Tetraose steroidal glycoalkaloids from potato can provide complete protection against fungi and insects

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Abstract

Plants with innate disease and pest resistance can contribute to more sustainable agriculture. Plant breeders typically focus on immune receptors or impaired susceptibility genes to develop resistant crops, but these can present challenges in terms of strength, durability or pleotropic effects. Although natural defence compounds produced by plants have the potential to provide a general protective effect against pathogens and pests, they are not a primary target in resistance breeding. The precise contribution of defence metabolites to plant immunity is often unclear and the genetics underlying their biosynthesis is complex. 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 can provide complete resistance to *Alternaria solani* and Colorado potato beetle through the production of tetraose steroidal glycoalkaloids. Moreover, we show that these compounds are active against a wide variety of fungi. This research provides a direct link between specific modifications to steroidal glycoalkaloids of potato and resistance against 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.

Introduction

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

Wild relatives of crop species are promising sources of natural disease resistance (9-12). Monogenic resistance caused by dominant resistance (*R*) genes, typically encoding immune receptors that belong to the class of nucleotide-binding leucine-rich repeat receptors (NLRs), are successfully employed by plant breeders to develop varieties with strong qualitative disease resistance. However, this type of resistance is usually restricted to a limited range of races and pathogens are often able to overcome resistance over time (13, 14).

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

A wide range of secondary metabolites with antimicrobial or anti-insect properties has been identified in diverse plant species (25-27), suggesting that secondary metabolites can play a direct role in plant immunity. However, there are only few studies to date in plants that demonstrate a direct link between secondary metabolites and disease resistance (28-31). Avenacin A-1, a triterpenoid saponin from oat, is a well-known example (32, 33). Saponins are compounds with soap-like properties that consist of a triterpenoid or steroidal aglycone linked to a variable oligosaccharide

chain (34). They are widely distributed in plants from different families and their effect stems from the ability to interact with membrane sterols, disrupting the cell integrity from target organisms (34-37). Saponins from the Solanaceae and Liliaceae families are characterized by a steroidal alkaloid aglycone (38, 39). Different studies show that steroidal glycoalkaloids (SGAs) from tomato, potato and lily have antimicrobial and anti-insect activity (40-50).

Early blight is an important disease of tomato and potato that is caused by the necrotrophic fungal pathogen *Alternaria solani* (51-53). In a previous study, we found a wild potato species, *Solanum commersonii*, with strong resistance to *A. solani* (54). We showed that resistance is likely caused by a single dominant locus and that it can be introgressed in cultivated potato (54). Resistance to necrotrophs is usually considered to be a complex, polygenic trait, or recessively inherited according to the *inverse gene-for-gene* model (55-60). It therefore surprised us to find a qualitative dominant resistance against early blight in *S. commersonii* (54).

In this study, we explored different accessions of *S. commersonii* and *S. malmeanum* (previously *S. commersonii* subsp. *malmeanum* (61)) and developed a population that segregates for resistance to early blight. Using a Bulked Segregant RNA-Seq (BSR-Seq) approach (62), we mapped the resistance locus to the top of chromosome 12 of potato. We sequenced the genome of the resistant parent of the population and identified two glycosyltransferases that can provide resistance to susceptible *S. commersonii*. We show that resistance is based on the production of tetraose SGAs. Interestingly, these SGAs are active against a wide variety of pathogens and even pests. As a result, plants producing the compounds have a broad-spectrum disease and insect resistance.

Results

Early blight resistance maps to chromosome 12 of potato

To find suitable parents for a mapping study targeting early blight resistance, we performed a disease screen with *A. solani* isolate altNL03003 (63) on 13 different accessions encompassing 37 genotypes of *S. commersonii* and *S. malmeanum* (S1 Table). The screen showed clear differences in resistance

83 phenotypes between and within accessions (Fig 1A). Roughly half of the genotypes were completely
84 resistant (lesion diameters < 3 mm indicate that the lesions are not expanding beyond the size of the
85 inoculation droplet) and the other half was susceptible (displaying expanding lesions), with only a
86 few intermediate genotypes. CGN18024 is an example of an accession that segregates for resistance,
87 with CGN18024_1 showing complete resistance and CGN18024_3 showing clear susceptibility (Fig
88 1B). The fact that individual accessions can display such clear segregation for resistance suggests that
89 resistance is caused by a single gene or locus. Because of its clear segregation, *S. commersonii*
90 accession CGN18024 was selected for further studies.
91 To further study the genetics underlying resistance to early blight, we crossed resistant CGN18024_1
92 with susceptible CGN18024_3. Thirty progeny genotypes were sown out and tested with *A. solani*.
93 We identified 14 susceptible genotypes and 16 fully resistant genotypes, with no intermediate
94 phenotypes in the population (Fig 1C). This segregation supports a 1:1 ratio (X^2 (1, N = 30) = 0.133,
95 p=.72), which confirms that resistance to early blight is likely caused by a single dominant locus in *S.*
96 *commersonii*.
97 To genetically localize the resistance, we isolated RNA from each progeny genotype and the parents
98 of the population and proceeded with a BSR-Seq analysis (62). RNA from resistant and susceptible
99 progeny genotypes were pooled in separate bulks and sequenced next to RNA from the parents on
100 the Illumina sequencing platform (PE150). Reads were mapped to the DMv4.03 (64) and Solyntus
101 potato genomes (65). To find putative SNPs linked to resistance, we filtered for SNPs that follow the
102 same segregation as resistance (heterozygous in resistant parent CGN18024_1 and the resistant bulk,
103 but absent or homozygous in susceptible parent and susceptible bulk). The resulting SNPs localize
104 almost exclusively on chromosome 12 of the DM and Solyntus genomes, with most of them located
105 at the top of the chromosome (Fig 1D). We used a selection of SNPs distributed over chromosome 12
106 as high-resolution melt (HRM) markers to genotype the BSR-Seq population. This rough mapping
107 proves that the locus for early blight resistance resides in a region of 3 Mb at the top of chromosome
108 12 (S1 Fig).

