Tetraose steroidal glycoalkaloids from potato provide resistance against *Alternaria solani* and Colorado potato beetle

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Abstract

Plants with innate disease and pest resistance can contribute to more sustainable agriculture. Natural defence compounds produced by plants have the potential to provide a general protective effect against pathogens and pests, but they are not a primary target in resistance breeding. Here, we identified a wild relative of potato, *Solanum commersonii*, that provides us with unique insight in the role of glycoalkaloids in plant immunity. We cloned two atypical resistance genes that provide resistance to *Alternaria solani* and Colorado potato beetle through the production of tetraose steroidal glycoalkaloids. Moreover, we provide *in vitro* evidence to show that these compounds have potential against a range of different (potato pathogenic) fungi. This research links structural variation in steroidal glycoalkaloids to resistance against potato diseases and pests. Further research on the biosynthesis of plant defence compounds in different tissues, their toxicity, and the mechanisms for detoxification, can aid the effective use of such compounds to improve sustainability of our food production.

30 # Introduction

31 Worldwide, up to 20-40% of agricultural crop production is lost due to plant diseases and pests (1).
32 Many crops have become heavily dependent on the use of pesticides, but this is unsustainable as
33 these can negatively affect the environment and their use can lead to development of pesticide
34 resistance (2-7). The European Union's 'Farm to Fork Strategy' aims to half pesticide use and risk by
35 2030 (8), a massive challenge that illustrates the urgent need for alternative disease control
36 methods.

37 Wild relatives of crop species are promising sources of natural disease resistance (9-12). Monogenic
38 resistance caused by dominant resistance (*R*) genes, typically caused by immune receptors that
39 belong to the nucleotide-binding leucine-rich repeat (NLR) class, is successfully employed by plant
40 breeders to develop varieties with strong qualitative disease resistance. However, this type of
41 resistance is usually restricted to a limited range of pathogens (13, 14) and it is often not very
42 durable.

43 More robust resistance can be obtained by combining NLRs with different recognition specificities
44 (15-18), or by including pattern recognition receptors (PRRs), which recognize conserved (microbe- or
45 pathogen-derived) molecular patterns. Recent reports show that PRRs and NLRs cooperate to
46 provide disease resistance (19-21). Alternatively, susceptibility (*S*) genes provide recessive resistance
47 that can be both broad-spectrum and durable (22-24). Unfortunately, their recessive nature
48 complicates the use of *S* genes in conventional breeding of autopolyploids and many mutated *S*
49 genes come with pleiotropic effects.

50 Besides the defences mentioned above, most plants produce specialised metabolites with
51 antimicrobial or anti-insect activity, either constitutively (phytoanticipins) or in response to pathogen
52 attack or herbivory (phytoalexins) (25). These natural defence compounds are derived from a wide
53 range of building blocks, leading to a large structural diversity throughout the plant kingdom (26, 27).
54 Specific classes of compounds can be found in different plant families, e.g. glucosinolates are
55 typically found in Brassicaceae and benzoxyzinoids are widely distributed among Poaceae, with

56 further chemical diversification within plant families (28, 29). Examples in various pathosystems
57 show that the capacity for detoxification of plant defence compounds is important for pathogenicity,
58 especially for necrotrophic pathogens which encounter toxic plant metabolites as a consequence of
59 their lifestyle (30, 31). Similarly, plant defence compounds play an important role against herbivorous
60 insects, which have evolved various mechanisms to deal with toxic plant compounds (32-34).
61 While plant immune receptors can offer resistance against a restricted range of pathogens, plant
62 defence compound have the potential to provide a more general protection, depending on their
63 mode of action. Plants from the nightshade family (Solanaceae) produce saponins that are
64 characterised by a steroidal alkaloid aglycone, linked to a variable oligosaccharide chain (steroidal
65 glycoalkaloids – SGAs) (35, 36). The protective effect of SGAs stems from their ability to interact with
66 membrane sterols, disrupting the cell integrity from target organisms (37-40). In addition, they can
67 act on the nervous system of pests and herbivores through their inhibitory effect on cholinesterase
68 enzymes (41-43). As a consequence, SGAs can have both antimicrobial and anti-insect activity (44-
69 54).
70 Early blight is an important disease of tomato and potato that is caused by the necrotrophic fungal
71 pathogen *Alternaria solani* (55-57). In a previous study, we found a wild potato species, *Solanum*
72 *commersonii*, with strong resistance against potato pathogenic *Alternaria* isolates and species from a
73 number of different locations (58). We showed that resistance is likely caused by a single dominant
74 locus and that it can be introgressed in cultivated potato (58). Resistance to necrotrophs is usually
75 considered to be a complex, polygenic trait, or recessively inherited according to the *inverse gene-*
76 *for-gene* model (59-64). It therefore surprised us to find a qualitative dominant resistance against
77 early blight in *S. commersonii* (58).
78 In this study, we explored different accessions of *S. commersonii* and *S. malmeanum* (previously also
79 referred to as *S. commersonii* subsp. *malmeanum* (65)) and developed a population that segregates
80 for resistance to early blight. Using a Bulked Segregant RNA-Seq (BSR-Seq) approach (66), we
81 mapped the resistance locus to the top of chromosome 12 of potato. We sequenced the genome of

82 the resistant parent of the population and identified two glycosyltransferases that can provide
83 resistance to susceptible *S. commersonii*. We show that the resistance is based on the production of
84 tetraose SGAs and provide *in vitro* evidence to show that they can be effective against other fungi
85 besides *A. solani*. As SGAs can be involved in resistance against insects, we also tested if they can
86 protect against Colorado potato beetle (CPB). Combined, our results show that the tetraose SGAs
87 from *S. commersonii* have potential to provide resistance against a range of potato pathogens and
88 pests.

89 Results

90 Early blight resistance maps to chromosome 12 of potato

91 To find suitable parents for a mapping study targeting early blight resistance, we performed a disease
92 screen with *A. solani* isolate altNL03003 (67) on 13 different accessions encompassing 37 genotypes
93 of *S. commersonii* and *S. malmeanum* (S1 Table). The screen showed clear differences in resistance
94 phenotypes between and within accessions (Fig 1A). Roughly half of the genotypes were highly
95 resistant (lesion diameters < 3 mm indicate that the lesions are not expanding beyond the size of the
96 inoculation droplet) and the other half was susceptible (displaying expanding lesions), with only a
97 few intermediate genotypes. CGN18024 is an example of an accession that segregates for resistance,
98 with CGN18024_1 showing strong resistance and CGN18024_3 showing clear susceptibility (Fig 1B).
99 The fact that individual accessions can display such clear segregation for resistance suggests that
100 resistance is caused by a single gene or locus. Because of its clear segregation, *S. commersonii*
101 accession CGN18024 was selected for further studies.
102 Disease tests with an *A. solani* isolate from the US (ConR1H) and a recent Dutch isolate from the
103 Netherlands (altNL21001) confirm that the resistance of CGN18024_1 is effective against additional
104 *A. solani* isolates (S1 Fig). To further study the genetics underlying resistance to early blight, we
105 crossed resistant CGN18024_1 with susceptible CGN18024_3. Thirty progeny genotypes were sown
106 out and tested with *A. solani* isolate altNL03003. We identified 14 susceptible genotypes and 16

107 resistant genotypes, with no intermediate phenotypes in the population (Fig 1C). This segregation
108 supports a 1:1 ratio (X^2 (1, N = 30) = 0.133, p=.72), which confirms that resistance to early blight is
109 likely caused by a single dominant locus in *S. commersonii*.
110 To genetically localize the resistance, we isolated RNA from each progeny genotype and the parents
111 of the population and proceeded with a BSR-Seq analysis (66). RNA from resistant and susceptible
112 progeny genotypes were pooled in separate bulks and sequenced next to RNA from the parents on
113 the Illumina sequencing platform (PE150). Reads were mapped to the DMv4.03 (68) and Solyntus
114 potato genomes (69). To find putative SNPs linked to resistance, we filtered for SNPs that follow the
115 same segregation as resistance (heterozygous in resistant parent CGN18024_1 and the resistant bulk,
116 but absent or homozygous in susceptible parent and susceptible bulk). The resulting SNPs localize
117 almost exclusively on chromosome 12 of the DM and Solyntus genomes, with most of them located
118 at the top of the chromosome (Fig 1D). We used a selection of SNPs distributed over chromosome 12
119 as high-resolution melt (HRM) markers to genotype the BSR-Seq population. This rough mapping
120 proves that the locus for early blight resistance resides in a region of 3 Mb at the top of chromosome
121 12 (S2 Fig).

122 Improved genome assembly *of S. commersonii*

123 A genome sequence of *S. commersonii* is already available (70), but we do not know if the sequenced
124 genotype is resistant to *A. solani*. To help the identification of additional markers and to explore the
125 resistance locus of a genotype with confirmed resistance, we sequenced the genome of resistant
126 parent CGN18024_1. High-molecular-weight (HMW) genomic DNA (gDNA) from CGN18024_1 was
127 used for sequencing using Oxford Nanopore Technology (ONT) on a GridION X5 platform and for
128 sequencing using DNA Nanoball (DNB) technology at the Beijing Genomics Institute (BGI) to a depth
129 of approximately 30X. We used the ONT reads for the initial assembly and the shorter, more
130 accurate, DNBseq reads to polish the final sequence. The resulting assembly has a size of 737 Mb,
131 which is close to the size of the previously published genome of *S. commersonii* (730 Mb) (70). N50

132 scores and Benchmarking Universal Single-Copy Orthologs (BUSCO) score indicate a highly complete
133 and contiguous genome assembly of *S. commersonii* (Table 1).

