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Old Enzyme, New Role: The β-Glucosidase BglC of *Streptomyces scabiei* Interferes with the Plant Defense Mechanism by Hydrolyzing Scopolin

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Abstract: The beta-glucosidase BglC fulfills multiple functions in both primary metabolism and induction of pathogenicity of *Streptomyces scabiei*, the causative agent of common scab in root and tuber crops. Indeed, this enzyme hydrolyzes cellobiose and cellotriose to feed glycolysis with glucose directly and modifies the intracellular concentration of these cello-oligosaccharides, which are the virulence elicitors. The inactivation of *bglC* led to unexpected phenotypes such as the constitutive overproduction of thaxtomin A, the main virulence determinant of *S. scabiei*. In this work, we reveal a new target substrate of BglC, the phytoalexin scopolin. Removal of the glucose moiety of scopolin generates scopoletin, a potent inhibitor of thaxtomin A production. The hydrolysis of scopolin by BglC displayed substrate inhibition kinetics, which contrasts with the typical Michaelis–Menten saturation curve previously observed for the degradation of its natural substrate cellobiose. Our work, therefore, reveals that BglC targets both cello-oligosaccharide elicitors emanating from the hosts of *S. scabiei*, and the scopolin phytoalexin generated by the host defense mechanisms, thereby occupying a key position to fine-tune the production of the main virulence determinant thaxtomin A.

Keywords: common scab disease; beta-glucosidase; plant defense mechanism; virulence; pathogenicity; scopolin and scopoletin

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1. Introduction

Common scab disease in root and tuber crops is caused by a dozen bacterial strains belonging to the Gram-positive *Streptomyces* genus, with strain *Streptomyces scabiei* (syn. *S. scabies*) as the model species. The major virulence determinants are 4-nitroindol-3-yl-containing 2,5-dioxopiperazines called thaxtomins, which are potent cellulose synthesis inhibitors in higher plants [1]. Thaxtomin A is the most abundant toxin, and its biosynthesis, together with many other specialized metabolites that compose the "virulome" of *S. scabiei* [2–7], is triggered by cello-oligosaccharides, with cellotriose being the main elicitor emanating from the plant hosts [8–10]. Cellotriose is imported by the ABC-type transporter CebEFG-MsiK [8], and, once inside the cytoplasm, it prevents, together with cellobiose generated by cellotriose hydrolysis (see below), DNA-binding of the transcriptional repressor CebR, allowing the expression of the thaxtomin regulatory and biosynthetic genes [11,12].

One gene/protein intimately linked to the other players involved in the perception and transport of cello-oligosaccharides suggests that our current understanding of the virulence signaling pathway is partial. The piece that renders the jigsaw more complicated than initially thought is the beta-glucosidase BglC, whose primary function is to feed glycolysis with glucose released from the hydrolysis of cellotriose and cellobiose [13] (Figure 1, role ①). Hence, it is expected that complete degradation of cellotriose and cellobiose into glucose by BglC prevents *S. scabiei* from accumulating the elicitors of the virulome and, as a consequence, behaves like a saprophyte. How BglC manages to control the intracellular concentration of cello-oligosaccharides for determining the proper timing of the pivotal

switch from the saprophytic to the pathogenic lifestyles is currently unknown (Figure 1, role ②). The deletion of *bglC* led to an unpredicted phenotype where *S. scabiei* (strain Δ*bglC*) constitutively produced the thaxtomin phytotoxins in culture conditions free of cello-oligosaccharides and displaying a hypervirulent phenotype [13]. This unexpected cello-oligosaccharide-independent overproduction of thaxtomin A in strain Δ*bglC* suggests that in the wild-type strain *S. scabiei* 87–22, BglC must repress a yet unknown alternative path to pathogenicity (Figure 1, role ③). Finally, we discovered that the primary function of *bglC*/BglC, i.e., the utilization of cello-oligosaccharides, is safeguarded by a mechanism of genetic compensation that awakens the expression of alternative beta-glucosidases upon perceiving the loss of *bglC* [14]. When BglC is active, the transcription of these alternative beta-glucosidases is kept to a minimal level and is not induced by cello-oligosaccharides (Figure 1, role ④).

