Show me your secret(ed) weapons: a multifaceted approach reveals novel type III-secreted effectors of a plant pathogenic bacterium

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Abstract

Many Gram-negative plant and animal pathogenic bacteria employ a type III secretion system (T3SS) to secrete protein effectors into the cells of their hosts and promote disease. The plant pathogen *Acidovorax citrulli* requires a functional T3SS for pathogenicity. As with *Xanthomonas* and *Ralstonia spp.*, an AraC-type transcriptional regulator, HrpX, regulates expression of genes encoding T3SS components and type III-secreted effectors (T3Es) in *A. citrulli.* A previous study reported eleven T3E genes in this pathogen, based on the annotation of a sequenced strain. We hypothesized that this was an underestimation. Guided by this hypothesis, we aimed at uncovering the T3E arsenal of the *A. citrulli* model strain, M6. We carried out a thorough sequence analysis searching for similarity to known T3Es from other bacteria. This analysis revealed 51 *A. citrulli* genes whose products are similar to known T3Es. Further, we combined machine learning and transcriptomics to identify novel T3Es. The machine learning approach ranked all *A. citrulli* M6 genes according to their propensity to encode T3Es. RNA-Seq revealed differential gene expression between wild-type M6 and a mutant defective in HrpX. Data combined from these approaches led to the identification of seven novel T3E candidates, that were further validated using a T3SS-dependent translocation assay. These T3E genes encode hypothetical proteins, do not show any similarity to known effectors from other bacteria, and seem to be restricted to plant pathogenic *Acidovorax* species. Transient expression in *Nicotiana benthamiana* revealed that two of these T3Es localize to the cell nucleus and one interacts with the endoplasmic reticulum. This study not only uncovered the arsenal of T3Es of an important pathogen, but it also places *A. citrulli* among the "richest" bacterial pathogens in terms of T3E cargo. It also revealed novel T3Es that appear to be involved in the pathoadaptive evolution of plant pathogenic *Acidovorax* species.

Author summary

Acidovorax citrulli is a Gram-negative bacterium that causes bacterial fruit blotch (BFB) disease of cucurbits. This disease represents a serious threat to cucurbit crop production worldwide. Despite the agricultural importance of BFB, the knowledge about basic aspects of *A. citrulli*-plant interactions is rather limited. As many Gram-negative plant and animal pathogenic bacteria, *A. citrulli* employs a complex secretion system, named type III secretion system, to deliver protein virulence effectors into the host cells. In this work we aimed at uncovering the arsenal of type III-secreted effectors (T3Es) of this pathogen by combination of bioinformatics and experimental approaches. We found that this bacterium possesses at least 51 genes that are similar to T3E genes from other pathogenic bacteria. In addition, our study revealed seven novel T3Es that seem to occur only in *A. citrulli* strains and in other plant pathogenic *Acidovorax* species. We found that two of these T3Es localize to the plant cell nucleus while one partially interacts with the endoplasmic reticulum. Further characterization of the novel T3Es identified in this study may uncover new host targets of pathogen effectors and new mechanisms by which pathogenic bacteria manipulate their hosts.

67 ## Introduction

68 The genus *Acidovorax* (class Betaproteobacteria) contains a variety of species
69 with different lifestyles. While some species are well adapted to soil and water
70 environments, others have developed intimate relationships with eukaryotic organisms,
71 including as plant pathogens [1]. Among the latter, *Acidovorax citrulli* is one of the most
72 important plant pathogenic species [2]. This bacterium infects all aerial parts of cucurbit
73 plants, causing bacterial fruit blotch (BFB) disease. The unavailability of effective tools
74 for managing BFB, including the lack of resistance sources, and the disease's high
75 destructive potential, exacerbate the threat BFB poses to cucurbit (mainly melon and
76 watermelon) production [3, 4]. Despite the economic importance of BFB, little is known
77 about basic aspects of *A. citrulli*-plant interactions.

78 On the basis of genetic and biochemical features, *A. citrulli* strains are divided
79 into two main groups: group I strains have been generally isolated from melon and other
80 non-watermelon cucurbits, whereas group II strains have been mainly isolated from
81 watermelon [5-7]. *Acidovorax citrulli* M6 is a group I strain that was isolated in 2002
82 from a BFB outbreak of melons in Israel [5], and subsequently became a model group I
83 strain for investigation of basic aspects of BFB. The *A. citrulli* M6 genome has been
84 sequenced, first by Illumina MiSeq [8] and recently, by PacBio [9], which allowed its
85 complete closure.

86 As many Gram-negative plant and animal pathogenic bacteria, *A. citrulli* relies on
87 a functional type III secretion system (T3SS) to promote disease [10]. This complex
88 secretion system is employed by these pathogens to deliver protein effectors into target
89 eukaryotic cells. Collectively, type III-secreted effectors (T3Es) promote disease by
90 modulating a variety of cellular functions for the benefit of the pathogen [11-13]. In the
91 case of plant pathogenic bacteria, type III-secreted effectors (T3Es) were shown to

92 promote virulence through alteration of the plant cell metabolism and/or suppression of
93 host immune responses [14, 15]. As part of their defence mechanism, plants recognize
94 some effectors by corresponding disease resistance (R) proteins, mostly belonging to the
95 nucleotide-binding (NB)-leucine-rich repeat (LRR) type of immune receptors (NLRs)
96 [16, 17]. Upon effector recognition, the R protein elicits a battery of defense responses
97 collectively referred to as effector-triggered immunity (ETI). ETI is often accompanied
98 by the hypersensitive response (HR), a rapid death of plant cells at the infection site that
99 arrests pathogen spread in the plant tissue [18]. Therefore, elucidating the arsenal of
100 effectors and their contribution to virulence, are of critical importance for the
101 understanding of basic aspects of pathogenicity but also for translational research in the
102 crop protection field.

103 Due to the requirement of type III secretion (T3S) for pathogenicity in susceptible
104 plants and HR elicitation in resistant plants, the genes encoding key T3SS regulators and
105 structural components in plant pathogenic bacteria are named *hrp* genes (for HR and
106 pathogenicity) or *hrc* genes, in the case of *hrp* genes that are conserved among different
107 bacterial genera, including in animal pathogens [19]. On the basis of gene content, operon
108 organization and regulation, *hrp* clusters are divided into two classes: class I contains the
109 *hrp* clusters of *Pseudomonas syringae* and enteric plant pathogenic bacteria, while class
110 II contains the clusters of *Xanthomonas* species, *Ralstonia solanacearum* and plant
111 pathogenic *Acidovorax* spp. [10, 19, 20] .

112 In *Xanthomonas* spp. and *R. solanacearum*, the expression of *hrp*, *hrc* and *hrp*-
113 associated (*hpa*) genes, as well as of some T3E genes, is regulated by HrpG and
114 HrpX/HrpB (HrpX in *Xanthomonas* spp. and HrpB in *R. solanacearum*). HrpG belongs
115 to the OmpR family of two-component system response regulators and controls
116 expression of *hrpX*/*hrpB* [21-23]. *hrpX* and *hrpB* encode AraC-type transcriptional

117 activators that directly mediate the expression of most *hrp*/*hrc* operons and many T3E
118 genes, via binding to DNA motifs that are present in their promoter regions. These DNA
119 motifs are named plant-inducible promoter (PIP) box (TTCGB-N15-TTCGB; B being
120 any nucleotide except adenine) in *Xanthomonas* spp. [24] and hrp_{II} box (TTCG-
121 N16_TTCG) in *R. solanacearum* [25]. Recently, Zhang *et al.* showed that the *hrpG* and
122 *hrpX*/*hrpB* (thereafter *hrpX*) orthologous genes of the *A. citrulli* group II strain Aac5 are
123 required for pathogenicity [26]. They also showed that HrpG activates expression of
124 *hrpX*, which in turn, regulates the expression of a T3E gene belonging to the YopJ family.
125 Until recently, based on the annotation of the genome of the *A. citrulli* group II
126 strain AAC00-1, we were aware of eleven genes showing similarity to known T3E genes
127 from other bacteria [27]. Considering the higher numbers of T3E genes in several other
128 plant pathogenic bacteria, we hypothesized that this is an underestimation of the actual
129 number of T3Es in *A. citrulli*. We also hypothesized that *A. citrulli* may carry novel T3E
130 genes that were not previously described in other bacteria. Guided by these hypotheses,
131 we carried out a detailed sequence analysis of *A. citrulli* M6 open reading frames (ORFs)
132 to identify genes with similarity to known T3E genes from other bacteria. We also
133 combined machine-learning (ML) and RNA-Seq approaches to identify putative, novel
134 *A. citrulli* T3Es. Further, we adapted a T3E translocation assay to verify T3S-dependent
135 translocation of candidate effectors. Combining these approaches allowed identification
136 of seven new T3Es that appear to be unique to plant pathogenic *Acidovorax* species.
137 Subcellular localization of three of these T3Es in *N. benthamiana* leaves was also
138 determined by *Agrobacterium*-mediated transient expression.
139

140 **Results**

141 **Identification of new T3E genes of *A. citrulli* by genome annotation, machine**
142 **learning and sequence analyses**

143 Analysis of the genome of the group II *A. citrulli* strain AAC00-1 (GenBank
144 accession CP000512.1) revealed eleven genes similar to T3E genes of other plant
145 pathogenic bacteria [27]. These genes were present in all tested group II strains. In
146 contrast, all assessed group I strains, including M6, lacked the effector gene *Aave_2708*
147 (gene ID according to the AAC00-1 annotation), encoding a *Xanthomonas euvesicatoria*
148 XopJ homolog. Group I strains also had disrupted open reading frames (ORFs) in the
149 genes *Aave_3062*, encoding an effector similar to *Xanthomonas oryzae* pv. *oryzicola*
150 AvrRxo1, and *Aave_2166*, encoding a *X. euvesicatoria* AvrBsT homolog [27].

151 To identify new putative T3E genes of *A. citrulli* we applied a machine learning
152 (ML) approach, that was successfully utilized for identification of new T3E genes of *X.*
153 *euvesicatoria* [28] and *Pantoea agglomerans* [29]. Using this algorithm, all ORFs of a
154 bacterial genome are scored according to their propensity to encode T3Es. The scoring is
155 based on a large set of features including similarity to known T3E genes, genomic
156 organization, amino acid composition bias, characteristics of the putative N-terminal
157 translocation signal and GC content, among others (see Methods).

158 An initial ML run was used to classify all ORFs of strain AAC00-1 according to
159 their probability to encode T3Es. This strain, rather than M6, was used for learning and
160 prediction, because at the time this ML was conducted, the AAC00-1 genome was fully
161 assembled with better annotation. For training, the positive set included 12 AAC00-1
162 genes that encoded T3E homologs: the eleven genes described by Eckshtain-Levi *et al.*
163 [27] and one additional gene, *Aave_2938* that is identical to *Aave_2708*. The negative set
164 included genes that showed high sequence similarity to ORFs of a non-pathogenic
165 *Escherichia coli* strain. The output of this ML run was a list of all annotated genes of *A.*

166 *citrulli* AAC00-1 ranked by their propensity to encode T3Es (S1 Table). For each ORF,
167 we searched for the homolog in *A. citrulli* M6. Among the top predictions from AAC00-1,
168 many genes did not have homologs in M6. As expected, the aforementioned 12 positive
169 T3E genes of AAC00-1 were ranked high in this list (among the 36 highest scoring
170 predictions, with eight being ranked among the top 10, and eleven among the top 15; S1
171 Table). Results from this first ML run served, together with RNA-Seq data, as the basis
172 for selection of candidate T3E (CT3E) genes for experimental validation (see below).

173 In parallel, we performed an extensive homology search, using BlastP, to identify
174 additional putative T3E genes of *A. citrulli* M6. This analysis led to the identification of
175 many additional genes with significant similarity to T3E genes from other plant
176 pathogenic bacteria. Table 1 summarizes the arsenal of putative T3E genes of *A. citrulli*
177 M6, based on its genome annotation and sequence similarity analysis. Overall, we found
178 51 putative T3E genes in the *A. citrulli* M6 genome, in support of the notion that *A. citrulli*
179 has a larger T3E repertoire than previously estimated. Most of these genes also received
180 high scores in the ML search ranking among the top 100 ORFs (Table 1 and S1 Table).
181 With that said, ten genes encoding T3E homologs were ranked in very low positions in
182 the ML run (positions 231 to 1161; Table 1). On the other hand, many top ranked genes
183 were annotated as encoding hypothetical proteins, some of which could encode yet
184 unknown T3Es.

