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DOTTORATO DI RICERCA IN
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HOMEOSTASIS OF OLIGOGALACTURONIDES (OGs)
AND THEIR ACTIVITY AS DAMAGE ASSOCIATED
MOLECULAR PATTERNS (DAMPs)
(BIO/04)

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ABSTRACT

Cell walls are important features of plant cells that perform a number of essential functions, both during plant development and growth and in plant-microbe interactions. Recognition of endogenous molecules released during the infection process and acting as “damage-associated molecular patterns” (DAMPs) is a key feature of immunity in plants. Oligogalacturonides (OGs), generated from disruption of homogalacturonan by pathogen pectic enzymes act as DAMPs and their perception leads to a response cascade that leads to intracellular Ca^{2+} release, oxidative burst activation and transcriptional reprogramming. Recently transgenic *Arabidopsis* plants expressing a chimeric protein, named “OG-machine” (OGM), constituted by a fungal PG (FpPG) and a polygalacturonase-inhibiting protein (PGIP) from common bean (PvPGIP2) have been generated. OGM-expressing plants accumulate OGs in their tissue and exhibit enhanced resistance to a variety of pathogens, thereby providing direct

evidence for the function of OGs as *in vivo* elicitors of the plant defense responses (DAMPs).

Besides inducing immunity, OGs negatively affect plant growth and development, likely due to an auxin-antagonistic activity. This effect reflects the typical trade-off that exists between defence and growth and entails that the recognition of DAMPs poses the intrinsic risk of activating an exaggerated response that reduces or completely arrests plant growth. Thus, regulatory homeostatic mechanisms that prevent the deleterious effects of DAMP hyper-accumulation are essential for an optimal immune response. Starting from transgenic plants that express, in a β -estradiol-inducible manner, the OGM, we looked for such a regulatory mechanism in *Arabidopsis thaliana* by searching for elicitor-inactive OGs that may derive from active OGs through an enzymatic modification. In the presence of β -estradiol, these plants contain increased levels of elicitor-active OGs compared to wild-type plants and a large amount of modified OG-like fragments.

Mass spectrometry analyses showed that the oligomers indeed corresponded to modified OGs and are characterized by a galactaric acid residue at the reducing end, leading to the conclusion they are oxidized OGs (oxOGs). oxOGs

were tested for their ability to induce defense responses and antagonize auxin responses. In all experiments, they were inactive as compared to the corresponding typical OGs.

The enzyme with OG oxidizing activity was identified as Isoform 2 of Reticuline oxidase-like protein and was named OGOX1. OGOX1 produces elicitor-inactive oxidized OGs and H₂O₂ and is encoded by the gene At4g20830. This belongs to the superfamily of genes encoding the FAD-binding berberine-bridge enzyme-like proteins (BBELs).

In order to analyze the role of OGOX1, I have obtained an *ogox1* null insertional mutant and transgenic plants overexpressing OGOX1 under the CaMV 35S promoter (OGOX1-OE#1.9 and #11.8). Since additional T-DNA insertional mutants of *OGOX1* were not available, transgenic plants (amiR-OO1) expressing a β -estradiol-inducible artificial microRNA (amiR) against the *OGOX1* transcript (amiR-OO1 lines #2.5 and #3.4) were also generated. The characterization of these plants suggests that OGOX are responsible of an homeostatic mechanism involved in both development and immunity. In the last part of my work the characterization of closely related Arabidopsis BBELs has revealed the presence of at least three additional enzymes with OG oxidizing activity. Thus,

OGOX is a functional and redundant key element for regulating OG homeostasis and likely avoiding an exaggerated activation of plant defences.

1. INTRODUCTION

1.1 The plant cell wall

One of the main differences between plant and animal cells are the walls surrounding plant cells providing structural support during growth and development. The Cell Wall (CW) is also involved in many functions such as functional specialization of cell types, cell-to-cell communication and protection against biotic and abiotic stress.

CW is a dynamic and complex structure which has both structural and regulatory functions with different roles in the physiology and development of the plant (Vorwerk, S. et al. 2004). It is a structure composed for the majority of polysaccharides, phenolic compounds and highly glycosylated proteins. We can distinguish two different type of CWs, namely primary and secondary CW, which are deposited outside the plasma membrane. Primary and secondary CWs are microfibril-based nanocomposites differing in the arrangement, mobility and structure of matrix polymers.

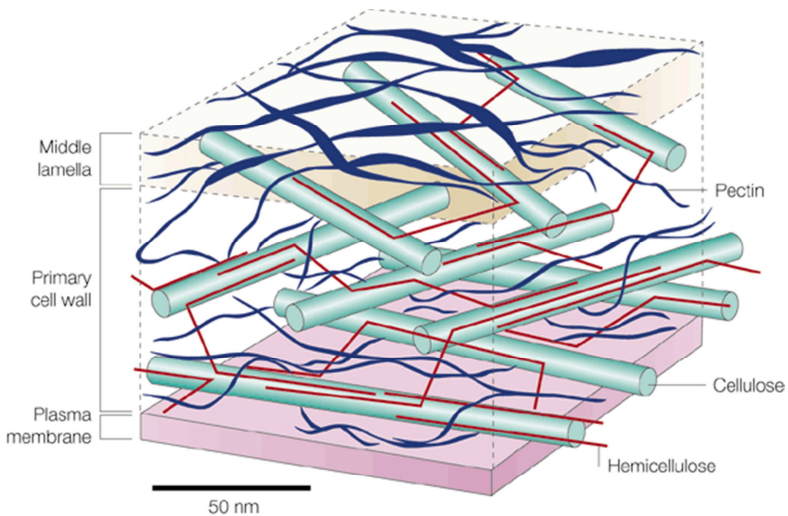
The first layer of CW to be deposited is the middle lamella, the outermost layer through which adjacent cells are in contact. At the end of cell division, the two daughter cells lay the next layer, the primary wall. Some specialized cells, during differentiation, lay a further layer, the secondary wall.

Primary CW, schematically represented in Figure 1.1, is a highly extensible structure synthesized during growth and is connected to neighbour cells through an outer portion, called middle lamella. It is relatively thin, pliant, highly hydrated structure (Smith, L. G. 2001). Nevertheless it must be strong to withstand the tensile forces arising from turgor pressure, extensible to allow wall stress relaxation, cell water uptake and physical enlargement of the cell, as well as capable of linking newly deposited wall polymers into the load-bearing structure.

The secondary CW is deposited at the end of development, during differentiation of some cell types (Cosgrove, Daniel and Jarvis, michael 2012), providing high tensile strength and rigidity in plant tissues that have ceased growing. Any tall terrestrial plant requires stems with bending strength and with water-conducting tissues that can withstand negative pressures (Koch, K. 2004). Secondary walls therefore need

compressive as well as tensile strength, but not extensibility. Under some conditions the secondary CW can undergo various deformation processes that resemble, to some degree, primary CW growth..

The CW consists of two main components: a microfibrillar component and a matrix. The relatively stiff cellulose microfibrils are embedded in a hydrated matrix of pectins, hemicellulose, phenolic compounds and structural protein.



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Figure 1.1. Structural representation of the plant cell wall. The primary CW is deposited outside the plasma membrane, and is composed by a complex network of cellulose, hemicellulose and pectins. The middle lamella connects adjacent cells and is mainly composed of pectins (Smith, L. G. 2001).

The main CW structural polysaccharide is cellulose, which is deposited by an enzymatic complex on the plasma membrane called cellulose synthase. Cellulose consists of a repetition of two units of D-glucopyranose bound by β -1-4 linkages and twisted by 180°. Cellulose aggregates in microfibrils connected through hydrogen bonds with hemicelluloses, forming a network embedded in a matrix of pectin. Hemicelluloses are heterogeneous and branched polysaccharides, comprising xyloglucans (XyGs), xylans and mannans. XyGs have a β -(1→4)-linked backbone of glucose with substitutions of galactose, mannose or xylose. Other hemicelluloses in the secondary CW include

- a) xylans, composed of a linear chain of xylose and ramifications in which the most abundant residues are glucuronosyl and methyl glucuronosyl residues;
- b) mannans, which are polymers of β -1-4-linked mannose;
- c) glucomannans, with a backbone of alternating β -1-4-linked mannose and galactose residues (Scheller, H. V. and Ulvskov, P. 2010).

Primary CWs and middle lamellas are rich in pectins, a heterogeneous group of polysaccharides consisting of rhamnogalacturonan I and II (RG-I and RG-II), xylogalacturonan (XGA) and homogalacturonan (HG). RG-

I has a backbone composed of galactose and rhamnose residues and lateral branches of galactan and arabinan that vary among cell types and developmental stages. RG-II has a linear backbone of galacturonic residues and side branches comprising 12 different type of sugars. HG is the simplest polymer consisting of a linear chain of galacturonic acid bound by an α -1-4 linkage. Methyl or acetyl groups can esterify carboxylic groups in the HG chain. Lastly, XGA is essentially a linear chain of HG that can be substituted by xylose. HG represents around 60% of total CW pectin and about 30% of the primary CW (Wolf, S. and Greiner, S. 2012); it plays different functions in plant life, including growth, development, defence, cell adhesion and signalling (Mohnen, D. 2008). HG is synthesized in the Golgi apparatus, where pectin methyltransferases (PMTs) transfer methyl groups to the native HG chain. Highly methylesterified HG is then secreted in the apoplast, where it is further remodelled by pectin methylesterases (PMEs) that remove methyl groups, leaving free carboxylic groups. In the presence of calcium ions, de-methylated HG chains form a particular structure, called “egg-box” (Fig. 1.2), that makes pectin more rigid (Lionetti, V. et al. 2010). Pectin may also form other kind of interactions, such as crosslinks

with phenolic compounds or structural proteins (Iiyama, K. et al. 1994),(Wolf, S. and Greiner, S. 2012).

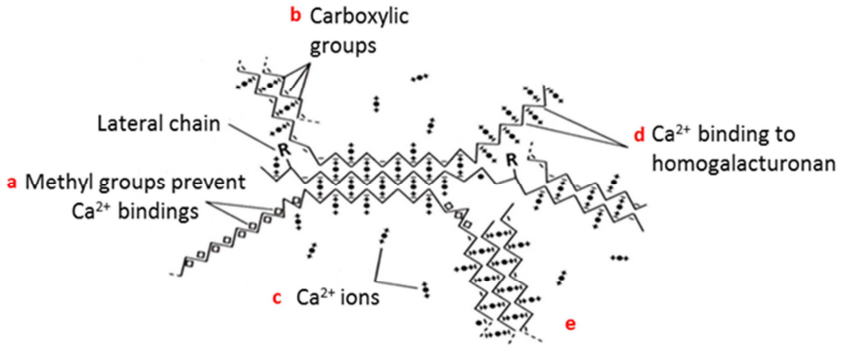


Figure 1.2. Homogalacturonan forms intermolecular crosslinks in the presence of calcium. a) Residues in HG chains are esterified, preventing Ca^{2+} binding. b) PME remove methyl-groups and c) Ca^{2+} in the apoplast d) binds to de-methylated galacturonic acid residues. e) Two close HG chains form the egg-box through calcium bridges.

Phenolic compounds are generally abundant in the secondary CW, sometimes present also in the primary CW and in the middle lamella. In the primary CW, ferulic acid mediates crosslinks with polysaccharides (via ester linkages) or between polysaccharides and lignin (via ether bonds); these bonds can be created by photo-oxidation or by the action of CW peroxidases. An important CW phenolic compound is lignin, a complex polymer produced by the quasi-random condensation of three hydroxycinnamyl

alcohols: p-coumaryl, coniferyl and sinapyl alcohol. When incorporated into the polymer, these hydroxycinnamyl alcohols form different types of lignin, such as guayacil (G) lignin, most abundant in softwood and syringyl (S) lignin, most abundant in hardwood (Rencoret, Jorge et al. 2011). Lignin is often deposited in secondary CWs, even though it is also found in the primary CW and in the middle lamella. Lignin deposition makes the CW more rigid, impermeable to water and resistant to enzymatic hydrolysis. Lignin deposition is particularly important for xylem functionality, since xylematic vessels need to transport water without collapsing as a consequence of the highly negative pressure. Lignin is also important for defence against pathogens, and is often deposited in the CW in response to infection.

Among CW structural elements, proteins, even if present in lower amounts, play important functions (Keller, B. 1993). They are divided into hydroxyproline-rich proteins (HRPGs), proline-rich proteins (PRPs), glycine-rich proteins (GRP), arabinogalactan proteins (AGPs) and lectins (Rumyantseva, N. I. 2005). Representative HRPGs are extensins, self-assembly amphiphiles that generate scaffolding networks able to interact with pectins (Lamport, Derek T. A. et al. 2011). In addition, extensin can contribute

to stiffen the CW through intra- or inter- crosslinks mediated by specific peroxidase called extensin peroxidase (Everdeen, D. S. et al. 1988). AGPs are highly glycosylated hydroxyproline-containing proteins, involved in vegetative growth and reproduction (Rumyantseva, N. I. 2005), that may directly bind to CW polymers. Another class of proteins that plays important roles in CW organization, though they are not structural proteins, are expansins that induce CW loosening through the cleavage of non-covalent bonds between cellulose and xyloglucans.

1.2 Wall sensing

CWs are not inert but highly dynamic structures involved in biological processes. Important functions of the plant CWs are protection against biotic and abiotic stress and structural support during growth and development. CWs are able to perform these functions because their composition and fine structure are modified in response to different type of stimuli originating in the environment or deriving from the plant itself. Examples for plant-derived chemical stimuli are the cell wall fragments oligogalacturonides (see below), metabolites or peptides while invading pathogens represent

sources of non-plant-derived stimuli (like FLG22, ELF) (Ferrari, S. et al. 2013). These two types of chemical stimuli can initiate specific defense responses (Dangl, J. L. et al. 2013). Changes in the mechanical characteristic of CW or changes in turgor pressure are physical stimuli that can be indicative also for abiotic stress.

The tethered model of the CW, according to which there is a load-bearing structure composed by xyloglucan and cellulose, embedded in pectin gel (Carpita, N. C. and Gibeaut, D. M. 1993), is now being challenged by several authors. First, also pectin seems to be connected to cellulose, though more weakly than hemicelluloses. Hemicelluloses bind cellulose only in specific hydrophobic region, called biomechanical hotspots, that control CW extensibility (Park, Yong Bum and Cosgrove, Daniel J. 2015).

On the other side the CW is strictly connected to the plasma membrane and the cytoskeleton (Humphrey, T. V. et al. 2007) and a dedicated system constantly monitors its integrity and transduces any change to trigger downstream responses (Hamann, T. 2015). The existence of a mechanism that perceives wall alterations, was firstly observed in *Saccharomices cerevisiae*. The plant CW

integrity (CWI) maintenance system is poorly understood, but it is likely that in plants, as in yeast, it involves perception of osmotic and damage stress (Hamann, T. and Denness, L. 2011). This CWI maintenance mechanism appears to modulate physiological growth and to regulate responses to stresses. To date, the proposed sensors for CWI are putative kinase receptors and proteins that can physically link the CW to the cytoskeleton, or also stretch-activated ion channels (Humphrey, T. V. et al. 2007);(Wolf, S. et al. 2012) (Fig. 1.3).

Among the proposed CWI receptors, Wall Associated Kinases (WAKs) have been reported to directly bind the CW pectins through an extracellular domain that contains Epidermal Growth Factor-like repeats (Decreux, A. and Messiaen, J. 2005).

The way CW components interact is likely very important for CWI maintenance. Damage of CW components may expose other polymers to hydrolytic activity and remodelling, with the consequent release of CW fragments allowing binding to specific proteins. This highlights the hypothesis that the CWI maintenance mechanism could form an essential component of plant immunity such as responses to damage associated molecular patterns

(DAMPs), since cell wall-derived fragments can behave like DAMPs.

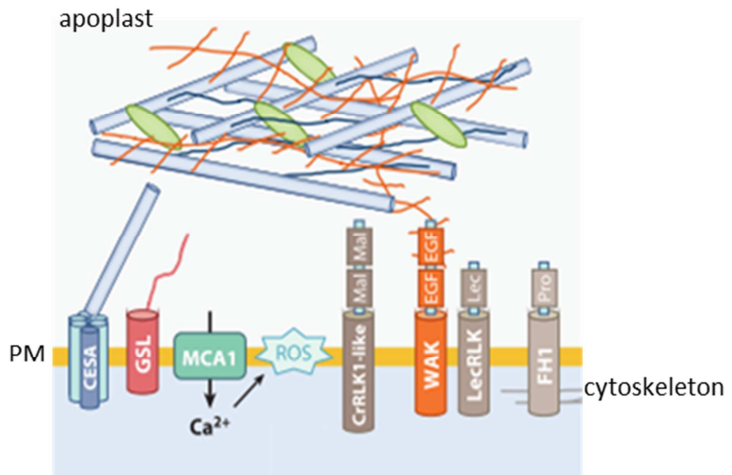


Figure 1.3. Proposed players in the CWI maintenance system. Plasma membrane-localized proteins may play a role in perception of cell wall alterations. They comprise proteins involved in cellulose and callose biosynthesis (CESA and GSL); mechanosensors (MCA1, a stretch-activated calcium channel); *Catharanthus roseus* (CrRLKs) and lectin-RLKs (LecRLK) receptor-like kinases (RLKs); proteins able to bind CW polymers (e.g. WAK1) or cell wall and cytoskeleton (FH1). Adapted by (Wolf, S. et al. 2012).

