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Short oligogalacturonides induce pathogen resistance-associated gene expression in *Arabidopsis thaliana*

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Abstract

Background: Oligogalacturonides (OGs) are important components of damage-associated molecular pattern (DAMP) signaling and influence growth regulation in plants. Recent studies have focused on the impact of long OGs (degree of polymerization (DP) from 10–15), demonstrating the induction of plant defense signaling resulting in enhanced defenses to necrotrophic pathogens. To clarify the role of trimers (trimeric OGs, DP3) in DAMP signaling and their impact on plant growth regulation, we performed a transcriptomic analysis through the RNA sequencing of *Arabidopsis thaliana* exposed to trimers.

Results: The transcriptomic data from trimer-treated *Arabidopsis* seedlings indicate a clear activation of genes involved in defense signaling, phytohormone signaling and a down-regulation of genes involved in processes related to growth regulation and development. This is further accompanied with improved defenses against necrotrophic pathogens triggered by the trimer treatment, indicating that short OGs have a clear impact on plant responses, similar to those described for long OGs.

Conclusions: Our results demonstrate that trimers are indeed active elicitors of plant defenses. This is clearly indicated by the up-regulation of genes associated with plant defense signaling, accompanied with improved defenses against necrotrophic pathogens. Moreover, trimers simultaneously trigger a clear down-regulation of genes and gene sets associated with growth and development, leading to stunted seedling growth in *Arabidopsis*.

Keywords: Plant signaling, *Arabidopsis thaliana*, Oligogalacturonides, OG, Trimers, Transcriptomics, Defense induction, Growth inhibition, Disease resistance, *Pectobacterium carotovorum*, *Botrytis cinerea*

Background

Necrotrophic phytopathogens, which are ubiquitous in nature, are represented among bacteria and fungi. Many agriculturally important diseases, such as soft rot and blackleg, are caused by bacterial necrotrophs [1, 2], while necrotrophic fungi are causative agents of devastating gray molds and rusts, among others [3, 4]. Enterobacterial soft-rot pathogens of the genus *Pectobacterium* include broad host-range pathogens that cause disease in a variety of plant species and economically important crops, such as potato [1, 5, 6]. Similar to many other necrotrophic brute force pathogens, resistance to broad host-range

Pectobacteria is complex and does not appear to involve single resistance genes [7–9]. Instead, general plant innate immunity systems, including salicylic acid (SA)- and jasmonic acid/ethylene (JA/ET)-mediated defenses, are triggered by conserved pathogen-associated molecular patterns (PAMPs) [10–15]. In addition to bacterial necrotrophs, also fungi cause severe pre- and post-harvest losses to crops worldwide [16]. Of these, the wide broad host-range gray mold pathogen *Botrytis cinerea*, capable of infecting more than 200 plant species, is one of the most comprehensively studied necrotrophic fungus. Similar to *Pectobacteria*, *B. cinerea* uses enzymes to break down cell walls to access the host tissue. In *Arabidopsis*, enhanced plant resistance to *Botrytis* seems to be independent of the phytohormones SA and JA, but rather dependent on ET, PAD3 and the accumulation of the phytoalexin camalexin [17, 18].

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In addition to PAMPs, damage-associated molecular patterns (DAMPs) play a vital role in defense activation against bacterial and fungal necrotrophs (i.e. *Pectobacteria* and *Botrytis*). Plants perceive DAMPs, such as plant cell wall fragments released by the action of plant cell wall-degrading enzymes (PCWDEs) secreted by these pathogens, as signals of damage or modified self [10, 19]. PCWDEs are among the central virulence determinants employed by necrotrophic phytopathogens for the maceration of host tissue and the release of nutrients [20]. Pectin is a major component of the primary plant cell wall matrix. It is a complex heteropolysaccharide composed of galacturonan residues and a prominent target of PCWDEs [21, 22]. Both *Pectobacteria* and *Botrytis* employ PCWDEs for the degradation of pectin, central of which are; pectin methylesterases (Pem) and pectin lyases (Pnl), which directly operate on pectin polymers and polygalacturonases (Peh) and pectate lyases (Pel), which operate on pectate (de-esterified pectin) [20–24].

Major end products of the degradation of pectin by PCWDEs are oligogalacturonides (OGs) with varying degrees of polymerization (DP). These OG fragments act as potent DAMPs, capable of triggering plant defense signaling [25–29]. In addition to plant responses to pathogens, OGs regulate plant growth and development [30]. Consequently, OGs play an important biological role as signaling molecules, but the complexity of both OG fragments and the responses generated has resulted in OGs being difficult to study [29, 31]. Early studies on plant response to OGs were often limited to specific molecular targets or processes, including; the production of plant hormones, such as ET and JA, or the expression of specific defense-related genes [26, 28, 32–34]. More recent transcriptomics and functional genetic analyses have enabled systems-level studies of plant responses to OGs and the characterization of the OG-responsive transcriptome in *Arabidopsis* [17, 30, 35]. These studies have suggested that long OGs (DP > 10) are the most effective in modulating signaling involved in plant defense responses, with short OGs having little or no effect [17, 30, 35, 36]. In part, recent studies have focused on long OGs because the only identified receptor, WAK1, is reported to bind to and be stimulated by long OGs [37, 38].

However, previous studies by us and others have suggested that also short OGs (DP < 10) impact plant defense [28, 39] and development [40]. For example, short OGs of DP2-5 induced a strong expression of a gene involved in JA biosynthesis in *Arabidopsis* [28]. Furthermore, short OGs (DP4-6, DP2 and DP1-7, respectively) induced genes involved in pathogen response and defense in potato and tomato and the synthesis of phytohormones in tobacco and tomato [28, 39, 41–43]. Moloshok et al. [44] demonstrated the induction of a proteinase inhibitor in response to dimeric OGs in tomato seedlings.

To clarify the impact of short OGs on plant signaling associated with defense and development, we performed an RNA sequencing assay using trimers (trimeric OGs, DP3) as models for short OGs. Trimers were chosen as model as during the natural growth phases of *Arabidopsis* trimers are present during senescence as well as after cell wall digestion by pectinases [45]. Furthermore, in plant pathogen interactions it has been demonstrated that trimers are produced in tomato fruit after infection with *B. cinerea* [46]. For bacterial pathogens it has been observed that the pectolytic enzymes from various species generate trimers from pectin [47–51], and we have previously shown that commercially available trimers have a similar effect when applied exogenously to plant tissue, as does culture filtrate from *P. carotovorum* and polygalacturonic acid degraded with pectolytic enzymes.

We further compared the sequencing results to those of previous studies detailing the global transcriptomic effects of long OGs (DP > 10) on *Arabidopsis* [17, 30, 35]. The transcriptomic data obtained by RNA sequencing suggests that trimers clearly induce the expression of a number of genes involved in plant defense, while genes involved in growth, biosynthetic pathways and development are down-regulated. The up-regulation of defense-associated and OG-responsive genes identified in this study was comparable to previous studies in which in vitro-grown *Arabidopsis* seedlings have been treated with long OGs [17, 35]. However, the modulation of gene expression induced by trimers was generally not as strong as that induced by long OGs. To confirm the transcriptomic data, we performed quantitative RT-PCR (qRT-PCR) analyses of genes previously associated with OG signaling [17, 35]. In addition, we conducted phenotypic analyses for the trimer-induced enhancement of pathogen resistance and the inhibition of plant growth. Importantly, pre-treatment with trimers or long OGs induced enhanced resistance to the necrotrophic pathogens *Pectobacterium carotovorum* at a similar degree, and retarded seedling growth in *Arabidopsis*. Further, both long OGs and trimers were able to induce phosphorylation of MPK3 and MPK6, indicating activation of signaling pathways involved in recognition of elicitors. These results suggest that trimers are potent elicitors of plant defenses, putatively leading to the re-allocation of plant resources from growth and development towards the induction of defense.

Results

Short OGs have a significant impact on the *Arabidopsis* transcriptome

Previous studies have characterized the responses triggered in *Arabidopsis* to exogenously applied oligogalacturonides with DP > 10 at the transcriptome level during the early stages of signaling [30, 35]. In contrast, with the

exception of the expression of a few specific genes, the potential of trimers to trigger plant defense signaling has not been characterized at the transcriptome level. In some studies, trimers have failed to elicit specific defense gene expression, whereas in other studies, the up-regulation of specific defense-related genes has been observed [28, 52]. To address this apparent controversy, we characterized the Arabidopsis transcriptome by RNA sequencing in response to trimers. The RNA sequencing data obtained in this study revealed significant differences in the gene expression of plants treated with trimers (183 genes down-regulated and 517 genes up-regulated) compared to plants subjected to mock treatment (Additional file 1: Table S2).

Comparative meta-data analysis of transcriptomes in response to trimers and long OGs

To elucidate differences and overlaps in plant responses to OGs with different DPs, we compared the transcriptome data obtained by RNA sequencing from trimer-treated samples with the corresponding published transcriptome data from microarray analyses of long OG (DP > 10)-treated samples [17, 30, 35]. We combined RNA-sequencing data from the 3 h trimer treatment with transcriptomic studies of the 1 h and 3 h treatments using long OGs and *in vitro* Arabidopsis seedlings as described by Denoux et al. [35] and Ferrari et al. [17]. Intriguingly, there was a significant degree of overlap between the studies (Fig. 1).

