A). As before experimental evolution, the difference in pathogenic fitness of the tomato isolate on potato and on tomato was still less obvious than for the potato isolate. However, it

could be shown here to be statistically significant (P = 0.02). Despite of this, the initial difference in pathogenic specialization between both isolates was unchanged during experimental evolution, as the evolved tomato isolate remained more generalist (B_A = 0.95; se = 0.06) than the evolved potato isolate (B_A = 0.62; se = 0.02).

Effector gene expression

State-specific markers and other genes are differentially expressed at 2 and 4 dpi. Transcript abundance differed between 2 and 4 dpi (Fig 3). As expected, transcript abundance of *SNE1* and *PexRD2* genes, involved in biotrophy, was clearly higher at 2 than at 4 dpi (Fig 3A). The same was true for the counter-defense genes *AVRblb2*, *EPIC2B*, *EPI1* and *Pi03192*. Transcripts of the stage-specific marker for necrotrophy *PiNPP*, but also of *INF1* and *CA*, were more abundant at 4 dpi than at 2 dpi (Fig 3B). Increased transcript abundance of these necrotrophy-related genes was associated with the presence of macroscopically visible necrotic lesions at 4 dpi.

Effector genes are differentially expressed between isolates and hosts. Transcript abundance differed significantly between isolates and host plants, revealing distinguishable patterns (Fig 4). Transcript abundance of *EPI1, EPIC2B, CA* and *PiNPP* was significantly higher in the potato isolate than in the tomato isolate, whatever the host (potato and tomato). Transcript abundance of *PiNPP* was also higher in the potato isolate growing on its alternative host, *i.e.* tomato, than on potato (PT > PP), contrasting with the similar transcript abundance found in the tomato isolate grown on each host. Transcript abundance of *PexRD2* was generally higher on potato than on tomato (PP and TP > PT and TT). Interestingly, transcript abundance of *SNE1* was high in the tomato isolate grown on potato compared to other isolate-host combinations (TP > PP, PT and TT). On the opposite, transcript abundance of *INF1* and *AVRblb2* was lower in the potato isolate grown on potato than in other

206 isolate-host combinations (PP < TP, PT and TT). Finally, transcript abundance of *Pi03192* did not
207 significantly differ among all tested isolate-host combinations.
208
209 **Gene expression patterns remain stable in experimental evolution on different hosts**. Overall,
210 similar levels of transcript abundance were measured in *P. infestans* lineages subcultured on potato,
211 tomato or on alternating hosts (Fig 4). However, it is noteworthy that after subculturing on tomato,
212 transcript abundance of the biotrophy related genes PexRD2 and SNE1 was slightly – but not
213 significantly – increased on potato relative to other subculturing hosts.
214
215 **Matching pathogenic fitness and effector gene expression**
216
217 **Subculturing on host tissue leads to isolate-specific phenotypic variation.** After nine passages on
218 their original host, the pathogenic fitness of the potato isolate was unchanged whereas that of the
219 tomato isolate significantly increased (Fig 5A). This isolate-specific increase of pathogenic fitness was
220 accompanied by a significantly lower transcript abundance of *AVRblb2* and *EPIC2B* but higher
221 transcript abundance of *Pi03192* and *PiNPP* (Fig 5B). No significant variation of these genes was
222 observed in the potato isolate, with unchanged pathogenic fitness. These isolate-specific effects of
223 subculturing were comparable on both tested hosts, and regardless of the host on which they had
224 been subcultured before. Transcript abundance of the other genes tested (*EPI1*, *SNE1, PexRD2, INF1*
225 and *CA*) was not significantly different before and after subculture (S1 Fig).
226
227 **Redundancy analysis points to isolate-specific reaction patterns on potato and tomato hosts.**
228 Variation of fitness and effector gene expression – generated by the study of two *P. infestans* isolates
229 on two hosts, before and after experimental evolution in different habitats – is summarized jointly by
230 redundancy analysis (RDA, Fig 6). The final RDA model explains 64.11 % of the total variance in the
231 dataset. The first and second constraint component axis represent 56.54 and 19.35 % of the

constrained variance (cumulative 75.89 %), respectively. A third constraint component axis (S2 Fig) represents an additional 15.48 % of the constraint variance (cumulative 91.36 %). The first axis separates isolates (Fig 6A; tomato isolate on the left, potato isolate on the right), the second axis displays a separation according to the host on which the isolates were tested (individuals tested on tomato on the top and individuals tested on potato below). The statistical significance of these arrangements – suggesting the phenotype of isolates to be distinct and to depend on the host – was confirmed by a permutation test for RDA ($P < 0.001$). However, the effect of the subculturing host is not significant ($P = 0.17$) and thus not illustrated. Furthermore, significant interaction terms (isolate x host, $P = 0.003$ and isolate x subculture, $P = 0.002$) point to isolate-specific reaction patterns. As obvious from arrangements on the factorial map, the tomato isolate phenotype is distinct before and after subculture (illustrated by open and filled boxes), but the potato isolate phenotype stayed unaltered (isolate x subculture interaction). On the contrary, the model suggests phenotypic variation of isolates on different hosts to be more pronounced for the potato isolate than for the tomato isolate (isolate x host interaction), consistent with the clear host specificity in this isolate.

Pathogenic fitness is correlated with effector gene expression. Vector alignments in the loading plot of RDA analysis (Fig 6B) indicate pathogenic fitness to be positively correlated with the expression of *SNE1* and *PexRD2* (grouped left arrows), but negatively correlated with that of *PiNPP*, *CA*, *EPI1* and *EPIC2B* (left arrow of fitness opposed to grouped right arrows of effector gene expression). Vectors of fitness and *INF1* are almost orthogonally aligned, indicating no correlation between both variables. The short vector length of *AVRblb2* and *Pi03192* illustrates their low contribution to the RDA model. For verification purpose, RDA analysis was also performed separately on data obtained before and after experimental evolution: vector alignments were consistent in both situations, with the exception of *EPIC2B*. Pairwise correlation tests on unconstrained data (correlations ignoring factor levels) confirmed the statistical significance of all correlative relationships mentioned above (Table 1).

Table 1: Pairwise correlations between fitness and effector gene expression of *P. infestans*. Pearson product-moment correlation coefficient r and p-values were calculated on unconstrained data (n = 12, grouped factor combinations). Asterisks indicate significance: p < 0.01 **, p < 001 ***.

gene	r	p-value	
EPI1	-0,65	> 0,0001	***
PiNPP	-0,59	> 0,0001	***
CA	-0,58	> 0,0001	***
EPIC2B	-0,45	0,0013	**
PexRD2	0,40	0,0048	**
SNE1	0,39	0,0068	**
Pi03192	0,15	0,2932	
AVRblb2	0,06	0,7018	
INF1	-0,02	0,8730	

Correlations between fitness and effectors are host- and isolate-specific. Pairwise correlation tests constrained to one host (potato and tomato) or to one isolate (15.P50.09 collected on potato and 15.TII.02 collected on tomato) were performed to explore within-group correlations (Fig 7). Correlations between fitness and effector gene expression were statistically significant on tomato but not on potato (except for *EPIC2B*, Fig 7A). Because a visual inspection of correlative data revealed the presence of split data clouds on tomato (two data groups with different means for each variable), each corresponding to data from one isolate, correlation tests performed separately on data from each isolate revealed isolate-specific correlations between pathogenic fitness and transcript abundance (Fig 7B). *PiNPP* and *INF1* were each positively correlated with pathogenic fitness in isolate 15.TII.02 (collected on tomato), but negatively correlated with fitness in isolate 15.P50.09 (collected on potato). *PexRD2* was not significantly correlated with pathogenic fitness in isolate 15.TII.02, but positively correlated in isolate 15.P50.09.

Discussion

In the present work, quantitative differences in host specialization of *Phytophthora infestans* to potato and tomato were analyzed in terms of stability over serial passages through different hosts and in relation to effector gene expression. The two *P. infestans* isolates included in this study were chosen to display distinct levels of host specialization [15]: the isolate from tomato performed equally well on both hosts (therefore called a generalist), but the isolate from potato, highly pathogenic on its original host, struggled on tomato (therefore called a potato-specialist). As such a pattern has also been consistently reported from other studies [17, 18, 44, 61], the quantitative differences in terms of specialization on potato and on tomato of the isolates studied here may be considered to be representative for *P. infestans* populations on these hosts.

Our work clearly illustrates the absence of ongoing specific adaptation of *P. infestans* to potato and to tomato in controlled conditions: initial differences in pathogenic specialization were maintained throughout the experimental evolution. This may not be intuitive *a priori* for at least two reasons. First, pathogenicity changes during experimental evolution has been documented for various pathosystems, and in many cases the increase of fitness/virulence/growth was specific for the alternative host on which the parasite had been passed and associated with attenuation of these traits on the original host [53]. Depending on the pathosystem, as little as one [54] or only few passages [55, 56] on alternative hosts may be sufficient to obtain such patterns. Second, *P. infestans* is best described as a highly adapted pathogen as it has been reported to be locally adapted to the host species potato and tomato [15, 18] and generally adapted to particular potato cultivars as well [57-59]. There are even evidences that tomato isolates could evolve from a local potato population and acquire specialization to tomato [43, 52]. However, to our knowledge, there is only timid evidence for the possibility of ongoing specific adaptation of *P. infestans* to a particular host cultivar or host species in controlled conditions [60]. Results from our experimental evolution experiment do not contribute on this, but are rather in line with other studies on *P. infestans* stating the absence of

ongoing specific adaptation to a particular host cultivar [59, 62] or host species [61, 63] in controlled conditions. We therefore conclude that the levels of quantitative adaptation of *P. infestans* to different host – and therefore the level of host specialization – is a stable trait, at least on the short term and in the conditions of the test. If the here realized number of nine serial passages equals as believed the number of pathogen generations during a growing season in the field, we may speculate that the observed isolate-specific levels of host specialization are not inverted in the field. Most obviously, potato isolates struggling on tomato are not expected to get adapted to tomato during one growing season. We cannot exclude the existence of additional evolutionary forces in natural environments that could accelerate the process of adaptation, but the durable population separation in the field [48, 64] and results from a competitive experiment in field plots [65] are in favor to our view that host specialization is a stable trait. Interestingly, stability of specific adaptation of *P. infestans* to a particular host during the experimental evolution experiment was related to unaltered patterns of concomitantly assessed expression of effector genes.

