TECHNICAL ADVANCE

Berberine Bridge Enzyme-like Oligosaccharide Oxidases Act as Enzymatic Transducers Between Microbial Glycoside Hydrolases and Plant Peroxidases

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Oligogalacturonide (OG)-oxidase 1 (OGOX1) and cellodextrin (CD)-oxidase (CELLOX) are plant berberine bridge enzyme-like oligosaccharide oxidases that oxidize OGs and CDs, cell-wall fragments with the nature of damage-associated molecular patterns. The oxidation of OGs and CDs attenuates their elicitor activity and concomitantly releases H_2O_2. By using a multiple enzyme–based assay, we demonstrate that the H_2O_2 generated downstream of the combined action between a fungal polygalacturonase and OGOX1 or an endoglucanase and CELLOX can be directed by plant peroxidases (PODs) either towards a reaction possibly involved in plant defense, such as the oxidation of monolignol or a reaction possibly involved in a developmental event, such as the oxidation of auxin (indole-3-acetic acid), pointing to OGOX1 and CELLOX as enzymatic transducers between microbial glycoside hydrolases and plant PODs.

Keywords: auxin, berberine bridge enzyme-like protein, DAMPs, glycoside hydrolase, H_2O_2, lignin, oligosaccharide oxidase, peroxidase, plant immunity

Plant cell walls are a complex mixture of polysaccharides, proteins, and phenolic compounds, fundamental for physiological processes including cell expansion, maintenance of turgor pressure and cell shape, as well as protection against microbial infection. In order to colonize the plant tissue, pathogens need first to dismantle the cell wall, whose degradation is achieved through the secretion of cell wall–degrading enzymes that include glycoside hydrolases (GHs), esterases, and oxidoreduc-tases (Benedetti et al. 2019; Giovannoni et al. 2020). The enzymatic hydrolysis of cell wall polysaccharides may result in the transient accumulation of cell wall fragments such as oligogalacturonides (OGs), cellodextrins (CDs), and other cell wall oligosaccharides that are quickly perceived by plants as danger signals, i.e., as damage-associated molecular patterns (Pontiggia et al. 2020).

How plants modulate the amplitude of defenses in response to the extent of cell wall hydrolysis is not known. Cell wall degradation occurs not only upon a microbial attack but is also necessary for remodelling during development. Therefore, cell wall fragments can also be generated by endogenous enzymes during the relaxation of the cell wall structures, pointing to the necessity of a system capable of discriminating an exogenous infection from an endogenous developmental stimulus. Thus, a system capable of measuring the entity of cell wall damage must exist.

Some berberine bridge enzyme-like (BBE-l) proteins from *Arabidopsis thaliana* have been recently identified as specific OG-oxidases (OGOXs) and CD-oxidases (CELLOXs). OGOXs include four isoforms (OGOX1 to OGOX4) encoded by paralogous genes that are capable of oxidizing galacturonic acid oligomers of different size (OGs), whereas CELLOX oxidizes CDs (Benedetti et al. 2018; Locci et al. 2019). Structural data of two *Arabidopsis* BBE-l monolignol-oxidases (Daniel et al. 2015, 2016) as well as three-dimensional structural modeling and amino acid alignment of the four OGOXs, CELLOX, and other plant BBE-l carbohydrate oxidases allowed the identification of features important for oxidase activity, including the residue V155/157 of OGOX1/CELLOX (Benedetti et al. 2018; Locci et al. 2019) as the gatekeeper residue of the oxygen binding pocket [P(T/S)VGVGG] (Leferink et al. 2009; Zafred et al. 2015). Indeed, OGOXs and CELLOX inactivate the elicitor nature of OGs and CDs by concomitantly releasing H_2O_2, a molecule with multiple functions in cell wall strengthening and signalling (Smirnoff and Arnaud 2019). The oxidized oligosaccharides are characterized by an increased recalcitrance to enzymatic hydrolysis (Benedetti et al. 2018), but nothing is known about their involvement in other physiological processes. Recently, the combined use of *Arabidopsis* OGOX1 and a peroxidase (POD) allowed the measurement of OGOX1 activity, suggesting that possible physiological processes could be driven by the OGOX-generated H_2O_2 in the presence of POD (Scortica et al. 2021).

e-**X**tra: Supplementary material is available online.

The author(s) declare no conflict of interest.

RESULTS

Working hypothesis: BBE-l oligosaccharide oxidases act as enzymatic transducers between microbial GHs and plant PODs.