1.3 The plant immune system

The battle between plants and microbes is evolutionarily ancient and highly complex. This struggle is often described in terms of an “arms race”. Unlike animals, plants have not evolved an adaptive immunity system (Jones, J. D. and

Dangl, J. L. 2006), but their ability to fend off microbial infection is based on different layers of defence that can be observed at the level of each cell.

Plants respond to pathogen attack through constitutive and inducible mechanisms. Structural barriers or reservoirs of antimicrobial compounds represent performed constitutive defences against tissue colonization. The frontline of the plant defense system consists of physical and chemical barriers such as the cuticle and the cell wall. If these obstacles are overcome, the pathogen is still confronted by elaborate surveillance system in which molecular sentinels operate to activate resistance responses (Jones, J. D. and Dangl, J. L. 2006).

Induced defense responses include the activation of defence-related genes, leading to the reinforcement of the plant cell wall and the accumulation of phytoalexin and pathogenesis related protein (PR-protein).

Most plant pathogenic microorganisms actively penetrate the plant apoplast for access to intracellular nutrients. To gain this access, bacterial and fungal pathogens possess a range of enzymatic and/or physical tools. On the other hand plants respond to attempted penetration by a battery of wall-associated defense reactions. The plants often recognize

microbial attacks through a nonself recognition that operates through the perception of microbial molecules or by surveillance of host cellular integrity. The presence of microbes in most cases is detected by pattern recognition receptors that respond to specific pathogen-associated molecular patterns (PAMPs). PAMPs are usually molecular components that serve essential function for the fitness or survival of microbes and are often highly conserved and are not present in the host.

In addition to sensing PAMPs, sensing a compromised “self” by detecting damage-associated molecular patterns (DAMPs) such as released plant cell wall fragments is a central part of plant the defense (Boller, T. and Felix, G. 2009). Indeed both PAMP- and DAMP-recognition activates an innate immune response commonly named PAMP-Triggered Immunity (PTI) (Fig. 1.4).

Well known examples of PAMPs are bacterial peptidoglycans, the bacterial flagellin, the bacterial Elongation Factor Tu (EF-Tu) and the fungal chitin. PAMP perception is mediated by ligand-binding surface-exposed transmembrane pattern-recognition receptors (PRRs) of either the receptor-like kinase (RLK) or receptor-like proteins (RLPs) families. RLKs have an intracellular kinase

domain while RLPs lack this cytosolic signalling domain (Monaghan, Jacqueline and Zipfel, Cyril 2012).

The archetypical bacterial PRRs are the elongation factor receptor EFR, a leucine-rich repeat RLK (LRR-RLK) that recognizes the elongation factor cytoplasmic protein EF-Tu and the related flagellin sensing 2 (FLS2) that recognizes flagellin, the principal component of bacterial flagella. These two PAMPs are often characterized by their minimal requirement peptide epitopes, elf18 and flg22.

PTI induction leads to a series of early and late responses and the final consequence of PTI is the induction of resistance responses that will prevent microbial colonization. The early responses occur within minutes to hours, and consist of rapid ion fluxes across the plasma membrane, an oxidative burst, activation of mitogen/activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs), and induction of defense-related genes. Deposition of callose and resistance are later responses that occur within days (Boller, T. and Felix, G. 2009). Callose deposition like the papillae are an important feature of immunity and are thought to reinforce the cell wall at the microbial penetration sites to impede infections. PTI is effective against a broad spectrum of

microbes, but many successful pathogens to escape detection adopt a strategy secreting a range of effector proteins that modulate the PTI components (Jones, J. D. and Dangl, J. L. 2006). The microbial effectors can in turn be counteracted in the plant by an intracellular surveillance system consisting of an array of nucleotide-binding leucine-rich-repeat proteins that detect the presence of such effector proteins and enable induction of effector-triggered-immunity (ETI) (Fig 1.4).

Defence responses activated during PTI and ETI also include production of hormones, for example salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). In many cases, ETI blocks the establishment of disease by inducing a programmed plant cell death called “hypersensitive response” (HR). ETI also causes a massive increase, in the infected tissues, of SA, which is systemically translocated via the phloem. Accumulation of SA induces defence responses in distal tissues that are responsible for the development of a systemic acquired resistance (SAR). SA also negatively regulates growth, and for this reason its production is tightly regulated (Seyfferth, Carolin and Tsuda, Kenichi 2014). While SA is mostly involved in mounting defences against biotrophic pathogens, JA is

required against insects and, in combination with ET, against necrotrophic pathogens, with some exceptions (Denancé, Nicolas et al. 2013).

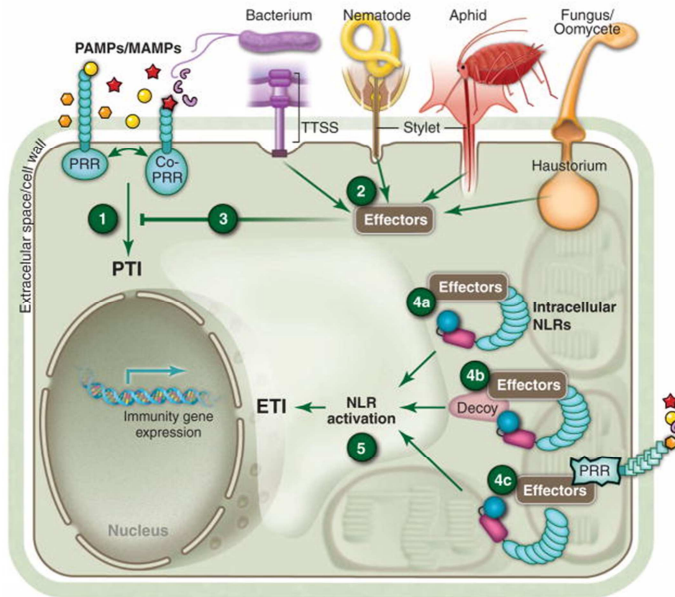


Figure 1.4. The plant immune system. During pathogenic events, PAMPs are recognized by host PRRs and their co-receptors. (1) Activation of PTI is effective against a broad spectrum of pathogens. (2) Successful pathogens release effectors that (3) block PTI. (4) During an incompatible interaction, plant NLRs perceive effectors through (4a) direct binding, (4b) recognition of altered structure of proteins that mimic effector targets (decoy) and (4c) recognition of alteration in effector target proteins (e.g. intracellular domains of PRR). NLR activation leads to ETI (Dangl, J. L. et al. 2013).

1.4 Oligogalacturonides are pectin-derived endogenous elicitor

Pathogen have evolved a diversity of strategies to breach CW, including the secretion of numerous glycosyl hydrolase. In response to an attack, plants may deposit reinforcing polymers like callose as well as phenolic complexes and toxic compounds. However, the physical and chemical responses are only one part of the defense played by cell wall. The release of degradation fragments that are monitored by plants also is an important defense mechanism.

Endogenous elicitors are released from cellular components during pathogen attack or abiotic stresses, and have been indicated as damage-associated molecular patterns (DAMPs). DAMPs typically appear in the apoplast and, similarly to PAMPs, serve as danger signals to activate the immune response (De Lorenzo, G. et al. 2011).

Homogalacturonan (HGA), a main component of pectin in the cell wall, can be considered the plant counterpart of the vertebrate hyaluronan (HA) (Cyphert, Jaime M. et al. 2015). Both are linear and acidic high molecular weight polysaccharides and have been describes as “the most simply designed, yet most versatile biological molecules in nature” (Cyphert, Jaime M. et al. 2015).

Oligogalacturonides (OGs), derived from the fragmentation of HGA, are the first and probably the best characterized plant DAMPs ever discovered. OGs are oligomers of galacturonic acids that accumulate in the apoplast as a consequence of HGA hydrolysis by polygalacturonases (PGs) (Ferrari, S. et al. 2013).

PGs from microbes are highly active and rapidly degrade HGA into monomers, dimers and trimers. The complete hydrolysis of homogalacturonan by fungal PGs is hampered by the apoplastic polygalacturonase-inhibiting proteins (PGIPs); the PG-PGIP interaction favors the accumulation of elicitor-active oligogalacturonides (De Lorenzo, G. et al. 2011) (Fig. 1.5).

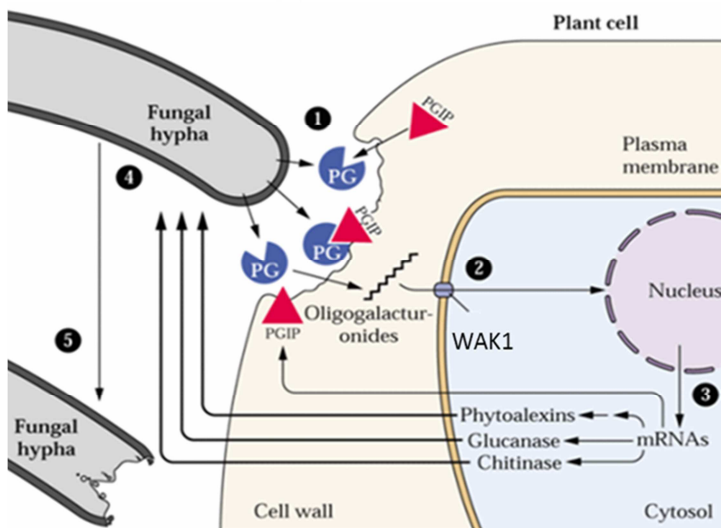


Figure1.5. Production of OGs during plant-pathogen interactions. During infection, pathogens release PGs that degrade HGA. Activity of PGs is attenuated by PGIPs released by the host, and their interaction leads to OG accumulation in the apoplast. OGs are perceived by their receptor WAK1 and induce transcriptional reprogramming and accumulation of antimicrobial compounds.

The elicitor activity of OGs is related to their molecular size, being OGs with a degree of polymerization between 10 and 15 the most active elicitors (Cote, F. and Hahn, M. G. 1994). This size is optimal for the formation of Ca^{2+} -mediated intermolecular cross-links resulting in structures called “egg boxes” (Braccini, I. and Perez, S.

2001);(Cabrera, J. C. et al. 2008) that are thought to be necessary for OG activity (Fig. 1.6).

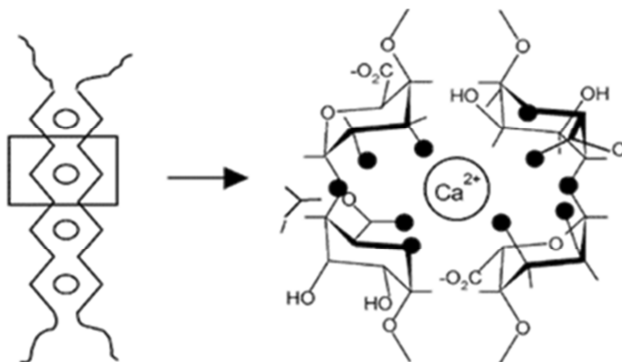


Figure 1.6. Egg boxes formation. Ionic bridges between the carboxyl groups (COO^-) of the galacturonic acid residues and calcium ions leads to the formation of intermolecular complexes called "egg-boxes".

OGs can bind the extracellular domain of the Arabidopsis WAK1, a member of the WAK family (Decreux, A. and Messiaen, J. 2005, He, Z. H. et al. 1996, Wagner, T. A. and Kohorn, B. D. 2001). Recent evidence suggests that activation of WAK1 by OGs triggers downstream defence responses (Brutus, A. et al. 2010), indicating that this protein acts as a OG receptor. The signal transduction pathway linking OG perception to the activation of the immune response has been extensively studied. OGs perception induce ROS accumulation through the activation of the NADPH oxidase AtRbohD, nitric oxide production,

callose deposition, and MAPK-mediated activation of defence gene expression (Fig. 1.7).

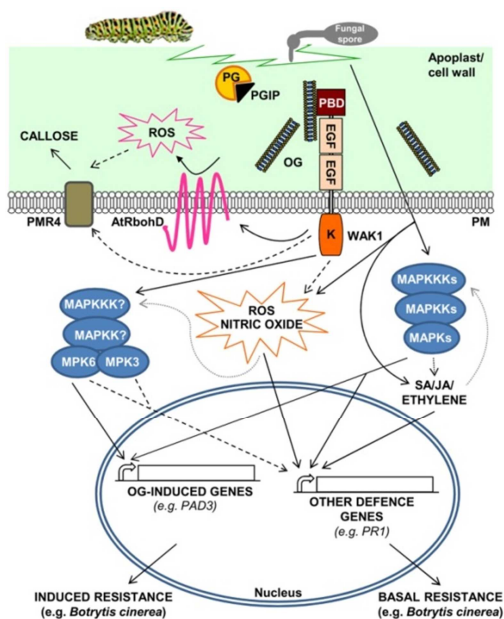


Figure 1.7. Model of defence responses triggered by oligogalacturonides in *Arabidopsis thaliana*. OGs are released from the cell wall after degradation of homogalacturonan by mechanical damage or by the action of hydrolytic enzymes, secreted by pathogens, such as PGs. In the apoplast PGIPs modulate PG activity, favouring the accumulation of elicitor-active OGs. OGs are perceived by the receptor WAK1 (Wall-associated kinase 1) and trigger defence responses such as ROS accumulation through the activation of the NADPH oxidase AtRbohD, nitric oxide production, callose deposition, and MAPK-mediated activation of defence gene expression. Pathogen invasion or mechanical damage also cause an increase of hormones levels (JA, SA, and ethylene), mediated by MAPK cascades, triggering defence responses independently of OGs. DAMP- and hormone-mediated defence responses result, respectively, in induced and basal resistance to necrotrophic pathogens, such as *Botrytis cinerea*. Dashed lines indicate hypothetical cascades; dotted gray lines indicate over simplification of the complex and still partially uncharacterized roles of MAPKs in the regulation of hormone and ROS synthesis/response (Ferrari, S. et al. 2013).

The WALL-ASSOCIATED KINASES (WAKs) are thought to be DAMP sensor that monitor the integrity of pectin by detecting the presence of OGs (Ferrari, S. et al. 2013). In the context of degradation by microbial enzymes the level of HGA methyl esterification is critically important. Endo-PGs and pectate lyases (PLs) preferentially cleave non-esterified HGA and these enzyme frequently act in concert with pectin methyl esterases (PMEs) to create endo-PG and PL cleavage sites.

OGs have been proposed as important signals also in the wound response (Bishop, P. D. and Ryan, C. A. 1987);(Rojo, E. et al. 1999). Wounding is one of the most common dangers faced by plants, as the injured tissue represents an easy entry point for pathogen. Plants are able to perceive wounded tissues as an altered self and activate localized defences similar to those activated by pathogen infection, such as ROS production (Bradley, D. J. et al. 1992, Brisson, L. F. et al. 1994), expression of defence genes (Reymond, P. et al. 2000) and the synthesis of pathogenesis-related proteins. Moreover several genes induced by wounding are also regulated in response to pathogens (Reymond, P. and Farmer, E. E. 1998); (Reymond, P. et al. 2000);(Durrant, W. E. et al. 2000). A

study on local and systemic response to wounding in tomato showed that OGs induce proteinase inhibitor accumulation (Ryan, C. A. and Jagendorf, A. 1995) suggesting a role in the wound response of these DAMPs.

Pre-treatments with exogenous OGs confer protection against subsequent infection with the necrotrophic fungus *Botrytis Cinerea*. The protection lasts at least 72 h after spraying suggesting a “primed” status in which the plant immune system determines an enhanced capability of restricting pathogen growth. Protection is also induced in distal tissues when OGs are infiltrated locally in a single leaf.

