**INTRODUCTION**

*Streptomyces scabies* is the causative agent of common scab on tuber and root plants via the production of the phytotoxin thaxtomin A amongst other virulence factors (Bignell et al., 2010; Lerat et al., 2009; Loria et al., 2008). The onset of thaxtomin A is triggered on transport of the cello-oligosaccharides cellobiose [(Glc)$_2$] and cellotriose [(Glc)$_3$], which involves the ATP-binding cassette (ABC) transporter system CebEFG-Msk (Jourdan et al., 2016). Once inside the cell, mainly (Glc)$_2$, but also (Glc)$_3$, can interact with the cellulose utilization repressor CebR, preventing it from binding to its operator sequences associated with the thaxtomin biosynthetic gene cluster, and therefore allowing the production of the phytotoxin (Francis et al., 2015). Adjacent to the *cebR-cebEFG* divergern and 146 nucleotides downstream of *cebG*, *scab57721 (bglC)* encodes a putative $\beta$-glucosidase (BG) of the glycosyl hydrolase (GH) GH1 family, which is expected to catalyse the hydrolysis of terminal, non-reducing $\beta$-D-glucosyl residues, with the release of $\beta$-D-glucose from $\beta$-D-glucosides and oligosaccharides (Henrissat, 1991; ENZYME entry: EC 3.2.1.21). The presence of a gene coding for an intracellular GH within the cluster of a sugar ABC transporter is a common feature which allows the co-transcription of the genes required for carbohydrate import and their subsequent enzymatic degradation in the cytoplasm. The use of molecules that are also common (most likely the most recurrent) soil carbohydrate nutrients for the onset of pathogenicity is very intriguing (Jourdan et al., 2017). In non-pathogenic *Streptomyces*, the coordinated expression of genes for BG and cello-oligosaccharide transport is appropriate for feeding the glycolysis pathway with glucose (Fig. 1). However, as stated earlier, in the plant pathogen *S. scabies*, (Glc)$_2$ and (Glc)$_3$ are not only perceived as nutrients used during the course of saprophytic behaviour, but are, above all, signalling molecules eliciting its pathogenic lifestyle (Johnson et al., 2007; Jourdan et al., 2016; Wach et al., 2007). Enzymes with a BG activity could thus potentially play an important role in controlling the onset of the virulence of

---

**SUMMARY**

Common scab disease on root and tuber plants is caused by *Streptomyces scabies* and related species which use the cellulose synthase inhibitor thaxtomin A as the main phytotoxin. Thaxtomin production is primarily triggered by the import of cello-oligosaccharides. Once inside the cell, the fate of the cello-oligosaccharides is dichotomized: (i) the fuelling of glycolysis with glucose for the saprophytic lifestyle and (ii) elicitation of the pathogenic lifestyle by the inhibition of CebR-mediated transcriptional repression of thaxtomin biosynthetic genes. Here, we investigated the role of *scab57721*, encoding a putative BG (*bglC*), in the onset of the pathogenicity of *S. scabies*. Enzymatic assays showed that *bglC* was able to release glucose from cellobiose, cellotriose and all other cello-oligosaccharides tested. Its inactivation resulted in a phenotype opposite to that expected, as reduced production of thaxtomin was monitored when the mutant was cultivated on medium containing cello-oligosaccharides as unique carbon source. This unexpected phenotype could be attributed to the highly intriguing (Jourdan et al., 2009; Loria et al., 2008). The onset of thaxtomin A is triggered on transport of the cello-oligosaccharides cellobiose [(Glc)$_2$] and cellotriose [(Glc)$_3$], which involves the ATP-binding cassette (ABC) transporter system CebEFG-Msk (Jourdan et al., 2016). Once inside the cell, mainly (Glc)$_2$, but also (Glc)$_3$, can interact with the cellulose utilization repressor CebR, preventing it from binding to its operator sequences associated with the thaxtomin biosynthetic gene cluster, and therefore allowing the production of the phytotoxin (Francis et al., 2015). Adjacent to the *cebR-cebEFG* divergern and 146 nucleotides downstream of *cebG*, *scab57721 (bglC)* encodes a putative $\beta$-glucosidase (BG) of the glycosyl hydrolase (GH) GH1 family, which is expected to catalyse the hydrolysis of terminal, non-reducing $\beta$-o-glucosyl residues, with the release of $\beta$-o-glucose from $\beta$-o-glucosides and oligosaccharides (Henrissat, 1991; ENZYME entry: EC 3.2.1.21). The presence of a gene coding for an intracellular GH within the cluster of a sugar ABC transporter is a common feature which allows the co-transcription of the genes required for carbohydrate import and their subsequent enzymatic degradation in the cytoplasm. The use of molecules that are also common (most likely the most recurrent) soil carbohydrate nutrients for the onset of pathogenicity is very intriguing (Jourdan et al., 2017). In non-pathogenic *Streptomyces*, the coordinated expression of genes for BG and cello-oligosaccharide transport is appropriate for feeding the glycolysis pathway with glucose (Fig. 1). However, as stated earlier, in the plant pathogen *S. scabies*, (Glc)$_2$ and (Glc)$_3$ are not only perceived as nutrients used during the course of saprophytic behaviour, but are, above all, signalling molecules eliciting its pathogenic lifestyle (Johnson et al., 2007; Jourdan et al., 2016; Wach et al., 2007). Enzymes with a BG activity could thus potentially play an important role in controlling the onset of the virulence of