The comparison of effector gene expression among host-isolate combinations also points to isolate- and host-specific patterns. First, the effectors *EPI1*, *EPIC2B*, *CA* and *PiNPP1.1* provided a highly similar profile: their expression was rather similar on both hosts, but differed greatly between both isolates. Second, *PexRD2* and *INF1* were rather similarly expressed in both isolates but their expression level differed between both hosts. These patterns of effector gene expression may reflect isolate and host properties and possibly contributes to variability of pathogenic fitness among host-isolate combinations. Following a validation step on more than two isolates, the observed isolate-specific pattern of *EPI1*, *EPIC2B*, *CA* and *PiNPP1.1* could also be used as markers to distinguish both isolate types (isolates from potato, isolates from tomato).

Despite the absence of specific adaptation of *P. infestans* to a particular host, we observed pathogenic fitness after the experimental evolution experiment to be generally increased compared to fitness of the same isolates before experimental evolution. Increasing fitness due to serial passages on a given host has also been observed elsewhere for *P. infestans* on different potato cultivars [66] and from other fungal pathosystems [67, 68]. The general increase of pathogenic fitness could be due to the fact that *P. infestans* was maintained as axenic culture before experiments. It has been established that *P. infestans* loses pathogenicity when maintained on artificial culture medium [60]. The reason for the loss of pathogenicity during axenic culture has not yet been established, but it may be linked to the absence of living host tissue. In fact, in natural conditions, *P. infestans* has a biotrophic lifestyle and no or only limited saprophytic survival ability in the absence of a living host [14, 69]. The increase of pathogenic fitness was only significant for the isolate from tomato. We could thus speculate that the tomato isolate, highly biotrophic on tomato, has lost more pathogenicity than the potato isolate during axenic culture. This could explain the stronger rate of recovery during serial passages on living host tissue. Increased fitness of the isolate at the end of the experimental evolution experiment was related to an altered expression of some of the tested effector genes (*AVRblb2*, *EPIC2B*, *PiNPP1.1*, *Pi03192*). Among these genes, the expression of the protease inhibitor *EPIC2B* was most noticeably altered: it was strongly reduced after experimental evolution in the fitter tomato isolate. Interestingly, no change in effector gene expression was observed for the potato isolate whose fitness was unaltered by experimental evolution. These results show that – even in absence of specific adaptation of *P. infestans* to a particular host – fitness may vary over time and is accompanied by variation of effector gene expression.

Correlation analysis of data for pathogenic fitness and effector gene expression showed biotrophy to be a major clue to explain quantitative differences in specialization of *P. infestans* to potato and tomato. Fitness was found to be positively related with the expression of *SNE1* and

350 *PEXRD2*, two effector genes that have been previously associated with biotrophy in regard to their
351 timely expression and their ability to oppose cell death [36, 70]. As expected by its documented
352 necrosis-triggering activity [37], effector *PiNPP1.1* was also negatively related with fitness in the
353 present study. The antagonistic activity on cell death of *SNE1* and *PiNPP1.1*, supported by our data,
354 has actually been proposed as a potential mechanism for *P. infestans* to control the duration of
355 biotrophy [38, illustrated model, 71-76]. Comparing gene expression in the two isolates included in
356 this study on their respective original and alternative hosts also suggests a similar antagonistic
357 interaction between *PiNPP1.1* and *PexRD2*. Consistent with this view, the potato-specialized isolate
358 coordinately increased expression of *PiNPP1.1* and reduced expression of *PexRD2* on tomato, relative
359 to the expression of both genes on its original host. The resulting stronger host cell death and shorter
360 biotrophic period were associated with a drastically lower pathogenic fitness. This relationship
361 between reduced biotrophy and reduced pathogenic fitness is consistent with the view that *P.*
362 *infestans* is a biotrophic pathogen that requires living cells to feed on.
363
364 Why would potato-specialized isolates of *P. infestans* trigger early host cell death on their
365 alternative tomato host by increased secretion of apoptosis-related effectors (i.e. *PiNPP1.1*) if this is
366 negatively related with fitness? A biological explanation refers to the "increasing plant defense"
367 theory [reviewed by 7], which claims that (hemi-)biotrophic pathogens eventually kill the host cells
368 they infected to protect themselves as a last resort against steadily increasing host defenses.
369 Particularly strong evidence for this theory is provided by work on the fungal pathogens
370 *Magnaporthe oryzae* [77] and *Colletotrichum graminicola* [78]: the authors found that the duration
371 of the biotrophic stage fits the speed of increasing host defense. Our results, as well as literature
372 reports on *P. infestans* [37, 79], suggest that highly expressed host defenses also force this otherwise
373 biotrophic pathogen to actively trigger host cell death (Fig. 8). Literature reports on steadily
374 increasing host defense during *P. infestans* infectious process [80, 81], and their more or less
375 successful effector-mediated suppression [72, 82] strengthen this view.

In addition to the cell death antagonistic effectors *PiNPP1.1* - *SNE1*/*PexRD2*, we also studied the expression of the counter-defense protease inhibitors *EPI1* and *EPIC2B* [27, 30], that were both found to be negatively related to pathogenic fitness of *P. infestans* on tomato. This relation may be explained through the programmed cell death in hypersensitive reactions (HR PCD), a pathogen-triggered host response to infection that is associated with strong immune responses [83]. The direct implication of *EPI1* and *EPIC2B* in host cell death has not yet been conclusively established, but we argue – based on evidence from literature – that *EPI1* and *EPIC2B* protease inhibitor activity of these effectors could modulate *P. infestans* interactions by blocking immunity related programmed cell death in the host (Fig. 9 and S1 Appendix). This hypothesis is based on two main facts: (i) *EPI1* and *EPIC2B* jointly inhibit protease activity in the tomato apoplast [30] and (ii) at least one of these inhibited proteases (*PIP1*) is required for immunity-related PCD in tomato [84]. We thus hypothesize that *P. infestans* could address PCD during the HR by secreting proteases inhibitors that target cell death-related host proteases. On that condition, expression of proteases inhibitors by *P. infestans* may reflect the intensity of host immune signaling for HR PCD encountered in the tested host-isolate combinations. There is indeed literature evidence that *P. infestans* protease inhibitors are specifically up-regulated in host tissue compared to culture medium [25], and are expressed concomitantly to their respective host targets [27, 30]. We can thus expect a strong host immune signaling for HR PCD in situations where *EPI1* and *EPIC2B* expression is increased, and only a low host immune signaling for HR PCD in situations with low expression of these protease inhibitors. The negative correlation with fitness may be explained by the continuous increase of other immune responses lowering pathogenic fitness. It is now well established that HR is not limited to PCD, but also involves the strong accumulation of various defense compounds and the rapid expression of defense-related genes [85, 86-88]. This apparently also applies to *P. infestans*, where HR PCD is ubiquitous [10, 11, 71, 72, 89] and positively related to timing and/or intensity of defense [71-76]. We therefore speculate that the short duration of biotrophy in the potato isolate-tomato interaction results from a

strong host immune response. In the absence of experimental evidence, the severe and fast cell death observed may be either plant-controlled HR PCD and/or pathogen-controlled cell death in response to overwhelming defenses (increasing defense theory, Fig. 8). In both cases, necrosis would be associated with low pathogenic fitness of *P. infestans*.

To conclude, the set of results reported here clearly shows that the level of pathogenic specialization of *P. infestans* to potato and tomato is a biotrophy-related trait, and that is unaltered by the different tested conditions of our experimental evolution. Our data strongly support the idea that pathogenic fitness of *P. infestans* is positively related with the duration of biotrophy, itself finely regulated by the balance in the expression of antagonistic necrosis-inducing (PiNPP) and necrosis–opposing (SNE1, PexRD2) effectors, and that this balance itself depends on the time required for host defenses to reach a threshold level. Future research may address this hypothesis, by studying inducible host defenses in response to a range of *P. infestans* isolates collected from different hosts. As they stand, these conclusions and hypotheses however carry wide-ranging consequences for the biology and management of late blight, and possibly of similar pathogens (downy mildews in the first place). Indeed, they show that *P. infestans*, often called a hemibiotroph [15-18], should rather be seen as an 'impecfect biotroph', since its basic infectious process and trophic mode is biotrophic, but eventually fails to keep its host alive like 'perfect biotrophs' (rust or powdery mildews, for instance) do for extended periods of time. The negative association between host damage and parasitic fitness, clearly evident from our data, tend to revert the commonly held equation that increased aggressiveness (*i.e.* faster and more extensive necrosis of host tissue) equates with greater fitness and invasion potential in *P. infestans* ([see e.g. 19]. This, together with the stability of adaptation patterns over serial passages though different hosts, open new and important ways to better control late blight through host genetic resistance.

Material and Methods

Plant material

The potato cultivar Bintje and the tomato cultivar Marmande – both highly susceptible to late blight – were grown in a glasshouse as described by Kröner et al. [15]. Fully developed young leaflets were collected from seven week-old plants for experiments.

