

Sclerotinia sclerotiorum Circumvents Flavonoid Defenses by Catabolizing Flavonol Glycosides and Aglycones^{1[OPEN]}

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Flavonols are widely distributed plant metabolites that inhibit microbial growth. Yet many pathogens cause disease in flavonol-containing plant tissues. We investigated how *Sclerotinia sclerotiorum*, a necrotrophic fungal pathogen that causes disease in a range of economically important crop species, is able to successfully infect flavonol-rich tissues of *Arabidopsis thaliana*. Infection of rosette stage *Arabidopsis* with a virulent *S. sclerotiorum* strain led to the selective hydrolysis of flavonol glycosidic linkages and the inducible degradation of flavonol aglycones to phloroglucinol carboxylic and phenolic acids. By chemical analysis of fungal biotransformation products and a search of the *S. sclerotiorum* genome sequence, we identified a quercetin dioxygenase gene (*QDO*) and characterized the encoded protein, which catalyzed cleavage of the flavonol carbon skeleton. *QDO* deletion lines degraded flavonols with much lower efficiency and were less pathogenic on *Arabidopsis* leaves than wild-type *S. sclerotiorum*, indicating the importance of flavonol degradation in fungal virulence. In the absence of *QDO*, flavonols exhibited toxicity toward *S. sclerotiorum*, demonstrating the potential roles of these phenolic compounds in protecting plants against pathogens.

Flavonoids are multifunctional phenolic natural products that are ubiquitous in plants (Williams and Grayer, 2004; Buer et al., 2010). With a C₆-C₃-C₆ core structure, they are synthesized through the phenylpropanoid pathway and are divided into subclasses depending on their structure, including flavonols, flavanones, isoflavones, flavones, flavan-3-ols, and anthocyanins (Winkel-Shirley, 2001; Pereira et al., 2009). Flavonoids function in attraction of pollinators, protection against UV radiation, and regulation of auxin transport (Li et al., 1993; Mol et al., 1998; Peer and Murphy, 2007). Flavonoids are also much discussed as nutraceuticals because of their antioxidant and anticancer activity (Rice-Evans et al., 1995; Cook and Samman, 1996). Another frequently cited role for flavonoids in plants is as protection against pathogenic microbes. There

are many reports of flavonoid antimicrobial activity, such as the strong inhibition of *Fusarium culmorum* growth in barley (*Hordeum vulgare*) by dihydroquercetin (Skadhauge et al., 1997). Moreover, pathogen infection often triggers local accumulation of flavonoids, such as the elevated levels of isoflavones, daidzein, and genistein, in soybean (*Glycine max*) after *Sclerotinia sclerotiorum* infection (Wegulo et al., 2005). However, it is unclear why many pathogens appear to grow well in flavonoid-rich plant tissues.

One of the most aggressive and widespread necrotrophic plant pathogens is *S. sclerotiorum*, which has a host range comprising more than 400 species, including many agriculturally important crops on which it causes substantial economic losses (Boland and Hall, 1994). Owing to the virulence of *S. sclerotiorum* and its long-term survival ability under harsh environmental conditions (Riou et al., 1991; Bolton et al., 2006), there are currently no sustainable strategies for controlling this fungus. *S. sclerotiorum* is known to overcome host plant defenses by producing pectinolytic and cellulolytic enzymes to break down the host cell wall and oxalic acid to suppress host defense responses, such as the oxidative burst and the hypersensitive response (Morrall et al., 1972; Riou et al., 1991; Cessna et al., 2000). In addition, *S. sclerotiorum* transforms plant defense compounds. For example, the indole-sulfur phytoalexins of the Brassicaceae, such as camalexin, brassinin, cyclobraassinin, and brassillexin, are detoxified by the fungus by glycosylation (Pedras and Ahiahonu, 2005; Pedras and Hossain, 2006).

¹This work was supported by the China Scholarship Council (CSC; 201406170041) and the Max Planck Society (MPG).

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J.C., J.G., and A.H. designed the research. J.C. performed the experiments and analyzed the data, assisted by C.U., M.R., and A.H. C.U. did the phylogenetic analysis. J.C., J.G., and A.H. wrote the article. All authors read and approved the manuscript.

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www.plantphysiol.org/cgi/doi/10.1104/pp.19.00461

However, the molecular mechanisms underlying these detoxification reactions are poorly studied, and the effort to understand their significance for plant-pathogen interactions is still in its infancy.

In addition to indole-sulfur compounds, the Brassicaceae produce ample quantities of flavonols, a common group of flavonoids with a 3-OH function, which may also serve in antimicrobial defense. Flavonols such as quercetin, kaempferol, and isorhamnetin are mainly present in leaves as glycosides (Onyilagha et al., 2003; Cartea et al., 2010). However, it is not known if these compounds play a role in defense against pathogen infection and whether pathogens can circumvent them. In this study, we investigated the role of flavonols in the interaction between *S. sclerotiorum* and the Brassicaceae species *Arabidopsis*, which is very susceptible to this fungal pathogen. Leaves infected by *S. sclerotiorum* had reduced levels of flavonol glycosides due to fungal catabolism. Analysis of *S. sclerotiorum* flavonol degradation products resulted in the identification of a key enzyme in the breakdown of the flavonol ring system, a quercetin 2,3-dioxxygenase. Characterization of this enzyme in *S. sclerotiorum* using molecular as well as biochemical methods confirmed its importance in flavonol degradation and in fungal virulence on *Arabidopsis*.

RESULTS

Flavonol Glycosides Are Metabolized by *S. sclerotiorum* In *Planta* and in Culture

To investigate the flavonoid defenses of the host plant against *S. sclerotiorum* infection, the levels of flavonol glycosides (K3(R2'G)7R, kaempferol 3-*O*-rhamnosyl 2'-glucoside-7-*O*-rhamnoside; Q3(R2'G)7R, quercetin 3-*O*-rhamnosyl 2'-glucoside-7-*O*-rhamnoside; K3G7R, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside; Q3G7R, quercetin 3-*O*-glucoside-7-*O*-rhamnoside; K3R7R, kaempferol 3-*O*-rhamnoside-7-*O*-rhamnoside; Q3R7R, quercetin 3-*O*-rhamnoside-7-*O*-rhamnoside) in *Arabidopsis* leaves were analyzed after inoculation with the fungus. Flavonol glycoside concentrations decreased significantly by about 50% in *S. sclerotiorum*-inoculated *Arabidopsis* leaves ($P < 0.05$, Student's *t* test) when compared with mock-inoculated plants 3 d postinoculation (Fig. 1A).

To investigate if the fungus can also reduce the levels of flavonol glycosides in vitro, a liquid culture of *S. sclerotiorum* was supplemented with a flavonol glycoside extract from *Arabidopsis*. Analysis of the medium collected at 0, 24, and 48 h after addition of the flavonol glycosides showed that the concentrations of these compounds were reduced significantly, approximately 70% in 2 d after inoculation (ANOVA, $P < 0.001$; Fig. 1B), suggesting that the fungus had rapidly degraded these metabolites. Liquid medium that contained the same amount of flavonol glycoside extract but no fungus was used as a negative control. The flavonol glycosides did not degrade at all, showing that

flavonol glycosides are not degraded spontaneously in vitro (Supplemental Fig. S1).

The degradation products of the major flavonol glycosides from *Arabidopsis* accumulating during incubation with *S. sclerotiorum* were identified using LC-ESI-MS operated in the alternating ionization mode. *Arabidopsis* (Col-0) produces mainly K3(R2'G)7R ([M-H]⁻ = 739), K3G7R ([M-H]⁻ = 593), and K3R7R ([M-H]⁻ = 577; Veit and Pauli, 1999; Kerhoas et al., 2006; Saito et al., 2013). After 48 h incubation in medium with *S. sclerotiorum*, two new peaks appeared in the chromatogram with an [M-H]⁻ of 593 and 431, respectively (peaks 4 and 5; Fig. 1C). According to mass spectral collision induced dissociation fragmentation data (MS²), peak 4 appeared to be K3(R2'G), derived from K3(R2'G)7R by hydrolysis of the 7-*O*-rhamnose residue (Fig. 1D). Although no standard of K3(R2'G) is commercially available, an extract containing this compound from *Clitoria ternatea*, where it is reported to be the major flavonol glycoside (Kazuma et al., 2003), gave a peak with the same retention time and mass spectrum as that produced by the *S. sclerotiorum* culture, confirming its identity (Supplemental Fig. S2A). Peak 5 was identified as K3R by comparison with a pure standard and is produced by the fungus from K3R7R (Fig. 1D; Supplemental Fig. S2B). These results suggested that *S. sclerotiorum* can readily cleave a 7-*O*-rhamnose or 7-*O*-glucose substituent. However, kaempferol 3-*O*-glucoside (K3G), which would be produced from K3G7R, was not detected in the medium. When we amended the *S. sclerotiorum* culture with K3G and K3R, only K3G was depleted in the medium after 15 h incubation, and no traces of kaempferol were detected (Supplemental Fig. S3A).

Flavonol Aglycones Are Also Metabolized by *S. sclerotiorum* in an Inducible Manner

The lack of flavonol aglycone accumulation suggested that these compounds were also degraded by *S. sclerotiorum*. To confirm this supposition, quercetin and kaempferol were added to the fungal culture and were shown to be degraded using LC-ESI-MS over a time course of 48 h. Approximately 25% of the initial quercetin was degraded in 24 h by the fungus, while the contents of kaempferol decreased by up to 90% during the same time interval, indicating that kaempferol is degraded more rapidly than quercetin (Fig. 2A).