---

**Keywords:** $\beta$-glucosidase, CebR, cello-oligosaccharides, common scab disease, thaxtomin.

---

*Correspondence: Email: srigali@uliege.be
†These authors contributed equally to this work.
‡These authors jointly supervised this work.*
S. scabies by limiting the intracellular accumulation of signals triggering thaxtomin A biosynthesis (Fig. 1). As a consequence, intracellular BG(s) of S. scabies might have evolved to display specific/unique properties to ensure that the microorganism adopts the appropriate behaviour—saprophytic versus pathogenic—according to the environmental conditions (Fig. 1). In this work, we define the enzymatic properties, assess the expression control mechanism and investigate the role of scab57721 (bglC) in thaxtomin A production, and therefore in the onset of the virulence of S. scabies.

RESULTS AND DISCUSSION

Enzymatic properties of BglC of S. scabies

The gene scab57721 encodes a 480-amino-acid peptide orthologous to the well-characterized intracellular GH1 family BG BglC of Thermobifida fusca (53% and 67% amino acid identity and similarity, respectively) which also lies downstream of the cebEFG operon (Spiridonov and Wilson, 2001). BglC of S. scabies contains the MYVTENGAA sequence (amino acids 376–384) which
matches the GH1 family active site signature [LIVMFSTC]-[LIVFYW]-[LIV]-[LIVMST]-[E-N-G]-[LSMVFR]-[CSAGN] (PROSITE accession number PS00572). In order to assess the substrate specificity and enzymatic properties of the predicted intracellular BG, scab57721 (bglC) was cloned into pET28a (Table 1) for heterologous expression in *Escherichia coli* with a six histidine tag fused to the N-terminus part of the protein (6His-BglC). Purification through Ni²⁺-nitrilotriacetic acid (Ni-NTA) affinity chromatography enabled the recovery of 6His-BglC with an apparent molecular weight (MW) of ~54 kDa which corresponds well to the calculated MW of 54.121 kDa (Fig. 2A).

The kinetic parameters of 6His-BglC were determined by measuring the initial rate of (Glc)₂ hydrolysis (glucose release) at various concentrations of (Glc)₂. The maximum rate of the reaction (Vₘₐₓ) was 7.3 μmol/min/mg. The Kₘ and kₘₐₓ values were 0.77 mM and 400 min⁻¹, respectively (Fig. 2B). The activity of 6His-BglC at different temperatures (from 20 to 55 °C) and pH values (pH 5–10) was measured using p-nitrophenyl-β-L-glucopyranoside (p-NPgB) as substrate [mimicking (Glc)₂]. The activity of the enzyme gradually increased from 20 to 30 °C, remained constant up to 37 °C and declined abruptly to 10% of the maximal activity at 42 °C (Fig. 51, see Supporting Information). The optimal pH of BglC is around pH 7.5 as the enzyme maintained high activity between pH 6.5 and pH 8.5, and declined rapidly to 30% and 50% of its optimum at pH 5.5 and pH 9, respectively (Fig. 1A).

To determine the substrate specificity of BglC, the recombinant protein was incubated with (Glc)₂, various cello-oligosaccharides ranging from (Glc)₃ to cellohexaose [(Glc)₆] and different disaccharides unrelated to cellulose degradation (lactose, saccharose, maltose, trehalose and turanose). Samples collected after increasing incubation times were spotted on a thin layer chromatography plate which revealed that 6His-BglC was able to generate glucose from (Glc)₂ and all other cello-oligosaccharides tested (Fig. 2C). 6His-BglC was not able to release glucose from disaccharides unrelated to cellulose, except for lactose, but with much lower efficiency relative to (Glc)₂ or any of the other cello-oligosaccharides (data not shown). If BglC displayed activity *in vitro* against (Glc)₆, it was unlikely to occur inside the cytoplasm, as the extracellular ABC transporter component CebE of