A subsequent comparative analysis of data from all three treatments (trimers at 3 h and long OGs at 1 h and 3 h) identified 140 genes that exhibited significant expression changes in response to treatment with long OGs and trimers (Additional file 2: Table S3, Fig. 2). Of the 140 genes with altered expression across all of the OG treatments, 24 genes were down-regulated and 108 genes were up-regulated. A total of 8 genes differed in up/down-regulation in at least one experiment. We further performed hierarchical clustering (complete agglomeration) based on the expression values of genes triggered by both long and short OGs. Gene expression

triggered by the 3 h treatment with trimers and long OGs at 3 h was the most similar across all 3 treatments. These data provide further support for a considerable overlap in the effect of the trimers and long OGs on gene expression.

Moscatiello et al. [30] studied the effects of long OGs (DP > 10) on Arabidopsis in cell culture. When the transcriptomic data from this study were included in our comparative analysis, only the expression of ten genes was significantly altered across all four experiments (Table 1). The ten genes with altered expression included defense-associated genes, such as peroxidases. Although all of the ten common OG-associated genes were down-regulated in the study by Moscatiello et al. [30], they were up-regulated in the other studies.

In conclusion, a comparative gene expression analysis of studies using short and long OGs suggests that several defense-associated genes are up-regulated by both types of OGs in similar experiments. Furthermore, the expression of genes altered both in this study and those published by Ferrari et al. [17] and Denoux et al. [35] at the 3 h time point was generally similar to data from the 1 h time point. In addition to time point-dependent changes, the impact of OGs depends on the type of experiment, as suggested by the gene expression analysis by Moscatiello et al. [30], which was performed using Arabidopsis cells in a suspension culture. In our study, the results from the global transcriptomic analysis resemble the previously characterized effects of long OGs, suggesting that trimers have a significant impact on the expression of hundreds of plant genes, many similar to those affected by long OGs.

Gene sets associated with pathogen response and hormone signaling are up-regulated while those associated with metabolic and developmental pathways are down-regulated across studies of OG treatment

A gene set enrichment analysis (GSEA) was performed using transcriptome data from our RNA sequencing and from previous microarray studies characterizing plant

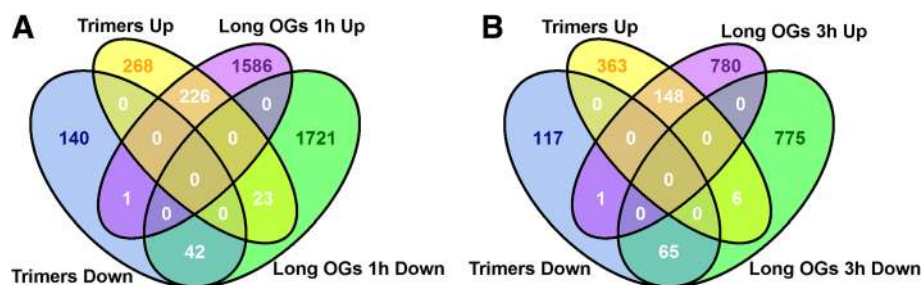


Fig. 1 Global gene expression data from trimer-treated seedlings exhibit similarities and differences with those from long-OG-treated seedlings at different time points. Venn diagram comparison of the up- and down-regulated genes based on the RNA sequencing data between trimer (trimers in the figure) pretreatment and treatment with long OGs at 1 h **a** and long OGs at 3 h **b** compared to mock treatment

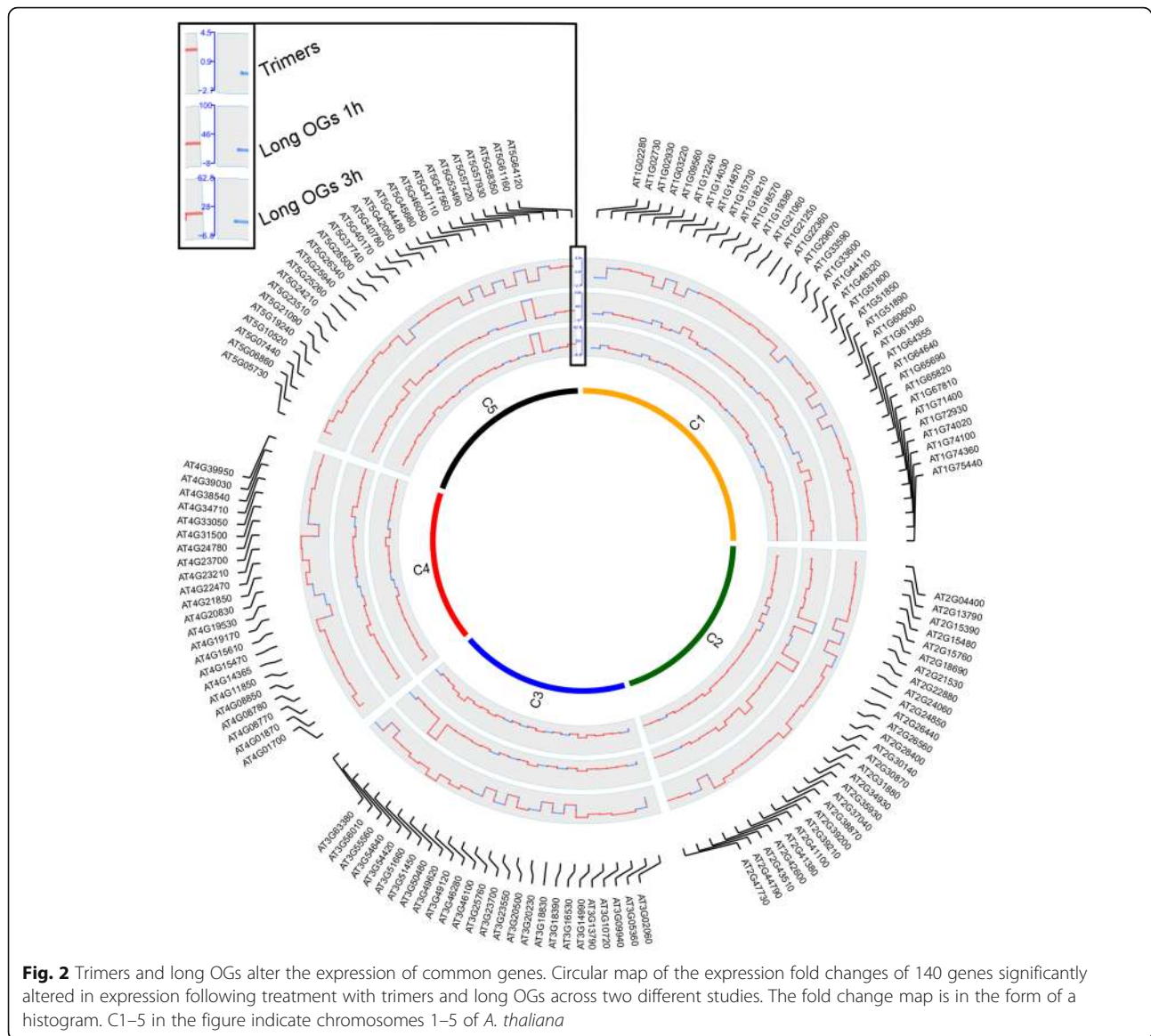


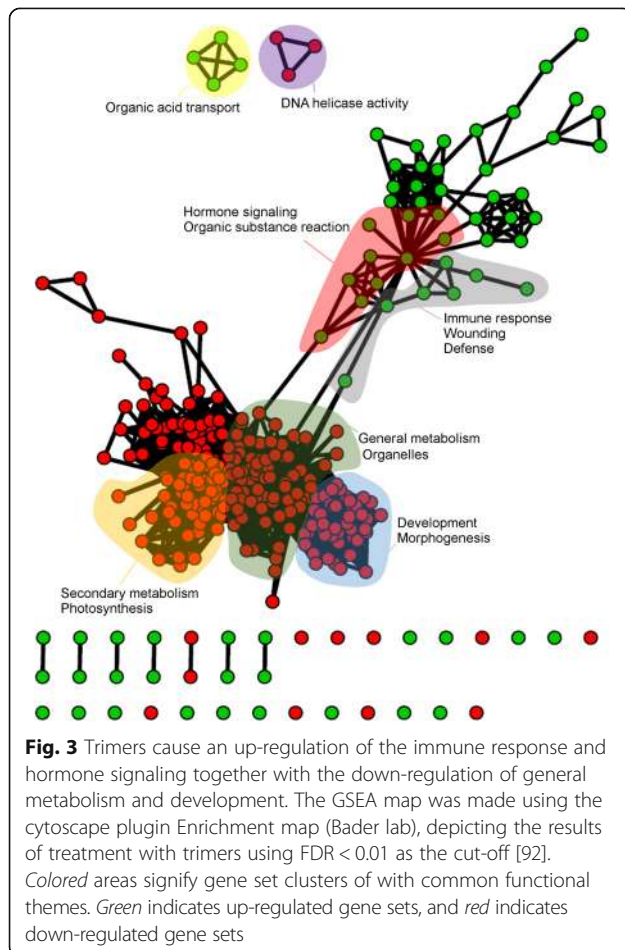
Table 1 Genes with altered expression across different studies that examined the response to trimers and long OGs