134 **Table 1. Genome assembly metrics of *S. commersonii* cmm1t (70) and CGN18024_1**

Genome	CMM1t[a)]	CGN18024_1
Total size (Mb)	730	737
Contig number	278460	637
Largest contig (Mb)	0,17	21,2
N50 (Mb)	0,007	4,02
Complete BUSCO (%)	81,9	95,7

135 [a)] Aversano et al (2015)

136 Identification of two glycosyltransferase resistance genes

137 To identify candidate genes that can explain the resistance of *S. commersonii*, it was necessary to
138 further reduce the mapping interval. By aligning the ONT reads to the CGN18024_1 genome
139 assembly, we could identify new polymorphisms that we converted to additional PCR markers (S3-6
140 Figs). We performed a recombinant screen of approximately 3000 genotypes from the population to
141 fine-map the resistance region to a window of 20 kb (S7 and S8 Figs).
142 We inferred that the resistance locus is heterozygous in CGN18024_1 from the segregation in the
143 mapping population. We used polymorphisms in the resistance region to separate and compare the
144 ONT sequencing reads from the resistant and susceptible haplotype. This comparison showed a
145 major difference between the two haplotypes. The susceptible haplotype contains a small insertion
146 of 3.7 kb inside a larger region of 7.3 kb. The larger region is duplicated in the resistant haplotype (Fig
147 2A). As a result, the resistance region of the resistant haplotype is 27 kb, 7 kb larger than the
148 corresponding region of the susceptible haplotype (20 kb).
149 Two genes coding for putative glycosyltransferases (GTs) are located within the rearrangement of the
150 resistant haplotype. The corresponding allele from the susceptible haplotype contains a frameshift
151 mutation, leading to a truncated protein (S9 Fig). Several other short ORFs with homology to
152 glycosyltransferases were predicted in the resistant haplotype, but *ScGTR1* (*S. commersonii*
153 glycosyltransferase linked to resistance 1) and *ScGTR2* are the only full-length genes in the region.

154 Reads from the BSR-Seq experiment show that both genes are expressed in bulks of resistant
155 progeny and not in susceptible progeny (Fig 2B), suggesting a putative role for these genes in causing
156 resistance. *ScGTR1* and *ScGTR2* are homologous genes with a high similarity (the predicted proteins
157 that they encode share 97% amino acid identity). We compared the predicted amino acid sequences
158 with previously characterised GTs (71-78) and found that they share some similarity with GTs with a
159 role in zeatin biosynthesis (79-81) and with GAME17, an enzyme involved in biosynthesis of the SGA
160 α-tomatine typically found in tomato (76) (S10 Fig, S2 Table).
161 To test whether the identified candidate genes are indeed involved in resistance, we transiently
162 expressed both alleles of the resistant haplotype (*ScGTR1* and *ScGTR2*) as well as the corresponding
163 allele from the susceptible haplotype (*ScGTS*), in leaves of resistant CGN18024_1 and susceptible
164 CGN18024_3 and *S. tuberosum* cultivar Atlantic, using agroinfiltration (82). Following agroinfiltration,
165 the infiltrated areas were drop-inoculated with a spore suspension of *A. solani* altNL03003. Transient
166 expression of *ScGTR1* as well as *ScGTR2* significantly reduced the size of the *A. solani* lesions in
167 susceptible CGN18024_3, compared to *ScGTS* and the empty vector control. Resistant CGN18024_1
168 remained resistant, whereas susceptible Atlantic remained susceptible regardless of the treatment
169 (Fig 2C). We conclude that both *ScGTR1* and *ScGTR2* can affect resistance in susceptible *S.*
170 *commersonii* CGN18024_3, but not in *S. tuberosum* cv. Atlantic.

171 Leaf compounds from resistant *S. commersonii* inhibit growth of 172 diverse fungi, including pathogens of potato

173 Glycosyltransferases are ubiquitous enzymes that catalyse the transfer of saccharides to a range of
174 different substrates. To test if resistance of *S. commersonii* to *A. solani* can be explained by a host-
175 specific defence compound, we performed a growth inhibition assay using crude leaf extract from
176 resistant and susceptible *S. commersonii*. Leaf material was added to PDA plates to equal 5% w/v and
177 autoclaved (at 121 °C) or semi-sterilised at 60 °C. Interestingly, leaf material from resistant
178 CGN18024_1 strongly inhibited growth of *A. solani* isolate altNL03003, while we did not see any

179 growth inhibition on plates containing leaves from susceptible CGN18024_3 (Fig 3A). Remarkably,
180 ample contamination with diverse fungi appeared after a few days on the plates containing semi-
181 sterilised leaves from susceptible *S. commersonii* but not on plates with leaves from CGN18024_1
182 (Fig 3A). Thus, leaves from CGN18024_1 contain compounds that can inhibit growth of a variety of
183 fungi besides *A. solani*.
184 To quantify the inhibitory effect of leaves from *S. commersonii* against different fungal pathogens of
185 potato, we performed a growth inhibition assay with *A. solani* (altNL03003 (67)), *Botrytis cinerea*
186 (B05.10 (83)) and *Fusarium solani* (1992 vr). As before, we added 5% (w/v) of leaf material from
187 CGN18024_1 or CGN18024_3 to PDA plates and we placed the fungi at the centre of the plates. We
188 measured colony diameters in the following days and compared it with the growth on PDA plates
189 without leaf extract. Indeed, growth of all three potato pathogens was significantly reduced on
190 medium containing leaf material from CGN18024_1 (Fig 3B), compared to medium containing
191 material from CGN18024_3 or on normal PDA plates. These results indicate that phytoanticipins from
192 the leaves of resistant *S. commersonii* have the potential to protect against diverse fungal pathogens
193 of potato.

194 Tetraose steroidal glycoalkaloids from *S. commersonii* provide
195 resistance to *A. solani* and Colorado potato beetle

196 *Solanum* leaves usually contain SGAs, which are known phytoanticipins against fungi and other plant
197 parasites (84). *S. tuberosum* typically produces the triose SGAs α-solanine (solanidine-Gal-Glu-Rha)
198 and α-chaconine (solanidine-Glu-Rha-Rha), while five major tetraose SGAs were previously identified
199 in *S. commersonii*: commersonine (demissidine-Gal-Glu-Glu-Glu), dehydrocommersonine (solanidine-
200 Gal-Glu-Glu-Glu), demissine (demissidine-Gal-Glu-Glu-Xyl), dehydrodemissine (solanidine-Gal-Glu-
201 Glu-Xyl) and α-tomatine (tomatidine-Gal-Glu-Glu-Xyl) (84-89). To test if SGAs can explain resistance
202 of *S. commersonii*, we measured SGA content in leaves from Atlantic and susceptible/resistant *S.*
203 *commersonii* by Ultra High Performance Liquid Chromatography (UPLC) coupled to mass

204 spectrometry (MS). As expected, we could detect the triose SGAs α-solanine and α-chaconine in
205 susceptible *S. tuberosum* cv. Atlantic, but we found a remarkable difference in the SGA profile of
206 resistant and susceptible *S. commersonii*. We detected tetraose SGAs in resistant *S. commersonii*
207 CGN18024_1, whereas susceptible *S. commersonii* CGN18024_3 accumulates triose SGAs (Fig 4A and
208 S3 and S4 Tables). The mass spectra of the four major tetraose SGAs from *S. commersonii* correspond
209 to (dehydro-) commersonine and (dehydro-) demissine, matching the data from previous studies(85,
210 87-89). Notably, the mass spectra of the two major SGAs from susceptible CGN18024_3 correspond
211 to the triose precursors of these SGAs (solanidine-Gal-Glu-Glu and demissidine-Gal-Glu-Glu
212 respectively) (S3 and S4 Tables). These results suggest that the triose SGAs present in susceptible
213 CGN18024_3 are modified to produce the tetraose SGAs in resistant CGN18024_1, by addition of an
214 extra glucose or xylose moiety.
215 To investigate a possible role for *ScGTR1* and *ScGTR2* in the production of tetraose SGAs from
216 CGN18024_1 and their link to resistance, we generated stable transformants of *ScGTR1* and *ScGTR2*
217 in triose SGA producing CGN18024_3 (S11 Fig). UPLC-MS analysis showed that both *ScGTR1* and
218 *ScGTR2* transformants accumulate tetraose SGAs, while the amount of triose SGAs is markedly
219 reduced (Fig 4A). Strikingly, ScGTR1 and ScGTR2 appear to have different specificities.
220 Overexpression of *ScGTR1* resulted in the addition of a hexose to the triose SGAs from CGN18024_3
221 (corresponding to a commertetraose), while overexpression of *ScGTR2* caused the addition of a
222 pentose (corresponding to a lycotetraose) (Fig 4A and D). This *in planta* evidence suggests that
223 *ScGTR1* is a glucosyltransferase and that *ScGTR2* is a xylosyltransferase. However, we detect a slight
224 overlap in activity. In addition to the lycotetraose products, we detected small amounts of
225 commertetraose product in *ScGTR1* transformants and vice versa in the *ScGTR2* transformants (Fig
226 4A and S3 Table). A multivariate Principal Components Analysis (PCA) on the full metabolic profile
227 consisting of all 1,041 detected mass peaks revealed that ScGTR1 and ScGTR2 are highly specific
228 towards SGAs since 75% of the metabolic variation between the transformants and the wild types
229 could be explained by the SGA modifications (S12 Fig). Modifications catalysed by both enzymes can

230 lead to resistance, as *ScGTR1* and *ScGTR2* transformants are both resistant to *A. solani* isolate
231 altNL03003 (Fig 4B). Atlantic *ScGTR1* and *ScGTR2* transformants did not show differences in SGA
232 profile, probably because they contain different triose SGA substrates than found in *S. commersonii*
233 CGN18024_3 (S3 and S4 Tables).
234 Leptine and dehydrocommersonine SGAs from wild potato relatives have previously been linked to
235 resistance to insects such as CPB (44, 47-51, 90). To see if the SGAs from *S. commersonii* can protect
236 against insects as well, we performed a test with larvae of a CPB genotype collected in the
237 Netherlands on wildtype CGN18024_1/CGN18024_3 and on CGN18024_3 transformed with *ScGTR1*
238 or *ScGTR2* (Fig 4B). Wildtype CGN18024_3 is susceptible to the CPB genotype that was tested, but
239 CGN18024_1 and CGN18024_3 transformed with *ScGTR1* or *ScGTR2* are resistant, as illustrated by a
240 very low larvae weight and survival (Fig 4C). Thus, the conversion of triose SGAs from CGN18024_3 to
241 tetraose SGAs produced by CGN18024_1, carried out by both ScGTR1 and ScGTR2, can provide
242 protection against *A. solani* as well as CPB (Fig 4A-D).