### Table 1 Primes and plasmids used and generated in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’–3’)*</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>scab57721_+3 Ndel</td>
<td>TTGATGATGACATCCCAGAGGAGGAGGCTGCATTCA</td>
<td>PCR for cloning scab57721 in pET28a</td>
</tr>
<tr>
<td>scab57721_+1458 HindIII</td>
<td>CCGTCGGTCGACACGACCACCAATGGGAGCGCTTCCATG</td>
<td>PCR verification of Δscab57721</td>
</tr>
<tr>
<td>imf298</td>
<td>AAGCTTCCGTCGGTCGACACGACCACCAATGGGAGCGCTTCCATG</td>
<td>scab57721 (bglC) expression analysis</td>
</tr>
<tr>
<td>imf299</td>
<td>GAAGACGACGGTGAGGAAGC</td>
<td>Complementation of Δscab57721</td>
</tr>
<tr>
<td>imf300</td>
<td>ATGCTGATGACATCCCAGAGGAGGAGGCTGCATTCA</td>
<td>Source or reference</td>
</tr>
<tr>
<td>imf301</td>
<td>AAGCTTCCGTCGGTCGACACGACCACCAATGGGAGCGCTTCCATG</td>
<td>Δscab57721</td>
</tr>
<tr>
<td>imf302</td>
<td>AAACTTACAGATGCTGATGTCGGTCGACACGACCACCAATGGGAGCGCTTCCATG</td>
<td>Δscab57721</td>
</tr>
<tr>
<td>imf303</td>
<td>GTGCTGATGACATCCCAGAGGAGGAGGCTGCATTCA</td>
<td>Δscab57721</td>
</tr>
<tr>
<td>imf304</td>
<td>GAAGACGACGGTGAGGAAGC</td>
<td>Δscab57721</td>
</tr>
<tr>
<td>imf305</td>
<td>ATGCTGATGACATCCCAGAGGAGGAGGCTGCATTCA</td>
<td>Δscab57721</td>
</tr>
<tr>
<td>imf306</td>
<td>AAGCTTCCGTCGGTCGACACGACCACCAATGGGAGCGCTTCCATG</td>
<td>Δscab57721</td>
</tr>
<tr>
<td>imf307</td>
<td>AAACTTACAGATGCTGATGTCGGTCGACACGACCACCAATGGGAGCGCTTCCATG</td>
<td>Δscab57721</td>
</tr>
<tr>
<td>plJ790</td>
<td>Δ. Red plasmid (tIII, CmlR)</td>
<td>Gust et al. (2003)</td>
</tr>
<tr>
<td>plZ8002</td>
<td>Supplies transfer functions for mobilization of oriT-containing vectors from <em>E. coli</em> to Streptomyces (KanR)</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td>SuperCos1</td>
<td>SuperCos1 derivative containing the S. scabies 87-22 cellobiose utilization regulator CebR locus (KanR, AmpR)</td>
<td>Stratagene, Amsterdam, The Netherlands</td>
</tr>
<tr>
<td>Cosmid 833</td>
<td>SuperCos1 derivative containing the S. scabies 87-22 cellobiose/cellobiose ABC transporter locus (KanR, AmpR)</td>
<td>Francis et al. (2015)</td>
</tr>
<tr>
<td>pCR™-BluntII-TOPO</td>
<td>Cloning vector for PCR products (KanR)</td>
<td>Invitrogen, Carlsbad, Canada</td>
</tr>
<tr>
<td>pAU3–45</td>
<td>pSET152 derivative, integrates into the <em>ΦC31 attB</em> site in <em>Streptomyces</em> (AprR, ThioR)</td>
<td>Bignell et al. (2005)</td>
</tr>
<tr>
<td>pMF001</td>
<td>pAU3–45 derivative containing scab57721 and its upstream region cloned into the XbaI site</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Non-homologous extensions are shown in bold and engineered restriction sites are indicated in italics.*

AmpR, ampicillin resistance; AprR, apramycin resistance; CmlR, chloramphenicol resistance; KanR, kanamycin resistance; ThioR, thiostrepton resistance; tIII, temperature sensitive.
2.13) overexpression of cebR resulted in an 85-fold (wild-type 0.025 vs. ces Project medium 4 (ISP4). This revealed that the deletion of allowed the evaluation of the effect of the deletion of chromatography-multiple reaction monitoring (LC-MRM) analysis cebR deletion mutant production in polymerase chain reaction (qPCR) was performed on RNA zation repressor CebR. Quantitative reverse transcription-std, standard cello-oligosaccharides; Glc, glucose; (Glc)2, cellobiose; (Glc)3, only displayed a high binding affinity to (Glc)2 and (Glc)3 (Jourdan et al., 2016). In addition, targeted liquid hydrolysis reaction performed in 50 mM HEPES buffer, pH 7.5, at 25 °C. Data were fitted to the Henri–Michaelis–Menten equation using GraphPad Prism 5 software in order to obtain Vmax, Km and kcat. (C) Substrate specificity of 6His-BglC for cello-oligosaccharides. Cello-oligosaccharides (6.25 mM) were incubated with pure 6His-BglC (0.4 µg) at 30 °C for 0, 15, 30 and 60 min. std, standard cello-oligosaccharides; Glc, glucose; (Glc)2, cellobiose; (Glc)3, cellotriose; (Glc)4, cellotetraose.

S. scabies only displayed a high binding affinity to (Glc)2 and (Glc)3 (Jourdan et al., 2016).

**bgIC expression is repressed by CebR and induced by (Glc)2**

In order to ascertain that BglC is indeed involved in the catabolism of (Glc)2, and (Glc)3 in vivo, we assessed whether its expression/production in S. scabies is under the control of the cellulose utilization repressor CebR. Quantitative reverse transcription-polymerase chain reaction (qPCR) was performed on RNA extracted from the wild-type strain of S. scabies 87-22, and its cebR deletion mutant ΔcebR, grown on International Streptomyces Project medium 4 (ISP4). This revealed that the deletion of cebR resulted in an 85-fold (wild-type 0.025 vs. cebR mutant 2.13) overexpression of bgIC (Fig. 3A). In addition, targeted liquid chromatography-multiple reaction monitoring (LC-MRM) analysis allowed the evaluation of the effect of the deletion of cebR, as well as the presence of (Glc)3, on BglC production in S. scabies. Quantitative analyses of two specific tryptic peptides of BglC (LVDELLAK and TDPVASLR) showed that the protein was more abundant in the total intracellular protein extracts of the ΔcebR mutant (2.3-fold more abundant relative to 87-22), as well as in extracts of the S. scabies wild-type strain grown in (Glc)2-containing medium [2.9-fold more abundant relative to the condition without (Glc)3] (Fig. 3B). The observed transcriptional repression exerted by CebR and the (Glc)2-dependent induction of bgIC/BglC are mediated through direct binding of CebR to the CebR-binding site (TGGaAGCGCTCCCA) identified at position −14 nucleotides upstream of bgIC (Fig. 3C). The results deduced from the targeted proteomic approach are in agreement with the early and constitutive overall intracellular BG activity measured as a consequence of cebR deletion, whereas the S. scabies 87-22 wild-type only displayed measurable BG activity when grown in the presence of (Glc)2 (Fig. 3D).

