Title

Effector loss and gain drives pathogen host range at a fitness cost.

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Abstract

The genetic constraints for microbial host adaptation to emerge for generalist and specialist behaviors remain unclear but is key for epidemiological preparedness. Here, we show that generalist cereal pathogen *Xanthomonas translucens* arose from a specialist ancestor via the loss of a single effector gene, *xopAL1*. Deleting barley-specialist *X. translucens xopAL1* recapitulated the host jump to wheat and demonstrates risk across each globally distributed genetic lineages. However, this niche expansion via XopAL1 loss incurs a significant pathogenic fitness cost on barley. Moreover, the specialist lineage gained additional effector genes, such as *xopAJ*, enhancing virulence on barley while restricting oat infection, thereby reinforcing niche specialization. We further identified and validated key host defense genes that restrict host infection, providing new, undefined targets for crop improvement. Our work provides a validated evolutionary framework of intergenera host jump, demonstrating that single loss and gain events create a dynamic trade-off between niche breadth and specialization.

Teaser

Evolutionary events of gene loss and gain shape the ecological niche of *Xanthomonas translucens* for host plant range.

MAIN TEXT

Introduction

Disease outbreaks often result directly from pathogen evolution, leading to changes in microbial niche specialization. Changes in niche-specificity may occur from single genetic loss or gain events (*1, 2*) or the movement of genomic islands and plasmids that dictate the transitional

behaviors such as niche shift between microbial pathogens and their hosts (*3, 4*). Niche alterations are universal and widespread in plant, human and animal epidemiology and have significant impact on host health outcomes (*5*). Some pathogens have generalist behaviors with broad host ranges while others are highly specialized on a particular host or niche (*6, 7*). However, the underlying evolutionary and biological mechanisms often remain undefined for most niche changes, especially in plant agricultural systems.

Host range is a particular type of niche-specificity, and many pathogenic organisms can be found across the spectrum from generalist to specialist. The Gammaproteobacterial genus *Xanthomonas* limits agricultural production by causing diseases in over 400 identified plant species, although these pathogens as a whole infect close to 50 plant families. *Xanthomonas* bacteria in the generalist spectrum cause disease in a broad range of host genera with access to a breadth of potential hosts for resources, while specialists remain restricted to a particular species or genus (Fig. 1A and Fig. S1) (*8-15*). Curiously, differences in niche-specificity of closely related plant pathogenic species and strains are often due to small changes in genome content (*16-19*) but the evolutionary mechanisms for niche specialization of *Xanthomonas* spp. remains poorly understood. Ultimately, understanding the spectrum and evolution of host range specificity will benefit prediction and mitigation of disease epidemics (*6, 7, 20*).

Bacterial organisms regularly secrete proteins and other molecules into the extracellular environment or directly into host cells to interact with their niche aiming at promoting growth, survival, colonization and virulence (*21, 22*). Many Gram-negative animal and plant bacterial pathogens, including *Xanthomonas spp.*, depend on a Type III secretion system (T3SS) for injecting effector proteins into the host cells to aid in pathogenesis (*9, 23-25*). Our previous work revealed that frequent loss and gain of a single gene encoding a Type II secreted cellulase effector, CbsA, promoted lifestyle switch for changes in tissue-specific host adaptation (*2*). Here, we used a similar framework to define the evolution of host range shift in specialist and generalist subgroups (e.g. respectively, narrow and broad host range) of *Xanthomonas translucens*, arguably the most important re-emergent bacterial pathogen of cereals beyond rice (*26-28*). We have determined that evolutionary loss and gain of Type III effectors govern the phenotypic switch between specialist and generalist behaviors of *X. translucens* subgroups for host range. Specifically, we determined that the broad host range lineage lost a single effector, XopAL1, to evade host defenses but at fitness cost on a primary host. Moreover, we assessed the potential for contemporary *X. translucens* specialist pathogens to emerge providing a framework for epidemiological tracking of particular lineages of risk.