243 Discussion

244 In this study, we set out to characterise resistance of *S. commersonii* to *A. solani*. We showed that it
245 is caused by a single dominant locus containing two GT candidate resistance genes. Both GTs are
246 involved in the production of tetraose SGAs in *S. commersonii*, but they transfer distinct sugars. Both
247 modifications can cause resistance to *A. solani*. We provide *in vitro* evidence to show that the
248 tetraose SGAs from *S. commersonii* have the potential to protect against other fungi besides *A. solani*
249 and we demonstrate that plants producing the compounds are resistant to CPB. Collectively, our data
250 link the tetraose SGAs from *S. commersonii* to disease and pest resistance.
251 It is known that specialised metabolites from plants can act in plant defence and compounds with
252 antimicrobial effects have been characterised in many different plant species (26, 27, 91). They may
253 also influence other aspects of the crop, such as flavour or taste and they can have dietary benefits
254 or be toxic to humans. SGAs from potato can cause risks for human health, but a total SGA content of
255 less than 200 mg/kg is generally considered to be safe for human consumption (84, 92-94). Potato

256 breeders generally try to reduce SGA content in tubers, to prevent problems with toxicity and to
257 meet safety regulations, but they do not usually consider the effect on disease resistance. There is
258 not much known about how modifications to SGAs of potato affect human toxicity and resistance to
259 biotic stress, but additional knowledge on this topic could help breeders to optimize the metabolite
260 profile of their cultivars (95).

261 Biosynthesis of SGAs in *Solanum* is controlled by many genes. The discovery of *S. commersonii*
262 genotypes with and without tetraose SGAs provides us with unique insight in the role of these
263 compounds in plant immunity. Similar compounds are produced in *Solanum* species such as *S.*
264 *chacoense*, *S. chomatophilum*, *S. oplocense*, *S. paucisectum* and *S. piurae*, which may explain why
265 these (or their descendants) display resistance to *A. solani* or CPB (48-50, 96, 97). The compounds
266 that are found in resistant *S. commersonii* are an interesting combination of a solanidine or
267 demissidine aglycone and a lycotetraose or commertetraose sugar moiety. Solanidine forms the
268 aglycone backbone of α-solanine and α-chaconine from potato, while the lycotetraose decoration is
269 found on α-tomatine from tomato (87, 98). The biosynthesis pathways leading to the production of
270 these major SGAs from cultivated potato and tomato have largely been elucidated in recent years
271 and it was found that the underlying genes occur in conserved clusters (76, 98). This knowledge and
272 the similarities between SGAs from *S. commersonii* and cultivated potato/tomato will help to identify
273 the missing genes from the pathway through comparative genomics.

274 The broad-spectrum activity of tetraose SGAs is attractive, but this non-specificity also presents a
275 risk. The antifungal and anti-insect activity of SGAs from *S. commersonii* is not restricted to potato
276 pathogens and pests, but could also affect beneficial or commensal micro-organisms or other
277 animals that feed on plants (43, 99). In tomato fruit, α-tomatine is converted to esculeoside A during
278 fruit ripening in a natural detoxification process from the plant (100, 101) to facilitate dispersal of the
279 seeds by foraging animals. Unintended toxic effects of SGAs should also be taken into account when
280 used in resistance breeding.

281 Studies on α-tomatine and avenacin A-1 show that changes to the sugar moiety of these saponins
282 from tomato and oat respectively, can affect their toxicity (46, 102-104). Tomato and oat pathogens
283 produce enzymes that can detoxify these compounds through removal of one or more glycosyl
284 groups (46, 52, 53, 105-107). The degradation products of saponins can also suppress plant defence
285 responses (108, 109). Conversely, here we show that the resistance of *S. commersonii* is based on the
286 addition of a glycosyl group to a triose saponin from *S. commersonii.* There is large variation in both
287 the aglycone and the sugar moiety of SGAs from wild *Solanum*, with likely over 100 distinct SGAs
288 produced in tubers (87) (110). This diversity suggests a pressure to evolve novel molecules, possibly
289 to resist detoxification or other tolerance mechanisms, reminiscent of the molecular arms race that
290 drives the evolution of plant immune receptors (14). Thus, wild *Solanum* germplasm is not only a rich
291 source of immune receptors, it also provides a promising source of natural defence molecules.
292 Studies of how pathogens that naturally occur on *S. commersonii*, or other *Solanum* species
293 producing tetraose SGAs, can tolerate SGAs produced by their hosts could help judge the durability
294 of this type of resistance.
295 As crops are usually affected by multiple diseases and pests, significant reduction of pesticide use can
296 only be achieved if plants are naturally protected against a range of pathogen species and pests.
297 Different strategies towards this goal have been proposed and our study underlines the potential of
298 defence compounds that are naturally produced by plants. The fact that genes for specialised plant
299 metabolites can occur in biosynthetic gene clusters (76, 111-113), means that introgression breeding
300 could help to move these compounds from wild relatives to crop species. We had already created *S.*
301 *commersonii* x *S. tuberosum* hybrids with resistance to early blight in a previous study (58), but it is
302 clear that potential negative effects of SGA variants on human health and the environment should be
303 considered before these can be developed into a cultivar.
304 Additional insight in the biosynthesis pathway of the tetraose SGAs produced by *S. commersonii*
305 would make it possible to employ them through metabolic engineering and allow for a more precise
306 control of the amounts that are produced and in which tissues (91). Alternatively, the defence

307 compounds could be produced in non-crop plants or other organisms and applied on crops as
308 biological protectants. Studies on how natural defence compounds are produced in different plant
309 tissues, their toxicity and how they are detoxified, combined with studies on how different
310 modifications ultimately affect plant immunity and toxicity, are essential to employ them in a safe
311 and effective manner. Such studies at the interface of plant immunity and metabolism can help to
312 design innovative solutions to complement existing resistance breeding strategies and improve
313 sustainability of our food production.

314 Methods

315 A brief method description is given below, full details on methods can be found in S2 File. The
316 primers used in this study are listed in S5-7 Tables.

317 Genome assembly and separation of haplotypes covering resistance 318 region

319 ONT reads were filtered using Filtlong v0.2.0 (https://github.com/rrwick/Filtlong) with --min_length
320 1000 and --keep_percent 90. Adapter sequences were removed using Porechop (114). Fastq files
321 were converted to Fasta using seqtk v1.3 (https://github.com/lh3/seqtk). Assembly was performed
322 with smartdenovo (https://github.com/ruanjue/smartdenovo/) and a k-mer size of 17, with the
323 option for generating a consensus sequence enabled. ONT reads were mapped back to the assembly
324 using minimap2 v2.17 (115) and used for polishing with racon v1.4.3 (116) using default settings.
325 DNBseq reads were mapped to the resulting sequence using bwa mem v0.7.17 (117) and used for a
326 second round of polishing with racon v1.4.3. This procedure to polish the assembly using DNBseq
327 reads was repeated once. ONT reads were mapped back to the polished CGN18024_1 assembly using
328 minimap2 v2.17 (115). The alignment was inspected using IGV v2.6.3 (118) to identify polymorphisms
329 for new markers and marker information was used to identify ONT reads representative for both
330 haplotypes spanning the resistance region of CGN18024_1. Bedtools v2.25.0 (119) was used extract
331 the resistance region from the reads and to mask the corresponding region from the original

332 CGN18024_1 assembly. The extracted resistance regions from both reads were appended to the
333 assembly and the polishing procedure described above was repeated to prepare a polished genome
334 assembly of CGN18024_1, containing a sequence of both haplotypes covering the resistance region.
335 Quality of the genome was assessed using quast v5.0.2 with --eukaryote --large (120).

336 Transient disease assay

337 Agroinfiltration was performed as described previously using *Agrobacterium tumefaciens* strain AGL1
338 (82, 121). Agrobacterium suspensions were used at an OD_{600} of 0.3 to infiltrate fully expanded leaves
339 of 3-week-old CGN18024_1, CGN18024_3 and *S. tuberosum* cv. Atlantic. *ScGTR1*, *ScGTR2*, *ScGTS* and
340 pK7WG2-empty were combined as four separate spots on the same leaf and the infiltrated areas
341 were encircled with permanent marker. The plants were transferred to a climate cell 48 h after
342 agroinfiltration and each infiltrated area was inoculated with *A. solani* by pipetting a 10 µl droplet of
343 spore suspension (1 x 10^5 conidia/ml) at the centre of each spot. Lesion diameters were measured 5
344 days post inoculation. Eight plants were tested of each genotype, using three leaves per plant.

345 Fungal growth inhibition assays

346 Mature leaf material from 5-week-old plants was extracted in phosphate-buffered saline (PBS) buffer
347 using a T25 Ultra Turrax disperser (IKA) and supplemented to obtain a 5% w/v suspension in PDA and
348 autoclaved (20 min at 121C), or added to PDA after autoclaving, followed by an incubation step for
349 15 min at 60C to semi-sterilise the medium. The medium was poured into Petri dishes. Small agar
350 plugs containing mycelium from *A. solani* (altNL03003) or *F. solani* (1992 vr) were placed at the
351 centre of each plate and the plates were incubated at 25C in the dark. Similarly, approximately 100
352 spores of *B. cinerea* B05.10 (83) were pipetted at the centre of PDA plates containing the different
353 leaf extracts and the plates were incubated at room temperature in the dark. 3 plates per fungal
354 isolate/leaf extract combination were prepared and colony diameters were measured daily using a
355 digital calliper.

356 Data analysis

357 Data were analysed in RStudio (R version 4.02) (122, 123), using the tidyverse package (124). Most
358 figures were generated using ggplot2 (125), but genomic data were visualised using Gviz and
359 Bioconductor (126). PCA was performed using PAST3 software (https://past.en.lo4d.com/windows).
360 *P* values for comparisons between means of different groups were calculated in R using Welch's Two
361 Sample t-test.

362 Data availability

363 RNAseq data from the BSR-Seq experiment was deposited in the NCBI Sequence Read Archive with
364 BioProject ID PRJNA792513 (Sequencing Read Archive accession IDs SRR17334110, SRR17334111,
365 SRR17334112 and SRR17334113). Raw reads used in the assembly of the CGN18024_1 genome were
366 deposited with BioProject ID PPRJNA789120 (Sequencing Read Archive accession IDs SRR17348659
367 and SRR17348660). The assembled genome sequence of CGN18024_1 was archived in GenBank
368 under accession number JAJTWQ000000000. Sequences of *ScGTR1* and *ScGTR2* were deposited in
369 GenBank under accession numbers OM830430 and OM830431. Numerical data underlying the
370 figures of this manuscript are included in S1 File.