Phytophthora infestans isolates

The *P. infestans* isolates 15.P50.09 (from potato, EU_13_A2_86 clonal lineage) and 15.TII.09 (from tomato, EU_23_A1 clonal lineage) were collected in 2015 in France from naturally infected potato and tomato crops. They were selected for this study on the basis of previously available genotypic and phenotypic data [15]: they belong to distinct clonal lineages that are – at least in France – typically separated by the host (potato/tomato) and provide the usual pattern of pathogenic fitness *in vitro* on these hosts (tomato isolates perform overall well, but potato isolates struggle on tomato). During six months, both isolates were maintained by serial transfers on pea broth agar medium in darkness at 15°C [90].

Experimental evolution experiment design

The experimental evolution experiment (S3 Fig) consisted in subculturing *P. infestans* isolates on detached leaflets: either on the original host (from which the isolate had been collected), on the alternative host (e.g. on tomato leaflets if the isolate had been collected on potato), or on alternating hosts (in turns on potato and on tomato). The same inoculum source was used to initiate these conditions for experimental evolution. For each condition, three replicated lineages were subcultured nine times at intervals of seven days. Inoculum (3.10^4 sporangia.mL^{-1}) was prepared in sterile water from sporangia produced on previously infected leaflets. New leaflets were inoculated on the abaxial side with four drops (each 20 µL) of this suspension. A detailed description of

453 inoculum preparation is available in Kröner et al. [15]. During the experimental evolution experiment,
454 temperature was maintained at 18/15°C (day/night) and the incubation chamber was illumined to
455 obtain 16 h day length. Fitness and effector gene expression of *P. infestans* were assessed
456 simultaneously, before and after experimental evolution.
457

458 **Estimation of pathogenic fitness**

459 A single fitness estimate, representing the mean fitness of *P. infestans* from three lineages (biological
460 replicates), was calculated as proposed by Montarry et al. [91]. Relevant life-history traits of *P.*
461 *infestans* (latency, lesion size and sporangia production) were measured on potato and tomato
462 leaflets. Experimental conditions equal those encountered during the experimental evolution
463 experiment, with the exception that only one drop of inoculum (instead of four) was placed next to
464 the center of a detached leaflet. To determine latency period, six replicate leaflets per replicated
465 lineage (6 leaflets x 3 lineages = 18 leaflets in total) were inspected for sporangia formation at 24 h
466 intervals, beginning two days post inoculation (dpi). At five dpi, lesion size and sporangia production
467 were assessed on the same leaflets, as described by Montarry et al. [90]. The two constants of the
468 fitness model were fixed as follows: the underlying leaflet size of potato and tomato (X) was
469 determined experimentally to 20.3 cm^2, and the assumed time available to exploit the leaf ($1/\mu$) was
470 set to five days. Fitness data (S1 Table) was used to calculate the Levin's measure of standardized
471 niche breath B_A on a scale from zero to one [15, 92]. For these calculations, potato and tomato were
472 considered as possible hosts of *P. infestans*. A low niche breath points to a high level of specialization
473 on one of these hosts, and *vice versa*.
474

475 **Effector gene expression**

476 **Sample preparation.** Effector gene expression was assessed at the same time as fitness in order to
477 assure identical conditions: same batch of leaflets, sporangia suspension and inoculation conditions.
478 Leaf discs (22 mm in diameter, centered on the site of inoculation) were sampled at 2 dpi and 4 dpi.

479 For each replicated lineage (three biological replicates), six leaf discs (one leaf disc per infected
480 leaflet) were grouped, shock frozen in liquid nitrogen and lyophilized. The sample preparation system
481 FastPrep-24 (MPbio) was used to obtain a homogenous powder. This was achieved by three
482 successive 30-second runs at speed 4. The homogenous powder was aliquoted to obtain two 5 mg
483 samples (two technical replicates of crushed tissue) that were stored at – 80 °C prior to RNA
484 extraction.
485
486 **RNA extraction, quality control and cDNA synthesis.** For each replicated lineage (three biological
487 replicates), total RNA was extracted separately from two 5 mg crushed-tissue samples by using the
488 SV Total RNA Isolation System (Promega), according to the manufacturer's instructions. The
489 suitability of this protocol for high quality RNA extraction – in the present experimental conditions –
490 was initially confirmed by calculating the RNA Integrity Number (RIN) from random samples by using
491 the 2100 Bioanalyzer (Agilent Technologies). During routine operation, total RNA was quantified by
492 the NanoDrop 1000 spectrophotometer (Thermo Scientific) and RNA quality was confirmed by
493 agarose gel electrophoresis. Synthesis of cDNA was performed with 1 µg of total RNA by using the
494 GoScriptTM Reverse Transcription System (Promega), according to the manufacturer's
495 recommendations. The absence of genomic DNA contamination was confirmed by amplification of an
496 intron-spanning region of the elongation factor 1 alpha (EF-1α, GenBank: DQ284495), as described by
497 Rosati et al. [93]. The cDNA samples were stored at -80 °C.
498
499 **qPCR primers, amplification conditions and calculations.** qPCR primers for eight *P. infestans* effector
500 genes (*AVRblb2*, *EPIC2B*, *EPI1*, *PexRD2*, *SNE1*, *PiNPP1.1*, *INF1*, *Pi03192*), the candidate effector gene
501 coding Carbonic Anhydrase (*CA*) and three reference genes (*ACTA*, *BTUB*, *EF2A*) have been designed
502 and optimized for this study (Table S2). Specificity of these primers was checked by sequencing
503 amplicons in both directions. Real-Time qPCR was performed twice on each cDNA sample (two
504 technical replicates of analysis) by using Lightcycler 480 SYBR Green I Master (ROCHE) chemistry in

505 combination with the Lightcycler 480 II system (ROCHE). PCR reaction volumes of 10 µL contained:
506 1.5 µL PCR-grade H_20, 0.5 µL of the forward and 0.5 µL of the reverse primers at a concentration of
507 10 µM respectively, 5 µL Master Mix (2X conc.) and 2.5 µL of cDNA template. Amplifications were
508 performed in 384-well plates under the following cycling conditions: 15 min at 95°C; 39 cycles of 15 s
509 at 95°C, 30 s at 62°C (but 64°C for *EPI1*, *ACTA* and *BTUB*) and 30 s at 72 °C. Melting curve analysis
510 was performed from 62°C to 96°C. Transcript abundance of effector genes was calculated by using
511 the relative quantification method described by Pfaffl [94]. To take into account for possible
512 variations of PCR efficiency, separate standard curves from template dilution series were included for
513 each PCR run x primer set x isolate combination (e.g. PCR run 1 x *AVRblb2* primer x isolate
514 15.P50.09). To calculate transcript abundance, Ct (threshold cycle) values were first transformed to
515 linear scale expression quantities. Expression of the target gene was then normalized in respect to
516 the geometric mean of the three reference genes. These normalized transcript abundance data (S3
517 Table) are expressed in this article as the means from three replicated lineages of *P. infestans* (three
518 biological replicates).
519
520 **Statistical analyses**
521 Statistical analyses were performed using the statistical software R version 3.1.1 [95]. The means of
522 fitness and transcript abundance of effector coding genes were calculated from three replicated
523 lineages of *P. infestans* (three biological replicates), after having averaged – for each replicate lineage
524 – results from technical replicates (for fitness: life-history traits measured on six leaflets; for
525 transcript abundance: two crushed-tissue samples from six leaflets x two PCR analysis).
526
527 Null hypotheses were rejected if $P < 0.05$. Data were transformed to logarithms or square roots when
528 necessary before performing analysis of variance (ANOVA), analysis of covariance (ANCOVA),
529 multiple comparisons of means with adjusted *P*-values (Student's t-tests), tests for association
530 between paired samples (Pearson's product moment correlation coefficient) and Redundancy

Analysis (RDA, data prepared for multivariate analysis is available in S4 Table). A permutation test for constrained multivariate analyses was performed to test the significance of discrimination between factors included in the RDA full model (experiment, isolate, subculturing host, and tested host). The full model was then simplified by excluding the factor "subculturing host", as model comparison revealed no significant difference.

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References

1. Kemen AC, Agler MT, Kemen E. Host-microbe and microbe-microbe interactions in the evolution of obligate plant parasitism. New Phytol. 2015;206(4):1207-28.

2. Oliver RP, Ipcho SVS. *Arabidopsis* pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens. Mol Plant Pathol. 2004;5(4):347-52.

3. Lewis DH. Concepts in fungal nutrition and the origin of biotrophy. Biol Rev. 1973;48(2):261-77.

555 4. Schulze-Lefert P, Panstruga R. Establishment of biotrophy by parasitic fungi and reprogramming of
556 host cells for disease resistance. Annu Rev Phytopathol. 2003;41:641-67.
557 5. van Kan JAL. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci.
558 2006;11(5):247-53.
559 6. Govrin EM, Levine A. The hypersensitive response facilitates plant infection by the necrotrophic
560 pathogen *Botrytis cinerea*. Curr Biol. 2000;10(13):751-7.
561 7. Kabbage M, Yarden O, Dickman MB. Pathogenic attributes of *Sclerotinia sclerotiorum*: Switching
562 from a biotrophic to necrotrophic lifestyle. Plant Sci. 2015;233:53-60.
563 8. Münch S, Lingner U, Floss DS, Ludwig N, Sauer N, Deising HB. The hemibiotrophic lifestyle of
564 *Colletotrichum* species. J Plant Physiol. 2008;165(1):41-51.
565 9. Kankanala P, Czymmek K, Valent B. Roles for rice membrane dynamics and plasmodesmata during
566 biotrophic invasion by the blast fungus. Plant Cell. 2007;19(2):706-24.
567 10. Kamoun S, Huitema E, Vleeshouwers V. Resistance to oomycetes: a general role for the
568 hypersensitive response? Trends Plant Sci. 1999;4(5):196-200.
569 11. Vleeshouwers V, van Dooijeweert W, Govers F, Kamoun S, Colon LT. The hypersensitive response
570 is associated with host and nonhost resistance to *Phytophthora infestans*. Planta. 2000;210(6):853-
571 64.
572 12. Kamoun S, Furzer O, Jones JDG, Judelson HS, Ali GS, Dalio RJD, et al. The Top 10 oomycete
573 pathogens in molecular plant pathology. Mol Plant Pathol. 2015;16(4):413-34.
574 13. Fry WE, Birch PRJ, Judelson HS, Grunwald NJ, Danies G, Everts KL, et al. Five reasons to consider
575 *Phytophthora infestans* a reemerging pathogen. Phytopathology. 2015;105(7):966-81.
576 14. Andrivon D, Montarry J, Corbière R, Pasco C, Glais I, Marquer B, et al. The hard life of
577 *Phytophthora infestans*: when trade-offs shape evolution in a biotrophic plant pathogen. Plant
578 Pathol. 2013;62(Suppl. 1):28-35.