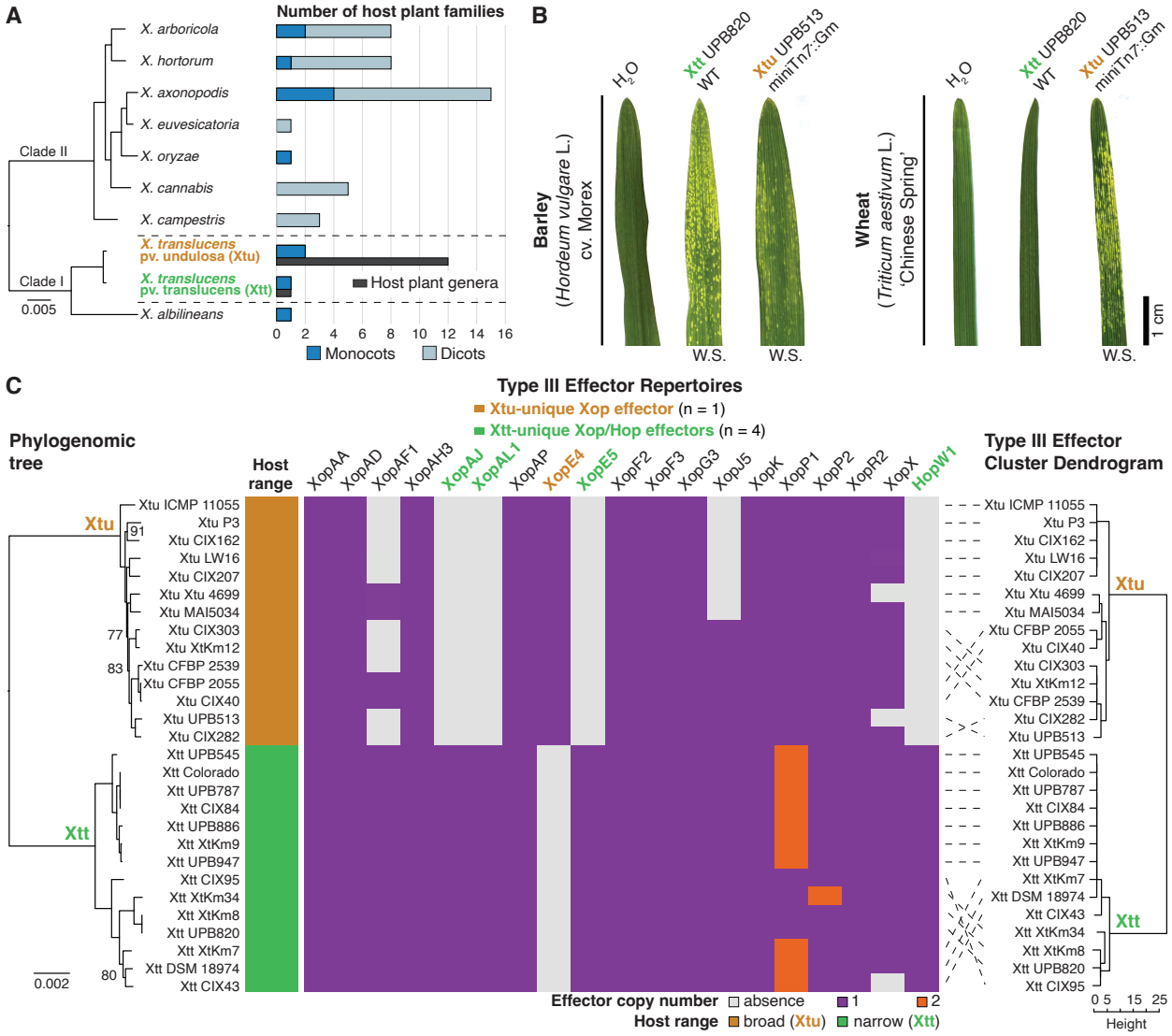


Fig. 1. *X. translucens* pathovars with differential host range breadth encode significantly different effector repertoires. **A.** Phylogenomics and host range of representative *Xanthomonas* spp. The number of plant families that are known hosts (natural or experimentally determined) for each analyzed *Xanthomonas* spp. are shown in the chart to the right side of the panel. The number of plant genera that are known hosts for *X. translucens* pathovars translucens and undulosa is also included in the chart. **B.** Symptom development in barley (*Hordeum vulgare* L. cv. Morex) and wheat (*Triticum aestivum* L. genotype 'Chinese Spring') leaves inoculated (spray) with Xtu and Xtt strains (OD_{600} 0.5) at six days post inoculation. Water soaking symptoms were visualized using a light box and appear as lighter areas in the leaf. Scale bar is shown in figure. W.S. – water soaking. **C.** Effector repertoires of *X. translucens* pvs. translucens and undulosa (only non-TAL effectors are presented). A core genome-based tree of 28 *X. translucens* strains is shown to the left side of the panel. Putative type III secretion system effectors were identified via local Blastx against a database of known *Xanthomonas* effectors. Clustering of strains according to a hierarchical clustering of their encoded repertoires (type III effector cluster dendrogram) is shown to the right side of the panel. Trees were visualized via

FigTree, and mid-point rooted. Branches with bootstrap values below 98% are indicated in each tree. Xtt - *X. translucens* pv. translucens; Xtu - *X. translucens* pv. undulosa.

Results

Xanthomonas translucens subgroups with differential host range behaviors encode significantly different Type III effector repertoires

Bacteria in the *X. translucens* species primarily infect grass and cereal plants in the Poaceae family, causing bacterial leaf streak (BLS) and blight, and black chaff of cereals (*26, 28*). Early documentation from the 1900s described the broad host range of generalist *X. translucens* pv. undulosa (Xtu) (*10, 11*). This subgroup uses a wide range of metabolites upon infection of host plants (*29*) and causes disease in wheat, barley, rye, triticale, spelt, wild oat, ornamental asparagus, quackgrass, perennial ryegrass, canary grass, green foxtail, intermediate wheatgrass, and wild rice, totaling 12 plant genera (Fig. 1A and Fig. S1) (*10, 11, 28, 30-35*). In contrast, the closely related *X. translucens* pv. translucens (Xtt) is a specialist with limited metabolism that only causes disease in plants of the *Hordeum* genus (barley) (Fig. 1A-B and Fig. S1) (*10, 29, 31*). The main small grains affected by Xtt and Xtu are barley and wheat, respectively (*26, 31*). For example, upon spray inoculation for naturalistic entry and infection, Xtt and Xtu both caused water soaking symptom development in barley, but only generalist Xtu caused disease in wheat, while Xtt elicited no response in wheat (Fig. 1B).

To better understand differences in niche specificity between the closely related (>97% average nucleotide identity) (*36*) but phylogenomically distinct Xtt and Xtu (Fig. 1A,C), comparative genomic analyses were conducted and revealed that over 3,000 core proteins are shared between them (Table S1). On the other hand, each subgroup had over 100 unique conserved proteins (Table S2). Secreted proteins, which are often associated with virulence (*21, 22*), represented 3% and 3.5% of shared (core genome) and unique proteins (accessory genome), respectively (Tables S1-2). The number of unique, overall secreted proteins were not significantly different between Xtt and Xtu (Student's *t*-test; *P* value = 0.7772). However, specialist Xtt has a larger Type III-secreted effector repertoire than generalist Xtu (Wilcoxon rank-sum test; *P* value = 3.436 e-06), while their individual core genomes did not significantly differ in shared gene content (Student's *t*-test; *P* value = 0.3064).

Based on the biological importance of Type III effectors (*23, 24*), we aimed to determine if there is a link between host range of *X. translucens* and encoded Type III effector repertoires. Hierarchical clustering of strains according to their encoded effector repertoires was congruent with the subspecies tree (Fig. 1C), and Type III-secreted virulence effector abundance significantly related phylogenomically and with generalist and specialist pathogenic behaviors (Fig. 1C; Wilcoxon rank-sum test; *P* value = 3.436 e-06). Specialist Xtt genomes included an expanded effector repertoire that included XopAJ, XopAL1, XopE5, and HopW1 and an effector family expansion for XopP1 and XopP2. Only one unique Type III effector was identified in Xtu (XopE4), while seven were overall absent in this generalist subgroup (Fig. 1C). Moreover, Type III-secreted transcription activator-like (TAL) effectors were significantly more abundant in broad host range Xtu (Fig. S2; Student's *t*-test; *P* value = 0.02511). Highly virulent Xtu strains have previously been found to precisely manipulate plant hormone pathways via the Tal8 (synonym TalDC) effector to cause wheat bacterial leaf streak (*37*). This effector is not encoded by low-virulence strains of Xtu or any Xtt strain (Fig. S2) (*37*). We therefore hypothesize the