We next conducted an in vitro enzyme assay using total protein extracts from *S. sclerotiorum* that had been cultured either with or without flavonol supplementation. Total protein was extracted from mycelium that was pretreated with 0.01 mg mL⁻¹ quercetin or kaempferol for 2 h. This protein extract was then used in an in vitro enzyme assay with the substrates quercetin and kaempferol. Pretreatment with quercetin or kaempferol demonstrated that the activity for degradation of these flavonols increased from 2- to over 10-fold compared to protein extracts from nonpretreated fungus (Student's *t* test; $P < 0.01$; Fig. 2B).

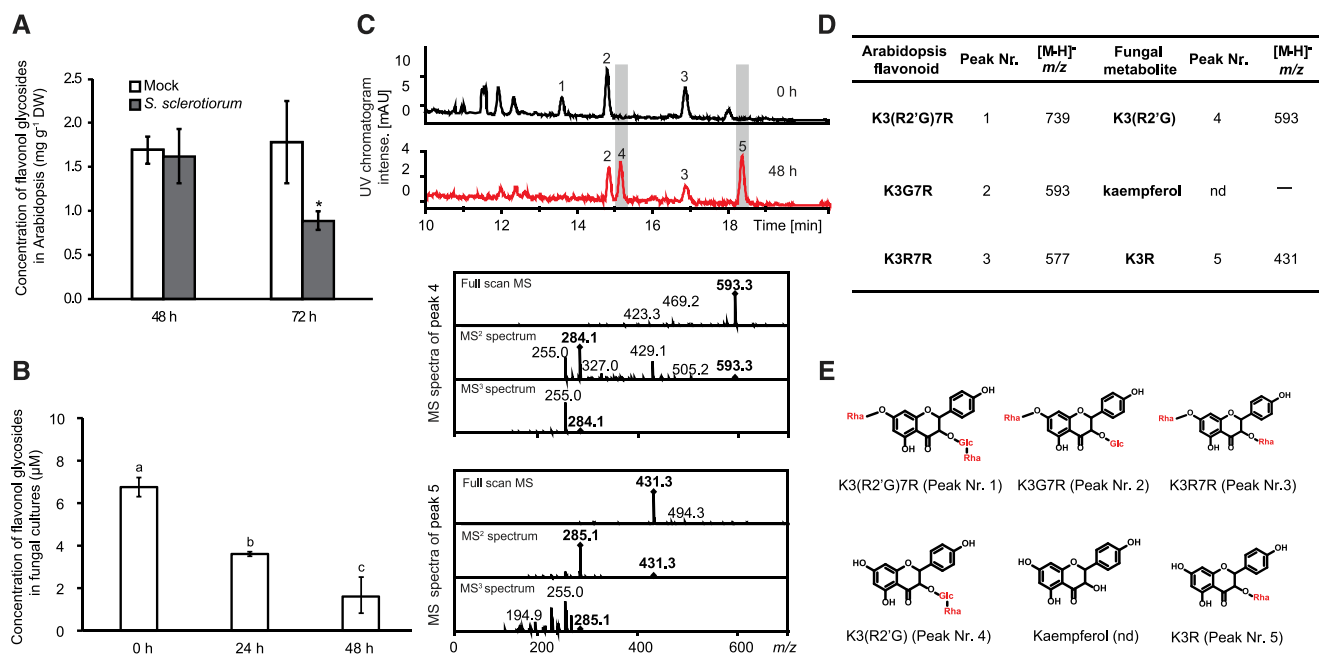


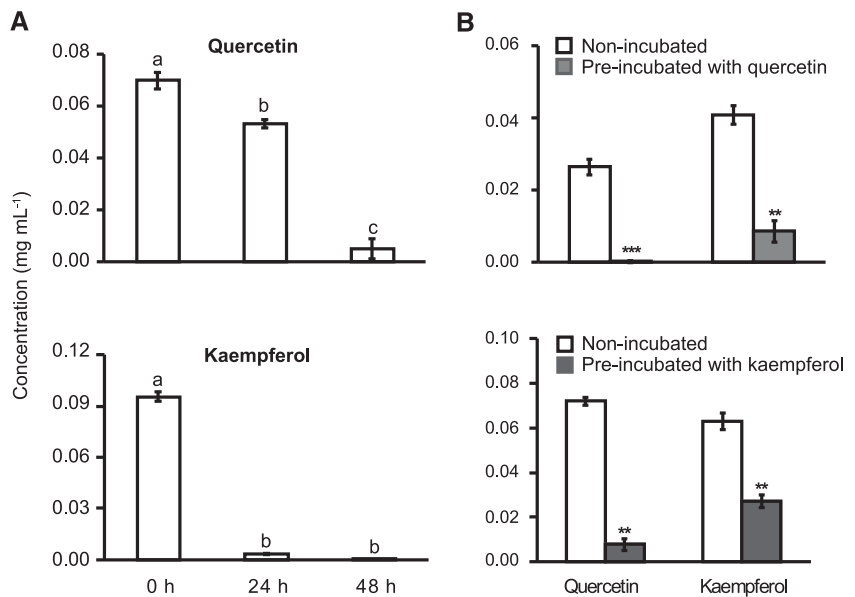
Figure 1. *S. sclerotiorum* degrades Arabidopsis flavonol glycosides. A, Total flavonol glycoside levels in Arabidopsis 3 d post-inoculation with *S. sclerotiorum* wild-type UF-70. Flavonol glycosides were analyzed using LC-UV (330 nm) and quantified using an external K3R7R calibration curve. Total flavonol glycosides represent the summed quantities of all the flavonol glycosides detected. Asterisk represents significant difference ($P < 0.05$, Student's *t* test). B, Degradation of the major flavonol glycosides in Arabidopsis extracts by *S. sclerotiorum* in artificial medium over a time course of 48 h. Samples were analyzed by LC-UV (330 nm). Different letters above the bars indicate significant differences in residual flavonol glycoside content over time ($P < 0.001$, one-way ANOVA followed by Tukey's post-hoc test). C, Representative HPLC chromatograms with UV detection (280 nm) from liquid cultures of *S. sclerotiorum* 0 and 48 h after addition of Arabidopsis flavonol glycoside extract. Peaks 1, 2, and 3 are the major flavonol glycosides in Arabidopsis. Cocultivation with the fungus for 48 h resulted in the appearance of peaks 4 and 5 (structures shown in E, ESI-MS² and MS³ spectra in negative ionization mode shown below the chromatograms, are comparable to spectra for authentic standards shown in Supplemental Fig. S2). D, Negative molecular ions (*m/z*) of major Arabidopsis (*Arabidopsis thaliana*) flavonol glycosides and their corresponding *S. sclerotiorum* deglycosylated derivatives as identified by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). E, Structures of major flavonol glycosides in Arabidopsis and suggested structures of the products produced by *S. sclerotiorum* in culture. All data represent means \pm se ($n = 3$). K3(R2'G)7R, kaempferol 3-*O*-rhamnosyl 2'-glucoside-7-*O*-rhamnoside; K3G7R, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside; K3R7R, kaempferol 3-*O*-rhamnoside-7-*O*-rhamnoside; K3(R2'G), kaempferol 3-*O*-rhamnosyl 2'-glucoside; K3R, kaempferol 3-*O*-rhamnoside. DW, dry weight.

Identification and Heterologous Expression of a *S. sclerotiorum* Flavonol Degradation Enzyme, Quercetin 2,3-Dioxygenase

Enzyme-mediated quercetin degradation was previously shown to be the key step in the catabolism of rutin (quercetin 3-*O*-rhamnosyl-6'-glucoside) in some fungi and bacteria (Schaab et al., 2006; Tranchimand et al., 2010). In this pathway, the disaccharide moiety, rutinose, is hydrolyzed from rutin, and the resulting quercetin moiety is then cleaved by quercetin 2,3-dioxygenase (QDO) at the 2,3-position to form 2-protocatechuoyl phloroglucinol carboxylic acid. This degradation product of quercetin can be further metabolized to protocatechuic acid and phloroglucinol carboxylic acid by an esterase (Fig. 3A). We hypothesized that *S. sclerotiorum* degrades quercetin through the same pathway as we detected protocatechuic acid in the culture medium of *S. sclerotiorum* supplemented with quercetin (Supplemental Fig. S3B).

We used the annotated QDO from *Penicillium olsonii* to search the National Center for Biotechnology Information (NCBI)/GenBank databases and found a very similar gene in *S. sclerotiorum* designated *SsQDO* (accession number, MK992913). *SsQDO* has a 1113-bp open reading frame (ORF) that encodes a predicted protein of 371 amino acids. The ORF was interrupted by a 63-bp intron. We constructed a maximum-likelihood phylogenetic tree with protein sequences of similar annotated QDO genes and nonannotated genes from different fungal species (Fig. 3B). QDO enzymes are present in the Sordariomycetes, Dothideomycetes, Leotiomycetes, and Eurotiomycetes. The putative QDO protein from *S. sclerotiorum* grouped closest to orthologs from *Sclerotinia borealis* and *Botrytis cinerea* with 86% and 84% similarity, respectively. Both *S. sclerotiorum* and *B. cinerea* belong to the class Leotiomycetes. QDO amino acid sequences from the Eurotiomycetes and Sordariomycetes were on average 53% and 46% similar, respectively, to *SsQDO*.

