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Mixed DAMP/MAMP oligosaccharides promote both growth and defense against fungal pathogens of cucumber

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ABSTRACT

Plants recognize a variety of environmental molecules, thereby triggering appropriate responses to biotic or abiotic stresses. Substances containing microbes-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) are representative inducers of pathogen resistance and damage repair, thus treatment of healthy plants with such substances can pre-activate plant immunity and cell repair functions. In this study, the effects of DAMP/MAMP oligosaccharides mixture (Oligo-Mix) derived from plant cell wall (cello-oligosaccharide and xylo-oligosaccharide), and fungal cell wall (chitin-oligosaccharide) were examined in cucumber. Treatment of cucumber with Oligo-Mix promoted root germination and plant growth, along with increased chlorophyll contents in the leaves. Oligo-Mix treatment also induced typical defense responses such as MAP kinase activation and callose deposition in leaves. Pretreatment of Oligo-Mix enhanced disease resistance of cucumber leaves against pathogenic fungi *Podosphaera xanthii* (powdery mildew) and *Colletotrichum orbiculare* (anthracnose). Oligo-Mix treatment increased the induction of hypersensitive cell death around the infection site of pathogens, which inhibited further infection and the conidial formation of pathogens on the cucumber leaves. RNA-seq analysis revealed that Oligo-Mix treatment upregulated genes associated with plant structural reinforcement, responses to abiotic stresses and plant defense. These results suggested that Oligo-Mix has beneficial effects on growth and disease resistance in cucumber, making it a promising biostimulant for agricultural application.

Keywords: Anthracnose, Biostimulant, Cucumber, Damage-associated molecular patterns, Elicitors, Microbe-associated molecular patterns, Oligosaccharides, Powdery mildew.

1. Introduction

Plants have an array of mechanisms for sensing and adapting to environmental stresses. Some breakdown molecules of plant cells are released by physical damage, pathogen infection, or insect attack and are recognized as signals by plants to promote cellular repair or defense responses. Such molecules are commonly called DAMPs (damaged-associated molecular patterns), including cello-oligosaccharides (COS), which are constituent molecules of cellulose derived from plant cell walls, and xylo-oligosaccharides (XOS) contained in hemicellulose, which cross-link cellulose and lignin in the cell wall (Souza et al., 2017; Claverie et al., 2018; Pring et al., 2023). Volatiles emitted by damaged plants are also involved in stress responses, thus considered as a type of DAMPs (Yamauchi et al., 2018). It is also known that specific peptides released externally by cell disruption are recognized as DAMPs by receptors on the surface of neighboring cells (Pearce et al., 1991; Yamaguchi et al., 2010; Wang et al., 2018; Hander et al., 2019).

Molecules specific to microorganisms are recognized by plant cells as alert signals of pathogen attack, and the epitope structures of such substances are called MAMPs (microbes-associated molecular patterns). Some MAMPs are highly conserved in large groups (i.e. bacteria, fungi or oomycetes), such as flg22 (a conserved peptide of the bacterial flagellar protein flagellin, Gómez-Gómez et al., 1999), peptidoglycan (the structural polymer of the bacterial cell wall, Gust et al., 2007), chitin and β-glucan (major cell wall components of fungi, Sharp and Albersheim, 1984; Felix et al., 1993) and 9-methyl-4,8-sphingadienine (9Me-Spd, a substructure of ceramides found in the cell membrane of fungi and oomycetes, Kato et al., 2022; Monjil et al., 2024). Other MAMPs are found in certain groups of pathogenic microbes, such as elicitins produced by oomycete pathogens (Derevnina et al., 2016), and fungal ethylene-inducing xylanases (EIXs, Dean et al., 1991). On the other hand, the recognition mechanisms of MAMPs by plant species are also diverse; for example, Solanaceae plants and rice can recognize different parts of bacterial flagellin other than flg22 (Fliegmann et al., 2016). Elicitin and EIX have also been reported to be recognized as MAMPs by a limited range of plant species (Kamoun et al., 1993; Ron and Avni, 2004; Takemoto et al., 2005).

While the understanding of the recognition mechanisms of DAMPs and MAMPs by plants is a subject of basic research, these substances are also anticipated to be utilized as materials to activate plant growth and immunity in agricultural production. Substances that activate beneficial plant responses are collectively referred to as biostimulants. The DAMPs and MAMPs derived from fundamental structures of plants and microbes are expected to be versatile as agricultural materials because they can activate the responses of diverse plant

species. The cell walls of plants and fungi are essentially composed of common materials (Cosgrove 2005; Gow and Lenardon 2023), thus substances in their cell walls are representative candidates for biostimulant.