Because pectin is among the first components that are modified when the wall undergoes physiological remodelling, OGs may be important not only in defense against pathogens, but also, under physiological conditions, may have effects on plant growth and development. One of the first described effects, is the induction of tomato fruit ripening through the induction of ethylene, which was later shown to be mediated by OGs in the size range of DP 4–6 (Simpson, S. D. et al. 1998). Physiological responses to auxins can be antagonized by OGs, as described for the first time by Branca et al. (Branca, C. et al. 1988), who showed

that auxin-induced elongation in pea stem segments is competitively inhibited by OGs. OGs have been subsequently shown to inhibit auxin-induced root formation in tobacco and *Arabidopsis* leaf explants as well as in thin cell-layer explants (Bellincampi, D. et al. 1993);(Savatin, D. V. et al. 2011) and to induce flower formation in explants that do not normally form organs (Marfà, V. et al. 1991). Although OGs do not simply act by inhibiting the action of IAA (Spiro, M. D. et al. 2002), most of the developmental effects of OGs may be explained with their ability to antagonize auxin responses (Fig. 1.8).

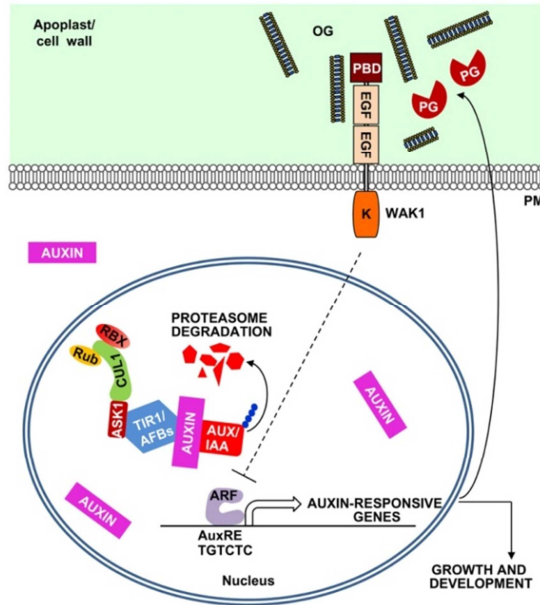


Figure 1.8. A model for the OG-mediated negative feedback regulation of the auxin responses. Plant cells sense auxin through the receptors TIR1/AFBs, F-box proteins that form a SCF E3 ubiquitin ligase complex together with SKP1 (ASK1) and CULLIN1 (CUL1). This complex is regulated by RUB1 conjugating enzyme (Rub) and RING BOX1 (RBX) proteins and, in the presence of auxin, leads to the ubiquitination of Aux/IAA repressors and their proteasome-mediated degradation. Aux/IAA degradation releases auxin response factors (ARFs) that initiate the transcription of auxin-responsive genes, characterized by the presence of auxin response elements (AuxREs) in their promoters. Auxin also induces the expression of plant PGs and other pectin-degrading enzymes (Laskowski, M. et al. 2006). The action of these enzymes may release in the apoplast OGs that can inhibit auxin-related responses, establishing a negative feedback loop (Ferrari, S. et al. 2013).

The activity of OGs has been mostly studied by using exogenous applications. Recently a chimeric protein was generated by fusing a PG from the fungal pathogen *Fusarium phyllophilum* (FpPG) to PvPGIP2, a PGIP from common bean (*Phaseolus vulgaris*) (Benedetti, M. et al. 2015). The PG-PGIP fusion, named OGMachine or OGM, was engineered under a β -estradiol inducible promoter and expressed in transgenic *Arabidopsis* plants to obtain enhanced OG levels in the tissue. Notably, expression of the OGM fusion protein was accompanied by the accumulation of OGs in the plant tissues (Fig. 1.9).

After induction, leaves expressing the chimera activated defence responses, including the accumulation of callose and the expression of two genes previously shown to be strongly up-regulated by OGs (Denoux, C. et al. 2008), RetOx and WRKY40 (Xu, X. et al. 2006). The same responses had been previously observed upon application of exogenous OGs (Denoux, C. et al. 2008, Galletti, R. et al. 2008).

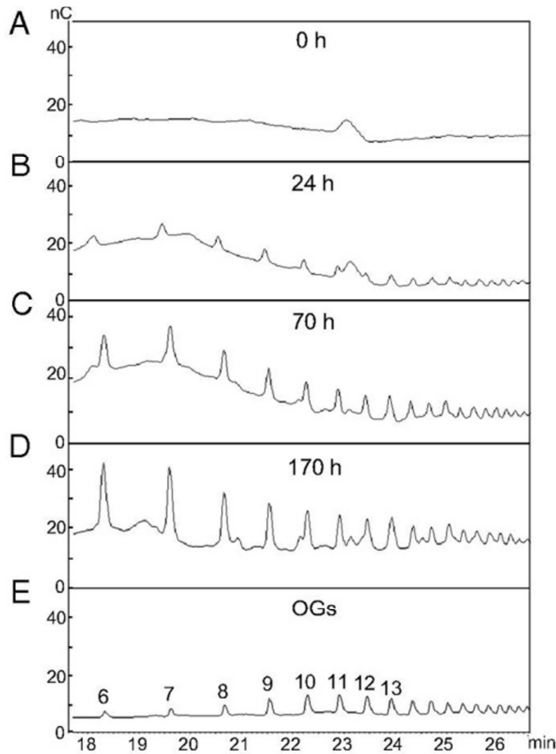


Figure 1.9. Accumulation of OGs in Arabidopsis plants expressing the OGM. Plants expressing the inducible OGM were treated for the indicated times with 50 μ M β -estradiol. (A-D) Oligosaccharides in the cell wall pectin fraction were analyzed by HPAEC-PAD. Chromatograms indicate signal intensity (nC) at each retention time (min). (E) Representative chromatogram of purified OGs; the numbers indicate the degree of polymerization (DP) (Benedetti, M. et al. 2015).

Expression of the OGM under the control of the promoter of the Arabidopsis *PATHOGENESIS RELATED-1* (*PR-1*) gene, which is strongly induced by a variety of pathogens (Cao, H. et al. 1994), leads to increased resistance to pathogens due to the prompt activation of immune responses. In particular after inoculation with the fungal pathogen *Botrytis cinerea* the number of lesions was significantly reduced in the transgenic plants compared to the wild type and the average area of the lesions was also significantly smaller. Transgenic plants also showed a strong reduction of symptoms in response to the bacterial necrotroph *Pectobacterium carotovorum* and of the bacterial hemibiotroph *Pseudomonas syringae* pv. *Tomato* (Benedetti, M. et al. 2015).

In an attempt to amplify the OG-mediated immune response via a feed-forward process, the expression of the OGM under the control of the promoter of the RetOx gene, which is strongly and rapidly induced by OGs (Denoux, C. et al. 2008), was generated. pRetOx:OGM plants displayed a marked dwarfism, curled leaves and reduced stem elongation, and died between two to four weeks after transfer to soil (Fig. 1.10).

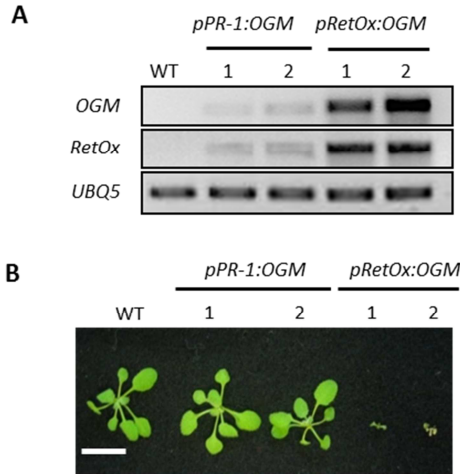


Figure 1.10. *pRetOx:OGM* plants show constitutive activation of the immune response. (A), levels of *OGM* and *RetOx* transcripts in two-week-old WT (WT) plants, *pPR-1:OGM* 1 and 2 plants, and in two representative *pRetOx:OGM* T1 plants. *UBQ5* was used as reference. (B) Pictures of the plants used in panel A. The white bar corresponds to 2 cm (Benedetti, M. et al. 2015).

These data supports the hypothesis that the accumulation of OGs during pathogen infection induces defence responses but also restricts plant growth.

To verify that a high expression of OGM is responsible of the impaired plant growth, the β -estradiol inducible OGM seeds were germinated in the presence of increasing concentrations of inductor. Transgenic seedlings showed reduced biomass proportional to the level of induction (Fig. 1.11), confirming that high levels of OGM expression negatively affect growth.

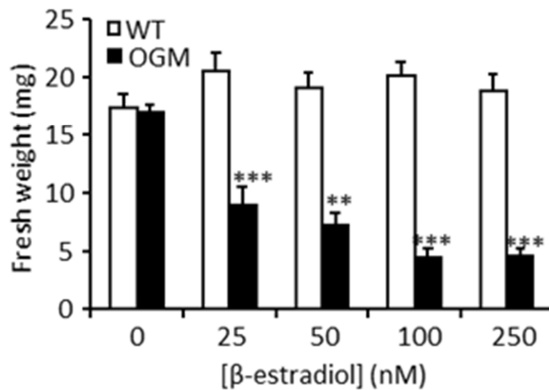


Figure 1.11. OGM levels correlate with a reduction of plant growth. OGM seeds were germinated in the presence of β -estradiol and fresh weight was measured after 10 days. Bars: average ($n>10$) $\pm$ SE. ***, statistical difference between control- and estradiol-treated seedlings (Student's t-test, $P<0.001$) (Benedetti, M. et al. 2015).

The observed reduction of growth in plants expressing high levels of OGM may be a consequence of an exaggerated activation of defence responses. In particular, it is known that the phytohormone SA, which plays a key role in the response to biotic stress, also has significant effects on growth (Rivas-San Vicente, Mariana and Plasencia, Javier 2011). When OGM were treated with the inducer, an almost ten-fold increase in SA levels was observed, both at the adult and at the seedling stage, within 24 h from the treatment (Benedetti, M. et al. 2015), supporting the

hypothesis that the reduced biomass of these plants is caused by the OG-mediated activation of an immune response.

These data show that hyperaccumulation of OGs, when the OGM is expressed under the control of the promoter of the RetOx gene or when growth on high doses of inductor (β -estradiol), severely affects growth, eventually leading to cell death and clearly pointing to OGs as player in the growth-defense trade-off (Benedetti, M. et al. 2015). Therefore, regulatory homeostatic mechanisms to prevent the deleterious effect of DAMP hyper accumulation must exist and are essential for an optimal immune response.

Searching for this mechanism and investigating the control of the level of OGs that occur in plants, has been the starting point of my Ph.D. work and the OGM plants have been a useful tool for my studies.

2. AIM OF THE WORK

The recognition of endogenous molecules released upon infection or injury and acting as Damage-Associated Molecular Patterns (DAMP) and the regulation of their signalling for optimal immunity is an important issue in both animals and plants (Irazoqui, J. E. et al. 2010);(Heil, M. and Land, W. G. 2014).

A main focus of my project is on oligogalacturonides (OGs), oligomers of alpha-1,4-linked galacturonosyl residues deriving from the fragmentation of the homogalacturonan (HGA), a main component of pectin in the cell wall. These molecules are able to elicit defense responses, including accumulation of reactive oxygen species and pathogenesis-related proteins, and protect plants against pathogen infections (Ferrari, S. et al. 2013).

Although OGs are the first DAMPs ever discovered, much is unknown about their biology and complexity of their mediated signalling due to the difficulty of isolating mutants defective in specific or general responses to OGs. In my lab a novel instrument based on the release of OGs *in planta* on

command by an engineered inducible molecular tool named OG-machine (OGM) has been developed (Benedetti, M. et al. 2015).

The accumulation of OGs, caused by the expression of OGM rather than the addition of exogenous OGs, better represents the formation of OGs *in planta* that naturally occurs during the physiological and pathological events of cell wall deconstruction and the consequent OG-dependent developmental processes and immunity responses.

In the OGM plants the OGs released at high levels trigger a deleterious hyper-immunity and lead to plant death, entailing that recognition of OGs as DAMP poses the intrinsic risk of activating an exaggerated response that may completely arrest plant growth. This effect is consistent with the current notion that trade-off occurs between growth potential and capacity for defense and suggests that OGs are critical signal molecules in this trade-off.

The aim of my work was to demonstrate that homeostatic mechanisms, essential for an optimal immune response and preventing hyper and autoimmunity exist. Inactivation played by a specific OG oxidases (OGOxS) belonging to the complex berberin bridge-like enzyme (BBIE) family is

one of such mechanisms, essential for controlling homeostasis of OGs, growth and immunity.

During my PhD thesis, the identification of modified OGs (oxidized OGs) that are unable to activate immune responses and the identification of the enzyme responsible of this oxidation and their role in plant immunity and development have been analysed.

3. MATERIALS AND METHODS

3.1 Biological material and growth conditions

All *Arabidopsis thaliana* plants used in this work are in the Columbia (Col-0) background. Wild-type (WT). Col-0 seeds were obtained from Lehle Seeds (Round Rock, TX, USA). The T-DNA-insertion null mutant (*ogox1*; WiscDsLox432E05) was obtained from the Salk Institute Genomic Analysis Laboratory (Alonso, J. M. et al. 2003), β -estradiol inducible OGM seeds were obtained in the laboratory where I worked (Benedetti, M. et al. 2015).

The inducible silenced lines (amiR-OO1, lines #3.4 and #2.5), generated by expressing an artificial miRNA (amiRNA) (Schwab, R. et al. 2006) specifically targeting *At4g20830* transcripts in Col-0 background, were previously obtained in the laboratory.

Plants were grown in soil (Einheitserde, Germany) in a growth chamber at 22°C, 70% of relative humidity and a photoperiod of 16 hours of light and 8 hours of dark, using fluorescent lamps (Osram, Germany). Light intensity was

about $120 \mu\text{mol m}^{-2} \text{s}^{-1}$. Before sowing, seeds were always stratified in sterile water for three days in the dark at $+4^{\circ}\text{C}$. For *in vitro* growth, seeds were surface-sterilized as described in (Ferrari, S. et al. 2007) and stratified as described above. For growth on solid medium, seeds were germinated on plates containing half strength Murashige and Skoog (MS) (Murashige, T. and Skoog, F. 1962) basal salts supplemented with 1% (w/v) sucrose and 0.7% (w/v) plant-agar unless otherwise stated, pH 5.0. Oligogalacturonides were prepared as previously described (Bellincampi, D. et al. 2000).

3.2 Preparation of leaf diffusates

For preparation of diffusates, leaves from 25-day-old OGM plants at 170 h after spraying with $25 \mu\text{M}$ -estradiol were sterilized in 1% (v/v) sodium hypochlorite for 3 min, and extensively washed with ultrapure water for at least 4 times. Leaves were cut in thin strips 0.25 cm^2 and about 90-100 mg of tissue were incubated in 50 mM ammonium acetate pH 5.0, 50 mM 1,2-DiaminocyclohexaneTetraacetic Acid (CDTA) and 50 mM oxalate for 20 h at 30°C , in presence or in absence of 10 mM Na_2SO_3 . After incubation, the medium

was collected and precipitated by adding ethanol to a final concentration of 80% (v/v); the sample was centrifuged at 15000xg 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 3000xg for 3 min and the supernatant was collected for HPAEC-PAD (High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection) and ESI-MS analyses.

For the hydrolysis of fragment in diffusates in tri-, di-, and monogalacturonic acid, the diffusates were treated with 5ug of pure *Fusarium phyllophilum* PG expressed in *Pichia pastoris* (Benedetti, M. et al. 2015) for 1 h.

3.3 Chromatographic and Mass Spectrometric analysis of standard and modified OGs.

The HPAEC-PAD analysis was carried out by using equipment and procedures already described (Pontiggia, D. et al. 2015). The oxidation to galactaric acid of the residue at the reducing end of OGs causes a delay in the retention time of each oligomer.

ESI-MS analyses on modified OGs were performed on LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific in Bremen, Germany) using positive electrospray as the ionization process. The OG samples were diluted in MeOH (80 : 20, v/v). Infusion was performed at a flow-rate of 5 $\mu\text{l min}^{-1}$. Nitrogen was used as sheath gas (20 arbitrary units). MS analyses were carried out using a needle voltage of 4.6 kV and a heated capillary temperature of 340 °C. The AGC target was set to 1e^6 and the maximum injection time to 50 ms. In the high resolution measurements with setting of 40 000, one microscan was recorded.

We performed MS^2 and MS^3 analyses for structural confirmation. The various parameters (collision energy, qz activation value and activation time) were adjusted in order to optimize the signal and obtain maximal structural information from the ion of interest. In the positive ionization mode, the MS^2 on modified OGs only produced glycosidic bond cleavage fragments, generating B- and Y-ions, according to the nomenclature proposed by Domon and Costello (Domon, B. and Costello, C. E. 1988).