In the present study, the capability of generating H_2O_2 by combinations of OGOX1 with a microbial polygalacturonase and CELLOX with a microbial endoglucanase was tested. The generated H_2O_2 can be utilized as a substrate by plant PODs for oxidative reactions possibly involved in defense and development (Fig. 1A). Indeed, cell wall GHs, OGOX1, CELLOX, and extracellular PODs perform their enzymatic function in the same cell compartment, i.e., the apoplast, and it is plausible to consider their activities as related in cell wall metabolism. To evaluate whether, during a plant-microbe interaction, the combined activity of a plant-derived BBE-l oxidase and a microbial GH generates H_2O_2 that can be sequentially utilized by PODs to start biologically relevant reactions involved in defense and growth and, therefore, in the defense/growth trade-off (Fig. 1B), we used OGOX1 (Benedetti et al. 2018) and CELLOX (Locci et al. 2019) in combination with a recombinant endopolygalacturonase from *Fusarium phyllophilum* (FpPG) and a commercial endoglucanase from *Aspergillus niger* (AnEG), respectively. The commercial horseradish POD VI-A type (HRP) that catalyzes the oxidative polymerization of guaiacol, here used as coniferyl alcohol analogue, and an anionic POD preparation from ripe tomato fruit (APOD) that utilizes H_2O_2 to oxidize indole-3-acetic acid (IAA) (Kokkinakis and Brooks 1979), a typical growth hormone, were used as representative plant PODs.

Heterologous expression of FpPG and BBE-l oligosaccharide oxidases in *Pichia pastoris* and evaluation of OGOX1 and FHS-CELLOX as reducing oligosaccharide-to-H_2O_2 converters.

OGOX1, CELLOX and FpPG were expressed in *P. pastoris* and purified to homogeneity. The expression of OGOX1, was achieved as reported in (Scortica et al. 2021), whereas the expression of CELLOX, due to the low yield and high protein instability, required a different expression strategy that consisted in the addition of a Flag-6xHis-SUMOstar tag upstream of the sequence encoding CELLOX (here referred to as FHS-CELLOX) (Supplementary Fig. S1A). *FHS-CELLOX* (Supplementary Data

S1) was cloned under the control of the methanol-inducible promoter AOX and was expressed in *P. pastoris*. Immuno-decoration analysis performed on the culture filtrates from four different transformants showed that FHS-CELLOX is expressed in a heavily glycosylated form (Supplementary Fig. S1B) and, upon de-glycosylation with PNGase F, appears as a unique polypeptide chain of 74 kDa (Supplementary Fig. S1C). OGOX1 was purified from the culture filtrate of *P. pastoris* by two hydrophobic interaction chromatography steps performed at two different pH values (5.0 and 7.0) (Scortica et al. 2021), whereas FHS-CELLOX was purified in a single step by immobilized metal ion affinity chromatography. The AnEG used in our experiments was a highly pure preparation from a commercial source, whereas FpPG was constitutively expressed in *P. pastoris* and was purified using a three-step purification procedure as reported by Benedetti et al. (2011). The protein yields were about 5, 0.5, and 15 mg per liter for OGOX1, FHS-CELLOX, and FpPG, respectively. Before proceeding with the enzymatic assays, the purity grade of the different protein preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining analysis (Supplementary Fig. S2). To evaluate the H_2O_2-conversion efficiency of recombinant OGOX1 and FHS-CELLOX, the amount of H_2O_2 released from the enzymatic oxidation of penta-galacturonic oligosaccharide and cello-triose, here used as model substrates of OGOX1 and FHS-CELLOX, respectively, was measured over reaction time. Our results indicated that both OGOX1 and FHS-CELLOX are efficient reducing oligosaccharide-to-H_2O_2 converters, with H_2O_2 conversion efficiencies ranging from 85 to 95% (Supplementary Fig. S3).

BBE-l oligosaccharide oxidases convert the hydrolysis of a cell wall polysaccharide into H_2O_2.

To demonstrate that BBE-l oligosaccharide oxidases can also produce H_2O_2 through the oxidation of degradation products released by microbial GHs from cell wall polysaccharides, the enzymatic combinations FpPG-OGOX1 and AnEG-(FHS-)CELLOX were tested against polygalacturonic acid and carboxy-methyl cellulose, here used as respective substrates of FpPG and AnEG. In accordance with our working model (Fig. 1B), both the enzymatic combinations produced H_2O_2 in