**Inactivation of bgIC results in reduced thaxtomin A production when S. scabies is grown with cello-oligosaccharides as the sole carbon source**

As we demonstrated that bgIC/BglC is induced by cello-oligosaccharides and displays BG activity against (Glc)2 and (Glc)3, we finally assessed whether the catabolic activity of BglC influenced the production levels of thaxtomin A and, as a consequence, the virulence of S. scabies. We generated a bgIC null mutant (ΔbgIC) by replacing orf scab57721 with the apramycin resistance cassette, as performed previously for cebR, cebE and msiK (Francis et al., 2015; Jourdan et al., 2016). Semi-quantitative analysis by high-performance liquid chromatography (HPLC) revealed that the ΔbgIC/Δ57721 mutant produced only 37% and 9% of the thaxtomin levels produced by the wild-type strain when cultivated in liquid minimal medium (MM) with (Glc)2 or (Glc)3 as sole carbon source, respectively (Fig. 4). This result was unexpected, as the deletion of bgIC should normally lower the catabolism of cello-oligosaccharides for glycolysis, and therefore would result in their higher intracellular accumulation as allosteric inhibitors of CebR and activators of the bgIC expression.

To tentatively explain the reduced thaxtomin A production as a result of the inactivation of bgIC, we monitored (Glc)2 and (Glc)3 consumption, as well as the total BG activity, of the ΔbgIC strain. For this purpose, S. scabies wild-type 87-22 and its bgIC null mutant were grown for 24 h in MM supplemented with 500 µM of (Glc)2 or (Glc)3 as sole carbon source. The concentration of cello-oligosaccharides remaining in the total intracellular protein extracts of the ΔbgIC/Δ57721 mutant were grown for 24 h in MM supplemented with 500 µM of (Glc)2 or (Glc)3 as sole carbon source. The concentration of cello-oligosaccharides remaining in the total intracellular protein extracts of the ΔbgIC/Δ57721 mutant was impaired in both (Glc)2 and (Glc)3, respectively, whereas the bgIC mutant was impaired in both (Glc)2 and (Glc)3 utilization, as the total consumption of these cello-oligosaccharides was significantly reduced in the ΔbgIC/Δ57721 mutant relative to the wild-type strain.
oligosaccharides required about 3 h longer than for the wild-type (Fig. 5A). This delayed import and consumption could possibly postpone the production of thaxtomin A in the \( bglC \) mutant, but should not be responsible for the observed massive reduction in the production of the phytotoxin.

Concomitant with the measurement of cello-oligosaccharide consumption, we assessed the intracellular and extracellular relative BG activity to evaluate to what extent the loss of \( bglC \) impacted the overall BG activity (Figs 5B and S2, see Supporting Information). Each soluble fraction (intra- and extracellular) was assayed at five different time points in both wild-type and \( \Delta bglC \) strains using p-NP\( \beta \)-G as substrate. Very low extracellular BG activities were obtained for both strains and under MM with (Glc)\(_2\) or (Glc)\(_3\) culture conditions (Fig. S2). Assessment of the intracellular BG activity against p-NP\( \beta \)-G revealed that, although the activities measured in \( S. scabies \) wild-type and \( \Delta bglC \) were similar in (Glc)\(_2\)-containing medium at the beginning of culture, the activity of the mutant strain increased dramatically after 3 hpi (Fig. 5B). At the end of the experiment, the wild-type strain presented only a slight increase in BG activity, reaching merely one-

---

**Fig. 3** Expression of \( bglC \) is repressed by CebR and induced by cellobiose. (A) Quantitative reverse transcription-polymerase chain reaction (qPCR) analysis of \( bglC \) expression levels in \( S. scabies \) 87-22 and in the \( \Delta cebR \) strain. Data were normalized using the \( gyrA \) and \( murX \) genes as internal controls and the \( cebE \), \( cebF \) and \( cebR \) genes as CebR repressed genes. Mean normalized expression levels (± standard deviations) from three biological repeats analysed in triplicate are shown, with the \( bglC \) expression level in the wild-type normalized to one-fold. (B) Relative normalized abundance of BglC peptides in response to the deletion of \( cebR \) (\( \Delta cebR \)) and/or cellobiose supply, determined by liquid chromatography-multiple reaction monitoring (LC-MRM) mass spectrometry (MS) on tryptic digests of protein extracts. Target peptides for BglC: LVDELLAK (BglC1) and TDPVASLR (BglC2). *Significant quantitative peptide overproduction \((P < 0.05)\) compared with the wild-type (WT) strain grown in International Streptomyces Project medium 4 (ISP4) without cellobiose supply. Statistical significance was assigned by performing two-sided Student’s \( t \)-tests and assuming groups of equal variances. AUC, area under the curve. (C) Electrophoretic mobility shift assays (EMSAs) showing the specific interaction of CebR with the cbs (CebR-binding site) element at position \(-14\) nucleotides upstream of \( bglC \). Probes with the DasR-responsive element (dre) upstream of nagKA (Tenconi et al., 2015) and with the cbs upstream of \( cebE \) were used as negative and positive controls, respectively. (D) Overall \( \beta \)-glucosidase activity of \( S. scabies \) 87-22 and its \( bglC \) null mutant grown in liquid ISP4 with or without cellobiose (0.5 mM) supply.

