the plant journal

SEB Society for Experimental Biology

The Plant Journal (2018) **94,** 260–273 doi: 10.1111/tpj.13852

Four Arabidopsis berberine bridge enzyme-like proteins are specific oxidases that inactivate the elicitor-active oligogalacturonides‡

Manuel Benedetti[1,†], Ilaria Verrascina[1,†], Daniela Pontiggia[1,†], Federica Locci[1], Benedetta Mattei[2], Giulia De Lorenzo[1,*] and Felice Cervone[1]

[1]*Department of Biology and Biotechnology 'Charles Darwin', Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy, and*

[2]*Department MESVA, University of L'Aquila, L'Aquila, Italy*

Received 28 July 2017; revised 19 December 2017; accepted 4 January 2018; published online 3 February 2018.

*For correspondence (e-mail giulia.delorenzo@uniroma1.it).

†These authors contributed equally to this work.

‡This paper is dedicated to the memory of Renato D'Ovidio.

SUMMARY

Recognition of endogenous molecules acting as 'damage-associated molecular patterns' (DAMPs) is a key feature of immunity in both animals and plants. Oligogalacturonides (OGs), i.e. fragments derived from the hydrolysis of homogalacturonan, a major component of pectin are a well known class of DAMPs that activate immunity and protect plants against several microbes. However, hyper-accumulation of OGs severely affects growth, eventually leading to cell death and clearly pointing to OGs as players in the growth-defence trade-off. Here we report a mechanism that may control the homeostasis of OGs avoiding their deleterious hyper-accumulation. By combining affinity chromatography on acrylamide-trapped OGs and other procedures, an *Arabidopsis thaliana* enzyme that specifically oxidizes OGs was purified and identified. The enzyme was named OG OXIDASE 1 (OGOX1) and shown to be encoded by the gene *At4g20830*. As a typical flavo-protein, OGOX1 is a sulphite-sensitive H_2O_2-producing enzyme that displays maximal activity on OGs with a degree of polymerization >4. OGOX1 belongs to a large gene family of mainly apoplastic putative FAD-binding proteins [Berberine Bridge Enzyme-like (BBE-like); 27 members], whose biochemical and biological function is largely unexplored. We have found that at least four BBE-like enzymes in Arabidopsis are OG oxidases (OGOX1–4). Oxidized OGs display a reduced capability of activating the immune responses and are less hydrolysable by fungal polygalacturonases. Plants overexpressing OGOX1 are more resistant to *Botrytis cinerea*, pointing to a crucial role of OGOX enzymes in plant immunity.

Keywords: *Arabidopsis thaliana*, berberine bridge enzyme BBE-like proteins, damage-associated molecular patterns, oligogalacturonides, oxidized OGs, polygalacturonase, polygalacturonase-inhibiting protein.

INTRODUCTION

Activation of the immune response in both plants and animals relies on the recognition of danger signals, mostly molecular patterns that are either exogenous, i.e. pathogen-derived, or endogenous. In the latter case, molecules normally encased in larger polymers or confined in compartments inside the cell are released upon mechanical damage and/or a microbial infection. The released molecules then act as indicators of an altered self, i.e. as Damage-Associated Molecular Patterns (DAMPs) that activate specific pattern recognition receptors (PRRs) (Duran-Flores and Heil, 2016). Oligogalacturonides (OGs), oligomers of alpha-1,4-linked galacturonosyl residues, released upon partial degradation of homogalacturonan, a major component of pectin in plant cell walls, are a well known class of DAMPs. OGs elicit a wide array of defence responses, including a robust apoplastic oxidative burst, synthesis of phytoalexins and up-regulation of defence-related and pathogenesis-related genes and, consequently, confer protection against pathogens. Most plant defences are induced by OGs with a high degree of polymerization (DP), but shorter oligomers have also been recently reported to activate some defence responses (Ferrari *et al.*, 2013; Davidsson *et al.*, 2017).

Upon wounding and mechanical damage, OGs accumulate in the extracellular matrix through the action of plant-

endogenous polygalacturonases (PGs) (Savatin *et al.*, 2014a), while during microbial infections they are released through the action of pathogen-encoded PGs. Generation of elicitor-active OGs is favored *in vitro* by plant-encoded PG-inhibiting proteins (PGIPs), which counteract fungal PGs by slowing down their activity and impeding the complete hydrolysis of homogalacturonan (Cervone *et al.*, 1987a; De Lorenzo *et al.*, 2001; Casasoli *et al.*, 2009; Kalunke *et al.*, 2015). The *in vivo* proof of concept that PGIPs promote the generation of OGs and, in turn, that OGs act as endogenous DAMPs during infection was recently obtained by expressing in an inducible manner a fusion protein made up of a fungal PG and a PGIP. Transgenic Arabidopsis plants that expressed the chimera, named OG-machine (OGM) plants, released OGs and, consequently, exhibited enhanced resistance against several pathogens (Benedetti *et al.*, 2015). In the same OGM plants, however, the prolonged release of OGs was deleterious and caused reduced plant growth. If high concentrations of OGs are induced in OGM plants, the level of activated defence responses may become lethal for the plant itself (hyper-immunity), confirming an early observation in which external treatment of plant tissues with high amounts of OGs were shown to cause tissue necrosis (Cervone *et al.*, 1987b). The deleterious effects of OG hyper-accumulation suggest that a mechanism for controlling the OG homeostasis must exist to prevent prolonged impairment of plant physiological functions.

To date, enzymes that specifically inactivate the elicitor activity of OGs and maintain their homeostasis have not been reported. This role could theoretically be ascribed to the many forms of pectic enzymes such as PGs and pectate lyases that exist in plants and have important roles in cell wall remodeling (Senechal *et al.*, 2014). However, the activity of these endogenous enzymes cannot destroy an excess of OGs without simultaneously affecting cell wall structure and the correlated functions. Indeed, the specific activity of plant-derived pectic enzymes, unlike that of the corresponding enzymes of microbial origin, is generally very low because they are devoted to fine precision remodeling of pectin polysaccharides rather than to their disruption.

The deleterious effects of hyper-immunity are an important consideration in both plants and animals in which dysfunction in controlling DAMPs accumulation is often associated with disease (Cook *et al.*, 2015; Liston and Masters, 2017). In plants, the metabolic costs of activating immunity greatly affect resources invested to maintain good performances, growth and fitness of the entire organism. This phenomenon, called 'growth–defence trade-off', implies that the defence responses may have negative consequences over crop productivity if they are not strictly programmed, regulated and swift. Accumulating evidence points to OGs as modulators of plant growth, on the one hand, and as important signals in growth–defence trade-off on the other hand (Ferrari *et al.*, 2013; Benedetti *et al.*, 2015). Disentangling their action may lead to the design of novel strategies to obtain plants with increased pathogen resistance, yet with normal and better growth performances.

In this study, we identified a possible mechanism by which OG homeostasis activity can be maintained. By using the OGM plants in which a high quantity of released OGs can be induced on command, we show in *Arabidopsis thaliana* that a mechanism for their oxidation and inactivation exists. Four berberine bridge enzyme-like (AtBBE-like) proteins (Daniel *et al.*, 2017) are specific oxidases that inactivate OGs.

RESULTS

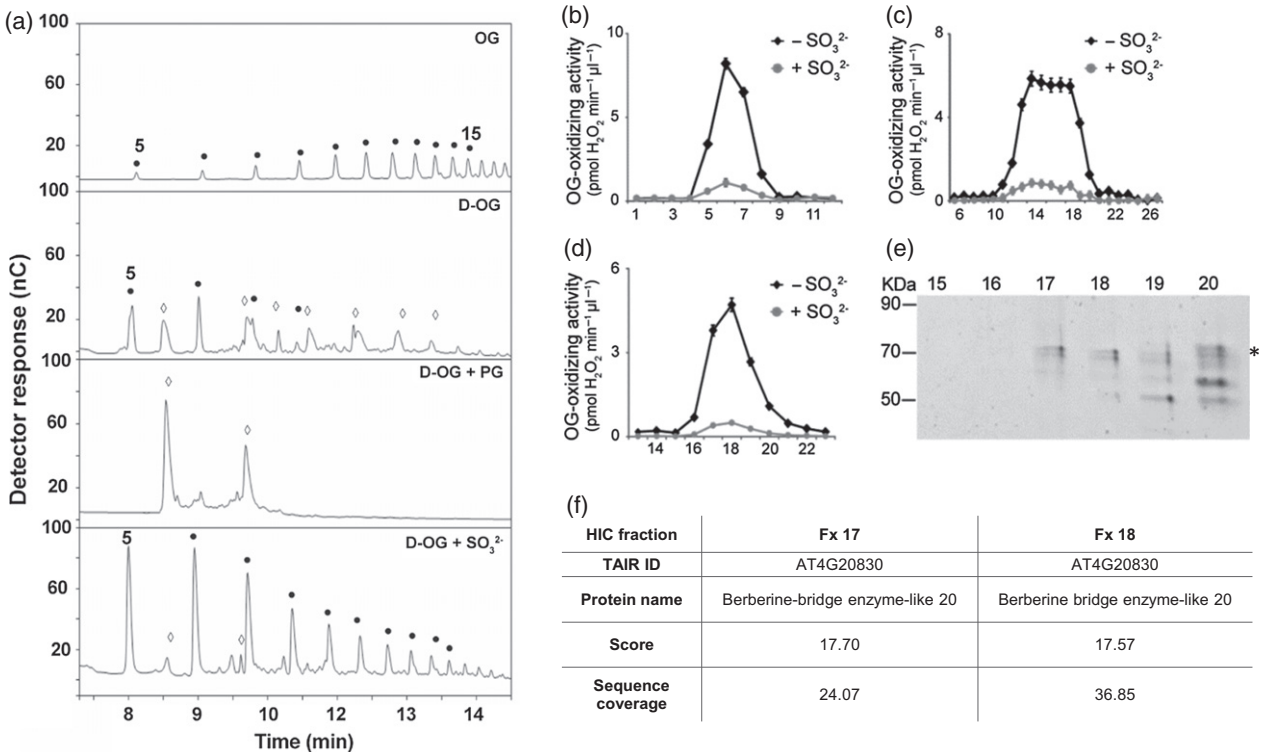
A specific OG-oxidizing activity exists in Arabidopsis

In order to search for modified and possibly inactive OGs, a non-disruptive method was used to extract pectin fragments from β-estradiol-induced OGM plants, which may accumulate high levels of OGs in their tissues, by incubating leaf strips in a strong chelating agent (Pontiggia *et al.*, 2015; Benedetti *et al.*, 2017). Chromatographic analysis of the leaf diffusates detected, in addition to small amounts of unmodified OGs, an abundant proportion of oligosaccharides with retention times that differed from those of standard OGs. These oligosaccharides were capable of being hydrolyzed by a fungal endoPG, indicating that they were OGs with a possible modified structure (Figure 1a). Their chemical structure was investigated by electrospray ionization mass spectrometry (ESI-MS) and showed that the reducing end residue had been oxidized to galactaric acid (Figure S1), probably by an enzyme present in the OGM plants.