145 TAL effector family expansion correlates with adaptation to new niches as these are highly
146 evolvable repetitive elements (*38-41*). While we only focused on non-TAL effectors in this
147 study, future work will explore host adaptation based on this special and abundant class of
148 effectors.
149
150 **The T3SS effector XopAL1 impacts both host range and fitness in *X. translucens***
151 We hypothesized that individual Type III-secreted effectors might be responsible for host range
152 differences between Xtt and Xtu. We first tested pathogenicity on both barley and wheat for
153 Δ*hrcT* mutants that lack a critical component for the *X. translucens* T3SS by inoculating leaves
154 via syringe infiltration (Fig. S3A) (*24*). Δ*hrcT* mutants of Xtt and Xtu did not cause symptoms in
155 barley or wheat. Unlike the lack of symptom development upon natural infection via spray (Fig.
156 1B), Xtt caused chlorosis in wheat when inoculated via syringe infiltration (Fig. S3A). This has
157 been described as a non-host or non-pathogenic response unlike the well-described
158 hypersensitive response and is an undefined plant response that prevents disease development by
159 Xtt (*14, 24, 42-45*).
160
161 We then aimed to define if a unique Xtt effector prevented wheat pathogenesis by screening for
162 loss of chlorosis. Xtt- and Xtu-unique effectors were individually deleted to investigate their
163 contribution to the differential host range breadth between these *X. translucens* subgroups.
164 Deletion of the four individual Xtt-unique or single Xtu-unique effector genes did not alter
165 pathogenicity in barley or chlorosis development in wheat upon infiltration inoculation except
166 for *xopAL1* (Fig. S3). Notably, *xopAL1* deletion in Xtt produced water soaking symptom
167 development on wheat similar to Xtu BLS (Fig. S3A). Moreover, complementation of the Xtt
168 Δ*xopAL1* mutant strain or *xopAL1* introduction into the true wheat pathogen Xtu (Xtu
169 miniTn7::*xopAL1*) triggered development of the non-pathogenic, chlorosis phenotype in wheat
170 (Fig. S3A). Introduction of the other three Xtt-unique effectors in Xtu did not trigger the wheat
171 chlorotic response (Fig. S3B).
172
173 These findings were further assessed by spray inoculation, an infection method more similar to
174 natural infections than syringe infiltration. Xtt Δ*xopAL1* caused BLS similar to Xtu on wheat and
175 barley (Fig. 2A). On the other hand, introduction of this effector into Xtu disrupted its ability to
176 cause disease in wheat (Fig. 2A). Curiously, deletion of *xopAL1* significantly decreased the
177 symptomatic leaf area of barley inoculated via spray (Fig. 2B). Because of the reduced virulence
178 of Xtt Δ*xopAL1* and Xtu although pathogenic is infrequently isolated from barley (*46*), we
179 suspected that XopAL1 plays a role in bacterial fitness beyond restricting the ability of Xtt to
180 cause disease in wheat. Significantly higher endophytic populations of Xtt wild-type (WT) and
181 Xtu miniTn7::*xopAL1* were observed in barley leaves at six days post spray inoculation in
182 comparison to non-*xopAL1*-encoding strains (Xtt Δ*xopAL1* and Xtu WT; Fig. 2C). Moreover, Xtt
183 Δ*xopAL1* had a similar population to Xtu in wheat upon syringe infiltration, whereas introduction
184 of this effector into Xtu significantly reduced its population to similar levels as Xtt WT (Fig. S4).
185 Altogether, these results demonstrate that *xopAL1* is a fitness factor upon which Xtt is dependent
186 for virulence and colonization on specialized host barley, but which restricts the ability of Xtt to
187 cause disease in wheat in an effector-dependent manner possibly via XopAL1 recognition.
188

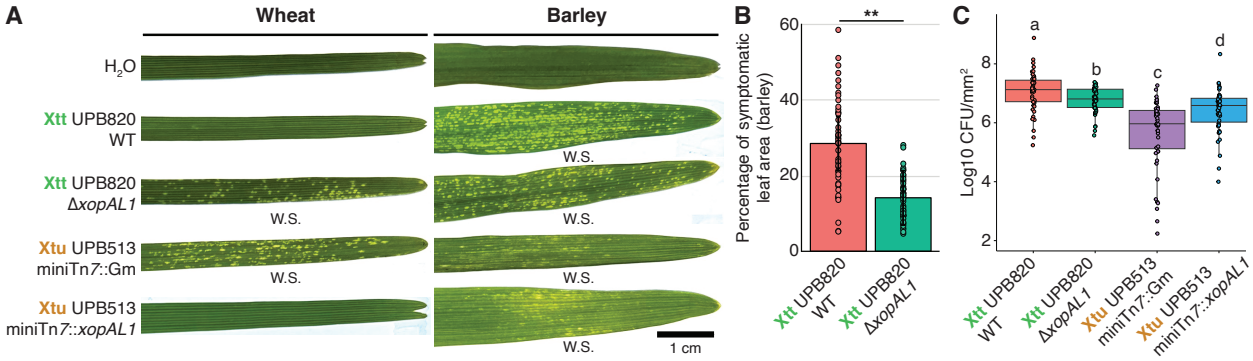


Fig. 2. Deletion of the type III secretion system effector gene *xopAL1* allows for host jump of Xtt from barley to wheat, while decreasing its pathogenic fitness in barley. **A.** Symptom development in barley and wheat leaves inoculated (spray) with Xtu and Xtt strains (wild-type, mutant, and insertion strains; OD_{600} 0.5) at six days post inoculation. Water soaking symptoms were visualized using a light box and appear as lighter areas in the leaf. Scale bar is shown in figure. **B.** Percentage of symptomatic leaf area of inoculated barley leaves (spray) with Xtt WT and Xtt Δ*xopAL1* shown in panel A. Original data of percentage of symptomatic leaf area (treatments; y-axis) was submitted to a transformation that combines the arcsine and square root functions. The Shapiro-Wilk test (W) was then applied to the transformed data, and it supported the assumption of normal distribution (W = 0.975; *P* value = 0.0671). ** indicates significant difference as determined by Student's *t*-test ($P<0.001$; n = two independent replicates with 23 to 24 biological replicates each). **C.** Xtt and Xtu (wild-type, mutant, and insertion strains) populations in barley leaves inoculated via spray (OD_{600} 0.5) at 144 hours post inoculation. Different letters indicate significant difference as determined by Wilcoxon rank-sum test ($P<0.05$; n = two independent replicates with 23 to 24 biological replicates, each with three internal replicates). Wheat – *Triticum aestivum* L. genotype 'Chinese Spring'; Barley – *Hordeum vulgare* L. cv. Morex; Xtt - *X. translucens* pv. translucens; Xtu - *X. translucens* pv. undulosa; W.S. – water soaking.