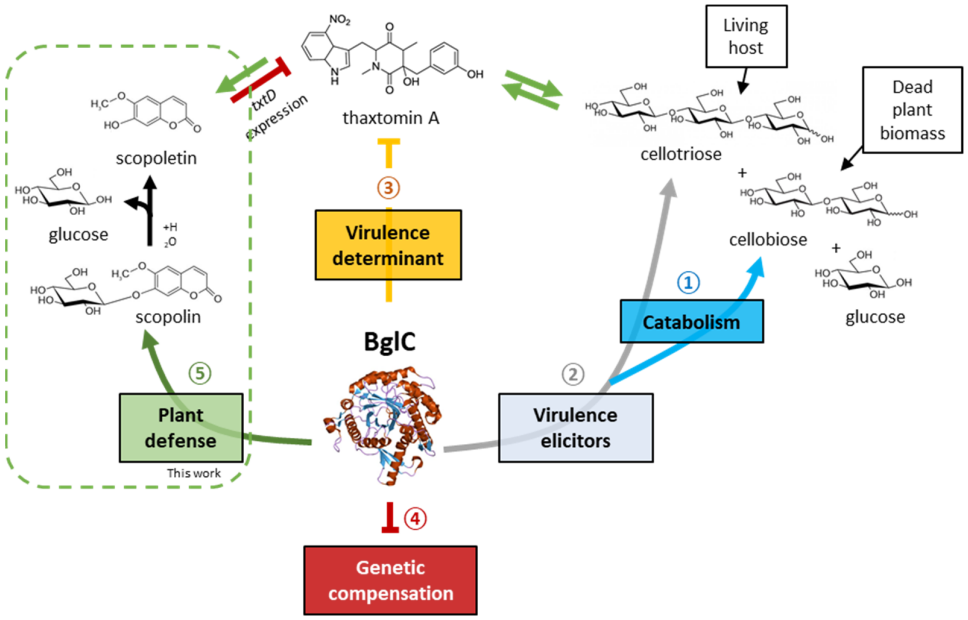

Figure 1. The five biological functions associated with BglC of *S. scabiei* 87–22. ① The primary function of BglC is to hydrolyze cellobiose, the main product resulting from the degradation of cellulose from decaying plant material, and cellotriose to directly feed glycolysis with glucose [13]; ② BglC degrades cellotriose as virulence elicitor released from living hosts [9] and therefore fine-tunes the strength of thaxtomin production, a crucial determinant for the onset of the pathogenic lifestyle of *S. scabiei*; ③ BglC represses the production of thaxtomin A, the main virulence determinant of *S. scabiei*, by a yet unknown cello-oligosaccharide-independent mechanism [13]; ④ The beta-glucosidase activity of BglC is safeguarded by a mechanism of genetic compensation that represses the expression/production of alternative beta-glucosidases [14]; ⑤ In this work, we showed that BglC also hydrolyzes the phytoalexin scopolin into scopoletin, a strong inhibitor of thaxtomin A produced by plants colonized by *S. scabiei* [15].

Because of its multiple roles as well as the unexpected phenotypes and physiological responses of the *bglC* null mutant, BglC is an untapped source to provide crucial information for properly understanding the lifestyle of *S. scabiei*. In this work, we unveil a fifth role for BglC. This beta-glucosidase can also hydrolyze the phytoalexin scopolin into scopoletin, the latter being a strong thaxtomin A inhibitor which is produced as a defense mechanism by plants upon colonization by *S. scabiei*.

2. Materials and Methods

2.1. Heterologous Production of His$_6$-Tagged Proteins and Purification

BglC of *S. scabiei* 87–22 with a six-histidine tag fused to the N-terminus part of the protein (His$_6$-BglC) was produced in *Escherichia coli* BL21(DE3) Rosetta™ and purified by

nickel affinity chromatography as described in [13,14]. The pure protein was stored and used in HEPES buffer (50 mM, pH 7.5).

2.2. TLC for Hydrolysis of Carbohydrates

Semi-quantitative substrate degradation was assessed by thin-layer chromatography (TLC). Reactions were carried out with His_6-BglC (1 µM) and the substrates (5 mM) in HEPES 50 mM pH 7.5 at 40 °C for 10 min. At the end of the reaction, the mixture was incubated for 5 min in a boiling water bath to inactivate the enzyme. 1-µL samples of the inactivated reaction mixtures were spotted next to undigested standards on aluminum-backed TLC plates (Silica gel Matrix, Sigma-Aldrich) and thoroughly dried. The protocol, adapted from [16], consisted of eluting the loaded TLC plate in a TLC chamber filled with an elution buffer (Chloroform–Methanol–Acetic acid–Water (50:50:15:5 (*v/v*)). After air-drying the eluted plate, sulfuric acid (5%) in ethanol was sprayed onto the TLC plate, and the excess liquid was drained. The visualization of the spots was finally conducted by heating the TLC plate.