Uronic acid oxidase activity, previously described in orange (Riov, 1975) and peach fruits (Cantu *et al.*, 2006) and in commercial horse-radish peroxidase preparations (Marsh, 1985), is characterized by the concomitant production of hydrogen peroxide and sensitivity to sulphites, typical of flavo-proteins. A total protein extract from OGM plants showed an OG-oxidizing activity that was sulphite sensitive (Figure S2a) and accompanied by production of H_2O_2; activity was five times lower in extracts from wild-type plants (Figure S2b). The addition of Na_2SO_3 to the chelating agent solution prevented the appearance of oxidized OGs in the leaf diffusates (Figure 1a, bottom panel) suggesting the involvement of a FAD-dependent activity in this oxidative reaction.

The OG-oxidizing enzyme (OGOX) is encoded by a member of the berberine bridge enzyme-like (BBE-like) family

In order to purify the OG-oxidizing activity, a substrate affinity chromatography was performed by passing an



(f)

HIC fraction	Fx 17	Fx 18
TAIR ID	AT4G20830	AT4G20830
Protein name	Berberine-bridge enzyme-like 20	Berberine bridge enzyme-like 20
Score	17.70	17.57
Sequence coverage	24.07	36.85

Figure 1. Leaves of OGM plants induced with β-estradiol contain an OG-oxidizing activity that is accompanied by a sulphite-sensitive H_2O_2 production.
(a) Chromatographic (HPAEC-PAD) analysis of standard OGs (OG), diffusates obtained from leaf strips of the OGM plants, at 170 h after spraying with β-estradiol, incubated in a strong chelating agent (D-OG), diffusates treated for 1 h with pure *Fusarium phyllophilum* PG expressed in *Pichia pastoris* (D-OG+PG) and diffusates obtained in the presence of 10 mM Na_2SO_3 (D-OG+SO_3^{2-}). Numbers above the peaks indicate the DP of the single oligomers. Full circles and white diamonds indicate peaks with retention time corresponding or different, respectively, from that of standard OGs. Signal intensity (nC) at each retention time (min) is shown.
(b–d) OG-oxidizing activity was followed by supplying OGs to the fractions eluted from the HGA-affinity column (b), the SP-Sepharose column (c) and the Phenyl-Sepharose column (d) in the presence (+) and in the absence (–) of 1 mM SO_3^{2-}.
(e) SDS-PAGE analysis of fractions from 15 to 20 eluted from the Phenyl-Sepharose column. The asterisk indicates the apparent molecular weight (70 kDa) of the proteins that co-elute with the highest OG-oxidizing activity detected in fractions 17–19.
(f) Protein identification, as determined by LC-MS analysis, carried out on the gel slice at 70 kDa of fractions 17 and 18.

OGM leaf protein extract through a column containing a cross-linked polyacrylamide gel entrapping polygalacturonic acid (Figure 1b). Several putative FAD-binding enzymes were identified in the retained fraction, with one berberine bridge enzyme-like encoded by *At4g20830* [BBE-like 20 or BBE20, according to the nomenclature reported in Daniel *et al.* (2015)] showing the highest score (Table S1). In parallel, non-affinity-based purification of the OG-oxidizing activity was undertaken by following the sulphite-sensitive OG-dependent H_2O_2 production. OGM leaf protein extracts were subjected to cationic exchange (CE) (Figure 1c) and hydrophobic interaction (HI) chromatography (Figure 1d). In SDS-PAGE of the HI fractions that displayed the highest OG-oxidizing activity (i.e. Fx17 and Fx18), a clear doublet of about 70 kDa (Figure 1e) was visible. The corresponding gel slices were digested with trypsin and the resulting peptides were subjected to LC-MS analysis. A major protein was identified in both fractions, with a higher sequence coverage in fraction 18 and that corresponded to the putative flavoprotein BBE20 (Figure 1f). In accordance to the SDS-PAGE analysis, protein identification carried out on the corresponding total fractions revealed a high abundance of the same protein (Table S2). Among the several proteins identified in fractions 17 and 18, BBE20 encoded by *At4g20830* was the only FAD-dependent enzyme that was highly abundant in both fractions. This protein was therefore considered to be the enzyme responsible for the sulphite-sensitive OG-dependent H_2O_2 production activity detected in the protein extract of the OGM plants.

At4g20830 belongs to the superfamily of FAD-binding berberine bridge enzyme-like genes that in Arabidopsis comprises 27 members. Two different transcripts are ascribed to this gene in the TAIR databank and both are strongly induced upon pathogen perception or elicitation; this finding was in agreement with the level of OG-oxidizing activity that we observed in the induced OGM plants (Figure S3). The transcripts encode two putatively secreted protein isoforms differing in the C-terminal region and with a length of 540 (BBL20) and 570 amino acids (BBE19)

(Figure S3c). The latter included a predicted glycosylphosphatidylinositol (GPI)-anchoring site, in agreement with the observation that At4g20830 is released from plasma membranes by treatment with phospholipase D, generally used for proteomic investigations of GPI-anchored proteins in a variety of cells, tissues, and organisms (Elortza *et al.*, 2006).

For biochemical characterization, *Pichia pastoris* was used as a heterologous host for the expression of the short isoform as a secreted protein (Figure S4a) that was purified to homogeneity (Figure S4b). Moreover, the short isoform encoded by At4g20830 was fused at the C-terminus to the c-*myc* tag, which allowed detection of the enzyme by immuno-blotting analysis. The activity of the enzyme from *P. pastoris* was similar to that of the native plant enzyme (Figure 2a,b) and assessed by using, as a substrate, standard elicitor-active OGs as well as short OGs and oligomannuronides (epimers of OGs, which are inactive as elicitors) (Mathieu *et al.*, 1991; Ferrari *et al.*, 2013). The enzyme was also tested on cellopentaose and cellotreose and several sugar monomers, including galacturonic acid, and shown to be active only on OGs. The enzyme from *P. pastoris* was tested on individual purified OG oligomers and showed higher activity on oligomers with a DP $\geq$ 4 (Figure 2c). The pH optimum of the enzyme was 11, and K_M and k_{cat} were 8 μM and 329 min^{-1}, respectively (Data S1). Among the different carbohydrate oxidases characterized so far (Carter and Thornburg, 2004; Custers *et al.*, 2004; Vuong *et al.*, 2013) our enzyme displayed an affinity for OGs (K_M 8 μM) higher than that of the *F. graminearum* enzyme for chitotetraose (K_M 250 μM) or the *S. strictum* enzyme for cellobiose (40 μM). Based on its characteristics, the enzyme encoded by *At4g20830* was named OG oxidase no. 1 (OGOX1) (Figure 2d).

Four BBE-l proteins are OG oxidases

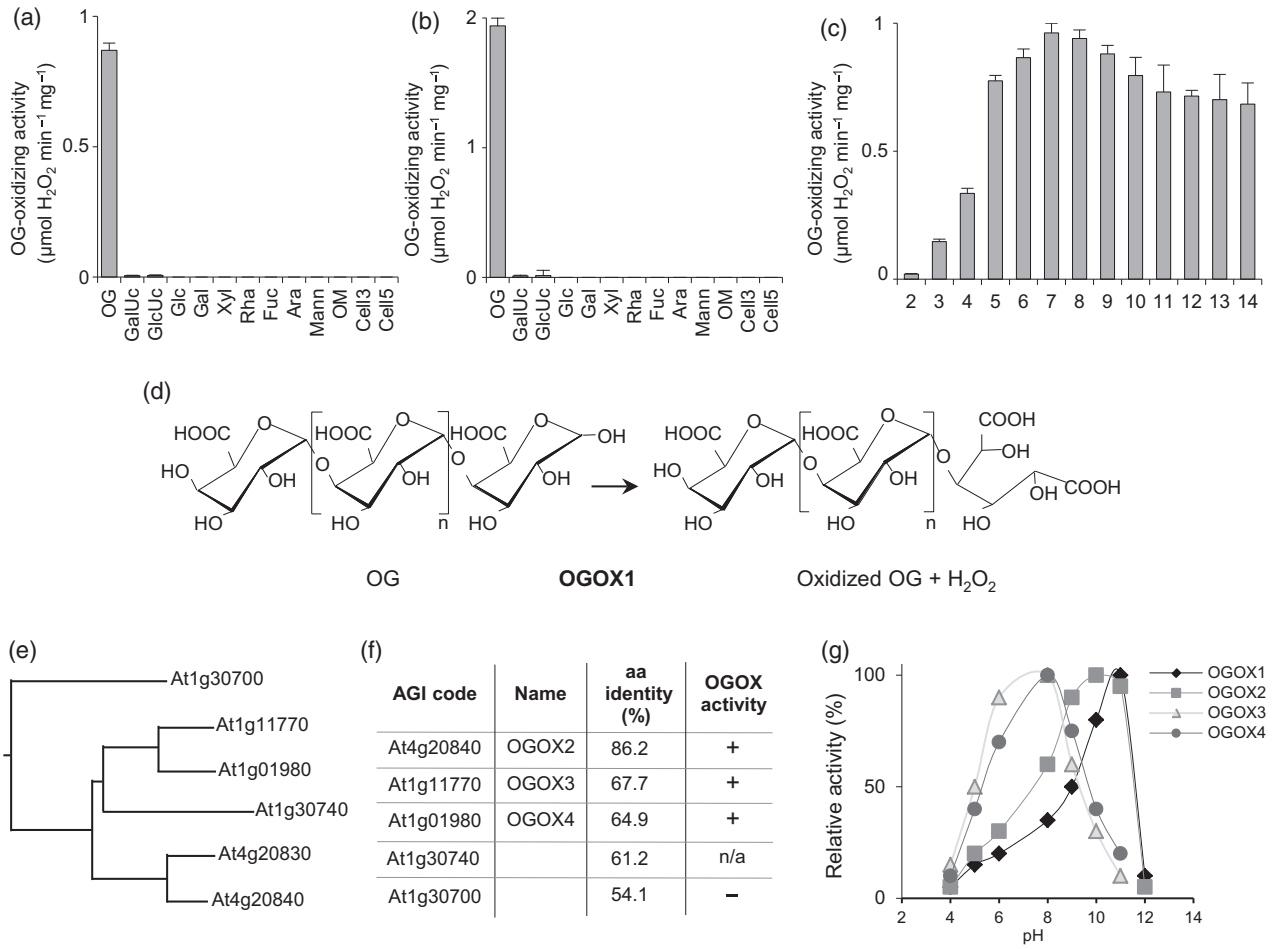
To assess whether other BBE-like members possessed OG-oxidizing activity, we expressed in *Pichia pastoris* the five closest OGOX1 paralogs (At4g20840/BBE21, At1g30740/BBE12, At1g01980/BBE1, At1g11770/BBE2, At1g30700/BBE8). All encoded products that share a similarity higher than 50% with OGOX1 (Figure 2e) and carry an N-terminal signal peptide for translocation into the endoplasmic reticulum (ER). Expression of all proteins, except At1g30740, in the yeast was successful (Figure S4c). Upon partial purification, only the three closest OGOX1 paralogs showed OG-oxidizing activity (Figure 2f,g) and all of these oxidized only OGs and, to a much lower extent, galacturonic acid (Figure S4d). The genes were named *OGOX2* (*At4g20840/BBE21*), *OGOX3* (*At1g11770/BBE2*) and *OGOX4* (*At1g01980/BBE1*). The protein encoded by *At1g30700/*BBE8, expressed in *P. pastoris* (Figure S4c), was inactive against all substrates tested.