Figure 2. *S. sclerotiorum* degrades flavonol aglycones in an inducible manner. A, Quercetin and kaempferol levels after treatment with *S. sclerotiorum* for 48 h in artificial medium. Different letters above the bars indicate significant differences ($P < 0.001$, one-way ANOVA followed by Tukey's post-hoc test). B, Degradation of both quercetin and kaempferol by total protein from *S. sclerotiorum* cultures preincubated with either quercetin or kaempferol or non-preincubated. Asterisks represent significant differences between protein extracts of preincubated and non-preincubated fungi (Student's *t* test, ** $P < 0.01$; *** $P < 0.001$). All data represent means \pm SE ($n = 3$) and were acquired by LC-ESI-MS analysis.



The coding region of the *SsQDO* gene was cloned and expressed in *E. coli* BL 21 (DE 3) using the Gateway expression vector pDest24. The crude protein extract of recombinant *E. coli* cleaved both quercetin and kaempferol (Fig. 3C). The quercetin oxidation product showed a molecular ion peak of mass-to-charge ratio (m/z) 305.0286 ($[M-H]^-$), which was consistent with the molecular formula $C_{14}H_{10}O_8$ ($C_{14}H_{10}O_8 - H$)⁻, the calculated monoisotopic mass m/z 305.029194 (Supplemental Fig. S4). This corresponds to the molecular formula of 2-protocatechuoyl phloroglucinol carboxylic acid. Kaempferol oxidation resulted in a molecular ion peak of m/z 289.0336 ($[M-H]^-$), which was consistent with the molecular formula $C_{14}H_{10}O_7$ ($C_{14}H_{10}O_7 - H$)⁻ calculated monoisotopic mass m/z 289.034279 (Supplemental Fig. S4). This corresponds to the molecular formula of 2,4-dihydroxy-6-[(4-hydroxybenzoyl)oxy]benzoic acid. Both enzyme assay products showed strong in-source fragmentation in negative mode ESI-MS to m/z 169. MS² spectra of this in-source fragment ion matched the MS² spectrum of an authentic standard of phloroglucinol carboxylic acid (Tronto Research Chemicals; Supplemental Fig. S4). However, the extracted ion of $m/z = 169$ from the *SsQDO* assay showed a different retention time compared to the authentic phloroglucinol carboxylic acid standard, suggesting that phloroglucinol carboxylic acid was not the direct product in the assay, but that m/z 169 is an in-source fragment of a larger molecule (Supplemental Fig. S4, A and C). We also tested the activity of *SsQDO* with other flavonol substrates, including fisetin, galangin, and an isoflavonol daidzein, but *SsQDO* did not show activity toward any of them (Supplemental Fig. S5). To compare the affinity of *SsQDO* to quercetin and kaempferol, we analyzed enzyme kinetics with heterologously expressed *SsQDO*, revealing that the enzyme has a higher affinity for kaempferol ($K_m = 0.12$ mM) than for quercetin ($K_m = 0.55$ mM; Supplemental Fig. S6).

This result is consistent with our observation that *S. sclerotiorum* degrades kaempferol more rapidly than quercetin in vitro (Supplemental Fig. S2A).

We also analyzed the expression of *SsQDO* in cultures grown with or without quercetin or kaempferol. Addition of flavonols for 2 h led to significant increases in *SsQDO* transcript levels compared to those in the nontreated mycelium, further supporting its role in flavonol degradation (ANOVA; $P < 0.001$; Supplemental Fig. S7).

QDO Knockout Mutants of *S. sclerotiorum* ($\Delta SsQDO$) Are Deficient in Flavonol Degradation Activity

To study the function of *SsQDO* in vivo and its role in the pathogenicity of the fungus, we generated *SsQDO* knockout mutants of *S. sclerotiorum* by polyethylene glycol (PEG)-mediated transformation and homologous recombination. A replacement vector containing a hygromycin cassette with upstream and downstream sequences flanking *SsQDO* was used to transform *S. sclerotiorum* protoplasts (Fig. 4A). PCR verification of *S. sclerotiorum* transformants showed that *SsQDO* was replaced by the hygromycin gene in three transformed lines (Fig. 4A). The other transformants were heterozygotes that contained both the *SsQDO* and the hygromycin gene, owing to the fact that *S. sclerotiorum* cells are multinuclear. The *SsQDO* knockout mutants were further verified by enzyme assay. After cocultivation with quercetin for 2 h, protein extracts from knockout mutants ($\Delta SsQDO$) showed no activity for degradation of flavonols, as no product was detected in the assay (Supplemental Fig. S8), while the enzyme extracts from the wild-type fungus degraded most of the substrates (ANOVA; $P < 0.001$; Fig. 4B).

We then measured growth rates of the wild-type and $\Delta SsQDO$ *S. sclerotiorum* on PDA plates, and there was

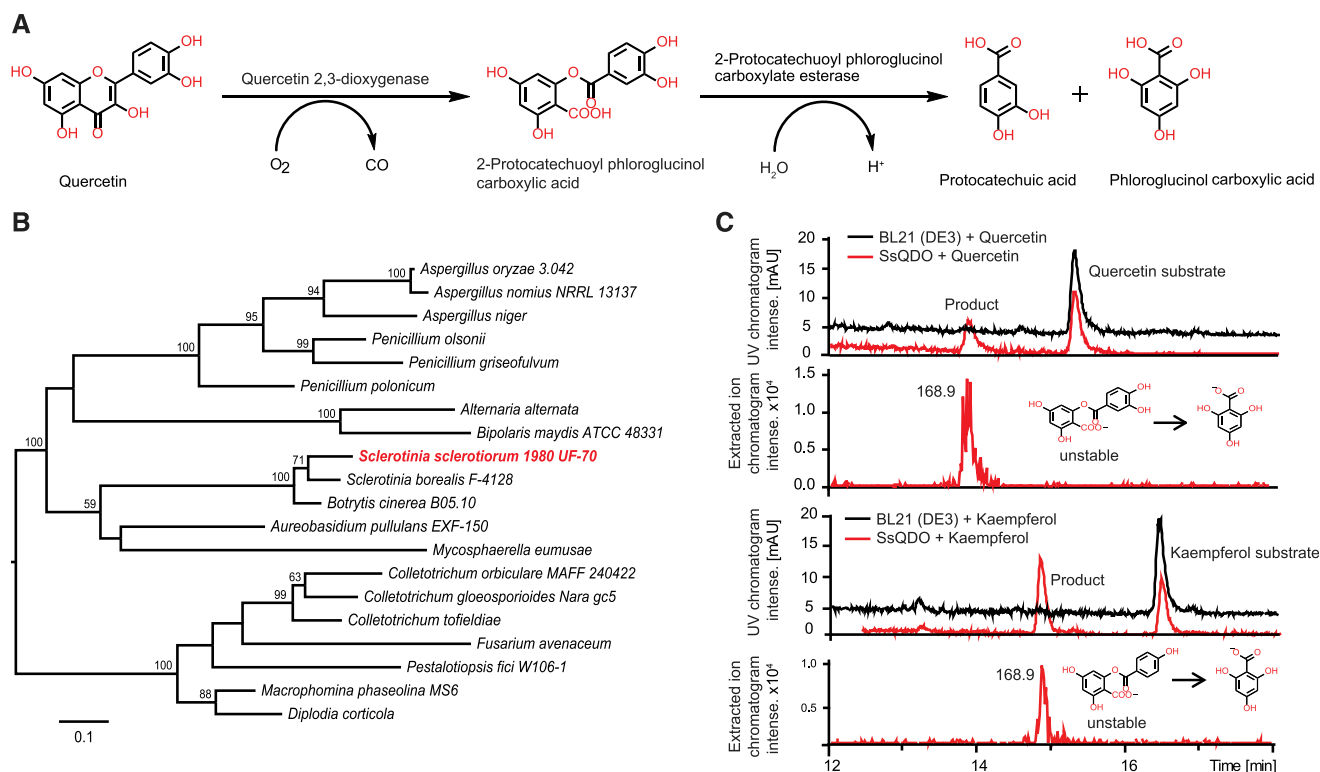


Figure 3. Identification and heterologous expression of *S. sclerotiorum* quercetin 2,3-dioxygenase (SsQDO). **A**, The catabolism of quercetin by QDO. **B**, Phylogenetic analysis of QDO from *S. sclerotiorum* and other fungi. The maximum-likelihood tree was constructed using PhyML-3.1 employing the amino acid substitution model LG (Le and Gascuel, 2008). Nonparametric bootstrap analysis was performed with 1000 replicates, and values next to each branch point indicate the branch support percentages (values >50 are shown). The scale bar indicates amino acid substitution per site. **C**, *S. sclerotiorum* SsQDO heterologously expressed in *Escherichia coli* cleaves both quercetin and kaempferol at the 2,3 position. Shown are the LC-UV chromatograms at 280 nm and the extracted ion traces of m/z 169 in negative ionization mode generated by a LC-ESI-IonTrap-MS (= in-source fragment of oxidation products with m/z 305 and m/z 289, respectively). Products were further identified by UHPLC-ESI-TOF-MS (see Supplemental Fig. S4).

no significant difference in the growth rate between the wild-type and the SsQDO deletion mutants (ANOVA; $P = 0.067$; Fig. 4C). However, when flavonols were added to the medium, Δ SsQDO strains grew slower compared to the wild-type fungus (ANOVA, $P < 0.01$; Fig. 4D). These results indicate that SsQDO is important for flavonol degradation, and its loss of function is deleterious in a flavonol-amended medium.