371 Competing interests

372 P.J.W, R.G.F.V. and V.G.A.A.V. are inventors on U.S. Patent Application No. 63/211,154 relating to
373 ScGTR1 and ScGTR2 filed by the J.R. Simplot company. The other authors declare no competing
374 interests.

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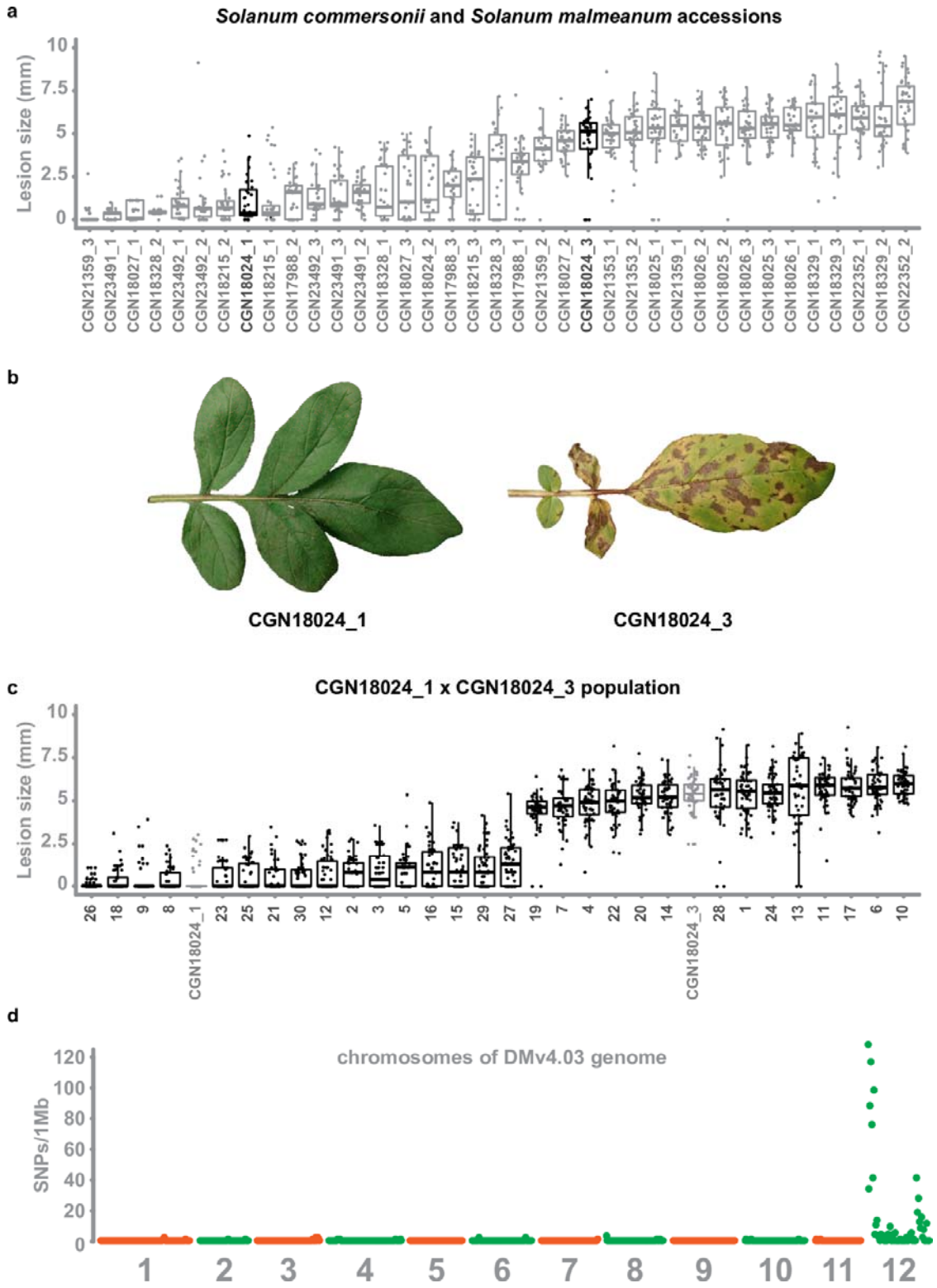
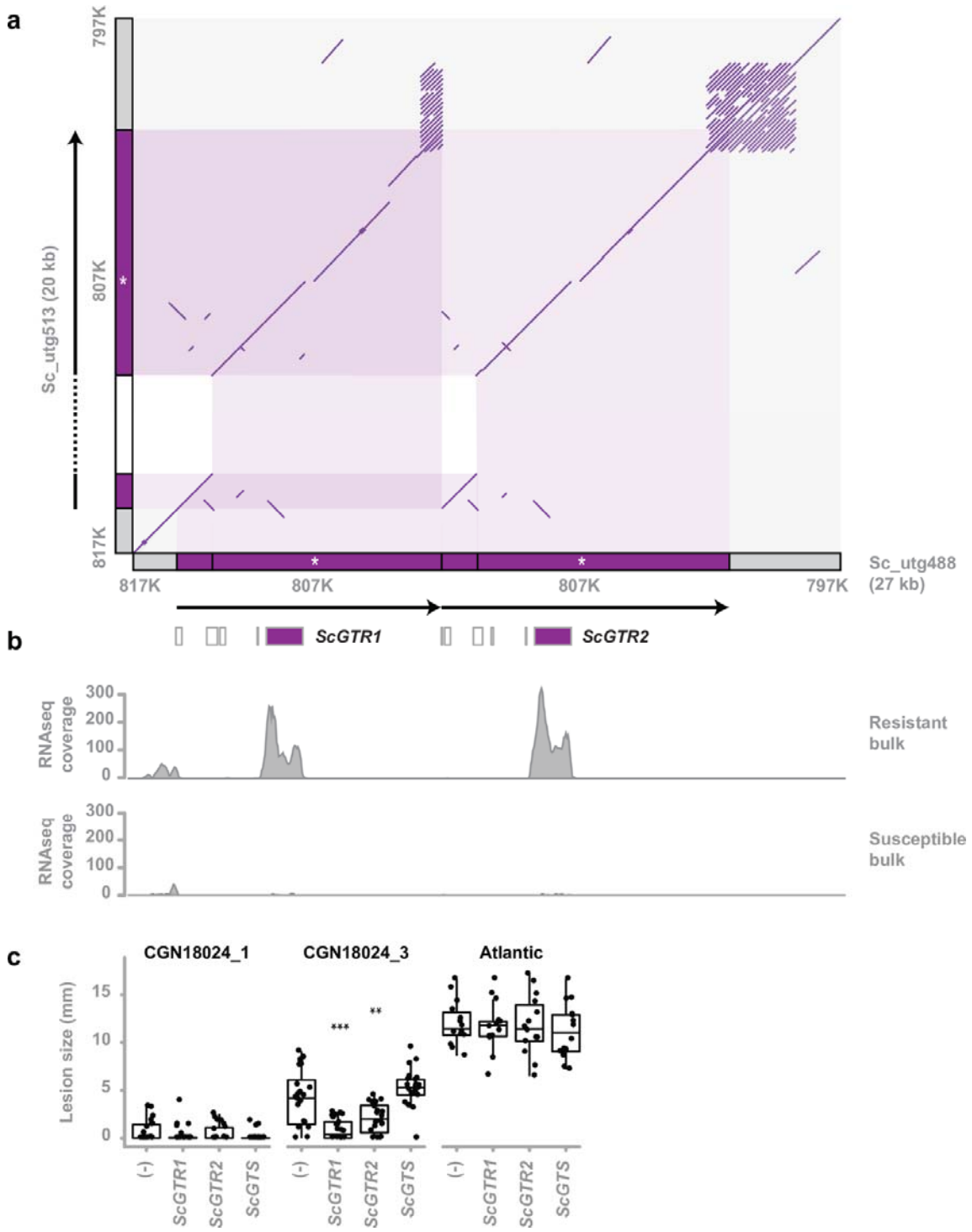


Fig 1. Early blight resistance maps to chromosome 12 of potato. A. 2-3 genotypes of 13 different accessions of *S. commersonii* and *S. malmeanum* were inoculated with *A. solani* altNL03003. 3 plants of each genotype were tested and 3 leaves per plants were inoculated with 6 10 µl droplets with spore suspension. Lesion diameters were measured 5 days post inoculation and visualised using boxplots, with horizonal lines indicating median values and individual measurements plotted on top. Non-expanding lesions (<3 mm) indicate resistance and expanding lesions indicate susceptibility. Some accessions segregate for resistance. **B.** Accession CGN18024 is an example of an accession that segregates for resistance to

390 *A. solani*, with CGN18024_1 displaying resistance and CGN18024_3 displaying susceptibility at 5 days after spray-
391 inoculation. **C.** Progeny from CGN18024_1 x CGN18024_3 was inoculated with *A. solani*. 3 plants of each genotype were
392 tested and 3 leaves per plants were inoculated with 6 10 µl droplets with spore suspension each. Lesion diameters were
393 measured 5 days post inoculation. 16 progeny genotypes are resistant (with lesion diameters < 2-3 mm) and 14 are
394 susceptible (with expanding lesions). This corresponds to a 1:1 segregation ratio (X^2 (1, N = 30) = 0.133, ρ= 0.72). **D.** SNPs
395 derived from a BSRseq analysis using bulks of susceptible and resistant progeny were plotted in 1 Mb windows over the 12
396 chromosomes of the potato DMv4.03 genome (68) They are almost exclusively located on chromosome 12.