3.4 Protein extraction from *A. thaliana* and enzymatic assay

Leaves from 25-day-old plants (about 100 mg) or seedlings (about 50 mg) were homogenized using liquid nitrogen. Pulverized samples were resuspended in the extraction buffer [20 mM Na-Acetate pH 5.0, 0.8 M NaCl; ratio 4:1 (mL : g of tissue)] and incubated for 30 min at 4°C. Samples were centrifuged at 9000xg for 10 min and supernatants collected and proteins quantified by the Bradford assay (Bradford, M. M. 1976).

For enzymatic activity, about 5 µg of total proteins were assayed in 50 mM Tris-HCl pH 8.8, 50 mM NaCl, in the presence and in the absence of 1 mM Na₂SO₃ using OGs (1 mg mL⁻¹) as a substrate. Hydrogen peroxide generated in the OG oxidation reaction was quantified using an xylenol orange-based colorimetric assay (Gay, C. et al. 1999). Enzyme activity was expressed as pmol H₂O₂ min⁻¹µg protein extract⁻¹.

For substrate specificity analyses, assays were performed in Na-Acetate pH 5.0, 50 mM NaCl using the purified protein at a concentration of 2.5 nM.. Monosaccharides, cellodextrins, oligomannuronides, standard OGs were used at a concentration of 0.4 mM. Single pure OG oligomers were used at a concentration of 0.04 mM. All

monosaccharides and cellodextrins were purchased from Sigma-Aldrich. Oligomannuronides were prepared as previously described (Haug, A. et al. 1974).

3.5 Purification of the OG-oxidizing activity

The OG-oxidizing activity was purified from leaves of OGM plants after a 170-h induction with β -estradiol (Benedetti, M. et al. 2015). Activity during purification was followed by using standard OGs as substrate and measuring the hydrogen peroxide generated in the presence and in the absence of sulphite (1 mM) as inhibitor. Proteins were extracted from 30 grams of leaves using 120 mL of extraction buffer [20 mM Na-Acetate pH 5.0, 0.8 M NaCl; ratio 4:1 (mL : g of tissue)]. The protein extract was diluted 20-fold in 10 mM Na-acetate pH 5.0 and loaded on a HGA-affinity column prepared as described in (Spadoni, S. et al. 2006). The HGA-affinity column was equilibrated with 30 mM Na-Acetate pH 5.0 and 5 mM CaCl_2 . Absorbed proteins were eluted with 20 mM Na-acetate pH 5.0 and 0.8 M NaCl. The fractions that displayed the highest OG-oxidizing activity were pooled and diluted 20-fold in 10 mM Na-acetate pH 5.0 and loaded on a 1 mL

SP-sepharose column (Amersham). Elution was carried out using a linear gradient of NaCl (from 0 to 0.8 M NaCl in 20 volumes of column). Fractions that displayed the highest OG-oxidase activity were pooled and brought to 2 M ammonium sulfate. The sample was loaded on a 1-mL Phenyl-Sepharose column (Amersham) and elution was performed by decreasing the ammonium sulfate concentration (from 2 to 0 M ammonium sulfate in 30 volumes of column). The fractions were subjected to SDS-PAGE (10% acrylamide w/v) and a protein band characterized by an apparent molecular weight of about 70 kDa was found to co-elute with OG-oxidizing activity, which eluted at an ammonium sulfate concentration ranging from 1.2 to 0.8 M. The band was picked from Coomassie blue stained gels and subjected to in gel-digestion for the identification by mass spectrometry.

3.6 Identification of the OG oxidase by proteomic analysis

The excised protein band was subjected to reduction by 5 mM DTT, alkylation by 2 mM iodoacetamide (IAA) and in-gel digestion with trypsin (Promega) at 30°C overnight. The tryptic peptides were acidified by adding TFA to a final

concentration of 0.5%, desalted by reversed phase-Stage Tips and analyzed by LC-MS/MS. Peptides were eluted over 180 min at 300 nl min⁻¹, using a 0–60% acetonitrile gradient in 0.1% formic acid using an Ultimate 3000 nano-chromatography pump (Thermo-Fisher Scientific) coupled to an LTQ Orbitrap Discovery mass spectrometer (Thermo-Fisher, Bremen, Germany) operated in a data dependent mode. MS was acquired at 30,000 FWHM resolution in the FTMS (using a target value of 5×10^5 ions) and MS/MS was carried out in the linear ion trap. Five MS/MS scans were obtained per MS cycle. Spectra were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.3.0.339). Sequest was set up to search the *Arabidopsis thaliana* proteome database (Uniprot.org) assuming trypsin as the enzyme with 2 missed cleavage allowed. Sequest was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 ppm. Fixed modification of carbamidomethyl cysteine and variable modifications of oxidized methionine were considered in the search.

3.7 Expression in *Pichia pastoris*

The DNA sequence encoding the mature short isoform *At4g20830.2* was amplified from *Arabidopsis* genomic DNA (gDNA) by using the PmlIAt4g20830Fw and XbaIAt4g20830mycRv primers (Table 3.1) and cloned in the PmlI-XbaI sites of the methanol-inducible expression vector pPICZαB, downstream of the sequence encoding the yeast alpha factor signal peptide for translocation into the ER, and upstream of the *c-myc* epitope-encoding sequence. The recombinant plasmid was introduced in *P. pastoris* by electroporation and transformants were grown in yeast extract (1%), peptone (2%) and glucose (2%) for 1 day. The culture was centrifuged and the cell pellet resuspended in a quarter of the starting volume of BMM medium (100 mM K-phosphate pH 6.0, 1.34 % v/v YNB, 4×10^{-5} % biotin and 0.5% methanol) for further growth and induction of protein expression. Culture filtrates at different times of induction (24-72 hours) with methanol were collected and the expression of *At4g20830.2* was evaluated by SDS-PAGE and immuno-blot by using a monoclonal anti-myc antibody (Santa Cruz Biotechnology, Inc.). Purification of the heterologous protein was performed by using two consecutive hydrophobic chromatography steps (Phenyl-

Sephacrose, Amersham), carried out at pH 4.6 and 7, respectively. Purity of the protein was assessed by SDS-PAGE/Coomassie blue staining and by measuring UV absorbance at a wavelength of 260 and 280 nm. For expression of At4g20840, At1g11770, At1g01980, At1g30740 and At1g30700, the corresponding DNA sequences encoding the mature proteins were amplified from Arabidopsis gDNA by using primers that introduced sites for the enzymes PstI and XbaI (Table 3.1). The genes were cloned in pPICZ α B previously digested with the same enzymes. Expression of the protein, immuno-detection and enzymatic assays were performed on the culture filtrates as described for At4g20830.

Table 3.1. Primers used for the construction of the different BBELs.

GENE	PRIMER NAME	PRIMER SEQUENCE
<i>At4g20830</i>	PmlIAt4g20830Fw	ATAAGCACGTGCAATCGAACT CTGTATACAACCTC
	XbaIAt4g20830mycRv	GTAAGTCTAGA GCTGCCTTA CTTTTCAAGGTAG
<i>At4g20840</i>	PstIAt4g20840Fw	ATAAGCTGCAGC ACCTCCGTCT TCTGATTC
	XbaIAt4g20840mycRv	CTAAGTCTAGAGCTGCCTTGC TAAGCACGGT AGG
<i>At1g11770</i>	PstIAt1g11770Fw	ATAAGCTGCAGCCGTCGAACC TCCGCCTGAAAC
	XbaIAt1g11770mycRv	CTAAGTCTAGAGAACGCCTAG CTGGCTTGCC
<i>At1g01980</i>	PstIAt1g01980Fw	ATAAGCTGCAGCGACCGCTCC GCCCAACACATC
	XbaIAt1g01980mycRv	CTAAGTCTAGACGACTCGAGG ATGGCTTGGTAGG
<i>At1g30740</i>	PstIAt1g30740Fw	ATAAGCTGCAGCCGATTCCGT AA TATCTACGAAG
	XbaIAt1g30740mycRv	CTAAGTCTAGAGATCTTCTAT TAGGAGGTAAAGTTGG
<i>At1g30700</i>	PstIAt1g30700Fw	ATAAGCTGCAGCGTCATCAGC AAACTCGGAGAC
	XbaIAt1g30700mycRv	CTAAGTCTAGACGTGGTAACA CAGGAATACTCTG

3.8 Expression in *N. benthamiana*

Since the *At1g30740* gene did not produce any detectable protein in *P. pastoris*, a construct was prepared for its expression *in planta*. The DNA sequence from the translation start to the termination codon (1602 bps) was amplified from Arabidopsis Col-0 gDNA using the XbaIAt1g30740Fw and SacIAt1g30740Rv primers (Table 3.2). The amplified fragment was cloned using the XbaI and SacI restriction sites of the binary vector pBI121 (Stratagene), replacing the β -glucuronidase gene sequence. The resulting plasmid (pBI121-At1g30740) was introduced into *Agrobacterium tumefaciens* GV3101 strain by electroporation. The transformed *Agrobacterium* bacteria were grown at 28°C overnight in presence of 50 $\mu\text{g ml}^{-1}$ kanamycin. Bacteria were suspended in 10 mM MgCl_2 and 150 $\mu\text{g ml}^{-1}$ acetosyringone with the final optical density of 0.1 nM. Cells were left at room temperature for 3 hours and then infiltrated into two-week-old leaves of *N. benthamiana*. *A. tumefaciens* GV3101 strain carrying the empty pBI121vector was used as negative control. Protein extraction and activity assay were performed as described above, at two and three days after agroinfiltration.

Table 3.2. Primers used for the construction of *At1g30740*

GENE	PRIMER NAME	PRIMER SEQUENCE
<i>At1g30740</i>	XbaIAt1g30740Fw	GAATGICTAGAAATGTATCTTATC TTTCTTCTATTC
	SacIAt1g30740Rv v	GATCCGAGCTCCTATCTTCTA TTAGGAGGTAAAG

3.9 Production of oxidized OGs (oxOGs)

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) from *P. pastoris*. 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 25000 x 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 performed using 20 mg of standard OGs in the absence of the enzyme

3.10 Plant treatment

For seedling assays, seeds were surface sterilized and germinated in multiwell plates (approximately 10 seeds/well) containing 0.5X Murashige and Skoog (MS; (Murashige, T. and Skoog, F. 1962) medium supplemented with 0.5% sucrose (2 mL/well). Seedlings were grown at 22°C and 70% relative humidity under a 16 h/8 h light/dark cycle (approximately 120 $\mu\text{mol}/\text{m}^2/\text{s}$). After 9 days, the medium was adjusted to 1 mL and treatments with OGs, oxOGs and OG+oxOGs were performed after 24 h.

3.11 Gene expression analysis

Seedlings were frozen in liquid nitrogen, homogenized with mixer mill MM301 (Retsch) for 2 min at 25 Hz, and total RNA was extracted with ISOL-RNA Lysis Reagent (5-Prime) according to the manufacturer's protocol. RNA was treated with RQ1 DNase (Promega) and first-strand cDNA was synthesized using ImProm-II reverse transcriptase (Promega) according to the manufacturer's instructions. Real-time PCR analysis was performed using a CFX96 Real-Time System (Biorad). One microliter of cDNA (corresponding to 50 ng of total RNA) was amplified in a 20

μ l reaction mix containing 1X GoTaq Real-Time PCR System (Promega) and 0.5 μ M of each primer. Three technical replicates were performed for each sample and data analysis was done using LinRegPCR software. Expression levels of each gene, relative to *UBQ5*, were determined using a modification of the Pfaffl method (Pfaffl, M. W. 2001) as previously described (Ferrari, S. et al. 2006). Primer sequences are shown in Table 3.3. Marker gene analysis was performed from at least 3 independent biological replicates, each composed by 20 seedlings or at least 4 adult leaves from different plants.

Table 3.3. Primer sequences used in gene expression analysis.

Gene	Forward Primer	Reverse Primer
<i>UBQ</i> (At3G62250)	GTTAAGCTCGCTGTT CTTCAGT	TCAAGCTTCAACTC CTTCTTTC
<i>RET-OX</i> (At1g26380)	CGAACCCTAACAAC AAAAAC	GACGACACGTAAG AAAGTCC
<i>CYP81F2</i> (At5g57220)	AAATGGAGAGAGAG CAACAACACAATG	ATCGCCCATTCCAA TGTTAC
<i>OGO1</i> (At4g20830)	GTCGCGACTGCGATT CCGAAGA	CAGTAGAGCTCCT AGAAGACATGTAA GACCACC

3.12 Measurement of oxidative burst

H₂O₂ generated by seedlings in response to OGs and oxOGs (50 µg mL⁻¹ each) was measured in the incubation medium after 30 minutes of treatment by a colorimetric assay based on the xylene orange dye (o-cresolsulfonephthalein 3',3''-bis[methylimino] diacetic acid, sodium salt; Sigma), as previously described (Galletti, R. et al. 2008). To determine H₂O₂ concentration, 100 µL of the incubation medium were added to 100 µL of assay reagent (500 mM ammonium ferrous sulfate, 50 mM H₂SO₄, 200 mM xylene orange, and 200 mM sorbitol). After 40 min of incubation, absorbance of the Fe³⁺-xylene orange complex (A₅₆₀) was detected. Standard curves of H₂O₂ were obtained for each independent experiment. Data were normalized and expressed as nM H₂O₂/mg fresh weight of seedlings. ROS measurement assays were performed from three independent biological replicates, each consisting of 40 seedlings.

3.13 Measurement of explanting forming roots in *Nicotiana tabacum*

Analysis of roots formation in *Nicotiana Tabacum* was performed as previously described in (Bellincampi, D. et al. 1996). Briefly ten explants (0.3 x 0.6-cm rectangles), were excised from leaves and placed abaxial side down in Petri dishes containing 10 mL of MS supplemented with 2% sucrose, 0.8% agar, and IAA(100µg/L). OGs or oxOGs, dissolved in distilled water and filter sterilized, were added to the culture medium to a final concentration 4-10 µg/ml. After 15 days of culture the number of roots per explant or the number of explants forming roots were scored.

3.14 Callose deposition

Analysis of callose deposition was performed as previously described with slight modifications (Brutus, A. et al. 2010). Briefly, leaves from 4-week-old plants were infiltrated with OGs, oxOGs and water as control. After 24 h about eight leaves from at least four independent plants, for each treatment, were cleared and dehydrated with 100% boiled ethanol. Leaves were fixed in an acetic acid: ethanol (1:3) solution for 2 h, sequentially incubated for 15 min in 75% ethanol, 50% ethanol, and in 150 mM phosphate buffer, pH 8.0, then stained in 150 mM phosphate buffer, pH 8.0,

containing 0.01% (w/v) aniline blue for 16 h at 4°C. After staining, leaves were mounted in 50% glycerol and examined by UV epifluorescence microscope (Nikon, Eclipse E200) using 4x or 10x magnification objective. Filter cubes used was the UV filter (ex. 330-380; em. 400) and the excitation was detected using a cooled charge-coupled device CCD camera (DS-Fi1C). Acquisition software is Nis Elements AR (Nikon). The relative callose units were calculated using ImageJ^R

3.15 Isolation and genotyping mutant

Genomic DNA from WiscDsLox432E05 and Col-o 25-day-old plants was extracted from rosette leaves as previously described (Edwards et al., 1991). Genomic DNA was subjected to PCR to detect the WT allele AT4G20830 using WISCDSLOX432E05L (GAATTCGAAACCACTGATTG) and WISCDSLOX432E05R: (ATCACGGAGATTTCGACATGTC) primers. To detect the insertion of the T-DNA, the P745 primer: (CAATGTGTTATTAAGTTGTC) was used. Samples were subjected to amplification using Taq DNA Polymerase (RCB-Bioscience) under the following conditions: 2 min at 94°C; 35 cycles as follows: 30 sec at 94°C, 30 sec at 60°C

and 45 sec at 72°C; 7 min at 72°C. PCR products were separated by agarose gel electrophoresis and visualized by EtBr staining.

Plants homozygous for the mutations were propagated and used for all subsequent experiments.

3.16 Generation of transgenic plants

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 genomic DNA extracted from Col-o 10-day-old seedlings using the BamHIA4g20830Fw and SacIA4g20830Rv primers (Table 3.4). The fragment was cloned using the BamHI and SacI restriction sites of pBI121 vector, which confers kanamycin resistance. The recombinant plasmid was introduced into *A. tumefaciens* GV3101 strain by electroporation and stable transgenic lines were obtained using the standard *Agrobacterium tumefaciens*-mediated gene transfer procedure (floral dip) (Clough, S. J. and Bent, A. F. 1998).

11 independent transgenic lines expressing the construct were selected based on their kanamycin resistance. From

these lines two homozygous plants of the T3 generation (OGOx1-OE #1.9 and #11.8), with a single transgene insertion, were chosen for experiments.