Structural and phylogenetic data are already available for two Arabidopsis BBE-like enzymes (Daniel *et al.*, 2015, 2016) as well as 3D structural modeling and amino acid alignment of the four OGOXs, At1g30700/BBE8 and three BBE-ls previously identified as carbohydrate oxidases (Figure 3 and Table S3). This allowed us to identify motifs and amino acids important for oxidase activity and to pinpoint putative residues responsible for specificity towards OGs. A 3D structural model of the short OGOX1 isoform (Data S2), obtained by homology-based molecular modelling using as the template the crystallographic structure of Arabidopsis At2g34790/BBE15 (UniProtKB – O64743) (Daniel *et al.*, 2015), confirmed the presence of a relatively open active site, designated as a type I active site, with characteristic features shared by the majority of Arabidopsis BBE-l enzymes and of other plant species (Daniel *et al.*, 2016, 2017). These features included one Tyr and one Gln residue engaged in a hydrogen bond as well as two Tyr and one Lys residues forming the catalytic base motif (Figure S5). Residue V155 in OGOX1 corresponded to the identified gatekeeper residue of the oxygen binding pocket (PTVGVGG) (Zafred *et al.*, 2015). Both these features are conserved in the plant BBE-l proteins that have been shown to act as carbohydrate oxidases as well as in OGOX1–4 (Figure 3a and Table S3). As expected, the residues involved in the covalent FAD attachment (Daniel *et al.*, 2015, 2016), are also conserved in these proteins (H90 and C152; Figures 3a and S5).

Three-D structural modelling also showed that OGOX1 contains a peculiar large number of positively charged patches on the protein surface compared with the non-OGOX enzymes (Figure 3b). Positively charged patches were absent also on the surface of monolignol oxidase (Daniel *et al.*, 2015) (Figure 3b). As shown by the multiple amino acid alignment, several positively charged residues are in the proximity of the active site and conserved in all BBE-like enzymes with carbohydrate oxidase activity (Figure 3a), probably being required to enhance the oxygen reactivity of flavoenzymes (Mattevi, 2006; Gadda, 2012). Additional positively charged residues (R41, R64, R273, K308, K377, K404, K486) are instead strikingly specific for OGOX1–4 (Figure 3a). Many of these OGOX-specific residues are in the proximity of the active site cleft (Figure 3b), surrounding the putative carbohydrate binding groove that may facilitate binding of oligosaccharides and is typical of the carbohydrate oxidases of this class (Huang *et al.*, 2005); these basic residues are likely to be important for OG binding.

Oxidized OGs are weaker elicitor than OGs

The effect of oxidation on the OG capability of acting as elicitors in the immune response was next tested by analyzing typical readouts of OG action. The oxidized oligosaccharides were unable to induce the expression of the early (maximum within 1 h) elicitor-induced genes such as *RetOx* (At1g26380/*BBE3*) and *CYP81F2* (At5g57220). *RetOx*, recently renamed *FOX1* (Boudsocq *et al.*, 2010), encodes a



AGI code	Name	aa identity (%)	OGOX activity
At4g20840	OGOX2	86.2	+
At1g11770	OGOX3	67.7	+
At1g01980	OGOX4	64.9	+
At1g30740		61.2	n/a
At1g30700		54.1	–

Figure 2. Characterization of the *myc*-tagged short At4g20830 isoform (BBE20) expressed in *Pichia pastoris*.
(a, b) (a) Enzyme activity of the pure short At4g20830 isoform expressed in *P. pastoris* and (b) the Arabidopsis enzyme purified from OGM plants, induced with β-estradiol as reported in Figure 1, towards different mono-, di- and oligosaccharides. (GalUc, galacturonic acid; GlcUc, glucuronic acid; Glc, glucose; Gal, galactose; Xyl, xylose; Rha, rhamnose; Fuc, fucose; Ara, arabinose; Mann, mannose; OM, oligomannuronides; Cell3, cellotriose; Cell5, cellopentaose).
(c) Enzyme activity of the pure short At4g20830 isoform expressed in *P. pastoris* towards single OG oligomers (DP between 2 and 14).
(d) Schematic representation of the reaction catalyzed by At4g20830/OGOX1.
(e) Homology tree of the mature products encoded by *At4g20830/OGOX1* and its five closest paralogs.
(f) Characteristics of the BBE-l members more closely related to At4g20830/OGOX1. Percentage of amino acid identity with OGOX1 is shown. + indicates detection of a sulphite-sensitive OG-oxidizing activity in culture filtrates of *P. pastoris* expressing the proteins; n/a: not applicable, because the protein was not expressed in the yeast; -, no OG-oxidizing activity.
(g) Relative H_2O_2-producing activity of the different OGOXs, tested using the *P. pastoris* culture filtrate and standard OGs as a substrate, at different pH values as indicated.

BBE-l enzyme that catalyzes the conversion of indole-3-acetaldoxime (IAOx) to indole-3-carbonyl nitrile, a metabolite with a role in defence (Rajniak *et al.*, 2015). *CYP81F2* (At5g57220) is a cytochrome P450 essential in pathogen-induced accumulation of indol-3-yl-methyl glucosinolate, a secondary metabolite whose activated form is involved in defense against fungi (Bednarek *et al.*, 2009; Clay *et al.*, 2009) (Figure 4a). Neither the oxidative burst (Figure 4b) nor the deposition of callose, a β-l,3-glucan with defensive role in plant–pathogen interactions (Figure 4c) was triggered by treatment with oxidized OGs. Moreover, oxidized OGs did not interfere with the elicitor activity of canonical OGs, being the co-treatment alone capable of inducing *RetOx* and *CYP81F2* at the same level as that observed with canonical OGs (Figure 4d). In addition, six other genes involved in different defence pathways and previously shown to be up-regulated by OGs (Galletti *et al.*, 2008; Gravino *et al.*, 2017) were analyzed after treatment with oxidized OGs. These genes included the early-induced *WRKY40* (At1g80840), encoding a transcription factor that acts as a negative regulator of basal defence (Xu *et al.*, 2006), and *FRK1*, the expression of which is considered to be strictly regulated by the MAPK cascade (Savatin *et al.*, 2014a; Gravino *et al.*, 2017). They also included the late 1 (maximal induction at 3 h after elicitation) genes, as defined in Gravino *et al.* (2017), *PAD3* (*At2g26830*),

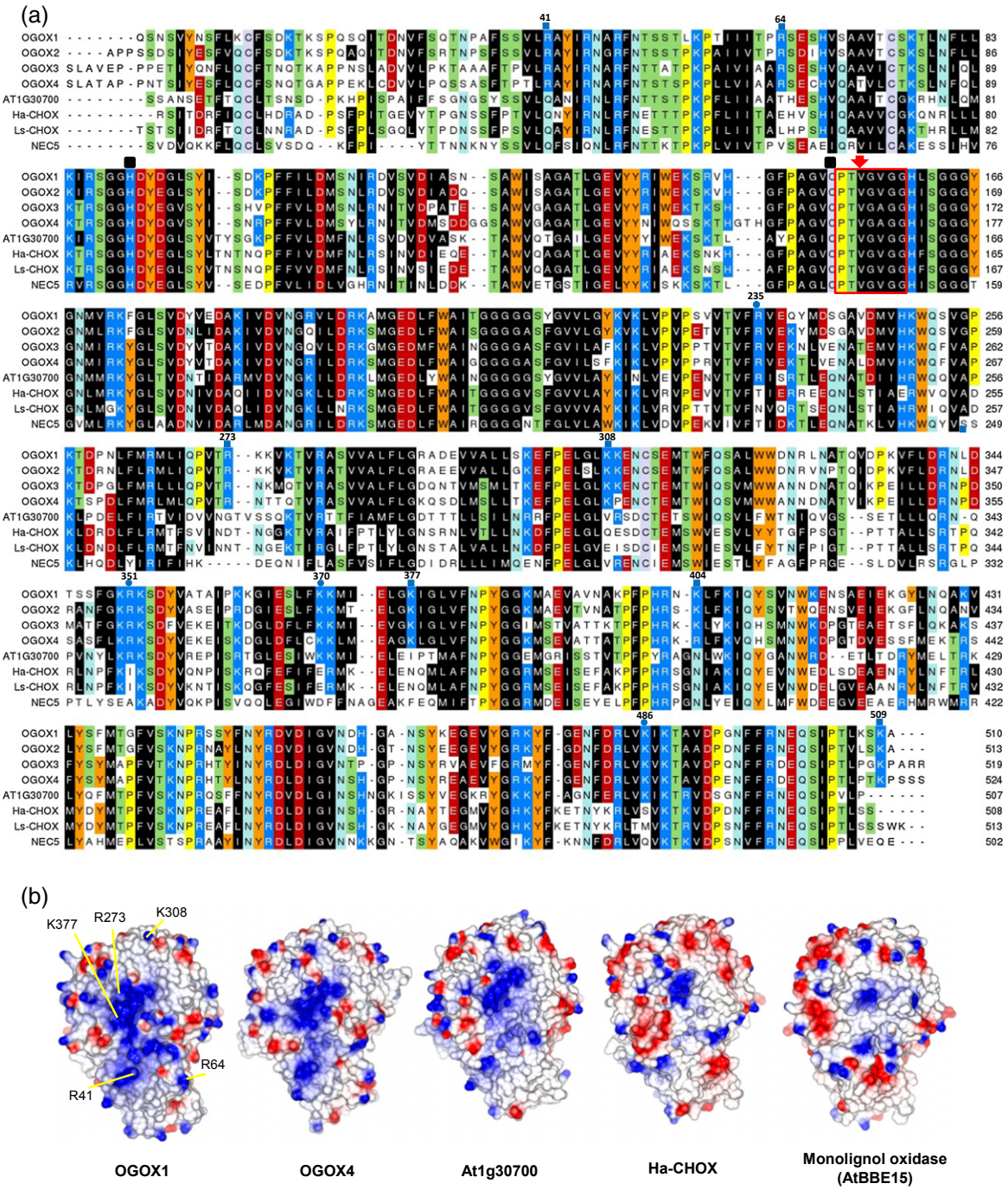


Figure 3. Shared and distinctive features between OGOXs and other BBE-I enzymes.