185

186 **Table 1. List of putative T3E genes of *Acidovorax citrulli* M6 based on genome**
187 **annotation and sequence similarity (BlastP) to known T3E genes from other plant**
188 **pathogenic bacteria.**

Locus_tag M6[1]	Annotation in M6[1]	Similarity[2]	ML1[3]	ML2[3]	Locus_tag AAC00-1[4]	X[5]	R[5]	P[5]
APS58_0030	*HP*	Type III effector HopBN1	171	8	*Aave_2531*	+	+	+
APS58_0167[6]	*avrBsT*	Avirulence protein AvrBsT	3	15	*Aave_2166*	+	+	+

APS58_0178	*HP*	Type III effector HopF2	not in ML1	131	-	+	(+)	+
APS58_0492	*avrPphE*	Avirulence protein AvrPphE family	14	32	*Aave_3452*	+	+	+
APS58_0502	*yopJ*	Type III effector YopP/ AvrRxv family	5	23	*Aave_3462*	+	+	(+)
APS58_0506	*HP*	Avirulence protein AvrPphE family	not in ML1	12	-	+	+	+
APS58_0542	*hopD2*	Type III effector HopD2/HopAO1	28	26	*Aave_3502*	+	+	+
APS58_0658	*HP*	Type III effector XopN	103	13	*Aave_3621*	+	+	+
APS58_0664	*HP*	Type III effector XopQ	86	107	*Aave_3626*	+	+	+
APS58_0885	*HP*	Type III effector (*R. solanacearum*)	231	81	*Aave_3847*	(+)	+	-
APS58_1000	*HP*	Type III effector protein	814	98	*Aave_3961*	+	+	-
APS58_1023	*xopD*	Type III effector XopD	265	38	*Aave_4359*	+	(+)	(+)
APS58_1209	*HP*	Type III effector YopP/ AvrRxv family	not in ML1	3	-	-	+	-
APS58_1255	*HP*	Type III effector XopAE	161	18	*Aave_4254*	+	-	-
APS58_1433	*HP*	Type III effector HopBD1	367	42	*Aave_4427*	+	-	+
APS58_1482	*HP*	Type III effector XopF1	241	28	*Aave_4472*	+	-	(+)
APS58_1627	*HP*	Type III effector protein	223	143	*Aave_4606*	-	+	-
APS58_1634	*HP*	Type III effector XopR	51	24	*Aave_4612*	+	-	-
APS58_1657	*HP*	LRR protein, type III effector PopP	73	110	*Aave_4631*	+	+	-
APS58_1658	*HP*	LRR protein, outer protein XopAC	43	52	*Aave_4632*	+	(+)	(+)
APS58_1676	*HP*	Type III effector protein	not in ML1	25	-	+	+	(+)
APS58_1760	*T3E protein*	Type III effector protein	8	16	*Aave_4728*	+	+	+
APS58_1921	*avrPph3*	Cysteine protease avirulence protein YopT/AvrPphB	61	4	*Aave_0085*	+	+	+
APS58_1966	*xopJ*	Type III effector XopJ	not in ML1	9	-	+	+	+
APS58_2045	*avrRpt2*	Cysteine protease avirulence protein AvrRpt2	156	14	*Aave_0201*	-	-	+
APS58_2122	*xopAG*	Type III effector HopG1/AvrGf1/XopAG	6	10	*Aave_0277*	+	+	+
APS58_2156	*HP*	Type III effector XopC2	951	100	*Aave_0310*	+	+	-
APS58_2228	*HP*	Type III effector SspH1 family	not in ML1	45	-	(+)	(+)	-
APS58_2229	*putative T3E, E3 ligase domain*	Type III effector SspH1 family	not in ML1	50	-	(+)	(+)	-
APS58_2287	*HP*	Type III effector XopK	235	11	*Aave_0433*	+	-	+

APS58_2313	*LRR ribonuclease inhibitor*	LRR type III effector protein (GALA5)	24	71	*Aave_0458*	-	+	-
APS58_2345	*HP*	Type III effector XopP	1161	17	*Aave_0588*	+	+	-
APS58_2589	*HP*	Type III effector YopP/ AvrRxv family	32	5	*Aave_0889*	+	+	(+)
APS58_2767	*HP*	Type III effector protein	not in ML1	64	-	+	+	-
APS58_2799	*HP*	Putative AWR type III effector protein	33	34	*Aave_1090*	-	+	-
APS58_3109	*HP*	Avirulence protein AvrXv3	40	36	*Aave_1373*	+	+	+
APS58_3252	*HP*	Outer protein XopAC	56	118	*Aave_1508*	+	+	-
APS58_3261	*HP*	Type III effector HopBF1	37	60	*Aave_1520*	-	(+)	+
APS58_3289	*hopW1-1*	Type III effector HopW1-1/HopPmaA	15	19	*Aave_1548*	+	+	+
APS58_3303	*HP*	Type III effector XopE2	not in ML1	1	-	+	+	+
APS58_3344	*HP*	Type III effector XopAI	27	93	*Aave_1647*	+	-	(+)
APS58_3751	*mltB_2*	Lytic murein transglycosylase, type III effector HopAJ2	36	285	*Aave_3237*	+	+	+
APS58_3909	*HP*	Type III effector XopV	193	21	*Aave_3085*	+	+	-
APS58_3930	*HP*	Type III effector AvrRxo1-ORF2	655	31	*Aave_3063*	+	-	-
APS58_3931[6]	-	Type III effector AvrRxo1	13	-	*Aave_3062*	+	-	-
APS58_3943	*HP*	Type III effector AvrPphF/HopF2	not in ML1	37	-	-	+	+
APS58_4070	*HP*	Type III effector HopH1	7	6	*Aave_2876*	+	+	+
APS58_4101	*HP*	Type III effector, lipase domain	22	22	*Aave_2844*	+	+	-
APS58_4112	*avrBs1*	Avirulence protein AvrBs1/AvrA1	4	7	*Aave_2173*	+	-	+
APS58_4113	*HP*	Avirulence protein AvrBs1/AvrA1	16	2	*Aave_2174*	+	-	+
APS58_4317	*HP*	Type III effector HopD1	34	33	*Aave_2802*	+	+	-

[1] Locus_tag and annotation according to GenBank accession CP029373 [9]. Bolded genes were found to be significantly regulated by HrpX based on RNA-Seq results (S2 Table). *HP*, hypothetical protein.

[2] Similarity based on BlastP analysis of the gene product.

[3] Ranking of the genes in machine learning (ML) runs 1 and 2. ML1 was done with ORFs of *A. citrulli* AAC00-1 (GenBank accession CP000512.1) and ML2 was done with the ORFs *of A. citrulli* M6 (GenBank accession CP029373). In column ML1, "not in ML1" means that this M6 gene was not detected in ML1 because it has no homologous gene in strain AAC00-1.

197 [4] Corresponding locus_tag in *A. citrulli* AAC00-1. Underlined genes are the T3E genes that were
198 known prior to this study, based on the annotation of the *A. citrulli* group II strain AAC00-1 [27],
199 in addition of gene *Aave_2708*, which is not present in strain M6.
200 [5] Similarity to gene products of *Xanthomonas* spp. (X), *Ralstonia* spp. (R) and *Pseudomonas*
201 *syringae* group (P). + indicates significant similarity to at least one gene product; (+) indicates
202 significant similarity to hits with relatively low query coverage (below 60%); – indicates that no
203 significant hits were detected.
204 [6] These genes are probably non-functional in strain M6 and in all group I strains assessed so far
205 [27].
206

207 An additional insight of this analysis was that most predicted T3E genes of *A.*
208 *citrulli* share levels of similarity with T3E genes of *Xanthomonas* spp. and *R.*
209 *solanacearum* (41 and 40 genes, respectively; Table 1). A smaller number of genes, 31,
210 shared similarity with T3E genes of *P. syringae* strains. We also assessed the occurrence
211 of these T3Es in other plant pathogenic *Acidovorax* species (S2 Table). Except for the
212 HopBD1 homolog APS58_1433 that could be detected only in *A. citrulli* strains, the other
213 predicted T3Es occur in other pathogenic *Acidovorax* species, with some of them being
214 widely distributed. For instance, the putative effectors APS58_0492, APS58_0506,
215 APS58_1482, APS58_1657, APS58_1658, APS58_2228, APS58_2313, APS58_2345,
216 APS58_2799, APS58_3303 and APS58_3751 could be detected, at different levels of
217 similarity, in all species. The other putative effectors were restricted to fewer species,
218 with most of them being detected in *A. avenae* strains. While this may reflect the close
219 relatedness between *A. citrulli* and *A. avenae* [30], it is important to consider that, at the
220 time of this analysis, the public database contained 7 and 18 genomes of *A. citrulli* and *A.*
221 *avenae* strains, respectively, but only two draft genomes of *A. oryzae* and one draft
222 genome for each of the other species.

223 Interestingly, of the 51 putative T3E genes of *A. citrulli* M6, ten were not present
224 in the genome of the group II strain AAC00-1 (Table 1). Besides M6 and AAC00-1, the

225 NCBI database includes draft genomes of one additional group II strain, KAAC17055,
226 and four group I strains (pslb65, tw6, DSM 17060 and ZJU1106). BlastN analyses
227 revealed that these ten genes are also absent in strain KAAC17055, but present in most
228 of the group I strains. The only exceptions were *APS58_0506* that was not detected in
229 strains tw6 and DSM 17060, *APS58_1209* that was not detected in tw6, and *APS58_2767*
230 that was not detected in DSM 17060. The inability to detect these T3E genes in the
231 genomes of strains tw6 and DSM 17060 could reflect true absence in these strains but
232 also could be due to the draft nature of these genomes. In any case, these results strongly
233 suggest that the ten M6 T3E genes that are absent in the group II strains AAC00-1 and
234 KAAC17055 could be specific to group I strains of *A. citrulli*. Yet, this assumption should
235 be verified on a larger collection of strains. Interestingly, among these ten T3E genes,
236 *APS58_0506*, *APS58_2228* and *APS58_3303*, were detected in strains of all other plant
237 pathogenic *Acidovorax* species (S2 Table). In the case of *APS58_2228*, it should be
238 mentioned that the group II strains AAC00-1 and KAAC17055 possess genes
239 (*Aave_0378* in AAC00-1) that encode short products (140 a.a.) and partially align with
240 the C-terminal region of the group I product (with predicted length of 538 a.a.). In our
241 analysis we did not consider them as ortholog genes.
242

243 **HrpX is required for pathogenicity of *A. citrulli* M6 and regulates the expression of**
244 **T3SS components and T3E genes**

245 In *Xanthomonas* spp. and *R. solanacearum*, the transcriptional regulator HrpX
246 (HrpB in *R. solanacearum*) plays a key role in regulation of *hrp* and T3E genes. We
247 hypothesized that this is also the case in *A. citrulli* M6. To assess this hypothesis, we first
248 generated an *A. citrulli* M6 strain mutated in *APS58_2298,* the *hrpX* orthologous gene.
249 This mutant lost the ability to cause disease in melon (Fig 1A) and induce HR in pepper

250 leaves (Fig 1B), as previously observed for a strain carrying a mutation in the *hrcV* gene,
251 which encodes a core component of the T3SS [10]. A similar loss of pathogenicity was
252 observed for a mutant defected in the *hrpG* homolog gene, *APS58_2299* (S1 Fig).
253 Complementation of both *hrpX* and *hrpG* mutations restored pathogenicity, although
254 necrotic symptoms induced by the complemented strains were less severe than those
255 induced by the wild-type strain (S1 Fig).

256

257 **Fig 1. HrpX is required for pathogenicity and regulates expression of T3S and T3E**
258 **genes in *Acidovorax citrulli* M6.** (A) Disease lesions produced in a melon leaf inoculated
259 with wild-type M6, but not with mutant strains defective in *hrpX* or *hrcV* (encoding a core
260 component of the T3SS) genes. The picture was taken at 3 days after infiltration (d.a.i.).
261 (B) Cell death observed in a pepper leaf following inoculation with wild-type M6, but not
262 with *hrpX* and *hrcV* mutants. The picture was taken at 4 d.a.i. In (A) and (B), leaves were
263 syringe-infiltrated with bacterial suspension of 10^8 CFU/ml. (C) Qualitative assessment
264 of differential gene expression between wild-type M6 and the M6 *hrpX* mutant after 72 h
265 of growth in XVM2 minimal medium at 28 °C. gDNA, amplification of genomic DNA.
266 cDNA, reverse-trancriptase (RT)-PCR of RNA extracts. Genes: *hrcV* (*APS58_2306*),
267 *hrcT* (*APS58_2309*), *hrcJ* (*APS58_2321*) and *hrcC* (*APS58_2331*), encoding core
268 components of the T3SS; *APS58_3289*, encoding a T3E similar to *Pseudomonas syringae*
269 *hopW1-1*; and *GADPH*, glyceraldehyde-3-phosphate dehydrogenase (*APS58_1610*;
270 control gene).

271

272 Further, we used reverse transcription-PCR (RT-PCR) to compare expression of
273 four genes encoding T3SS components and one T3E gene (*APS58_3289*, encoding a *P.*
274 *syringae hopW1-1* homolog of *hopW1-1*) between the *hrpX* mutant and wild-type M6
275 following growth in XVM2 medium. This medium was optimized for expression of T3S
276 genes in *X. euvesicatoria*, as it simulates, to some extent, the plant apoplast environment
277 [31]. After 72 h of growth, expression of the tested genes was reduced in the *hrpX* mutant
278 relative to wild-type M6 (Fig 1C).

279

280 **Identification of HrpX-regulated genes by RNA-Seq**

281 Based on RT-PCR results, we carried out RNA-Seq analysis to compare gene
282 expression between wild-type M6 and the *hrpX* mutant, after 72 h of growth in XVM2
283 medium. This approach revealed 187 genes showing significant differential expression
284 (significant fold-change of ± 2) between the strains (Fig 2A). Of these, 159 genes had
285 significantly reduced expression in the *hrpX* mutant relative to wild-type M6, while 28
286 genes showed the opposite pattern (S3A and S3B Tables). RNA-Seq results were
287 validated by qPCR experiments that confirmed lower expression of 10 tested genes in the
288 *hrpX* mutant under the same conditions (Fig 2B).