© 2017 BSPP AND JOHN WILEY & SONS LTD MOLECULAR PLANT PATHOLOGY (2017)
third of the overall BG activity displayed by the \( \text{bglC} \) null mutant (Fig. 5B). The corresponding activities measured in (Glc)\(_3\)-containing medium were more similar for both strains at the beginning of the culture, but the \( \text{bglC} \) null mutant presented a BG activity about four-fold higher than that of the wild-type at 24 hpi (Fig. 5C). This delay in the response of BG might be a consequence of the delay in (Glc)\(_3\) consumption observed for the \( \text{D} \text{bglC} \) strain (Fig. 5A), but also because (Glc)\(_3\) is a much weaker allosteric effector of CebR relative to (Glc)\(_2\) (Francis \textit{et al}., 2015). These observations demonstrate that BglC is not the only functional BG in \textit{S. scabies} to catabolize cello-oligosaccharides. The fact that the mutant displayed BG activity points to the presence of one or several additional/alternative BGs which are apparently overproduced, or for which the biosynthesis is awakened, when (Glc)\(_2\) or (Glc)\(_3\) is provided as the sole carbon source. The nature and pathway associated with the induction of the alternative BG(s) are currently unknown, but might involve CebR, as the response differed according to (Glc)\(_2\) or (Glc)\(_3\) supply. The contribution of BglC to the overall BG activity of the wild-type is another pending question.

The fact that the \( \text{bglC} \) null mutant displayed a much higher overall BG activity would result in a more rapid depletion of the incorporated thaxtomin-inducing cello-oligosaccharides, thus providing a possible explanation for the unexpected decreased thaxtomin A production of \textit{S. scabies} \( \Delta \text{bglC} \) compared with the wild-type when cello-oligosaccharides are provided as the only carbon source. Similarly reduced thaxtomin A production levels were also observed when assays were performed on solid MM. When inoculated on MM with (Glc)\(_2\) as sole carbon source (TDMc, Fig. 6), the \( \Delta \text{bglC} \) mutant displayed a growth delay during the first 24 h consistent with the absence of a major (Glc)\(_2\) hydrolysing enzyme. When incubated for a longer period, growth recovered, but the \( \Delta \text{bglC} \) strain could not reach the level of thaxtomin produced by the wild-type in TDMc, as described previously in liquid MM (Fig. 4).

\textbf{Inactivation of \( \text{bglC} \) results in overproduction or constitutive production of thaxtomin A when cello-oligosaccharides are not the only carbon source}

The capability of the mutant to produce thaxtomin A was also monitored on a series of solid media, including complex oat bran agar (OBA) medium, which naturally contains cello-oligosaccharides and other carbon sources (Johnson \textit{et al}., 2007; Fig. 6). When grown on OBA, the \( \Delta \text{bglC} \) mutant overproduced thaxtomin A compared with the wild-type strain (Fig. 6B). On this medium, the addition of (Glc)\(_2\) neither decreased nor further increased thaxtomin production, suggesting that the \( \text{bglC} \) mutant could have partially lost its capacity to respond to (Glc)\(_2\) when other carbon sources are available (Fig. 6B). Surprisingly, the \( \text{bglC} \) mutant also overproduced thaxtomin A when inoculated on ISP4 medium deprived of cello-oligosaccharides as nutrient sources.
Fig. 6 Thaxtomin A production by Streptomyces scabies wild-type (87-22) and the bglC null mutant grown on various minimal and complex solid media. (A) Photographs of media inoculated with S. scabies 87-22 and its bglC null mutant. Thaxtomin A production can be seen by its distinct yellow pigmentation. (B) Quantification of thaxtomin A extracted from plates shown in (A) after incubation for 7 days at 28 °C. Means and standard deviations were calculated on three biological replicates. The wild-type production level in thaxtomin defined medium + cellobiose [TDM + (Glc)2] was fixed to 100%. OBA, oat bran agar; PMA, potato mash agar; SFM, soy flour mannitol.
(Fig. 6). In order to ascertain the validity of this unexpected phenotype, the mutant was complemented by introducing plasmid pLMF001 (Table 1) containing the \(bg\) gene with its promoter into the \(\Delta bg\) mutant isolates. Complementation of \(\Delta bg\) restored the wild-type phenotype when bacteria were streaked out on ISP4 (Fig. S3, see Supporting Information), demonstrating that the observed alteration in thaxtomin production was indeed caused by the deletion of the \(bg\) gene and not by a possible unspecific event, such as a spontaneous mutation. The thaxtomin A overproduction phenotype was further confirmed on other media tested, regardless of the presence of (Glc)\(_2\) or other cello-oligosaccharides (Fig. 6).