QDO Knockout Lines of *S. sclerotiorum* Caused Fewer and Smaller Necrotic Lesions in Arabidopsis Leaves

To test whether *S. sclerotiorum* SsQDO mutants with impaired abilities to degrade flavonols have an altered virulence, both wild-type and Δ SsQDO were used to inoculate both detached Arabidopsis leaves and intact plants. Lesion areas on detached leaves calculated at 24 h postinoculation showed that the Δ SsQDO lines formed significantly smaller lesions, less than 50% as large as wild-type lines (ANOVA; $P < 0.01$; Fig. 5A). After inoculation of intact plants, the ratio of the fungal Histone H3-encoding gene and Arabidopsis *ACTIN* gene

transcript levels used to quantify fungal colonization in Arabidopsis at 24 h revealed that Δ SsQDO lines were less than half as pathogenic as the wild-type strain (ANOVA; $P < 0.01$; Fig. 5B).

Degradation of Flavonol Glycosides In Planta and in Culture Is Less Efficient in QDO Knockout Lines Than in Wild-Type *S. sclerotiorum*

To determine how SsQDO deletion affected flavonol glycoside levels during infection, the flavonol glycoside concentration in Arabidopsis leaves was measured 72 h postinoculation both with wild-type and Δ SsQDO strains. Consistent with previous results, a significant decrease in flavonol glycoside content was observed in Arabidopsis leaves inoculated with the wild-type fungus compared to mock-treated leaves (ANOVA; $P < 0.05$; Fig. 6A). On the other hand, leaves inoculated with the SsQDO deletion mutants accumulated significantly higher levels of flavonol glycosides than leaves inoculated with wild-type *S. sclerotiorum* (ANOVA; $P < 0.05$; Fig. 6A). However, leaves inoculated with Δ SsQDO did

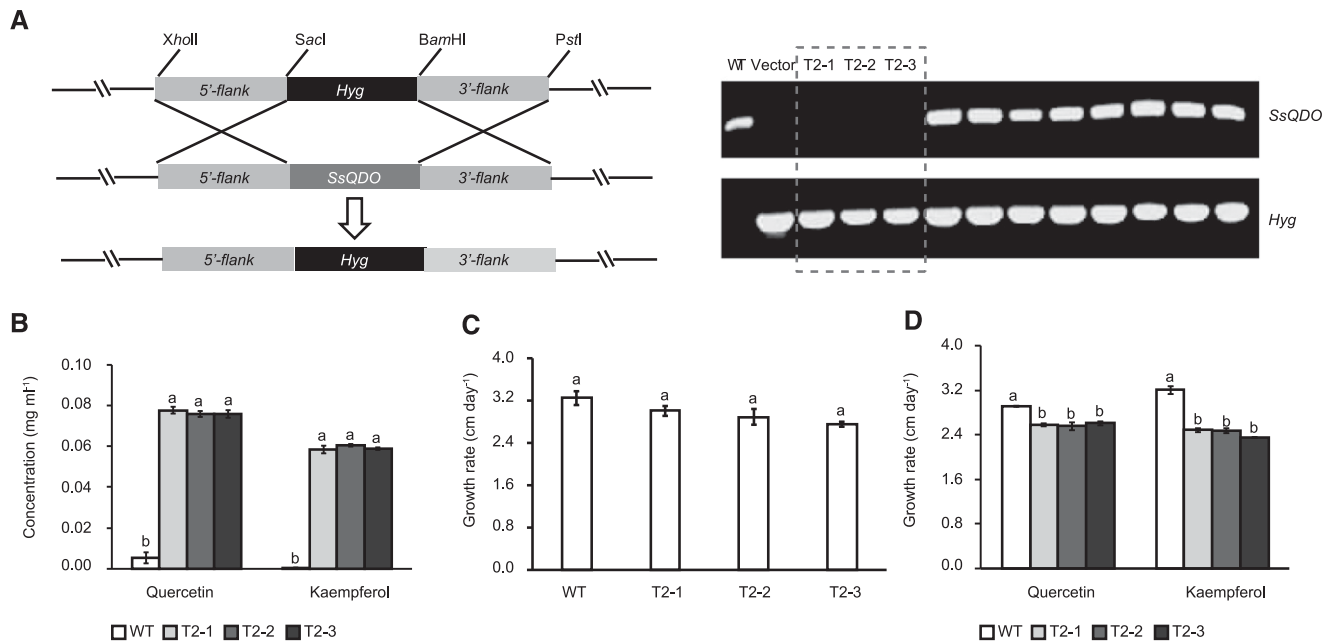


Figure 4. Deletion of *SsQDO* and phenotypic characterization of the *SsQDO* replacement mutants ($\Delta SsQDO$). A, Replacement of *SsQDO* with a hygromycin gene (*Hyg*) cassette by homologous recombination and PCR verification of transformants. The agarose gels depict (in order): wild type (WT), vector only, three transformed homozygotic lines, and eight heterozygotic lines. B, Flavonol degradation activity in $\Delta SsQDO$ and wild-type lines. Shown is 0.01 mg mL⁻¹ quercetin and kaempferol after 2 h incubation with protein from preinduced wild-type and $\Delta SsQDO$ mycelium. Different letters above the bars indicate significant differences ($P < 0.001$, one-way ANOVA followed by Tukey's post-hoc test). C, Growth rate of wild-type fungus and $\Delta SsQDO$ mutants on PDA medium. There were no significant differences in growth ($P = 0.067$; one-way ANOVA). D, Growth rate of wild-type fungus and $\Delta SsQDO$ mutants on PDA medium supplemented with 0.01 mg mL⁻¹ quercetin or kaempferol. Different letters above the bars indicate significant differences ($P < 0.01$, one-way ANOVA followed by Tukey's post-hoc test). All data represent means \pm SE ($n = 3$). T2-1, T2-2, and T2-3, $\Delta SsQDO$ mutants.

show a decrease in flavonol glycoside levels at 72 h postinoculation compared to the mock treatment (even if not significantly different), suggesting that deletion of the *SsQDO* gene did not disable all possible mechanisms of flavonol glycoside degradation in this fungus.

We next added flavonol glycoside extract from *Arabidopsis* to *S. sclerotiorum* cultures growing on artificial medium to validate the results of our in planta experiments. Media from both wild-type and $\Delta SsQDO$ cultures were analyzed at 0 and 48 h after flavonol glycoside extract addition. Higher levels of the major flavonol glycoside were recovered from medium colonized by the $\Delta SsQDO$ fungus compared to medium colonized by wild-type at 48 h (ANOVA; $P < 0.001$; Fig. 6B). We also quantified the biotransformed flavonols produced by the fungus from the compounds present in the extract. The kaempferol aglycone only appeared in the *SsQDO* mutant cultures after 48 h (Fig. 6C), confirming that further metabolism of this compound is blocked by the loss of QDO. Therefore, kaempferol aglycones are normally released by *S. sclerotiorum* from K3G7R, even though these were not detected in the wild-type culture. The reduction of K3R (peak 5; Fig. 1C) in the $\Delta SsQDO$ mutants compared to wild-type fungus (Fig. 6D), suggests that deletion of *SsQDO* not only disrupts cleavage of aglycones but also decreases

the actual upstream reactions in this catabolic pathway. Taken together, these data show that deletion of *SsQDO* results in reduced both flavonol glycoside and flavonol aglycone metabolism by the fungus.

DISCUSSION

Flavonols, a subclass of flavonoids, are thought to play important roles in plant defense against pathogens because of their antimicrobial activity and local accumulation after fungal infection (Snyder and Nicholson, 1990; Snyder et al., 1991; Padmavati et al., 1997; Skadhauge et al., 1997; McNally et al., 2003; Hammerbacher et al., 2014; Ullah et al., 2017). Thus, we investigated the role of flavonols during fungal infection using an economically important plant pathogen, *S. sclerotiorum*, and the model plant, *Arabidopsis*, which produces high levels of flavonol glycosides, including kaempferol and quercetin glycosides (Graham, 1998; Veit and Pauli, 1999).