289

290 **Fig. 2. Comparative transcriptomics analysis between *Acidovorax citrulli* M6 and the**
291 **M6 *hrpX* mutant.** (A) Relative gene expression profile as assessed by RNA-Seq of cells
292 grown for 72 h at 28 °C in minimal XVM2 medium (2 and 3 replicates for the *hrpX*
293 mutant and wild-type strain, respectively). The *A. citrulli* M6 genome map is represented
294 in the external circle. Internal red line shows differential gene expression between the
295 strains. Genes within the gray zone: no significant differences between the strains. The -
296 8 to 2 scale indicates relative expression of the mutant compared with the wild-type.
297 Genes with significantly reduced or increased expression in the mutant relative to the
298 wild-type strain are in the inner and outer regions relative to the gray zone, respectively.
299 Arrows indicate the Hrp-T3SS cluster as well as genes with homology to known effectors
300 from other plant pathogenic bacteria. (B) Relative expression of selected genes by qRT-
301 PCR following bacterial growth under identical conditions as for the RNA-Seq
302 experiment (3 biological replicates per strain). Asterisks indicate significant differences
303 between wild-type and *hrpX* mutant at $\alpha = 5\%$ by the Mann-Whitney non-parametrical
304 test. All tested genes except *APS58_2764* showed significantly reduced expression in the
305 mutant relative to strain M6 in the RNA-Seq analysis.

306

307 Most HrpX-regulated genes could not be assigned to Gene Ontology (GO)
308 categories using Blast2GO. Of the 159 genes that showed reduced expression in the *hrpX*
309 mutant, only 47 were assigned to at least one biological process category. Blast2GO
310 results are detailed in S3C and S3D Tables, and Fig 3 shows the number of biological
311 process categories of genes with reduced expression in the mutant. Among the most
312 frequent categories, 10 hits were found for transmembrane transport proteins, including
313 several ABC transporters and permeases, and 6 matched with regulation of transcription.
314 Nine hits belonged to protein secretion/protein secretion by the T3SS and these
315 corresponded to genes encoding Hrp/Hrc components. Notably, most T3S and T3E genes
316 could not be assigned to any specific GO biological process; this was the case for 11
317 *hrp*/*hrc*/*hpa* genes and for 24 T3E genes (S3C Table). Overall, RNA-Seq revealed 20
318 *hrp*/*hrc*/*hpa* genes and 27 genes encoding putative T3Es (including the seven new
319 effectors identified in this study; see below) that had significantly reduced expression in
320 the *hrpX* mutant relative to wild-type M6 (S3B and S3C Tables). Importantly, almost 60
321 genes that showed reduced expression in the *hrpX* mutant are annotated as hypothetical
322 proteins and did not show similarity to known T3E genes. It is possible that some of these
323 genes encode novel T3Es.
324
325 **Fig. 3. Distribution of *Acidovorax citrulli* M6 HrpX-regulated genes among**
326 **categories of biological processes.** Of the 159 genes that showed reduced expression in
327 the *hrpX* mutant relative to wild-type M6, only 47 could be assigned to at least one Gene
328 ontology (GO) biological process category (blue columns). HrpX-regulated genes
329 encoding T3S structural and accessory proteins (red column) and putative T3Es (green
330 column) were manually assigned to these categories.
331
332 Interestingly, the *hrpX* mutant also showed reduced expression of several genes
333 encoding proteins that are putatively secreted by the type II secretion system (T2SS). We

334 used SignalP, Pred-Tat and Phobius tools to detect putative Tat or Sec type II secretion
335 (T2S) signals in the ORFs of all genes that showed significantly lower expression in the
336 *hrpX* mutant relative to the wild-type strain. While T2S signals were predicted in 39 genes
337 by at least one of the tools (not shown), 14 genes were predicted to encode products with
338 T2S signals by the three different tools (S3E Table). Among these genes were
339 *APS58_0633* (*xynB*) encoding 1-4-β-xylanase, *APS58_2599* (*pelA_2*), encoding pectate
340 lyase, and *APS58_3722*, encoding a family S1 extracellular serine protease. These three
341 genes were also shown to contain PIP boxes in their promoter region (S3B Table).
342 Of the 28 genes showing increased expression in the *hrpX* mutant relative to wild-
343 type M6, only ten could be assigned to GO categories, most of which belonged to
344 regulatory genes (regulation of transcription, phosphorelay signal transduction system,
345 signal transduction; S3D Table).
346
347 **Identification of PIP boxes in HrpX-regulated genes**
348 We used fuzznuc to search for perfect PIP boxes in the *A. citrulli* M6 genome,
349 using the consensus sequence TTCGB-N15-TTCGB. Based on Koebnik *et al.* [32], we
350 considered only those cases for which the distance between the end of the PIP box and
351 the putative start codon was shorter than 650 nucleotides. This screen revealed a total of
352 78 PIP boxes (S4 Table), of which 41 correlated with significant regulation by HrpX
353 (Table 2 and S4 Table). We used the PIP boxes of the aforementioned 41 genes/operons
354 to determine the consensus PIP box of *A. citrulli* using the MEME suite (Fig 4).
355 Importantly, some of the PIP boxes are upstream of operons, thus probably regulating the
356 expression of more than one gene. We detected additional 25 genes [marked as (+) in the
357 PIP box column of S3B Table] that are likely in PIP box-containing operons and showing
358 higher expression in the wild-type strain relative to the *hrpX* mutant. It is also worth

359 mentioning that eleven additional genes (some of which encoding T3Es) carrying PIP
360 boxes showed higher expression values in the wild-type relative to the *hrpX* mutant in the
361 RNA-Seq experiment, but were slightly below the level of statistical significance (S3A
362 and S4 Tables).

363

364 **Table 2. Perfect plant-inducible promoter (PIP) box sequences in genes that were**
365 **shown to be regulated by HrpX in *Acidovorax citrulli* M6.**

Gene_ID[1]	Annotation[1]	Strand	PIP box[2]	Start of PIP box	End of PIP box	Gene start codon	Distance (bp)[3]
APS58_0030	*HP*	-	ttcgttttgttgattggaaattcgc	34554	34578	34553	1
APS58_0077	*HP*	-	ttcgcaattcgagaaatttgttcgg	93187	93211	93022	165
APS58_0185	*HP*	-	ttcgtgttgaaggcattcgtttcgg	216423	216447	216315	108
APS58_0197	*puuD_1*	-	ttcgtgcatcggctcttccattcgc	227069	227093	226515	554
APS58_0218	*HP*	-	ttcgcgtgtgcgtgaactctttcgc	254146	254170	254075	71
APS58_0500	*HP*	-	ttcgccccggcctgccggacttcgc	579574	579598	579502	72
APS58_0502	*yopJ*	-	ttcgcccggcaggcacccgtttcgc	583025	583049	582809	216
APS58_0543	*HP*	-	ttcgcatgcatgtgagcggattcgg	631454	631478	630839	615
APS58_0633	*xynB*	-	ttcgcttgctgcttcacgggttcgc	715951	715975	715863	88
APS58_0886	*HP*	-	ttcgcatcgccgtgcatggtttcgc	1015878	1015902	1015701	177
APS58_0986	*HP*	+	ttcgcattccgcgcgactgcttcgc	1113649	1113673	1113709	36
*APS58_1000**[4]	*HP*	+	ttcgccaccgggcgcacggcttcgt	1129783	1129807	1129817	10
APS58_1026	*HP*	-	ttcgtgcacgcgcctgccggttcgc	1161953	1161977	1161806	147
APS58_1255	*HP*	+	ttcgcgcgccacggccccgcttcgc	1409795	1409819	1410119	300
APS58_1340	*HP*	+	ttcgcatgtccgcggagtcgttcgg	1511860	1511884	1512052	168
APS58_1448	*HP*	-	ttcgcgaggccacgcattgcttcgc	1632395	1632419	1632309	86
APS58_1483	*HP*	-	ttcgcattcccgtggccggcttcgg	1669735	1669759	1669644	91
APS58_1760	*T3E protein*	+	ttcgtgcctgcgggcacgtattcgc	1970321	1970345	1970408	63
APS58_1954	*HP*	+	ttcgcaagttctccagctttttcgg	2174442	2174466	2174654	188
APS58_1986	*HP*	-	ttcgcgccagcgcgcgggacttcgc	2212320	2212344	2212063	257
APS58_2304	*hrcQ*	-	ttcgccttacgcgatgagccttcgg	2546196	2546220	2546073	123
APS58_2307	*hrcU*	-	ttcgcgcggggcggaaccgcttcgc	2550224	2550248	2550146	78
APS58_2308	*hrpB7*	+	ttcgcattccggtgcgcggcttcgg	2550284	2550308	2550387	79
APS58_2312	*hrpW*	+	ttcgcatccgctgcgccgccttcgc	2553056	2553080	2553423	343
APS58_2314	*HP*	+	ttcgcgatgccgcatgcagcttcgc	2556756	2556780	2556924	144
APS58_2329	*HP*	-	ttcgcaagccatgaagcaacttcgt	2567741	2567765	2566734	7
APS58_2331	*hrcC*	-	ttcgcaagccgtcgcgcggcttcgc	2569879	2569903	2569803	76
APS58_2345	*HP*	+	ttcgcgcaaaggtgagcggcttcgc	2581209	2581233	2581585	352
APS58_2347	*HP*	+	ttcgcaccgccgtgcaggggttcgc	2585239	2585263	2585399	136
APS58_2599	*pelA_2*	-	ttcggctgcatggccgccgcttcgc	2862541	2862565	2862501	40
APS58_2771	*HP*	+	ttcggaccgctgcgccggcattcgc	3052001	3052025	3052432	407
APS58_2974	*HP*	-	ttcgttccaggcaggctgtcttcgc	3262651	3262675	3262590	61
APS58_3261	*HP*	+	ttcgcctggcgcaatgcgggttcgc	3574633	3574657	3574825	168
APS58_3289	*hopW1-1*	-	ttcgccggggagggcagtttttcgc	3605458	3605482	3605197	261
APS58_3297	*HP*	-	ttcgcggggtggcactccgcttcgg	3611673	3611697	3611575	98
APS58_3344	*HP*	-	ttcgcagccctccccggcacttcgc	3656278	3656302	3656147	131
APS58_3685	*HP*	-	ttcgcacgttggacatgcatttcgc	3989762	3989786	3989700	62
APS58_3722	*HP*	+	ttcgttttaagacgaagaaattcgc	4030992	4031016	4031209	193
APS58_4095	*HP*	+	ttcgcatccatggggccggcttcgc	4442672	4442696	4443213	517
APS58_4116	*HP*	-	ttcgcgcaggcgcatgcgcgttcgc	4476037	4476061	4475953	84
APS58_4317	*HP*	-	ttcgcaccgtcggccatcgcttcgc	4692889	4692913	4692529	360

366 [1] Locus_tag and annotation according to GenBank accession CP029373. *HP*, hypothetical protein.
367 [2] PIP box consensus: TTCGB-N15-TTCGB (where B is any nucleotide except adenine).

368 [3] Distance between the end of the PIP box and the first nucleotide of the start codon.
369 [4] Gene *APS58_1000**: this gene was not annotated in the new M6 annotation. It is located
370 between genes *APS58_0999* and *APS58_1000* (positions 1129817-1130383), and its expression
371 was confirmed by RNA-Seq.

372

373 **Fig. 4. Sequence logo of the *Acidovorax citrulli* M6 plant-inducible promoter (PIP)**
374 **box motif.** The logo was generated with MEME-ChiP based on multiple alignment of the
375 41 perfect PIP boxes that were found to be associated with HrpX-regulated genes by
376 RNA-Seq (see Table 2).

377

378 **Establishment of a translocation assay for validation of *A. citrulli* T3Es**

379 A critical prerequisite for the discovery of new T3Es is the availability of a
380 suitable translocation assay. We assessed the possibility of exploiting the *avrBs2-Bs2*
381 gene-for-gene interaction to test translocation of predicted *Acidovorax* T3Es into plant
382 cells. The *X. euvesicatoria* AvrBs2 effector elicits an HR in pepper plants carrying the
383 *Bs2* resistance gene [33]. A truncated form of this effector, carrying amino acids 62-574
384 ($AvrBs2_{62-574}$), lacks the N-terminal T3S translocation signal, but retains the ability to
385 elicit the HR when expressed in *Bs2* pepper cells [34]. The *avrBs2-Bs2* translocation
386 assay is thus based on generation of plasmids carrying the candidate T3E (CT3E) genes
387 fused upstream and in frame to the $AvrBs2_{62-574}$. The plasmid is then mobilized into a *X.*
388 *euvesicatoria* 85-10 *hrpG**Δ*avrBs2* strain, that constitutively expresses *hrpG* and lacks
389 *avrBs2*. The resulting strain is used to inoculate leaves of the pepper line ECW20R that
390 carries the *Bs2* gene. If the $AvrBs2_{62-574}$ domain is fused with a T3E gene, this elicits a
391 *Bs2*-dependent HR [34]. Teper *et al.* recently used this reporter system to validate novel
392 T3Es of the *X. euvesicatoria* strain 85-10 [28].