Improved genome assembly *of S. commersonii*

A genome sequence of *S. commersonii* is already available (66), but we do not know if the sequenced genotype is resistant to *A. solani*. To help the identification of additional markers and to explore the resistance locus of a genotype with confirmed resistance, we sequenced the genome of resistant parent CGN18024_1. High-molecular-weight (HMW) genomic DNA (gDNA) from CGN18024_1 was used for sequencing using Oxford Nanopore Technology (ONT) on a GridION X5 platform and for sequencing using DNA Nanoball (DNB) technology at the Beijing Genomics Institute (BGI) to a depth of approximately 30X. We used the ONT reads for the initial assembly and the shorter, more accurate, DNBseq reads to polish the final sequence. The resulting assembly has a size of 737 Mb, which is close to the size of the previously published genome of *S. commersonii* (730 Mb) (66). N50 scores and Benchmarking Universal Single-Copy Orthologs (BUSCO) score indicate a highly complete and contiguous genome assembly of *S. commersonii* (Table 1).

Table 1. Genome assembly metrics of *S. commersonii* cmm1t (66) 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

[a)] Aversano et al (2015)

Identification of two glycosyltransferase resistance genes

To identify candidate genes that can explain the resistance of *S. commersonii*, it was necessary to further reduce the mapping interval. By aligning the ONT reads to the CGN18024_1 genome assembly, we could identify new polymorphisms that we converted to additional PCR markers (S2-5 Figs). We performed a recombinant screen of approximately 3000 genotypes from the population to fine-map the resistance region to a window of 20 kb (S6 and S7 Figs).

We inferred that the resistance locus is heterozygous in CGN18024_1 from the segregation in the mapping population. We used polymorphisms in the resistance region to separate and compare the

ONT sequencing reads from the resistant and susceptible haplotype. This comparison showed a major difference between the two haplotypes. The susceptible haplotype contains a small insertion of 3.7 kb inside a larger region of 7.3 kb. The larger region is duplicated in the resistant haplotype (Fig 2A). As a result, the resistance region of the resistant haplotype is 27 kb, 7 kb larger than the corresponding region of the susceptible haplotype (20 kb).

Two genes coding for putative glycosyltransferases (GTs) are located within the rearrangement of the resistant haplotype. The corresponding allele from the susceptible haplotype contains a frameshift mutation, leading to a truncated protein (S8 Fig). Several other short ORFs with homology to glycosyltransferases were predicted in the resistant haplotype, but *ScGTR1* (*S. commersonii* glycosyltransferase linked to resistance 1) and *ScGTR2* are the only full-length genes in the region. Reads from the BSR-Seq experiment show that both genes are expressed in bulks of resistant progeny and not in susceptible progeny (Fig 2B), suggesting a putative role for these genes in causing resistance to *A. solani*. *ScGTR1* and *ScGTR2* are homologous genes with a high similarity (the predicted proteins that they encode share 97% amino acid identity). We compared the predicted amino acid sequences with previously characterized GTs (67-74) and found that they share some similarity with GTs with a role in zeatin biosynthesis (75-77) and with GAME17, an enzyme involved in biosynthesis of α-tomatine, a steroidal glycoalkaloid typically found in tomato (72) (S9 Fig, S2 Table).

To test whether the identified candidate genes are indeed involved in resistance, we transiently expressed both alleles of the resistant haplotype (*ScGTR1* and *ScGTR2*) as well as the corresponding allele from the susceptible haplotype (*ScGTS*), in leaves of resistant CGN18024_1 and susceptible CGN18024_3 and *S. tuberosum* cultivar Atlantic, using agroinfiltration (78). Following agroinfiltration, the infiltrated areas were drop-inoculated with a spore suspension of *A. solani*. Transient expression of *ScGTR1* as well as *ScGTR2* significantly reduced the size of the *A. solani* lesions in susceptible CGN18024_3, compared to *ScGTS* and the empty vector control. Resistant CGN18024_1 remained resistant, whereas susceptible Atlantic remained susceptible regardless of the treatment (Fig 2C). We

conclude that both *ScGTR1* and *ScGTR2* can affect resistance in susceptible *S. commersonii* CGN18024_3, but not in *S. tuberosum* cv. Atlantic.

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

Glycosyltransferases are ubiquitous enzymes that catalyse the transfer of saccharides to a range of different substrates. To test if resistance of *S. commersonii* to *A. solani* can be explained by a host-specific defence compound, we performed a growth inhibition assay using crude leaf extract from resistant and susceptible *S. commersonii*. Leaf material was added to PDA plates to equal 5% w/v and autoclaved (at 121 °C) or semi-sterilised at 60 °C. Interestingly, leaf material from resistant CGN18024_1 strongly inhibited growth of *A. solani*, while we did not see any growth inhibition on plates containing leaves from susceptible CGN18024_3 (Fig 3A). Remarkably, on the plates containing semi-sterilised leaves from susceptible *S. commersonii,* ample contamination with diverse fungi appeared after a few days, but not on plates with leaves from CGN18024_1 (Fig 3A). Thus, leaves from CGN18024_1 contain compounds that can inhibit growth of a variety of fungi, not just *A. solani*.