As \(\Delta bg\) showed the constitutive production of thaxtomin A, its virulence capacity was evaluated on Arabidopsis thaliana and radish seedlings. No different outcome was observed between radish seedlings infected with the wild-type or the mutant (Fig. 7A). However, as the outcome of the radish assay is mostly influenced by the effect of thaxtomin on the plant's growth and development, and thaxtomin is active in nanomolar concentrations (King et al., 2001), it is hard to determine any difference between the production levels of the wild-type and a potential thaxtomin overproducer using radish as host. Assays were also performed using slightly older seedlings (48 h instead of 30 h after sowing) or a lower inoculum [200 \(\mu\)L of a mycelial stock with an optical density at 600 nm (OD\(_{600}\)) of 0.1 instead of OD\(_{600}\) of 1.0], but no difference could be observed. Yet, when thaxtomin was extracted from the agar–water support with the radish seedlings, a significantly higher concentration of thaxtomin A was measured for the assays performed with the \(\Delta bg\) isolates compared with the wild-type strain (Fig. S4, see Supporting Information). Arabidopsis thaliana (ecotype Col-0) as the plant model was more suitable than radish seedlings for the monitoring of hypervirulent phenotypes, as observed previously for the cebR mutant which also overproduces thaxtomin A (Francis et al., 2015). Arabidopsis thaliana seeds grown on Murashige–Skoog (MS) agar were inoculated with spores of \(S\). scabies 87-22 (wild-type) and its \(\Delta bg\) mutant. After 7 days of growth, seedlings inoculated with the \(bg\) mutant presented stronger growth and developmental defects compared with those inoculated with the wild-type strain (Fig. 7B). Closer inspection of individual plants revealed stronger root and shoot stunting as a consequence of the \(bg\) deletion (Fig. 7B).

**CONCLUSION AND PERSPECTIVES**

In this work, we have demonstrated that the protein encoded by the gene \(scab\) located downstream of the cebEFG operon is a BG active against different cello-oligosaccharides, including the best inducers of thaxtomin A production, i.e. (Glc)\(_2\) and (Glc)\(_3\). Expression of \(bg\) is also repressed by CebR, the master regulator of pathogenicity in \(S\). scabies, and is induced by (Glc)\(_2\). As (Glc)\(_2\) and (Glc)\(_3\) consumption by \(S\). scabies correlates with an intracellular increase in BG activity, we assumed that Bgc (and any other enzyme with BG activity) would play an essential role in controlling the pool of imported elicitors to trigger the CebR regulon, and therefore thaxtomin production, as proposed in the model illustrated in Fig. 1. In line with the current model of the cello-oligosaccharide-mediated induction of thaxtomin A production, we were expecting that the inactivation of \(bg\) would simply result in an increased or prolonged production of thaxtomin under culture conditions supplemented with (Glc)\(_2\) or (Glc)\(_3\), as these CebR allosteric molecules would remain longer in the cytoplasm.

However, surprisingly, we observed that the presence of (Glc)\(_2\) and (Glc)\(_3\) as sole carbon source reduced the production levels of thaxtomin A, probably as a consequence of the awakening of alternative BG(s) encoded in the genome of \(S\). scabies as compensation for the loss of Bgc. The identification of the protein(s) responsible for the high BG activity in the \(bg\) mutant is currently under investigation.

Finally, the most striking phenotype observed for the \(\Delta bg\) strain was the loss of (Glc)\(_2\)-dependent induction of thaxtomin, and thus constitutive thaxtomin production, in complex media devoid of eliciting cellulose-related sugars (Fig. 6). That this mutant is able to produce thaxtomin without the presence of inducing molecules is difficult to explain based on the current model of the induction pathway of thaxtomin production, and
suggests that the role of BglC in the induction of S. scabies pathogenicity involves mechanisms that remain to be uncovered.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and culture conditions**

*Escherichia coli* strains [DH5α and Rosetta™ (DE3)] were cultured in Luria–Bertani (LB) medium at 37 °C. *Streptomyces* strains (wild-type 87-22 and mutant strains ΔbglC) were routinely grown at 28 °C in tryptic soy broth (TSB; BD Biosciences, Detroit, MI, USA) or on ISP4 (BD Biosciences). When required, the medium was supplemented with the antibiotics apramycin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (25 μg/mL), thistostrepton (25 μg/mL) and/or nalidixic acid (50 μg/mL). (Glc)2 and cello-oligosaccharides were purchased from Megazyme (Bray, Ireland). For the BG activity assays and the thaxtomin production assays, the *Streptomyces* strains were grown on the complex media OBA (Johnson et al., 2007), soy flour mannitol (SFM; Kieser et al., 2000), potato mash agar (PMA; 12.5 g potato flakes and 5 g agar per litre), as well as the MM thaxtomin defined medium (TDM), modified from Johnson et al. (2007) by omitting sorbose and using a final concentration of 1% of the carbon source of choice.