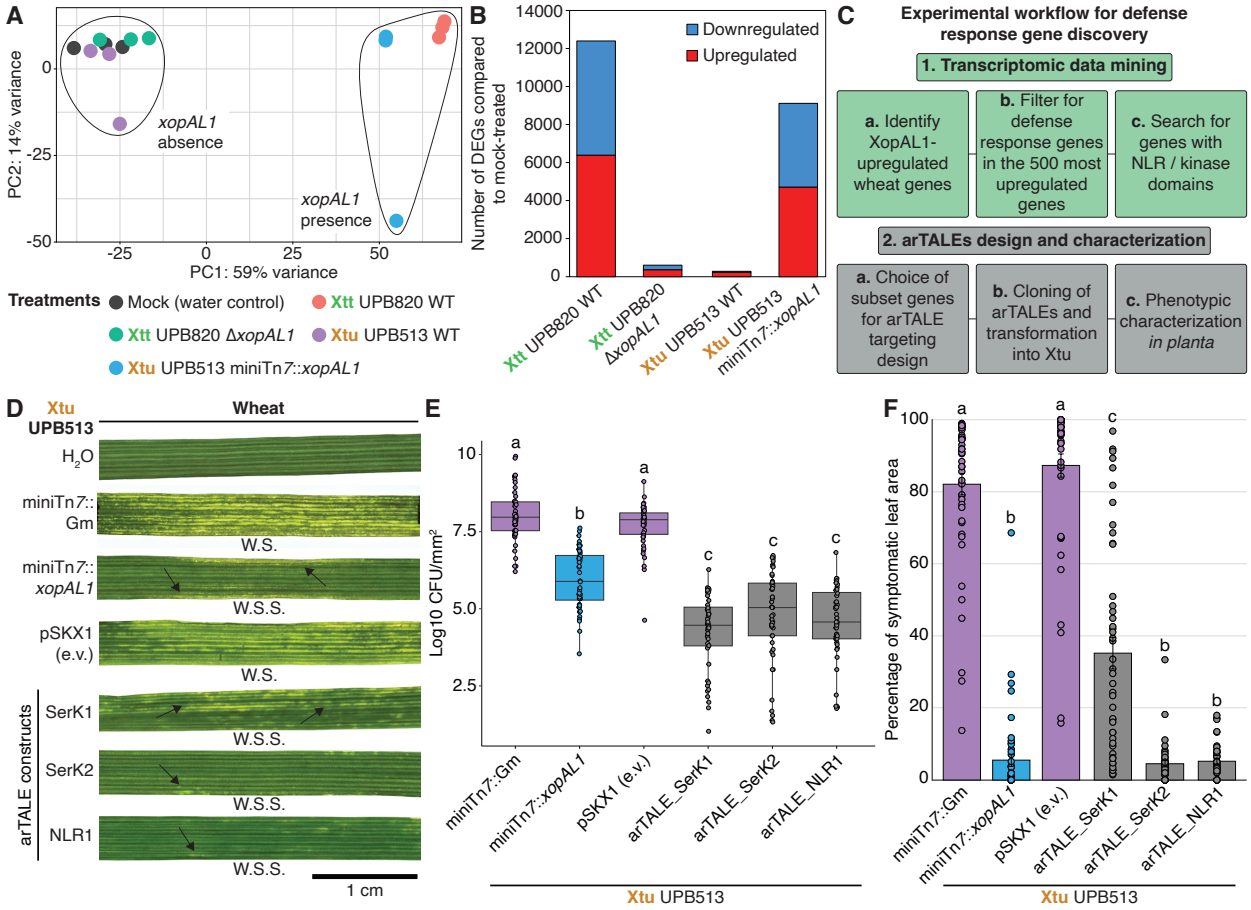
A cereal genotype diversity panel containing known susceptible hosts to Xtu was screened to assess if other plant species display a similar *xopAL1*-dependent resistance mechanism (Table S3). All screened hexaploid wheat genotypes (*Triticum aestivum* L., 2n = 6x = 42, BBAADD) presented a *xopAL1*-dependent resistance response against Xtt (Table S3). Diploid (einkorn wheat, AA genotype) and tetraploid (durum and emmer wheat, BBAA genotypes) wheat genotypes were overall more susceptible to Xtt, which was independent of XopAL1 (Table S3). Curiously, all tested Triticale lines (hexaploid genotype BBAARR) displayed a *xopAL1*-dependent resistance response against Xtt (Table S3). Despite Triticale being a wheat-rye hybrid (*47*), no *xopAL1*-dependent resistance was observed in rye, which remained resistant to Xtt (Table S3). Curiously, genomic introduction of *xopAL1* into Xtu did not convert this subgroup into a non-pathogen of Triticale, as opposed to wheat. We therefore hypothesize that wheat resistance against Xtt may be mostly encoded by the DD sub-genome, which has the annual grass *Aegilops tauschii* Coss. as the donor species (*48*).