2.3. Determination of Kinetic Parameters for His_6-BglC

The hydrolysis of scopolin was evaluated by glucose quantification using the D-Glucose HK Assay Kit (Megazyme) following the microplate procedure. His_6-BglC was mixed with scopolin at variable concentrations in HEPES 50 mM pH 7.5, and the incubation was conducted at 40 °C for 4 min. The reaction was terminated by a 5 min incubation in a boiling water bath. At least 10 different concentrations of scopoletin—if possible distributed around the K_m value—were tested in triplicate to estimate initial velocity values. The obtained data—initial velocity (V_i, mM/min) in function of scopolin concentration ([S], mM)—were fitted to the Substrate inhibition equation $V_i = (V_{max}*[S])/(K_m + [S]*(1 + [S]/K_i))$ using the GraphPad Prism (version 9.2.0) software.

3. Results

Due to the multiple functions fulfilled by BglC that cannot be explained by a single activity on its natural substrates, cellobiose and cellotriose, we investigated if this beta-glucosidase would target other carbohydrates with a terminal glucose attached by a β-1,4 linkage. The KEGG pathway [17] suggested a role for BglC of *S. scabiei* 87–22 in cyanoamino acid metabolism (https://www.genome.jp/kegg-bin/show_pathway?scb00460+SCAB_57721; accessed on 12 October 2021). Two cyanogenic glucosides were tested as possible targets of BglC, amygdalin and linamarin. The glycone moiety of amygdalin is the disaccharide gentiobiose (also called amygdalose), and its aglycone part is mandelonitrile, the cyanohydrin of benzaldehyde; linamarin is a glucoside of acetone cyanohydrin. According to the hypervirulent phenotype of the *bglC* null mutant [13], the scopolin heteroside was additionally selected as a possible substrate of BglC. Scopolin is the glucoconjugate of scopoletin, a phytoalexin produced by plants under colonization by *S. scabiei* or under application of pure thaxtomin A. The production of scopoletin and its glucoconjugate (e.g., scopolin) was observed in infected *Arabidopsis thaliana* seedlings, tobacco leaves [15], and potato tuber tissue [18,19]. Lerat and colleagues observed that, during *S. scabiei* infection, the production of scopoletin was responsive to thaxtomin A and that this compound acts as a powerful inhibitor of the expression of *txtD*, consequently limiting thaxtomin biosynthesis [15].

Semi-quantitative substrate degradation was first assessed by thin-layer chromatography (TLC) where amygdalin and its glucoside moiety gentiobiose, linamarin, and scopolin (5 mM) were incubated with 1 µM pure six-histidine tagged BglC (His_6-BglC) (see the Materials and Methods section for detailed protocols). Cellobiose was included in these degradation assays as a positive control to assess the correct activity of pure His_6-BglC. Reaction samples were spotted on TLC plates and migrated in an elution chamber to separate the glucose moiety (or possibly the gentiobiose moiety in the case of amygdalin) from the rest of the substrate. As shown in Figure 2, glucose was released from cellobiose

when the disaccharide was incubated with His_6-BglC, thereby confirming that the pure beta-glucosidase was active. His_6-BglC did not degrade D-amygdalin nor gentiobiose, the glycone moiety of amygdalin. Similarly, glucose was not released from linamarin upon incubation with His_6-BglC. In contrast, a clear spot with the same migration rate as glucose was observed when scopolin was incubated with His_6-BglC.