Id	Description	Trimers	Long OGs 1 h	Long OGs 3 h	Long OGs 2 h
AT2G30870	Glutathione S-transferase PHI 10/ERD13	1.43	2.11	2.19	-2.32
AT1G33590	Leucine-rich repeat (LRR) family protein	1.43	3.70	2.20	-5.01
AT4G08770	Peroxidase superfamily protein	1.50	3.56	4.59	-5.25
AT4G38540	FAD/NAD(P)-binding oxidoreductase family protein	1.54	2.10	1.94	-2.91
AT3G49120	Peroxidase CB	1.60	2.27	1.84	-4.66
AT5G40170	Receptor like protein 54	1.61	3.91	1.37	-2.21
AT3G05360	Receptor like protein 30	1.70	8.04	6.51	-2.15
AT4G08780	Peroxidase superfamily protein	1.77	1.92	2.48	-5.96
AT3G13790	Glycosyl hydrolases family 32 protein	1.81	3.70	4.88	-6.31
AT2G34930	Disease resistance family protein/LRR family protein	4.09	5.95	3.59	-4.57

Genes significantly affected by trimers and long OGs across three different *A. thaliana* transcriptome studies are presented: this study, long OGs at 1 h and 3 h and long OGs at 2 h in a cell-culture [17, 30, 35]. Numeric values indicate the fold change compared to a mock treatment

response to long OGs (DP > 10). Gene sets were obtained from the Plant Gene Set Enrichment Analysis Toolkit (<http://structuralbiology.cau.edu.cn/PlantGSEA/database/Ara.DetailInfo>), where references for all of the literature- and database-derived gene sets identified in the following GSEA can be obtained [53]. From the GSEA output of the trimer/mock RNA sequencing data, we identified 247 down-regulated gene sets and 73 up-regulated gene sets using an FDR of <0.01 as the cut-off (Additional file 3: Table S4, Fig. 3).

This data analysis suggests a trend of trimer-mediated up-regulation of biotic defense-related gene sets. These include gene sets induced in *Arabidopsis* after inoculation with *Xanthomonas campestris* pv. *campestris* and gene sets induced by *Myzus persicae* or *Plutella xylostella* [54–56]. Based on the gene ontology (GO) database (<http://geneontology.org/>, the Gene Ontology Consortium), the up-regulated gene sets included jasmonic acid biosynthesis (GO:0009695), ethylene response (GO:0009723), oxylipin biosynthesis (GO:0031408), response to wounding (GO:0009611) and immune effector processes (GO:0002252). The down-regulated gene sets included cellular component biogenesis (e.g., GO:004225



- ribosome biogenesis and GO:0070271 - protein complex biogenesis), organelle organization (e.g., GO:0009657 - plastid organization, GO:0006996 - organelle organization, GO:0009668 - plastid membrane organization and GO:0009658 - chloroplast organization), energy metabolism (e.g., GO:0015979 - photosynthesis, GO:0005996 - monosaccharide metabolic process and GO:0019684 - photosynthesis light reaction) and development (e.g., GO:0009790 - embryo development, GO:0048316 - seed development and GO:0044085 - cellular component biogenesis).

A GSEA analysis was performed on genes that were similarly regulated by trimers and long OGs at 3 h and using only gene sets from the GO database with a GSEA *P*-value cut-off of <0.05. A total of 213 gene sets were found to be up-regulated, and 244 were down-regulated (Additional file 4: Table S5). By further filtering the GSEA output by only including expression data of genes similarly triggered by long OGs at 1 h, we found 94 gene sets to be up-regulated and 92 to be down-regulated. In the filtered GSEA output, we detected a trend for the up-regulation of defense- (e.g., GO:0009620 - response to fungus, GO:0009626 - plant hypersensitive response and GO:0045087 - innate immune response) and JA-related gene sets (GO:0009694 - jasmonic acid metabolism and GO:0009695 - jasmonic acid biosynthesis). In contrast, gene sets involved in photosynthesis (GO:0015979) and in the biosynthesis of isoprenoids (GO:0008299), phospholipids (GO:0008654) and pigments (GO:0046148) were down-regulated (Tables 2 and 3, Additional file 5: Table S6, Fig. 4).

To examine differences in the responses to trimers or long OGs, the GSEA (FDR < 0.01) results for long OGs at 3 h (using a *P*-value of <0.01 and fold changes of >log₂ 0.5 and <log₂ 0.5) and trimers were compared by selecting gene sets that were affected by one but not the other (Additional file 6: Table S7). Trimer-specific gene sets that were not found to be significantly affected by the long OGs at 3 h were involved in, for example, developmental processes (GO:0048869-cellular developmental process, GO:0048364-root development and GO:0051301-cell division). The gene sets that were up-regulated by long OGs but not by trimers (Additional file 7: Table S8) were involved in respiratory burst (GO:0045730) and response to microbes (GO:0009617 - response to bacterium, GO:0009627 - systemic acquired resistance and GO:0009814 - defense response incompatible reaction).

A pathway analysis was performed for the additional characterization of how trimers and long OGs affect genes involved in the plant-pathogen response and hormone signaling. Both trimers and long OGs induce the expression of genes triggered by fungal and bacterial pathogens. According to the plant-pathogen pathway map of the KEGG database, these genes include *BAKI*

Table 2 Trimers and long OGs induce gene sets associated with phytohormones, stress and defense signaling

Gene set	ID	Size	NES	P-value
RESPONSE_TO_ORGANIC_SUBSTANCE	GO:0010033	59	2.11	0.00
OXYLIPIN_METABOLIC_PROCESS	GO:0031407	9	2.10	0.00
OXYLIPIN_BIOSYNTHETIC_PROCESS	GO:0031408	9	2.05	0.00
JASMONIC_ACID_METABOLIC_PROCESS	GO:0009694	8	1.92	0.00
JASMONIC_ACID_BIOSYNTHETIC_PROCESS	GO:0009695	8	1.90	0.00
RESPONSE_TO_CARBOHYDRATE_STIMULUS	GO:0009743	27	1.84	0.00
CELLULAR_RESPONSE_TO_CHEMICAL_STIMULUS	GO:0070887	33	1.78	0.02
RESPONSE_TO_CHITIN	GO:0010200	26	1.78	0.02
FATTY_ACID_BIOSYNTHETIC_PROCESS	GO:0006633	12	1.75	0.02
CELLULAR_RESPONSE_TO_STIMULUS	GO:0051716	49	1.74	0.02
IMMUNE_SYSTEM_PROCESS	GO:0002376	49	1.70	0.03
CELLULAR_RESPONSE_TO_STRESS	GO:0033554	45	1.67	0.03
RESPONSE_TO_ETHYLENE_STIMULUS	GO:0009723	14	1.66	0.03
IMMUNE_RESPONSE	GO:0006955	49	1.64	0.03
RESPONSE_TO_FUNGUS	GO:0009620	32	1.64	0.03
INNATE_IMMUNE_RESPONSE	GO:0045087	49	1.63	0.02
RESPONSE_TO_CYCLOPENTENONE	GO:0010583	11	1.61	0.04
PROGRAMMED_CELL_DEATH	GO:0012501	26	1.61	0.04
PLANT-TYPE_HYPERSENSITIVE_RESPONSE	GO:0009626	26	1.61	0.03
HOST_PROGRAMMED_CELL_DEATH_INDUCED_BY_SYMBIONT	GO:0034050	26	1.60	0.04

The 20 most significantly up-regulated gene sets based on the GO database are presented, as determined by a GSEA on the GenePattern platform using the combined trimer and long OG gene expression data

(encoding a leucine-rich receptor-like kinase with a central role in the PAMP-response triggered by flagellin), calmodulin-like (*CML*) genes and genes encoding calcium-dependent protein kinases (*CDPKs*; Additional file 8: Figure S1). Characterization of the hormonal signaling pathways induced by both 3 h treatments (trimers and long OGs) revealed the induction of genes associated with ET and JA responses and the SA response.

The results of the GSEA and pathway analyses of the effect of short and long OG treatments suggest a trade-off in the priorities for plant resource allocation. The expression of genes associated with the defense-related JA, ET and SA signaling pathways was enhanced, while the expression of genes involved in the gibberellic acid and cytokinin pathways, associated with development and growth, were mainly down-regulated (Additional file 9: Figure S2). Thus, the global transcriptomic data suggest that trimers induce a significant response from gene sets and pathways associated with pathogen defense, phytohormone production and plant development.

The expression of OG-responsive marker genes correlates with the short-OG-triggered induction of defense-related pathways

To confirm the trimer-triggered transcriptomic changes indicated by RNA sequencing and to further compare

the impact of trimers on the specific gene-expression changes induced by long OGs ($DP > 10$) in Arabidopsis, we performed qRT-PCR and assayed the expression of selected genes previously shown to be induced by long OGs, as well as those indicating the activation of phytohormone signaling pathways [17, 35]. Plants were mock treated, treated with trimers or treated with a commercial long-OG mix (DP8-19) (Fig. 5).