**Heterologous expression and purification of His-tagged BglC**

The open reading frame encoding SCABS7721 (BglC) was amplified by PCR using the primers scab_57721_+3_NdeI and scab_57721_+1458_HindIII (see Table 1 for primer sequences). The PCR product was subsequently cloned into the pET1.2/blunt cloning vector, yielding pSAJ021. After DNA sequencing to verify the correct amplification of scab57721, an NdeI–HindIII DNA fragment was excised from pSAJ021 and cloned into pET-28a digested with the same restriction enzymes, leading to pSAJ022. *Escherichia coli* Rosetta™ (DE3) cells carrying pSAJ022 were grown at 37 °C in 250 mL of LB medium containing 50 μg/mL of kanamycin until the culture reached an absorbance at 600 nm (A600) of 0.6. The production of 6His-tagged BglC (6His-BglC) was induced overnight (~20 h) at 16 °C by the addition of 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were collected by centrifugation and ruptured by sonication in lysis buffer (100 mM Tris–HCl buffer, pH 7.5, 250 mM NaCl and 20 mM imidazole) supplemented with ethylenediaminetetraacetic acid (EDTA)-free complete protease inhibitor cocktail (Roche, Indianapolis, USA). Soluble proteins were loaded onto a pre-equilibrated Ni-NTA-agarose column (bed volume, 5 mL) and 6His-BglC was eluted within the range 100–150 mM imidazole. Fractions containing the pure protein were pooled (Fig. 2A) and dialysed overnight in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5.

**Construction of the bglC mutant in S. scabies 87-22 and its genetic complementation**

The deletion of the bglC coding region was created as described previously (Francis et al., 2015; Jourdan et al., 2016). Specific primers used to generate and verify the gene deletion and to complement the bglC null mutant are listed in Table 1. A fragment containing the bglC coding region and the upstream region (379 bp) harbouring the promoter was generated by PCR using primers with engineered XbaI sites (Table 1), and cloned into pCR™BluntII-TOPO (Invitrogen, Carlsbad, Canada). After sequence confirmation, fragments were retrieved through an XbaI restriction digest, gel purified and cloned into an XbaI-linearized pAU3–45 (Bignell et al., 2005), resulting in plasmid pIMF001 (Table 1). Complementation constructs, as well as the empty pAU3–45 plasmid, were introduced into three bglC mutant isolates through intergeneric conjugation, similar to the gene deletion process as described previously (Francis et al., 2015; Jourdan et al., 2016).

**qPCR**

RNA was prepared from 72-h-old mycelia grown on ISP4 plates at 28 °C using the RNAeasy minikit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. Verification of the absence of contaminating genomic DNA, cDNA synthesis and qPCR were performed as described previously (Francis et al., 2015; Jourdan et al., 2016). The bglC-specific internal primers imf302 and imf303 were used to quantify the expression levels of the bglC gene (Table 1). The mux, hrdB and gna genes were used to normalize the amount of RNA in the samples (Joshi et al., 2007). Each measurement was performed in triplicate with three different cebR mutant isolates.

**Targeted proteomics**

*Streptomyces scabies* 87-22 and its cebR null mutant were grown on ISP4 plates with or without 0.7% (Glc)2. The mycelium was collected after 48 h of incubation at 28 °C, and resuspended in 50 mM NH4HCO3 buffer (pH 7.5). Crude intracellular extracts were obtained after sonication of the mycelium as described previously (Jourdan et al., 2016). Sample preparation for LC-MRM analysis and the LC-MRM analysis procedure were performed as described previously (Jourdan et al., 2016).

**BG activity assays**

The relative enzyme activity was determined using p-NPβGl as substrate. The reaction mixture (200 μL) containing 50 mM HEPES buffer (pH 7.5), 0.2 μM of purified 6His-BglC and the tested reagent was incubated for 10 min at 25 °C before the addition of 1 mM p-NPβGl. The reaction was carried out at 25 °C for 2 min and stopped by the addition of 100 μL of 2 μL Na2CO3. All assays were performed under these conditions, unless otherwise indicated. The release of p-nitrophenol (p-NP) was measured at 405 nm with a TECAN infinite® 200 PRO (Männedorf, Switzerland).

**Kinetic analysis**

Kinetic parameters of BglC (Km and kcat) were determined by measuring the glucose released at various (Glc)2 concentrations in 50 mM HEPES buffer, pH 7.5, at 26 °C. A reaction time of 7 min was chosen to ensure initial rates of hydrolysis. The glucose released was determined using the D-Glucose HK Assay Kit from Megazyme. Data were fitted to the Henri–Michaelis–Menten equation using GraphPad Prism 5 software.

**Hydrolysis of disaccharides and oligosaccharides**

The cleavage ability of BglC was tested against different cello-oligosaccharides [(Glc)2, (Glc)3 and celloolactose (Megazyme)] or different
disaccharides (lactose, saccharose, maltose, trehalose and turanose). Reaction mixtures (100 μL) containing 50 mu HEPES buffer, pH 7.5, 0.4 μm of purified 6HIs-BglC, 6.25 μm of cello-oligosaccharides or 12.5 μm of disaccharides were incubated at 30 °C. Samples (15 μL) of each were collected at 0, 15, 30 and 60 min, and heated at 98 °C for 5 min to stop the reaction. Each sample was spotted onto an aluminum-backed silica gel plate (Sigma, Steinheim, Germany). The plates were run with chloroform–methanol–acetic acid–water solvent (50 : 50 : 15 : 5, v/v), air dried, dipped in 5% H2SO4 in ethanol and heated over a hot plate until visualization of the carbohydrate spots, as described by Gao and Wakarchuk (2014).