Table 3.4. Primers used for the construction of *At4g20830*

GENE	PRIMER NAME	PRIMER SEQUENCE
<i>At4g20830</i>	BamHIA4g20830Fw	CATGAGGATCCATGCTCACGACA CCTCCACG
	SacIA4g20830Rv	CAGTAGAGCTCCTAGAAGACA TGTAAGACCACC

3.17 Arabidopsis root length assay

For root length determination, surface-sterilized seeds were germinated and grown vertically in plates containing 1/2 Murashige and Skoog medium containing 0.8% (w/v) agar and 1% (w/v) sucrose. For amiRNA inducible seedlings, β -estradiol (and DMSO) was supplied to the medium at a final concentration of 5 μ M. After seven days the root length was measured using ImageJ^R.

3.18 Infection assays

Pectobacterium carotovorum subsp. *carotovorum* (strain DSMZ 30169) was obtained by DSMZ GmbH (Braunschweig, Germany). Bacteria were cultivated in LB for 16-18 h at 28°C, 340 rpm. Next bacterial cells were collected by centrifugation (8000 x g for 10 min) and suspended in a 50 mM potassium-phosphate buffer (pH 7.0) at a final OD₆₀₀ = 0.05, corresponding to a concentration of 5×10^7 colony forming units mL⁻¹. Four-weeks old Arabidopsis rosette leaves were detached and placed in Petri dishes containing 0.7% plant agar with the petiole embedded in the medium.

Two lesions were made on the epidermis of the adaxial surface of each leaf, at the sides of the middle vein, using a sterile needle and 5 µL of the bacterial suspension were placed on each punctured site. Plates were covered with transparent plastic film to maintain the humidity and incubated at 22°C with a 12 h photoperiod for 16 h. Lesion size was then determined measuring the diameter of necrotic area by using ImageJ software. Infections were performed from three independent biological replicates.

P. syringae pv. tomato strain DC3000 was cultured in LB broth at 28°C for one day, and bacteria were

suspended in 10 mM MgCl_2 at a final concentration of 10^5 cfu/ml corresponding to 2.5×10^3 cfu cm^2 of leaf tissue.

Bacteria were inoculated by syringe infiltration on leaf previously marked so that it can be identified, approximately two site of infiltration for each leaves were done. After 3 days infected leaves are harvested and surface sterilized in a 70% ethanol solution (1 minute) and then rinsed in sterile (1 minute). Leaf disks for each sample are excised and placed in a 1.5-mL microfuge tube with 100 μL sterile distilled water. The tissue samples are ground with a microfuge tube plastic pestle and the pestle is rinsed with 900 μL of water, such that the sample is in a volume of approximately 1mL. The samples are thoroughly vortexed and A 100- μl sample is removed and diluted in 900 μl sterile distilled water. Four serial 1:10 dilution series were created for each sample.

10 ul of each diduition of each samples were spotted on LB medium supplemented with rifampicin, to select for the inoculated bacterial strain, and allowed to dry onto the surface.

The plates are placed at 28°C for 2 days and then the colony-forming units for each dilution of each sample are counted. The single spot should be used for estimating the

colony-forming units (CFU) only if it has >10 and <70 colonies present in the spotted sample dilution.

The CFU for each sample was calculated based on the dilution factor divided by the amount of tissue present in each sample.

Pathogenicity assays with *B. cinerea* were performed as previously described (Gravino, M. et al. 2015). *B. cinerea* was grown on 20 g l^{-1} malt extract, 10 g l^{-1} proteose peptone n. 3 (Difco, Detroit, USA), and 15 g l^{-1} agar for 7–10 days at 24°C with a 12 h photoperiod before collection of spores. Rosette leaves from Arabidopsis plants were placed in Petri dishes containing 0.7% agar, with the petiole embedded in the medium. Inoculation was performed by placing $5\text{ }\mu\text{l}$ of a suspension of 5×10^5 conidiospores ml^{-1} in 24 mg ml^{-1} potato dextrose broth (Difco, Detroit, USA) on each side of the middle vein. The plates were incubated at 22°C under constant light (about $80\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$) for 48 h. High humidity was maintained by covering the plates with a clear plastic lid and after 48 hours, lesion size of infection was determined by measuring the necrotic area by using ImageJ software.

4. RESULTS

4.1 Diffusates from OGM plants contain oxidized OGs

The control of the OGs levels through the expression of the β -estradiol inducible OGM represents a unique tool for elucidating the signaling processes mediated by these molecules. The expression of OGM causes the accumulation of OGs in planta (Benedetti, M. et al. 2015). However OGs at high levels trigger a deleterious hyperimmunity and homeostatic mechanism that prevent their hyper-accumulation must exist.

To investigate on the existence of regulatory homeostatic mechanisms in the perception and signalling of OGs, we searched differences in OG content or in OG structure using OGM plants because they can accumulate high amount of OGs in the tissues.

A procedure for *in vivo* OG extraction and analysis was set up (Materials and Methods), using OGM plants induced with 50 μ M β -estradiol for 170 hours. Upon this treatment, symptoms of hyper-immunity such as chlorosis and necrosis were visible. Leaf strips were prepared from the β -estradiol-

induced OGM plants and incubated in a strong chelating agent buffer (ChA) to promote the diffusion of pectin fragment into the medium. After 20 h, diffusates were collected and HPAEC-PAD analysis was performed. This showed an abundant proportion of oligosaccharides with chromatography characteristics different from that of standard OGs, i.e. with different retention times, as shown in figure 4.1 (a-b).

To elucidate the chemical nature of these atypical fragments, the diffusates were treated with a polygalacturonase that degrade homogalacturonan to tri-, di-, and monogalacturonic acid as final products. After incubation with pure *Fusarium phyllophilum* PG expressed in *Pichia. pastoris* for 1 h, the fragments in the diffusate were hydrolyzed (Fig. 4.1c) indicating that they had the typical alpha-1,4 homogalacturonan backbone. I concluded that the fragments were OGs with modified structural characteristics.

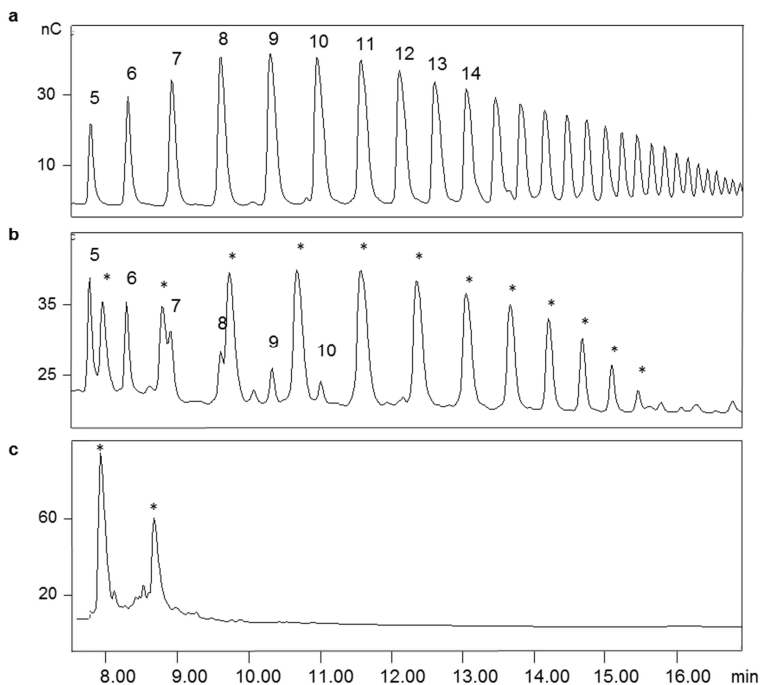


Figure 4.1. HPAEC-PAD analysis on the diffusates obtained from the β -estradiol-induced OGM plants. Adult plants were sprayed with 25 μ M β -estradiol and leaves were collected after 170 h for preparation of diffusates. Numbers above the peaks indicate the DP of the single oligomers. Asterisks indicate oligosaccharides with retention times different from those of standard OGs. **a)** standard OGs, **b)** fragments obtained from the diffusates of leaf strips incubated in ChA buffer for 20 h, **c)** fragments obtained from the diffusates as in b after treatment with PG.

The chemical nature of the modified OGs in the diffusates was determined by electrospray ionization mass spectrometry (ESI-MS) analysis. As shown in figure 4.2a the mass spectrum of the diffusate contains two envelopes

associated with two major series of singly protonated species. The first series encompasses molecules separated by an m/z interval of 176, which equals the mass of the galacturonic acid (GalUA) repeat unit, and therefore represent the standard unmodified OGs. The ions in this series were assigned to OGs with DPs ranging from 3 to 7. The second series includes eight species characterized by an increased mass (+ 16 m/z) with respect to the mass of standard unmodified OGs, indicated in figure with ox (Fig 4.2a) and with DP ranging from 2 to 9.

The assignment of the structures of these modified oligosaccharides were obtained by tandem mass spectrometry using the ion at m/z .409.17. Full MS2 on the $[M+H]^+$ molecular ion at m/z .409.17 produced glycosidic bond cleavage fragments, generating B- and Y-ions (Fig. 4.2b) with the molecular masses of B-ions corresponding to galacturonic acid and the molecular masses of Y-ions increased by 16 Da, indicating that an oxidation reaction had occurred in the reducing end with formation of galactaric acid (MM 210 Da).

Using pure galactaric acid as standard, we obtained by MS2 a fragmentation pattern (Fig. 4.2d) identical to that obtained by MS3 on the Y ion at m/z .233.0 (Fig. 4.2c), leading to the

conclusion that the oligosaccharides present in the diffusates were oxidized OGs.

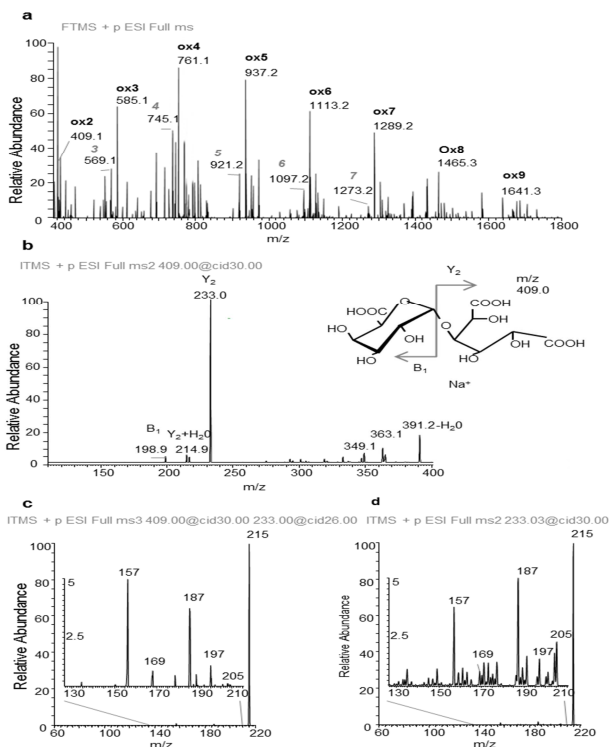


Fig 4.2. Structure and positive ion CID mass spectra of modified OGs by ESI-MS. **a**) Mass spectrum of the oligosaccharides detected in the diffusates from the OGM plants. Numbers above the peaks indicate the DP of the single OGs; ox+number indicates the DP of the oxidized oligomer, characterized by an increased mass (+16 m/z) with respect to the corresponding unmodified one, **b**) Full MS2 on the [M+ H]⁺ molecular ion at m/z.409.17 produced glycosidic bond cleavage fragments, generating B- and Y-ions. The fragment ion peaks are labeled according to the nomenclature proposed by Domon and Costello (Domon, B. and Costello, C. E. 1988). The molecular masses of Y-ions only were increased by 16 Da, corresponding to OGs oxidized at the reducing end. **c**) Full MS3 on the Y ion at m/z.233.0 produced a fragment ion pattern corresponding to that obtained by MS2 of the standard galactaric acid (**d**).

4.2 Proteins from OGM plants possess an OG-oxidizing activity

Because of the presence of chemically modified OGs in leaf strips of Arabidopsis plants expressing the OGM, the same plants were used to search for an enzymatic activity capable of oxidizing OGs.

The presence of an enzyme capable of catalyzing the oxidation of D-galacturonic acid (and D-glucuronic acid) to the corresponding hexaric acids, galactaric acid (and D-glucaric acid), in the presence of molecular oxygen as an electron acceptor, with the production of H_2O_2 , had been previously reported in citrus leaves. (Riov, Joseph 1975). A similar uronic acid oxidase activity was found in extracts from peach fruit (Cantu, D. et al. 2006) and in commercial plant peroxidase preparations (Marsh, C. A. 1985). These activities were inhibited by sulfites, suggesting that they are flavoproteins. Sulfite ions are thought to be bound at the active site of the enzymes, probably by forming a complex with the flavin prosthetic group and inhibit flavoprotein oxidases, but not flavoprotein dehydrogenases (Massey, V. et al. 1969).

We therefore examined whether the presence of sulfite could influence the presence of oxidized OGs in the

Arabidopsis leaf diffusates. Diffusates were prepared as described above in the presence or in the absence of 10 mM Na_2SO_3 and subjected to HPAEC-PAD analysis.

As shown in Figure 4.3, in the absence of sulfites the presence of oligosaccharides with a chromatography pattern different from that of standard OGs was confirmed, whereas in the presence of Na_2SO_3 , only non-oxidized OGs with retention times typical of standard OGs were observed (Fig. 4.3). These data suggested that the oxidized OGs observed in the diffusates of the leaf strips are formed by the action of endogenous flavoenzymes acting during incubation.

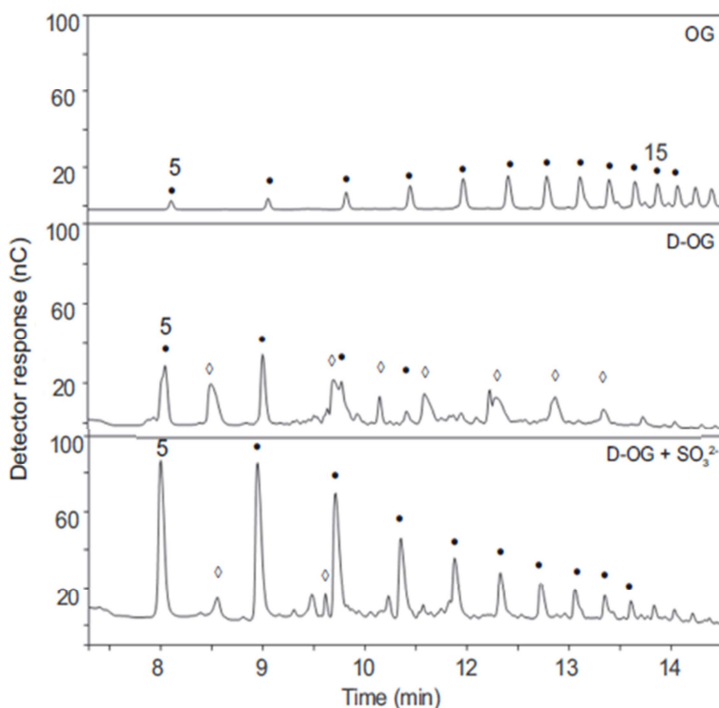


Fig 4.3. HPAEC-PAD analysis of β -estradiol-induced OGM leaf diffusates prepared in presence of sulphite. Chromatograms of standard OGs (OG), diffused (D-OG) and diffusate obtained in the presence of 10 mM Na₂SO₃ (D-OG + SO₃²⁻). Numbers above the peaks indicate the DP of the single oligomers. Black circles indicate the retention times of standard OGs. White diamonds indicate oligosaccharides with retention times different from those of standard OGs. Signal intensities (nC) at each retention time (min) are indicated.

This observation prompted to identify the OG-oxidizing flavoprotein-dependent enzyme in total protein extracts of induced OGM plants as well as in wild-type total protein

extracts to assess the presence of the same activity in physiological conditions. Analyses were performed in the presence and in the absence of 1 mM Na₂SO₃, by following the oxidation of a standard OG preparation by HPAEC-PAD. In addition, whether hydrogen peroxide was generated by the reaction was investigated using an xylenol orange-based colorimetric assay.

A marked OG-oxidizing activity that was inhibited by SO₃²⁻ was found in the OGM extracts, and the measurement of H₂O₂ allowed us to quantify this activity was about 5-times higher in the OGM plants with respect to Col-0 plants (Fig.4.4).