Results from the qRT-PCR analysis confirmed, in general, activity similar to that observed in the RNA sequencing data. The OG receptor *WAK1* was significantly up-regulated by trimers and to even greater extent by the long-OG mix at 1, 3 and 6 h [35, 37, 38]. The up-regulation of *WAK1* indicates that short OGs are also capable of inducing genes affected by long OGs (Fig. 5).

Since OGs are involved in DAMP signaling in Arabidopsis, we assayed the induction of glucosinolate defenses activated in response to tissue damage [57]. Accordingly, *CYP81F2*, an oxidase involved in glucosinolate modification, was rapidly induced by both trimers and the long-OG mix; trimers triggered stronger expression of this gene at the 3 h time point and the long-OG mix did so at 6 h (Fig. 5).

Furthermore, necrotrophs such as *B. cinerea*, damage plant tissues by secreting various PCWDEs, such as polygalacturonases, thereby releasing OGs from the cell

Table 3 Trimers and long OGs down-regulate gene sets associated with organelle organization, photosynthesis and metabolic processes

Gene set	Identity	Size	NES	P-value
RNA_METABOLIC_PROCESS	GO:0016070	13	-2.78	0
CHLOROPLAST_PART	GO:0044434	17	-2.69	0
PIGMENT_BIOSYNTHETIC_PROCESS	GO:0046148	6	-2.66	0
NCRNA_METABOLIC_PROCESS	GO:0034660	6	-2.66	0
PYRUVATE_METABOLIC_PROCESS	GO:0006090	11	-2.66	0
ISOPRENOID_METABOLIC_PROCESS	GO:0006720	11	-2.66	0
ISOPENTENYL_DIPHOSPHATE_BIOSYNTHETIC_PROCESS	GO:0009240	11	-2.65	0
CELLULAR_CARBOHYDRATE_METABOLIC_PROCESS	GO:0044262	22	-2.65	0
ISOPENTENYL_DIPHOSPHATE_BIOSYNTHETIC_PROCESS, MEVALONATE-INDEPENDENT_PATHWAY	GO:0019288	11	-2.64	0
PIGMENT_METABOLIC_PROCESS	GO:0042440	6	-2.64	0
PLASTID_PART	GO:0044435	17	-2.64	0
CELLULAR_GLUCAN_METABOLIC_PROCESS	GO:0006073	6	-2.64	0
PLASTID	GO:0009536	28	-2.63	0
ISOPENTENYL_DIPHOSPHATE_METABOLIC_PROCESS	GO:0046490	11	-2.61	0
CELLULAR_POLYSACCHARIDE_METABOLIC_PROCESS	GO:0044264	6	-2.60	0
GLUCAN_BIOSYNTHETIC_PROCESS	GO:0009250	6	-2.59	0
POLYSACCHARIDE_METABOLIC_PROCESS	GO:0005976	6	-2.59	0
GLYCERALDEHYDE-3-PHOSPHATE_METABOLIC_PROCESS	GO:0019682	11	-2.58	0
CHLOROPLAST	GO:0009507	27	-2.58	0

The 20 most significantly down-regulated gene sets based on the GO database are presented, as determined by a GSEA on the GenePattern platform using the combined trimer and long OG gene expression data

wall [58, 59]. Consequently, *PGIP1–2* (*Polygalacturonase Inhibiting Protein 1–2*) was up-regulated by both trimers and the long-OG mix from 1 to 6 h. Interestingly, compared to long OGs, trimers triggered a clearly stronger induction of this gene at 3 and 6 h. Furthermore, the anionic peroxidase *PER4*, which is required for basal plant resistance to *B. cinerea*, was rapidly induced in response

to both trimers and the long-OG mix [60]. However, the expression triggered by the long-OG mix was considerably higher than that induced by trimers, especially at 1 h. In Arabidopsis, the phytoalexin camalexin, originating from the tryptophan pathway, is central for OG-triggered defense to *B. cinerea* [60]. We noted up-regulation of *PAD3* (*Phytoalexin Deficient 3*), which

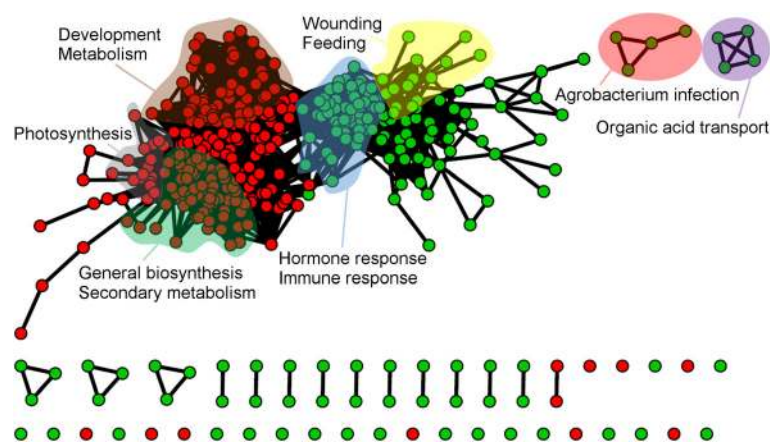


Fig. 4 Both trimers and long OGs trigger the up-regulation of immune and stress responses and the down-regulation of metabolism. The GSEA map was made using the cytoscape plugin Enrichment map (Bader lab), depicting the results of combined data for trimers and long OGs (combined gene expression data for trimers and long OGs at 3 h as reported by Denoux et al. 2008) using a *P*-value cut-off of <0.05. Colored areas indicate gene set clusters of with common functionality. Green indicates up-regulated gene sets, and red indicates down-regulated gene sets

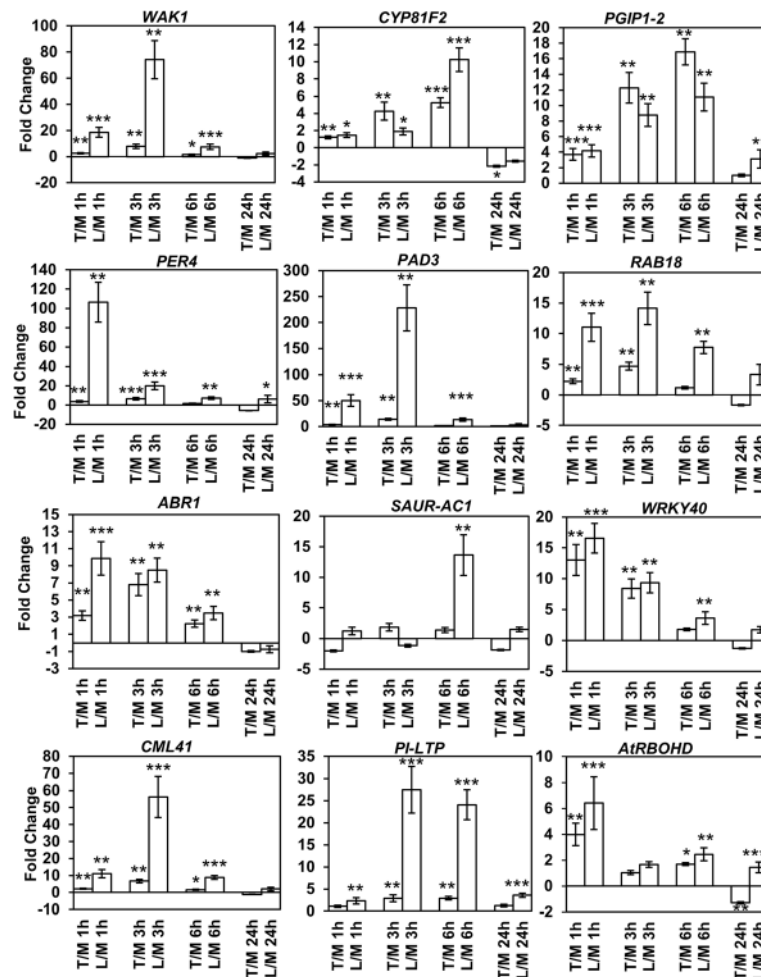


Fig. 5 Trimers trigger the up- and down-regulation of genes involved in defense, phytohormone signaling and the OG response, similarly to the long-OG mix (DP > 8). The results from the qRT-PCR gene expression analysis are presented. T/M indicates trimer-treated plants compared to mock treated plants, and L/M indicates plants treated with the long-OG mix (DP > 8) compared to those treated with the mock suspension. A total of 4 time points (1, 3, 6 and 24 h) were analyzed for each gene. Statistical analysis using Student's *t*-test is indicated by asterisks in the figure (1 asterisk = $P < 0.05$, 2 asterisks = $P < 0.01$, and 3 asterisks = $P < 0.001$)

encodes an enzyme required for the biosynthesis of camalexin in response to both trimers and the long-OG mix from 1 to 3 h (Fig. 5). However, the long-OG mix induced clearly higher and longer-lasting expression of this gene compared to induction by trimers.