(a) Multiple amino acid alignment between mature OGOXs, the close paralog At1g30700/BBE8, which lacks both OG- and cellulose fragment-oxidizing activity, and other known BBE-I proteins known as carbohydrate oxidases. Different colours highlight amino acids with different chemical properties; non-polar amino acids are shown in black. A red frame indicates the reactive oxygen motif (PTVGVGG) and the red arrow shows residue V155 as the oxygen reactivity gatekeeper residue. Black squares above the amino acid sequence indicate the sites involved in the covalent binding of the FAD cofactor (H90 and C152); blue symbols indicate positively charged residues at apoplastic pH (histidine, lysine and arginine) that appear only in OGOXs (square) and in both OGOXs and At1g30700 (circles); numbers refer to the position in OGOX1. The short OGOX1 isoform was used for the alignment. NEC5: *Nicotiana* sp. (Nectarin 5; Ha-CHOX and LsCHOX: *Helianthus annuus* and *Lactuca sativa* carbohydrate oxidase), respectively. Additional details on the examined proteins are in Table S3. Numbering is from the first amino acid of the mature proteins.

(b) Electrostatic surface potential representation of OGOX1, OGOX4, At1g30700 and HaCHOX, obtained by homology-based molecular modelling using as a template the crystallographic structure of the monolignol oxidase At2g34790/BBE15 from *A. thaliana*, shown on the right [code 4UD8 in the Protein Data Bank (http://wwpdb.org/)]. Red and blue indicate regions of negative and positive electrostatic potential, respectively. Positively charged residues that are specific of OGOXs are shown on the OGOX1 structure. Two additional OGOX-specific positively charged residues are on the opposite surface.

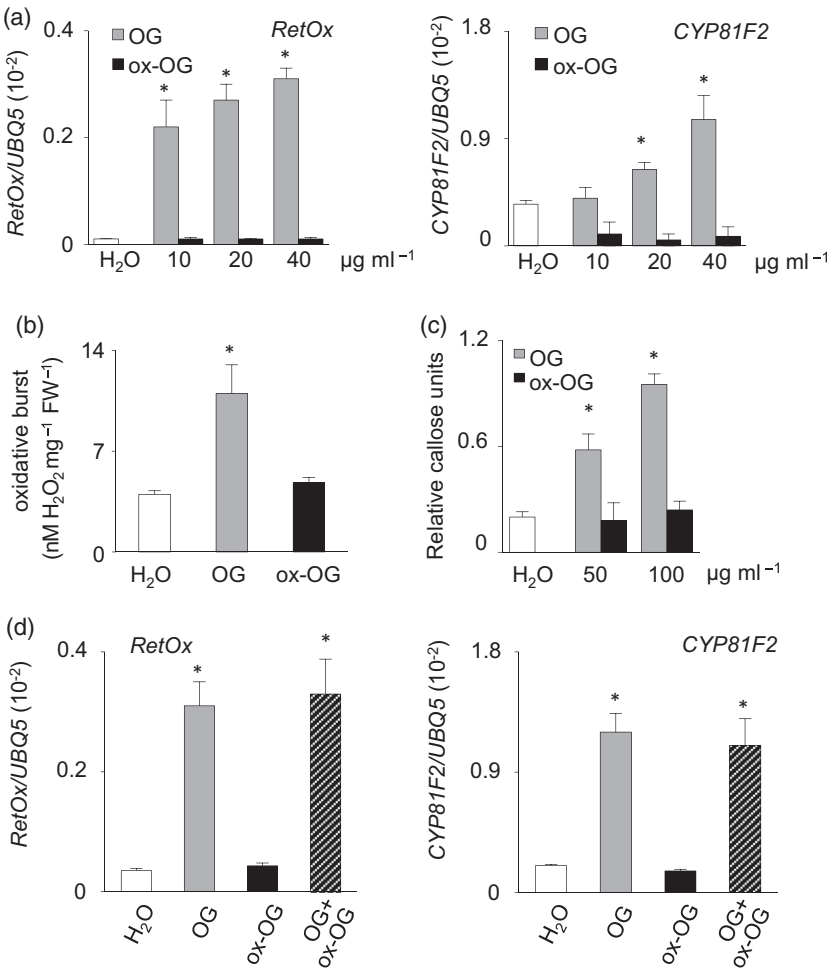


Figure 4. Oxidized OGs are weaker elicitors than non-oxidized OGs.
(a) Gene expression analysis of *RetOx* and *CYP81F2* in seedlings after 1 h treatment with different amounts of OGs and oxidized OGs (ox-OG). Analysis of additional defence genes is shown in Figure S6.
(b) Oxidative burst measured in seedlings after a 30-min treatment with 50 μg ml^{-1} OGs (OG) or ox-OGs.
(c) Callose deposition in adult leaves measured 24 h after infiltration with different amounts of OGs and ox-OGs.
(d) Gene expression analysis of *RetOx* and *CYP81F2* in seedlings after 1 h treatment with OGs or ox-OGs, or co-treatment with OGs plus ox-OGs (OG + ox-OG; 40 μg ml^{-1} each). Asterisk (*) indicates statistical significant difference between elicitor-treated and untreated plant according to Student's *t*-test (*$P < 0.05$).

involved in the synthesis of phytoalexins (Ferrari *et al.*, 2007) and *PGIP1* (At5g06860), encoding a polygalacturonase inhibitor (Ferrari *et al.*, 2003a), as well as the late 2 (maximal induction at 6–8 h) genes *PDF1.2* (AT5G44420) and *PR1* (AT2G14610), encoding an ethylene- and jasmonate-responsive plant defensin and the pathogenesis-related protein 1, respectively (Gravino *et al.*, 2015). All these genes were not induced by treatment with oxidized OGs (Figure S6), further indicating that oxidized OGs display a marked reduction in elicitor activity.

Oxidized OGs are more recalcitrant to hydrolysis by fungal endoPG

Oligogalacturonides formed during plant–pathogen interaction play a dual role: on the one hand, they are degraded to monomers and dimers to provide carbon for the invading microbes and, on the other hand, act as a danger signal for the plant to activate immune responses (Cervone *et al.*, 1989). A very early report described that chemical oxidation of the reducing end to galactaric acid of short oligomers prevents the release of galacturonic acid by a yeast endoPG, which attacks the bond closest to the reducing end (Patel and Phaff, 1959). We investigated the degradability of oxidized OGs by various endoPGs from fungal pathogens, i.e. PGI from *B. cinerea* (Sicilia *et al.*, 2005), PGI from *Colletotrichum lupini* (Bonivento *et al.*, 2008) and PG from *Fusarium phyllophilum* (Benedetti *et al.*, 2011). A pentamer was oxidized using purified OGOX1 from *P. pastoris* and the resulting oxidized product was used as the substrate of the endoPGs in the presence of OGOX1 to quickly oxidize the newly formed reducing ends generated by the ongoing digestion. HPAEC-PAD analysis showed

that, with all the PG tested, the resulting digestion products were approximately equal molar amounts of oxidized trimers and oxidized dimers. The oxidized trimer was, in all cases, completely recalcitrant to further hydrolysis. Conversely, the non-oxidized standard OGs as well as the non-oxidized pentamer and trimer were completely hydrolyzed to GalUA and di-GalUA by all enzymes tested (Figure 5a, representative data obtained with *F. phyllophilum* PG, FpPG).

Response to *B. cinerea* is altered in OGOX1-overexpressing plants

In order to assess if oxidized OGs can be fully degraded by the entire complex pectinolytic machinery of *B. cinerea*, which secretes exo-polygalacturonases and pectin/pectate lyases, as well as PGs, the fungus was grown in a minimal medium containing OGs or oxidized OGs as the sole carbon source. HPAEC-PAD analysis, carried out after 4 days of culture, revealed the presence of the oxidized dimer that could not be further degraded in medium supplied with oxidized OGs, whereas no sugars were detected in the culture filtrates of medium supplied with non-oxidized OGs (Figure 5b). After 4 days of culture, growth of the fungus in the medium supplied with oxidized OGs was less than fungal growth on standard OGs (Figure 5c). Indeed, these data indicated that the fungus was not able to completely degrade and utilize oxidized OGs and consequently did not grow well when OGs were the carbon source.

Following this indication, the effect of high levels of OGOX1 on the response to *B. cinerea* was investigated using two single-insertion homozygous overexpressing lines carrying a CaMV 35S::*OGOX1* construct (OGOX1-OE, #1.9 and #11.8). *OGOX1* transcripts and OG-oxidizing activity were increased in both overexpressing lines (Figure 6a, b). The overexpressing plants were more resistant to the fungus proportionally to the expression level of OGOX1 (Figure 6b,c) and showed a strongly reduced fungal biomass in their tissues at 36 and 48 h (Figure 6d). The overexpressing plants showed a slightly reduced induction of the defence genes *CYP81F2* and *PAD3*, whose expression has been demonstrated to positively correlate with the resistance against fungi (Ferrari *et al.*, 2003b; Bednarek *et al.*, 2009) (Figure 6e). Similarly, expression of *PR1*, a gene commonly used as marker of the salicylic acid-dependent defence pathway, which is also required for local resistance to *B. cinerea* (Ferrari *et al.*, 2003a,b) was slightly reduced in transgenic plants. These data suggest that the increased resistance against *B. cinerea*, exhibited by the OGOX1 overexpressing plants, is not due to a higher activation of defence responses, supporting the hypothesis that it can be ascribed to the difficult digestion of oxidized OGs by the fungus. Consequently, the overexpression of OGOX1 is likely to render plant tissue more recalcitrant to degradation on the one hand and, on the other hand, may contribute to dampen the deleterious hyperactivity of OGs.