To quantify the inhibitory effect of leaves from *S. commersonii* against different fungal pathogens of potato, we performed a growth inhibition assay with *A. solani* (altNL03003 (63)), *Botrytis cinerea* (B05.10 (79)) and *Fusarium solani* (1992 vr). As before, we added 5% (w/v) of leaf material from CGN18024_1 or CGN18024_3 to PDA plates and we placed the fungi at the centre of the plates. We measured colony diameters in the following days and compared it with the growth on PDA plates without leaf extract. Indeed, growth of all three potato pathogens was significantly reduced on medium containing leaf material from CGN18024_1 (Fig 3B), compared to medium containing material from CGN18024_3 or on normal PDA plates. These results indicate that constitutively produced defence compounds (phytoanticipins) from the leaves of resistant *S. commersonii* can have a protective effect against diverse fungal pathogens of potato.

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

Leaves from *Solanum* usually contain SGAs, which are known phytoanticipins against fungi and other plant parasites (80). *S. tuberosum* typically produces the triose SGAs α-solanine (solanidine-Gal-Glu-Rha) and α-chaconine (solanidine-Glu-Rha-Rha), while five major tetraose SGAs were previously identified in *S. commersonii*: commersonine (demissidine-Gal-Glu-Glu-Glu), dehydrocommersonine (solanidine-Gal-Glu-Glu-Glu), demissine (demissidine-Gal-Glu-Glu-Xyl), dehydrodemissine (solanidine-Gal-Glu-Glu-Xyl) and α-tomatine (tomatidine-Gal-Glu-Glu-Xyl) (80-85). To test if SGAs can explain resistance of *S. commersonii,* we measured SGA content in leaves from Atlantic and susceptible/resistant *S. commersonii* by Ultra High Performance Liquid Chromatography (UPLC) coupled to mass spectrometry (MS). As expected, we could detect the triose SGAs α-solanine and α-chaconine in susceptible *S. tuberosum* cv. Atlantic, but we found a remarkable difference in the SGA profile of resistant and susceptible *S. commersonii*. We detected tetraose SGAs in resistant *S. commersonii* CGN18024_1, whereas susceptible *S. commersonii* CGN18024_3 accumulates triose SGAs (Fig 4A and S3 and S4 Tables). The mass spectra of the four major tetraose SGAs from *S. commersonii* correspond to (dehydro-) commersonine and (dehydro-) demissine, matching the data from previous studies(81, 83-85). Notably, the mass spectra of the two major SGAs from susceptible CGN18024_3 correspond to the triose precursors of these SGAs (solanidine-Gal-Glu-Glu and demissidine-Gal-Glu-Glu respectively) (S3 and S4 Tables). These results suggest that the triose SGAs present in susceptible CGN18024_3 are modified to produce the tetraose SGAs in resistant CGN18024_1, by addition of an extra glucose or xylose moiety.

To investigate a possible role for *ScGTR1* and *ScGTR2* in the production of tetraose SGAs from CGN18024_1 and their link to resistance, we generated stable transformants of *ScGTR1* and *ScGTR2* in triose SGA producing CGN18024_3 (S10 Fig). UPLC-MS analysis showed that both *ScGTR1* and *ScGTR2* transformants accumulate tetraose SGAs, while the amount of triose SGAs is markedly

reduced (Fig 4A). Strikingly, ScGTR1 and ScGTR2 appear to have different specificities. Overexpression of *ScGTR1* resulted in the addition of a hexose to the triose SGAs from CGN18024_3 (corresponding to a commertetraose), while overexpression of *ScGTR2* caused the addition of a pentose (corresponding to a lycotetraose) (Fig 4A and D). This *in planta* evidence suggests that *ScGTR1* is a glucosyltransferase and that *ScGTR2* is a xylosyltransferase. However, we detect a slight overlap in activity. In addition to the lycotetraose products, we detected small amounts of commertetraose product in *ScGTR1* transformants and vice versa in the *ScGTR2* transformants (Fig 4 and S3 Table). A multivariate Principal Components Analysis (PCA) on the full metabolic profile consisting of all 1,041 detected mass peaks revealed that ScGTR1 and ScGTR2 are highly specific towards SGAs since 75% of the metabolic variation between the transformants and the wild types could be explained by the SGA modifications (S11 Fig). Modifications catalysed by both enzymes can lead to resistance, as *ScGTR1* and *ScGTR2* transformants are both resistant to *A. solani* (Fig 4B). Atlantic *ScGTR1* and *ScGTR2* transformants did not show differences in SGA profile, probably because they contain different triose SGA substrates than found in *S. commersonii* CGN18024_3 (S3 and S4 Tables).

Leptine and dehydrocommersonine SGAs from wild potato relatives have previously been linked to resistance to insects such as Colorado potato beetle (CPB) (40, 43-47, 86). To see if the SGAs from *S. commersonii* can protect against insects as well, we performed a test with larvae of CPB on wildtype CGN18024_1/CGN18024_3 and on CGN18024_3 transformed with *ScGTR1* or *ScGTR2* (Fig 4B). Wildtype CGN18024_3 is susceptible to CPB, but CGN18024_1 and CGN18024_3 transformed with *ScGTR1* or *ScGTR2* are completely resistant to CPB, as illustrated by a very low larvae weight and survival (Fig 4C). Thus, the conversion of triose SGAs from CGN18024_3 to tetraose SGAs produced by CGN18024_1, carried out by both ScGTR1 and ScGTR2, can provide protection against fungi and insects (Fig 4A-D).