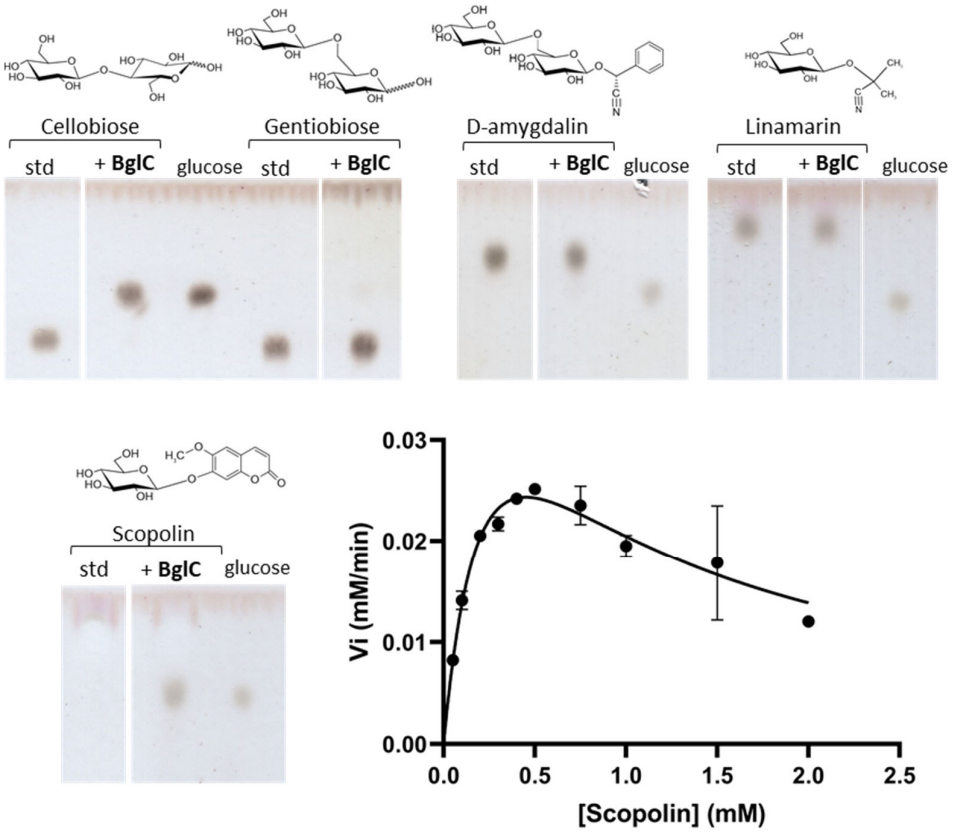

Figure 2. Beta-glucosidase activity of BglC on scopolin. Upper and bottom left panels: TLC plates revealing the release of glucose after incubation of a variety of substrates (5 mM) with His_6-BglC (1 μM) compared to the intact substrate (standard (std)). The respective chemical structures of the substrates are displayed above each name. Note that scopolin and its aglycone scopoletin both migrate almost at the same height as the solvent front, which makes it difficult to visualize these compounds. Bottom right panel: Plot of the initial velocity (V_i, mM/min) estimated by the rate of glucose released by His_6-BglC as a function of scopolin concentrations (in mM). Individual values were entered into the GraphPad Prism software (9.2.0), which fitted the data to the Substrate Inhibition model. Error bars display the standard deviation values determined for the V_i for three replicates at each substrate concentration.

Following these preliminary enzymatic assays through TLC, the hydrolysis of scopolin by His_6-BglC was further investigated by determining the kinetic parameters of the reaction. As deducted from the non-linear regression profile (Figure 2, bottom right panel), His_6-BglC is subjected to substrate inhibition when scopolin is provided at a concentration of 1 mM or higher. The inhibitory constant (K_i) for His_6-BglC was calculated at 0.68 mM as deducted using the equation $V_i = (V_{max}*[S])/(K_m + [S]*(1+[S]/K_i))$ proposed by the GraphPad Prism software (version 9.2.0). The K_m (affinity of the enzyme for the substrate) and k_{cat} (turnover of substrate molecules per second) values were 0.30 mM (0.77 mM for cellobiose), and 9.4 s^{-1} (6.7 s^{-1} for cellobiose), respectively.

4. Discussion

In this work, we revealed that the phytoalexin scopolin is a new substrate hydrolyzed by the beta-glucosidase BglC, further expanding the crucial biological roles played by this enzyme in *S. scabiei* (Figure 1, role ⑤). Although scopolin-hydrolyzing beta-glucosidases were previously identified in the roots of Arabidopsis [20], BglC of *S. scabiei* is the first example of a bacterial beta-glucosidase able to degrade this plant heteroside. By hydrolyzing the molecules (cellotriose and cellobiose) that activate thaxtomin production, as well as a molecule (scopolin) that generates an inhibitor of thaxtomin biosynthesis, BglC occupies a key position to fine-tune the production of the main virulence determinant of *S. scabiei* (Figure 3).