We unexpectedly found a significant decrease in flavonol glycoside levels in *Arabidopsis* 3 d after inoculation with *S. sclerotiorum*. Incubation of *S. sclerotiorum* with the major flavonol glycosides produced by *Arabidopsis* revealed that the fungus cleaves both 7-*O*-glucose and 7-*O*-rhamnose moieties to produce and accumulate K3(R'2G) and K3R. We further amended

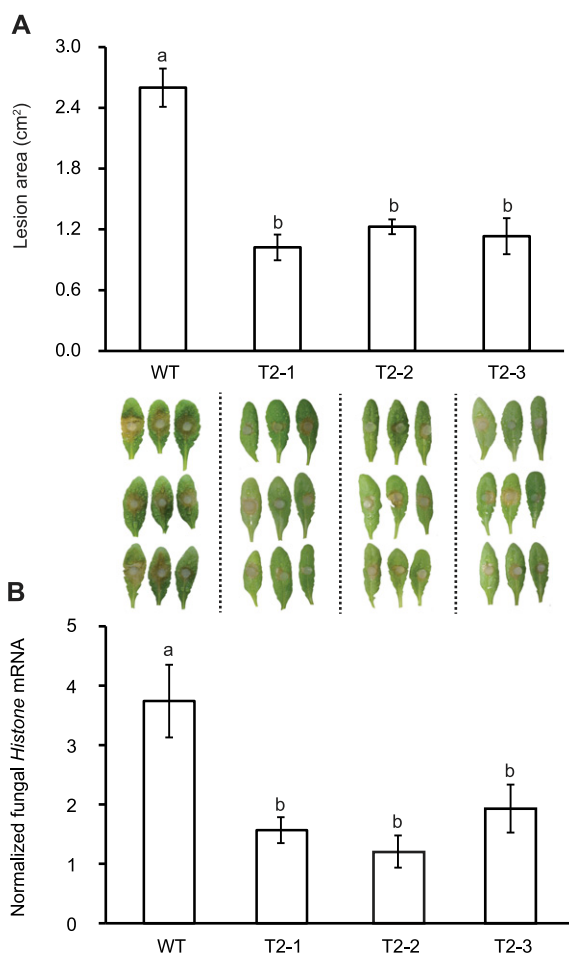


Figure 5. *SsQDO* deletion mutants showed less pathogenicity than wild-type (WT) *S. sclerotiorum*. A, Lesion area in Δ *SsQDO* and wild-type *S. sclerotiorum* lines 24 h after in vitro inoculation of detached *Arabidopsis* leaves. Leaf images have been digitally abstracted and made into a composite for comparison. B, Relative quantification of fungal Histone mRNA normalized to plant housekeeping gene *ACTIN* 24 h after in vivo inoculation of *Arabidopsis* leaves as determined by RT-qPCR. Different letters above the bars indicate significant differences ($P < 0.01$, one-way ANOVA followed by Tukey's post-hoc test). Data for the in vitro inoculation experiment represent means of nine biological replicates. Data represent means \pm SE ($n = 9$ for in vitro inoculation experiment, $n = 4$ for in vivo inoculation). T2-1, T2-2, and T2-3, Δ *SsQDO* mutants.

S. sclerotiorum cultures with K3R and K3G. We found that K3G was completely consumed by *S. sclerotiorum*, whereas K3R was not transformed by this fungus. In contrast, a previous study reported that *Aspergillus flavus* cleaves 3-*O*-rhamnose linkages of rutin (Krishnamurthy and Simpson, 1970). Due to the fact that both K3R and K3(R'2G) cannot be further transformed by *S. sclerotiorum*, we hypothesize that the specificity of this fungal glycosyltransferase depends on both sugar linkage and position of flavonol glycosides. Moreover, K3G was completely consumed by *S. sclerotiorum* after 15 h in the liquid medium without leaving any traces of kaempferol, suggesting that *S. sclerotiorum* selectively cleaved

glucoside moieties of flavonol glycosides and degraded the aglycones subsequently.

Plants also contain numerous glycoside hydrolases, which cleave flavonol glycosides during plant recovery from synergistic abiotic stresses, such as nitrogen deficiency and low temperature (Xu et al., 2004; Roepke and Bozzo, 2015; Roepke et al., 2017). The β -glucosidase BGLU15 characterized from *Arabidopsis* cleaves the 3-*O*- β -glucosides from K3G7R to produce kaempferol 7-*O*-rhamnoside (Roepke et al., 2017). Kaempferol 7-*O*-rhamnoside was, however, not found among the fungal degradation products of flavonol glycoside extracts in our study, suggesting a different hydrolysis pathway of glycosidic linkages in fungi. Whether plants regulate their flavonol glycoside metabolism in response to pathogen infections is also not known.

The degradation of glycosidic moieties from plant flavonol glycosides might be a carbohydrate assimilation process for *S. sclerotiorum* and beneficial for its expansion in host plants (Jobic et al., 2007), but the released flavonol aglycones are known to be more active antimicrobial compounds than the corresponding glycosylated derivatives (Liu et al., 2010). It was hypothesized that the basic structure of flavonol aglycones allows entry to microbial cells, and so individual functional groups may then target different components or functions of the cell (Cushnie and Lamb, 2005). For instance, quercetin can bind to the GyrB subunit of DNA gyrase and inhibits the enzyme's ATPase activity in *E. coli* (Plaper et al., 2003). Many studies have shown the direct toxicity of flavonols toward various microbes. The growth of the rice (*Oryza sativa*) pathogen *Xanthomonas oryzae* causing leaf blight was inhibited by naringenin (Padmavati et al., 1997). Dihydroquercetin strongly reduced *Fusarium* growth and macrospore formation (Skadhauge et al., 1997). Moreover, naringenin and tangeretin, two constitutive flavonoids in *Citrus aurantium*, showed antifungal activity against *Penicillium digitatum* (Arcas et al., 2000). Many studies have also been conducted on how flavonoids are involved in plant-microbial interactions in the rhizosphere (Shaw et al., 2006). For example, naringenin, quercetin, and kaempferol accumulated significantly in root galls of *Arabidopsis* after *Plasmodiophora brassicae* infection and modulated auxin efflux from root galls (Päsold et al., 2010). In legumes, the role of flavonols is known as a key signal in regulating nodulation-related genes and rhizobial nodulation (Cooper, 2004). Interestingly, flavonols stimulate arbuscular mycorrhizal fungal growth, despite the fact that these compounds have antimicrobial activity toward a broad spectrum of pathogens in vitro (Gianinazziperson et al., 1989; Padmavati et al., 1997).

Accordingly, we confirmed that the pathogenic fungus *S. sclerotiorum* is able to degrade flavonols, including kaempferol and quercetin. The degradation of flavonol aglycones in *S. sclerotiorum* is an inducible process, and the activity of enzymes involved in this process was significantly higher after exposure to quercetin or kaempferol. This suggests that the ability to degrade flavonols is not essential for development but for coping

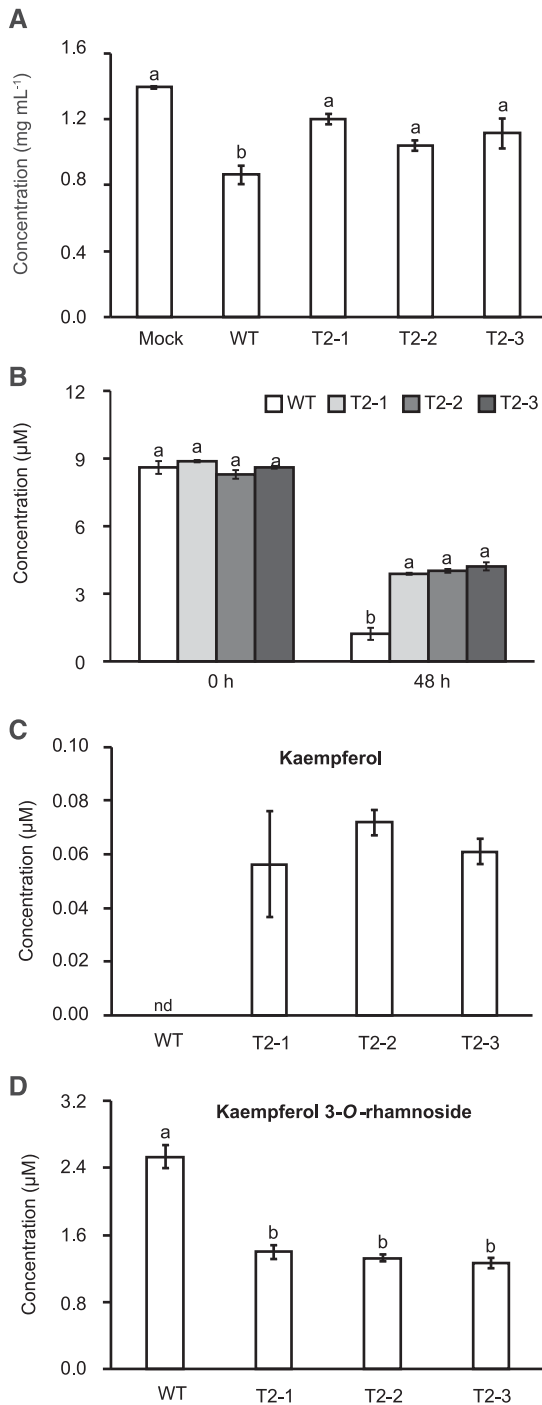


Figure 6. Deletion of *SsQDO* results in lower flavonoid catabolism in *S. sclerotiorum*. A, Flavonol glycosides in Arabidopsis (Col-0) inoculated with the wild-type (WT) fungus, in mock-inoculated control plants or in plants inoculated with Δ SsQDO mutants at 72 h. Different letters above the bars indicate significant differences ($P < 0.001$, one-way ANOVA followed by Tukey's post-hoc test). B to D, Major flavonoid levels in cultures of wild-type and Δ SsQDO strains to which a concentrated flavonol extract from Arabidopsis was added. B, Catabolism of major flavonoid glycosides in cultures of Δ SsQDO and wild type. Different letters above the bars indicate significant differences ($P < 0.001$, one-way ANOVA followed by Tukey's post-hoc test). C, Kaempferol