Discussion

In this study, we set out to characterise resistance of *S. commersonii* to *A. solani*. We showed that it is caused by a single dominant locus containing two GT candidate resistance genes. Both GTs are involved in the production of tetraose SGAs in *S. commersonii*, but they transfer distinct sugars. Both modifications cause resistance to *A. solani*. We demonstrate that the tetraose SGAs from *S. commersonii* can protect against other fungi besides *A. solani* and that plants producing the compounds are resistant to CPB. Collectively, our data establish a direct link between the tetraose SGAs from *S. commersonii* and resistance against different potato pathogens and pest insects.

It is known that specialized metabolites from plants have a role in plant defence and compounds with antimicrobial effects have been characterized in many different plant species (25-27). However, exact knowledge of how these compounds contribute to resistance and how they are produced is limited. As a result, saponins and other specialized defence metabolites are not targeted in resistance breeding. Instead, the current focus is on using immune receptors or *S* genes. These different strategies each come with their own challenges in terms of durability, specificity, pleiotropic effects and strength of the resulting resistance. The tetraose SGAs from *S. commersonii* compare favourably in many of these aspects, as they provide a strong and broad-spectrum resistance without any noticeable negative effects on the plant.

Biosynthesis of SGAs in *Solanum* is a complex trait that is controlled by many genes. The discovery of *S. commersonii* genotypes with and without tetraose SGAs provides us with unique insight in the role of these compounds in plant immunity. To make a practical use of them, it is necessary to identify the genes upstream of *ScGTR1* and *ScGTR2*. The compounds that are found in resistant *S. commersonii* are an interesting combination of a solanidine or demissidine aglycone and a lycotetraose or commertetraose sugar moiety. Solanidine forms the aglycone backbone of α-solanine and α-chaconine from potato, while the lycotetraose decoration is found on α-tomatine from tomato (83, 87). The biosynthesis pathways leading to the production of these major SGAs from cultivated potato and tomato have largely been elucidated in recent years and it was found that the underlying genes occur in conserved clusters (72, 87). This knowledge and the similarities between SGAs from *S.*

256 *commersonii* and cultivated potato/tomato will help to identify the missing genes from the pathway
257 through comparative genomics.
258 The broad-spectrum activity of tetraose SGAs is attractive, but this non-specificity also presents a
259 risk. The antifungal and anti-insect activity of SGAs from *S. commersonii* is not restricted to potato
260 pathogens and pests, but could also affect beneficial or commensal micro-organisms or other
261 animals that feed on plants (88, 89). In potato tubers, a total SGA content of less than 200 mg/kg is
262 generally considered to be safe for human consumption (90-92), but little is known about the toxicity
263 of individual SGAs. In tomato fruit, α-tomatine is converted to esculeoside A during fruit ripening in a
264 natural detoxification process from the plant (93, 94) to facilitate dispersal of the seeds by foraging
265 animals. Unintended toxic effects of SGAs should similarly be taken into account when used in
266 resistance breeding.
267 Studies on α-tomatine and avenacin A-1 show that changes to the sugar moiety can affect toxicity of
268 these saponins (42, 95-97). Tomato and oat pathogens produce enzymes that can detoxify these
269 compounds through removal of one or more glycosyl groups (42, 48, 49, 98-100). The degradation
270 products of saponins can also suppress plant defence responses (101, 102). Conversely, here we
271 show that the resistance of *S. commersonii* is based on the addition of a glycosyl group to a triose
272 saponin from *S. commersonii.* There is large variation in both the aglycone and the sugar moiety of
273 SGAs from wild *Solanum*, with likely over 100 distinct SGAs produced in tubers (83) (103). This
274 diversity suggests a pressure to evolve novel molecules, possibly to resist detoxification or other
275 tolerance mechanisms, reminiscent of the molecular arms race that drives the evolution of plant
276 immune receptors (14). Thus, wild *Solanum* germplasm is not only a rich source of immune
277 receptors, it also provides a promising source of natural defence molecules.
278 As crops are usually affected by multiple diseases and pests, significant reduction of pesticide use can
279 only be achieved if plants are naturally protected against a range of pathogen species and pests.
280 Different strategies towards this goal have been proposed and our study underlines the relatively
281 unexplored potential of defence compounds that are naturally produced by plants. The fact that

genes for specialized plant metabolites can occur in biosynthetic gene clusters (72, 104-106), means that introgression breeding could help to move these compounds from wild relatives to crop species. If the genes underlying the biosynthesis pathways are identified, it is also possible to employ them through metabolic engineering (27). Alternatively, the defence compounds could be produced in non-crop plants or other organisms and applied on crops as biological protectants. Studies on how natural defence compounds are produced in different plant tissues, their toxicity and how they are detoxified, combined with studies on how different modifications ultimately affect plant immunity, are essential to employ them in a safe and effective manner. Such studies at the interface of plant immunity and metabolism can help to design innovative solutions to complement existing resistance breeding strategies and improve sustainability of our food production.