397



398

399 **Fig 2. Identification of two glycosyltransferase resistance genes. A.** Comparison of the susceptible and resistant haplotype
400 of the *Solanum commersonii* CGN18024_1 resistance region (delimited by markers 817K and 797K) in a comparative dot
401 plot shows a rearrangement. Locations of markers used to map the resistance region are indicated in grey along the x- and
402 y-axis. The duplicated region of the resistant haplotype contains marker 807K (white asterisk) and two predicted
403 glycosyltransferase genes (*ScGTR1* and *ScGTR2*). Several short ORFs with homology to glycosyltransferase genes that were
404 predicted in the resistance region are indicated by white boxes, but *ScGTR1* and *ScGTR2* are the only full-length genes. As a
405 result of the rearrangement, the resistance region of the resistant haplotype (27 kb) is 7 kb larger than the corresponding

region of the susceptible haplotype (20 kb). **B.** Alignment of RNAseq reads from the BSRSeq analysis shows that *ScGTR1* and *ScGTR2* are expressed in bulks of resistant progeny, but not in bulks of susceptible progeny. **C.** *S. tuberosum* cv. 'Atlantic', *S. commersonii* CGN18024_1 and CGN18024_3 were agroinfiltrated with expression constructs for *ScGTR1* and *ScGTR2*, *ScGTS* and empty vector (-). *A. solani* is inoculated 2 days after agroinfiltration and lesion diameters are measured 5 days after inoculation. Lesion sizes were visualised with boxplots, with horizonal lines indicating median values and individual measurements plotted on top. Agroinfiltration with expression constructs for *ScGTR1* and *ScGTR2* results in a significant (Welch's Two Sample t-test, $^{**}P < 0.01$, $^{***}P < 0.001$) reduction of lesion sizes produced by *Alternaria solani* altNL03003 in *S. commersonii* CGN18024_3, but not in *S. tuberosum* cv. 'Atlantic'.

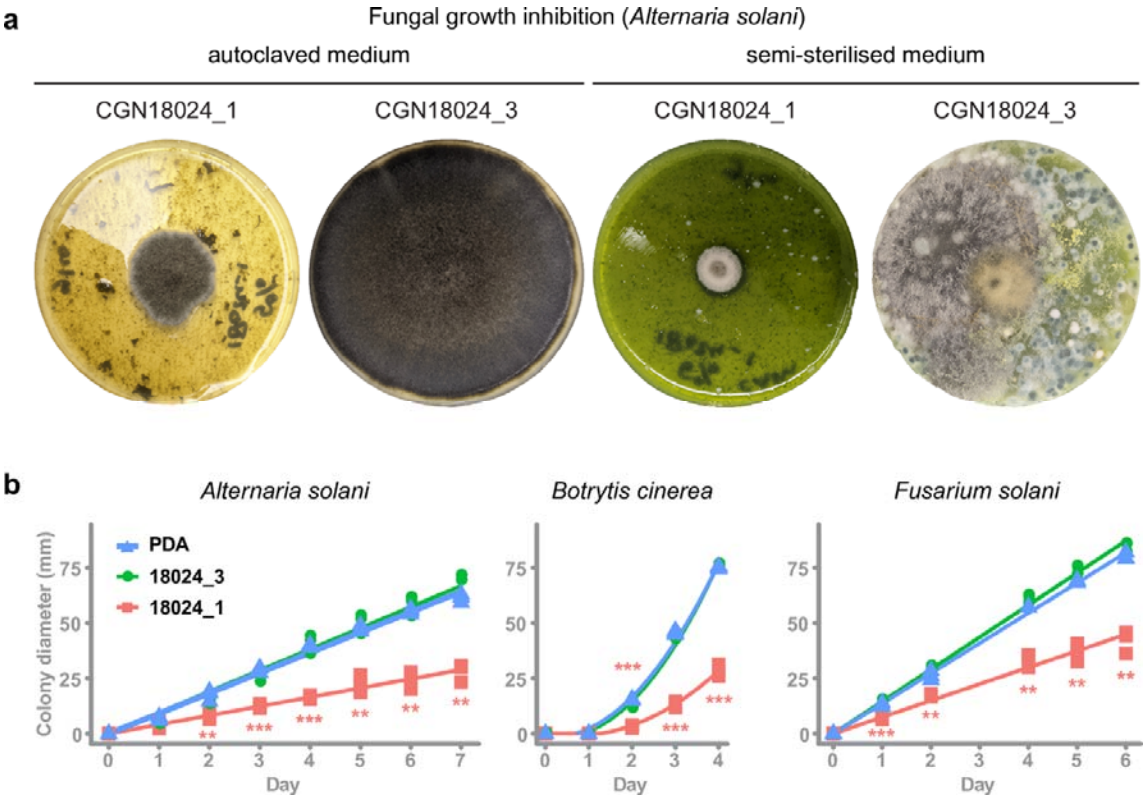


Fig 3. Leaf compounds from resistant *S. commersonii* inhibit growth of diverse fungi, including pathogens of potato. A. Crude leaf extract from CGN18024_1/CGN18024_3 was added to PDA plates (5% w/v) and autoclaved (left) or semi-sterilised for 15 min at 60 °C (right)**.** Growth of *Alternaria solani* altNL03003 was strongly inhibited on PDA plates with autoclaved leaf extract from CGN18024_1 compared to plates with CGN18024_3, as shown on the left two pictures taken at 7 days after placing an agar plug with mycelium of *A. solani* at the centre of each plate. Abundant fungal contamination appeared after 4 days on plates containing semi-sterilised leaf from CGN18024_3, but not on plates containing material from CGN18024_1 (right two pictures). **B.** Growth of potato pathogenic fungi *A. solani* (altNL03003), *B. cinerea* (B05.10) and *F. solani* (1992 vr) was followed by measuring the colony diameter on PDA plates containing autoclaved leaf material from CGN18024_1/CGN18024_3. Growth of all three fungi was measured on PDA plates containing CGN18024_1 (red squares), CGN18024_3 (green circles) or plates with PDA and no leaf material (blue triangles). Significant differences in growth on PDA plates containing plant extract compared to PDA plates without leaf extract are indicated with asterisks (Welch's Two Sample t-test, $^{**}P < 0.01$, $^{***}P < 0.001$).

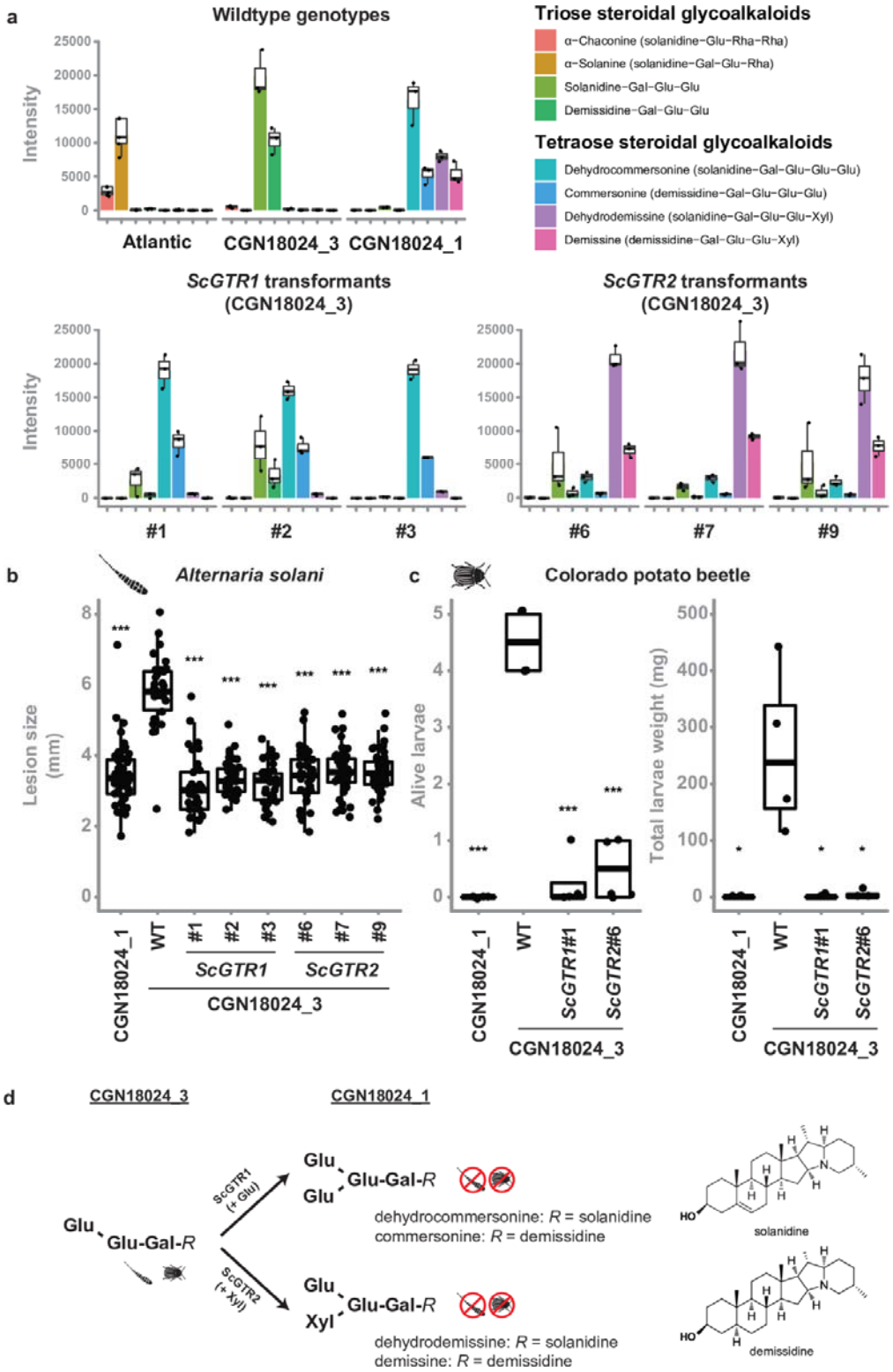


Fig 4. Tetraose steroidal glycoalkaloids from *Solanum commersonii* provide resistance to *Alternaria solani* and Colorado potato beetle. Data are visualised with boxplots, with horizonal lines indicating median values and individual measurements plotted on top. **A.** Tetraose steroidal glycoalkaloids (SGAs) were detected in resistant CGN18024_1 and in CGN18024_3 transformed with *ScGTR1*/*ScGTR2*. Susceptible *S. tuberosum* cv. 'Atlantic' and wildtype (WT) CGN18024_3 contain only triose SGAs. Overexpression of *ScGTR1* resulted in the addition of a hexose to the triose SGAs from CGN18024_3, resulting in a commertetraose (Gal-Glu-Glu-Glu), while overexpression of *ScGTR2* caused the addition of a