with specialized metabolites produced by the host plant. QDO was shown in previous studies to be responsible for flavonol degradation in numerous pathogenic fungi, such as *A. flavus*, *Aspergillus niger*, and *Penicillium olsonii* (Westlake and Simpson, 1961; Hund et al., 1999; Tranchimand et al., 2008). In our study, we found a homologous *QDO* gene in *S. sclerotiorum* as well as in its close relatives, such as *Botrytis cinerea*, suggesting that flavonol degradation might be a conserved function in fungal pathogens. The heterologously expressed SsQDO only showed activity toward quercetin and kaempferol but not toward other flavonoids. We analyzed the reaction products by LC-MS, whereas previous studies demonstrated enzyme activity only through detection of carbon monoxide released during catalysis using PdCl₂ (Tranchimand et al., 2008). Flavonoids can also be oxidized by plant enzymes, such as polyphenol oxidases (PPOs). These enzymes are involved in the formation of brown pigments when internal plant tissues are exposed to oxygen (Pourcel et al., 2007). For example, aurone synthase is a catechol oxidase that belongs to the PPO enzyme family. This enzyme catalyzes the conversion of chalcones to aurones and is responsible for yellow coloration of *Antirrhinum majus* (Ono et al., 2006). However, a PPO enzyme specifically involved in quercetin oxidation in planta has not been reported yet, and therefore it is unlikely that the flavonol oxidation we observed after fungal inoculation is related to PPO activity (Jiménez and García-Carmona, 1999).

The role of flavonol degradation in fungal virulence was unknown and raised our interest. We generated three independent *SsQDO* deletion mutants, Δ SsQDO, and confirmed that these mutant strains could not degrade flavonols added to artificial medium. The Δ SsQDO mutants grew at similar rates as the wild-type fungus on medium in the absence of flavonols. However, the mutants grew significantly slower than the wild-type on medium amended with flavonols as well as on Arabidopsis leaves. These phenotypes combined with the inducibility of protein activity in response to flavonols confirmed our hypothesis that flavonol degradation is an important detoxification response for *S. sclerotiorum* and contributes to its virulence.

The effects of Δ SsQDO on flavonol glycoside metabolism was also monitored in planta and in culture. Arabidopsis leaves inoculated with Δ SsQDO contained more flavonol glycosides compared to leaves inoculated with the wild-type. In culture, flavonol glycosides were consumed by Δ SsQDO at a slower rate than by wild-type strains. Additionally, when we measured individual compounds, the wild-type accumulated more

accumulation in Δ SsQDO and wild-type cultures. D, Kaempferol-3-O-rhamnoside production in cultures of Δ SsQDO and wild-type fungus. Different letters above the bars indicate significant differences ($P < 0.001$, one-way ANOVA followed by Tukey's post-hoc test). All data were acquired by LC-ESI-MS and represent means \pm SE ($n = 3$). nd, not detected. T2-1, T2-2, and T2-3, Δ SsQDO mutants.

K3R than the mutants, but the kaempferol aglycone was only detected in Δ SsQDO cultures and not in wild-type cultures. This shows that the *SsQDO* gene deletion reduced flavonol glycoside metabolism in addition to the degradation of flavonol aglycones, although flavonol glycoside cleavage is not directly inhibited by knockout of QDO activity. We speculate that the flavonol, which is released after assimilation of the sugar moiety, inhibits the growth of *S. sclerotiorum* and inhibits the metabolism of flavonol glycosides via a negative feedback loop. The reduced virulence of Δ SsQDO may thus be attributed not only to the toxicity of flavonol aglycones, but might also be due to the reduced availability of Glc and other catabolites derived from flavonol glycosides as carbon and energy sources. This observation might also explain the slower growth of Δ SsQDO in planta compared to growth in artificial medium, which was amended only with flavonols.

Evidence to support that flavonols can serve as phytoalexins or phytoanticipins in planta is still lacking. Therefore, the characterization of *SsQDO* mutants in our study not only demonstrates the toxicity of flavonols to the fungus in culture, but also reveals that these phenolics cause a significant decline in fungal pathogenicity. This result shows that flavonols serve as antifungal defenses in plants unless a pathogen possesses specific adaptations to circumvent the toxicity of these compounds.

CONCLUSION

Among the flavonoids, isoflavones and flavan-3-ols are often reported as antifungal defense compounds (Naim et al., 1974; Harborne et al., 1976; Hammerbacher et al., 2014; Ullah et al., 2017). In contrast, information on the defensive roles of flavonols against fungal infection is sparse, although these compounds commonly occur in plants. For this reason, we hypothesize that plant pathogens may have evolved mechanisms to circumvent the toxicity of these compounds. We have shown here that *S. sclerotiorum* minimizes the potential toxicity of flavonols in Arabidopsis by degrading these metabolites. This fungus can cleave glycosidic linkages and degrade the resulting aglycones by employing an inducible dioxygenase that attacks the flavonol's C ring. Future studies should focus on understanding how widespread this and other flavonol detoxification mechanisms are in the fungal kingdom. Given the broad distribution of flavonols in plants, the ability to detoxify these compounds or circumvent them in other ways could be an important determinant of fungal pathogenesis.

MATERIALS AND METHODS

Biological Materials

For inoculation experiments, the genome sequenced strain *Sclerotinia sclerotiorum* UF-70 (Amselem et al., 2011) was maintained on potato dextrose agar (PDA) at 25°C for 2 to 3 d. The fungus was cultured in potato dextrose broth (PDB) at 25°C, shaking at 150 rpm for 2 d for experiments in artificial medium.

The seeds of Arabidopsis (*Arabidopsis thaliana*) wild-type ecotype Columbia (Col-0) from the Arabidopsis Stock Centre (Nottingham, United Kingdom) were germinated on Murashige and Skoog medium. One-week-old seedlings were transferred to soil and grown under short-day conditions (10 h light/14 h dark cycle with fluorescent light at 120 to 180 $\mu\text{mol m}^{-2}\text{s}^{-1}$, 21°C, humidity 60%) for 4 to 5 weeks.

Plant Inoculation

Agar plugs (0.5 cm diameter) with actively growing mycelium were placed on five fully expanded leaves of 4- to 5-week-old Arabidopsis (Col-0) plants. Agar plugs without mycelium were used as controls (mock inoculation). Inoculation experiments were performed under the short-day conditions described above, and each treatment had three biological replicates unless otherwise stated. Both challenged and unchallenged Arabidopsis leaves were harvested 48 h and 72 h after inoculation and flash frozen.

Extraction and Analysis of Flavonoids of Arabidopsis

Inoculated plant samples were lyophilized using an Alpha 1-4 LD Plus freeze dryer (Martin Christ) for 2 d. Lyophilized leaves were ground with zirconium oxide beads in a shaking ball mill. Extraction was performed on 20 mg of tissue in 1 mL 80% (v/v) methanol agitated for 10 min on a horizontal shaker at room temperature. After centrifugation at 18,000g for 10 min, supernatant (800 μL) was transferred onto a DEAE-Sephadex A-25 (Sigma-Aldrich) column conditioned with 800 μL distilled water and then with 500 μL 80% (v/v) methanol in a 96-well filter plate. An aliquot of the flow-through (100 μL) collected in 96-well plates was diluted with 300 μL distilled water and used for analysis of flavonoids. The flavonol glycosides were identified as the major flavonol glycosides previously reported in Arabidopsis leaves (Veit and Pauli, 1999; Kerhoas et al., 2006; Saito et al., 2013). Analysis of flavonol glycosides was achieved on an HPLC 1100 (Agilent) with a Nucleodur Sphinx RP C-18 column (250 \times 4.6 mm, 5 μm , Macherey-Nagel). Formic acid (0.2%) in water (solvent A) and acetonitrile (solvent B) were used as mobile phases for separation at a flow rate of 1 mL min⁻¹ using a gradient as follows: 0 to 5 min, 100% A; 5 to 15 min, 0% to 45% B; 16 to 17 min, 100% B; 17 to 21 min, re-equilibration with 0% B. Flavonol glycosides were detected using a UV detector with wavelength 330 nm. K3R7R (High-Purity Compound Standard) was used as an external standard for quantification of flavonol glycosides. Quantification of flavonol glycosides was based on the UV (330 nm) absorption peak with the assumption that K3(R2'G)7R, Q3(R2'G)7R, K3G7R, Q3G7R, and Q3R7R had the same molar response as K3R7R.

Bioassays Using Crude Flavonol Glycoside Extract from Arabidopsis

Crude flavonol glycoside extracts from Arabidopsis were prepared for addition to experiments in artificial medium by extraction of lyophilized Arabidopsis leaf tissues (1 g) with 20 mL 80% (v/v) methanol and agitation for 20 min on a horizontal shaker. The contents were centrifuged at 50,000g for 15 min (Avanti J25, Beckman). The supernatant was loaded onto a DEAE-Sephadex column (to eliminate glucosinolates from the raw extract), and flow-through was collected and concentrated to approximately 4 mL by using a rotary evaporator. *S. sclerotiorum* 2-d-old cultures were supplied with the concentrated crude flavonol glycoside extracts using 1 volume of extract per 1000 volumes of medium. Liquid media without fungus supplied with the same amount of flavonol glycosides were used as controls. A 200- μL quantity of medium was collected 0, 24, and 48 h after addition to the fungal culture, and liquid medium was mixed with the same amount of methanol. Samples were centrifuged at 14,000g for 10 min, and supernatants were analyzed by LC-ESI-IonTrap-MS.