Methods

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

Genome assembly and separation of haplotypes covering resistance region

ONT reads were filtered using Filtlong v0.2.0 (https://github.com/rrwick/Filtlong) with --min_length 1000 and --keep_percent 90. Adapter sequences were removed using Porechop (107). Fastq files were converted to Fasta using seqtk v1.3 (https://github.com/lh3/seqtk). Assembly was performed with smartdenovo (https://github.com/ruanjue/smartdenovo/) and a k-mer size of 17, with the option for generating a consensus sequence enabled. ONT reads were mapped back to the assembly using minimap2 v2.17 (108) and used for polishing with racon v1.4.3 (109) using default settings. DNBseq reads were mapped to the resulting sequence using bwa mem v0.7.17 (110) and used for a second round of polishing with racon v1.4.3. This procedure to polish the assembly using DNBseq reads was repeated once. ONT reads were mapped back to the polished CGN18024_1 assembly using minimap2 v2.17 (108). The alignment was inspected using IGV v2.6.3 (111) to identify polymorphisms

for new markers and marker information was used to identify ONT reads representative for both haplotypes spanning the resistance region of CGN18024_1. Bedtools v2.25.0 (112) was used extract the resistance region from the reads and to mask the corresponding region from the original CGN18024_1 assembly. The extracted resistance regions from both reads were appended to the assembly and the polishing procedure described above was repeated to prepare a polished genome assembly of CGN18024_1, containing a sequence of both haplotypes covering the resistance region. Quality of the genome was assessed using quast v5.0.2 with --eukaryote --large (113).

Transient disease assay

Agroinfiltration was performed as described previously using *Agrobacterium tumefaciens* strain AGL1 (78, 114). Agrobacterium suspensions were used at an OD_{600} of 0.3 to infiltrate fully expanded leaves of 3-week-old CGN18024_1, CGN18024_3 and *S. tuberosum* cv. Atlantic. *ScGTR1*, *ScGTR2*, *ScGTS* and pK7WG2-empty were combined as four separate spots on the same leaf and the infiltrated areas were encircled with permanent marker. The plants were transferred to a climate cell 48 h after agroinfiltration and each infiltrated area was inoculated with *A. solani* by pipetting a 10 µl droplet of spore suspension (1 x 10^5 conidia/mL) at the centre of each spot. Lesion diameters were measured 5 days post inoculation. Eight plants were tested of each genotype, using three leaves per plant.

Fungal growth inhibition assays

Mature leaf material from 5-week-old plants was extracted in phosphate-buffered saline (PBS) buffer using a T25 Ultra Turrax disperser (IKA) and supplemented to obtain a 5% w/v suspension in PDA and autoclaved (20 min at 121C), or added to PDA after autoclaving, followed by an incubation step for 15 min at 60C to semi-sterilise the medium. The medium was poured into Petri dishes. Small agar plugs containing mycelium from *A. solani* (CBS 143772) or *F. solani* (1992 vr) were placed at the centre of each plate and the plates were incubated at 25C in the dark. Similarly, approximately 100 spores of *B. cinerea* B05.10 (79) were pipetted at the centre of PDA plates containing the different leaf extracts and the plates were incubated at room temperature in the dark. 3 plates per fungal

isolate/leaf extract combination were prepared and colony diameters were measured daily using a digital calliper.

Data analysis

Data were analysed in RStudio (R version 4.02) (115, 116), using the tidyverse package (117). Most figures were generated using ggplot2 (118), but genomic data were visualised using Gviz and Bioconductor (119). PCA was performed using PAST3 software (https://past.en.lo4d.com/windows). *P* values for comparisons between means of different groups were calculated in R using Welch's Two Sample t-test.

Data availability

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

Competing interests

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 ScGTR1 and ScGTR2 filed by the J.R. Simplot company. The other authors declare no competing interests.

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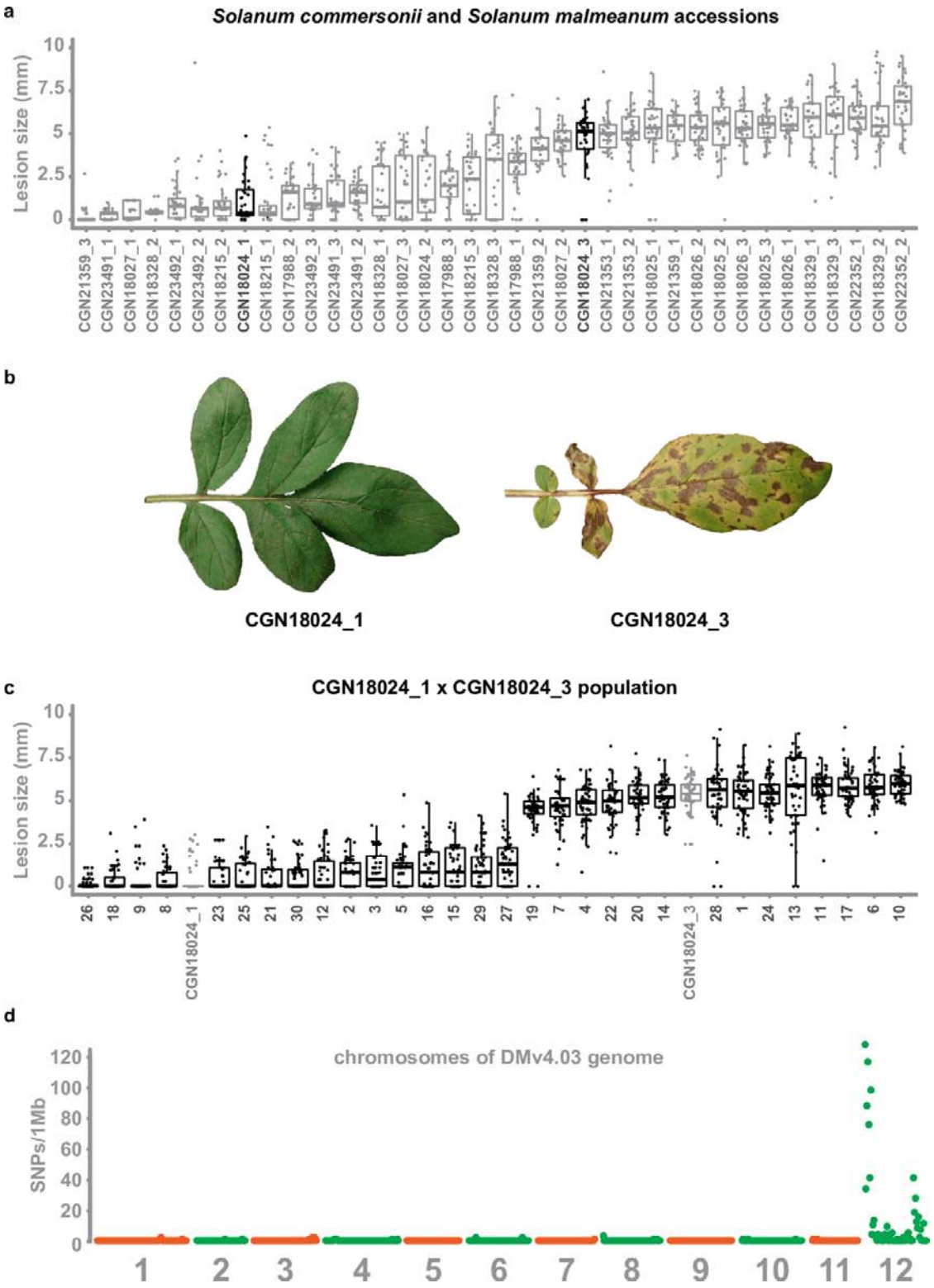