**Monitoring of (Glc)2 and (Glc)3 consumption and glucose production**

Glucose, (Glc)2 and (Glc)3 consumption measurements were performed by HPLC (Alliance, Waters, Milford, MA, USA) on a lead-form Aminex HPX-87P column (300 mm × 7.8 mm; particle size, 9 μm; supplied by Bio-Rad, Richmond, Canada) in combination with two Micro-Guard columns (dashing refill cartridge, 30 mm × 4.6 mm, supplied by Bio-Rad) heated to 80 °C with Milli-Q (18.2 MΩ cm) distilled–deionized H2O in an isocratic mode (flow rate, 0.6 mL/min). Peaks were detected by a refractive index detector (Waters 2414) and processed with Empower 3 software (Waters).

**Thaxtomin production assays**

Thaxtomin production assays were performed as described previously (Francis et al., 2015; Jourdan et al., 2016). Briefly, plates were inoculated with equal amounts of mycelial suspensions of the S. scabies 87-22 wild-type and its bgIC null mutant, and incubated for 7 days at 28 °C. Thaxtomin was extracted from the agar and quantified by reverse-phase HPLC was performed as described previously (Francis et al., 2015; Jourdan et al., 2016). For liquid cultures, thaxtomin was extracted from 1 mL of the culture supernatant with 0.3 mL of ethyl acetate, and quantified by HPLC using a NUCLEODUR® 100-5 C18ec column (Macherey-Nagel, Duren, Germany). Samples were eluted at a flow rate of 0.8 mL/min, and A245 was monitored using a Multi λ fluorescence detector (2475, Waters). All experiments were repeated using three different biological and technological replicates for each S. scabies strain.

**Virulence assays**

Virulence assays on Arabidopsis seedlings were performed as follows. Seeds of the Col-0 ecotype were surface sterilized for 15 min in bleach solution (40% v/v bleach, 0.05% v/v Tween-20), thoroughly rinsed with sterile H2O and stratified for 3 days at 4 °C in the dark before sowing. Arabidopsis seeds (300–400) were sown in each well of a six-well plate containing half-concentrated MS medium (Sigma M5513) supplemented with 1% sucrose. Each well was inoculated with 250 μL of spore suspension of the S. scabies 87-22 wild-type and bgIC mutant (5 × 106 spores/μL), or sterile water as the control. The plates were incubated at 25 ± 0.5 °C under a 16-h photoperiod for 7 days.

Virulence phenotypes on radish seedlings were performed as described previously (Jourdan et al., 2016). Thaxtomin was extracted from the combination of the radish seedlings and the water–agar medium by cutting the material into small pieces and soaking in 15 mL of methanol for 10 min. The liquid phase was dried down and resuspended in 1 mL of methanol. These samples were analysed by HPLC as described above.

**ACKNOWLEDGEMENTS**

The work of S.J. and Be.D was supported by Aspirant grants from the FNRS. S.R. is an Fonds de la Recherche Scientifique (FRS-FNRS) research associate. This work was supported in part by the Belgian program of Interuniversity Attraction Poles initiated by the Federal Office for Scientific Technical and Cultural Affairs (PAI no. P7/44) to Ba.D. and S.R., and by the FNRS (research project T.0006.14-PDR [FRFC]) to S.R. I.M.F. was supported by the Agriculture and Food Research Initiative Competitive Grants Program (grant 2010–65110-20416 from the US Department of Agriculture’s National Institute of Food and Agriculture to R.L.). Ba.D. was also supported by a Bijzonder Onderzoeksfonds (BOF)-basic equipment and Geconcerteerde onderzoeksacties (GOA) grant from the Ghent University special research funds. The authors declare that they have no conflicts of interest with regard to the publication of this article.

**REFERENCES**


**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Fig. S1** Effect of temperature and pH on BglC activity. The optimal temperature was determined by measuring the relative enzyme activity of BglC (0.2 μM) in HEPES (50 mM, pH 7.5) at 20, 25, 30, 37 and 42 °C. The effect of pH on the relative activity of BglC was assessed in the ranges pH 5.0–6.5 (50 mM 2-(N-morpholino)ethanesulfonic acid [MES] buffer), pH 7.0–8.5 (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] buffer) and pH 9–10 (50 mM N-Cyclohexyl-2-aminoethanesulfonic acid [CHES] buffer) at 25 °C.

**Fig. S2** Weak overall extracellular β-glucosidase activity of *Streptomyces scabies* compared with its overall intracellular β-glucosidase activity. Overall intra- and extracellular β-glucosidase activities of *S. scabies* wild-type and *bglC* null mutant grown in minimal medium (MM) supplemented with cellobiose (A) and cellotriose (B).

**Fig. S3** Complementation of the *bglC* mutant. The *bglC* mutant complemented with plasmid pIMF001 carrying the *Streptomyces scabies bglC* gene and its upstream region restored thaxtomin production to a similar level as produced by the wild-type, demonstrating that the phenotype of the mutant is indeed caused by the chromosomal deletion of the *S. scabies bglC* gene.

**Fig. S4** Thaxtomin A production of *Streptomyces scabies* wild-type and the *bglC* mutant when inoculated on radish seedlings. High-performance liquid chromatography (HPLC) analysis of thaxtomin extracted from the corresponding radish assays showing that, although there is no visual difference in virulence on radish between wild-type and mutant strains (Fig. 7A), the *bglC* mutant isolates produced significantly more thaxtomin than the wild-type bacteria when inoculated on plants.