435 pentose, resulting in a lycotetraose (Gal-Glu-Glu-Xyl). **B.** WT CGN18024_1/CGN18024_3 and CGN18024_3 transformants
436 were inoculated with *Alternaria solani* altNL03003. 3 plants of each genotype were tested and 3 leaves per plants were
437 inoculated with 6 10 µl droplets with spore suspension each. Lesions diameters were measured 5 days post inoculation.
438 *ScGTR1* and *ScGTR2* can both complement resistance to *A. solani* in CGN18024_3, as the lesion sizes produced on
439 CGN18024_3 transformants are comparable to resistant CGN18024_1. **C.** 3 plants per genotype were challenged with 5
440 Colorado potato beetle larvae each. The tetraose SGAs produced by *ScGTR1* and *ScGTR2* can provide resistance to Colorado
441 potato beetle, as indicated by reduced larvae survival and total larvae weight. Significant differences with WT CGN18024_3
442 are indicated with asterisks (Welch's Two Sample t-test, $*P < 0.05$, $***P < 0.001$). **D**. Putative structures of SGAs detected in
443 CGN18024_1 and CGN18024_3, based on previous studies (85, 87-89). CGN18024_3 produces triose SGAs and is
444 susceptible to Colorado potato beetle and *A. solani*. ScGTR1 and ScGTR2 from CGN18024_1 convert these triose SGAs from
445 susceptible *S. commersonii* to tetraose SGAs, through the addition of a glucose or xylose moiety respectively. Both sugar
446 additions can provide resistance to Colorado potato beetle and *A. solani*.

447

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Supporting information

S1 Fig. Resistance of *S commersonii* genotypes CGN18024_1 and CGN18024_3 against different isolates of *A. solani*. CGN18024_1 and CGN18024_3 were inoculated with *A. solani* altNL03003 (the reference isolate used throughout the manuscript), altNL21001 (isolated from a potato field in the Netherlands in 2021) and ConR1H (harvested from a potato field in Idaho, USA in 2015). CGN18024_1 is resistant against all three isolates, whereas CGN18024_3 is susceptible

S2 Fig. Resistance from *S. commersonii* to *A. solani* is mapped to the top of chromosome 12. Filtered SNPs from bulked segregant RNAseq analysis (BSA-RNAseq) are plotted in 100 kb windows on chromosome 12 of the DMv4.03 genome at the top of the figure. A selection of SNPs ('A1'-'A10' and 'B1'-'B4') was used as markers in high resolution melting (HRM) analysis to genotype resistant *S commersonii* parent CGN18024_1 and susceptible parent CGN18024_3 from the AJW12 mapping population as well as progeny used in BSA-RNAseq. HRM analysis led to the identification of recombinants AJW12_13, AJW12_18, AJW12_23 and AJW12_29. Recombinant AJW12_13 (susceptible to ***A.*** *solani*) and recombinant AJW12_29 (resistant to *A. solani*) are used to map the resistance locus from *S. commersonii* to a window of approximately 3 Mb at the top of chromosome 12, delimited by marker 'B3'.

S3 Fig. Overview of marker 817K. Integrated Genomics Viewer (IGV) snapshot of Oxford Nanopore Technology (ONT) reads aligned to the genome of *S. commersonii* CGN18024_1. An Insertion/Deletion (InDel) of 254 bp is observed at approximately 817 kb of contig utg1998 that covers the resistance region. Primers were designed flanking the InDel to develop marker 817K.

S4 Fig. Overview of marker 807K. Integrated Genomics Viewer (IGV) snapshot of Oxford Nanopore Technology (ONT) reads aligned to the genome of *S. commersonii* CGN18024_1. An Insertion/Deletion (InDel) of 310 bp is observed at approximately 807 kb of contig utg1998 that covers the resistance region. Primers were designed flanking the InDel to develop marker 807K.

S5 Fig. Overview of marker 797K. Integrated Genomics Viewer (IGV) snapshot of Oxford Nanopore Technology (ONT) reads aligned to the genome of *S. commersonii* CGN18024_1. An Insertion/Deletion (InDel) of 6 bp is observed at approximately 797 kb of contig utg1998 that covers the resistance region. Primers were designed flanking the InDel to develop marker 797K.

S6 Fig. Overview of marker 764K. Integrated Genomics Viewer (IGV) snapshot of Oxford Nanopore Technology (ONT) reads aligned to the genome of *S. commersonii* CGN18024_1. An Insertion/Deletion (InDel) of 47 bp is observed at approximately 764 kb of contig utg1998 that covers the resistance region. Primers were designed flanking the InDel to develop marker 764K.

S7 Fig. Fine mapping the resistance locus in CGN18024_1. New markers based on the Solyntus and CGN18024_1 genome were used to screen for recombinants among progeny from a cross between resistant CGN18024_1 and susceptible CGN18024_3. Physical locations of the markers on the DMv4.04, Solyntus and CGN18024_1 genome are indicated at the top of the figure. Recombinants that were identified were tested for resistance to *A.* solani to fine map the resistance region. Recombinants 2-G10 (resistant, R), 14-F06 and 14-C12 (both susceptible, S) are used to delimit the resistance region between markers 817K and 797K, corresponding to a region of 20 kb in the CGN18024_1 genome.

S8 Fig. Early blight disease symptoms on key recombinants. The picture shows lesions of representative leaves of key recombinants at 5 days post drop-inoculation with spores of *A. solani*.

S9 Fig. Alignment of putative *S. commersonii* glycosyltransferases (ScGTs) linked to resistance. ScGTR1, ScGTR2 and ScGTS show high similarity, but the GT encoded by the susceptible haplotype (ScGTS) contains a mutation that leads to a truncated protein.

S10 Fig. Comparative phylogenetic analysis of glycosyltransferases with a known function (S2 Table). The phylogenetic tree is constructed using the maximum likelihood method (100 bootstraps). ScGTR1 and ScGTR2 are indicated with arrows and GTs with a previously characterised role in SGA biosynthesis are marked with

asterisks. Direct homologs of these SGA GTs (based on identity and synteny) derived from the CGN18024_1 genome are included in the analysis (names starting with 'SCM').

S11 Fig. Validation of *ScGTR1* and *ScGTR2* transformants using PCR. Gel electrophoresis of PCR amplicons produced by primer combinations p35S + ScGTR1sr3 (*ScGTR1*), p35S + ScGTR2sr3 (*ScGTR2*) and ef1αF1 + ef1αR1 (*EF1α*) using genomic DNA template of wildtype CGN18024_3 (WT) and *ScGTR1*/*ScGTR2* CGN18024_3 transformants.

S12 Fig. Principal Component Analysis (PCA) on *Solanum commersonii* genotypes and transformants. PCA based on 1041 mass peaks detected by UPLC-MS in leaves of ScGTR1 (red dots) and ScGTR2 (red squares) transformants compared to the corresponding susceptible wildtype Solanum commersonii CGN18024_3 (blue circles) and resistant CGN18024_1 (yellow circles). 75% of the total metabolic variation between the groups is explained by the 1st and the 2nd PC, mostly loaded by variation between tri- and tetraglycosylated steroidal glycoalkaloids. S/DhS - Solanidine/demissidine.

S1 Table. *Solanum commersonii* and *Solanum malmeanum* accessions used in this study. Accessions were obtained from the Centre for Genetic Resources, the Netherlands (CGN WUR). 2-3 genotypes from each accession were used in the disease screen with *A. solani*.

S2 Table. Overview of characterised glycosyltransferases used in comparative phylogenetic analysis (S9 Fig). Glycosyltransferases (GTs) with a known function are taken from Bowles et al. (2005), McCue et al. (2005, 2006, 2007), Masada et al. (2009), Itkin et al. (2011, 2013) and Tikunov et al. (2013) (71-78)

S3 Table. Putative identities and relative contents of SGAs in different potato genotypes. Average signal intensities (3 replicates per genotype) are presented as a percentage of the maximum signal intensity.

S4 Table. Overview of the steroidal glycoalkaloids detected in our study. RT - retention time, [M-H+FA]- - mass of a molecular ion at negative ionization mode (all alkaloids were represented by formic acid adduct ions); [M+H]+ - mass of a molecular ion at negative ionization mode; Putative structure - putative combination of aglycones and sugar moieties deduced by comparing the fragmentation spectrum derived at positive ionization with previous studies (85, 87-89); Fragmentation spectra derived using positive ionization: P - parent ion or P-fragment(s) loss.