579 15. Kröner A, Mabon R, Corbière R, Montarry J, Andrivon D. The coexistence of generalist and
580 specialist clonal lineages in natural populations of the Irish Famine pathogen *Phytophthora infestans*
581 explains local adaptation to potato and tomato. Mol Ecol. 2017:DOI: 10.1111/mec.14004.
582 16. Danies G, Small IM, Myers K, Childers R, Fry WE. Phenotypic characterization of recent clonal
583 lineages of *Phytophthora infestans* in the United States. Plant Dis. 2013;97(7):873-81.
584 17. Smart CD, Myers KL, Restrepo S, Martin GB, Fry WE. Partial resistance of tomato to *Phytophthora*
585 *infestans* is not dependent upon ethylene, jasmonic acid, or salicylic acid signaling pathways. Mol
586 Plant Microbe Interact. 2003;16(2):141-8.
587 18. Vega-Sanchez ME, Erselius LJ, Rodriguez AM, Bastidas O, Hohl HR, Ojiambo PS, et al. Host
588 adaptation to potato and tomato within the US-1 clonal lineage of *Phytophthora infestans* in Uganda
589 and Kenya. Plant Pathol. 2000;49(5):531-9.
590 19. Cooke DEL, Cano LM, Raffaele S, Bain RA, Cooke LR, Etherington GJ, et al. Genome analyses of an
591 aggressive and invasive lineage of the Irish potato famine pathogen. PLoS Pathog.
592 2012;8(10):e100294.
593 20. Jones JDG, Dangl JL. The plant immune system. Nature. 2006;444(7117):323-9.
594 21. Fawke S, Doumane M, Schornack S. Oomycete interactions with plants: infection strategies and
595 resistance principles. Microbiol Mol Biol Rev. 2015;79(3):263-80.
596 22. Thines M, Kamoun S. Oomycete-plant coevolution: recent advances and future prospects. Curr
597 Opin Plant Biol. 2010;13(4):427-33.
598 23. Hein I, Gilroy EM, Armstrong MR, Birch PRJ. The zig-zag-zig in oomycete-plant interactions. Mol
599 Plant Pathol. 2009;10(4):547-62.
600 24. Zuluaga AP, Vega-Arreguín JC, Fei Z, Ponnala L, Lee SJ, Matas AJ, et al. Transcriptional dynamics of
601 *Phytophthora infestans* during sequential stages of hemibiotrophic infection of tomato. Mol Plant
602 Pathol. 2015;17(1):29-41.
603 25. Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker RE, Cano LM, et al. Genome sequence and
604 analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature. 2009;461(7262):393-8.

605 26. Dong SM, Raffaele S, Kamoun S. The two-speed genomes of filamentous pathogens: waltz with
606 plants. Curr Opin Genet Dev. 2015;35:57-65.
607 27. Tian MY, Huitema E, da Cunha L, Torto-Alalibo T, Kamoun S. A Kazal-like extracellular serine
608 protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease
609 P69B. J Biol Chem. 2004;279(25):26370-7.
610 28. Tian MY, Benedetti B, Kamoun S. A second kazal-like protease inhibitor from *Phytophthora*
611 *infestans* inhibits and interacts with the apoplastic pathogenesis-related protease P69B of tomato.
612 Plant Physiol. 2005;138(3):1785-93.
613 29. Song J, Win J, Tian M, Schornack S, Kaschani F, Ilyas M, et al. Apoplastic effectors secreted by two
614 unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3. Proc Natl Acad Sci
615 USA. 2009;106(5):1654-9.
616 30. Tian MY, Win J, Song J, van der Hoorn R, van der Knaap E, Kamoun S. A *Phytophthora infestans*
617 cystatin-like protein targets a novel tomato papain-like apoplastic protease. Plant Physiol.
618 2007;143(1):364-77.
619 31. Bozkurt TO, Schornack S, Win J, Shindo T, Ilyas M, Oliva R, et al. *Phytophthora infestans* effector
620 AVRblb2 prevents secretion of a plant immune protease at the haustorial interface. Proc Natl Acad
621 Sci USA. 2011;108(51):20832-7.
622 32. McLellan H, Boevink PC, Armstrong MR, Pritchard L, Gomez S, Morales J, et al. An RxLR effector
623 from *Phytophthora infestans* prevents re-localisation of two plant NAC transcription factors from the
624 endoplasmic reticulum to the nucleus. PLoS Pathog. 2013;9(10):e1003670.
625 33. Wang X, Boevink P, McLellan H, Armstrong M, Bukharova T, Qin Z, et al. A host KH RNA-binding
626 protein is a susceptibility factor targeted by an RXLR effector to promote late blight disease. Mol
627 Plant. 2015;8(9):1385-95.
628 34. van West P, de Jong AJ, Judelson HS, Emons AMC, Govers F. The *ipiO* gene of *Phytophthora*
629 *infestans* is highly expressed in invading hyphae during infection. Fungal Genet Biol. 1998;23(2):126-
630 38.

631 35. Pieterse CMJ, Derksen A, Folders J, Govers F. Expression of the *Phytophthora infestans ipiB* and
632 *ipiO* genes *in planta* and *in vitro*. Mol Gen Genet. 1994;244(3):269-77.
633 36. Kelley BS, Lee S-J, Damasceno CMB, Chakravarthy S, Kim B-D, Martin GB, et al. A secreted effector
634 protein (SNE1) from *Phytophthora infestans* is a broadly acting suppressor of programmed cell death.
635 The Plant Journal. 2010;62(3):357-66.
636 37. Kanneganti TD, Huitema E, Cakir C, Kamoun S. Synergistic interactions of the plant cell death
637 pathways induced by *Phytophthora infestans* Nep1-like protein PiNPP1.1 and INF1 elicitin. Mol Plant
638 Microbe Interact. 2006;19(8):854-63.
639 38. Lee S-J, Rose JKC. Mediation of the transition from biotrophy to necrotrophy in hemibiotrophic
640 plant pathogens by secreted effector proteins. Plant Signal Behav. 2010;5(6):769-72.
641 39. Raffaele S, Farrer RA, Cano LM, Studholme DJ, MacLean D, Thines M, et al. Genome evolution
642 following host jumps in the Irish Potato Famine pathogen lineage. Science. 2010;330(6010):1540-3.
643 40. Champouret N, Bouwmeester K, Rietman H, van der Lee T, Maliepaard C, Heupink A, et al.
644 Phytophthora infestans isolates lacking Class I *ipiO* variants are virulent on *Rpi-blb1* potato. Mol Plant
645 Microbe Interact. 2009;22(12):1535-45.
646 41. Devictor V, Clavel J, Julliard R, Lavergne S, Mouillot D, Thuiller W, et al. Defining and measuring
647 ecological specialization. J Appl Ecol. 2010;47(1):15-25.
648 42. Bolnick DI, Svanback R, Fordyce JA, Yang LH, Davis JM, Hulsey CD, et al. The ecology of individuals:
649 Incidence and implications of individual specialization. Am Nat. 2003;161(1):1-28.
650 43. Legard DE, Lee TY, Fry WE. Pathogenic specialization in *Phytophthora infestans*: aggressiveness on
651 tomato. Phytopathology. 1995;85(11):1356-61.
652 44. Michalska AM, Sobkowiak S, Flis B, Zimnoch-Guzowska E. Virulence and aggressiveness of
653 *Phytophthora infestans* isolates collected in Poland from potato and tomato plants identified no
654 strong specificity. Eur J Plant Pathol. 2016;144(2):325-36.
655 45. Hutchinson GE. Population studies -Animal ecology and demography - Concluding remarks. Cold
656 Spring Harbor Symposium. Quantitative Biology. 1957;22:415-27.