Fig. 1. OGOX1 (oligogalacturonide-oxidase 1 from *Arabidopsis thaliana*) and CELLOX (cellodextrin-oxidase from *A. thaliana*) as transducers between microbial glycoside hydrolases (GHs) and plant peroxidases (PODs). **A,** Schematic representation showing the transducing role of OGOX1 and CELLOX between microbial GHs and plant PODs and their potential involvement in different plant processes. **B,** A working model of OGOX1/CELLOX-POD machinery. Step 1, microbial GHs hydrolyze the cell-wall polysaccharides by generating reducing end free oligomers. Step 2, specific BBE-l (berberine bridge enzyme-like) oligosaccharide oxidases (OGOX1 and CELLOX) oxidize such reducing ends by concomitantly releasing H_2O_2. Step 3, H_2O_2 is used by plant PODs to oxidize monolignols or indole-3-acetic acid (IAA). oxIAA = oxidized IAA.
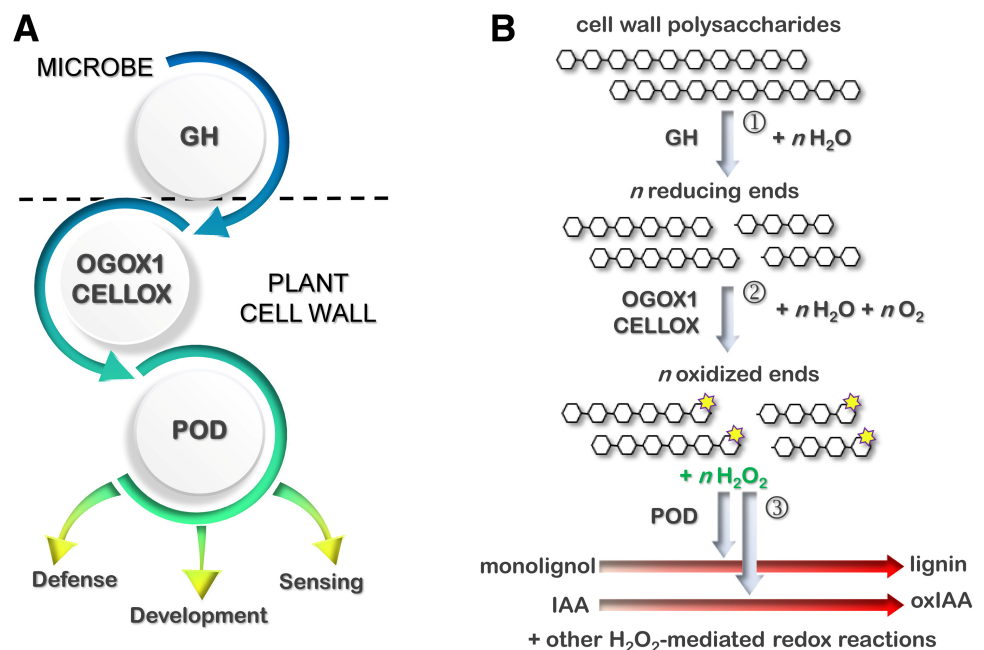
a time-dependent manner (Fig. 2), clearly demonstrating that H_2O_2 can be generated through the combined activity of specific GH-BBE-l oligosaccharide oxidase pairs on pectin and cellulosic substrates.

A BBE-l oligosaccharide oxidase-POD machinery converts the hydrolysis of a cell wall polysaccharide into tetra-guaiacol polymerization and IAA oxidation.

Polygalacturonic acid and carboxy-methyl cellulose were added to obtain the two enzymatic combinations FpPG-OGOX1-HRP and AnEG-(FHS-)CELLOX-HRP. In both reaction mixtures, HRP utilized the generated H_2O_2. The degrading activity of FpPG and AnEG was converted into tetra-guaiacol polymerization in a time-dependent manner, allowing us to monitor the activity of both GHs over the entire reaction time (Fig. 3). The absence of GH or HRP or BBE-l oligosaccharide oxidase in the reaction mixture prevented the guaiacol oxidation, whereas the addition of exogenous H_2O_2 in the presence of HRP resulted in a rapid tetra-guaiacol polymerization (Fig. 3). Taken together, these results indicated the central role of H_2O_2 as the molecule linking the activity of microbial GHs and plant PODs.

Ripe tomato fruit was used as the source of APOD (Kokkinakis and Brooks 1979). Before proceeding with the assays in combination with the OGOX1/(FHS-)CELLOX pairs, APOD activity was quantified using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and H_2O_2 (Supplementary Fig. S4). The same substrates, i.e., polygalacturonic acid and carboxy-methyl cellulose, were added to two enzymatic combinations FpPG-OGOX1-APOD and AnEG-(FHS)-CELLOX-APOD, respectively. In both reaction mixtures, APOD utilized the generated H_2O_2. The degrading activity of FpPG and AnEG was converted into oxidized IAA in a time-dependent manner, and activities of both GHs could be monitored by following the amount of residual (non-oxidized) IAA over reaction time (Fig. 4). The lack of GH or APOD or

BBE-l oligosaccharide oxidase in the reaction mixture prevented the IAA oxidation, whereas the addition of exogenous H_2O_2 in the presence of APOD resulted in a rapid IAA oxidation (Fig. 4). These results, taken together, clearly demonstrate that the H_2O_2 generated downstream of the GH/BBE-l oligosaccharide oxidase pair is successfully used by plant PODs as oxidant in two different processes, i.e., tetra-guaiacol polymerization and IAA oxidation.