Damage and the subsequent release of OGs from plant cell walls also modulate phytohormone signaling, such as that mediated by ABA, JA and auxin [61]. We detected clear induction of *RAB18*, a marker for ABA signaling, in response to the long-OG mix and, to a lesser extent, to trimers [62]. On the other hand, *ABR1*, a repressor of ABA responses, was also induced by both trimers and the long-OG mix at 1 and 6 h post-treatment, indicating OG-triggered down-regulation of ABA signaling [63]. Furthermore, *NCED4*, involved in ABA signaling, was down-regulated according to the sequencing data. The rapid induction of the transcription factor *WRKY40* by both

types of OGs suggested the same, as well as the simultaneous activation of JA-signaling associated with DAMP response. Here, the trimer treatment displayed a greater effect on the fold change than did the longer OGs. In addition to defense signaling, OGs have also been shown to influence processes involved in plant growth and development [64]. This was also suggested by the RNAseq data, in which the processes related to growth and development were clearly down-regulated. Accordingly, both trimers and the long-OG mix triggered the down-regulation of *SAUR-AC1*, a marker gene for auxin signaling during the early OG response. This, along with the down-regulation of another gene involved in Auxin production, *TAR2*, indicates crosstalk between different hormonal signaling pathways during the early stage of the DAMP response. However, this signaling seems complex in its nature, as *IAA2* was up-regulated.

In response to long OGs, the calmodulin-like gene *CML41* is significantly up-regulated at early time points and is putatively involved in dampening the immune response [35]. In this study, the gene was rapidly up-regulated by trimers at 1, 3 and 6 h, similarly to the long-OG mix, indicating that both short and long OGs trigger this gene [35].

PI-LTP, a lipid transfer protein, was up-regulated by the trimers at 3 and 6 h and by the long-OG mix from 1 to 24 h. Lipid transfer proteins have been identified as antimicrobial peptides and significant contributors to plant defense [65].

In conclusion, the qRT-PCR expression analysis of genes associated with the activation of OG-triggered DAMP responses or those considered markers for phytohormone signaling revealed that the plant response to trimers and longer OGs is overall quite similar, especially at the earlier time points. While there seems to be a difference in the amplitude of gene expression, i.e. plant treatment with the long-OG mix seems to result in stronger induction, the trimers are nevertheless also potent elicitors of genes involved in defense.

Trimers elicit plant defense responses leading to enhanced resistance to infection by *P. carotovorum*

The perception of a phytopathogen triggers the synthesis and accumulation of specific phytohormones that mediate associated defense signaling. In Arabidopsis, resistance to necrotrophic pathogens, such as *B. cinerea* and *P. carotovorum*, is affected by the phytohormones JA, ET and SA [13, 66, 67]. Because our RNA sequencing analysis indicated that JA/ET-related signaling is induced in response to trimers, we sought to determine whether pretreatment with this compound could elicit plant resistance to subsequent infection by a necrotrophic pathogen. We treated in vitro plants with trimers (20 μ M and 0.025% Silwet) or long OGs (20 μ M and 0.025% Silwet) for 24 h before inoculation with *P. carotovorum*. Compared to mock-treated (MQ and 0.025% Silwet) plants, there was a significant reduction of bacterial growth 24 h post-inoculation in plants treated with the trimers and long OGs, but no significant difference between the different OGs (Fig. 6).

This clearly indicates that trimers can prime plant defenses to *P. carotovorum*.

Trimers inhibit growth in Arabidopsis

The activation of plant defenses diverts resources from plant growth [68]. Consistently, our transcriptomic data indicate that GO-based gene sets related to plant growth, organelle organization, cellular component biogenesis and photosynthesis were down-regulated in plants treated with trimers (Additional file 3: Table S4). To determine whether trimers were indeed capable of inhibiting growth,

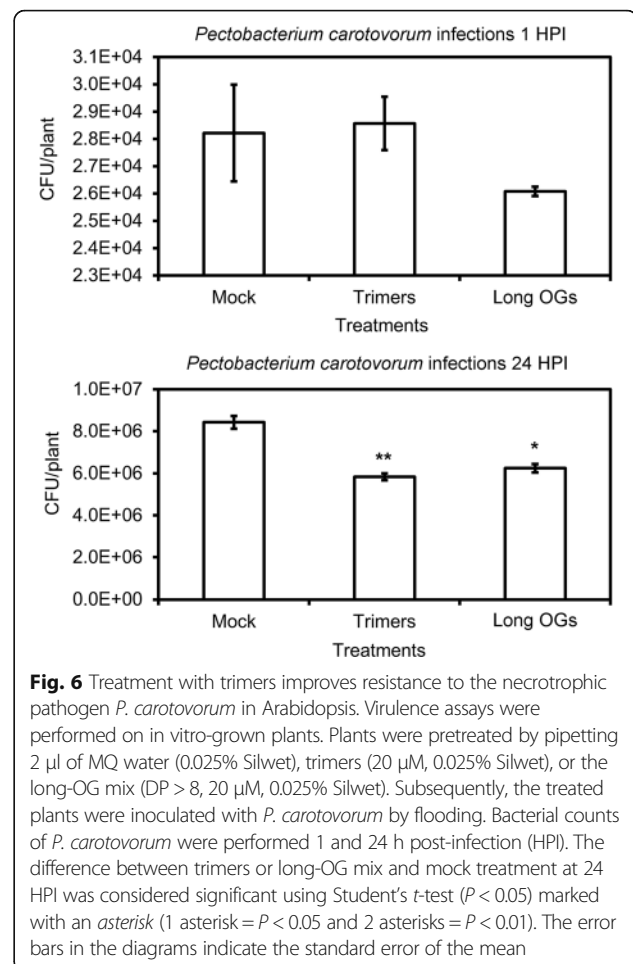


Fig. 6 Treatment with trimers improves resistance to the necrotrophic pathogen *P. carotovorum* in Arabidopsis. Virulence assays were performed on in vitro-grown plants. Plants were pretreated by pipetting 2 μ l of MQ water (0.025% Silwet), trimers (20 μ M, 0.025% Silwet), or the long-OG mix (DP > 8, 20 μ M, 0.025% Silwet). Subsequently, the treated plants were inoculated with *P. carotovorum* by flooding. Bacterial counts of *P. carotovorum* were performed 1 and 24 h post-infection (HPI). The difference between trimers or long-OG mix and mock treatment at 24 HPI was considered significant using Student's *t*-test ($P < 0.05$) marked with an asterisk (1 asterisk = $P < 0.05$ and 2 asterisks = $P < 0.01$). The error bars in the diagrams indicate the standard error of the mean

Arabidopsis plants were grown in vitro in medium containing trimers (200 μ M), long-OG mix (DP > 8, 200 μ M), or Mock. Arabidopsis seedlings treated with either of the OG preparations displayed significant growth retardation (Fig. 7). However, treatment with trimers produced growth retardation to a significantly greater extent than treatment with the long-OG mix.

Long OGs trigger a ROS burst, whereas trimers do not

Plant defense activation by long OGs and other elicitors in Arabidopsis is typically associated with the induction of a ROS burst from the plasma membrane NADPH oxidase *AtRBOHD* [64]. The induction of *AtRBOHD* has previously been observed upon treatment with long OGs [17, 30, 35]. Here, the transcriptomic data show a transient induction also after treatment with trimers (Fig. 5). To determine whether short OGs could also induce an initial ROS burst, we measured the early ROS induced by treating plants with trimers. In accordance with previous data [69], only the long-OG mix (DP > 8) elicited a ROS burst significantly more intense than that of the mock treatment at early time points (Fig. 8). Even higher

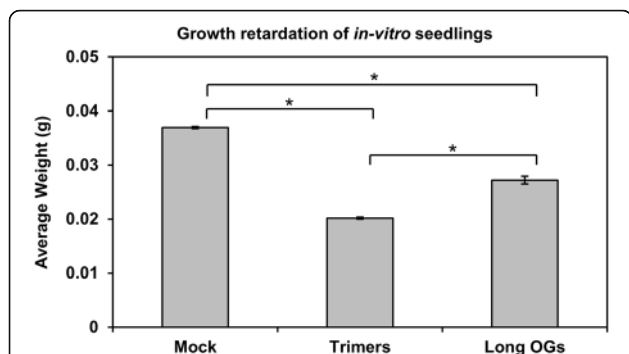


Fig. 7 Trimers and the long-OG mix (DP > 8) induce growth retardation in Arabidopsis. Each biological replicate is a pool of four plants grown in individual wells on a 12-well plate. At least six biological replicates were used for each treatment. Differences between the trimers (200 μM), long-OG mix (200 μM) and mock (1/2 MS) treatments were statistically significant for $P < 0.05$ using ANOVA and Scheffe's *post hoc* test, indicated by asterisks in the figure. The data shown represent three replicate experiments with similar outcomes. The error bars indicate the standard error

concentrations (up to 10 mM) of trimers did not elicit a ROS burst (data not shown). However, the *AtRBOHD*-mediated oxidative burst elicited by longer OGs is not required for the expression of several genes typically considered to be OG-responsive, nor for OG-induced resistance to *B. cinerea* [52]. Thus, while trimers do not induce a detectable oxidative burst in plants, this does not necessarily diminish their role in eliciting plant defense. Furthermore, trimers were found to influence

the expression of a somewhat different set of peroxidases than did long OGs (Additional file 1: Table S2), possibly effecting ROS homeostasis.