393 Given the close similarity between the T3SSs of *A. citrulli* and *Xanthomonas* spp.,
394 we hypothesized that the *X. euvesicatoria* T3S apparatus would recognize and translocate

395 *A. citrulli* T3Es, and therefore, that the *avrBs2-Bs2* reporter system would be suitable for
396 validating *A. citrulli* CT3E genes. To assess this hypothesis, we tested translocation of
397 eight T3Es of *A. citrulli* showing similarity to known T3Es of other plant pathogenic
398 bacteria. All tested fusions were translocated into pepper cells in a T3S-dependent manner
399 and induced a *Bs*2-dependent HR in ECW20R pepper leaves. In contrast, HR was not
400 detected when the fusions were tested in ECW30 leaves (lacking the *Bs2* gene), and when
401 a *X. euvesicatoria hrpF* mutant (impaired in T3S) was used in these assays (Fig 5A).
402 Overall, these results demonstrated the suitability of the *avrBs2-Bs2* assay for validation
403 of *A. citrulli* CT3Es.
404
405 **Fig. 5. Translocation assays of T3Es of *Acidovorax citrulli* M6.** (A) Selected T3Es
406 based on sequence similarity to T3Es from other plant pathogenic bacterial species (see
407 Table 1). (B) Candidate T3Es (CT3Es) selected from ML and RNA-Seq analyses.
408 T3E/CT3E ORFs were cloned in plasmid pBBR1MCS-2 upstream to the AvrBs2$_{62\text{-}574}$
409 domain, which elicits HR in ECW20R pepper plants carrying the *Bs2* gene, but not in
410 ECW30R pepper plants that lack this gene. The plasmids were transformed into
411 *Xanthomonas euvesicatoria* 85-10-*hrpG*-ΔavrBs2*, and the resulting strains were used to
412 inoculate pepper plants. All known T3Es (A) and seven among eleven tested CT3Es (B)
413 elicited HR in ECW20R but ECW30R leaves, similarly to the positive control XopS-
414 AvrBs2$_{62\text{-}574}$. Infiltrated areas are surrounded by red circles. No HR was induced when
415 leaves were inoculated with a *X. euvesicatoria* mutant impaired in T3S (*ΔhrpF*)
416 expressing T3E/CT3E-AvrBs2$_{62\text{-}574}$ fusions. Also, no HR was induced following
417 inoculation with *X. euvesicatoria* 85-10-*hrpG*-ΔavrBs2* without any plasmid (not
418 shown) or with a plasmid expressing the AvrBs2$_{62\text{-}574}$ domain alone (ΔN-terminal).
419 Numbers at the top correspond to the locus_tag in strain M6 (for example, 0492 is gene
420 *APS58_0492*).
421
422 **Seven novel T3Es of *A. citrulli* are translocated into plant cells**

423 Following validation of the *avrBs2-Bs2* reporter assay for *A. citrulli* T3Es, we
424 selected seven CT3Es based on results from the first ML run and RNA-Seq analysis. Four
425 genes that were ranked relatively low in the ML were also included in these experiments
426 to evaluate the quality of the ML prediction (Table 3 and S1 Table). All seven CT3E
427 genes, but not the low-ranked ML genes, were translocated (Fig 5B). The validated genes
428 were annotated as hypothetical proteins, had a predicted PIP box, were shown to be
429 positively regulated by HrpX, and ranked high in the ML run (Table 3 and S1 Table).
430 Importantly, the gene *APS58_1340*, which contains a PIP box in its promoter region and
431 its expression is regulated by HrpX (Table 3) was not translocated, indicating that these
432 two parameters alone are not sufficient for accurate prediction of T3Es.
433

434 **Table 3. Candidate T3E genes of *Acidovorax citrulli* M6 that were tested in the**
435 ***avrBs2-Bs2* translocation assays.**

Gene ID[1]	Product	ML[2]	PIP[3]	RSEQ[4]	TRA[5]
APS58_0500	Hypothetical protein[7]	39/48	+	+	+
APS58_0705	GrxD, glutaredoxin-4	91/2203	-	-	-
APS58_0863	Hypothetical protein	64/749	-	-	-
APS58_1000*[6]	Hypothetical protein[7]	21/*	+	+	+
APS58_1340	Hypothetical protein	84/535	+	+	-
APS58_1448	Hypothetical protein[7]	17/104	+	+	+
APS58_2974	Hypothetical protein[7]	19/29	+	+	+
APS58_3297	Hypothetical protein[7]	20/61	+	+	+
APS58_4095	Hypothetical protein[7]	31/46	+	+	+
APS58_4116	Hypothetical protein[7]	11/43	+	+	+
APS58_4399	Hypothetical protein	174/739	-	-	-

436 [1] Gene IDs are according to the annotation of the *A. citrulli* M6 chromosome (GenBank accession
437 CP029373).
438 [2] ML: rankings in first/second machine learning (ML) runs. *, gene *APS58_1000** was not
439 included in the second ML run (see below).
440 [3] PIP: presence (+) or absence (-) of perfect plant-inducible promoter (PIP) box in the promoter
441 region.

442 [4] RSEQ: significantly reduced expression in the *hrpX* mutant relative to the wild type (+)/no
443 significant differences between strains (-).
444 [5] TRA: translocated (+)/non-translocated (-) in *avrBs2-Bs2* translocation assays (rows of validated
445 genes are shaded with gray).
446 [6] *APS58_1000**: this gene ranked high in the first ML but was not annotated in the recent
447 annotation of *A. citrulli* M6, although its expression was confirmed by RNA-Seq. Its ORF is
448 located between genes *APS58_0999* and *APS58_1000* (positions 1129817-1130383).
449 [7] These genes were detected only in plant pathogenic *Acidovorax* species.
450

451 BlastP analyses of the seven newly identified T3E genes revealed strong similarity
452 only to hypothetical proteins of plant pathogenic *Acidovorax* species. The fact that no
453 homologs for these genes were detected in non-pathogenic *Acidovorax* strains (despite
454 the availability of more than 70 genomes of such spp.) or in other plant pathogenic
455 bacterial species suggests a specific and unique role for their products in *Acidovorax*
456 pathogenicity. These seven genes were detected also in AAC00-1 (S2 Table) and in all
457 other group I and II genomes available in NCBI. Some of them were widely distributed
458 among other plant pathogenic *Acidovorax* species. For instance, *APS58_4095* was also
459 detected in *A. oryzae* and in *A. cattleyae*, and homologs with less than 60% query
460 coverage were also present in *A. konjaci*, *A. anthurii* and *A. valerianellae*. In contrast,
461 *APS58_2974* was not detected in *Acidovorax* spp., other than *A. citrulli* and *A. avenae*
462 (S2 Table). Searches for conserved domains in these T3Es did not provide any insight.
463

464 **Assessment of localization of three of the newly identified T3Es**

465 We attempted to assess the subcellular localization of three of the newly identified
466 T3Es, APS58_0500, APS58_1448 and APS58_4116. Prediction of subcellular
467 localization using the Plant-mPLoc server indicated that the three effectors could localize
468 to the nucleus. Browsing these T3Es with the LogSigDB server revealed endoplasmic

469 reticulum (ER) localization signals in the three effectors, and nuclear localization signals
470 in APS58_0500 and APS58_4116.

471 We assessed localization of these effectors fused to the yellow fluorescent protein
472 (YFP) in *Nicotiana benthamiana* leaves following transient expression by
473 agroinfiltration. Based on the aforementioned predictions, in first experiments the leaves
474 were co-infiltrated with *A. tumefaciens* carrying the ER marker mRFP-HDEL, and were
475 also stained with DAPI for nucleus localization. Representative images from these
476 experiments are shown in Fig 6. The results suggested that the three effectors could
477 interact with the ER, but only APS58_0500 and APS58_1448 partially localized to the
478 nucleus, including in clearly visible nuclear foci (Fig 6).

479

480 **Fig. 6. Transient expression of *Acidovorax citrulli* T3Es in *Nicotiana benthamiana*.**
481 The T3E genes *APS58_0500*, *APS58_4116* and *APS58_1448*, identified by ML and
482 RNA-Seq and validated in translocation assays, were cloned in the binary vector
483 pEarleyGate101, fused to the C-terminus of YFP. The plasmids were transformed into
484 *Agrobacterium tumefaciens* GV3101, and the resulting strains were used for transient
485 expression in *N. benthamiana*. Leaves were co-inoculated with *A. tumefaciens* GV3101
486 carrying the mRFP-HDEL endoplasmic reticulum marker and stained with DAPI for
487 visualization of plant cell nuclei. Samples were visualized in a Leica SPE confocal
488 microscope 48 h after inoculation. Bars at the right bottom of each picture, 20 µm.

489

490 In a second set of experiments, the YFP-fused effectors were co-infiltrated with
491 free-mCherry, localized mainly in the cytosol and in the nucleus, HDEL-mCherry,
492 localized to the ER, and the membrane-bound protein SlDRP2A (L. Pizarro and M. Bar,
493 unpublished results). Representative images from these experiments are shown in S2-S4
494 Figs for APS58_0500, APS58_1448 and APS58_4116, respectively. The three effectors
495 partially co-localized with the membrane-bound protein SIDRPA, as evidenced by the
496 Pearson correlation coefficients (0.40±0.024 for APS5_0500, 0.49±0.040 for

497 APS58_1448, and 0.53±0.037 for APS58_4116). Since APS58_0500 appeared to have a
498 stronger membrane localization, we used the classical plasma membrane microdomain
499 protein Flot1 [35] as an additional membrane control marker. Indeed, APS58_0500 had
500 an expression pattern that was highly similar to that of Flot1 (compare top and bottom
501 panels in S2 Fig). In agreement with the first set of experiments (Fig 6), APS58_0500 (S2
502 Fig) and APS58_1448 (S3 Fig) partially localized to the nucleus. On the other hand, these
503 experiments confirmed that only APS58_4116 partially interacted with the ER, mostly at
504 the nuclear envelope (Fig 6 and S4 Fig; Pearson coefficient with HDEL-mCherry
505 0.53±0.037). None of the effectors was shown to have a significant cytosolic presence:
506 the Pearson coefficient with mCherry was lower than 0.12 for APS_0500 and APS_4116,
507 while for APS_1448 the coefficient was 0.53±0.037, due to the strong nuclear presence
508 of this effector, as indicated above. Overall, we can conclude that the three effectors are
509 associated with the plasma membrane, APS58_0500 and APS58_1448 partially localize
510 to the nucleus, and APS_4116 partially interacts with the ER.
511
512 **Generating an improved list of candidate T3Es of *A. citrulli* M6 with a second ML**
513 **run**
514 Machine learning can be improved after refinement of features specific to the
515 studied pathogen. Thus, we carried out a second ML run using the *A. citrulli* M6 genome.
516 The main differences between the first and second ML runs were: (i) the second run was
517 done on the M6 genome [9], which by this time was fully assembled; (ii) we included the
518 seven novel T3Es identified in this study in the positive set and the four ORFs that were
519 found not to be translocated were added to the negative set; (iii) in the positive set we
520 included ORFs with high sequence similarity to known effectors from other bacteria,
521 based on our homology search results (Table 1); and (iv) we used HrpX-mediated

522 regulation as an additional feature used to train the classifier. The results are summarized
523 in S5 Table. Most known/validated T3Es ranked among the top 100 hits, and among the
524 top 40 hits, 34 were known/validated T3Es. Importantly, some genes with high propensity
525 to encode T3Es (ranking among the top 60 in the second ML run) did not appear among
526 the top 200 hits in the first ML list (Table 1 and S1 Table), thus supporting the higher
527 reliability of the new list relative to the first prediction.

528 Among the top 100 hits of the second ML run, there were 37 genes that matched
529 to hypothetical proteins from the public database, with no similarity evidence to suggest
530 a T3E nature. Since this was the case of the seven T3Es validated in this study, it is
531 possible that some of these genes encode previously undiscovered T3Es. In this regard, it
532 is worth mentioning genes *APS58_1954*, *APS58_1986*, *APS58_3685*, *APS58_0987* and
533 *APS58_1694* (ranking at positions 20, 27, 57, 62 and 83 in the second ML, respectively).
534 While *APS58_1694* shares similarity only with hypothetical proteins of plant pathogenic
535 *Acidovorax* species, the first four also share similarities to hypothetical proteins of other
536 plant pathogenic genera (eg., *Xanthomonas*, *Ralstonia*, *Pseudomonas* and/or *Erwinia*).
537 These genes also showed increased expression in wild-type M6 relative to the *hrpX*
538 mutant, and have PIP boxes in their promoter region. Therefore, these genes are strong
539 candidates for further experimental validations.

540

541 **Discussion**

542 Type III effectors (T3Es) play a dual role in the interaction between many Gram-
543 negative plant pathogenic bacteria and plants: while they collectively promote virulence
544 on susceptible plants, some may induce effector-triggered immunity (ETI) in plants
545 carrying the corresponding resistance (*R*) genes. *R* genes provide resistance against
546 economically important pathogens and have been mobilized to commercial crop varieties

547 by breeding programs. Thus, this is one of the most important means of disease
548 management [36, 37].

549 *Acidovorax citrulli* requires a functional type III secretion (T3S) system for
550 pathogenicity [10]. The main objective of this study was to significantly advance the
551 current knowledge about the arsenal of T3Es of *A. citrulli*. Among well-investigated plant
552 pathogenic bacteria, the pools of T3Es vary from only few effectors in phytopathogenic
553 bacteria from the *Enterobacteriaceae* family, to approximately 20 to 40 in strains of *P.*
554 *syringae* and *Xanthomonas* spp. [21, 28, 40-44] and an average of over 75 in *R.*
555 *solanacearum* isolates [45, 46]. Thus, we hypothesized that the repertoire of *A. citrulli*
556 T3Es could be much larger than the eleven T3E genes identified in the group II strain
557 AAC00-1 [27].

558 As a first approach to uncover the arsenal of *A. citrulli* T3Es, we used a genome-
559 wide machine learning (ML) algorithm to determine the propensity of ORFs to encode
560 T3Es. In parallel, we looked carefully at the annotation of the group I model strain of *A.*
561 *citrulli*, M6, and carried out BlastP analyses of the genes encoding hypothetical proteins
562 or functions that could infer effector activity. These analyses revealed 51 putative T3E
563 genes that shared different levels of similarity with known effector genes from
564 *Xanthomonas* spp., *R. solanacearum* and/or *P. syringae* strains (Table 1). Homologs for
565 most of these T3E genes and for those identified in the present study were also detected
566 in other plant pathogenic *Acidovorax* species (S2 Table).