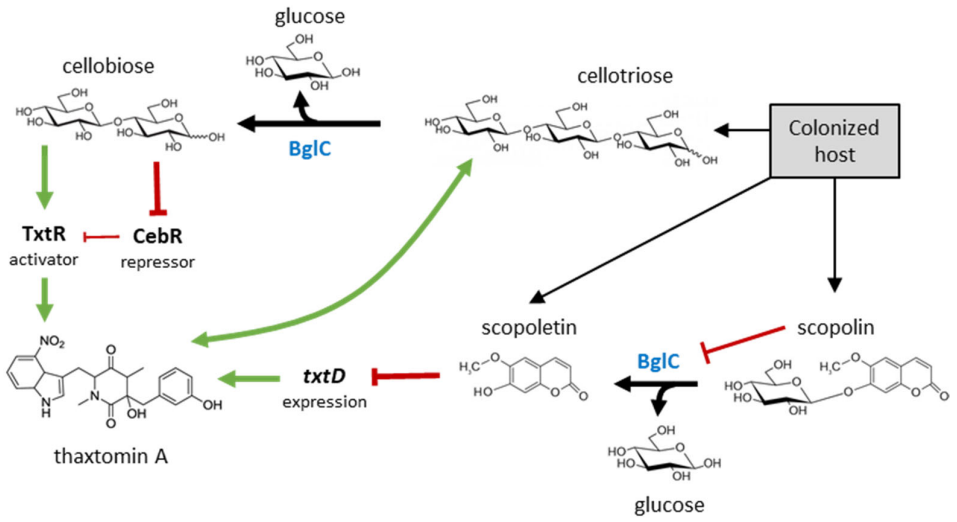

Figure 3. BglC-mediated fine-tuning of thaxtomin production in *S. scabiei*. Cellotriose emanating from expanding plant tissue triggers thaxtomin A production which in turn causes more release of cellotriose from the host [9]. Perception of thaxtomin A by the colonized host induces the production of the scopolin and scopoletin phytoalexins [15], the latter being a strong repressor of thaxtomin A production by reducing the expression of *txtD* (also called *nos*, *scab31841*) [15]. Hydrolysis of cellotriose by BglC generates cellobiose, the best allosteric effector of CebR—the repressor of the thaxtomin biosynthetic gene cluster—which also directly represses the expression of the thaxtomin pathway-specific activator TxtR [12]. High concentrations of scopolin would inhibit the activity of BglC, resulting in (i) less accumulation of scopoletin (a thaxtomin A inhibitor) and (ii) limited degradation of cellotriose (the thaxtomin A inducer); both consequences are presumed to cause increased thaxtomin A production.

The kinetic values for the hydrolysis of cellobiose and scopolin are in the same order of magnitude. The major difference between the two substrates is that for scopolin, the activity of BglC is inhibited at high concentrations of substrate, whereas in the case of cello-oligosaccharides, BglC remains fully active at the highest concentration tested, and the kinetic analysis follows a classic Michaelis–Menten curve [13]. As many GH1-family beta-glucosidases are inhibited by their product, which would be glucose in the case of BglC, it is tempting to interpret the enzymatic curve presented in Figure 2 as a result of product inhibition rather than substrate inhibition. However, the protocol used to generate our enzymatic data is the quantification of the glucose released and not the degradation of the substrate scopolin with increasing concentrations of glucose. Moreover, BglC of *S. scabiei* is one of the most glucose-resistant beta-glucosidase characterized so far [21]. In the proposed model presented in Figure 3, high concentrations of scopolin would inhibit the activity of BglC, which would result in (i) less accumulation of scopoletin (a thaxtomin production inhibitor), and (ii) limited degradation of cellotriose (the thaxtomin production inducer). Both consequences are presumed to cause an increase in thaxtomin production.

Finally, an important question that remains is how scopolin can reach the cytosol of the bacterium since BglC is intracellular. Streptomycetes possess a huge number of sugar transporters [22], but investigation of plant heteroside-specific importers has never been performed. However, the identification and characterization of an intracellular GH3 family beta-glucosidase (BcpE2, see [14] for its discovery) with high promiscuity for plant heterosides (scopolin included) strongly supports the existence of such transporters in these bacteria [23].

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