A subset of this cereal genotype diversity panel was screened with individual mutant strains for the other Xtt-unique effectors (*xopAJ*, *xopE5*, and *hopW1*; Table S4). While no change in host range was observed for Xtt Δ*xopE5* and Xtt Δ*hopW1*, our analysis revealed a *xopAJ*-dependent resistance response of oat (*Avena sativa* L., AACCDD hexaploid genome) against Xtt (Table S4;

Fig. S5). Results thus suggest that this unique effector of the specialist subgroup Xtt determines its host range for causing disease in oat, in which recognition of XopAJ triggers defense response by this plant. Nevertheless, our downstream analyses described here focused mainly on XopAL1 due to its role in determining the ability of *X. translucens* to cause disease in its two main economic host plants, wheat and barley (*26, 31*).

Transcriptomic analysis identified genes in the XopAL1-dependent defense response of wheat

As XopAL1 limits Xtt to cause disease in wheat, we carried out RNA sequencing (RNA-seq) to determine the molecular pathways that restrict bacterial infection. Expression profiles were compared between hexaploid wheat leaves inoculated with *xopAL1*-encoding strains (Xtt WT and Xtu miniTn7::*xopAL1*), non-encoding strains (Xtt Δ*xopAL1* and Xtu WT), and mock-inoculated wheat leaves. A principal component analysis (PCA) of the RNA-seq count data demonstrated that samples clustered in two primary transcriptional profiles in a *xopAL1*-dependent manner, reflecting the ability of strains to cause disease in wheat or not (Fig. 3A). Accordingly, much higher numbers of significantly differentially expressed genes (DEGs) were identified in plants inoculated with Xtt and Xtu encoding *xopAL1* when compared to mock-treated samples (12,397 and 9,118 DEGs, respectively; Table S5) and in comparison to non-encoding strains of these subgroups (607 and 285 DEGS, respectively; Table S5) (Fig. 3B). These findings indicated that recognition of XopAL1 in wheat results in extensive transcriptional changes that ultimately lead to a non-host response.



250 **Fig. 3.** XopAL1 mediates global transcriptional changes in inoculated wheat plants, allowing for
251 discovery of specific downstream defense response genes. **A.** Principal component analysis
252 (PCA) of the normalized RNAseq data transcripts per million of wheat leaf tissue infected with
253 different *X. translucens* strains and mock (treatments). Ellipses are arbitrarily drawn to indicate
254 clustering of treatment based on *xopAL1* presence and absence. **B.** Number of significantly
255 differentially expressed genes (DEGs; downregulated and upregulated) in wheat from each
256 bacterial treatment compared to mock. **C.** Experimental workflow for discovery of defense
257 response genes in wheat against XopAL1-encoding strains. **D** and **E.** Symptom development (**D**)
258 and pathogen population (**E**) in wheat leaves inoculated with Xtu UPB513 transformed with
259 arTALEs, miniTn*7*::*xopAL1*, and respective empty vector controls at 4-days post inoculation
260 (spray; OD_{600} 0.5). **F.** Percentage of symptomatic leaf area of inoculated wheat leaves (spray) as
261 shown in panel D. Different letters in panels E and F indicate significant difference using one-
262 way ANOVA and Tukey's post-hoc test ($P<0.05$; n = two independent replicates with 20 to 24
263 biological replicates each). arTALE constructs are named based on the wheat genes targeted for
264 expression induction (see table S6 for genes description). NLR – Nucleotide-binding leucine-rich
265 repeat; SerK – Serine-threonine kinase; e.v. – empty vector; W.S. – water soaking; W.S.S. –
266 water soaking spots. Arrows are pointing to water soaking spots in panel D.
267
268 A gene ontology (GO) term enrichment analysis was performed to investigate the specific plant
269 processes in wheat that led to the non-host response. Our analysis revealed that upregulated
270 DEGs in response to inoculation with Xtt and Xtu expressing *xopAL1* were significantly
271 enriched in GO terms associated with immunity, induced defense response, defense signaling,
272 and programmed cell death (Fig. S6; Table S5). On the other hand, significant overrepresentation
273 of GO terms associated with photosynthetic pathways, including chloroplast and chlorophyll
274 processes, were observed among the downregulated DEGs in response to strains expressing
275 *xopAL1* (Fig. S6; Table S5).
276
277 The significant upregulation of genes associated with defense response in response to XopAL1
278 may explain the restriction in host-specificity (Fig. S6 and Table S5). To determine specific
279 components of the XopAL1-triggered defense response of wheat, we developed an experimental
280 workflow for defense response gene discovery (Fig. 3C, see materials and methods for details).
281 Briefly, we mined the transcriptomic dataset for XopAL1-upregulated genes, filtered for those
282 with defense-related functions, and specifically focused on genes with nucleotide-binding
283 leucine-rich repeat domains (NLRs) and kinase domains due to their critical roles in effector-
284 triggered defense signaling (*45, 49, 50*). Candidate genes were then artificially induced during
285 infection with Xtu, a non-*xopAL1* encoding strain, to evaluate their role in the XopAL1-triggered
286 nonhost response. To do this, artificial TAL effectors (arTALEs) were designed and cloned to
287 target individual induction of identified candidate XopAL1-induced defense response genes. By
288 hijacking the modular DNA-binding and transcriptional activation system of native TALEs,
289 arTALEs provide a powerful tool to test gene function in planta and have been used to
290 ectopically activate resistance genes and trigger hypersensitive responses (*9, 51, 52*). When
291 wheat plants were spray-inoculated (Fig. 3D), Xtu transformants carrying the constructs
292 arTALE_SerK1, arTALE_SerK2, and arTALE_NLR1 showed significantly reduced bacterial
293 populations (Fig. 3E) and milder symptom development (Fig. 3F) compared to the empty vector
294 (pSKX1) control, closely resembling the response observed with Xtu encoding *xopAL1*
295 (miniTn*7*::*xopAL1*) (Fig. 3D-F). Significant transcriptional induction of target genes by arTALE