567 To identify new T3E genes of *A. citrulli*, we also used RNA-Seq to identify HrpX-
568 regulated genes. Based on the knowledge accumulated with *Xanthomonas* spp. and *R.*
569 *solanacearum* [22, 32, 47, 48], we expected that most genes encoding T3SS components
570 and some T3Es of *A. citrulli* would be under the direct regulation of HrpX. This
571 assumption was strengthened in preliminary experiments comparing gene expression

572 between a wild-type and a *hrpX* mutant strain (Fig 1C). As previously mentioned, Zhang
573 *et al.* recently showed that HrpX controls the expression of one T3E gene in the group II
574 strain, Aac5 [26].

575 The RNA-Seq approach revealed 159 genes showing significantly reduced
576 expression in the *hrpX* mutant, while 28 genes had significantly increased expression in
577 the mutant (S3 Table). These numbers are similar to those reported in gene expression
578 studies carried out with *Xanthomonas* spp. HrpX and with *R. solanacearum* HrpB. For
579 instance, microarray analyses of *Xanthomonas axonopodis* pv. *citri* (*Xac*) in XVM2
580 medium revealed that 181 genes were up-regulated by HrpX, while 5 to 55 genes
581 (depending on the time point) were down-regulated by this transcriptional regulator [47].
582 Occhialini *et al.* found 143 HrpB up-regulated genes and 50 HrpB down-regulated genes
583 in *R. solanacearum* [48]. In these, as well as in several other studies, HrpX/HrpB was
584 found to regulate the expression of most genes encoding T3S components and accessory
585 proteins as well as several T3E genes [21, 22, 49]. In line with this background, among
586 the 159 HrpX up-regulated genes found in our study, 20 encoded *hrp*/*hrc*/*hpa* genes and
587 27 encoded T3E genes. Interestingly, *hrcC* was a member of the *A. citrulli* HrpX regulon.
588 *hrcC* expression in *X. euvesicatoria* is directly regulated by HrpG, in an HrpX-
589 independent manner [31]. In contrast, in *R. solanacearum*, *hrcC* is regulated by HrpX
590 [49, 50] as we found in *A. citrulli* M6.

591 In *Xanthomonas* spp. and in *R. solanacearum*, the HrpX/HrpB regulon includes
592 many genes that are not involved in T3S [21, 47, 49]. A similar picture emerged from our
593 study, where HrpX was shown to regulate genes involved in transmembrane transport,
594 including several ABC transporters and permeases as well as transcriptional regulators.
595 Among the HrpX up-regulated genes we also detected several genes whose products are
596 putatively secreted by type II secretion (T2S). These included genes encoding 1-4-β-

597 xylanase (*xynB*), pectate lyase and a protein with similarity to a family of S1 extracellular
598 serine proteases (S3E Table). HrpX regulation of genes encoding type II-secreted
599 enzymes was also demonstrated in *Xanthomonas* spp. and in *R. solanacearum* [22, 47,
600 51-54].

601 Among the 159 HrpX up-regulated genes *in A. citrulli*, more than 60 carried
602 perfect PIP boxes in their promoter region or were part of operons carrying perfect PIP
603 boxes (Table 2; S3 and S4 Tables). Although some other genes may carry imperfect PIP
604 boxes and may be directly regulated by HrpX, this result suggests that many of the HrpX
605 up-regulated genes are indirectly regulated by this transcriptional factor. This is a
606 reasonable assumption, considering that among the genes that are up- and down-regulated
607 by HrpX, there are several transcriptional regulators. For instance, genes encoding
608 transcriptional factors belonging to the LysR (*APS58_0949* and *APS58_2039*), IclR
609 (*APS58_1263*), FmbD (*APS58_1340*) and TetR (*APS58_3638*) families were shown to
610 be up-regulated by HrpX. In contrast, two genes encoding DNA-binding response
611 regulators, homologous to PhoP (*APS58_0821*) and FixJ (*APS58_1682*) were HrpX-
612 down-regulated (S2B Table).

613 After demonstrating the suitability of the *avrBs2-Bs2* T3E translocation assay with
614 eight known T3Es, we used the data obtained from the first ML run and the RNA-Seq
615 analysis to select seven *A. citrulli* M6 ORFs for experimental validation (Fig 5). We
616 validated translocation of the seven candidates, thus demonstrating the strength of
617 combining ML and RNA-Seq for identifying T3E genes. Importantly, the lack of
618 translocation of the four ORFs that received relatively low scores in the first ML run
619 strengthened the suitability of our combined computational/experimental approach.

620 Remarkably, the seven effectors identified in this study were up-regulated by
621 HrpX and carried PIP boxes in their promoter regions, while among the four non-

622 validated genes, only one had these traits (Table 3). An interesting trait of the seven new
623 T3Es was that they share significant similarity only with hypothetical proteins of other
624 plant pathogenic *Acidovorax* strains (Table 3 and S2 Table). This strongly supports that
625 these effectors are unique to plant pathogenic *Acidovorax*. Importantly, a second ML run,
626 informed by the knowledge accumulated from this study, revealed additional genes that
627 were ranked in relatively high positions and encoded hypothetical proteins that occur only
628 in plant pathogenic *Acidovorax* or in other plant pathogenic bacteria (S5 Table). These
629 represent high priority CT3Es for future experimental validation assays. This emphasizes
630 one benefit of the ML approach: its ability to integrate novel knowledge in the prediction
631 algorithm.

632 Another interesting characteristic of the new T3Es discovered in this study is their
633 relatively small size. Based on the annotation of the M6 genome, the average and median
634 lengths of *A. citrulli* M6 T3Es are 387.7 and 345 amino acids (a.a.), respectively. Except
635 for *APS_4116* that encodes a 347-a.a. protein, the size of the six other new T3Es ranged
636 from 113 a.a. (APS58_4095) to 233 a.a. (APS58_0500) (S5 Fig). In the public database
637 (GenBank), there are several examples of small T3Es from plant pathogenic bacteria,
638 including *Xanthomonas* AvrXv3 (most having 119 a.a.), *Pseudomonas syringae* HopAF1
639 (112-291 a.a.), HopBF1 (125-207 a.a.), HopF2 (177-280 a.a.), HopH1 (201-218 a.a.) and
640 AvrRpt2 (222-255 a.a.), and the *R. solanacearum*/*Xanthomonas* HopH1 homologs (155-
641 218 a.a.).

642 In this study we assessed plant cell localization of three of the new T3Es validated
643 in translocation assays, APS58_0500, APS58_1448 and APS58_4116. Utilization of
644 subcellular localization prediction tools and confocal microscopy of *N. benthamiana*
645 agro-infiltrated leaves strongly suggest that the three tested effectors interact with the
646 plasma membrane (S2-S4 Figs), with APS58_0500 remarkably mimicking the

647 localization of the classical non-clathrin mediated endocytic system protein, Flot1 [35].
648 While APS58_4116 interacted with the endoplasmic reticulum (Fig 6 and S2 Fig),
649 effectors APS58_0500 and APS58_1448 partially localized to the nucleus (Fig 6; S3 and
650 S4 Figs). Interestingly, and in line with the predicted nuclear localization of these
651 effectors, BlastP showed that APS58_0500 has low similarity with an ATP-dependent
652 RNA helicase of the Metazoa organism *Clonorchis sinensis* (query cover, 38%; e-value,
653 0.23), while APS58_1448 has low similarity to a transcriptional regulator of the
654 bacterium *Hoeflea halophila* (query cover, 54%; e-value, 0.19-0.53), suggesting possible
655 functions the *Acidovorax* effectors might execute upon entrance into the plant cell
656 nucleus.

657 In conclusion, we have combined sequence analysis, ML and RNA-Seq
658 approaches to uncover the arsenal of T3Es of the group I model strain of *A. citrulli*, M6,
659 including discovery of new T3Es that appear to be unique to plant pathogenic *Acidovorax*
660 spp. Further characterization of the novel T3Es identified in this study may uncover new
661 host targets of pathogen effectors and new mechanisms by which pathogenic bacteria
662 manipulate their hosts. We also demonstrated the suitability of a translocation reporter
663 system for validation of *A. citrulli* T3Es, which we expect, will be very helpful to the
664 *Acidovorax* research community. Until recently it was assumed that *A. citrulli* strains (and
665 in general plant pathogenic *Acidovorax* strains) possess little over ten T3E genes.
666 However, from this study it is clear that the *A. citrulli* pan-genome encodes more than 50-
667 60 T3Es. Therefore, the *A. citrulli* T3E repertoire is larger than those of most well-
668 characterized plant pathogenic bacteria, including plant pathogenic *Enterobacteria*, *P.*
669 *syringae* pathovars, *Xanthomonas* spp. and, and closer in numbers to the T3E repertoires
670 of *R. solanacearum*. Moreover, the second ML run suggested that *A. citrulli* may possess
671 yet unrevealed T3E genes. Importantly, among the 58 known T3E genes of *A. citrulli* M6,

672 ten (17.2%) appear to be unique to group I strains. On the other hand, the group II model
673 strain, AAC00-1, carries T3E genes that are absent or non-functional in group I strains as
674 shown in our previous report [27] and in this study (Table 1 and S1 Table). Thus, it is
675 logical to assume that the variability in T3E content between group I and II strains plays
676 a critical role in shaping the differences in host-preferential association between the
677 groups. Despite this, more research is needed to test this hypothesis, and to understand
678 the mode of action and contribution of individual effectors to the virulence of *A. citrulli*.
679

680 # Methods

681 **Bacterial strains and plasmids**

682 Bacterial strains and plasmids used in this study are listed in S6 Table. Unless
683 stated otherwise, *Acidovorax citrulli* strains were grown at 28 ºC in nutrient broth (NB;
684 Difco Laboratories, Detroit, Michigan) or nutrient agar (NA; NB containing 15 g/L agar).
685 For RT-PCR, qRT-PCR and RNA-seq experiments, *A. citrulli* strains were grown in
686 XVM2 medium [31]. *Xanthomonas euvesicatoria*, *Agrobacterium tumefaciens* and
687 *Escherichia coli* strains were cultured on Luria-Bertani (LB) medium [55] at 28 ºC for *X.*
688 *euvesicatoria* and *A. tumefaciens*, and 37 ºC for *E. coli*. When required, media were
689 supplemented with the following antibiotics: ampicillin (Ap, 100 µg/mL for *E. coli* and
690 200 µg/mL for the others), rifampicin (Rif, 50 µg/mL), kanamycin (Km, 50 µg/mL), and
691 gentamycin (Gm, 50 µg/mL for *A. citrulli* and 10 µg/mL for the others).
692

693 **Molecular manipulations**

694 Routine molecular manipulations and cloning procedures were carried out as
695 described [55]. T4 DNA ligase and restriction enzymes were purchased from Fermentas
696 (Burlington, Canada). AccuPrep® Plasmid Mini Extraction Kit and AccuPrep® PCR

697 Purification Kit were used for plasmid and PCR product extraction and purification,
698 respectively (Bioneer Corporation, Daejeon, Republic of Korea). DNA was extracted
699 with the GeneElute bacterial genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA).
700 PCR primers were purchased from Sigma-Aldrich and are listed in S7 Table. PCR
701 reactions were performed with the Readymix Red Taq PCR reactive mix (Sigma-Aldrich)
702 or with the Phusion high-fidelity DNA polymerase (Fermentas, Waltham, MA, USA)
703 using an Eppendorf (Hamburg, Germany) thermal cycler. Sequencing of PCR fragments
704 and constructs was performed at Hy Laboratories (Rehovot, Israel). *Escherichia coli* S17-
705 1 λpir, DH5-α and DB3.1 strains were transformed using an Eppendorf 2510
706 electroporator according to manufacturer's instructions. Plasmid mobilizations to *A.*
707 *citrulli* and *X. euvesicatoria* strains were done by bi-parental mating as described [56]. *A.*
708 *tumefaciens* cells were transformed by the heat shock method [57].
709
710 **Machine learning classifications**
711 In order to predict T3Es, we applied ML classification algorithms, which are
712 similar to the ones we have previously described [28, 29, 58, 59]. The first ML run was
713 used to search for T3Es in the AAC00-1 genome (GenBank accession CP000512.1). The
714 training data included 12 ORFs that were known as T3Es (see in Results). The negative
715 set included 2,680 ORFs that had high similarity (E-value less than 0.001) to ORFs in the
716 non-pathogenic *E. coli* K12 genome (accession number NC_000913.3). The positive and
717 negative ORFs are marked in S1 Table. For this ML, 71 features were used, including
718 homology (to known effectors or to bacteria without T3SS), composition (amino acid
719 composition, GC content), location in the genome (e.g., distance from known T3Es), and
720 the presence of a PIP box in the promoter region. The complete list of features is given in
721 S8 Table. Features were extracted using in-house Python scripts. The outcome of the ML

722 run is a score for each ORF, reflecting its likelihood to encode a T3E. We evaluated
723 several classification algorithms: random forest [60], naïve Bayes [61], support vector
724 machine (SVM; [62]), K nearest neighbors (KNN), linear discriminate analysis (LDA),
725 logistic regression (all three described in Hastie *et al*. [63]), and Voting, which aims to
726 predict averaging over all other ML algorithms. For each run, feature selection was
727 performed. The ML algorithms and feature selection were based on the Scikit-learn
728 module in Python [64]. The area under the curve (AUC) score over 10-fold
729 cross-validation was used as a measure of the classifier performance. The first ML run
730 was based on the random forest classifier which gave the highest AUC (0.965).