DISCUSSION

We have uncovered a biochemical mechanism by which oligogalacturonides are oxidized and inactivated, possibly to prevent the deleterious effects of their excessive

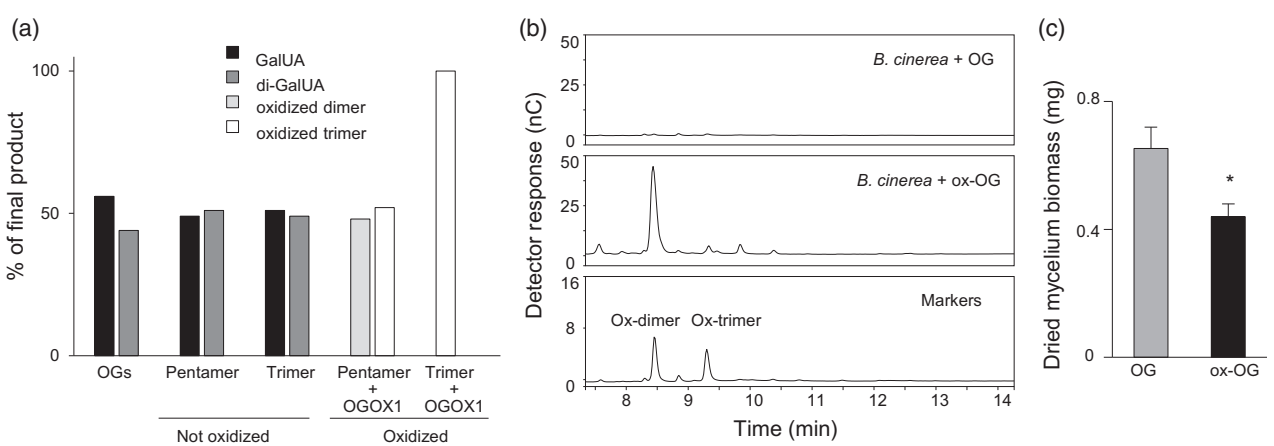


Figure 5. Oxidized OGs are not efficiently hydrolyzed by fungal endoPGs.
(a) Sugars released from the indicated oligosaccharides (not oxidized or oxidized) after digestion with *Fusarium phyllophylum* polygalacturonase (FpPG) as determined by HPAEC-PAD analysis. Sugars released from the oxidized pentamer and trimer by FpPG in the presence of OGOX1 are also shown. Sugars are indicated as percentage of final product. Similar results were obtained using *Colletotrichum lupini* PGI and *B. cinerea* PGI. A representative experiment is here reported.
(b) HPAEC-PAD analysis of culture filtrate of *B. cinerea* grown for 96 h in a medium supplemented with 0.15% OGs or 0.15% oxidized OGs (in panel b, *B. cinerea* + OG and *B. cinerea* + ox-OG, respectively). Signal intensities (nC) at each retention time (min) are indicated; standard oxidized oligomers are also shown (markers).
(c) Biomass of *B. cinerea* grown as in (b); bars indicate mean ± SD, and an asterisk (*) indicates statistically significant differences according to Student's *t*-test ($P < 0.05$).

accumulation. A FAD-dependant oxidase, specific for these oligosaccharides and inactive on other polysaccharides or sugars, was identified, purified and named OGOX1. OGOX1 is encoded by a member of the so-called berberine bridge enzyme-like (BBE-like) family (Daniel *et al.*, 2017). OGOX1 activity is inhibited by sulphites and exhibits a high pH optimum compatible with increasing apoplastic pH that generally occurs when tissue is treated with OGs. Subsequently, by sequence homology and expression in *P. pastoris*, three other OG oxidases belonging to the same BBE-like family were identified. All four OGOX enzymes are FAD-dependent and sulphite-sensitive and presumably have different and/or complementary functions, as suggested by their different pH dependence. Exploring the function and physiological role of the four OGOXs will be the object of future investigations.

Among the flavoproteins, family pfam 08031, characterized by an unusual bicovalent attachment of the FAD cofactor, was named as the 'BBE-like protein family' after the identification in *Eschscholzia californica* of an enzyme involved in the biosynthesis of isoquinoline alkaloids (Kutchan and Dittrich, 1995; Facchini *et al.*, 1996) that catalyzes the formation of the so-called 'berberine bridge' by oxidation of the *N*-methyl group of (*S*)-reticuline (Winkler *et al.*, 2006). A well characterized BBE-like protein is nectarin 5 (NEC5) from *Nicotiana* sp., which oxidizes glucose (Carter and Thornburg, 2004). BBE-like enzymes from *Helianthus annus* and *Lactuca sativa* have been reported to oxidize cellulose-derived fragments as well as cellobiose and glucose (Custers *et al.*, 2004). In Arabidopsis, the BBE-like family comprises 27 members (Carter and Thornburg, 2004) in which *At4g20830*, now named *OGOX1*, encodes two different isoforms, thus increasing the number of BBE-like enzymes to 28 (Hille *et al.*, 2012; Daniel *et al.*, 2015). So far, a function has been ascribed only to *At2g34790/BBE15*, which encodes a monolignol oxidase (Daniel *et al.*, 2015) and *At1g26380/BBE3/FOX1*, routinely exploited as a defence marker gene, whose product catalyzes the conversion of indole-3-acetaldoxime (IAOx) to indole-3-carbonyl nitrile (ICN), a metabolite with a role in inducible pathogen defence (Rajniak *et al.*, 2015). Therefore, the overall involvement of the BBE-l family in immunity and defence is markedly increasing. A knowledge of the single and combined function of the family members may open new perspectives and scenarios in the field and have in the future a great effect on the biotechnological improvement of crop disease resistance.

BBE-like enzymes have also been characterized in phytopathogenic fungi. In both *Sarocladium strictum* and *Microdochium nivale* (Vuong *et al.*, 2013) enzymes acting on mono-, oligo-, or polymeric saccharides and preferentially on cello-oligosaccharides, but not on uronic acids, have been characterized, whereas a chito-oligosaccharide oxidase has been described in *Fusarium graminearum* (Heuts *et al.*, 2008).

The 3D structure of the *S. strictum* glucose oxidase (Foumani *et al.*, 2011; Vuong *et al.*, 2013), the Arabidopsis monolignol oxidase At2g34790/BBE15 (Daniel *et al.*, 2015) and of the At5g44440/BBE28 (Daniel *et al.*, 2016) have been solved. Positive charges have been identified in glucose oxidase as well as in other FAD-dependent enzymes such as monomeric sarcosine oxidase, *N*-methyltryptophan oxidase and fructosamine oxidase that electrostatically stabilize the transition state for the initial single electron transfer that generates the $O_2^{-\bullet}$/flavin semiquinone radical pair (Gadda, 2012). Additional positively charged residues such as arginine or lysine are present in OGOXs that may facilitate binding of the enzyme and the correct positioning of the OG substrate, by forming salt bridges with the negatively charged carboxylic acid groups. Similarly, a cluster of four regularly spaced positively charged amino acids (arginine and/or lysine) has been identified as a pectin binding site on the surface of cationic peroxidases and PGIP (Carpin *et al.*, 2001; Spadoni *et al.*, 2006). Interestingly, positive patches, present at the entrance of the cleft in hyaluronan lyases from *Streptococcus pneumoniae* and *Streptococcus agalactiae*, have been proposed to assist in drawing the substrate into the cleft (Jedrzejas *et al.*, 2002).

Microbial enzymes quickly degrade homogalacturonan to dimers and monomers utilized by pathogens as a carbon source for their growth. It is well known that the presence in the cell wall of inhibitors like PGIPs retards the degradation of homogalacturonan (Jolie *et al.*, 2010; Kalunke *et al.*, 2015). In this paper we report that modification of OGs by OGOXs occurs at the reducing galacturonic acid that is oxidized to galactaric acid. Our results suggest that the OG oxidation by OGOXs provides an additional mechanism by which degradation of homogalacturonan fragments is retarded and the final utilization of the hydrolysis products by microbes is impeded. According to our results, the enzymatic degradation of oxidized OGs mainly produces oxidized trimers and dimers, which *in vitro* cannot be further metabolized by the endoPG of fungal pathogens, including *B. cinerea*. If oxidized OGs are utilized as substrates for *B. cinerea* growth, accumulation of oxidized dimer and no appearance of oxidized trimer was observed, probably as a result of an exo-polygalacturonase (exoPG) activity that may convert the oxidized trimer in oxidized dimer. It is known that this enzyme acts at the non-reducing end of OGs, thus avoiding the steric hindrance due to the galactaric acid at the opposite end (Rha *et al.*, 2001).