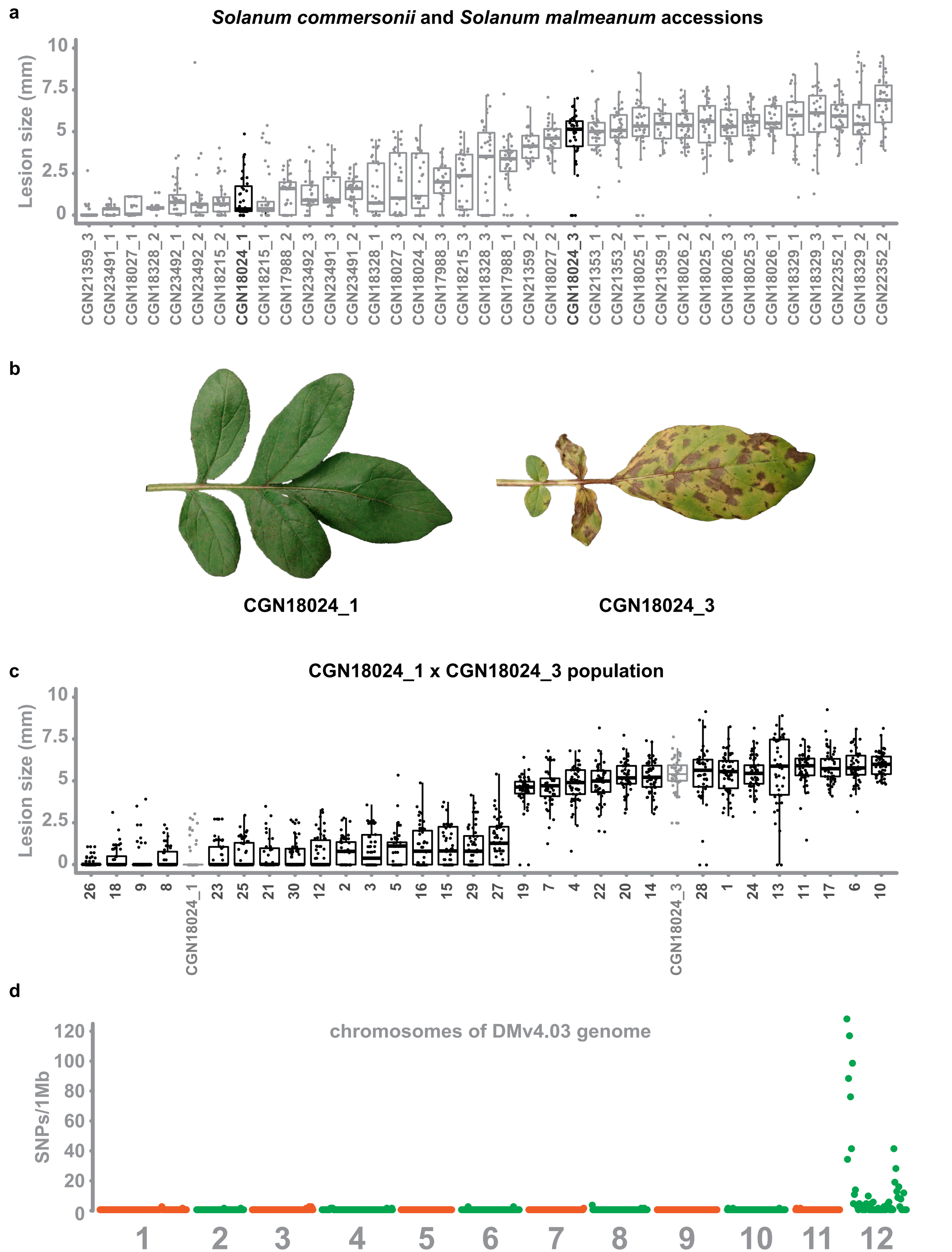
S5 Table. Overview of primers used to map the resistance region.

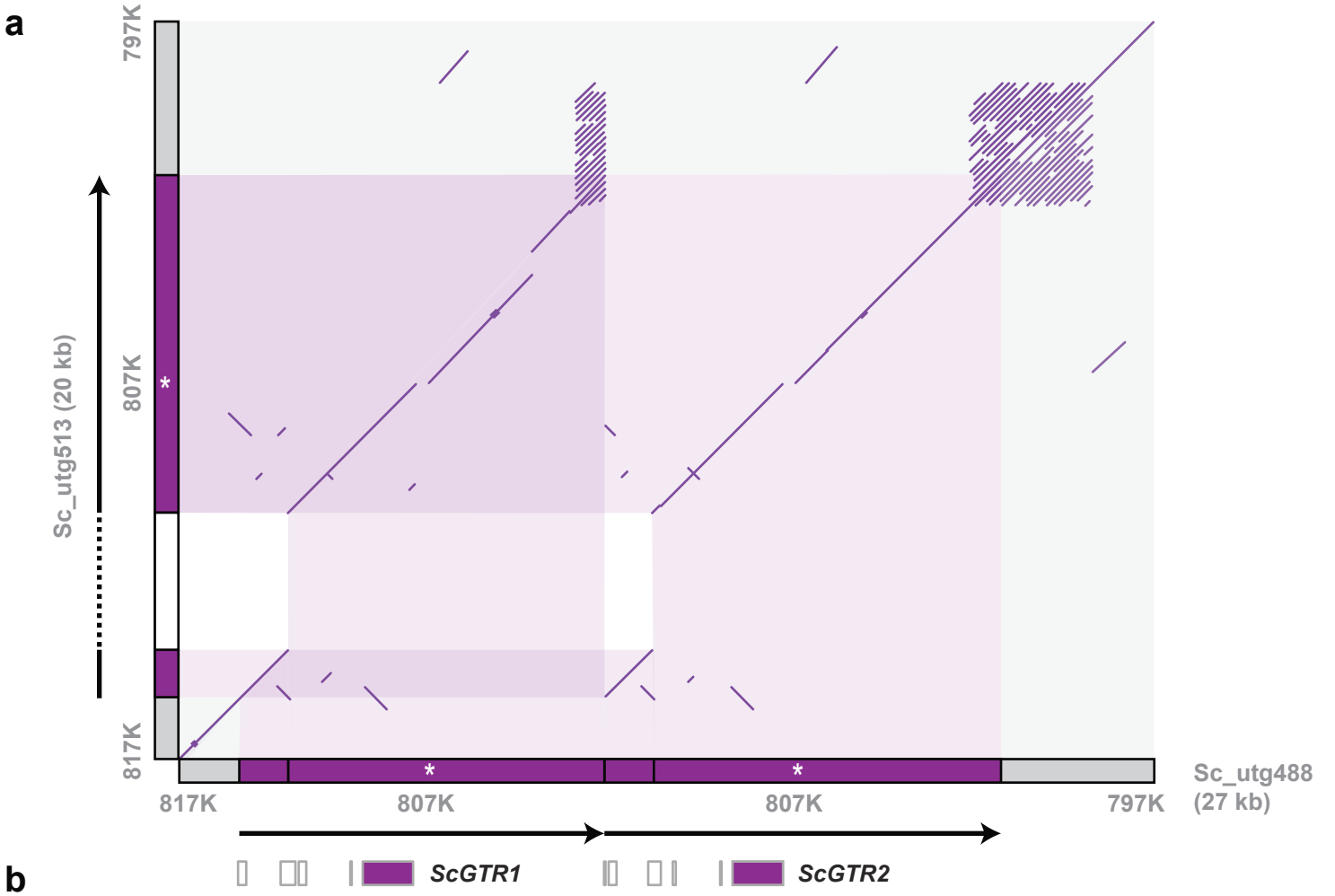
S6 Table. Overview of primers used to clone candidate resistance genes.

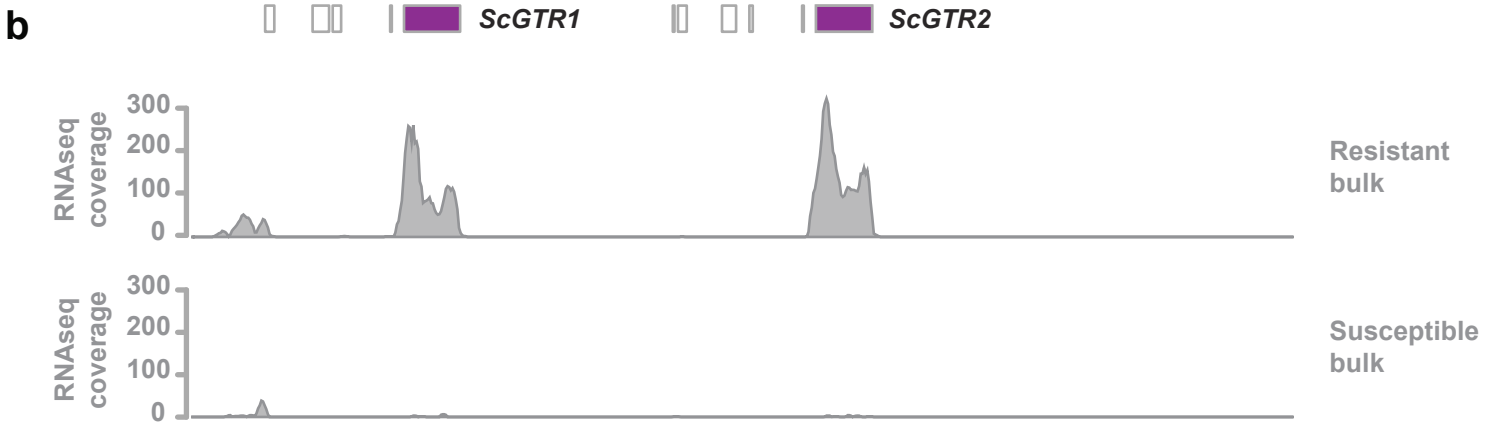
S7 Table. Overview of primers used to validate transformants.

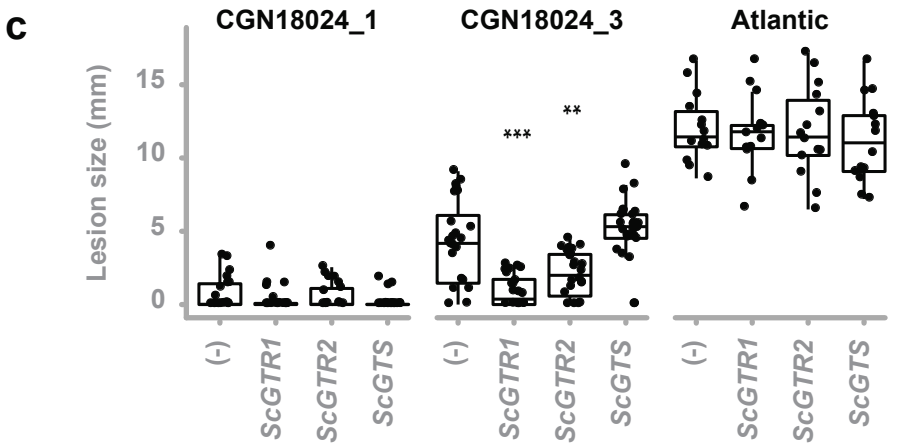
S1 File. Numerical data underlying the figures of this manuscript.