657 46. Kassen R. The experimental evolution of specialists, generalists, and the maintenance of diversity.
658 J Evol Biol. 2002;15(2):173-90.
659 47. Levins R. Evolution in changing environments. Princeton, New York: Princeton Univerity Press;
660 1968.
661 48. Lebreton L, Andrivon D. French isolates of *Phytophthora infestans* from potato and tomato differ
662 in phenotype and genotype. Eur J Plant Pathol. 1998;104(6):583-94.
663 49. Chowdappa P, Kumar BJN, Madhura S, Kumar SPM, Myers KL, Fry WE, et al. Severe outbreaks of
664 late blight on potato and tomato in South India caused by recent changes in the *Phytophthora*
665 *infestans* population. Plant Pathol. 2015;64(1):191-9.
666 50. Oyarzun PJ, Pozo A, Ordonez ME, Doucett K, Forbes GA. Host specificity of *Phytophthora infestans*
667 on tomato and potato in Ecuador. Phytopathology. 1998;88(3):265-71.
668 51. Suassuna ND, Maffia LA, Mizubuti ESG. Aggressiveness and host specificity of Brazilian isolates of
669 *Phytophthora infestans*. Plant Pathol. 2004;53(4):405-13.
670 52. Cohen Y. Populations of *Phytophthora infestans* in Israel underwent three major genetic changes
671 during 1983 to 2000. Phytopathology. 2002;92(3):300-7.
672 53. Ebert D. Experimental evolution of parasites. Science. 1998;282(5393):1432-5.
673 54. Akinsanmi OA, Chakraborty S, Backhouse D, Simpfendorfer S. Passage through alternative hosts
674 changes the fitness of *Fusarium graminearum* and *Fusarium pseudograminearum*. Environ Microbiol.
675 2007;9(2):512-20.
676 55. Montarry J, Cartier E, Jacquemond M, Palloix A, Moury B. Virus adaptation to quantitative plant
677 resistance: erosion or breakdown? J Evol Biol. 2012;25(11):2242-52.
678 56. Agudelo-Romero P, de la Iglesia F, Elena SF. The pleiotropic cost of host-specialization in Tobacco
679 etch potyvirus. Infect Genet Evol. 2008;8(6):806-14.
680 57. Andrivon D, Pilet F, Montarry J, Hafidi M, Corbière R, Achbani EH, et al. Adaptation of
681 *Phytophthora infestans* to partial resistance in potato: evidence from French and Moroccan
682 populations. Phytopathology. 2007;97(3):338-43.

683 58. Flier WG, van den Bosch GBM, Turkensteen LJ. Stability of partial resistance in potato cultivars
684 exposed to aggressive strains of *Phytophthora infestans*. Plant Pathol. 2003;52(3):326-37.
685 59. Caten CE. Intra-racial variation in *Phytophthora infestans* and adaptation to field resistance for
686 potato blight. Ann Appl Biol. 1974;77(3):259-70.
687 60. Jinks JL, Grindle M. Changes induced by training in *Phytophthora infestans*. Heredity.
688 1963;18(3):245-64.
689 61. Berg A. Tomato late blight and its relation to late blight of potato. Bull West Virginia Agrl Expt Sta.
690 1926;25:1-31.
691 62. Paxman GJ. Variation in *Phytophthora infestans*. European Potato Journal. 1963;6(1):14-23.
692 63. Molot PM, Leroux JP, Conus M, Burgerjeonglandard A. Use of transformed roots to preserve the
693 pathogenicity of fungal cultures. Agronomie. 1990;10(2):121-6.
694 64. Knapova G, Gisi U. Phenotypic and genotypic structure of *Phytophthora infestans* populations on
695 potato and tomato in France and Switzerland. Plant Pathol. 2002;51(5):641-53.
696 65. Lebreton L, Lucas JM, Andrivon D. Aggressiveness and competitive fitness of *Phytophthora*
697 *infestans* isolates collected from potato and tomato in France. Phytopathology. 1999;89(8):679-86.
698 66. Spiglazova S, Kuznetsova M, Smetanina T, Filippov A. Changes in *Phytophthora infestans*
699 aggressiveness as a result of repeated reproduction on different potato cultivars. PPO-Special Report.
700 2012;(15):225-8.
701 67. Guidot A, Jiang W, Ferdy JB, Thebaud C, Barberis P, Gouzy J, et al. Multihost experimental
702 evolution of the pathogen *Ralstonia solanacearum* unveils genes involved in adaptation to plants.
703 Mol Biol Evol. 2014;31(11):2913-28.
704 68. Wang B, Brubaker CL, Tate W, Woods MJ, Burdon JJ. Evolution of virulence in *Fusarium*
705 *oxysporum* f. sp *vasinfectum* using serial passage assays through susceptible cotton. Phytopathology.
706 2008;98(3):296-303.
707 69. Andrivon D. Biology, ecology, and epidemiology of the potato late blight pathogen *Phytophthora*
708 *infestans* in soil. Phytopathology. 1995;85(10):1053-6.

709 70. King SRF, McLellan H, Boevink PC, Armstrong MR, Bukharova T, Sukarta O, et al. *Phytophthora*
710 *infestans* RXLR effector PexRD2 interacts with host MAPKKK epsilon to suppress host cell death. Plant
711 Cell. 2014;26(3):1345-59.
712 71. Chen Y, Halterman DA. Phenotypic characterization of potato late blight resistance mediated by
713 the broad-spectrum resistance gene *RB*. Phytopathology. 2011;101(2):263-70.
714 72. Wang X, El Hadrami A, Adam LR, Daayf F. Differential activation and suppression of potato
715 defence responses by *Phytophthora infestans* isolates representing US-1 and US-8 genotypes. Plant
716 Pathol. 2008;57(6):1026-37.
717 73. Panina YS, Gerasimova NG, Chalenko GI, Vasyukova NI, Ozeretskovskaya OL. Salicylic acid and
718 phenylalanine ammonia lyase in potato plants infected with the causal agent of late blight. Russ J
719 Plant Physiol. 2005;52(4):511-5.
720 74. Kolomiets MV, Chen H, Gladon RJ, Braun EJ, Hannapel DJ. A leaf lipoxygenase of potato induced
721 specifically by pathogen infection. Plant Physiol. 2000;124(3):1121-30.
722 75. Joos HJ, Hahlbrock K. Phenylalanine ammonia lyase in potato (*Solanum tuberosum* L.) - genomic
723 complexity, structural comparison of two selected genes and modes of expression. Eur J Biochem.
724 1992;204(2):621-9.
725 76. Cuypers B, Hahlbrock K. Immunohistochemical studies of compatible and incompatible
726 interactions of potato leaves with *Phytophthora infestans* and of the nonhost response to
727 *Phytophthora megasperma*. Can J Bot. 1988;66(4):700-5.
728 77. Marcel S, Sawers R, Oakeley E, Angliker H, Paszkowski U. Tissue-adapted invasion strategies of
729 the rice blast fungus *Magnaporthe oryzae*. Plant Cell. 2010;22(9):3177-87.
730 78. Vargas WA, Martin JMS, Rech GE, Rivera LP, Benito EP, Diaz-Minguez JM, et al. Plant defense
731 mechanisms are activated during biotrophic and necrotrophic development of *Colletotricum*
732 *graminicola* in maize. Plant Physiol. 2012;158(3):1342-58.
733 79. Abramovitch RB, Martin GB. Strategies used by bacterial pathogens to suppress plant defenses.
734 Curr Opin Plant Biol. 2004;7(4):356-64.

735 80. Yao KN, Deluca V, Brisson N. Creation of a metabolic sink for tryptophan alters the
736 phenylpropanoid pathway and the susceptibility of potato to *Phytophthora infestans*. Plant Cell.
737 1995;7(11):1787-99.
738 81. Friend J, Reynolds SB, Aveyard MA. Phenylalanine ammonia lyase, chlorogenic acid and lignin in
739 potato tuber tissue inoculated with *Phytophthora infestans*. Physiol Plant Pathol. 1973;3(4):495-507.
740 82. Zuluaga AP, Vega-Arreguin JC, Fei ZJ, Matas AJ, Patev S, Fry WE, et al. Analysis of the tomato leaf
741 transcriptome during successive hemibiotrophic stages of a compatible interaction with the
742 oomycete pathogen *Phytophthora infestans*. Mol Plant Pathol. 2016;17(1):42-54.
743 83. Heath MC. Hypersensitive response-related death. Plant Mol Biol. 2000;44(3):321-34.
744 84. Xu QF, Cheng WS, Li SS, Li W, Zhang ZX, Xu YP, et al. Identification of genes required for Cf-
745 dependent hypersensitive cell death by combined proteomic and RNA interfering analyses. J Exp Bot.
746 2012;63(7):2421-35.
747 85. Wu L, Chen H, Curtis C, Fu ZQ. Go in for the kill How plants deploy effector-triggered immunity to
748 combat pathogens. Virulence. 2014;5(7):710-21.
749 86. Meng X, Zhang S. MAPK cascades in pant disease resistance signaling. Annu Rev Phytopathol.
750 2013; 51:245-66.
751 87. Coll NS, Epple P, Dangl JL. Programmed cell death in the plant immune system. Cell Death Differ.
752 2011;18(8):1247-56.
753 88. Hofius D, Tsitsigiannis DI, Jones JDG, Mundy J. Inducible cell death in plant immunity. Semin
754 Cancer Biol. 2007;17(2):166-87.
755 89. Freytag S, Arabatzis N, Hahlbrock K, Schmelzer E. Reversible cytoplasmic rearrangements precede
756 wall apposition, hypersensitive cell-death and defense-related gene activation in potato
757 *Phytophthora infestans* interactions. Planta. 1994;194(1):123-35.
758 90. Montarry J, Corbière R, Lesueur S, Glais I, Andrivon D. Does selection by resistant hosts trigger
759 local adaptation in plant-pathogen systems? J Evol Biol. 2006;19(2):522-31.

760 91. Montarry J, Hamelin FM, Glais I, Corbière R, Andrivon D. Fitness costs associated with
761 unnecessary virulence factors and life history traits: evolutionary insights from the potato late blight
762 pathogen *Phytophthora infestans*. BMC Evol Biol. 2010;10:283.
763 92. Donovan TM, Welden CW. Spreadsheet exercises in ecology and evolution. Sunderland,
764 Massachusetts: Sinauer Associates, Inc.; 2002.
765 93. Rosati C, Cadic A, Duron M, Renou JP, Simoneau P. Molecular cloning and expression analysis of
766 dihydroflavonol 4-reductase gene in flower organs of *Forsythia x intermedia*. Plant Mol Biol.
767 1997;35(3):303-11.
768 94. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic
769 Acids Res. 2001;29(9):e45.
770 95. R Development Core Team. R: A language and environment for statistical computing. Vienna,
771 Austria: R Foundation for Statistical Computing; 2014.