As far as the activity of OGs is concerned, structural modifications of OGs at the reducing end by chemical methods, i.e. oxidation by diluted nitric acid, have already been shown to alter their biological activity (Spiro *et al.*, 1998). This paper demonstrates that such an oxidation and consequent inactivation of biological activity occurs enzymatically *in vivo*. Oxidized OGs possess a markedly lower eliciting activity than non-oxidized OGs. At the same time,

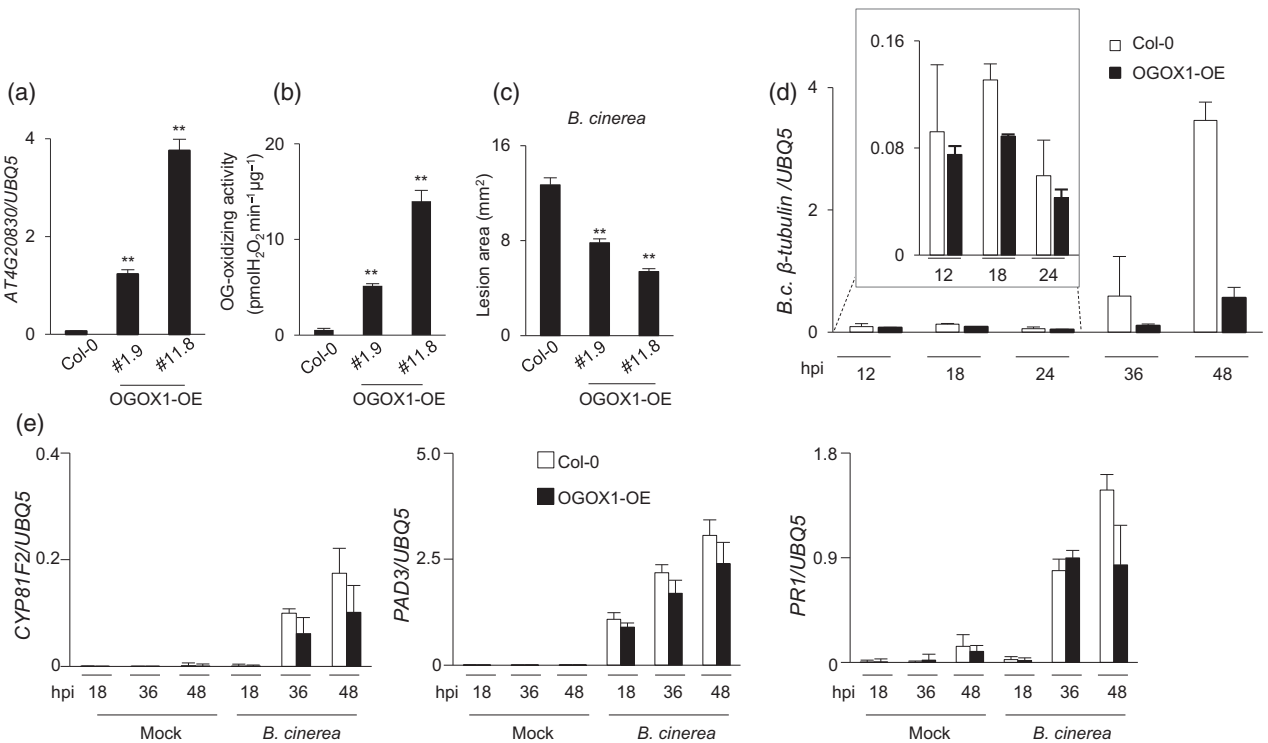


Figure 6. OGOX1 plays a role in plant defence.

(a–c) Levels of (a) OGOX1 transcripts and (b) OG-oxidizing activity in leaves in the OGOX1 overexpressing plants (OGOX1-OE #1.9 and #11.8). (c) Lesion area in adult excised leaves of the overexpressing lines drop-inoculated with *Botrytis cinerea* conidia. In (a–c), asterisks indicate statistically significant differences against control (Col-0), according to Student's *t*-test (**$P < 0.01$).

(d) Expression level of *B. cinerea* β-tubulin, normalized against Arabidopsis *UBQ5*, in infected wild-type and OGOX1-OE#11.8 leaves at different hours post-infection (hpi).

(e) Gene expression analysis in excised adult leaves of wild-type and OGOX1-OE#11.8 plants. Leaves were drop-inoculated with *B. cinerea* conidia or PDB (mock treatment) as a control. *CYP81F2, PAD3* and *PR1* transcripts levels were analyzed by qRT-PCR after 18, 36 and 48 h post inoculation. Transcripts levels of the genes were normalized on the *UBQ5* transcript levels.

as uncovered in this study, they are less efficiently hydrolysed by microbial endoPGs into the typical end-reaction products, i.e. di-galacturonic and galacturonic acid utilized by microbes as a carbon source. The latter feature probably renders the transgenic OGOX1-overexpressing plants more resistant to *B. cinerea*. So, we can speculate that, if expressed at the right timing, OGOXs may convert the elicitor-active OGs into elicitor-inactive OGs to prevent the deleterious effects of their hyper-accumulation and, at the same time, this can be beneficial for the plant because oxidized OGs display high tolerance to hydrolysis by the fungal pectinolytic machinery.

In accordance with our results, overexpression of carbohydrate oxidase from sunflower in transgenic tobacco conferred resistance against the bacterial necrotroph *P. carotovorum* ssp. *carotovorum*, confirming a role in active defence for this class of plant oxidoreductases (Custers *et al.*, 2004). It is also worth noting that the oxidase activity of OGOXs is accompanied by the production of H_2O_2 and this molecule may play different roles such as a defensive role, a cell wall strengthen-factor and a second messenger in signaling (Quan *et al.*, 2008). If locally produced at the sites of the cell wall ruptures, H_2O_2 can contribute to promptly repair the wounds occurring during microbial attacks as well as the micro-lesions formed during plant growth and development. Further studies will clarify the role of the four OGOXs, and their interplay, in defence against several pathogens as well as in plant growth and development.

The identification of four members of the Arabidopsis BBE-like family as OG oxidases opens to further study the other possible oligosaccharide oxidases comprised in the family and involved in the turnover/metabolism of cell wall-derived signals like, for example, cellodextrins and cellobiose shown to possess a DAMP activity in grapevine (Aziz *et al.*, 2007) and more recently in Arabidopsis (Souza *et al.*, 2017). As mentioned above, BBE-like enzymes capable of oxidizing cellodextrins and cellobiose occur in sunflower (Custers *et al.*, 2004). Indeed, the diversity of oligosaccharide fragments embedded in the cell wall has been hypothesized as a possible reservoir of signal molecules for a variety of biological functions (Hahn, 1981; Hamann, 2015). Also the involvement in sensing the cell wall integrity of receptors like Theseus 1 and others (Van

der Does *et al.*, 2017), which at the moment remain orphan receptors, may indicate that other cell wall fragments may act as signals. The knowledge about the existence and the action of these molecules, however, is still very limited. Much is unknown about how many oligosaccharides other than OGs are released from the cell wall for specific signalling purposes. Further characterization of the BBE-like family may help to elucidate this aspect of cell wall biology.

EXPERIMENTAL PROCEDURES

Plant material, treatments and preparation of leaf diffusates

Plant material and treatments are described in Methods S1. Diffusates were prepared as described in Benedetti *et al.* (2017) with some modifications. Leaves were excised from 25-day-old OGM plants 170 h after spraying with 25 μM-estradiol, sterilized in 3 ml of 1% sodium hypochlorite for 3 min, and extensively washed with ultrapure water for at least four times. Leaves were cut in thin strips and about 90–100 mg of tissue were incubated in 50 mM ammonium acetate pH 5.0, 50 mM CDTA and 50 mM ammonium oxalate for 16 h at 30°C. After incubation, the medium was collected and precipitated by adding ethanol to a final concentration of 80% (v/v); the sample was directly centrifuged at 15 000 ***g*** for 30 min. Subsequently, the pellet was solubilized in 0.2 ml ultrapure water and incubated at 65°C for 20 min in order to eliminate any residual enzymatic activity. Finally, the sample was centrifuged at 3000 ***g*** for 3 min and the supernatant was collected for high-performance anion-exchange chromatography coupled with pulsed electrochemical detection (HPAEC-PAD) (Pontiggia *et al.*, 2015) and ESI-MS analyses, as described in Methods S2.

Enzymatic assay and purification of the OG-oxidizing activity

For enzyme assay, total proteins (5 μg) were assayed in 50 mM Tris–HCl pH 8.8, 50 mM NaCl, using OGs (1 mg ml^{-1}) as a substrate. Hydrogen peroxide generated in the OG oxidation reaction was quantified through a xylenol orange assay (Gay *et al.*, 1999). Enzyme activity was expressed as μmol H_2O_2 min^{-1} μg protein $extract^{-1}$. Preparation of total protein extracts from leaves or seedlings for detection of the OG-oxidizing activity is described in Methods S3 and identification of the OG oxidase by proteomic analysis in Methods S4. OG oxidase activity was purified from leaves of β-estradiol-induced OGM plants as reported in Methods S5.

Expression in *Pichia pastoris* and characterization of the OGOXs

Expression of the mature short isoform At4g20830.2/OGOX1, At4g20840, At1g11770, At1g01980, At1g30740 and At1g30700 in *P. pastoris*, and purification of OGOX1 from culture filtrates are described in Methods S6. Enzyme assays and substrate specificity analyses are described in Methods S7. Bioinformatic analyses are detailed in Methods S8.

Preparation of oxidized OGs and biological and degradation assays

Standard OGs (20 mg) were dissolved in 5 ml of 50 mM Tris–HCl pH 8.8, 50 mM NaCl. The mixture was incubated at 28°C for 16 h with 5 μg of pure OGOX1 (short isoform) expressed in *P. pastoris* as described in Methods S6. After incubation, a small aliquot was analysed by HPEAC-PAD in order to verify the complete oxidation. The sample was incubated at 65°C for 20 min in order to inactivate the enzyme and ethanol was added to a final concentration of 20%. After 2 h at 4°C, the sample was centrifuged at 25 000 ***g*** for 20 min and the supernatant was discarded. The pellet was dried by a Savant SpeedVac Concentrator and dissolved in 200 μl ultrapure water. As a control, the same procedure was carried out on 20 mg of standard OGs in the presence of the boiled enzyme. For the preparation of single oxidized OG oligomers, trigalacturonic acid (1 μg) and pentagalacturonic acid (3 μg) were dissolved separately in 0.1 ml of 50 mM Na-acetate pH 5.0 and 50 mM NaCl. The reaction mixtures were incubated with 0.1 μg of OGOX1 from *P. pastoris* for 16 h at 28°C. After incubation, an aliquot was analysed by HPEAC-PAD to verify the complete oxidation. The sample was incubated at 65°C for 20 min in order to inactivate the enzyme. Oxidized digalacturonic acid, which was used as a reference marker in the subsequent analyses, was purified from leaf diffusates of 25-day-old OGM plants induced with β-estradiol by HPEAC and checked by ESI-MS analyses. Biological activity and *in vitro* hydrolysis by pectic enzymes of the oxidized OGs were carried out as described in Methods S9 and S10, respectively.