Trimers trigger MAPK3 and MAPK6 phosphorylation in Arabidopsis seedlings

Mitogen-activated protein kinase (MAPK) cascades participate in defense responses triggered by various elicitors, for example flg22 and OGs have been shown to cause phosphorylation of MPK3 and MPK6 [35, 70–72]. To determine whether trimers could also induce MPK3 and MPK6 phosphorylation we used immunoblotting to assay the phosphorylation levels 10 min after treatment with trimers. In accordance with previous data the long-OG mix (DP > 8) and flg22 triggered phosphorylation of MPK3 and MPK6 [71, 35]. Trimers also triggered phosphorylation, possibly somewhat less with 200 μM than 1 mM. Long OGs caused similar amount of phosphorylation independently of concentration, implying saturation of the response at 200 μM. Flg22 treatment resulted in more phosphorylation than either OG treatment (Fig. 9).

Discussion

Oligogalacturonides (OGs) are oligomers of alpha-1,4-linked galacturonosyl residues released from plant cell walls upon pathogen attack or mechanical wounding. OGs act as damage-associated molecular patterns (DAMPs) and elicit plant defense responses similar to those of well-characterized pathogen-associated molecular patterns (PAMPs), such as the bacterial flagellin [64, 73, 74].

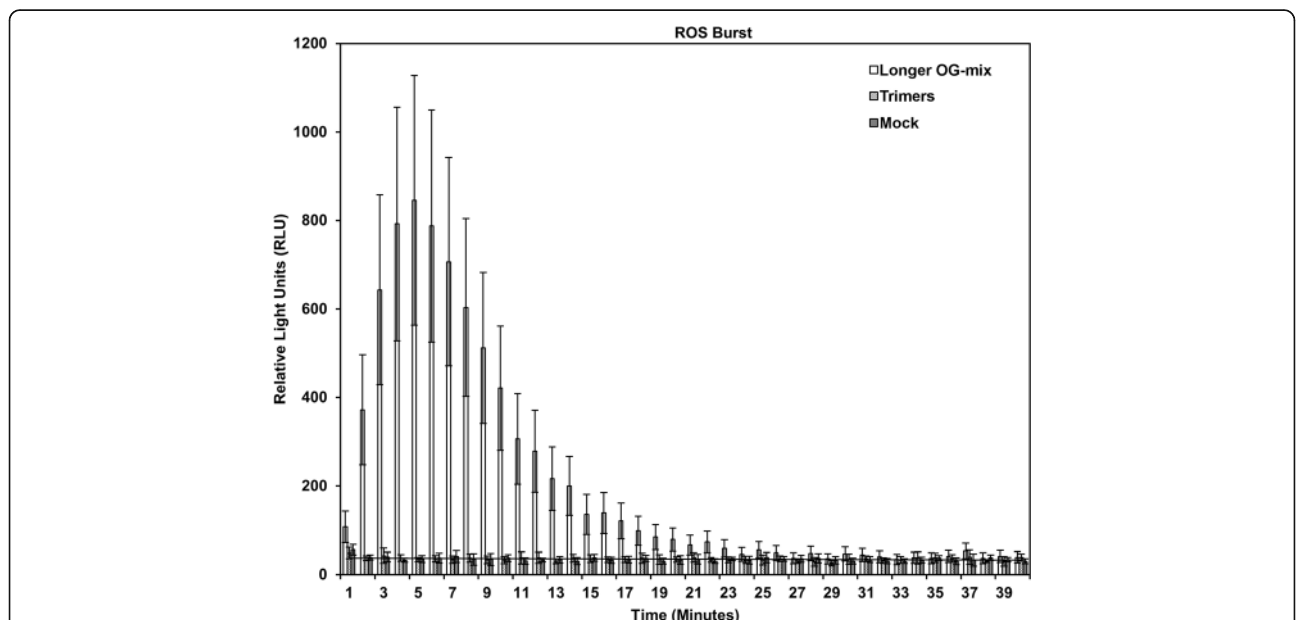
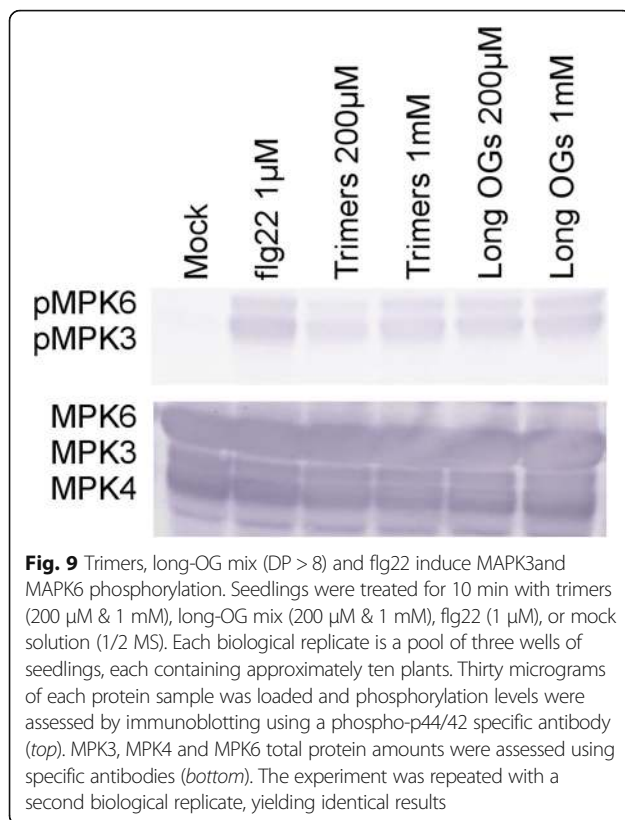


Fig. 8 The long-OG mix (DP > 8) induce a rapid ROS burst in leaf discs, whereas trimers do not. Reactive oxygen species (ROS) burst in RLU as measured by EnSpire® Multimode Plate Reader for leaf discs exposed to the long-OG mix (200 μM), trimers (200 μM) or mock (MQ water). The error bars depict the standard deviation at each time point



DAMPs, such as OGs, trigger the accumulation of reactive oxygen species (ROS), defense-associated proteins and callose, which contribute to enhanced pathogen resistance in plants [17, 52, 75, 76]. This enhanced resistance has been associated with long OGs (DP > 10) [17, 30, 35, 36]. However, there are several indications that also short OGs are potent elicitors of plant defense gene expression and may act as DAMPs to trigger plant innate immunity [28, 39, 41]. Furthermore, short OGs play a role in modulating plant development [30, 40]. To elucidate the putative functional divergence between OGs of different DPs, we explored the capacity of trimers to elicit plant defenses and to control plant growth and development. This was performed by characterizing the transcriptome using RNA sequencing from trimer-treated *Arabidopsis*, combined with an analysis of the physiological and phenotypic consequences of the same treatment. Furthermore, we compared the gene expression data obtained from trimer-treated *Arabidopsis* seedlings with data from previous microarray studies of *Arabidopsis* responses to long OGs [17, 30, 35]. The resulting comparative transcriptomic data reveal that trimers induce the expression of many defense-related genes in *Arabidopsis*. Additionally, treatment with short OGs leads to the down-regulation of genes involved in plant growth and development.

The impact of short OGs on plant defense and development was further analyzed using a GSEA. GO-based gene

sets involved in stimulus (immunity and hormonal (mainly JA and ET)) responses were induced by trimers, while gene sets involved in photosynthesis, general metabolism, development and transcription were down-regulated (Figs. 4 and 5). In conclusion, RNA sequencing data from plants exposed to trimers are generally qualitatively, if not quantitatively, similar to the plant gene expression effects triggered by long OGs. Overall, the GSEA results, similar to the gene expression data, suggest that long OGs may have a greater impact on the transcriptome, but trimers still have a significant global impact.

The trimer-triggered induction of defense-associated genes and the down-regulation of genes involved in morphogenesis and development is generally consistent with the effects that long OGs induce in plants [35]. The trimers described here regulate more than 120 genes (Additional file 2: Table S3) similarly to the long OGs reported in other studies [17, 35]. A GSEA of common trimer and long-OG-regulated genes at 3 h exhibited a similar gene set map as that for trimers alone (Figs. 3 and 4).

The qRT-PCR data, together with the common genes and gene sets identified in the transcriptomic analyses as modulated in expression in response to both trimers and long OGs, indicate that both long and short OGs induce significant changes in the expression of genes involved in signaling associated with plant defense and growth. In general, while trimers and the long-OG mix induced similar expression of the corresponding marker genes, some differences in the timing and amplitude of the response were evident, particularly at the early time points.

Here, trimers triggered a stronger expression of *PGIP1-2* than that observed in response to the long-OG mix. This could reflect varying plant responses to the appearance of OGs with different DPs during infection. Trimers supposedly appear at the point when the infection has already proceeded further; therefore, the plant responds with a higher induction of *PGIP1-2* to inhibit the degrading enzymes of the pathogen.