296 constructs was confirmed via quantitative PCR (qPCR; Fig. S7). Candidate wheat genes targeted
297 by these arTALE constructs that decreased Xtu virulence and fitness include two genes encoding
298 serine-threonine kinases and one gene encoding a NLR protein (Table S6). These results define
299 critical downstream defense components activated by XopAL1 recognition in wheat and
300 highlight promising targets for enhanced resistance to *X. translucens*.
301
302 **Development of the chlorotic non-host response in wheat is related to lower chlorophyll**
303 **content in inoculated leaves**
304 Our phenotypic and transcriptomic data revealed downregulation of photosynthetic pathways.
305 Despite being an artifact of artificially inoculating cells of a non-pathogenic strain directly into
306 the inner leaf tissue via infiltration, the basis of chlorosis development in a non-host response is
307 widespread in *Xanthomonas*-plant interactions but remains unclear (*14, 42, 43*). This XopAL1-
308 mediated response was also triggered in wheat by a distant *Xanthomonas campestris* pv.
309 campestris ortholog (Fig. S8) when transformed in Xtu (Fig. S9). Based on our RNA-seq data
310 (Fig. S6) and phenotypic characterization (Fig. S3), we hypothesized that chlorosis is the result
311 of reduced photosynthetic activity and chlorophyll content in wheat upon XopAL1 recognition.
312 Indeed, we observed significantly lower chlorophyll concentration in wheat leaves inoculated
313 with *xopAL1*-encoding Xtt and Xtu in comparison to those inoculated with non-encoding strains
314 and mock-inoculated leaves (Fig. S10).
315
316 ***xopAL1* has been lost in Xtu and presents an inherent risk for host jump by Xtt**
317 Plant pathogen populations may shift and diversify the content of their encoded virulence factor
318 repertoires to avoid recognition by the host immune system (*1, 53*). We therefore tested if the
319 emergence of broad host range Xtu was correlated with the loss of *xopAL1*. Xtu was suggested to
320 have diverged from a Xtt ancestor (*54*), and we examined *xopAL1* evolution with maximum
321 likelihood phylogenetic comparisons of whole genomes and the *xopAL1* locus neighborhood
322 (Fig. 4A-B; Figs. S11-12), and by reconciling with the *xopAL1* tree (Fig. S8A). Specific
323 neighborhoods of *xopAL1* were defined across the *X. translucens* species (Fig. 4A and Fig. S13).
324 Our phylogenetic hypothesis testing of the *X. translucens xopAL1* locus determined that this
325 effector was lost in Xtu (neighborhood type 1) during its divergence from a common ancestor
326 with Xtt (neighborhood type 2; Fig. 4B-C).
327