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774 Supporting information captions

775

776 **S1 Appendix. Hypothesis - The proteases inhibitors EPIC2B/EPI1 block immunity-related**
777 **programmed cell death.**

778

779 **S1 Fig. Effector genes equally expressed before and after experimental evolution.** Two *P. infestans*
780 isolates collected on potato (P-isolate 15.P50.09) and on tomato (T-isolate 15.TII.02) had been
781 subcultured nine times on their original host: the P-isolate on the potato cv. Bintje and the T-isolate
782 on the tomato cv. Marmande. Effector gene expression was assessed before (dark grey bars) and
783 after subculturing (white bars). Both measurements were performed on the potato cv. Bintje and on
784 the tomato cv. Marmande, but results were averaged as the effects of subculturing were similar on

785 both tested hosts. For effector genes shown here, pairwise t-tests revealed no significant differences
786 in transcript abundance before and after nine times subculture (n=6, significance level $\alpha = 0.05$, n.s. =
787 not significant).
788
789 **S2 Fig. Loading plot of redundancy analysis.** Correlations among effector genes and fitness are
790 displayed on the first and the third constrained component axis, representing respectively 56.54 and
791 15.48 % (cumulative 72.02 %) of the constraint variance.
792
793 **S3 Fig. Experimental evolution experiment design.** *Phytophthora infestans* inoculum was prepared
794 on the host from which the isolate had been collected. This base inoculum served to start
795 experimental evolution, consisting in nine times subculture on detached leaflets: either on the
796 original host (from which the isolate had been collected), on the alternative host (e.g. on tomato
797 leaflets if the isolate had been collected on potato), or on alternating hosts (in turns on potato and
798 tomato). Each of these lineages was replicated three times. Fitness and effector gene expression of *P.*
799 *infestans* on potato and tomato were assessed simultaneously, before and after experimental
800 evolution.
801
802 **S1 Table. *Phytophthora infestans* pathogenic fitness.**
803
804 **S2 Table. RT-PCRq primer sequences for genes of interest and reference genes, amplicon length**
805 **and primer efficiency.**
806 **S3 Table. *Phytophthora infestans* effector gene expression (normalized transcript abundance).**
807
808 **S4 Table. *Phytophthora infestans* mean fitness and effector gene expression (normalized transcript**
809 **abundance) for RDA analysis.**

Figures

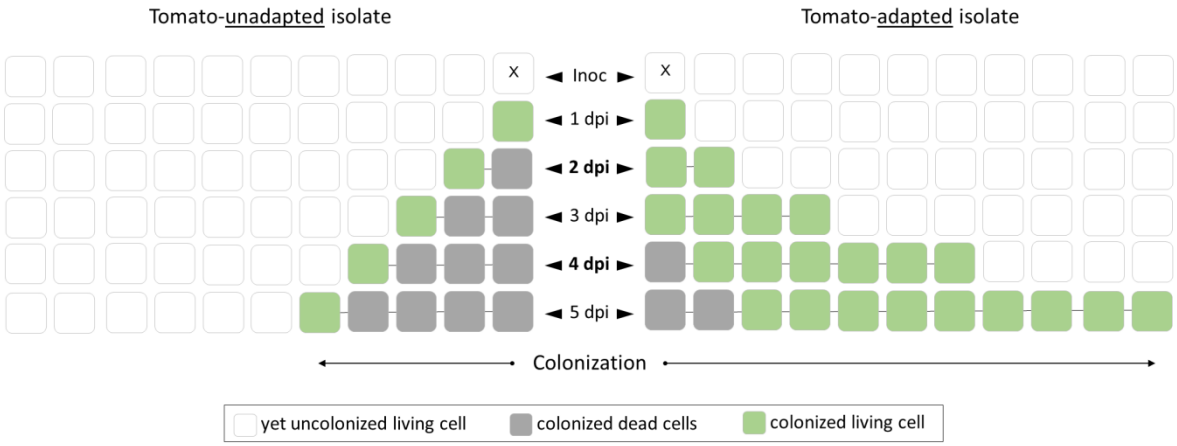

Fig 1. Colonization of tomato leaf tissue by adapted and unadapted *Phytophthora infestans* isolates. This schematic view illustrates spatio-temporal changes of host cell status (uncolonized/colonized, living/dead) in tomato leaf tissue typically observed during colonization by differentially adapted *P. infestans* isolates. Horizontal reading (line-by-line) illustrates the spatial aspect of colonization at one to five dpi respectively. Vertical reading (column-by-column) illustrates the temporal status of a given host cell.

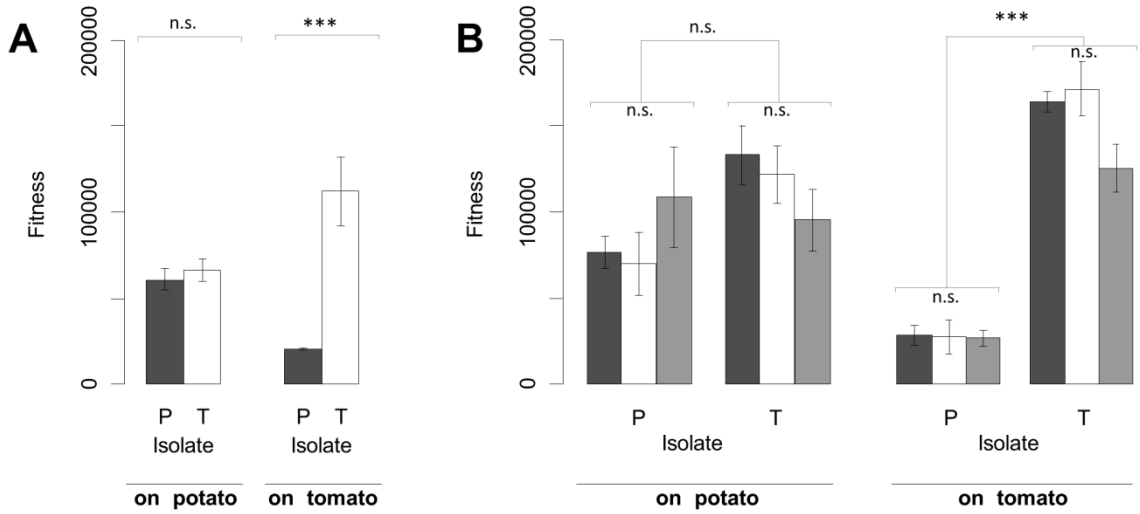

Fig 2. Pathogenic fitness of *Phytophthora infestans* before and after experimental evolution. Two *P. infestans* isolates collected on potato (P-isolate, 15.P50.09) and on tomato (T-isolate, 15.TII.02) were cross-inoculated on detached leaflets of the potato cv. Bintje and the tomato cv. Marmande. Pathogenic fitness at five dpi has been assessed two times: before experimental evolution **(A)** and after experimental evolution **(B).** Experimental evolution consisted of subculturing the initial inoculum nine times on potato (dark grey bars), tomato (white bars) and alternately on both hosts (light grey bars). Significance of differences was assessed by ANOVA followed by pairwise t-tests (significance level $\alpha = 0.05$, *** $P < 0.001$, n.s. not significant).

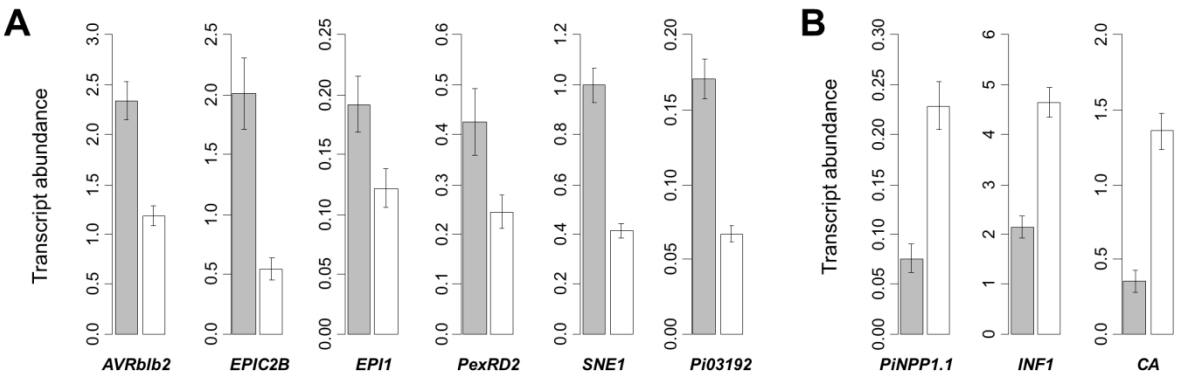

Fig 3. Effector gene expression in *Phytophthora infestans* at two infection stages. Transcript abundance has been assessed at the early biotrophic (2 dpi, grey bars) and at the later transitory/necrotrophic stage of infection (4 dpi, white bars). Some genes were found to be more expressed at 2 dpi **(A)**, but others at 4 dpi **(B)**. The relative timing of gene expression was the same for both isolates (15-P50.09 and 15-TII-02) tested on both hosts (the potato cv. Bintje and the tomato cv. Marmande), before and after nine times subculturing. Results are thus presented as the global mean of these factors (n = 96).