Tissue damage, triggered for example by wounding or infection by necrotrophic pathogens, induces specific defense responses, such as the activation of glucosinolate defenses. This is accompanied with the rapid activation of JA synthesis and signaling. The fast induction of *CYP81F2* (Fig. 5) suggests that trimers are indeed prominent inducers of DAMP signaling. The clear up-regulation of the positive regulator of JA-signaling *WRKY40* further supported this notion (Fig. 5). The phytohormones auxin and ABA have a complex role in defense and growth signaling, as well as cross talk between each other [73, 77, 78]. Their activity according to marker genes such as *RAB18*, *ABR1*, *TAR2*, *NCED4* and *IAA2* was found in the RNASeq data to be ambiguous due to their contrasting up and down-regulation. However, trimers were observed to have a clearly retarding

effect on *Arabidopsis* seedling growth (Fig. 7), which was mirrored by the down-regulation of the auxin marker gene *SAUR-AC1* according to qPCR in response to trimers mirrored this growth retardation, underlining the negative effect defense signaling has on processes associated with growth.

Short OGs have previously been linked to an inability to induce a ROS-burst in *Nicotiana tabacum* cv. Petit Havana [69]. Accordingly, while we detected a clear ROS burst in *Arabidopsis* leaves treated with the long-OG mix, this was not noticed for trimers. The same has been observed by Legendre et al. (1993), who showed that the induction of H₂O₂ accumulation requires OGs with DP > 3 in suspension-cultured soybean cells [77]. Interestingly, we observed the induction of *AtRBOH* in response to both trimers and the long-OG mix. However, the expression induced by trimers appeared to be much shorter in duration (Fig. 5). Thus, trimers and long OGs differ in their ability to induce *AtRBOHD* and to trigger the initial ROS burst in *Arabidopsis*. Typically the ROS burst is associated with HR-associated cell death, which is considered to promote susceptibility to necrotrophs [68]. It would be tempting to speculate that at the early stages of infection, when mostly long OGs are present, the ROS burst and subsequent signaling could be beneficial in fighting off the pathogen. At the later stages of infection, when shorter OGs are supposedly dominating, the initiation of ROS production and cell death might be detrimental. Furthermore, trimers may still contribute to altered ROS homeostasis, possibly by affecting the induction of peroxidase genes (Additional file 1: Table S2).

Our results show that plants grown in the presence of trimers or the commercial long-OG mix exhibited reduced growth (Fig. 7). Furthermore, the *in vitro* treatment of seedlings with trimers induced plant defense against *P. carotovorum* (Fig. 6). The resource allocation from growth- to defense-related processes results from the rapid activation of DAMP signaling in response to trimer treatment; the binding of PAMPs or DAMPs to their corresponding receptors has previously been shown to trigger defense-associated mechanisms, such as ET biosynthesis, ROS burst, callose deposition and seedling growth inhibition [78]. The pretreatment of plants with known DAMPs, such as long OGs or the protein Pep1, has been demonstrated to elicit resistance to pathogens, such as *P. syringae* or *B. cinerea* [64, 76].

Although both long and short OGs produce a significant phosphorylation response in MAPKs at micromolar concentrations, the bacterial flg22 produces significantly more phosphorylation, indicating that the maximum capacity for phosphorylation of these two proteins is not reached by OGs, or possibly that it is reached at a difference in timing. This is in accordance with what was reported by [35] who observed a stronger and longer

lasting activity of MPK3 in *Arabidopsis* mesophyll protoplasts treated with OGs, than those treated with flg22. In conclusion, these data demonstrate that in addition to long OGs (DP > 10), also short OGs (DP3) are biologically active and capable of inducing defense gene expression, triggering defense related pathways, inhibiting seedling growth, as well as eliciting defense against the necrotrophic pathogen *P. carotovorum* in *Arabidopsis*.

Conclusions

This study provides a novel characterization of the general impact of trimers on the global *Arabidopsis* transcriptome. The RNA sequencing data from this study were compared with data from previous studies characterizing the global transcriptomic effects of long OGs in *Arabidopsis* [35, 64]. From this comparison, it became clear that although trimer treatment had less of an overall impact on the transcriptome than did the long OGs at early time points, trimers constitute a biologically active DAMP signal. This conclusion is in agreement with previous studies suggesting that there is not necessarily any minimum DP limit for OG activity [39]. In future studies, it will be of interest to perform a more encompassing analysis of the distinct plant responses to OGs with specific DPs.

Methods

Plant material and growth conditions

All of the experiments in this study used plant material from *Arabidopsis thaliana* Columbia-0 (Col-0) ecotype (*Arabidopsis*), obtained by the Nottingham *A. thaliana* Stock Centre. Seeds were surface sterilized using chlorine gas [79]. Briefly, the seeds were placed in 2 ml microcentrifuge tubes inside a desiccator. A glass container with 150 ml of sodium hypochlorite (Sigma-Aldrich, 10%) and 4.2 ml of HCl (Sigma-Aldrich, 37%) was placed in the desiccator. The lid was immediately sealed with tape, and the seeds were left for 68 h before the gas was allowed to dissipate. The plants used in virulence assays were grown in a growth room in pots containing a 1:1 mixture of soil and peat. The light period was 12 h, and the temperature was 22 °C. After 3–4 weeks, the plants were used for physiological assays.

Analysis of oligogalacturonide DP

Commercially available trimers (Trigalacturonic acid, Sigma-Aldrich) and a long-OG mix with a stated DP10-15 (Elicityl) were run on Silica gel 60_{F254} TLC plates and analyzed by mass spectrometry to confirm the degree of polymerization. Trimers were found to consist of only DP3, whereas the long-OG mix was found to consist of primarily DP8-19, with an average DP of approximately 14 (Additional file 10: Figure S3, Additional file 11: Figure S4 and Additional file 12: Figure S5).

Growth inhibition assay

For the growth inhibition assay, *Arabidopsis* seedlings were placed in individual wells with 1 ml of liquid 1/2 MS medium in 12-well plates [80]. The plates were sealed with Parafilm to minimize evaporation. Seeds were stratified for 3 days then allowed to grow in a 12 h light cycle at 20 °C. After 8 days, the medium was replaced with fresh liquid 1/2 MS, and the seedlings were grown for two more days before treatment with OG-containing suspensions of either trimers (200 µM) or the commercial long-OG mix (200 µM). As a mock treatment, 1/2 MS medium was used. After treatment, the plants grew for 8 days before the total plant weight was assessed. For all of the subsequent assays, 4 plants were combined into one biological replicate, and 12 biological replicates were tested per treatment. All of the experiments were performed a minimum of three times. The average plant weight per treatment was compared and statistically analyzed using an ANOVA combined with Scheffe's *post hoc* test.

RNA extraction and purification

Plant seedlings were grown *in vitro*, as described by Ferrari et al. (2007) except that the growth medium base that was used in this study was 1/2 MS and the photoperiod was 12 h [17]. Additionally, higher equimolar concentrations of trimers were used compared to those used in previously studies using long OGs [17, 35]. The concentrations used in this study were primarily set such that a significant induction of growth retardation and induction of defense genes could be observed. Briefly, the protocol for treating plants was similar to that used in growth inhibition studies except that 12 seeds were placed in each well for germination. The seedlings were treated with trimers (200 µM), the long-OG mix (200 µM) or mock solution (1/2 MS) for 3 h prior to RNA extraction. For each biological replicate, plants were harvested from 3 wells, rinsed in MQ water and blotted dry before freezing in liquid nitrogen. The plant material was crushed using metal beads in a shaker. The RNA was extracted using the GenJET Plant RNA Purification mini kit (ThermoScientific) following the manufacturer's protocol. The sample quality was assessed by gel electrophoresis and measured using a NanoDrop (ThermoScientific). Then, 2 µg of total RNA was treated with DNaseI and RNase-free (ThermoScientific) and used for cDNA synthesis using Maxima Reverse Transcriptase and Ribolock RNase Inhibitor (ThermoScientific) following the manufacturer's protocol. The primers used for cDNA synthesis were random hexamers and oligo dT primers.

RNA Sequencing

The total RNA extracted from 3 biological replicates per treatment (trimers and mock) was purified from rRNA

using an Epicenter Ribo-Zero rRNA removal kit (Plant leaf) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were assayed with a Qubit fluorometer (Life Technologies) and a NanoDrop spectrophotometer before sequencing. Sequencing was performed on the SOLiD 5500XL platform. Each sample was part of a pool and run in two separate lanes, generating approximately 9 million single-end reads with a length of 75 bp that were mapped to the genome using the SHRiMP platform [81]. A total of 33,597 gene sequences were used to align reads from sequencing. Gene expression was considered to be significantly different for FDR values <0.05 and a log₂ fold change of ≥0.5 or ≤-0.5 as calculated by the R package DESeq2 [82]. The raw RNA sequencing data can be found in the NCBI GEO repository under the accession number GSE69538.