Generation of transgenic plants and infection assay

The At4g20830 DNA sequence from the translation initiation codon to the termination codon of the At4g20830.1 isoform, therefore encompassing the intron, was amplified from Arabidopsis gDNA using the *Bam*HIAt4g20830Fw and *Sac*IAt4g20830Rv primers (Table S4). The fragment was cloned using the *Bam*HI and *Sac*I restriction sites of pBI121, replacing the β-glucuronidase gene sequence. The recombinant plasmid was introduced into *A. tumefaciens* GV3101 strain by electroporation and *A. thaliana* plants were transformed using the floral-dip method. From 11 independent transformed lines, two T3 homozygous lines (OGOX1-OE #1.9 and #11.8) containing a single insertion carrying the CaMV 35S:OGOX1 cassette were selected for further analyses. *Botrytis cinerea* infection was performed and analyzed as previously described (Benedetti *et al.*, 2015). Gene expression analyses of *B. cinerea*-infected leaves was performed by qRT-PCR and transcripts levels of the marker genes were normalized on the *UBQ5* transcript levels.

Botrytis cinerea growth assay and analysis

Botrytis cinerea growth assays were performed in a 24-well MULTIWELL plate (Falcon, Becton Dickinson Labware) containing 0.5 ml of a modified Pectic Zymogram (PZ) medium [20 mM $(NH_4)_2SO_4$, 2.5 mM KH_2PO_4 and 0.6 mM $MgSO_4$]; for growth in presence of OGs, PZ was supplied with 0.15% (w/v) of OGs and oxidized OGs (DP4–10, prepared as described in Methods S11) and pH was adjusted to 4.7 in all cases. Each well was inoculated with 7×10^4 conidiospores. Twelve replicates were prepared for each sample. Plates were incubated at 22°C for 96 h at 75 rpm. For fungal biomass determination, three pools of four replicates were obtained, dried and weighted. Standard deviation was calculated by the mean of the three different pools. For determination of sugar content, culture filtrate (0.1 μl or 1 μl) was analyzed by HPAEC-PAD analysis.

ACCESSION NUMBERS

At4g20830, At4g20840, At1g01980, At1g11770.

ACKNOWLEDGEMENTS

This work was supported by the European Research Council (Advanced Grant no. 233083 'FUEL-PATH' awarded to F.C.), by the Ministero delle Politiche Agricole, Alimentari e Forestali (grant BIOMASSVAL awarded to F.C. and grant FITOLISI awarded to G.D.L.), by the Ministero dell'Università e della Ricerca Scientifica (grant PRIN2009 awarded to G.D.L.) and by the Institute Pasteur Fondazione Cenci Bolognetti.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The atypical OGs in the diffusates contain a galactaric acid.

Figure S2. The OG-oxidizing activity from OGM plants is accompanied by H_2O_2 production.

Figure S3. The gene *At4g20830* encodes two different OGOX1 isoforms.

Figure S4. Expression of *myc*-tagged BBEl-proteins in *Pichia pastoris*.

Figure S5. Overall topology of OGOX1 and AtBBE-like 15.

Figure S6. Oxidized OGs display a markedly reduced capability of eliciting defence responses.

Table S1. Proteins identified in the OGM leaf protein extract after elution from polygalacturonic acid affinity chromatography as determined by LC-MS analysis.

Table S2. Proteins identified by LC-MS analysis in the fractions Fx17 and Fx18, eluted from the hydrophobic interaction chromatography.

Table S3. Sequences used for the amino acid alignment of the BBE-l enzymes described as carbohydrate oxidases in plants.

Table S4. Primers for the construction of cassettes for BBE-l protein expression in *Pichia pastoris* and for the overexpression of OGOX1 in *Arabidopsis thaliana* (in bold).

Data S1. Calculation of K_M and V_{max} of the short OGOX1 isoform (excel file).

Data S2. The 3D structural model of the short OGOX1 isoform (pdb file). The model was obtained by homology-based molecular modelling using as template the crystallographic structure of Arabidopsis At2g34790/BBE15.

Methods S1. Plant material and treatments.

Methods S2. Chromatographic and mass spectrometric analysis of standard and modified OGs.

Methods S3. Detection of the OG-oxidizing activity in plant extracts.

Methods S4. Identification of the OG oxidase by proteomic analysis.

Methods S5. Purification of the OG-oxidizing activity.

Methods S6. Expression in *Pichia pastoris*, protein purification and biochemical characterization.

Methods S7. Biochemical characterization of OG oxidases.

Methods S8. Bioinformatics analyses.

Methods S9. Gene expression analysis, oxidative burst and callose measurements of *Arabidopsis thaliana*.

Methods S10. *In vitro* hydrolysis of OG oligomers.

Methods S11. Preparation of oxidized OGs for *Botrytis cinerea* growth assays.

REFERENCES

Aziz, A., Gauthier, A., Bezler, A., Poinssot, B., Joubert, J.M., Pugin, A., Heyraud, A. and Baillieul, F. (2007) Elicitor and resistance-inducing activities of beta-1,4 cellodextrins in grapevine, comparison with beta-1,3 glucans and alpha-1,4 oligogalacturonides. *J. Exp. Bot.* **58**, 1463–1472.

Bednarek, P., Pislewska-Bednarek, M., Svatos, A. *et al.* (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science*, **323**, 101–106.

Benedetti, M., Leggio, C., Federici, L., De Lorenzo, G., Pavel, N.V. and Cervone, F. (2011) Structural resolution of the complex between a fungal polygalacturonase and a plant polygalacturonase-inhibiting protein by small-angle X-ray scattering. *Plant Physiol.* **157**, 599–607.

Benedetti, M., Pontiggia, D., Raggi, S., Cheng, Z., Scaloni, F., Ferrari, S., Ausubel, F.M., Cervone, F. and De Lorenzo, G. (2015) Plant immunity triggered by engineered in vivo release of oligogalacturonides, damage-associated molecular patterns. *Proc. Natl Acad. Sci. USA*, **112**, 5533–5538.

Benedetti, M., Mattei, B., Pontiggia, D., Salvi, G., Savatin, D.V. and Ferrari, S. (2017) Methods of isolation and characterization of oligogalacturonide elicitors. *Methods Mol. Biol.* **1578**, 25–38.

Bonivento, D., Pontiggia, D., Di Matteo, A., Fernandez-Recio, J., Salvi, G., Tsernoglou, D., Cervone, F., De Lorenzo, G. and Federici, L. (2008) Crystal structure of the endopolygalacturonase from the phytopathogenic fungus *Colletotrichum lupini* and its interaction with polygalacturonase-inhibiting proteins. *Proteins*, **70**, 294–299.

Boudsocq, M., Willmann, M.R., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, S.H. and Sheen, J. (2010) Differential innate immune signalling via Ca^{2+} sensor protein kinases. *Nature*, **464**, 418–422.

Cantu, D., Greve, L.C., Lurie, S. and Labavitch, J.M. (2006) Detection of uronic oxidase activity in ripening peaches. *Phytochemistry*, **67**, 13–18.

Carpin, S., Crevecoeur, M., de Meyer, M., Simon, P., Greppin, H. and Penel, C. (2001) Identification of a Ca(2+)-pectate binding site on an apoplastic peroxidase. *Plant Cell*, **13**, 511–520.

Carter, C.J. and Thornburg, R.W. (2004) Tobacco nectarin V is a flavin-containing berberine bridge enzyme-like protein with glucose oxidase activity. *Plant Physiol.* **134**, 460–469.

Casasoli, M., Federici, L., Spinelli, F., Di Matteo, A., Vella, N., Scaloni, F., Fernandez-Recio, J., Cervone, F. and De Lorenzo, G. (2009) Integration of evolutionary and desolvation energy analysis identifies functional sites in a plant immunity protein. *Proc. Natl Acad. Sci. USA*, **106**, 7666–7671.

Cervone, F., De Lorenzo, G., Degrà, L., Salvi, G. and Bergami, M. (1987a) Purification and characterization of a polygalacturonase- inhibiting protein from *Phaseolus vulgaris* L. *Plant Physiol.* **85**, 631–637.

Cervone, F., De Lorenzo, G., Degrà, L. and Salvi, G. (1987b) Elicitation of necrosis in *Vigna unguiculata* Walp. by homogeneous *Aspergillus niger* endo-polygalacturonase and by α-D-galacturonate oligomers. *Plant Physiol.* **85**, 626–630.

Cervone, F., Hahn, M.G., De Lorenzo, G., Darvill, A. and Albersheim, P. (1989) Host-pathogen interactions. XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. *Plant Physiol.* **90**, 542–548.

Clay, N.K., Adio, A.M., Denoux, C., Jander, G. and Ausubel, F.M. (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science*, **323**, 95–101.

Cook, D.E., Mesarich, C.H. and Thomma, B.P. (2015) Understanding plant immunity as a surveillance system to detect invasion. *Annu. Rev. Phytopathol.* **53**, 541–563.

Custers, J.H., Harrison, S.J., Sela-Buurlage, M.B. *et al.* (2004) Isolation and characterisation of a class of carbohydrate oxidases from higher plants, with a role in active defence. *Plant J.* **39**, 147–160.

Daniel, B., Pavkov-Keller, T., Steiner, B. *et al.* (2015) Oxidation of monolignols by members of the berberine-bridge enzyme family suggests a role in plant cell wall metabolism. *J. Biol. Chem.* **290**, 18770–18781.

Daniel, B., Wallner, S., Steiner, B., Oberdorfer, G., Kumar, P., van der Graaff, E., Roitsch, T., Sensen, C.W., Gruber, K. and Macheroux, P. (2016) Structure of a berberine bridge enzyme-like enzyme with an active site specific to the plant family brassicaceae. *PLoS ONE*, **11**, e0156892.

Daniel, B., Konrad, B., Toplak, M., Lahham, M., Messenlehner, J., Winkler, A. and Macheroux, P. (2017) The family of berberine bridge enzyme-like

enzymes: a treasure-trove of oxidative reactions. *Arch. Biochem. Biophys.* **632**, 88–103. https://doi.org/10.1016/j.abb.2017.06.023.

Davidsson, P., Broberg, M., Kariola, T., Sipari, N., Pirhonen, M. and Palva, E.T. (2017) Short oligogalacturonides induce pathogen resistance-associated gene expression in *Arabidopsis thaliana*. *BMC Plant Biol.* **17**, 19.

De Lorenzo, G., D'Ovidio, R. and Cervone, F. (2001) The role of polygacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu. Rev. Phytopathol.* **39**, 313–335.

Duran-Flores, D. and Heil, M. (2016) Sources of specificity in plant damaged-self recognition. *Curr. Opin. Plant Biol.* **32**, 77–87.