DISCUSSION

To date, OGOX1-4 and CELLOX are the only plant BBE-l proteins with proven oxidizing activities towards cell wall oligosaccharide fragments with an elicitor nature, i.e., OGs and CDs. However, due to the large number of members constituting the different plant BBE-l families (Daniel et al. 2017; Pontiggia et al. 2020), it is plausible that other BBE-l enzymes with unknown substrate specificity may act as specific oxidases of other cell wall–derived oligosaccharides. During the reaction catalyzed by OGOXs and CELLOX, OGs and CDs are inactivated and H_2O_2 is formed. Unlike with other extracellular H_2O_2-producing enzymes, such as the membrane bound NADPH oxidase (Kadota et al. 2015), H_2O_2 produced by OGOXs and CELLOX is produced only locally from the reducing end of OGs and CDs enzymatically liberated, either by an endogenous enzyme or, as in the case of a pathogenic attack, by microbial GHs at the site of infection where one molecule of H_2O_2 is generated from one free reducing end. During the degradation of the plant cell wall, the resulting OGs and CDs and possibly other cell wall fragments can be converted by OGOX and CELLOX and possibly other BBE-l oligosaccharide oxidases into H_2O_2 that, in turn, may be used by extracellular PODs to promptly reinforce the cell wall in a proportional opposite direction to the occurring degradation, i.e., more degradation is performed by microbes, more lignification occurs (Fig. 5).
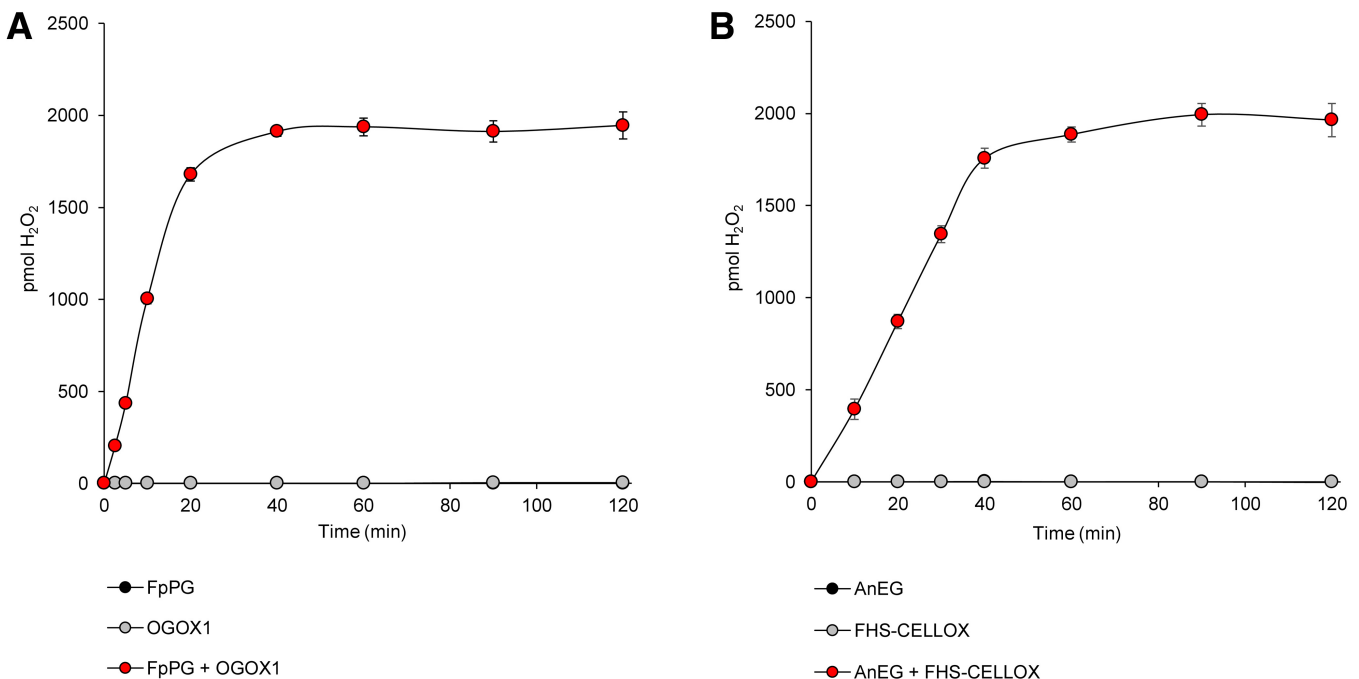