Quantitative RT-PCR analysis

Total RNA was extracted from plants that were treated with the trimers, long-OG mix or mock solution and were raised similarly to plants used for RNA sequencing. RNA for qRT-PCR analysis was extracted 1, 3, 7, 12 and 24 h post-treatment. For each time point, three biological replicates per treatment were used to generate RNA samples. For qPCR, HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biotec) was used with an estimated 8 ng of cDNA in a total reaction volume of 10 µl. The amplification program used on the BioRad CFX is as follows: an initial activation cycle of 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s, ending with a melt curve analysis. The resulting qRT-PCR data were analyzed using GeNorm software [83]. Housekeeping genes used as references in the qRT-PCR analysis were AT4G05320 (*UBQ10*), AT5G09810 (*ACT7*), AT5G12250 (*TUB6*), AT5G60390 (*EF1α*) and AT3G13920 (*eIF4A*). These genes were assessed for stability across cDNA samples using the GeNorm M-value principle in the GenEx 6 software (MultiD Analyses AB, Gothenburg, Sweden). The two most stable reference genes (*EF1α* and *eIF4A*) had an M-value <0.3 across all samples and were used for the subsequent normalization [83, 84]. All of the analyzed genes, along with their respective primers, are listed in Additional file 13: Table S1. Statistically significant differences between treatments were determined by Student's *t*-test comparing dCt values.

Infection assays

In vitro plant infections were performed by the flood-inoculation method [85]. *A. thaliana* seedlings were grown under axenic conditions on 1/2 MS [86] agar plates for 10-12 days (12 h day/night cycle at 20 °C) prior to pretreatment. The plants were pre-treated with the application of a 2 µl drop of either sterile MQ water

(0.025% Silwet), trimers (20 μ M, 0.025% Silwet), or the long-OG mix (20 μ M, 0.025% Silwet) to two rosette leaves per plant approximately 24 h before infection. *Pectobacterium carotovorum* sp. *carotovorum* SCC1 was cultured overnight in liquid Luria-Bertani (LB) medium at 28 °C. Bacterial cultures were washed twice in 10 mM MgSO₄ and resuspended in 35 ml of a solution containing 10 mM MgSO₄ and 0.025% silwet. The OD₆₀₀ of the bacterial suspension was adjusted to 0.001 before flooding the *A. thaliana* seedlings for approximately 2.5–3 min. After the bacterial suspension was decanted, the plants were incubated overnight under conditions consistent with those before inoculation. Each plate contained 6 plants that were combined to produce one biological replicate. Plants were crushed, and the resulting liquid was diluted and spotted onto LB plates. For each dilution and sample, five drops of 20 μ l each were plated for bacterial counting. Four biological replicates were used for each treatment at the 2 h time point, and 12 biological replicates were used for the 24 h time point. The experiment was repeated with similar results. Statistical significance was calculated using a Student's *t*-test ($P < 0.01$).

Quantification of ROS production

Prior to treatment, leaf discs (approximately 2 mm in diameter) were floated on MQ water for 24 h in 96-well plates. Next, the water was replaced with 100 μ l of a solution containing 20 mM luminol, 10 μ g/ml peroxidase and either MQ water, trimers (200, 400 or 800 μ M) or the long-OG mix (200 μ M). The relative light units (RLUs) produced in each well were measured every minute for 40 min using an EnSpire® Multimode Plate Reader [87].

Bioinformatics analysis

RNA sequencing data from plants exposed to short OGs, together with previously published microarray data from plants treated with long OGs (using a *P*-value of <0.01 as a cut-off, as in the corresponding studies), were analyzed for gene set enrichment using the GenePattern software (Broad Institute). Software parameters included 1000 permutations and a gene set size range from 5 to 1000 [17, 35, 88, 89]. A *P*-value cut-off of <0.05 was used to determine gene sets with significantly altered expression. Furthermore, a Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>)-based pathway analysis was performed using the R package Pathview [90].

Total protein extraction

Plant seedlings were grown as those used for RNA extraction. The seedlings were treated with trimers (200 μ M and 1 mM), the long-OG mix (200 μ M and 1 mM), flg22 (1 μ M), or mock solution (1/2 MS) for

10 min prior to protein extraction. For each biological replicate, plants were harvested from 3 wells, rinsed in MQ water and blotted dry before freezing in liquid nitrogen. The plant material was crushed using metal beads in a shaker. Total protein was extracted in extraction buffer (50 mM Tris-Hcl pH 7.5, 200 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100, 1 mM Pefabloc, 1 mM DTT, 1 x Protease inhibitor (Roche Diagnostics) and 1 x Phosphatase inhibitor (Thermo Fisher Scientific)), incubated on ice for 30 min and then centrifuged for 15 min at 21 500 g at 4 °C.

MPK3 and MPK6 immunoblotting

Immunodetection was performed similarly to Ortiz-Morea et al. 2016 [91]. Briefly, 30 μ g of protein from each sample was boiled for 3 min in Laemmli buffer, resolved on a 12% polyacrylamide gels before being transferred onto a PVDF membrane (Immobilion). The membrane was blocked for 2 h with 5% BSA before being incubated with primary antibodies (MPK3-Sigma M8318 Anti-AtMPK3 antibody produced in rabbit 1:1000, MPK6-Sigma A7104 Anti-AtMPK6 antibody produced in rabbit 1:3000, MPK4-Sigma A6979 Anti-AtMPK4 antibody produced in rabbit 1:1500, or Phospho-p44/42 MAPK-(Erk1/2) (Thr202/Tyr204) Antibody #9101 Cell Signaling Technology. 1:1000) overnight. Incubation with secondary antibody (Anti-Rabbit IgG Alkaline Phosphatase Conjugate-Promega 1:10 000) was done for 1 h. Visualization was performed using the AP Conjugate Substrate Kit (Bio-Rad Laboratories).

Additional files

Additional file 1: Table S2. RNA-sequencing gene expression data of plants treated by trimers compared to plants treated by a mock.

Corresponding data is presented from plants treated with microarray results from long OG treatment at 1 h (long OGs 1 h - Denoux et al. 2008), 3 h (long OGs 3 h - Denoux et al. 2008) and 2 h (long OGs 2 h, cell culture - Moscatiello et al. 2006). #N/A = not significantly affected in expression. (XLSX 242 kb)

Additional file 2: Table S3. Genes significantly affected in expression (Fold Change) by treatment with trimers at 3 h (Trimers), long OGs at 1 h in the study by Denoux et al. 2008 (Long OGs 1 h) and long OGs at 3 h in the study by Denoux et al. 2008 (Long OGs 3 h). (XLSX 51 kb)

Additional file 3: Table S4. GSEA results based on gene expression data from trimer treated plants compared to mock treatment. (XLSX 31 kb)

Additional file 4: Table S5. GSEA result of genes affected similarly in expression by both trimers and long OGs at 3 h (Denoux et al. 2008). (XLSX 36 kb)

Additional file 5: Table S6. GSEA result of genes affected in expression by both Trimers (3 h) and long OGs (1 h and 3 h-Denoux et al. 2008). (XLSX 26 kb)

Additional file 6: Table S7. GSEA results based on gene expression data from trimer treated plants, disregarding genesets also affected by long OGs at 3 h (Denoux et al. 2008). (XLSX 11 kb)

Additional file 7: Table S8. GSEA results based on gene expression data of long OGs at 3 h (Denoux et al. 2008) disregarding genesets also affected by trimers. (XLSX 41 kb)

Additional file 8: Figure S1. Pathway analysis of plant hormone signal transduction pathways using RNA-sequencing data of plants treated by trimers compared to plants treated by a mock (Trimers). Also used is corresponding data from plants treated by long OGs at 1 h (long OGs 1 h) and 3 h (long OGs 3 h) (Denoux et al. 2008). (TIF 1568 kb)

Additional file 9: Figure S2. Pathway analysis of plant-pathogen interaction pathways using RNA-sequencing data of plants treated by trimers compared to plants treated by a mock (Trimers). Also used is corresponding data from plants treated by long OGs at 1 h (long OGs 1 h) and 3 h (long OGs 3 h) (Denoux et al. 2008). (TIF 2399 kb)

Additional file 10: Figure S3. Mass spectrum graph for the trimeric OGs used in this study (Trimers). (TIF 759 kb)

Additional file 11: Figure S4. Mass spectrum graph for the long OGs with a degree of polymerization >8 used in this study (long-OG mix). (TIF 1425 kb)

Additional file 12: Figure S5. Results of TLC plate analysis of Trimers and long-OG mix (longer OG mix). (TIF 973 kb)

Additional file 13: Table S1. The qRT-PCR primers (nucleotide sequences) used in this study, their target gene ID, gene name and applicable references. (XLSX 11 kb)

Abbreviations

ABA: Abscisic Acid; DAMP: Damage Associated Molecular Pattern; DP: Degree of Polymerization; ET: Ethylene; GO: Gene Ontology; GSEA: GeneSet Enrichment Analysis; JA: Jasmonic Acid; OG: Oligogalacturonides; PAMP: Pathogen Associated Molecular Pattern; PCWDE: Plant Cell Wall-Degrading Enzyme; qRT-PCR: Quantitative RT-PCR; ROS: Reactive Oxygen Species; SA: Salicylic Acid; Trimers: Trigalacturonic acid

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Not Applicable.

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Availability of data and materials

Sequencing data can be found under the NCBI GEO series accession number GSE69538 at the website <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69538>.

Author's contributions

PD, MB, TK, NS, MP and TP designed the research. PD, MB and NS carried out the experiments. PD, MB, TK, NS, MP and TP wrote the paper. MB and PD analyzed the data. All of the authors have read and approved the final manuscript, and agreed to be accountable for all aspects of the work.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not Applicable.

Ethics approval and consent to participate

Not Applicable.

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