Elortza, F., Mohammed, S., Bunkenborg, J., Foster, L.J., Nuhse, T.S., Brodbeck, U., Peck, S.C. and Jensen, O.N. (2006) Modification-specific proteomics of plasma membrane proteins: identification and characterization of glycosylphosphatidylinositol-anchored proteins released upon phospholipase D treatment. *J. Proteome Res.* **5**, 935–943.

Facchini, P.J., Penzes, C., Johnson, A.G. and Bull, D. (1996) Molecular characterization of berberine bridge enzyme genes from opium poppy. *Plant Physiol.* **112**, 1669–1677.

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F. and De Lorenzo, G. (2003a) Tandemly duplicated Arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. *Plant Cell*, **15**, 93–106.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G. and Ausubel, F. (2003b) Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J.* **35**, 193–205.

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F.M. and Dewdney, J. (2007) Resistance to *Botrytis cinerea* induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. *Plant Physiol.* **144**, 367–379.

Ferrari, S., Savatin, D.V., Sicilia, F., Gramegna, G., Cervone, F. and De Lorenzo, G. (2013) Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Front. Plant Sci.* **4**, 49. https://doi.org/10.3389/fpls.2013.00049.

Foumani, M., Vuong, T.V. and Master, E.R. (2011) Altered substrate specificity of the gluco-oligosaccharide oxidase from *Acremonium strictum*. *Biotechnol. Bioeng.* **108**, 2261–2269.

Gadda, G. (2012) Oxygen activation in flavoprotein oxidases: the importance of being positive. *Biochemistry*, **51**, 2662–2669.

Galletti, R., Denoux, C., Gambetta, S., Dewdney, J., Ausubel, F.M., De Lorenzo, G. and Ferrari, S. (2008) The AtrbohD-mediated oxidative burst elicited by oligogalacturonides in Arabidopsis is dispensable for the activation of defense responses effective against *Botrytis cinerea*. *Plant Physiol.* **148**, 1695–1706.

Gay, C., Collins, J. and Gebicki, J.M. (1999) Hydroperoxide assay with the ferric-xylenol orange complex. *Anal. Biochem.* **273**, 149–155.

Gravino, M., Savatin, D.V., Macone, A. and De Lorenzo, G. (2015) Ethylene production in *Botrytis cinerea* and oligogalacturonide-induced immunity requires calcium-dependent protein kinases. *Plant J.* **84**, 1073–1086.

Gravino, M., Locci, F., Tundo, S., Cervone, F., Savatin, D.V. and De Lorenzo, G. (2017) Immune responses induced by oligogalacturonides are differentially affected by AvrPto and loss of BAK1/BKK1 and PEPR1/PEPR2. *Mol. Plant Pathol.* **18**, 582–595.

Hahn, M.G. (1981) *Fragments of plant and fungal cell wall polysaccharides elicit the accumulation of phytoalexins in plants*. PhD Thesis, University of Colorado.

Hamann, T. (2015) The plant cell wall integrity maintenance mechanism-concepts for organization and mode of action. *Plant Cell Physiol.* **56**, 215–223.

Heuts, D.P., Winter, R.T., Damsma, G.E., Janssen, D.B. and Fraaije, M.W. (2008) The role of double covalent flavin binding in chito-oligosaccharide oxidase from *Fusarium graminearum*. *Biochem. J.* **413**, 175–183.

Hille, R., Miller, S., Palfey, B., *et al.* (2012) Handbook of flavoproteins: oxidases, dehydrogenases and related systems, vol. **1**. Berlin: de Gruyter.

Huang, C.H., Lai, W.L., Lee, M.H., Chen, C.J., Vasella, A., Tsai, Y.C. and Liaw, S.H. (2005) Crystal structure of glucooligosaccharide oxidase from *Acremonium strictum* – a novel flavinylation of 6-S-cysteinyl, 8 alpha-N1-histidyl FAD. *J. Biol. Chem.* **280**, 38831–38838.

Jedrzejas, M.J., Mello, L.V., de Groot, B.L. and Li, S. (2002) Mechanism of hyaluronan degradation by *Streptococcus pneumoniae* hyaluronate lyase. Structures of complexes with the substrate. *J. Biol. Chem.* **277**, 28287–28297.

Jolie, R.P., Duvetter, T., Van Loey, A.M. and Hendrickx, M.E. (2010) Pectin methylesterase and its proteinaceous inhibitor: a review. *Carbohydr. Res.* **345**, 2583–2595.

Kalunke, R.M., Tundo, S., Benedetti, M., Cervone, F., De Lorenzo, G. and D'-dio, R. (2015) An update on polygalacturonase-inhibiting protein (PGIP), a leucine-rich repeat protein that protects crop plants against pathogens. *Front. Plant Sci.* **6**, 146.

Kutchan, T.M. and Dittrich, H. (1995) Characterization and mechanism of the berberine bridge enzyme, a covalently flavinylated oxidase of benzophenanthridine alkaloid biosynthesis in plants. *J. Biol. Chem.* **270**, 24475–24481.

Liston, A. and Masters, S.L. (2017) Homeostasis-altering molecular processes as mechanisms of inflammasome activation. *Nat. Rev. Immunol.* **17**, 208–214.

Marsh, C.A. (1985) Identification of uronic-acid oxidase in plant peroxidase preparations. *Phytochemistry*, **24**, 1649–1652.

Mathieu, Y., Kurkdijan, A., Xia, H., Guern, J., Koller, A., Spiro, M., O'Neill, M., Albersheim, P. and Darvill, A. (1991) Membrane responses induced by oligogalacturonides in suspension-cultured tobacco cells. *Plant J.* **1**, 333–343.

Mattevi, A. (2006) To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes. *Trends Biochem. Sci.* **31**, 276–283.

Patel, D.S. and Phaff, H.J. (1959) On the mechanism of action of yeast endopolygalacturonase on oligogalacturonides and their reduced and oxidized derivatives. *J. Biol. Chem.* **234**, 237–241.

Pontiggia, D., Ciarcianelli, J., Salvi, G., Cervone, F., De Lorenzo, G. and Mattei, B. (2015) Sensitive detection and measurement of oligogalacturonides in Arabidopsis. *Front. Plant Sci.* **6**, 258.

Quan, L.J., Zhang, B., Shi, W.W. and Li, H.Y. (2008) Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *J. Integr. Plant Biol.* **50**, 2–18.

Rajniak, J., Barco, B., Clay, N.K. and Sattely, E.S. (2015) A new cyanogenic metabolite in Arabidopsis required for inducible pathogen defence. *Nature*, **525**, 376–379.

Rha, E., Park, H.J., Kim, M.O., Chung, Y.R., Lee, C.W. and Kim, J.W. (2001) Expression of exo-polygalacturonases in *Botrytis cinerea*. *FEMS Microbiol. Lett.* **201**, 105–109.

Riov, J. (1975) Metabolism of uronic acids in plant tissues: partial purification and properties of uronic acid oxidase from citrus leaves. *Plant Physiol.* **55**, 602–606.

Savatin, D.V., Gramegna, G., Modesti, V. and Cervone, F. (2014a) Wounding in the plant tissue: the defense of a dangerous passage. *Front. Plant Sci.* **5**, 470. https://doi.org/10.3389/fpls.2014.00470.

Savatin, D.V., Gigli, B.N., Marti, L., Fabbri, C., Cervone, F. and De Lorenzo, G. (2014b) The Arabidopsis NPK1-related protein kinases ANPs are required for elicitor-induced oxidative burst and immunity. *Plant Physiol.* **165**, 1188–1202.

Senechal, F., Wattier, C., Rusterucci, C. and Pelloux, J. (2014) Homogalacturonan-modifying enzymes: structure, expression, and roles in plants. *J. Exp. Bot.* **65**, 5125–5160.

Sicilia, F., Fernandez-Recio, J., Caprari, C., De Lorenzo, G., Tsernoglou, D., Cervone, F. and Federici, L. (2005) The polygalacturonase-inhibiting protein PGIP2 of *Phaseolus vulgaris* has evolved a mixed mode of inhibition of endopolygalacturonase PG1 of *Botrytis cinerea*. *Plant Physiol.* **139**, 1380–1388.

Souza, C.A., Li, S., Lin, A.Z., Boutrot, F., Grossmann, G., Zipfel, C. and Somerville, S.C. (2017) Cellulose-derived oligomers act as damage-associated molecular patterns and trigger defense-like responses. *Plant Physiol.* **173**, 2383–2398.

Spadoni, S., Zabotina, O., Di Matteo, A., Mikkelsen, J.D., Cervone, F., Dl, G., Mattei, B. and Bellincampi, D. (2006) Polygalacturonase-inhibiting protein (PGIP) interacts with pectin through a binding site formed by four clustered residues of arginine and lysine. *Plant Physiol.* **141**, 557–564.

Spiro, M.D., Ridley, B.L., Eberhard, S., Kates, K.A., Mathieu, Y., O'Neill, M.A., Mohnen, D., Guern, J., Darvill, A. and Albersheim, P. (1998) Biological activity of reducing-end-derivatized oligogalacturonides in tobacco tissue cultures. *Plant Physiol.* **116**, 1289–1298.

Van der Does, D., Boutrot, F., Engelsdorf, T. *et al.* (2017) The Arabidopsis leucine-rich repeat receptor kinase MIK2/LRR-KISS connects cell wall

integrity sensing, root growth and response to abiotic and biotic stresses. *PLoS Genet.* **13**, e1006832.

Vuong, T.V., Vesterinen, A.H., Foumani, M., Juvonen, M., Seppala, J., Tenkanen, M. and Master, E.R. (2013) Xylo- and cello-oligosaccharide oxidation by gluco-oligosaccharide oxidase from *Sarocladium strictum* and variants with reduced substrate inhibition. *Biotechnol. Biofuels*, **6**, 148.

Winkler, A., Hartner, F., Kutchan, T.M., Glieder, A. and Macheroux, P. (2006) Biochemical evidence that berberine bridge enzyme belongs to a novel family of flavoproteins containing a bi-covalently attached FAD cofactor. *J. Biol. Chem.* **281**, 21276–21285.

Xu, X., Chen, C., Fan, B. and Chen, Z. (2006) Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell*, **18**, 1310–1326.

Zafred, D., Steiner, B., Teufelberger, A.R., Hromic, A., Karplus, P.A., Schofield, C.J., Wallner, S. and Macheroux, P. (2015) Rationally engineered flavin-dependent oxidase reveals steric control of dioxygen reduction. *FEBS J.* **282**, 3060–3074.