Fig. 2. His-tagged OGOX1 (oligogalacturonide-oxidase 1 from *Arabidopsis thaliana*) and FHS-CELLOX (Flag-His-SUMOstar-tagged cellodextrin-oxidase from *A. thaliana*) convert a polysaccharide hydrolysis into H_2O_2. Production of H_2O_2 over time using a glycoside hydrolase-berberine bridge enzyme-like (GH-BBE-l) oligosaccharide oxidase pair formed by **A,** FpPG (endopolygalacturonase from *Fusarium phyllophilum*) and OGOX1 against polygalacturonic acid and **B,** AnEG (endoglucanase from *Aspergillus niger*) and FHS-CELLOX against carboxy-methyl cellulose. The single enzymes of each combination were used as control reactions. The kinetics relative to the samples (OGOX1) and (FHS-CELLOX) superpose, respectively, with those of the samples (FpPG) and (AnEG). Values are mean ± standard deviation ($n = 3$).

During the pathogen attack, the same enzymatic interplay may also cause inhibition of plant growth through an oxidation of the extracellular IAA (Fig. 5). The APOD-mediated oxidation of IAA could play a role in the defense/growth trade-off when plants are required to redirect their metabolic energy from primary to secondary metabolism during pathogen infection (Pontiggia et al. 2020). Thus, the type of molecule that will be oxidized by H_2O_2 will depend on the substrate specificity of the available plant POD. Considering that 73 different class III plant PODs exist in *A. thaliana* (Almagro et al. 2009) and that most of them are localized in the extracellular space, the oxidizing activity of H_2O_2 can be sorted with different catalytic efficiencies towards several metabolic pathways. Transcriptomic data obtained from the GENEVESTIGATOR database (Zimmermann et al. 2004) indicate that *Arabidopsis* BBE-l oligosaccharide oxidases (OGOX1 and CELLOX) and several class III PODs are positively co-expressed during fungal infection, corroborating their involvement in a potential enzymatic interplay in plant defense (Supplementary Fig. S5; Supplementary Table S1). Our in-vitro experiments clearly demonstrate that apparently unrelated enzymes such as glycoside hydrolases, the flavoenzymes OGOX1 and CELLOX, and metallo-oxidoreductases (POD) can work together under the same apoplastic conditions (pH 5.5) and transduce the cell wall hydrolysis performed by microbial GHs into biochemical reactions potentially involved both in defense and growth. This aspect may allow the plants to mount a balanced response by lowering the metabolic costs and deleterious effects deriving from an exaggerated activation of their immunity (Benedetti et al. 2015). It is also worth mentioning that H_2O_2 is, per se, an important transduction signal and the recent identification of the extracellular H_2O_2 sensor

HPCA1 from *A. thaliana* reinforces its role as a cell-to-cell signal in plant immunity. Here, H_2O_2-mediated modification of the cysteine residues localized in HPCA1 ectodomain leads to stomatal closure, a well-known defense response against pathogenic bacteria (Wu et al. 2020). Similarly to the successful employment of the glucose oxidase-POD pair for the measurement of glucose in clinical and food analyses (Dubey et al. 2017; Trinder 1969), different BBE-l oligosaccharide oxidase-POD pairs may be exploited as biosensors of specific GH activities for the early detection of microbial contaminants in plant crops.

Interestingly, oligosaccharide oxidases are also produced by phytopathogens and saprotrophs. In this case, H_2O_2 produced from their activity may be used by microbial lytic polysaccharide monooxygenases (LPMOs) to degrade cellulose, xylan and pectin (Couturier et al. 2018; Sabbadin et al. 2021; Villares et al. 2017), since the copper-containing active site of LPMOs can be reactivated through a H_2O_2-mediated reduction (Müller et al. 2018).