731 The second ML run was similar to the first, with the following modifications.
732 First, the classifiers were run on the M6 genome (GenBank accession CP029373).
733 Second, the positive set included ORFs that were validated as T3Es in this study and
734 ORFs with high sequence similarity to known effectors as described in Table 1. The
735 negative set included 2,570 ORFs (S5 Table). The four ORFs that were experimentally
736 shown not to be T3Es were also included in this negative set. Third, the expression data
737 from the RNA-Seq analysis (HrpX regulation) were added as a feature. Fourth, the PIP
738 box feature was updated to reflect the PIP box as inferred from promoter regions of
739 *Acidovorax* T3Es (see additional bioinformatics tools below). The second ML run was
740 based on Voting classifier, which included all the classifiers specified above, as it gave
741 the highest AUC among all the classifiers. The AUC for this second ML run was 0.999.

742

743 **Generation of *A. citrulli* mutants and complemented strains**

744 *Acidovorax citrulli* M6 mutants disrupted in *hrpX* (*APS58_2298*) and *hrpG*
745 (*APS58_2299*) genes were generated by single insertional mutagenesis following single
746 homologous recombination. Internal fragments of the *hrpX* (383 bp) and *hrpG* (438 bp)

747 ORFs carrying nucleotide substitutions that encode early stop codons, were PCR-
748 amplified and inserted into the *Bam*HI/*Eco*RI site of the suicide plasmid pJP5603 [65].
749 The resulting constructs were transformed into *E. coli* S17-1 λpir, verified by sequencing,
750 and mobilized into *A. citrulli* M6 by bi-parental mating. Transconjugants were selected
751 by Km selection. Disruption of the target genes by single homologous recombination and
752 plasmid insertion was confirmed by PCR and sequencing of amplified fragments. To
753 generate complemented strains for mutants disrupted in *hrpX* and *hrpG* genes, the full
754 ORFs of these genes (1407 pb and 801 bp, respectively) were PCR-amplified and cloned
755 into the *Eco*RI/*Bam*HI sites of pBBR1MCS-5 [66]. The generated plasmids were
756 transformed into *E. coli* S17-1 λpir, verified by sequencing, and transferred by bi-parental
757 mating into the corresponding M6 mutant strains. Complemented strains were selected
758 by Gm resistance and validated by PCR.
759
760 **Infiltration of melon and pepper leaves with *A. citrulli* strains**
761 Melon (*Cucumis melo*) cv. HA61428 (Hazera Genetics, Berurim, Israel) plants
762 were grown in a greenhouse at ~28 °C. Pepper (*Capsicum annum*) cv. ECW20R and
763 ECW30 [67] plants were grown in a growth chamber (16 h/26 °C in the light; 8 h/18 °C
764 in the dark; relative humidity set to 70%). The three youngest, fully expanded leaves of
765 3-week-old melon and 5-week-old pepper plants were syringe-infiltrated in the abaxial
766 side with bacterial suspensions of *A. citrulli* strains containing 10^8 colony forming units
767 (cfu)/mL in 10 mM $MgCl_2$. Phenotypes were recorded 3 and 4 days after inoculation
768 (d.a.i.), for melon and pepper leaves, respectively. For a better visualization of HR
769 symptoms in pepper leaves, the infiltrated leaves were soaked in an acetic
770 acid:glycerol:water solution (1:1:1 v/v) for 4 h and then transferred to ethanol and boiled
771 for 10 min. Experiments were repeated twice with similar results.

772

773 **RNA isolation, cDNA synthesis and reverse transcription-PCR (RT-PCR)**

774 *Acidovorax citrulli* M6 and *hrpX* mutant were grown at 28 °C in 5 mL of XVM2
775 medium for 72 h. Total RNA was isolated using TRI reagent (Sigma-Aldrich) and Direct-
776 zol RNA miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's
777 instructions. Samples were treated with RNase free DNase using Turbo DNA-free kit
778 (Invitrogen, Carlsbad, CA, USA). RNA concentration was quantified using a Nanodrop
779 DS-11 FX (Denovix, Wilmington, Delaware) and RNA integrity was assayed on 1%
780 agarose gels. RNA was reverse transcribed into cDNA using a High Capacity cDNA
781 Reverse Transcription Kit (Applied Biosystems). Semiquantitative RT-PCR analysis was
782 performed using 1 µg of cDNA or gDNA (as positive control for amplification), 0.6 pmol
783 of selected primer, the Phusion High-Fidelity DNA Polymerase (ThermoFisher
784 Scientific, Waltham, MA, USA), and the following conditions: 98 ºC for 15 min, followed
785 by 35 cycles of 98 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 15 s. The *A. citrulli GADPH*
786 housekeeping gene [68] was used as reference. The relative amount of amplified DNA
787 was assayed on 2% agarose gels.

788

789 **RNA-Seq and quality analysis**

790 Total RNA of wild-type M6 and *hrpX* mutant strains was isolated as described
791 above for RT-PCR experiments. Three independent RNA extractions were obtained for
792 each strain. Ribosomal RNA was depleted using the MICROB Express Bacterial mRNA
793 Purification kit (Ambion, Foster City, CA, USA). The integrity and quality of the
794 ribosomal depleted RNA was checked by an Agilent 2100 Bioanalyzer chip-based
795 capillary electrophoresis machine (Agilent Technologies, Santa Clara, CA, USA). RNA
796 sequencing was carried out at the Center for Genomic Technologies at The Hebrew

797 University of Jerusalem (Jerusalem, Israel). The samples were used to generate whole
798 transcriptome libraries using the NextSeq 500 high output kit (Illumina, San Diego, CA,
799 USA) with a NextSeq 2000 sequencing instrument (Illumina). The cDNA libraries were
800 quantified with a Qubit 2.0 Fluorometer (Invitrogen) and their quality was assessed with
801 an Agilent 2200 TapeStation system (Agilent Technologies). One of the *hrpX* mutant
802 libraries was removed from further analysis due to low quality. Raw reads (fastq files)
803 were further inspected with FastQC v0.11.4 [69]. They were trimmed for quality and
804 adaptor removal using Trim Galore default settings: trimming mode, single-end; Trim
805 Galore version 0.4.3; Cutadapt version 1.12; Quality Phred score cutoff, 20; quality
806 encoding type selected, ASCII+33; adapter sequence, AGATCGGAAGAGC (Illumina
807 TruSeq, Sanger iPCR; auto-detected); maximum trimming error rate; 0.1; minimum
808 required adapter overlap (stringency), 1 bp. An average of 0.6% of the reads were quality
809 trimmed and 57% of the reads were treated for adaptor removal.
810
811 **Mapping of RNA-Seq reads on the *A. citrulli* M6 genome and differential expression**
812 **analysis**
813 Cleaned reads (~20 million per sample) were mapped against the latest version of
814 the *A. citrulli* M6 genome (CP029373) using STAR v 2.201 [70]. Mapping files were
815 further processed for visualization by Samtools Utilities v 0.1.19 [71]. The resulting Bam
816 files were used to improve gene and operon predictions along the genome using cufflinks
817 v2.2.1 followed by cuffmerge without a guiding reference file [72]. Uniquely mapped
818 reads per gene were counted twice [once using the original submitted annotation file
819 (orig.gff), and then using the merged annotations by cufflinks-cuffmerge (merged.gff)]
820 using HTSeq-count [73]. Differential expression analysis was performed using the

821 DESeq2 R package [73]. Differentially expressed genes were defined as those genes with
822 a fold-change higher than 2, and a *P* value lower than 0.05.
823
824 **Validation of RNA-Seq results by quantitative real-time PCR (qRT-PCR)**
825 RNA-seq data were verified by qRT-PCR using specific primers of selected genes
826 (S7 Table). Bacterial growth, RNA isolation and cDNA synthesis were as described
827 above for RT-PCR and RNA-Seq experiments. qRT-PCR reactions were performed in a
828 Light Cycler 480 II (Roche, Basel, Switzerland) using 1 µg of cDNA, 0.6 pmol of each
829 primer and the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia),
830 and the following conditions: 95 ºC for 15 min (1 cycle); 95 ºC for 15 s, 60 ºC for 20 s
831 and 72 ºC for 20 s (40 cycles); melting curve profile from 65 to 97 ºC to verify the
832 specificity of the reaction. The *A. citrulli GADPH* gene was used as an internal control to
833 normalize gene expression. The threshold cycles (Ct) were determined with the Light
834 Cycler 480 II software (Roche) and the fold-changes of three biological samples with
835 three technical replicates per treatment were obtained by the ΔΔCt method [74].
836 Significant differences in expression values were evaluated using the Mann-Whitney non-
837 parametrical test (α = 5%).
838
839 **Additional bioinformatics tools**
840 BlastP analyses for search of T3E homologs were done at the NCBI server against
841 the non-redundant protein sequences (nr) database, selecting the organisms *Acidovorax*
842 (taxid: 12916), *Xanthomonas* (taxid: 338), *Ralstonia* (taxid: 48736) or *Pseudomonas*
843 *syringae* group (taxid: 136849), with default parameters. Gene ontology (GO)
844 assignments were done using Blast2GO software v5.2 (https://www.blast2go.com/).
845 SignalP4.1 [75], Phobious [76] and Pred-Tat [77] were used for detection of N-terminal

846 type II secretion signal peptides. The program fuzznuc (EMBOSS package;
847 http://www.bioinformatics.nl/cgi-bin/emboss/fuzznuc) was used to detect perfect PIP box
848 sequences (TTCGB-N15-TTCGB; [32]) in the *A. citrulli* M6 genome. A logo of the PIP
849 box motif of *A. citrulli* M6 was done with MEME-ChiP [78] at the MEME Suite website
850 (http://meme-suite.org/). Domain search of T3Es was carried out using the following
851 databases/tools: Protein Data Bank (PDB) and UniProtKB/Swiss-Prot (through NCBI
852 Blast), PFAM (https://pfam.xfam.org/), Prosite (https://prosite.expasy.org/) and InterPro
853 (https://www.ebi.ac.uk/interpro/search/sequence-search). LogSidDB [79] and Plant-
854 mPLoc [80] were used for detection of protein localization signals and for prediction of
855 subcellular localization of T3Es, respectively.
856
857 **Translocation assays**
858 The ORFs without the stop codon of candidate genes were amplified using
859 specific primers (S7 Table) and cloned into the *Sal*I/*Xba*I sites of pBBR1MCS-
860 2*::avrBs2*$_{62\text{-}574}$, upstream to and in frame with the *avrBs2*$_{62\text{-}574}$ HR domain of *avrBs2* and
861 an haemagglutinin (HA) tag [28], except for ORFs of genes *APS58_0500* and
862 *APS58_1760*, which were cloned into the *Xho*I/*Xba*I sites of the same vector. The
863 resulting plasmids were mobilized into *X. euvesicatoria* strains 85-10 *hrpG**Δ*avrBs2* [81]
864 and 85-10 *hrpG**Δ*hrpF* [82]. Expression of recombinant T3E/CT3E-AvrBs2$_{62\text{-}574}$-HA
865 proteins was verified by Western blot using the iBlot Gel Transfer Stacks Nitrocellulose
866 kit (Invitrogen), and anti- hemagglutinin (HA)-tag and horseradish peroxidase (HRP)
867 antibodies (Cell Signaling Technology, Danvers, MA, USA) (S6 Fig). For translocation
868 assays, *X. euvesicatoria* strains were grown overnight in LB broth with Km, centrifuged
869 and resuspended in 10 mM $MgCl_2$ to a concentration of 10^8 cfu/mL. These suspensions
870 were used to infiltrate the three youngest, fully expanded leaves of 5-week-old ECW20R

871 and ECW30R [83] pepper plants, carrying and lacking the *Bs2* gene, respectively, using
872 a needleless syringe. The plants were kept in a growth chamber at 25 °C, ~50% relative
873 humidity, 12 h day/12 h night. HR was monitored 36 h after inoculation (h.a.i.). For
874 visualization of cell death, the infiltrated leaves were treated as described above for
875 pepper leaves infiltrated with *A. citrulli* strains. Each candidate gene was tested in three
876 independent experiments with at least three plants, with similar results being obtained
877 among replicates and experiments.
878
879 ***Agrobacterium*-mediated transient expression and confocal imaging**
880 The ORFs of genes *APS58_0500*, *APS58_1448* and *APS58_4116* were amplified
881 with specific primers (S7 Table) and cloned into pEarlyGate101 binary vector [84],
882 upstream of a Yellow Fluorescence Protein (YFP) encoding gene and an HA tag using
883 the Gateway cloning system (ThermoFisher Scientific). The resulting plasmids were
884 verified by sequencing and mobilized into *A. tumefaciens* GV3101 as indicated above.
885 Transient expression experiments were performed following the protocol described by
886 Roden *et al.* [81] with few modifications. Briefly, overnight cultures of *A. tumefaciens*
887 GV3101 carrying the different plasmids were centrifuged, and pellets were resuspended
888 in induction solution containing 10 mM $MgCl_2$, 10 mM 2-(N-morpholino)-ethanesulfonic
889 acid (MES), and 200 mM acetosyringone (pH 5.6). The suspensions were incubated at 25
890 ºC without shaking for 3 h. Bacterial cultures were then diluted to OD_{600nm}~0.6 and
891 infiltrated with a needleless syringe into leaves of 4-week-old *N. benthamiana* plants [85]
892 that were grown in a growth chamber (16 h/26 °C in the light, 8 h/18 °C in the dark;
893 relative humidity set to 70%). Subcellular localization of tested T3Es coupled to YFP
894 were investigated by co-infiltration with *A. tumefaciens* GV3101 carrying monomeric
895 Red Fluorescence Protein fused in frame with the endoplasmid reticulum (ER) marker

896 HDEL (mRFP-HDEL; [86, 87]), the membrane associated SlDRP2A (L. Pizarro and M.
897 Bar, unpublished results) fused to monomeric Cherry fluorescent protein, and by staining
898 with 1 mg/mL 4′,6-diamidino-2-phenylindole (DAPI), that was used to detect the nucleus
899 of the plant cells [88]. As controls, plants were infiltrated with *A. tumefaciens* GV3101
900 carrying pEarlyGate104 (YFP-encoding gene). Infiltrated plants were kept in the growth
901 chamber at similar conditions as above, and 48 h.a.i., functional fluorophores were
902 visualized using a SPE (Leica Microsystems, Wetzlar, Germany) or a LSM 780 (Zeiss,
903 Oberkochen, Germany) confocal microscope. Images were acquired using two tracks:
904 track 1 for YFP detection, exciting at 514 nm and collecting emission from the emission
905 range 530-560 nm; track 2 for RFP and mCherry detection, exciting at 561 nm and
906 collecting from the emission range 588-641 nm. Images of 8 bits and 1024X1024 pixels
907 were acquired using a pixel dwell time of 1.27, pixel averaging of 4 and pinhole of 1 airy
908 unit. Analysis of colocalization was conducted with Fiji-ImageJ using the Coloc2 tool.
909 For calculating the Pearson correlation coefficient, 15-18 images were analysed. Signal
910 profiles were analysed using the Plot Profile tool [89].