The flavonol glycosides in the extracts and their biotransformed products were identified via analysis on an Agilent HPLC1200 (Agilent Technologies) coupled to a Bruker Esquire 6000 ESI-IonTrap-MS system. Compounds were separated on a Nucleodur Sphinx RP C-18 column (250 \times 4.6 mm, 5 mm, Macherey-Nagel) at a flow rate of 1 mL min⁻¹. Formic acid (0.2%) in water (solvent A) and acetonitrile (solvent B) were used as mobile phases with the following gradient: 0 to 25 min, 95% to 45% A; 25 to 27 min, 100% B; 27 to 31 min, 95% A. An Esquire 6000 ESI-IonTrap mass spectrometer (Bruker Daltonik) was used to detect these compounds by scanning an *m/z* between 60 and 1100 with an optimal target mass of *m/z* 405 in alternating mode. The mass spectrometer parameters were set as follows: Skimmer voltage, -40 eV;

capillary exit voltage, -121 eV; capillary voltage, ± 3000 V; nebulizer pressure, 35 psi; drying gas, 11 L min^{-1} ; gas temperature, 330°C . The AutoMS function of Bruker Esquire Control software was used for MS² analysis of enzyme assay products. Standard compounds K3G and K3R were bought from TransMIT PlantMetaChem (Giessen).

In Vitro Flavonol Biotransformation by *S. sclerotiorum*

The fungus *S. sclerotiorum* was cultured in PDB medium containing quercetin or kaempferol at a concentration of 0.1 mg mL^{-1} under the conditions described above. Medium (200 μL) was collected from each treatment at 0, 24, and 48 h for LC-MS analysis. Chromatography of flavonols was performed on the same equipment described for flavonol glycoside analysis with the following elution profile: 0 to 1 min, 100% A; 1 to 15 min, 0% to 65% B in A; 15 to 18 min, 100% B, 18.1 to 22 min, 100% A. Detection and quantification of flavonols was achieved using LC-ESI-IonTrap-MS in negative ionization mode scanning a range from m/z 100 to 1600 with an optimal target mass of m/z 405. The mass spectrometer parameters were set as follows: capillary voltage, ± 3000 V; nebulizer pressure, 35 psi; drying gas, 11 L min^{-1} and gas temperature, 330°C . Capillary exit potential was kept at -121 V. The molecular ion peaks $[\text{M}-\text{H}]^-$ of the analytes (kaempferol m/z 285; quercetin m/z 301) were monitored in negative mode (extracted ion chromatograms), and the authentic external kaempferol (Carl Roth) and quercetin (Sigma-Aldrich) standards were used for quantification.

Total Protein Extraction from *S. sclerotiorum* and Enzyme Assays

S. sclerotiorum was cultured in liquid medium under the conditions described above. Mycelium was harvested 2 h after induction with 0.01 mg mL^{-1} quercetin or kaempferol, lyophilized for 1 d, and homogenized with steel beads in a shaking ball mill. After addition of 1 mL HEPES buffer (1 M sorbitol, 10 mM HEPES, 50 mM EDTA, 0.1 M KCl, 0.2% (v/v) Triton X-100, pH 7) to 10 mg of the homogenate, the solution was incubated on ice for 30 min. Following centrifugation at $18,000g$ for 10 min at 4°C , the supernatant was collected for enzyme assays. Protein concentration was determined with a 2-D Quant Kit according to the manufacturer's instructions (GE Healthcare).

Enzyme assays were carried out as follows: 90 μL total protein (1 μg μL^{-1}) was added to 10 μL of a 1 mg mL^{-1} quercetin or kaempferol solution. The reaction was incubated at 25°C for 30 min. The reactions were terminated by adding 100 μL methanol. A 10 - μL portion from each reaction was used for LC-MS analysis described above for in vitro flavonol biotransformation assays.

Sequence and Phylogenetic Analysis of SsQDO

The protein sequence of an identified quercetin 2,3-dioxigenase from *Penicillium olsonii* (accession number, ABV24349) was used to search the *S. sclerotiorum* genome using BLASTp on NCBI (Tranchimand et al., 2008). Only one protein (SS1G_11412 hypothetical protein) with 53% identity was found in *S. sclerotiorum*, which was later named SsQDO. Full-length homologous amino acid sequences of putative QDO enzymes from different fungal species were retrieved from the NCBI/GenBank databases. The sequences were aligned using a multiple sequence alignment program MAFFT v. 7 (Katoh and Standley, 2013) by employing the highly accurate method I-INS-I. The aligned sequences were then verified and manually adjusted using Mesquite 3.04 (<http://mesquiteproject.org>). The maximum likelihood tree was constructed with the aligned sequences (20) using the software PhyML v. 3.0 (Guindon et al., 2010). The LG amino acid substitution model was used (Le and Gascuel, 2008). The best optimized random starting tree was obtained out of five random trees using tree topology search BEST, which estimates the phylogeny using both NNI (nearest neighbor interchange) and SPR (subtree pruning and regrafting). To estimate branch support, a nonparametric bootstrap analysis was carried out ($n = 1000$). The tree with the highest log likelihood (-8862.81266) was viewed using Figtree (<http://tree.bio.ed.ac.uk/software/figtree/>) and rooted at the midpoint. The tree readability was improved using Adobe Illustrator CS5. Accession numbers of all protein sequences used in this phylogenetic analysis are provided at the end of the "Materials and Methods" section.

Cloning, Expression, and Enzyme Characterization of SsQDO in *Escherichia coli*

For heterologously expressing the ORF of SsQDO, the 1113-bp protein coding region was cloned using Gateway recombination technology (Invitrogen, Life

Technologies Corporation). SsQDO was PCR amplified with Gateway compatible primers (Supplemental Table S1) from *S. sclerotiorum* cDNA using high-fidelity Phusion taq (New England BioLabs) and cloned into the pDONR 201 vector using BP clonase II (Invitrogen) to generate an entry clone. After sequencing, the protein-coding gene was transferred to a destination expression vector pDEST24, which enables the production of a recombinant protein with a C-terminal GST tag using LR clonase II (Invitrogen). This plasmid was transformed into BL 21 (DE 3) *E. coli* for protein expression. A single colony of the expression clone was cultured in Luria Bertani broth and incubated at 37°C . After an OD₆₀₀ of 0.3 to 0.5 was reached, protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside at 37°C for 5 h. Bacteria were harvested by centrifugation and resuspended in $1\times$ PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4) containing 10% (v/v) glycerol, sonicated ($2\times$ 10% cycle, 60% power, 2 min), and centrifuged at $75,000g$ for 30 min at 4°C . The supernatant was used for enzyme assays. Total protein concentrations were determined according to the method described above.

Enzyme activity was performed as follows: 100 μM substrate, 0.5 mM phosphate buffer (pH 7.0), 50 μg crude protein, and deionized water to make a final volume 100 μL . Reaction time was decided by a linearity test with quercetin that showed that product formation exhibited a linear increase within 12 h. The reaction was incubated at 25°C for 2 h and terminated by adding 100 μL methanol. LC-MS analysis was conducted using the same equipment, gradient, and parameters described above for in vitro flavonol biotransformation assays. Protein from an untransformed BL21 (DE3) strain was used as a negative control.

The determination of the exact mass of enzyme assay products was performed on an ultra-HPLC-electrospray ionization-time-of-flight spectrometer (UHPLC-ESI-TOF-MS) system with a Dionex UltiMate 3000 rapid separation LC system (Thermo Fisher) and a timsTOF MS system (Bruker Daltonik). A Zorbax Eclipse XDB-C18 column (50×4.6 mm, 1.8 μm , Agilent) was used, and separation was accomplished using a mobile phase consisting of 0.1% (v/v) formic acid in ultrapure water as solvent A and acetonitrile as solvent B with a flow rate of 300 μL min^{-1} at 25°C . The gradient was as follows: 5% to 60% (v/v) B (6 min), 100% B (1 min), 100% to 5% B (0.1 min), 5% B (2.4 min). ESI source parameters were set to 3500 V for capillary voltage, dry temperature 330°C , and a dry gas flow of 11 L min^{-1} . Samples were measured in negative ionization mode at a mass range of m/z 50 to 1400. Instrument control, data acquisition, and reprocessing were performed using HyStar 4.1 (Bruker Daltonik).

Recombinant protein was further used for assays with quercetin and kaempferol in serial concentrations to compare the affinity of SsQDO. Each concentration was tested with three replicates. Enzyme reactions were set up as described above, and K_m values were calculated using SigmaPlot (Systat Software).