Although based on in vitro–assembled assays, our study provides a novel perspective on how the cell wall hydrolysis can be perceived and managed by plants to balance growth and defense (Fig. 5). The high number of PODs in plants and the possible occurrence of many other BBE-l oligosaccharide oxidases in addition to OGOX1 and CELLOX pose major challenges in elucidating their role not only in plant-microbe interactions but also in plant development, morphogenesis, and growth. In this regard, the enzymatic system described here will require a validation in vivo, e.g., by evaluating the plant responses in the presence of specific GH-BBE-l oligosaccharide oxidase pairs.
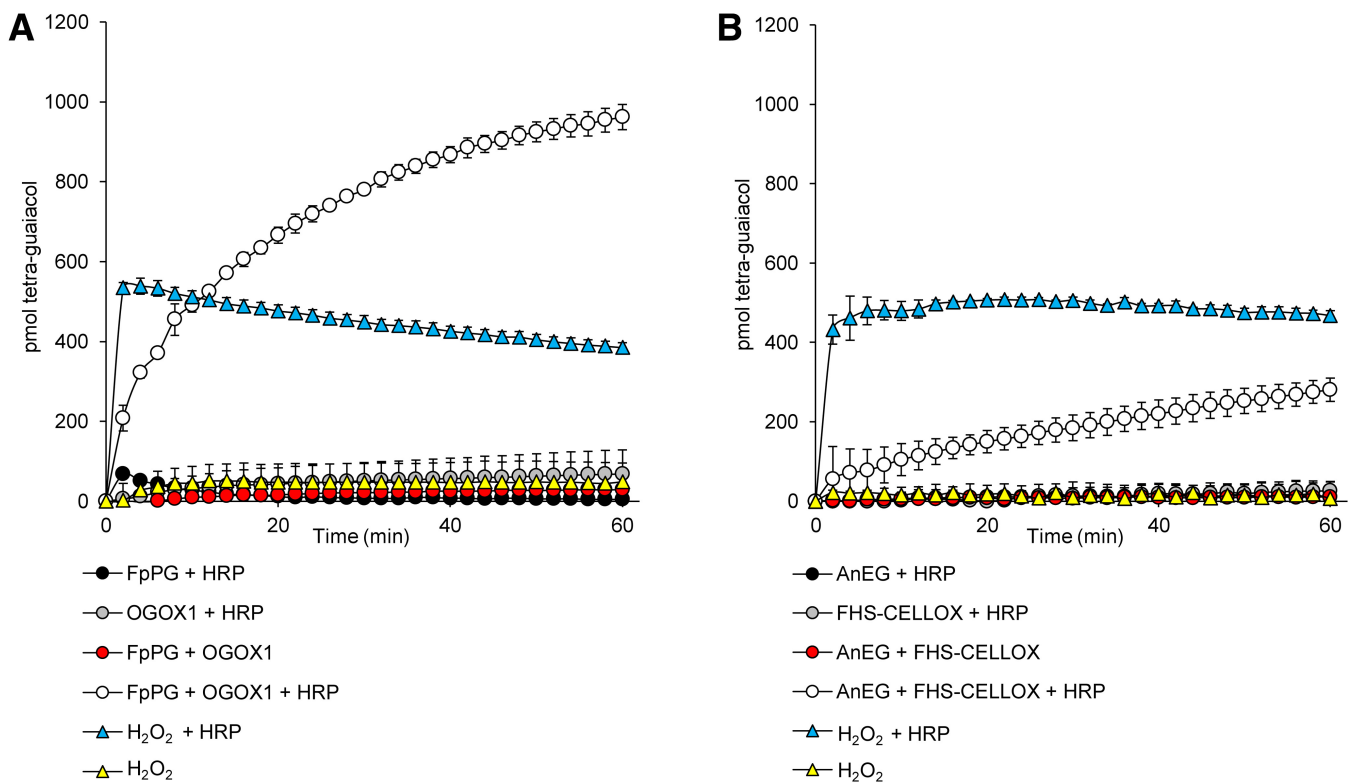

Fig. 3. OGOX1 (oligogalacturonide-oxidase 1 from *Arabidopsis thaliana*) and CELLOX (cellodextrin-oxidase from *A. thaliana*) machinery converts a polysaccharide hydrolysis into tetra-guaiacol polymerization. Tetra-guaiacol polymerization over time, using OGOX1/CELLOX-POD machinery formed by **A,** FpPG (endopolygalacturonase from *Fusarium phyllophilum*), His-tagged OGOX1, and HRP (horseradish peroxidase VI-A type) against polygalacturonic acid and **B,** AnEG (endoglucanase from *Aspergillus niger*), Flag-His-SUMOstar-tagged (FHS)-CELLOX, and HRP against carboxy-methyl cellulose. For each enzymatic machinery, different combinations of enzymes were used. (H_2O_2 + HRP) and (H_2O_2) were used as positive and negative control, respectively, of HRP-mediated tetra-guaiacol polymerization. Values are mean ± standard deviation (n = 3).

MATERIALS AND METHODS

GH-BBE-l oligosaccharide oxidase assay.

The GH-BBE-l oligosaccharide oxidase assay was performed in 20 mM Na acetate, pH 5.5, containing 0.5% (wt/vol) polygalacturonic acid (Sigma-Aldrich, Saint Louis) or 0.5% (wt/vol) carboxy-methyl cellulose (P-CMC4M; Megazyme, Dublin, Ireland) as respective substrates of FpPG and AnEG. The FpPG-OGOX1 pair was composed of FpPG (2 mg per liter) and OGOX1 (1 mg per liter), whereas the AnEG-(FHS-)CELLOX pair was composed of AnEG (0.06 mg per liter [E-CELAN; Megazyme]) and FHS-CELLOX (1 mg per liter). The activity assay was performed in a reaction volume of 0.1 ml at 25°C, whereas the measurement of H_2O_2 was determined by the orange-xylenol assay in accordance with Benedetti et al. (2018).