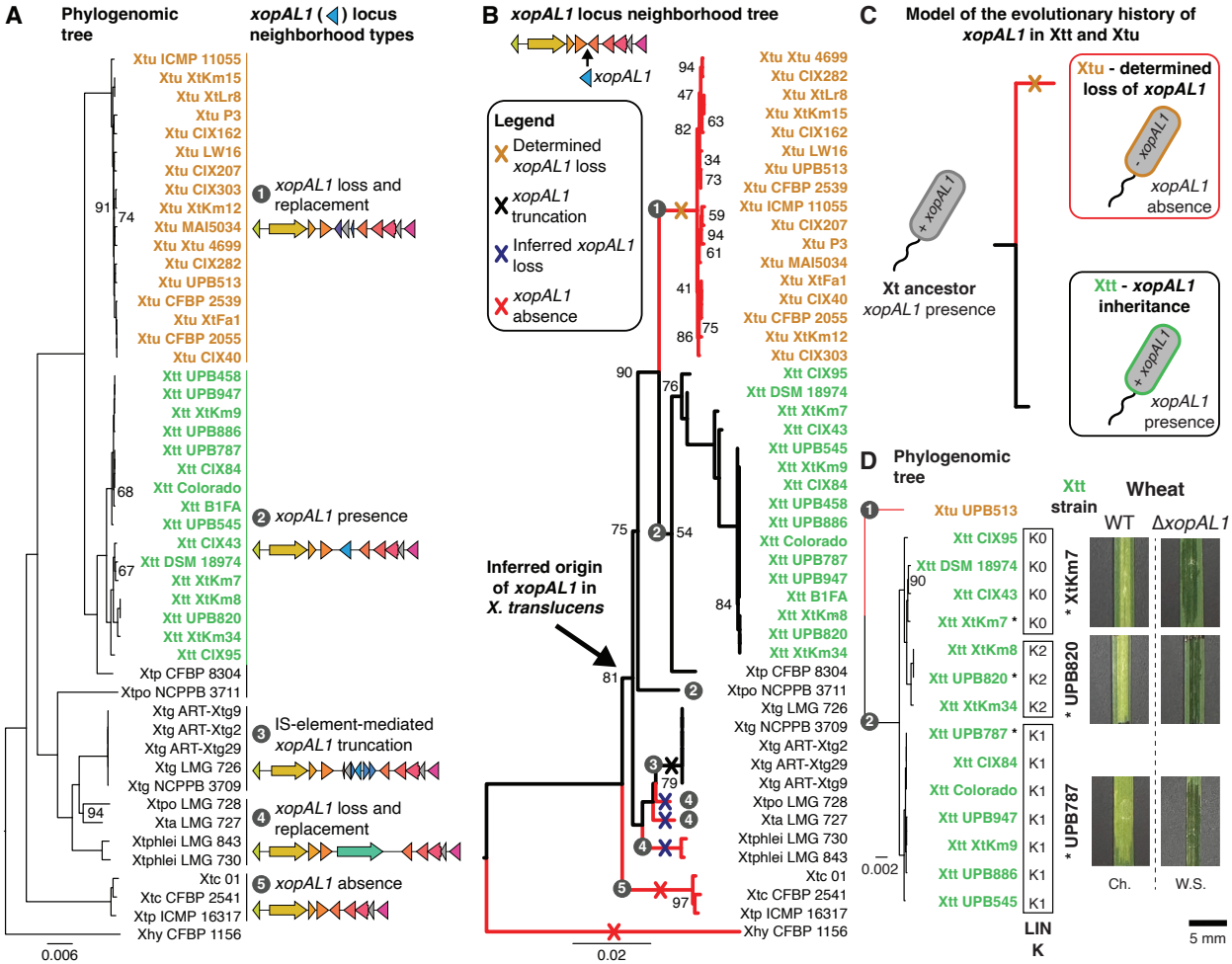


Fig. 4. Loss of *xopAL1* promoted host jump of *X. translucens* pv. undulosa to wheat. **A.** Core genome-based tree of *X. translucens* strains and their *xopAL1* locus neighborhood types. A complete list of genes composing each neighborhood is shown in figure S13. **B.** Tree of the concatenated sequences of the genes flanking *xopAL1* in *X. translucens*. The *xopAL1* locus neighborhood types are indicated in each branch. Red-colored branches indicate subgroups that do not encode *xopAL1*, with determined and inferred loss or absence being shown. *X. hyacinthi* strain CFBP 1156 was included as an outgroup to root the trees in panels A and B. **C.** Model of the evolutionary history of *xopAL1* in Xtt and Xtu. **D.** Deletion of *xopAL1* promotes host jump of all Xtt genomic subgroups. A core genome-based tree including the K0, K1 and K2 genomic subgroups of Xtt is shown. Symptom development in wheat (*Triticum aestivum* L. genotype 'Chinese Spring') leaves inoculated (syringe infiltration) with representative Xtt strains (OD_{600} = 0.1) belonging to the three K genomic subgroups and their respective *xopAL1* mutants at three days post inoculation is shown to the right side of the panel. Scale bar is shown in figure. Trees were visualized via Figtree. Branches with bootstrap values below 98% are indicated in each tree. Ch. – chlorosis; W.S. – water soaking. *X. translucens* pathovars: Xtc – pv. cerealis; Xtp – pv. pistaciae; Xtg – pv. graminis; Xtpo – pv. poae; Xta – pv. arrhenateri; Xtphlei – pv. phleipratensis; Xtu – pv. undulosa; Xtt – pv. translucens; Xhy – *X. hyacinthi*.

Loss of *xopAL1* was common across other *X. translucens* lineages. Three other inferred losses of *xopAL1* with acquisition of different genes replacing this effector were observed in subgroups

with neighborhood type 4, as similarly determined for Xtu (Fig. 4B). The origin of *xopAL1* (neighborhood type 2) was parsimoniously inferred in *X. translucens* to occur after divergence from a common ancestor with pathovar cerealis and strain ICMP 16317 in pathovar pistaciae (Xtp; neighborhood type 5; Fig. 4B). In addition, there was truncation of *xopAL1* in pathovar graminis (Xtg) by direct transposon insertion 5' of the remaining sequence fragment (neighborhood type 3; Fig. 4B and Fig. S8B).

Risk assessment is key for epidemic preparedness, but there is often a lack of precise knowledge about what genetic events lead to emergence, and about the potential of organisms to change their niche. Because loss of *xopAL1* allowed Xtu to jump host to wheat, we examined if deletion of this effector gene could lead to host jump for the three described, globally-distributed Xtt genomic subgroups (K0, K1, and K2) (*46, 55*). The deletion of *xopAL1* in representative K0, K1 and K2 Xtt strains led to host expansion to wheat (Fig. 4D). Xtt are globally important in barley production systems (*55*), and all lineages have an inherent pathogenic capacity in wheat production systems based on *xopAL1* loss.

Gene gain is also a mechanism for niche shift in *Xanthomonas* (*2*). As observed by screening a subset of the cereal genotype diversity panel tested here, XopAJ was another effector that regulated host specificity of *X. translucens* (Table S4; Fig. S5). We determined that the oat-limiting effector gene *xopAJ* was originated in Xtt (neighborhood 2) after divergence with Xtu (neighborhood 1; Figs. S14-15). This gene was also gained in related pistachio-infecting *X. translucens* (Xtp) strain CFBP 8304 but further truncated via transposon insertion (neighborhood 3; Figs. S14-15). The gene *xopAJ* is not encoded by any other *X. translucens* subgroup besides Xtt and the closely related Xtp strain CFBP 8304 (Figs. S14-15). In addition, this gene is not encoded in the same locus in comparison to other *xopAJ*-encoding *Xanthomonas* spp. (Fig. S16), and Xtt-encoded *xopAJ* is more phylogenetically closely related to *xopAJ* encoded by a distant species, *Paracidovorax citrulli*, than to *xopAJ* encoded by clade II *Xanthomonas* spp (Fig. S17). These results suggest that *xopAJ* evolution is dynamic and was gained in Xtt by horizontal gene transfer but lost by dicot pathogen Xtp. We hypothesized that gain of *xopAJ* by Xtt increases its adaptive fitness to infect barley. Deletion of this effector gene in Xtt significantly decreased the symptomatic leaf area of barley upon spray inoculation (Fig. S18A-B), but no significant differences were observed between endophytic populations of Xtt WT and Xtt Δ*xopAJ* (Fig. S18C). Altogether, results show that gain of *xopAJ* enhanced virulence of Xtt in barley, but it did not affect fitness for plant colonization.

Discussion

Emerging infectious diseases often result from microbial niche shift. Multiple genetic mechanisms control emergence including gene loss and gain. Genome and episome reduction are strongly associated with host specialization and gain can promote adaptation to a particular environment or resist control interventions (e.g. antibiotics) (*56, 57*). Here, were have determined that loss and gain of single Type III effectors control specialist and generalist behaviors of Xtt and Xtu for host range. Specifically, our results collectively demonstrate that a) XopAL1 is both a fitness and virulence requirement for Xtt; b) this effector is a determinant of host range since its loss contributed for host expansion of Xtu to wheat by circumventing an otherwise effector-triggered defense response of this host plant; and c) gain of XopAJ by Xtt limited its ability to cause disease in oat but contributes for full virulence in barley.

Bacterial pathogens commonly colonize host plants by delivering secreted factors that alter plant cellular processes to promote virulence, but these same factors can also trigger plant immune responses that restrict infection if recognized by cognate plant resistance (R) proteins (*9, 21, 23, 45*). Absence or presence of specific Type III-secreted effectors dictates host range of plant pathogens in an effector-triggered immunity (ETI) -dependent manner. Examples of effectors impacting host range include the XopAG, XopQ, and AvrBsT effectors of *Xanthomonas* spp. (*16, 17, 19, 43*), and HopQ1-1 effector of *Pseudomonas syringae* (*18*). Moreover, effectors have demonstrated fitness requirements for leaf colonization in particular hosts (*53, 58*). For instance, absence of AvrBsT expands the host range of *Xanthomonas perforans* while decreasing the fitness of this pathogen in tomato fields (*58*).