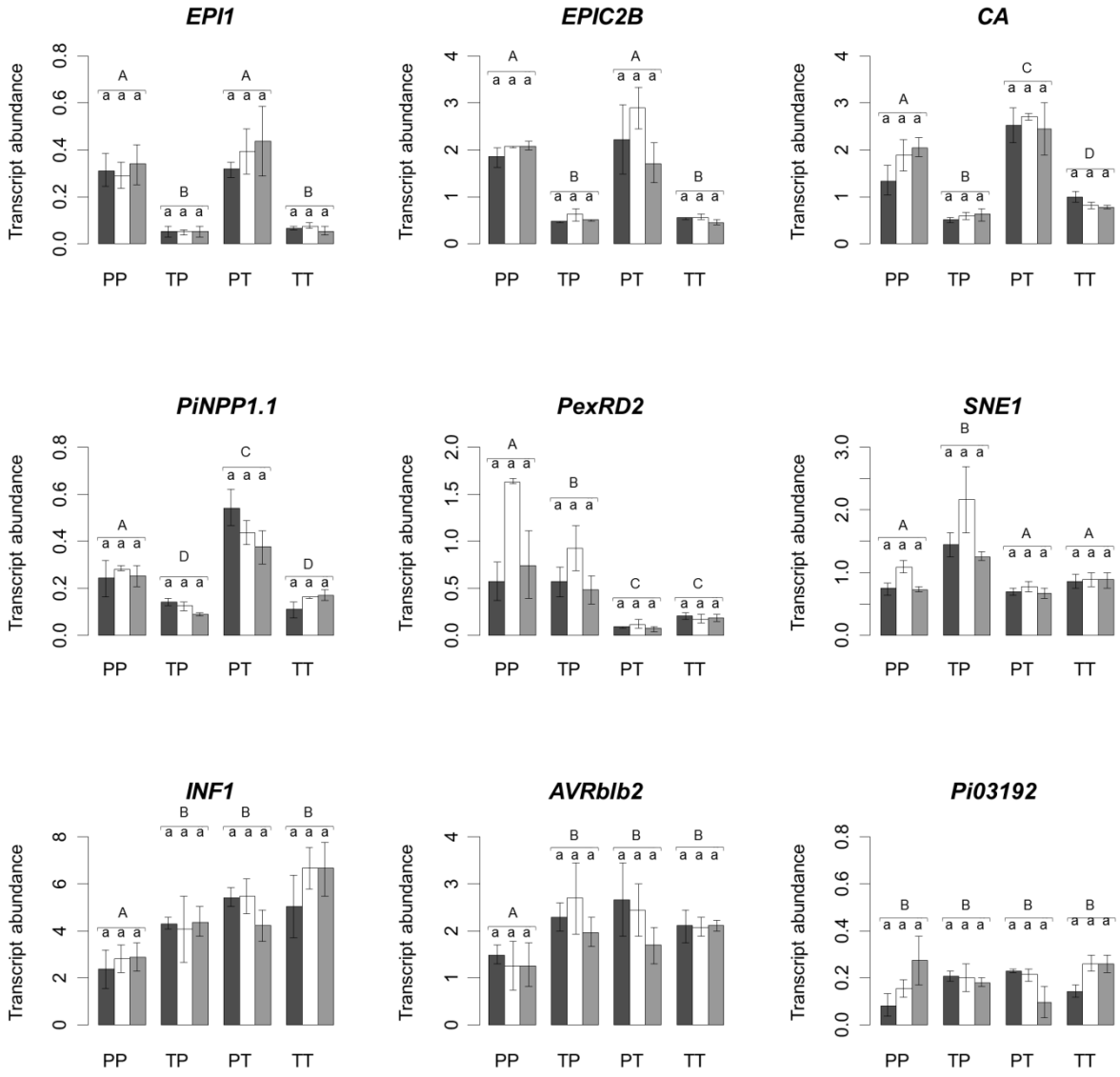

Fig 4. Effector gene expression of *Phytophthora infestans* after experimental evolution. Two *P. infestans* isolates collected on potato (P-isolate 15.P50.09) and on tomato (T-isolate 15.TII.02) have been subcultured nine times on potato (dark grey bars, n=3), on tomato (white bars, n=3) and alternately on both hosts (light grey bars, n=3). Transcript abundance of these experimentally evolved isolates was assessed on the potato cv. Bintje and the tomato cv. Marmande at two dpi (*INF1*, *CA* and *PiNPP* at four dpi), resulting in the following isolate-host combinations: P-isolate on potato (PP), T-isolate on potato (TP), P-isolate on tomato (PT) and T-isolate on tomato (TT). Different capital letters above bars indicate significant mean differences between isolate-host combinations

847 (pairwise t-test, n=9, significance level α = 0.05). There were no significant differences in transcript
848 abundance among subculturing hosts (see minuscule letters above bars).

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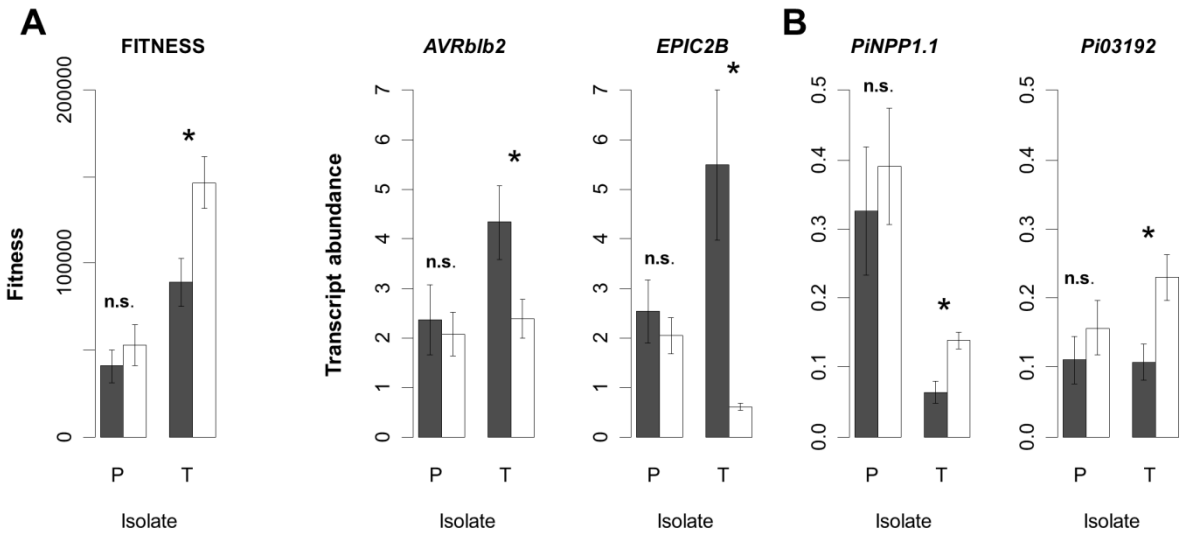
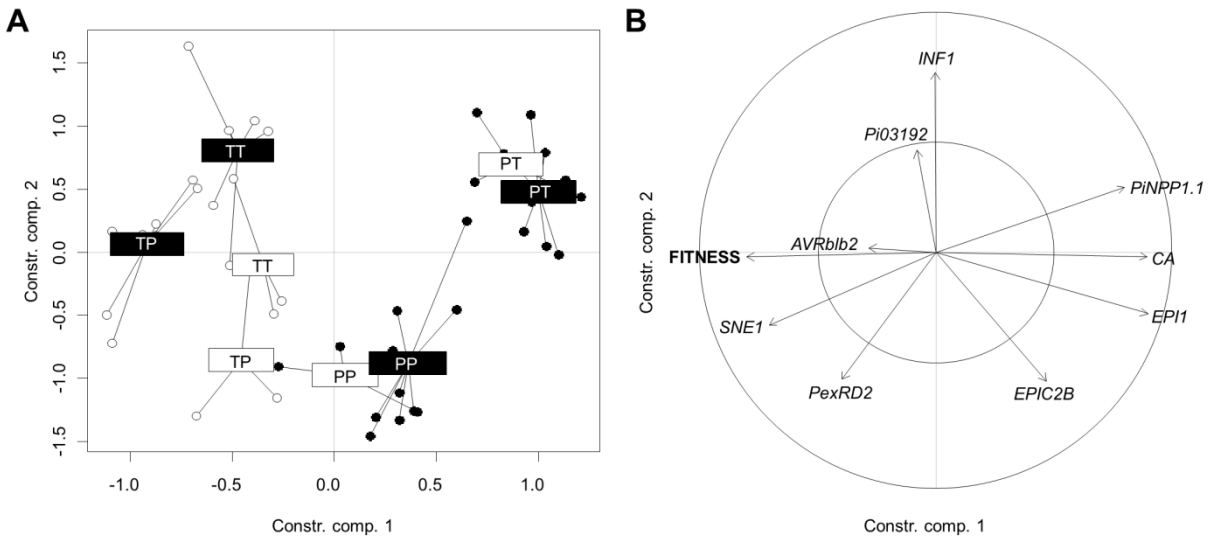


851

852 **Fig 5. Isolate-specific effects of subculturing on fitness and effector gene expression.** Two *P.*
853 *infestans* isolates collected on potato (P-isolate 15.P50.09) and on tomato (T-isolate 15.TII.02) had
854 been subcultured nine times on their original host: the P-isolate on the potato cv. Bintje and the T-
855 isolate on the tomato cv. Marmande. Fitness **(A)** and effector gene expression **(B)** were assessed
856 before (dark grey bars) and after subculturing (white bars). Fitness was assessed at five dpi and gene
857 expression at two dpi (or at four dpi for *PiNPP*). Both measurements were performed on potato and
858 on tomato. Results were averaged as the effects of subculturing were similar on both tested hosts.
859 Significance of isolate-specific mean differences (t-test comparing before and after subculture, n=6,
860 significance level α = 0.05) is indicated above bars (n.s. = not significant, * $P < 0.05$).