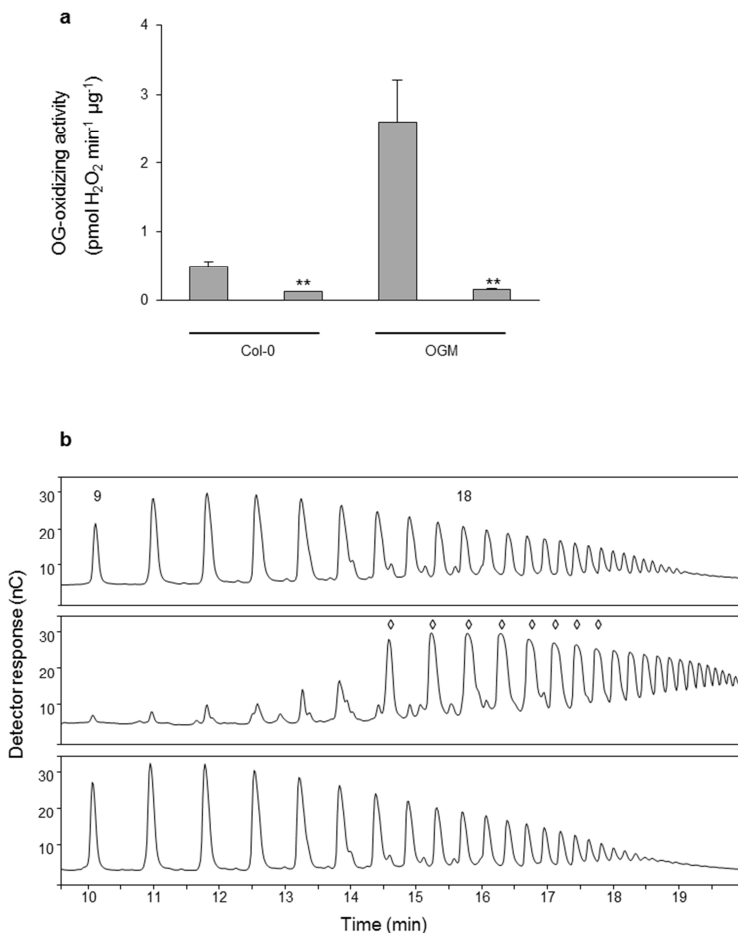


Figure 4.4. Total protein preparations from β -estradiol-induced OGM plants possess a marked OG-oxidizing activity that is inhibited by SO_3^{2-} . **a)** Enzyme activity, expressed as $\text{pmol H}_2\text{O}_2 \text{ min}^{-1} \mu\text{g}^{-1}$, detected in total protein preparations from Col-0 and induced OGM leaves, using OGs as a substrate, in the presence and in the absence of 1 mM Na_2SO_3 . Asterisks (**) indicate statistically significant differences according to Student's t-test ($P < 0.01$). **b)** HPAEC-PAD analysis of standard OGs (OG), standard OGs incubated with total protein preparation from OGM plants (OG + TPOGM) and standard OGs incubated with total protein preparation from OGM plants in the presence of 1 mM Na_2SO_3 (OG + TPOGM + Na_2SO_3). Numbers above the peaks indicate the DP of the single oligomers. White diamonds indicate oxOGs.

The purification of the OG-oxidizing activity was undertaken. As a first step, an affinity chromatography was performed using a polygalacturonic acid-polyacrylamide gel column (Spadoni, S. et al. 2006) (Fig. 4.5a), followed by cationic exchange (CE) (Fig. 4.5b) and hydrophobic interaction (HI) chromatography (Fig. 4.5c). In every purification step the OG-oxidizing activity was evaluated by supplying OGs as substrate to each fraction eluted, in the presence and in the absence of 1mM SO_3^{2-} , and by detecting H_2O_2 as a reaction product. SDS-PAGE analysis showed a prominent 70 kDa band of fractions displaying the highest oxidase activity eluted from the Phenyl-sepharose column (fractions 17-19; Fig. 4.5d). Gel slices corresponding to 70 kDa of fraction 17 and 18 were analyzed by LC-MS that identified a putative flavoprotein encoded by At4g20830 and referred to as reticuline oxidase-like protein.

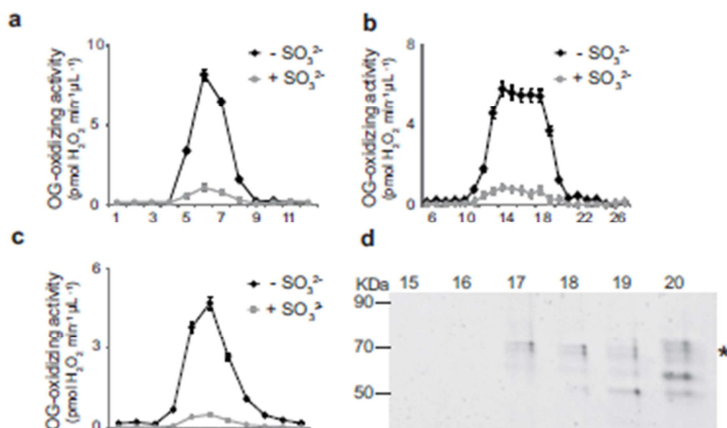


Fig 4.5. β -estradiol-induced OGM plants possess an OG-oxidizing activity. **a-c)** OG-oxidizing activity was evaluated by supplying OGs to the fraction eluted from the HGA-affinity column **a)**, from the SP-sepharose column **b)** and from the Phenyl-sepharose column **c)** in presence (+) and in absence (-) of 1mM SO₃²⁻. **d)** SDS-PAGE analysis of fractions (15-20) eluted from the Phenyl-sepharose column. The asterisk indicates the apparent molecular weight (70KDa) of the protein that co-elutes with the highest OG_oxidizing activity detected in fractions 17-19.

4.3 The AT4G20830 gene

The AT4G20830 gene, belongs to the superfamily of genes encoding the FAD-binding berberine-bridge enzyme-like proteins (BBELs). The Arabidopsis BBEL superfamily comprises 28 members (see below).

The AT4G20830 gene is located in chromosome 4 and presents two exons and one intron (379 nt) (Fig. 4.6a). Two different transcripts and two secreted protein isoforms have

been found for this gene, presumably produced by alternative splicing and/or alternative transcription termination (Fig. 4.6b). A longer one (AT4G20830.1) derives from the proper splicing of the intron; the other (AT4G20830.2) comprises part of the intron and lacks the sequences corresponding to the exon 2. In the latter transcript, the stop codon lies inside the intron (at nt 9 from its start) and the 3'UTR includes 214 nt of the intron sequence (Fig. 4.6b). The longer transcript is predicted to encode a precursor protein of 570 amino acids, whereas the shorter one encodes a protein of 540 aa. The amino acid sequence of the C-terminal portion that distinguishes the At4g20830 protein isoforms is characterized by an additional 30-amino acid hydrophobic tail (Fig. 4.6c). The isoforms AT4G20830.1 and AT4G20830.2 are hereon indicated as Long and Short, respectively.

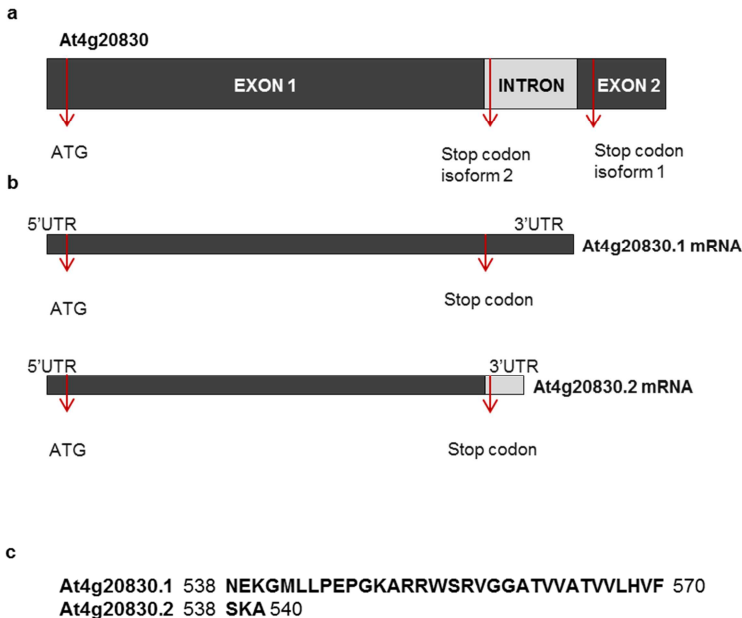


Figure 4.6. The gene *At4g20830* encodes two different OGOX1 isoforms. a) Schematic representation of the gene *At4g20830*. The gene is 2092 bps long (according to TAIR) and is characterized by the presence of an intron (379 bp), located 1856 bp downstream of the A of the translation initiation codon. **b)** Two different transcripts, *At4g20830.1* and *At4g20830.2*, are produced presumably by alternative splicing/termination, and encode 570- and 540-amino acid products, respectively. **c)** Amino acid sequence of the C-terminal portion that distinguishes the *At4g20830* protein isoforms. *At4g20830.1* is characterized by an additional 30-amino acid hydrophobic tail.

4.4 *At4g20830* encodes an OG oxidase (OGOX1)

For characterization, the short isoform was tagged at the C-terminus with the c-myc tag, expressed in *Pichia pastoris* using a pPICZalpha B vector (INVITROGEN see material and methods) for methanol-inducible expression. The

secreted heterologous protein was purified from medium collected after 48 h of growth in the presence of methanol (Fig. 4.7a). by using two consecutive hydrophobic chromatography steps (Phenyl-Sepharose, Amersham), with different pH (pH 4.6 and 7). During the purification, the OG-oxidizing activity was evaluated by supplying OGs as substrate to an aliquot of each fraction eluted and detecting H_2O_2 as a reaction product. Purity of the protein was assessed by SDS-PAGE/Coomassie blue staining (Fig 4.7b).

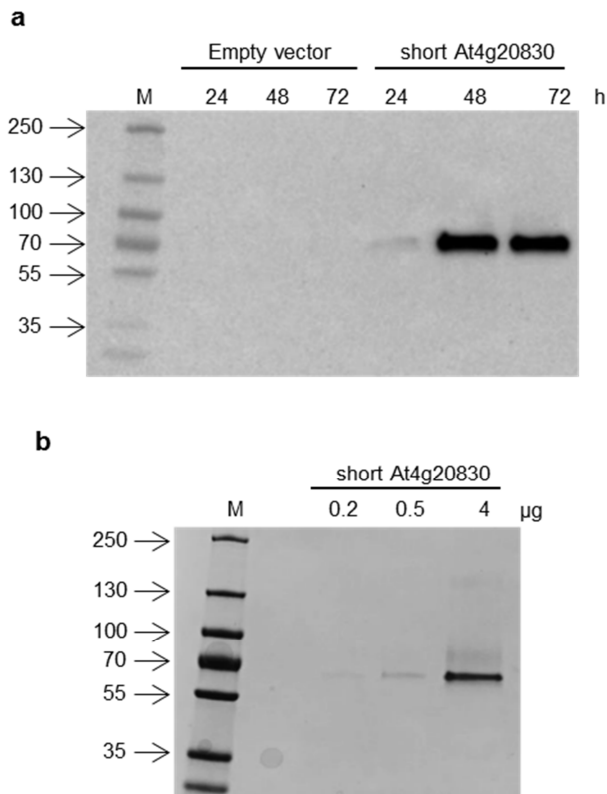


Figure 4.7. Expression of the myc-tagged short At4g20830 isoform in *P. pastoris*. **a)** Presence of the short At4g20830 protein was determined by immuno-blot using an α -myc antibody in culture filtrates of transformed *P. pastoris* at different times (24, 48 and 72 h) after induction with methanol. Culture filtrates of *P. pastoris* transformed with the empty vector were used as a negative control. Short At4g20830 is characterized by an apparent molecular weight of 70 KDa. **b)** Pure *P. pastoris*-expressed short At4g20830; different amounts were analyzed by SDS-PAGE (Coomassie blue staining).

The specificity of the enzyme purified from both plants (as described previously) and *P. pastoris* was determined by using different substrates: standard elicitor-active OGs as well as short OGs and oligomannuronides (epimers of OGs which are inactive as elicitors of most defense responses (Ferrari, S. et al. 2013, Mathieu, Y. et al. 1991), cellotriose and cellopentaose as well as several sugar monomers component known to be present in plant cell walls (galacturonic and glucuronic acid, glucose, galactose, xylose, rhamnose, fucose, arabinose, mannose) (Fig. 4.8a,b). Both the plant and *Pichia*-expressed enzyme were active only on OGs and, due to this feature, the enzyme was named OG oxidase 1 (OGOX1). In Figure 4.8c a schematic representation of the reaction catalyzed by OGOX1 is shown.

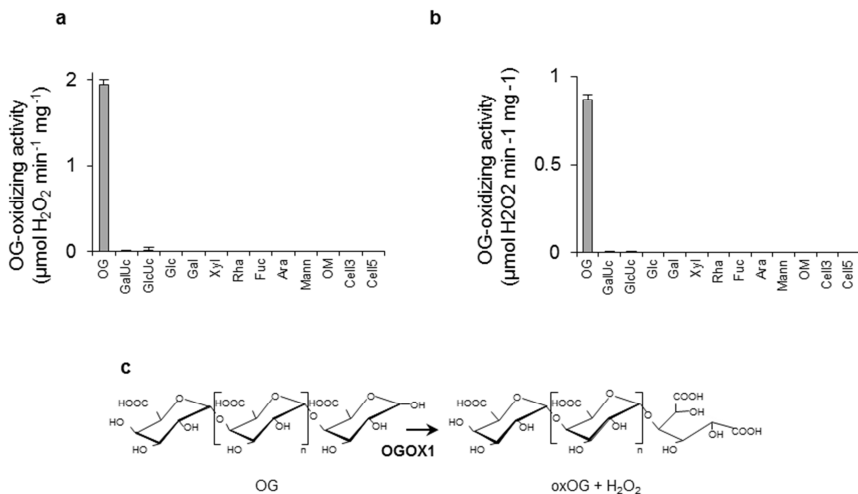


Figure 4.8. Characterization of the short At4g20830 isoform. **a)** Enzyme activity of the protein encoded by *At4g20830* purified from *Arabidopsis* leaves towards different monosaccharides and oligosaccharides [GalUc: galacturonic acid, GlcUc: glucuronic acid, Glc: glucose, Gal: galactose, Xyl: xylose, Rha: rhamnose, Fuc: fucose, Ara: arabinose, Mann: mannose, OM: oligomannuronides, Cell3: cellotriose, Cell5: cellopentaose]. **b)** Enzyme activity of the pure *P. pastoris*-expressed short At4g20830 towards different monosaccharides and [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)** Schematic representation of the reaction catalyzed by OGox1.

The enzyme from *P. pastoris* was also tested on individual OG oligomers, previously purified from the mixture of standard OGs and quantified and showed higher activity on oligomers with a DP ≥ 5 (Fig. 4.9).

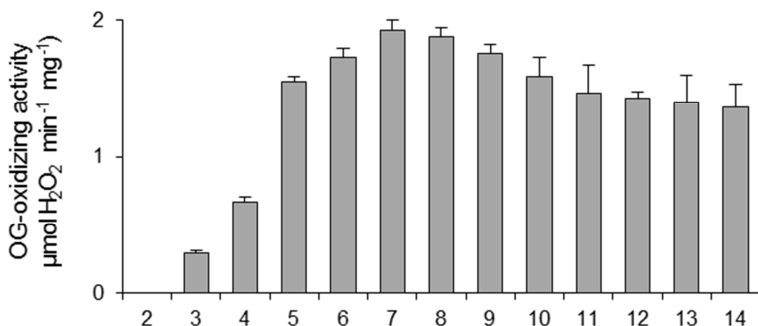


Figure 4.9. Characterization of the myc-tagged short At4g20830 isoform in *P. pastoris*. Enzyme activity of OGOX1 purified from *P. pastoris* towards single OG oligomers (DP between 2 and 14).

4.5 Oxidized OGs (oxOGs) are elicitor-inactive molecules

To investigate the biological function of OGOX1, I tested the effect of the oxidation on the biological activity of OGs. To this aim, purified OGOX1 from *P. pastoris* was used to oxidize to completion a mixture of standard OGs and the resulting oxidized OGs (oxOGs) were tested for their biological activity. The oxOGs were unable to induce responses that are typical readouts of the action of elicitor-active OGs i.e. the expression of the defence markers *RetOx* (At1g26380) (Fig. 4.10a) and *CYP81F2* (Fig. 4.10b). Moreover, oxOGs did not interfere with the elicitor activity of un-oxidized OGs, indicating that they do not compete with the active molecules at the receptor level (Fig. 4.10c).