Tetra-guaiacol polymerization.

The oxidative polymerization of guaiacol to tetra-guaiacol was measured by following the increase in absorbance at 470 nm (=26.6 mM^{-1} cm^{-1}) (Koduri and Tien 1995). The OGOX1/(FHS-)CELLOX-HRP assay was performed in 20 mM Na acetate, pH 5.5, containing 0.5% (wt/vol) polygalacturonic acid (Sigma-Aldrich) or 0.5% (wt/vol) carboxy-methyl cellulose (P-CMC4M; Megazyme), 150 µM guaiacol (2-methoxyphenol [Sigma-Aldrich]) and 0.05 g of HRP (P6782, Sigma-Aldrich) per liter in a reaction volume of 0.2 ml. The mixture also included a GH enzyme (7 mg of FpPG or 0.2 mg of AnEG per liter) and the appropriate BBE-l oligosaccharide oxidase (3 mg of OGOX1 or 3 mg of FHS-CELLOX per liter). The addition of exogenous H_2O_2 (2 nmol) to the reaction mixture in the presence or absence of HRP was used as positive and negative controls, respectively, of HRP-mediated tetra-guaiacol polymerization.

The activity of the OGOX1/(FHS-)CELLOX-HRP machinery was spectrophotometrically measured at 25°C, using an Infinite M Nano200 spectrophotometer (Tecan AG; Männedorf, Switzerland) in continuum mode for 60 min.
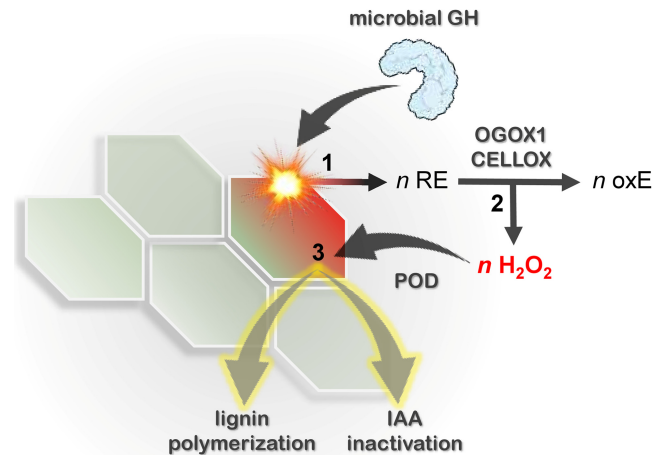