861

862 **Fig 6. Redundancy analysis (RDA) for transcript abundance and fitness of *Phytophthora infestans*.**

863 **(A)** A factorial map separating factor combinations. White boxes represent centroids before

864 experimental evolution and black boxes centroids after experimental evolution. Isolate-host

865 combinations are abbreviated as follows: P-isolate on Potato (PP), T-isolate on Potato (TP), P-isolate

866 on Tomato (PT) and T-isolate on Tomato (TT). Subculturing habitats (on potato, on tomato,

867 alternately on both hosts) are not tagged because model comparison with and without the

868 subculturing host variable revealed no significant difference (P = 0.82). **(B)** A loading plot showing

869 correlations among effector genes and fitness.

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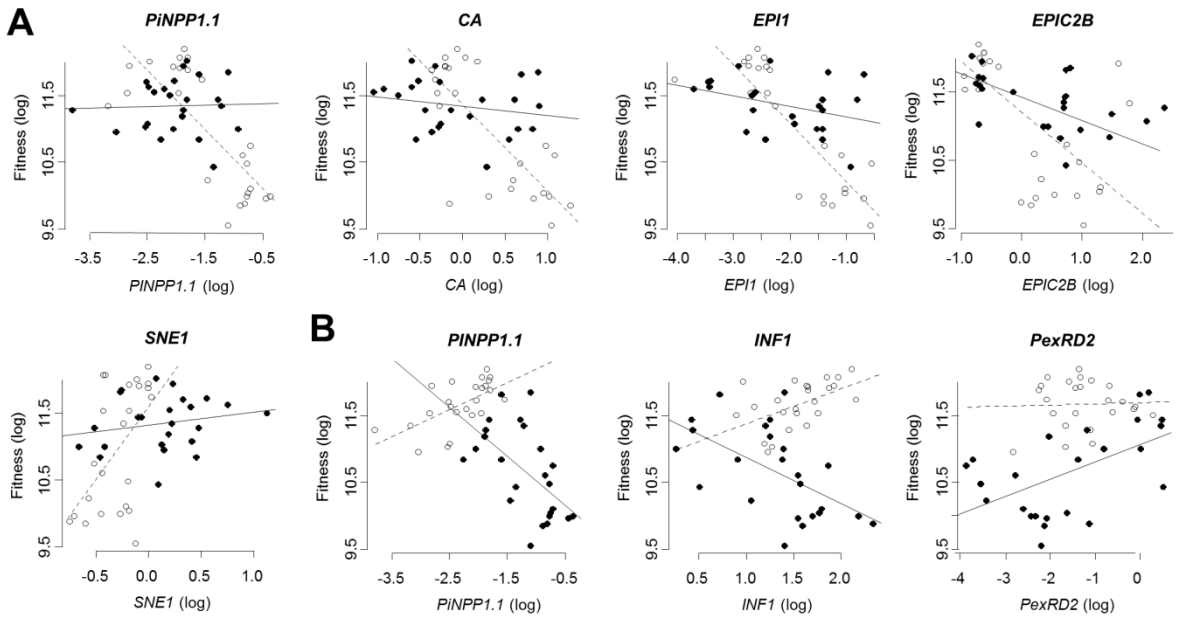

871

872 **Fig 7. Within-group correlations between fitness and effector genes of *Phytophthora infestans*. (A)**
873 Data were grouped by tested host. Black circles represent data obtained on potato (cv. Bintje,
874 correlations not significant at $\alpha = 0.05$, except for *EPIC2B*) and white circles represent data on tomato
875 (cv. Marmande, correlations highly significant). (**B)** Data were grouped by isolate. Black circles
876 represent data obtained for isolate 15.P50.09 (collected from potato, significant negative
877 correlations for *PiNPP* and *INF1* but positive correlation for *PexRD2*) and white circles represent data
878 for isolate 15.TII.02 (collected from tomato, highly positive correlations except for *PexRD2*).

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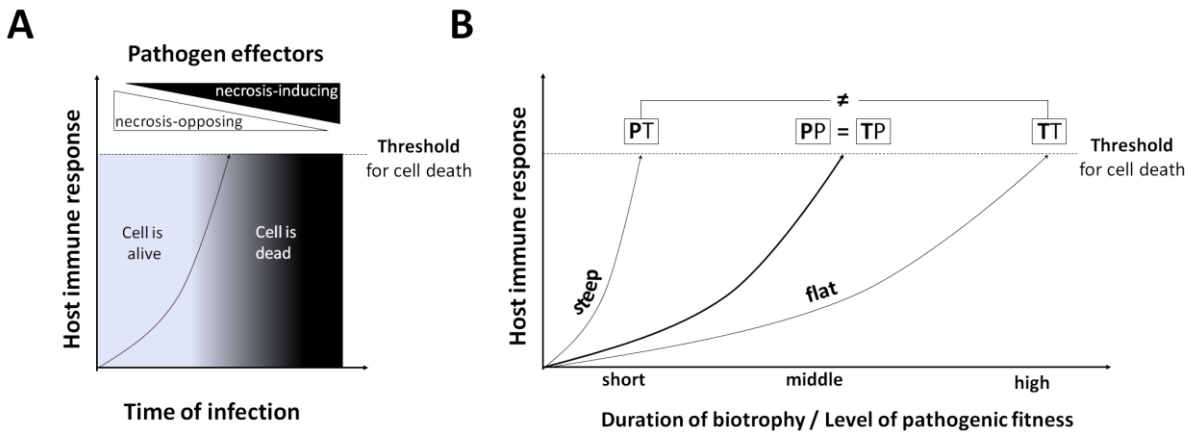

Fig 8. Host immune responses could limit the duration of biotrophy in *Phytophthora infestans* interactions. (A) Host immune responses force *P. infestans* to kill its host. It is well established that *P. infestans* secretes effectors into the parasitized host cell adnd that secretion is timely regulated. Biotrophy related effectors are secreted in the early state of the interaction to maintain the host cell alive. With progressing time of infection, secretion of pro-life effectors decreases while necrotrophy related effectors with cell-death inducing activity are increasingly secreted. As the parasitized cells die concomitantly to this switch, a tightly controlled and timely secretion of necrosis-opposing and necrosis-favoring effectors is regarded as a mean of *P. infestans* to regulate the duration of biotrophy. However, the reasons why *P. infestans* – an almost obligate parasite – actively kills host cells is not understood and may even appear counterintuitive. The here advocated explanatory approach refers to the "increasing host defense" theory (see explanations in the text) suggesting biotrophic pathogens to actively kill the host cell as a last resort against an intolerable level (threshold) of host defenses. Literature reports about steadily increasing host defenses in *P. infestans* interactions in conjunction with the here and previously observed timely regulated expression of pro-life/pro-death effector genes (e.g. SNE1/PiNPP1.1) support this view. **(B)** Duration of biotrophy in *P. infestans* interactions is positively related with host adaptation. It has been proposed that timing and intensity of host immune responses impact on the outcome of interactions between *P. infestans* and its hosts. Results from a range of studies show that immune responses have to be induced timely and strongly to be effective against *P. infestans*. Considering these results and with regard to the theory

that strong host immune responses force *P. infestans* to rapidly kill its host (see A), we would expect duration of biotrophy to be positively related with the level of host adaptation. Consistent to this prediction, similar pathogenic fitness of both tested isolates on potato matched macroscopically assessments of lesions. Furthermore, higher fitness of the tomato isolate on tomato in respect to the potato isolate matched a visibly more biotrophic interaction. Isolate-host combinations are abbreviated as follows: P-isolate on Potato (PP), T-isolate on Potato (TP), P-isolate on Tomato (PT) and T-isolate on Tomato (TT).

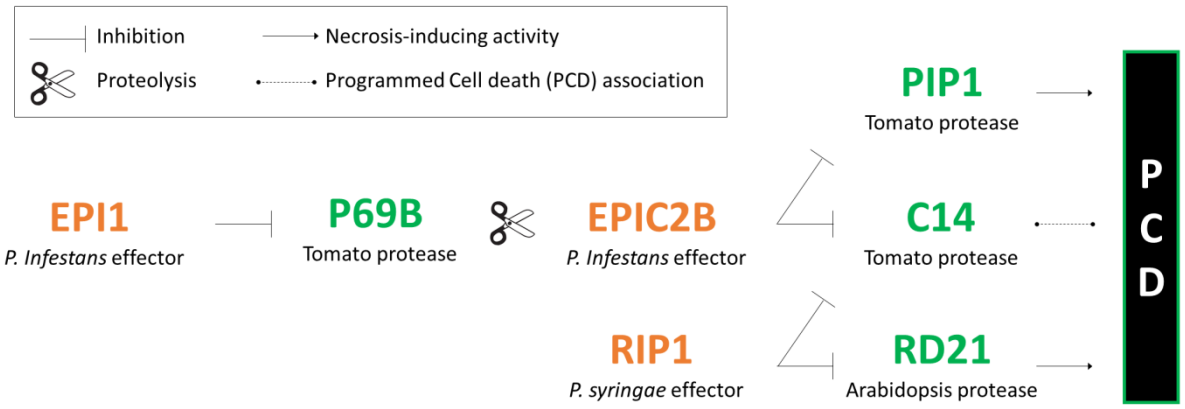

Fig 9. Protease inhibitors EPI1 and EPIC2B could block immunity-related programmed cell death. The *P. infestans* effector EPI1 prevents degradation of EPIC2B by inhibiting the tomato protease P69B. The thereby guarded EPIC2B inhibits in turn the tomato protease C14 that has been reported to be associated with stress-induced programmed cell death (PCD). EPIC2B also inhibits the tomato protease PIP1 that has been shown to be required for HCD. Tomato C14 is furthermore inhibited by the *Pseudomonas syringae* effector RIP1 that also inhibits in Arabidopsis the pro-death protease RD21. Altogether, these literature findings show that *P. infestans* could take control about the tomato host cell death machinery by secreting proteases inhibitors targeting cell death-related host proteases (e.g. PIP1, C14).