These studies demonstrate how Type III effector repertoires may vary in response to resistance within individual cropping or model plant systems but lack an evolutionary framework. Here, we determined the evolutionary history and ecological function of Type III effector loss and gain events related to fitness and host range. Using the generalist model Xtu, we posit that *xopAL1* was one of multiple loss events that led to emergence of this pathogen generalist subgroup. This generalist has lost other niche-specific factors for tissue-specificity. Our previous work demonstrated that loss of the vascular-associated pathogen gene *cbsA* is frequently associated with non-vascular tissue colonization. This gene is encoded by the vascular, specialist Xtt but was lost in non-vascular, generalist Xtu via transposon insertion, which led to changes in tissue colonization (*2*). Here, we have also observed truncation of *xopAL1* in Xtg, and *xopAJ* in Xtp via transposon insertion. Transposons shape microbial niche-specificity, and these insertion sequence (IS) elements are major drivers of ecological shifts of bacterial and fungal species (*2, 59-61*). Overall, we hypothesize that gene loss, frequently mediated by IS elements, is a common strategy for niche shifts across behaviors.

Interestingly, XopAJ, which we have determined here as a host range-limiting factor for *X. translucens* in oat, was gained in Xtt and contributes for virulence in barley. We speculate that gain of effectors upon host specialization (e.g. gain of *xopAJ* in specialist Xtt) provides resource access on a preferred host. XopAJ (synonym of AvrRxo1) was initially described as a host range-limiting factor of the rice pathogen *X. oryzae* pv. oryzicola (Xoc) when inoculated into maize lines carrying the cognate resistance gene Rxo1 (*62*). This effector has been shown to suppress pattern-triggered immunity (PTI) responses of host and non-host plants (*63*), and to enhance virulence of Xoc (*64*). Xtt is limited to barley (*Hordeum* spp.), and it remains to be determined whether XopAJ displays similar molecular functional roles for host colonization and pathogen virulence as observed in other *Xanthomonas* spp.

Hosts respond dramatically different to specialized and generalist pathogens and could be used to inform resistance trait discovery. The screening of a panel of cereal genotype diversity suggested that wheat resistance against Xtt is mostly encoded by the DD sub-genome. However, the defense response genes identified here are all located in the AA sub-genome, which originates from the diploid wild species of wheat *Triticum urartu* (*48*). We hypothesize that wheat resistance against Xtt is quantitative, involving multiple loci. Quantitative resistance is considered more durable than qualitative resistance that relies on single R genes (*65, 66*), thus correlating with emergence of the generalist Xtu that has lost multiple virulence factors such as

441 *xopAL1* and *cbsA*. Although NLR proteins make up the main class of R proteins (*44, 45*), we
442 currently have no evidence to determine if the NLR identified here is the cognate R protein that
443 recognizes XopAL1 and initiates the wheat defense response. We thus took an alternate approach
444 to determine the understudied downstream functions that govern triggered resistance. We
445 developed an experimental workflow for discovery of XopAL1-dependent defense response
446 genes in wheat by mining our transcriptomic dataset and then inducing the expression of
447 candidate genes via arTALEs. This analysis identified three genes, which were two serine-
448 threonine kinases and one NLR, involved in the downstream XopAL1-triggered defense
449 response of wheat against Xtt. These identified genes are a valuable source for generating
450 resistance against *X. translucens*. Cloning and expression of these genes in barley could generate
451 a XopAL1-dependent resistance against Xtt, as similarly described for R gene transfer between
452 crops against other *Xanthomonas* pathogens (*67-69*). On the other hand, resistance could be
453 generated in wheat by artificially converting the identified defense response genes into executor
454 R genes (*9*) by editing their promoters for targeting by conserved TAL effectors of Xtu. The use
455 of multiple defense response genes, a strategy known as R gene pyramiding, could generate
456 durable resistance against *X. translucens* in barley and wheat (*66*). We speculate that the use of
457 defense response genes acting downstream of R proteins will also be a powerful tool for
458 generating resistance in desired crops.
459
460 Preparedness for mitigation of new epidemics, especially when host jump occurs, depends on
461 strong knowledge of the evolution of niche-shifting. The research described here provides a
462 framework for reconstructing the evolutionary history of host jump in an important cereal
463 pathogen and allows us to predict pathogen emergence based on gene loss and gain. Based on the
464 results described here, we developed a tested evolutionary model for *X. translucens* intergenera
465 host jump centered on effector gene loss and gain (Fig. S19). Xtu is a generalist that has fewer
466 Type III effectors, and loss of *xopAL1* from a common ancestor with Xtt likely promoted its
467 broad host range behavior. On the other hand, gain of *xopAJ* in the specialist Xtt restricted its
468 ability to cause disease in oat. A practical implication of our model is that management of BLS
469 of wheat and oat must include surveillance of all lineages of Xtt in addition to Xtu due to their
470 inherent risk for jumping hosts based on loss of single genes. Moreover, our experimental
471 framework identified specific genes involved in the downstream XopAL1-triggered defense
472 response of wheat, which is of key importance for generating resistance in both barley and wheat
473 host plants. Overall, these findings have implications for mitigating epidemics and generating
474 resistance into economically important crops affected by this pathogen. Ultimately, our work
475 demonstrated that the shift between specialist and generalist subgroups of *X. translucens* for host
476 range is modulated by presence and absence of single genes, with gene loss and gain being major
477 drivers for changing niches and behaviors of pathogens.
478
479 **Materials and Methods**
480 Bacterial strains and plasmids used in this study are shown in Table S7. Whole genome
481 sequencing of strains and their comparative genomics and evolutionary phylogenetic analyses,
482 together with recombinant DNA techniques, and inoculation of assayed plants are described in
483 Supplementary Materials. RNA-seq was performed on an Illumina NextSeq 2000 and details are
484 included in Supplementary Materials. Artificial TAL effectors were designed and assembled via
485 a modular cloning strategy named Golden TAL technology (*70*), and induction of target genes
486 was assessed via qPCR, as described in Supplementary Materials. Chlorophyll concentration in

assayed wheat leaves was measured using the MC-100 Chlorophyll Concentration Meter (Apogee Instruments, Inc.; details in Supplementary Materials). Data from independent experiments were tested for normality using the Shapiro-Wilk test and then analyzed using the appropriate statistical analysis (see Supplementary Materials).

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