911

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917 Jerusalem for her assistance with the RNA-Seq data.

918

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1163

1164 ## Supporting information

1165 **S1 Fig. HrpX and HrpG are required for pathogenicity of *Acidovorax citrulli* M6.**
1166 Lesions induced in a melon (cv. HA61428) leaf syringe-infiltrated with 10^8 cfu/mL
1167 suspensions of wild-type M6, but not of M6 mutants defective in *hrpX* and *hrpG* genes.
1168 Partial restoration of the wild-type phenotype was observed following transformation of
1169 the mutants with plasmids pBBR1MCS-5::*hrpX* and pBBR1MCS-5::*hrpG*
1170 (complementation plasmids), respectively. The picture was taken 3 days after infiltration.
1171 **S2 Fig. Subcellular localization of APS_0500.** (A) Confocal microscopy images of *N.*
1172 *benthamiana* epidermal cells transiently expressing APS_0500-YFP and different
1173 endomembrane compartment markers as indicated. Representative images show
1174 APS_0500-YFP (green), the subcellular marker: HDEL-RFP, Free-mCherry or SlDRP2A
1175 (magenta) and the superimposed image of both channels (merge). Pearson correlation
1176 coefficient of the co-localization between APS_0500-YFP and the markers (N=15-18)
1177 was determined using the Coloc2 function from ImageJ. Data represented as mean ±
1178 SEM. (B) Confocal microscopy images of *N. benthamiana* epidermal cells transiently
1179 expressing the plasma membrane protein Flot1-GFP and Free-mCherry. All the images
1180 were acquired 48 h after *A. tumefaciens* infiltration using Zeiss LSM780 (40x/1,2 W
1181 Korr). Scale bar 20 µm.
1182 **S3 Fig. Subcellular localization of APS_1448.** Confocal microscopy images of *N.*
1183 *benthamiana* epidermal cells transiently expressing APS_1448-YFP and different
1184 endomembrane compartment markers as indicated. Representative images show
1185 APS_1448-YFP (green), the subcellular markers HDEL-RFP, Free-mCherry or
1186 SlDRP2A (magenta), and the superimposed image of both channels (merge). Pearson
1187 correlation coefficient of the co-localization between APS_1448-YFP and the markers
1188 (N=15–18) was determined using the Coloc2 function from ImageJ. Data represented as

1189 mean ± SEM. All the images were acquired 48 h after *A. tumefaciens* infiltration using
1190 Zeiss LSM780 (40x/1,2 W Korr). Scale bar, 20 µm.

1191 **S4 Fig. Subcellular localization of APS_4116.** Confocal microscopy images of *N.*
1192 *benthamiana* epidermal cells transiently expressing APS_1448-YFP and different
1193 endomembrane compartment markers as indicated. Representative images show
1194 APS_1448-YFP (green), the subcellular markers HDEL-RFP, Free-mCherry or
1195 SlDRP2A (magenta), and the superimposed image of both channels (merge). Pearson
1196 correlation coefficient of the co-localization between APS_1448-YFP and the markers
1197 (N=15–18) was determined using the Coloc2 function from ImageJ. Data represented as
1198 mean ± SEM. All the images were acquired 48 h after *A. tumefaciens* infiltration using
1199 Zeiss LSM780 (40x/1,2 W Korr). Scale bar, 20 µm.

1200 **S5 Fig. Distribution of *Acidovorax citrulli* M6 type III effectors (T3Es) according to**
1201 **their amino acid length.** The data are from the annotation (GenBank accession
1202 CP029373) of the *A. citrulli* M6 ORFs**.**

1203 **S6 Fig. Expression of effector-AvrBs2$_{62\text{-}574}$::HA fusion proteins of T3Es that were**
1204 **tested in translocation assays.** Total protein was extracted from overnight cultures of
1205 *Xanthomonas euvesicatoria* 85-10-*hrpG*-ΔavrBs2* expressing CT3E-AvrBs2$_{62\text{-}574}$-HA
1206 fusions in plasmid pBBR1MCS-2*::avrBs2*$_{62\text{-}574}$. Proteins were analysed by Western blot
1207 using HA-tag antibody. XopS (*X. euvesicatoria* effector)-AvrBs2$_{62\text{-}574}$::HA was included
1208 as positive control. Asterisks indicate the size of the expected bands.

1209 **S1 Table.** Ranking and prediction scores of open reading frames of *Acidovorax citrulli*
1210 AAC00-1 (GenBank accession CP000512.1) in the first machine learning run.

1211 **S2 Table.** Occurrence of *Acidovorax citrulli* M6 type III effectors in other plant
1212 pathogenic *Acidovorax* species.

1213 **S3 Table.** Differential gene expression as determined by RNA-Seq between *Acidovorax*
1214 *citrulli* M6 and an M6 mutant strain defective in *hrpX* gene, after 72 h of growth in XVM2
1215 minimal medium at 28 °C.

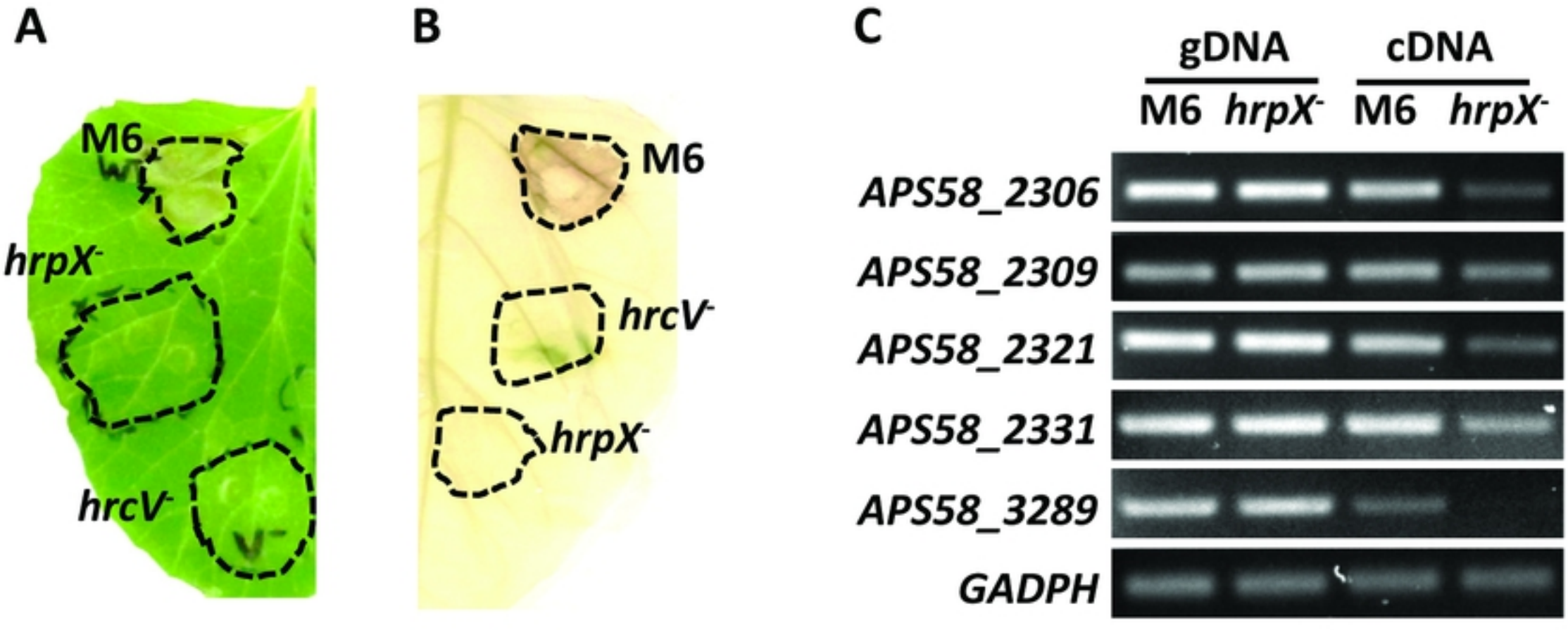
1216 **S4 Table.** Perfect plant-inducible promoter (PIP) boxes in the *Acidovorax citrulli* M6
1217 genome.

1218 **S5 Table.** Ranking and prediction scores of open reading frames of *Acidovorax citrulli*
1219 M6 (GenBank accession CP029373) in the second machine learning run.

1220 **S6 Table.** Bacterial strains and plasmids used in this study.

1221 **S7 Table.** DNA oligonucleotide primers used in this study.

1222 **S8 Table.** List and description of the features used for the first and second machine
1223 learning runs.


A
M6
hrpX-
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B
M6
hrcV-
hrpX-
C
gDNA
cDNA
M6
hrpX-
M6
hrpX-
APS58_2306
APS58_2309
APS58_2321
APS58_2331
APS58_3289
GADPH

Figure 1

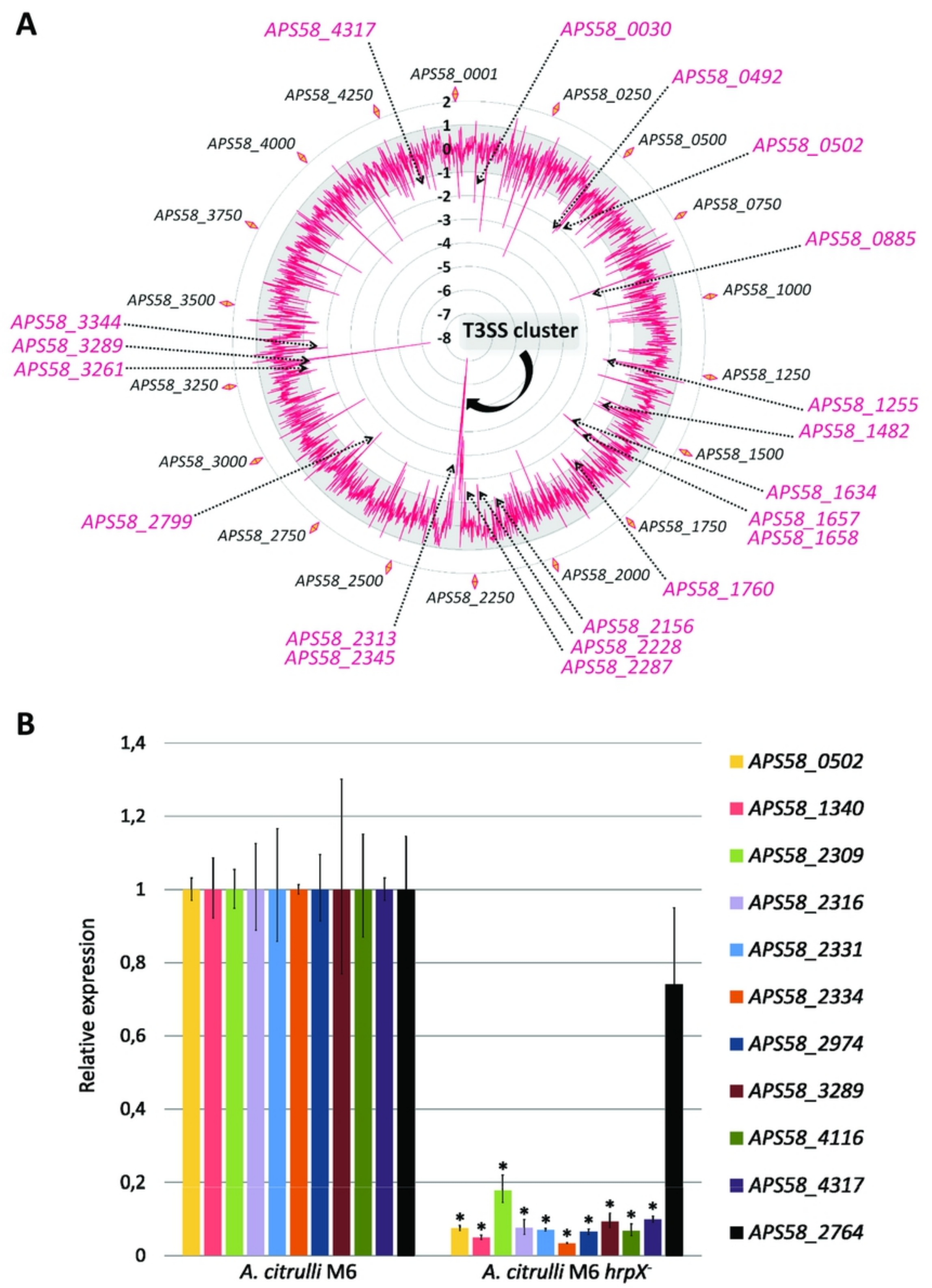
A
APS58_4317
APS58_0030
APS58_0492
APS58_0502
APS58_0885
APS58_3344
APS58_3289
APS58_3261
APS58_2799
APS58_2313
APS58_2345
APS58_2156
APS58_2228
APS58_2287
APS58_1760
APS58_1634
APS58_1657
APS58_1658
APS58_1482
APS58_1255
APS58_0001
APS58_0250
APS58_0500
APS58_0750
APS58_1000
APS58_1250
APS58_1500
APS58_1750
APS58_2000
APS58_2250
APS58_2500
APS58_2750
APS58_3000
APS58_3250
APS58_3500
APS58_3750
APS58_4000
APS58_4250
T3SS cluster
B
Relative expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
A. citrulli M6
A. citrulli M6 hrpX-
APS58_0502
APS58_1340
APS58_2309
APS58_2316
APS58_2331
APS58_2334
APS58_2974
APS58_3289
APS58_4116
APS58_4317
APS58_2764