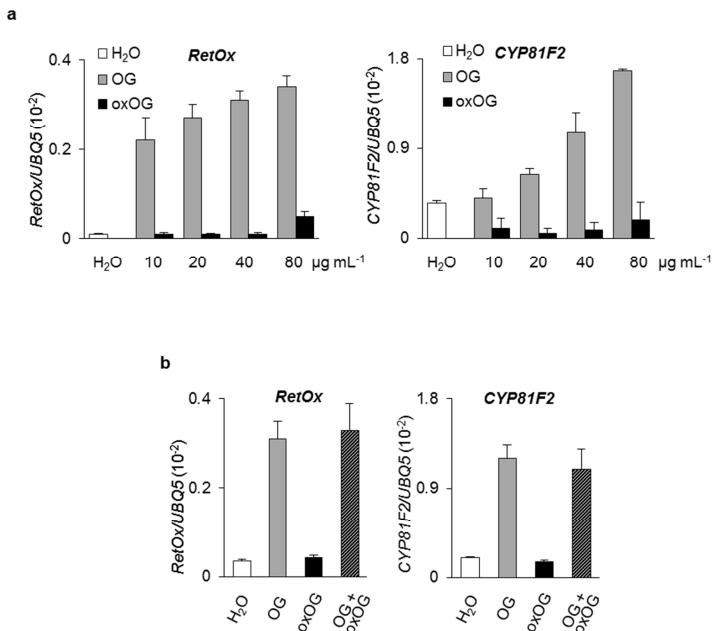


Figure 4.10 Oxidized OGs are not active in eliciting the defense responses. **a)** Gene expression analysis of *RetOx* and *CYP81F2* in 10-days old-seedlings after 1 h treatment with different amounts of OGs and oxOGs; **b)** Gene expression analysis of *RetOx* and *CYP81F2* in seedlings after 1 h of co-treatment with OGs and oxOGs (OG + oxOG) (40 µg mL⁻¹ each).

oxOGs were also tested for other typical readouts of the OGs action. They were unable to induce the oxidative burst (Fig. 4.11a), did not exhibit the typical antagonism with auxin displayed by the standard OGs (Fig. 4.11b) and did not induce the production of callose upon infiltration (Fig. 4.11c-d).

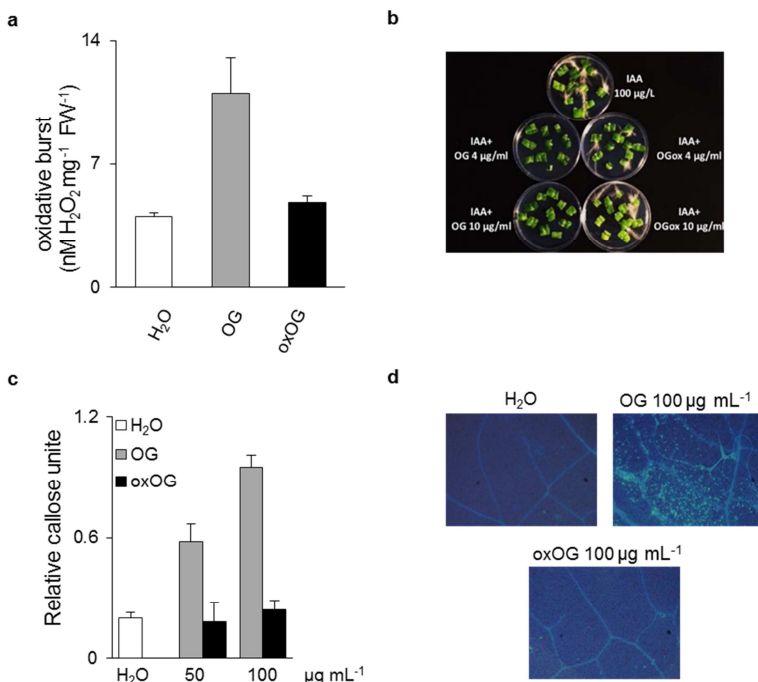


Figure 4.11 Oxidized OGs are not active in eliciting the defense responses. **a)** oxidative burst measured in seedlings after a 30-min treatment with OGs or oxOGs (50 µg mL⁻¹ each); **b)** Effect of treatment with IAA(100µg/L), IAA and OGs (4-10µg/ml), IAA and oxOGs (4-10µg/ml) on the formation of roots in *Nicotiana tabacum* leaf explants; **c)** callose deposition measured after leaf infiltration with H₂O, OGs and oxOGs; representative callose deposition for treatment is shown in **d**.

4.6 Identification of OGOX2-3-4 in the Arabidopsis (BBE) superfamily

In the genome of *Arabidopsis thaliana* are present 28 BBE-like proteins. Their relationship is shown in the tree

presented in Fig. 4.12 (obtained with Cobalt http://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi).

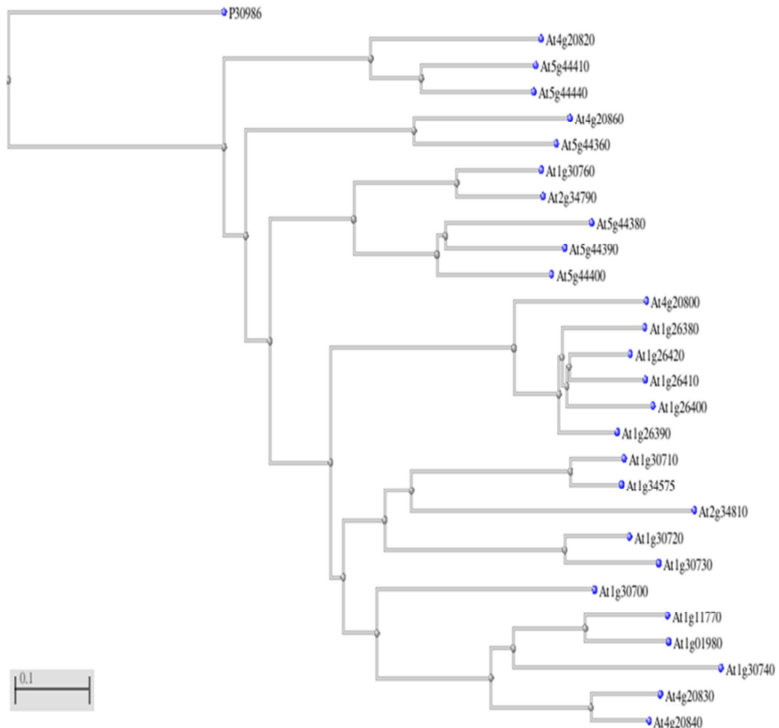


Figure 4.12 Similarity tree for *AtBBEs*. The tree is based on similarity among the *AtBBE* family protein members at the amino acid level; P30986 (Reticuline oxidase BBE from *Eschscholzia californica*), a true Berberine Bridge Enzyme, has been included in the analysis as an outgroup.

To search for other BBE members with OG-oxidizing activity, the five closest *OGOX1* paralogs (At4g20840, At1g30740, At1g01980, At1g11770, At1g30700) were

selected, which, like *OGOX1*, encode products carrying an N-terminal signal peptide for translocation into the ER and sharing a similarity > 45% with OGOX1/At4g20830 (Fig. 4.14a). The coding sequences of At1g30740, At1g01980, At1g11770, At1g30700 are not interrupted by introns.

As for OGOX1, for methanol-inducible expression of At4g20840, At1g11770, At1g01980, At1g30740 and At1g30700 in *Pichia pastoris*, the corresponding DNA sequences encoding the mature proteins were amplified from Arabidopsis genomic DNA (gDNA). The genes were cloned in pPICZ α B, fused to the sequence encoding the myc tag, and the constructs were introduced into the yeast by electroporation. The culture media obtained at different times of induction with methanol were tested for the presence of the heterologous protein by immunoblot assay.

The products of At4g20840, At1g01980, At1g11770, At1g30700, but not that of At1g30740, were all expressed as secreted proteins. In figure 4.13 the immunoblot assay performed on the culture filtrates at different times after induction with methanol are shown. For all proteins the culture medium at 48 h of induction was selected to test the OG oxidizing activity.

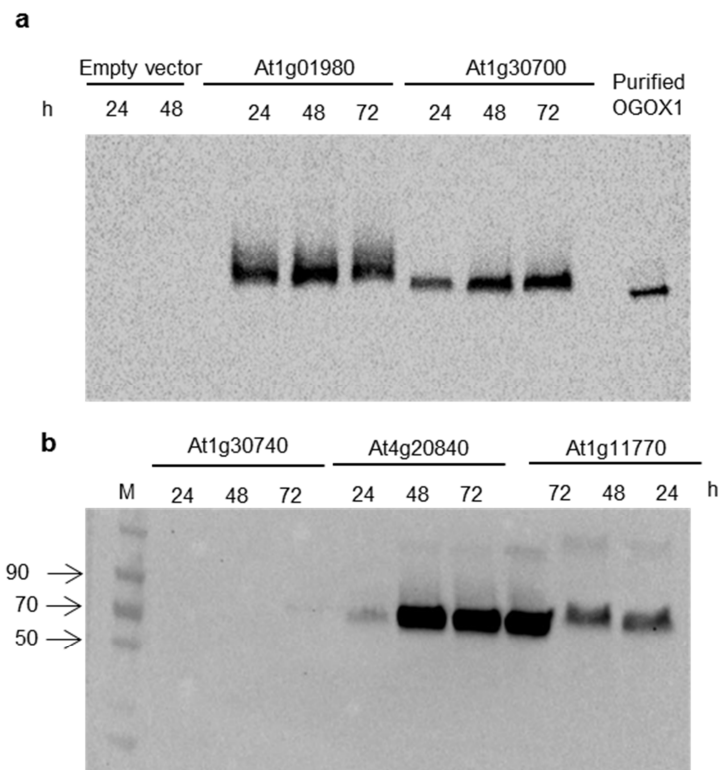


Figure 4.13. Expression of the myc-tagged of the closest *OGOX1* paralogs in *P. pastoris*. Presence of the proteins was determined by immuno-blot using an α -myc antibody in culture filtrates of transformed *P. pastoris* at different times (24, 48 and 72 h) after induction with methanol. **a)** detection of At1g01980 and At1g30700; culture filtrates of *P. pastoris* transformed with the empty vector were used as a negative control. Purified OGOX1 was used as control of molecular weight (70 Kda). **b)** detection of At1g30740, At4g20840 and At1g11770.

OG-oxidizing activity was detected only for the three closest OGOX1 paralog and these genes were named *OGOX2* (*At4g20840*), *OGOX3* (*At1g01980*) and *OGOX4* (*At1g11770*) (Fig. 4.14b). No activity was detected for At1g30700. The same preparations were tested for enzymatic activity using different substrate as previously described and none of them oxidized substrates other than OGs.

Because the At1g30740 gene did not produce any detectable protein in *P. pastoris*, we used a plant-based transient expression system for its expression. The DNA sequence from Arabidopsis Col-0 gDNA was amplified and cloned in PBI121 vector under the control of the CaMV 35S promoter. No tag was added to the encoded product. The resulting plasmid (pBI121-At1g30740) was introduced into *Agrobacterium tumefaciens* GV3101 and the transformed bacteria were used for agroinfiltration of *N. benthamiana* leaves. Two and three days after agroinfiltration the proteins were extracted and the activity assay performed. No activity was detected for At1g30740.

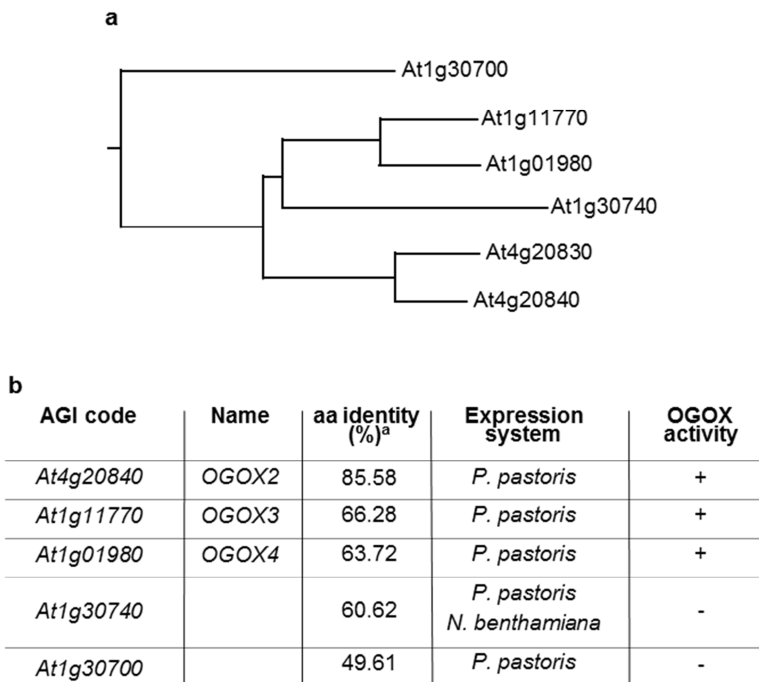


Figure 4.14 Four Arabidopsis BBLEs are OG-oxidizing enzymes. a) Homology tree of the mature products encoded by *At4g20830* and its five closest paralogs. **b)** Characterization of the OG-oxidizing activity of *OGOX1* close paralogs.

4.7 Overexpressing and knock out plant

Next, a reverse genetic approach was undertaken in order to investigate the biological role of OGOX1. I generated transgenic Arabidopsis Col-0 plants overexpressing the genes under the control of the CaMV 35S promoter.(CaMV 35S::*OGOX1*). From 11 independent transformed lines, two

T3 homozygous single-insertion transgenic lines expressing 35S::*OGOX1* were obtained (OGOX1-OE, #1.9 and #11.8). Analysis of *OGOX1* transcripts showed a higher expression in the line #11.8 and showed that in leaves of OGOX1-OE #11.8 and #1.9 the gene was overexpressed by 60- and 20-fold, respectively, compared to Col-0 (Fig.4.15b).

To evaluate the functionality of the 35S::*OGOX1* construct and the correct expression of the protein, I analyzed the activity of the protein using OGs as substrate and the detection of H₂O₂ after reaction. In agreement with gene expression analysis, the OG oxidizing activity was higher in the OGOX1-OE #11.8 (Fig.4.15c).

Moreover, I analyzed a homozygous single-T-DNA-insertion null mutant (*ogox1*) (Fig.4.15a) and two independent single-insertion homozygous lines expressing a β -estradiol-inducible specific artificial miRNA (amiR-OO1, lines #3.4 and #2.5) capable of targeting *OGOX1*, previously obtained in the laboratory. Transcripts (Fig. 4.15b) and OG-oxidizing activity (Fig. 4.15c) were undetectable in the *ogox1* mutant, while the reduction of both transcript and activity was observed in the two amiR-OO1, lines (#3.4 and #2.5) when germinated and grown in the presence of β -estradiol.

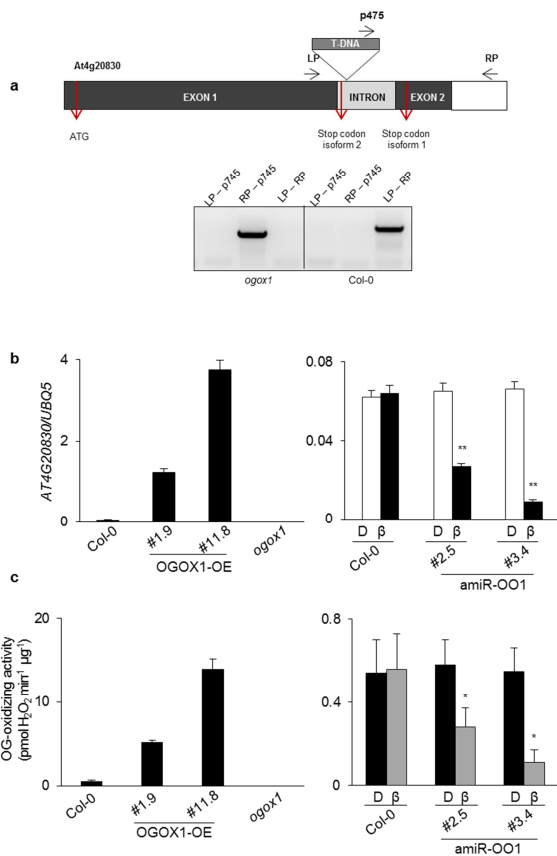


Figure 4.15. Characterization of OGOX1 transcript level and activity in transgenic plants. **a)** Schematic representation of the single T-DNA insertion in the *ogox1* null mutant and genotype analysis of the *ogox1* insertion mutant. The *OGOX1* gene was analyzed by PCR, using gene-specific primers flanking the T-DNA insertion using wild-type (Col-0) and *ogox1* gDNA as a template. **b-c)** Levels of *OGOX1* transcripts in **b** and of OG-oxidizing activity in **c** in leaves of the two *OGOX1* overexpressing plants (OGOX1-OE#1.9 and #11.8), the single-T-DNA-insertion null mutant (*ogox1*) and the two lines (amiR-OO1#2.5 and #3.4) expressing a β -estradiol-inducible specific artificial miRNA, germinated and grown for 10 days in the presence of β -estradiol. Asterisks indicate statistically significant differences with DMSO-treated plants, according to Student's t-test (*, $P < 0.05$).