Fig 1. Early blight resistance maps to chromosome 12 of potato. A. 2-3 genotypes of 13 different accessions of *S. commersonii* and *S. malmeanum* were inoculated with *A. solani* altNL03003. 3 plants of each genotype were tested and 3 leaves per plants were inoculated with 6 10 µl droplets with spore 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 (<2-3 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

A. solani, with CGN18024_1 displaying complete resistance and CGN18024_3 displaying susceptibility at 5 days after spray-inoculation. **C.** Progeny from CGN18024_1 x CGN18024_3 was inoculated with *A. solani*. 3 plants of each genotype were tested and 3 leaves per plants were inoculated with 6 10 µl droplets with spore suspension each. Lesion diameters were measured 5 days post inoculation. 16 progeny genotypes are resistant (with lesion diameters < 2-3 mm) and 14 are susceptible (with expanding lesions). This corresponds to a 1:1 segregation ratio (X^2 (1, N = 30) = 0.133, ρ= 0.72). **D.** SNPs derived from a BSRseq analysis using bulks of susceptible and resistant progeny were plotted in 1 Mb windows over the 12 chromosomes of the potato DMv4.03 genome (64) They are almost exclusively located on chromosome 12.

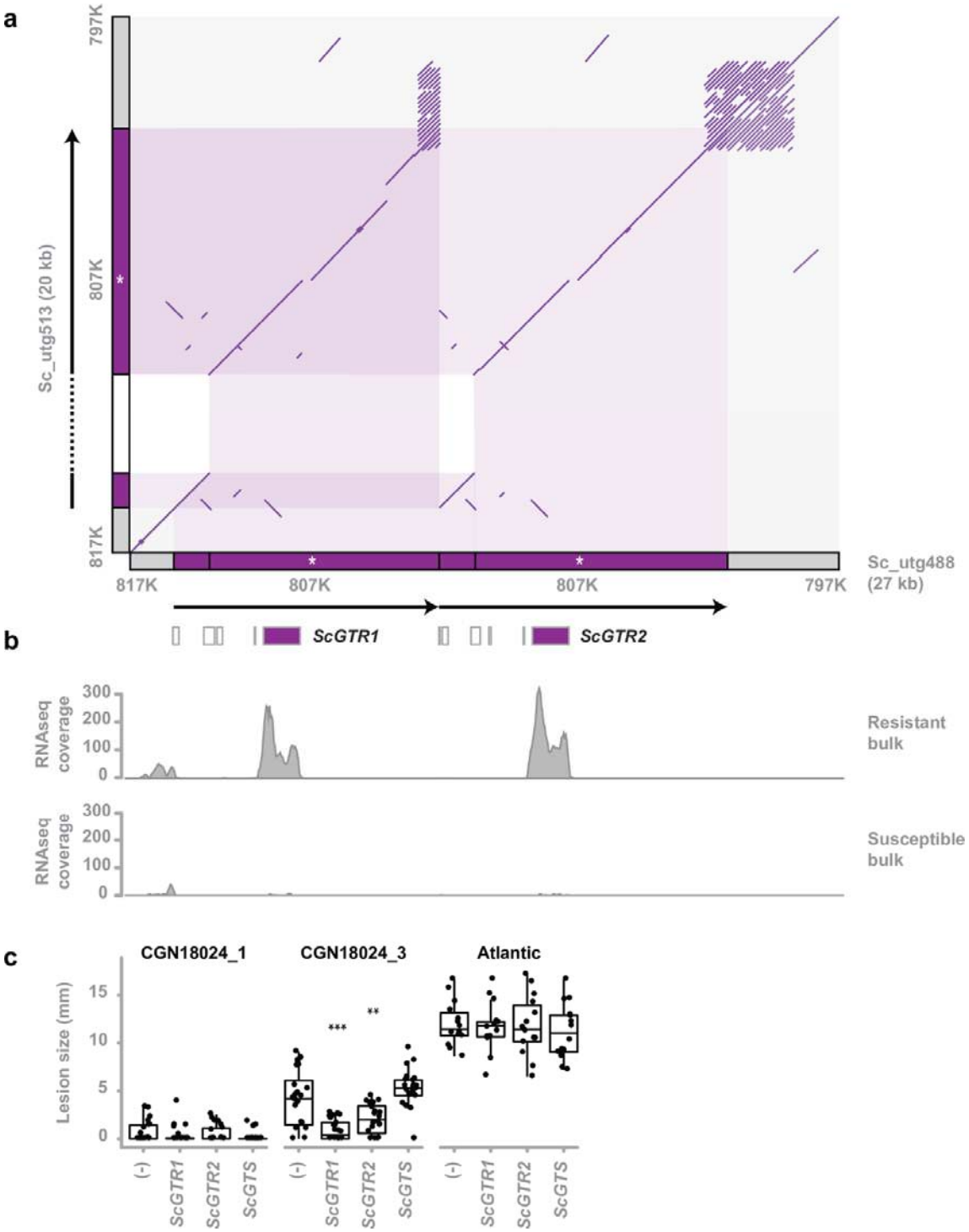


Fig 2. Identification of two glycosyltransferase resistance genes. A. Comparison of the susceptible and resistant haplotype of the *Solanum commersonii* CGN18024_1 resistance region (delimited by markers 817K and 797K) in a comparative dot plot shows a rearrangement. Locations of markers used to map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

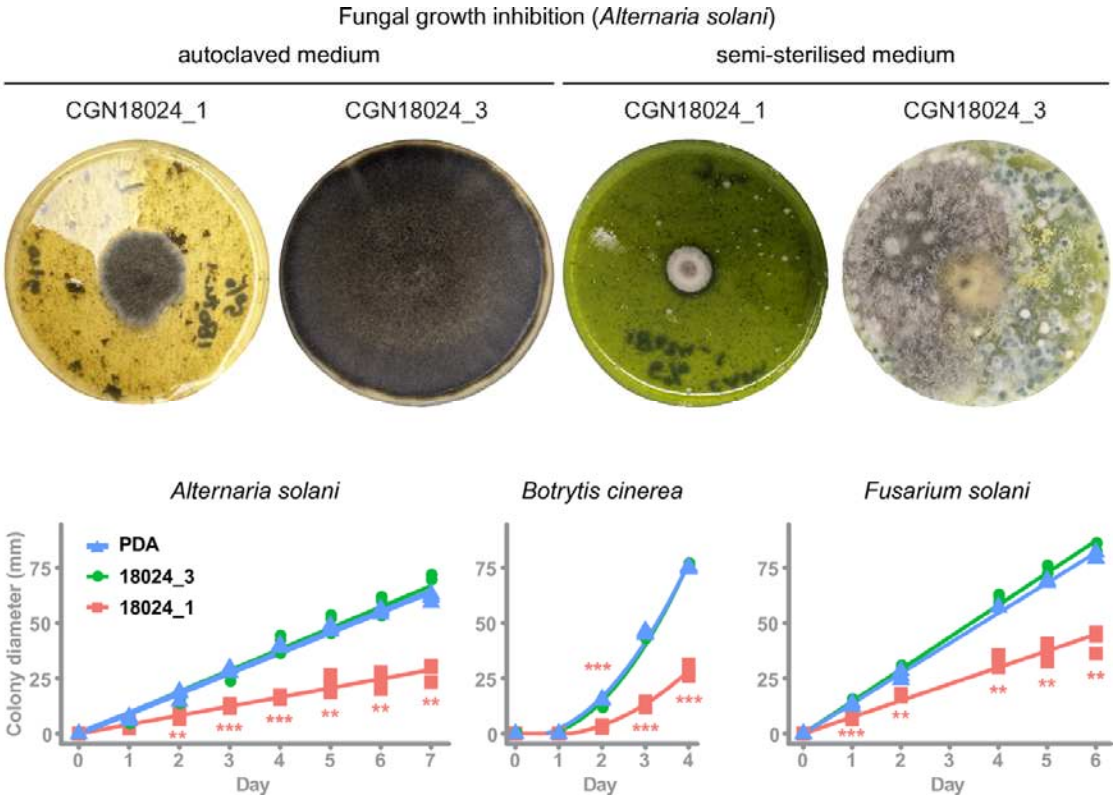
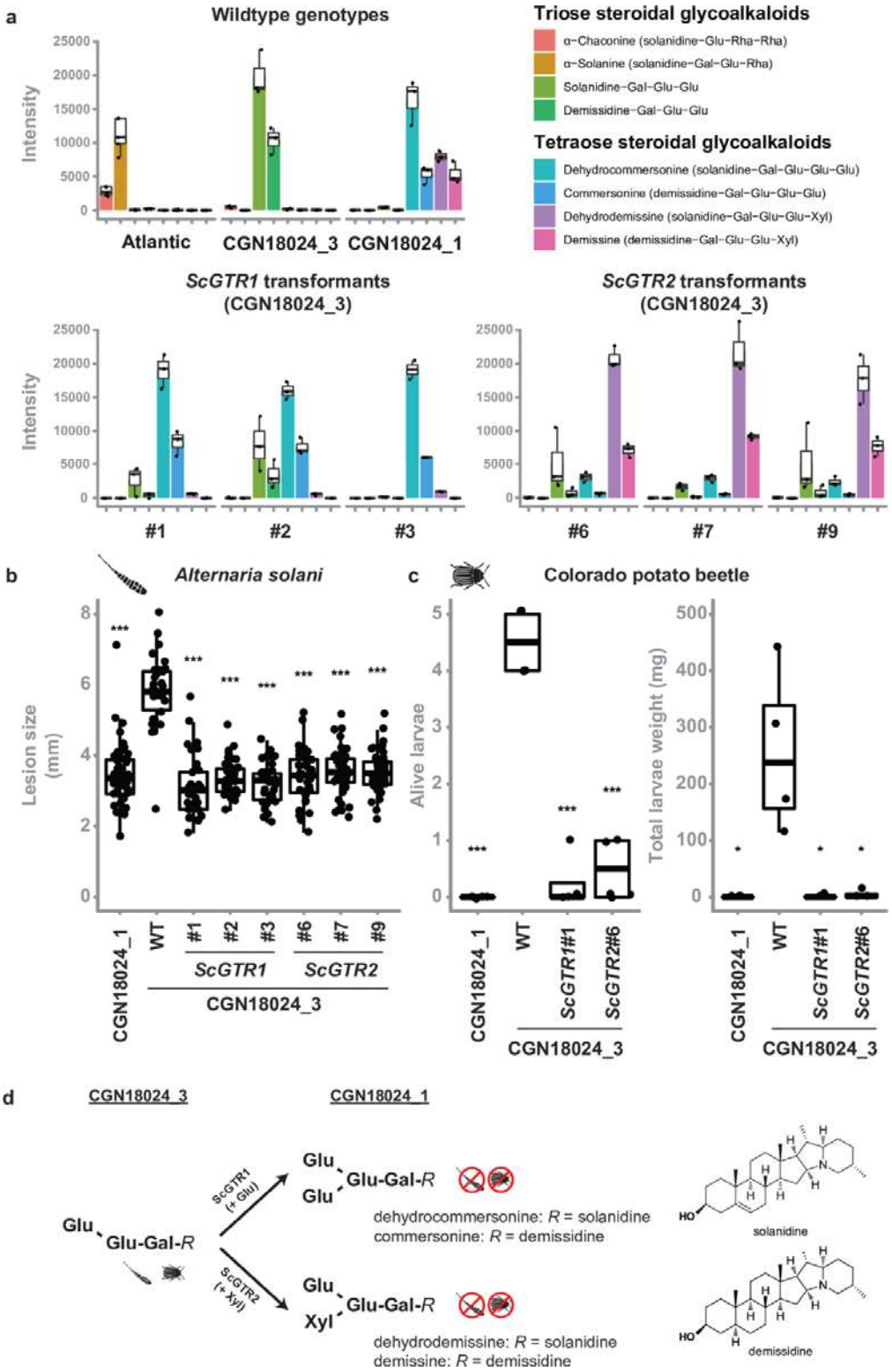