Construction of SsQDO Replacement Vector

To delete the SsQDO gene in *S. sclerotiorum*, genomic DNA of this fungus was isolated with the Stratec Plant DNA Kit (Birkenfeld, Germany) following the manufacturer's protocol. For cloning the flanking regions of SsQDO, a 1087-bp fragment (5' flank-SsQDO) upstream of SsQDO was amplified with primers 5' flank-SsQDO-forward (*Xho*I) and 5' flank-SsQDO-reverse (*Sac*I) using Phusion taq (New England BioLabs), following the manufacturer's instructions. A 1346-bp downstream fragment (3' flank-SsQDO) was amplified with primers 3' flank-SsQDO-forward (*Bam*HI) and 3' flank-SsQDO-reverse (*Pst*I). The purified upstream PCR product and the pXEH vector with a hygromycin phosphotransferase (hph) cassette containing a trpC promoter (Wang et al., 2016) were digested using *Xho*I and *Sac*I and ligated using the Quick Ligation Kit (New England Biolabs). The final replacement vector pXEH-5' flank-SsQDO-3' flank was obtained by digesting the recombinant vector pXEH-5' flank-SsQDO and the downstream flanking region with *Bam*HI and *Pst*I and ligating vector and fragments. Primers are listed in Supplemental Table S1.

Preparation of Protoplasts from *S. sclerotiorum* Mycelium

Protoplasts from *S. sclerotiorum* were prepared according to the protocol described by Rollins (2003). In short, wild-type *S. sclerotiorum* 1980 mycelium grown for 2 d in PDB medium was harvested by centrifugation and digested using a lysing enzyme from *Trichoderma harzianum* (Sigma). The lysing enzyme (0.4 mg) was first dissolved in 6 mL Novozyme buffer (1 M sorbitol, 50 mM sodium citrate, pH 5.8) and then added into 34 mL protoplast buffer (0.8 M $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.2 M sodium citrate $\cdot 2\text{H}_2\text{O}$; pH 5.5). Mycelium was digested with the enzyme mixture for 3 h at 28°C . Protoplasts were washed with 20 mL 0.6 M KCl and then with 10 mL STC (1 M sorbitol, 50 mM Tris-HCl, pH 8, 50 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$) twice. The final concentration was adjusted to 1×10^8 protoplasts

per mL STC. One milliliter protoplast stock was stored with 125 μL 60% PEG4000 ($v v^{-1}$), 125 μL KTC (1.8 M KCl, 150 mM Tris-HCl, pH 8.0, 150 mM CaCl_2), 1% DMSO, and 0.3 mg heparin at -80°C .

PEG-Mediated Protoplast Transformation and Analysis of Transformants

Approximately 1.5 μg chilled plasmid of the replacement vector pXEH-5' flank-SsQDO-3' flank was mixed with 100 μL ($1 \times 10^7 \text{ mL}^{-1}$) protoplast stock solution and incubated on ice for 30 min. One milliliter PEG solution (two parts KTC, one part 60% PEG4000) was added to the protoplast-DNA suspension and mixed gently. The mixture was incubated at room temperature for 20 min and evenly spread on the surface of 20 mL bottom agar (239.6 g of Suc, 0.5 g of yeast extract, 15 g of agar per liter), which did not contain the selective agent. After overnight (15–20 h) regeneration of protoplasts in the dark at room temperature, 10 mL of top agar (239.6 g of Suc, 0.5 g of yeast extract, 8 g of agar per liter) containing 3000 μg hygromycin was overlaid on the plate (to make a final concentration at 100 $\mu\text{g mL}^{-1}$). Transformants carrying the hygromycin gene were allowed to grow through the top agar for 5 to 7 d and then transferred at least three times to PDA medium containing 100 $\mu\text{g mL}^{-1}$ hygromycin (Rollins, 2003).

PCR and enzyme assays were performed for verification of transformants. Genomic DNA of transformants was isolated, and PCR reactions with both SsQDO primers and hygromycin resistance gene primers (Supplemental Table S1) were performed. The transformants with the hygromycin gene but without the SsQDO gene were further verified by assaying the QDO activity of both wild-type and transformants after induction with quercetin.

Growth Rate Assay

An agar plug with actively growing mycelium was inoculated on PDA at the center of a 9-cm petri dish. Growth measurements were taken at 24 and 48 h postinoculation in two vertical directions on each plate. The average results from three replicates for each strain were calculated. The growth rate of both wild-type and mutants on PDA amended with 0.01 mg mL^{-1} quercetin or kaempferol was measured using the same method.

Pathogenicity Assay with Detached Leaves

Arabidopsis (Col-0) plants were cultivated under the conditions described above. Detached leaves with similar ages were placed on water-saturated filter paper in a 9-cm petri dish and inoculated with a 5-mm agar plug of actively growing mycelium from both wild-type *S. sclerotiorum* and the SsQDO knockout mutants. Petri dishes were maintained at 22°C to 25°C in the laboratory. Nine replicates were used for each fungal strain, and the experiment was repeated twice. Lesion areas were determined at 24 h after inoculation by photographing each leaf together with a 2×2 cm reference square. Image analysis was conducted with Photoshop CS5, using the magic wand tool to select symptomatic regions and reference regions. The pixel values of the symptomatic regions and the 4 cm^2 reference region were used to calculate the lesion areas.

RT-qPCR

S. sclerotiorum was cultured in liquid medium under the conditions described above. Mycelium was harvested 2 h after induction either with 0.01 mg mL^{-1} quercetin or kaempferol. RNA was isolated from both non-pretreated and flavonol-pretreated mycelium. Fungal mycelium was ground in liquid nitrogen to a fine powder. Total RNA was isolated with the Stratec Plant RNA Mini Kit following the manufacturer's protocol, and DNA was eliminated with DNase (Qiagen) using the modifications described by Wadke et al. (2016). Approximately 1 μg DNA-free RNA was used for cDNA synthesis with the Superscript II kit (Invitrogen) following the protocols of the manufacturer. Two-step RT-qPCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) and a Bio-Rad CFX Connect Real-Time System with the following cycling conditions: 95°C for 5 min, 40 cycles at 95°C for 15 s, and extension at 60°C for 30 s. Primers used for RT-qPCR of SsQDO are listed in Supplemental Table S1. Primer efficiency and calibration curves were calculated with serially diluted templates using the same cycling ($R^2 > 0.99$). Primer specificity was tested with cycling melting curve analysis from 65°C to 95°C at 0.5°C s^{-1} melting rate.

Relative quantification of wild-type and ΔSsQDO pathogenicity on Arabidopsis was also achieved through RT-qPCR of the fungal *Histone* gene (Li et al., 2018) against the Arabidopsis *ACTIN* gene (Czechowski et al., 2005;

Supplemental Table S1). Plant inoculation, RNA isolation, cDNA synthesis, and RT-qPCR were conducted following the protocol described above.

Statistical Analysis

Data were analyzed using R version 3.4.0. Data normality and variances were verified using the Shapiro-Wilk and Levene's test, respectively. To meet the assumptions of parametric tests, some data were subjected to square root or log transformation. Data were then analyzed either by Student's *t* test or one-way ANOVA, depending on overall number of populations.

Accession Numbers

Accession numbers are as follows: EIT80042.1 (*Aspergillus oryzae* 3.042), XP_015403930.1 (*Aspergillus nomius* NRRL 13137), GAQ33184.1 (*Aspergillus niger*), ABV24349.1 (*Penicillium olsonii*), KXG50735.1 (*Penicillium griseofulvum*), OQD69664.1 (*Penicillium polonicum*), XP_018385410.1 (*Alternaria alternata*), XP_014075683.1 (*Bipolaris maydis* ATCC 48331), XP_001587420.1 now fully annotated MK992913 (*S. sclerotiorum* 1980 UF-70), ESZ96362.1 (*Sclerotinia borealis* F-4128), XP_001552378.1 (*Botrytis cinerea* B05.10), KEQ89945.1 (*Aureobasidium pullulans* EXF-150), KXS95020.1 (*Mycosphaerella eumusae*), ENH80076.1 (*Colletotrichum orbiculare* MAFF 240422), XP_007273525.1 (*Colletotrichum gloeosporioides* Nara gc5), KZL76304.1 (*Colletotrichum tofieldiae*), KIL83777.1 (*Fusarium avenaceum*), XP_007826906.1 (*Pestalotiopsis fici* W106-1), EKG13902.1 (*Macrophomina phaseolina* MS6), XP_020132085.1 (*Diplodia corticola*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Major flavonol glycoside extracts were not degraded spontaneously in liquid medium within 48 h.

Supplemental Figure S2. Identification of *S. sclerotiorum* degradation products of Arabidopsis flavonol glycosides formed in artificial medium.

Supplemental Figure S3. Flavonol glycosides and aglycones were degraded by *S. sclerotiorum*.

Supplemental Figure S4. Identification of SsQDO degradation products of flavonols by UHPLC-ESI-TOF-MS in negative ionization mode.

Supplemental Figure S5. Enzyme assays of SsQDO with other flavonoids as substrates including daidzein, fisetin, and galangin.

Supplemental Figure S6. Michaelis-Menten constants (K_m) of SsQDO with flavonols as substrates.

Supplemental Figure S7. Expression of SsQDO in *S. sclerotiorum* induced with quercetin and kaempferol.

Supplemental Figure S8. Quercetin degradation product protocatechuic acid was only detected in assay with protein from wild type, but not the ΔSsQDO mutant.

Supplemental Table S1. List of primers used in this study.

ACKNOWLEDGMENTS

The authors thank Bettina Raguschke for all sequencing and for her assistance in the laboratory and all members of the greenhouse team, especially Andreas Weber and Elke Goschala, for growing Arabidopsis for this study. The pXEH vector was kindly provided by Prof. Hongyu Pan (Jilin University, Changchun, Jilin).

Received April 29, 2019; accepted May 26, 2019; published June 20, 2019.

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