All these lines showed no macroscopic morphological and growth alteration in the aerial part. To evaluate if OGOX1 influences specifically the growth of below-ground organs, primary root length was measured in seven-day-old seedlings grown vertically on agar plates. As shown in Figure 4.16a, statistically relevant differences were observed: increased root growth was observed in the seedlings over-expressing OGOX1, more evident in the line showing the highest expression; conversely, a reduced root growth was observed in the *ogox1* mutant and in both silenced lines when grown in the presence of β -estradiol. This result is in agreement with the high level of OGOX1 expression in in the root elongation and maturation zones (Fig. 4.16b).

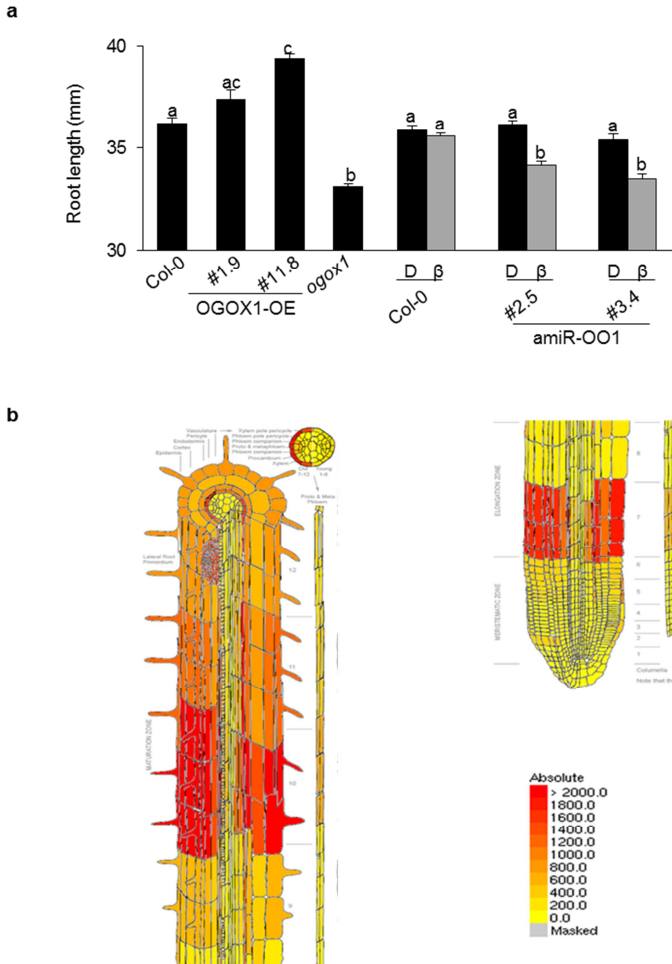


Figure 4.16 OGOX1 plays a role in development. **a)** Determination of the root length in 7-day-old seedlings of the two OGOX1 overexpressing plants (OGOX1-OE#1.9 and #11.8), the single-T-DNA-insertion null mutant (*ogox1*) and the two lines (amiR-OO1#2.5 and #3.4). amiR-OO1 seedlings were germinated and grown in presence of β -estradiol ($5 \mu\text{M}$) and DMSO (as a control). Statistical differences between root lengths were analyzed by One way ANOVA Tuckey HSD test ($n=30$). Bars indicate standard error. **b)** *OGOX1* is highly expressed in the root elongation and maturation zones (e-FP browser).

To investigate the role of OGOX1 in defense against pathogens, all the mutant/transgenic plants altered in *OGOX1* expression mentioned above were subjected to infection with different phytopathogenic microbes: the necrotrophic bacterial plant pathogen *Pectobacterium carotovorum*, the bacterial hemibiotrophic pathogen *Pseudomonas syringae* and the necrotrophic fungus *B. cinerea*.

The overexpressing plants showed a moderate but significant increase in susceptibility to the bacterial pathogens *Pectobacterium carotovorum* (Fig. 4.17a) and *Pseudomonas syringae* pv. *tomato* DC30000 (*Pst* DC3000) (Fig. 4.17b). In both infection the null mutant showed no difference compared to wild-type plants, indicating that overexpression of OGOX1 confers increased susceptibility to a necrotrophic and hemibiotrophic bacterial pathogen. These results are in agreement with the evidence that oxOGs lose their activity as DAMP. On the other hand a more conspicuous alteration was observed against the necrotrophic fungus *Botrytis cinerea*, where the area of the lesions caused by the fungus significantly differed among different genotypes. In overexpressing lines, proportionally to expression of OGOX1, lesion areas were reduced in

comparison to the wild-type, whereas the loss-of-function or silenced mutants were more susceptible (Fig.4.17c).

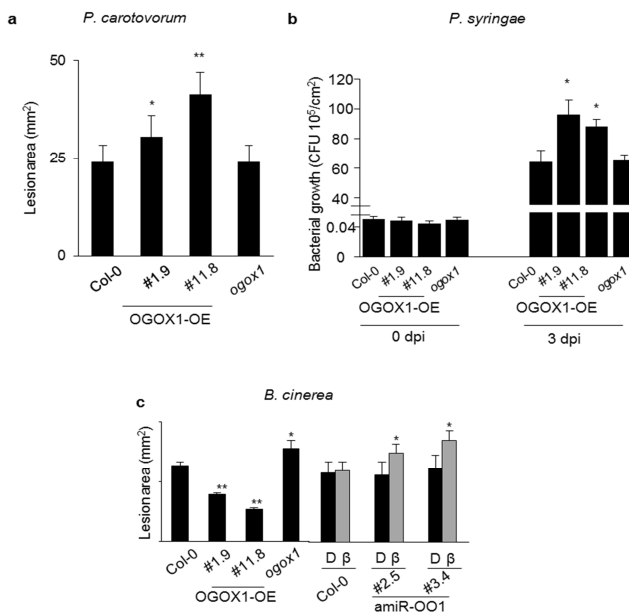


Figure 4.17 OGX1 plays a role in immunity. **a)** Four-week-old excised leaves of OGX1-OE#1.9 and #11.8 and *ogox1*, were drop-inoculated with *P. carotovorum* cells. Lesion size was measured after 16h using ImageJ^R. Bars indicate average lesion area $\pm$ SE of at least three independent experiments (n=20, in each experiment). **b)** Four-week-old leaves of OGX1-OE#1.9 and #11.8 and *ogox1*, were infiltrated with *P. syringae* DC3000 cells. CFU were evaluated at 0 and 3 days after infiltration. Bars indicate average CFU $\pm$ SE of at least three independent experiments (n=6, in each experiment). Asterisks indicate statistically significant differences against control (Col-0), according to Student's t test (*, $P < 0.05$) **c)** Four-week-old excised leaves of the two OGX1 overexpressing plants (OGX1-OE#1.9 and #11.8), the single-T-DNA-insertion null mutant (*ogox1*) and the two lines (amiR-OO1#2.5 and #3.4), were drop-inoculated with *B. cinerea* conidia. The amiR-OO1#2.5 and #3.4 plants were sprayed with β -estradiol (10 μ M) and DMSO (as a control) three times within a week before infection assay. Lesion size was measured at 48 h post-infection using ImageJ^R. Bars indicate average lesion area $\pm$ SE of at least three independent experiments (n=20, in each experiment); asterisks indicate statistically significant differences against control (Col-0), according to Student's t test (*, $P < 0.05$; **, $P < 0.01$).

5. DISCUSSION

The formation of cell wall fragments that act as signals of cell wall alteration occurs in multiple ways during the plant life as, for example, during the attack of microbes and herbivores, following a wide range of abiotic stresses or, simply, as a consequence of normal growth and development processes. Often the cell wall fragments accumulated during the attacks of microbes activate the plant defense responses and, therefore, behave as damage associated molecular patterns (DAMPs). OGs, derived from the fragmentation of HGA, are the first and best characterized plant DAMP. Because pectin is among the first components that are modified both during wall physiological remodelling and during pathogen attack the formation and the perception of OGs are likely to be crucial for plant survival

The construction of a molecular tool called OG-machine (OGM) and its inducible expression in transgenic plants has demonstrated the importance of these fragments in the

activation of defence responses and in the down-regulation of growth, reflecting the well-known phenomenon of growth-defence trade-off (Benedetti, M. et al. 2015). The hyper-accumulation of OGs severely affects growth, eventually leading to cell death. Therefore a mechanism to prevent the deleterious effect of DAMP hyper accumulation must exist.

In my thesis I have tried to support this hypothesis. A novel procedure for OG extraction was set up using leaf strips of OGM plants displaying symptoms of hyper-immunity such as chlorosis and necrosis and this procedure allowed me to identify the presence in these plants of oxidized OGs (oxOGs). I also assessed that the formation of oxOGs is due to the activity of a flavoprotein enzyme that utilizes O_2 as electron acceptor and produces oxOGs and H_2O_2 . This activity appear to be similar to enzymatic activities reported previously (Riov, Joseph 1975). The protein responsible for this reaction was identified and its specificity for the OGs, tested using different substrates, led us to discover the first OG oxidase named OGOX1, encoded by the At4g20830 gene.

The gene shows low expression levels in the shoot apex of adult plants and highest levels of expression in the

elongation zone of roots. It is moderately induced by ACC, IAA, ABA and strongly induced by several pathogens (*Pseudomonas*, *Botrytis cinerea*, *Phytophthora*) and by elicitors (both MAMPs and DAMPs). Its co-expression network (<http://atted.jp/cgi-bin/locus.cgi?loc=At4g20830>) highlights genes encoding important signal transduction elements such as a receptor-like kinase 7 (RLK7) involved in immunity and tolerance to oxidative stress, BRI1 (BRI-associated receptor kinase) and SERK3/BAK1, co-receptors of BRI1, involved in sensing the hormone brassinolide. Notably, BAK1 is also a well-known co-receptor involved in MAMP sensing.

OGOX1 overexpressing, null mutant and silenced plants were obtained that showed no macroscopical defects in the aerial part of the plants. However, the effect of both overexpression and down regulation of OGOX1 in radical growth was demonstrated and this is probably an important evidence of the role of OGs in a growing tissue where OGs are formed. The importance of OGOX1 was also demonstrated during pathogenic events where the overexpression of the gene increases susceptibility to bacterial pathogens, whereas unexpectedly, increases resistance to *B. cinerea*.

oxOGs have been shown to be unable to induce expression of the defence responses typically induced by the action of elicitor-active OGs; moreover, they do not compete with OGs at the receptor level. Therefore the oxidation of OGs probably leads to inactivation of their elicitor activity in exaggerated signalling conditions where growth is severely affected. This is in agreement with results regarding root growth that suggest a role of OGOX1 in this developmental stage. A low level of elicitor active OGs in the OGOX1 overexpressing plants probably occurs in the elongation zones causing less antagonism with auxin and a higher radical growth. Conversely the lack of OGOX1 causes less root growth, probably due to higher amount of elicitor-active OGs that antagonize the effect of auxin on growth.

The role of OGOX1 in disease resistance is rather complex. The results obtained with bacterial infections support the hypothesis that overexpressing plants possess higher levels of OG oxidase and less elicitor active OGs and therefore are less capable of activating a prompt defense response. OGOX1 overexpressing plants are more susceptible to *Pectobacterium carotovorum* and *Pseudomonas syringae* pv. *tomato* DC30000. The results obtained with knock out

plants do not confirm this conclusion probably due to a redundancy of the OGOX genes.

A different and more complex scenario was observed with the necrotrophic fungus *Botrytis cinerea*, where the area of the lesions caused by the fungus in overexpressing lines were reduced at an extent depending on the level of expression of OGOX1, i.e. higher expression produced smaller lesions. The loss-of-function or silenced mutants were instead more susceptible in comparison to the wild-type. These results do not fit with the model of “dampening the elicitor activity of OGs” and show that in this case the loss of only one OGOX enzyme could be crucial for the plant response to the fungus. The unexpected behaviour of the mutant/overexpressing plants toward this pathogen may reflect a complex interplay of plant OGOXs, the native and oxidized OGs and the H₂O₂ formed by the enzymes and requires further studies.

The inactivation played by a specific oxidase probably represents a mechanisms essential for controlling homeostasis of OGs, growth and immunity. OGOX1 belongs to a superfamily of BBEI that comprises 28 members, in this work we demonstrated that four of these enzymes have OG oxidizing activity. The Berberine Bridge

Domain located in the C-terminal region with only one exception, is always associated with a FAD-binding domain present in the N-terminal region, that binds the adenine part of the FAD. All OGOX have a predicted signal peptide sequence for translocation into the endoplasmic reticulum (SIGNALP-4.1), the presence of a GPI-anchor site is predicted in the longer isoform encoded by At4g20830 (DGPI and GPI-SOM softwares) that may localize the protein on the cell membrane. This prediction is in agreement with proteomics studies (Elortza, F. et al. 2006) that have shown the presence of the protein in a pool of potentially glycosylphosphatidylinositol-anchored proteins (GPI-APs) after treatment of *Arabidopsis thaliana* plasma membrane fractions with phospholipase D .

The name of this protein family derives from the berberine-bridge enzyme from *Eschscholzia californica* (California poppy), which catalyzes the oxidative ring-closure reaction from (S)-reticuline to (S)-scoulerine. The C-C-bond formed in this reaction is called “berberine bridge”. This reaction marks a branch point in the biosynthesis of isoquinoline alkaloids (Facchini, P. J. et al. 1996). Recently, many genes encoding BBEs have been identified in bacteria, fungi and plants; in the latter the number of these genes in individual

species varies considerably from a single gene in the moss *Physcomitrella patens* to 28 in *Arabidopsis* and 64 in the western poplar (*Populus trichocarpa*). Despite the widespread occurrence and abundance of BBEI protein families in the plant kingdom, most of these enzyme are thought not to synthesize alkaloids.

The first biochemical characterization of this kind of proteins was carried out in the fungus *Acremonium strictum*, where a flavoprotein catalyzing the oxidation of a variety of carbohydrates (glucose, maltose, lactose) was reported (Lin, Shuen Fuh et al. 1991). Another well-characterized fungal BBEI is a chitooligosaccharide oxidase (ChitO) from *Fusarium graminearum*.(Heuts, Dominic P. H. M. et al. 2007).

In plants a carbohydrate oxidase activity was found for a BBEI expressed in the nectary gland of tobacco, and identified as nectarin V (NINEC5) (Carter, C. J. and Thornburg, R. W. 2004). The enzyme has glucose oxidase activity with H₂O₂ production (Carter, C. J. and Thornburg, R. W. 2004). Moreover, two BBEIs with carbohydrate oxidase activity, respectively Ha-CHOX and Ls-CHOX, were identified in *Helianthus annuus* and *Lactuca. sativa*, with a broad substrate specificity (monosaccharides,

disaccharides and cellulose fragments from cellobiose to cellopentaose) and also in this reaction the enzyme is capable of producing hydrogen peroxide as a reaction product (Custers, J. H. et al. 2004). Ha-CHOX and Ls-CHOX are likely to play a role in defense (Custers, J. H. et al. 2004), as the two proteins were found in extracts from salicylic acid (SA)-treated lettuce and from sunflower leaves that displayed very potent antimicrobial activity against a set of phytopathogens.

Recently, (Daniel, B. et al. 2015) two BBEI enzymes from *Arabidopsis thaliana* have been shown to oxidize monolignols to their corresponding aldehydes, suggesting their involvement in the modification of the extracellular monolignol pool, that may affect plant cell wall metabolism with as yet unknown implications for lignin formation. The 28 AtBBEIs are divided in 7 phylogenetic group where all OGOX are regrouped in the group 5. Looking at the amino acid residues of the active site it is clear that the active site differs markedly among AtBBEI proteins. However, specific groups of residues, important for substrate binding and catalysis, are strictly conserved in 14 members of the AtBBEIs (including all the OGOXs) indicating that their

catalytic mechanism and substrate preference will be similar.

The identification of four members of the Arabidopsis BBE1 superfamily as OG oxidases opens many questions on their role in plant growth and defence and prompts further studies also on other putative oligosaccharide oxidases comprised in the family and involved in the turnover/metabolism of cell wall-derived signals other than OGs.

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