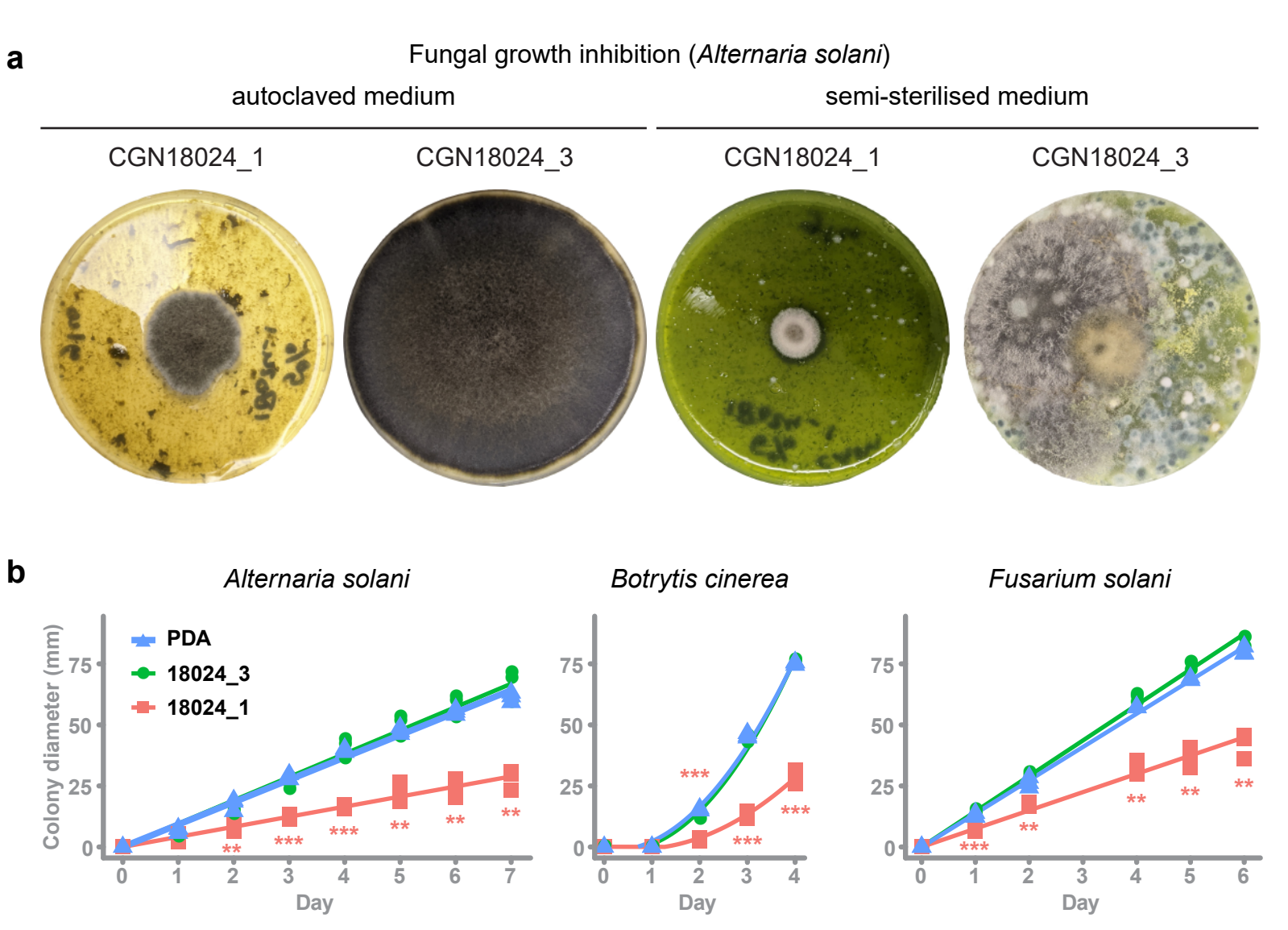
S2 File. Full information on methods.

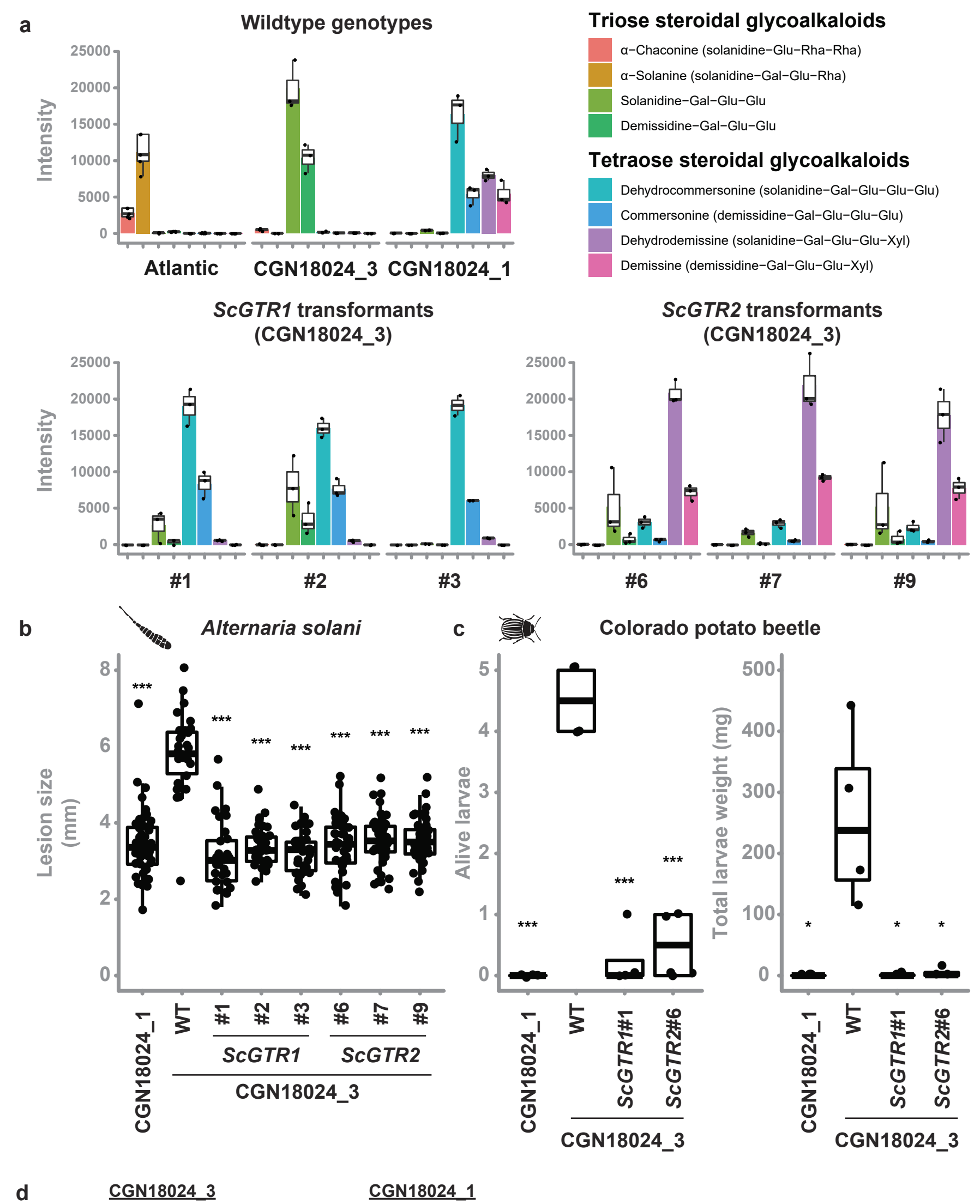

a
Solanum commersonii and Solanum malmeanum accessions
Lesion size (mm)
10
7.5
5
2.5
0
GN21359_3
GN23491_1
GN18027_1
GN18328_2
GN23492_1
GN23492_2
GN18215_2
GN18024_1
GN18215_1
GN17988_2
GN23492_3
GN23491_3
GN23491_2
GN18328_1
GN18027_3
GN18024_2
GN17988_3
GN18215_3
GN18328_3
GN17988_1
GN21359_2
GN18027_2
GN18024_3
GN21353_1
GN21353_2
GN18025_1
GN21359_1
GN18026_2
GN18025_2
GN18026_3
GN18025_3
GN18026_1
GN18329_1
GN18329_3
GN22352_1
GN18329_2
GN22352_2
b
CGN18024_1
CGN18024_3
c
CGN18024_1 x CGN18024_3 population
Lesion size (mm)
10
7.5
5
2.5
0
26
18
9
8
CGN18024_1
23
25
21
30
12
2
3
5
16
15
29
27
19
7
4
22
20
14
CGN18024_3
28
1
24
13
11
17
6
10
d
chromosomes of DMv4.03 genome
SNPs/1Mb
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12


a
797K
807K
817K
Sc_utg513 (20 kb)
*
*
*
817K
807K
807K
797K
Sc_utg488
(27 kb)
ScGTR1
ScGTR2


b
RNAseq coverage
300
200
100
0
Resistant bulk
RNAseq coverage
300
200
100
0
Susceptible bulk


c
CGN18024_1
CGN18024_3
Atlantic
Lesion size (mm)
15
10
5
0
***
**
(-)
ScGTR1
ScGTR2
ScGTS
(-)
ScGTR1
ScGTR2
ScGTS
(-)
ScGTR1
ScGTR2
ScGTS


a
Fungal growth inhibition (Alternaria solani)
autoclaved medium
semi-sterilised medium
CGN18024_1
CGN18024_3
CGN18024_1
CGN18024_3
b
Alternaria solani
Botrytis cinerea
Fusarium solani
PDA
18024_3
18024_1
Colony diameter (mm)
Day


a
Wildtype genotypes
Intensity
Atlantic
CGN18024_3
CGN18024_1
Triose steroidal glycoalkaloids
α−Chaconine (solanidine−Glu−Rha−Rha)
α−Solanine (solanidine−Gal−Glu−Rha)
Solanidine−Gal−Glu−Glu
Demissidine−Gal−Glu−Glu
Tetraose steroidal glycoalkaloids
Dehydrocommersonine (solanidine−Gal−Glu−Glu−Glu)
Commersonine (demissidine−Gal−Glu−Glu−Glu)
Dehydrodemissine (solanidine−Gal−Glu−Glu−Xyl)
Demissine (demissidine−Gal−Glu−Glu−Xyl)
ScGTR1 transformants (CGN18024_3)
ScGTR2 transformants (CGN18024_3)
#1
#2
#3
#6
#7
#9
b
Alternaria solani
Lesion size (mm)
CGN18024_1
WT
ScGTR1
ScGTR2
CGN18024_3
c
Colorado potato beetle
Alive larvae
Total larvae weight (mg)
ScGTR1#1
ScGTR2#6
d
CGN18024_3
CGN18024_1
Glu
Glu-Gal-R
ScGTR1 (+ Glu)
ScGTR2 (+ Xyl)
Xyl
dehydrocommersonine: R = solanidine
commersonine: R = demissidine
dehydrodemissine: R = solanidine
demissine: R = demissidine
solanidine
demissidine