Fig. 5. Proposed model of OGOX1 (oligogalacturonide-oxidase 1 from *Arabidopsis thaliana*) and CELLOX (cellodextrin-oxidase from *A. thaliana*) as transducers between microbial glycoside hydrolases (GHs) and plant peroxidases (PODs). The combined action of a microbial GH, a specific berberine bridge enzyme-like oligosaccharide oxidase (OGOX1, CELLOX) and a plant POD succeeded in converting the hydrolysis of a cell-wall polysaccharide into lignin polymerization and auxin inactivation over degradation time. Black numbers (1, 2, and 3) indicate the sequential order of the enzymatic reactions. IAA = indole-3-acetic acid, oxE = oxidized end, and RE = reducing end.
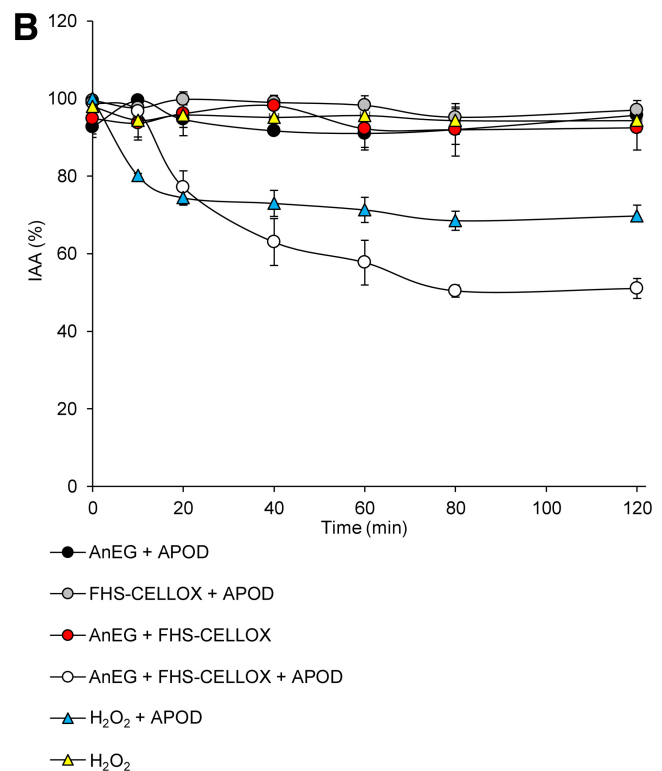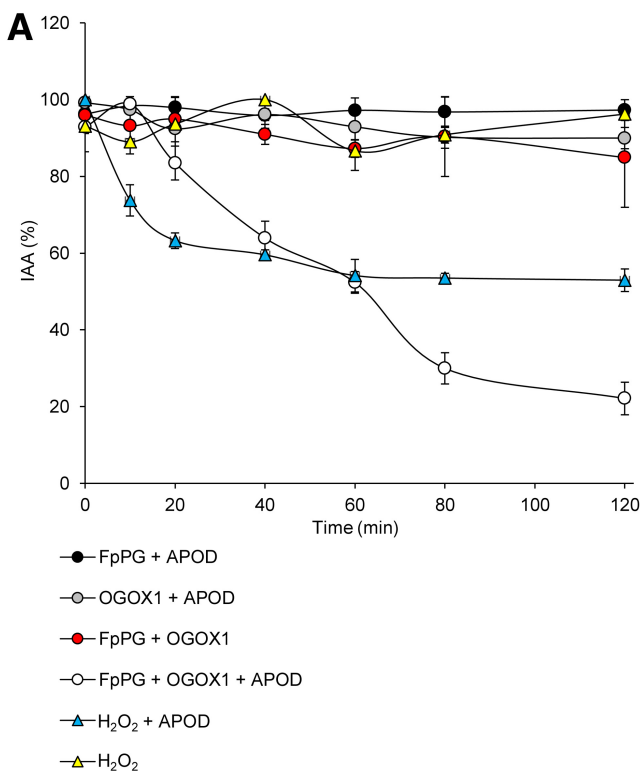

Fig. 4. OGOX1 (oligogalacturonide-oxidase 1 from *Arabidopsis thaliana*) and CELLOX (cellodextrin-oxidase from *A. thaliana*) machinery converts a polysaccharide hydrolysis into indole-3-acetic acid (IAA) oxidation. IAA oxidation over time using a OGOX1/CELLOX-POD machinery formed by **A,** FpPG (endopolygalacturonase from *Fusarium phyllophilum*), His-tagged OGOX1, and APOD (anionic POD preparation from ripe tomato fruit) against polygalacturonic acid and **B,** AnEG (endoglucanase from *Aspergillus niger*), Flag-His-SUMOstar-tagged (FHS)-CELLOX, and APOD against carboxy-methyl cellulose. For each enzymatic machinery, different combinations of enzymes were used. (H_2O_2 + APOD) and (H_2O_2) were used as positive and negative control, respectively, of APOD-mediated IAA oxidation. Values are mean ± standard deviation ($n = 3$).

IAA oxidation.

IAA oxidation was measured using the modified Salkowski method described by Gang et al. (2019). The OGOX1/(FHS)-CELLOX-APOD assay was performed in 20 mM Na acetate, pH 5.5, containing 0.5% (wt/vol) polygalacturonic acid (Sigma-Aldrich) or 0.5% (wt/vol) carboxy-methyl cellulose (P-CMC4M; Megazyme), 500 μM IAA (indole-3-acetic acid, auxin [Sigma-Aldrich]) and 0.14 g of APOD per liter in a reaction volume of 0.1 ml. The mixture also included a GH enzyme (7 mg of FpPG or 0.2 mg of AnEG per liter) and the appropriate BBE-l oligosaccharide oxidase (3 mg of OGOX1 or 3 mg of FHS-CELLOX per liter). The addition of exogenous H_2O_2 (50 nmol) to the reaction mixture in the presence or absence of APOD was used as positive and negative controls, respectively, of APOD-mediated IAA oxidation. IAA oxidation was measured at 25°C by following the decrease in absorbance at 536 nm, using an Infinite M Nano200 spectrophotometer (Tecan AG; Männedorf, Switzerland). For each reaction, the absorption values obtained from seven different timepoints were first converted into micromoles IAA by interpolation with the IAA-calibration curve and then into percentage of residual IAA (% IAA) respect to the highest IAA concentration measured in the same reaction (i.e., 100% IAA corresponds to $500 \pm 50\,\mu M$).

Availability of data and materials.

All relevant data are included in the article and the supplementary material.

AUTHOR-RECOMMENDED INTERNET RESOURCE

GENEVESTIGATOR database: https://genevestigator.com

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