Figure 2

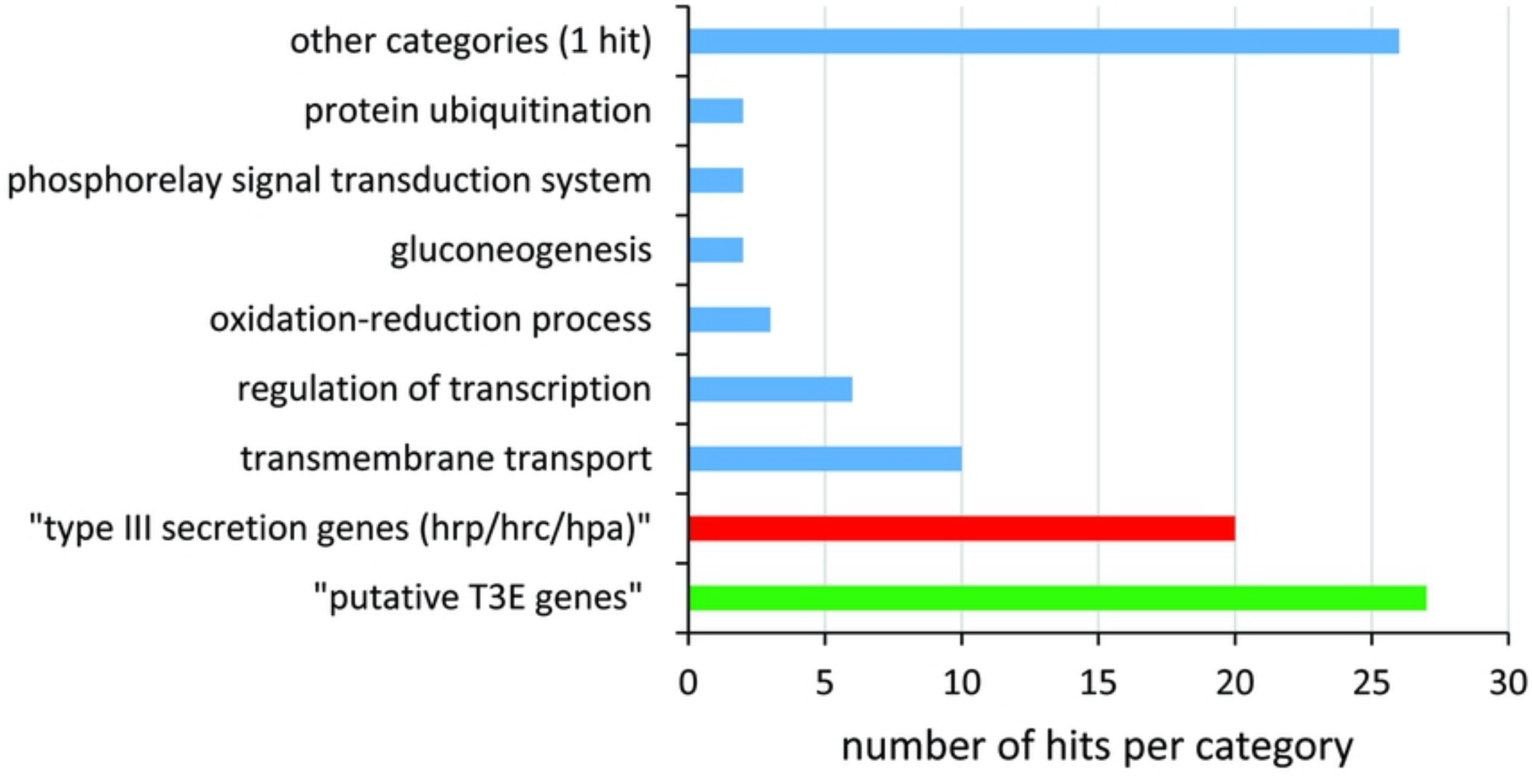
other categories (1 hit)
protein ubiquitination
phosphorelay signal transduction system
gluconeogenesis
oxidation-reduction process
regulation of transcription
transmembrane transport
"type III secretion genes (hrp/hrc/hpa)"
"putative T3E genes"
0
5
10
15
20
25
30
number of hits per category

Figure 3

Figure 4

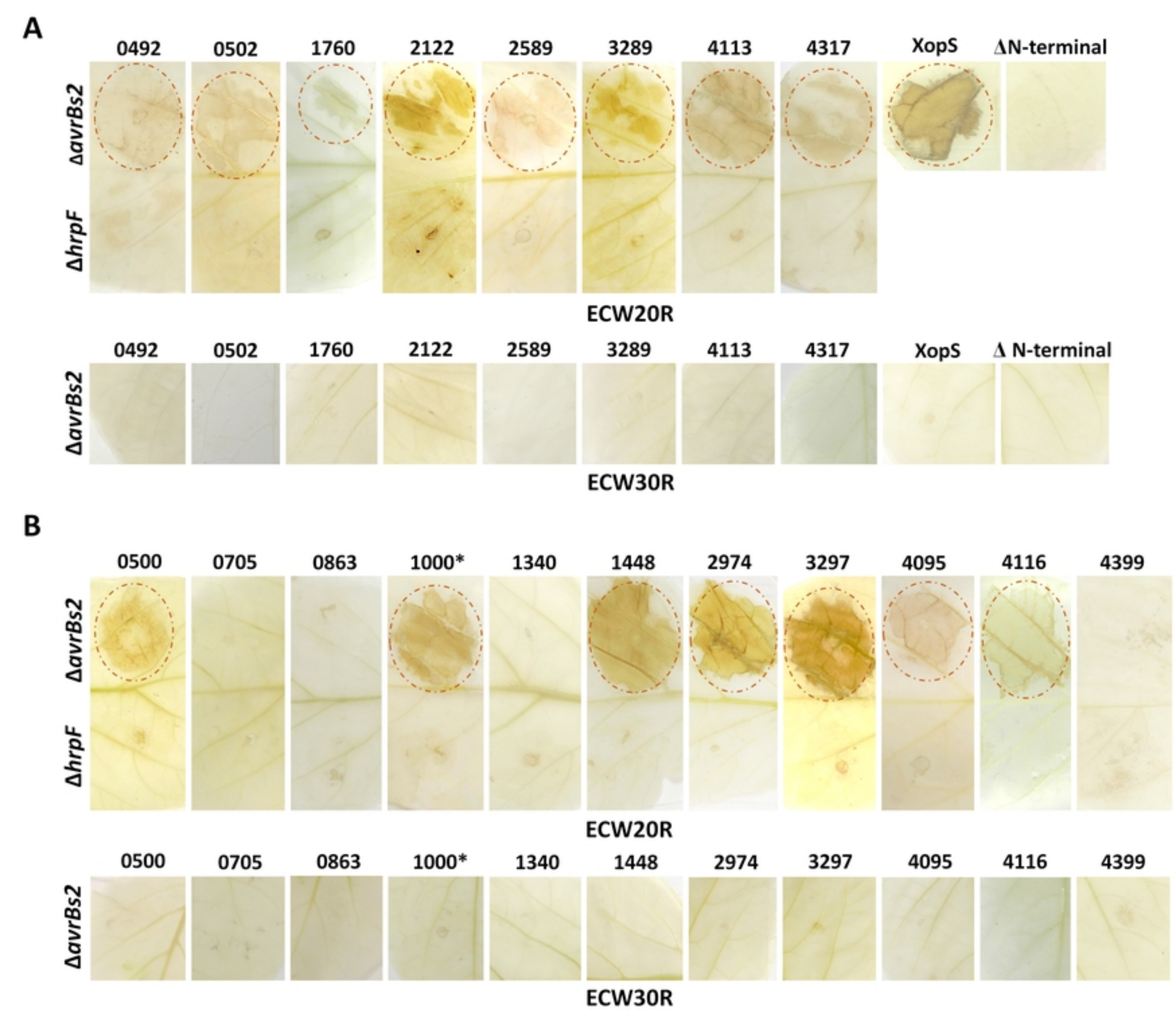
A
0492
0502
1760
2122
2589
3289
4113
4317
XopS
ΔN-terminal
ΔavrBs2
ΔhrpF
ECW20R
0492
0502
1760
2122
2589
3289
4113
4317
XopS
Δ N-terminal
ΔavrBs2
ECW30R
B
0500
0705
0863
1000*
1340
1448
2974
3297
4095
4116
4399
ΔavrBs2
ΔhrpF
ECW20R
0500
0705
0863
1000*
1340
1448
2974
3297
4095
4116
4399
ΔavrBs2
ECW30R

Figure 5

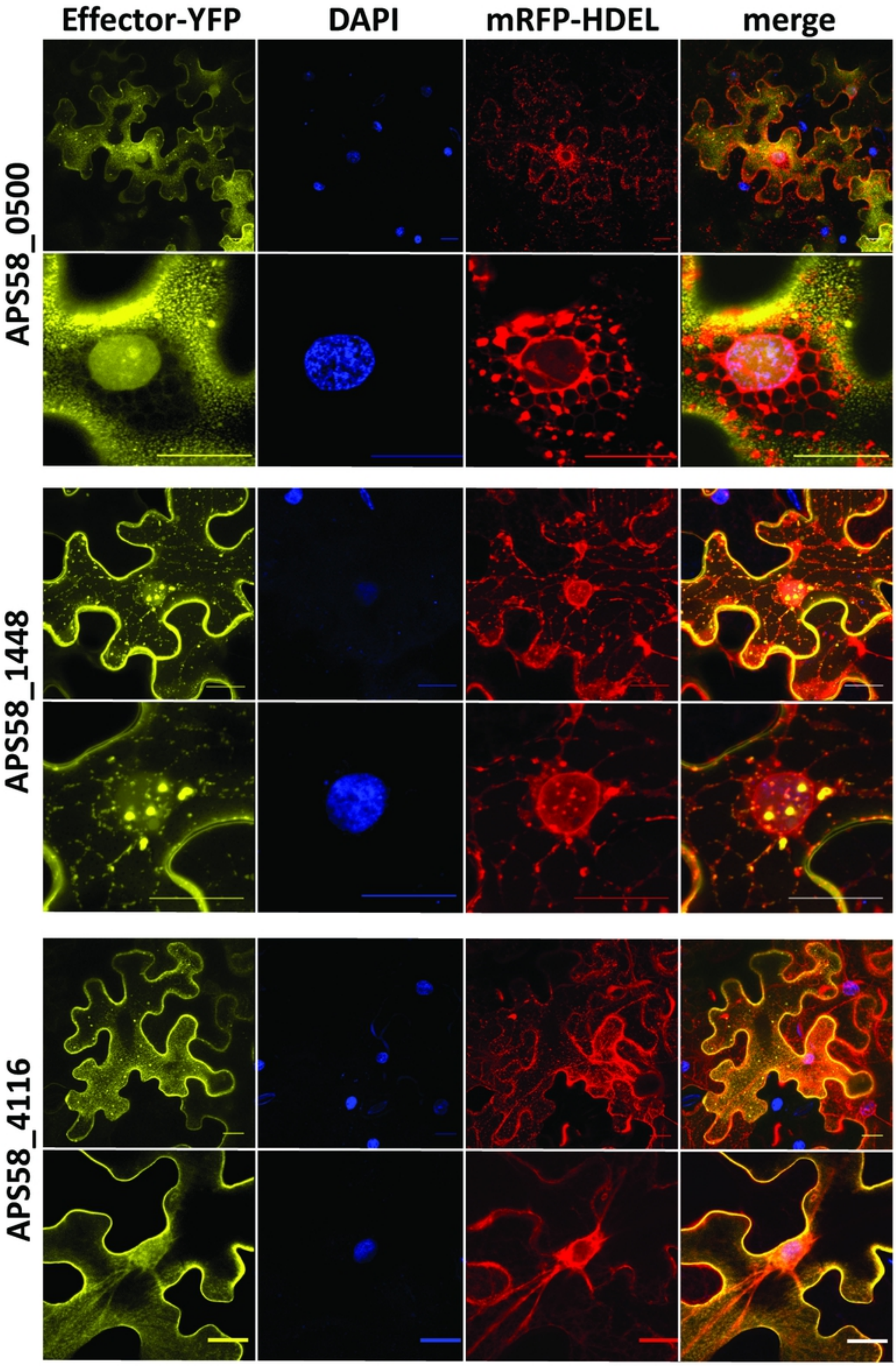
Effector-YFP
DAPI
mRFP-HDEL
merge
APS58_0500
APS58_1448
APS58_4116

Figure 6