Fig 3. Leaf compounds from resistant *S. commersonii* inhibit growth of diverse fungi, including pathogens of potato. A. Crude leaf extract from CGN18024_1/CGN18024_3 was added to PDA plates (5% w/v) and autoclaved (left) or semi-sterilised for 15 min at 60 °C (right). Growth of *Alternaria solani* altNL03003 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-sterilized leaf from CGN18024_3, but not on plates containing material from CGN18024_1 (right two pictures). **B.** Growth of potato pathogenic fungi *A. solani*, *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, $^{**}P < 0.01$, $^{***}P < 0.001$).



406

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

pentose, resulting in a lycotetraose (Gal-Glu-Glu-Xyl). **B.** WT CGN18024_1/CGN18024_3 and CGN18024_3 transformants were inoculated with *Alternaria solani* altNL03003. 3 plants of each genotype were tested and 3 leaves per plants were inoculated with 6 10 µl droplets with spore suspension each. Lesions diameters were measured 5 days post inoculation. *ScGTR1* and *ScGTR2* can both complement resistance to *A. solani* in CGN18024_3, as the lesion sizes produced on CGN18024_3 transformants are comparable to resistant CGN18024_1. **C.** 3 plants per genotype were challenged with 5 Colorado potato beetle larvae each. The tetraose SGAs produced by *ScGTR1* and *ScGTR2* can provide resistance to Colorado potato beetle, as indicated by reduced larvae survival and total larvae weight. Significant differences with WT CGN18024_3 are indicated with asterisks (Welch's Two Sample t-test, $*P < 0.05$, $***P < 0.001$). **D**. Putative structures of SGAs detected in CGN18024_1 and CGN18024_3, based on previous studies (81, 83-85). CGN18024_3 produces triose SGAs and is susceptible to Colorado potato beetle and *A. solani*. ScGTR1 and ScGTR2 from CGN18024_1 convert these triose SGAs from susceptible *S. commersonii* to tetraose SGAs, through the addition of a glucose or xylose moiety respectively. Both sugar additions can provide resistance to Colorado potato beetle and *A. solani*.

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Supporting information

S1 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'.

S2 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.

S3 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.

S4 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.

S5 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.

S6 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.

S7 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.

S8 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.

S9 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 characterized 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').

S10 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.

S11 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 characterized 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) (67-74)

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 (81, 83-85); 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.