Previously, we have shown that DAMP and MAMP oligosaccharides, COS prepared from cotton linters, XOS prepared from corn cobs as well as chitin-oligosaccharide (CHOS) from crustacean shells can trigger typical defense responses of Arabidopsis such as production of reactive oxygen species (ROS), MAP kinases phosphorylation and callose depositions (Pring et al., 2023). Gene ontology enrichment analysis of RNA-seq data revealed that simultaneous treatment of COS, XOS and CHOS (Oligo-Mix) effectively activate genes for disease resistance. Generally, it is considered that activating disease resistance in plants suppresses their growth, referred to as "trade-off effect" (Karasov et al., 2017). However, the treatment of Oligo-Mix had no significant defects on the expression of photosynthesis genes. In practice, treatment of the Oligo-Mix enhanced resistance of tomato against powdery mildew pathogen, and moreover, tomato growth was rather promoted by Oligo-Mix treatment (Pring et al., 2023).

In this study, the effects of Oligo-Mix treatment on cucumber were investigated. Cucumber (*Cucumis sativus* L.) is one of the most important vegetable crops cultivated worldwide. Besides the varieties with higher resistance to pathogens, in general, cucumbers are susceptible to a variety of destructive pathogens. Anthracnose, caused by a fungal pathogen *Colletotrichum orbiculare*, is a major foliage disease that reduces the yield and quality of cucumber and other Cucurbitaceae plants such as watermelon and melon. *C. orbiculare* is a hemi-biotrophic pathogen that employs both biotrophic and necrotrophic phases to infect its hosts (Matsuo et al., 2022). Powdery mildew of cucumber is widely distributed, rapidly spreading to cause destructive disease by obligate parasites *Golovinomyces cichoracearum* or *Podosphaera xanthii* (Lebeda et al., 2011). While there are some fungicides accessible for managing these pathogens, the emergence of drug-resistant fungal strains has become a major problem (Vielba-Fernández et al., 2020). Here, we evaluated the influence of the Oligo-Mix treatment on cucumber growth, development, and resistance against anthracnose and powdery mildew pathogens.

2. Material and methods

2.1. Plant material and growth condition

Cucumber (cv. Shimoshirazu Jibai, Takii seed, Kyoto, Japan) were grown in autoclaved commercial soil (Sakata Super Mix A, Sakata seed, Yokohama, Japan) in a plant growth room at 23˚C with 16 h of light per day. For the greenhouse test, two-week-old seedlings grown in a

118 controlled growth chamber were transferred to pots (28 cm diameter x 22 cm height, filled with
119 Sakata Super Mix A) and grown in a greenhouse in Togo field, Nagoya University (University
120 farm, Togo, Aichi, Japan).
121
122 *2.2. Oligosaccharide mixture (Oligo-Mix)*
123 Oligo-Mix (undiluted solution) used in this study consists of 20 mg/ml cello-oligosaccharide
124 (COS, prepared from cotton linter), 40 mg/ml xylo-oligosaccharide (XOS, from corn cob), and
125 20 mg/ml chitin-oligosaccharide (CHOS, from shrimp shell) (Sreynich et al., 2023). Both
126 Oligo-Mix and the control solutions contain water-soluble P (P_2O_5, 4.7% w/v) and K (K_2O,
127 3.4% w/v). Oligo-Mix and control solution were diluted to 1/1000 and used for the treatment
128 of plants by spray or infiltration using a needleless syringe. Oligo-Mix and control solutions
129 were provided by Resonac Corporation (Tokyo, Japan).
130
131 *2.3. Determination of root number and length*
132 Cucumber seeds were placed on laboratory tissue soaked with distilled water in Petri dishes
133 and incubated at 26 ˚C with a light/dark cycle set at 16 h/8 h. The cucumber seeds germinated
134 at same day were collected and sprayed with Oligo-Mix or control solution (250 µl per petri
135 dish), and the number of roots per seed and the length of longest root were measured 2 days
136 after the treatment.
137
138 *2.4.Determination of cucumber growth*
139 The individual seedlings, treated with control solution or Oligo-Mix as described above,
140 were transferred into 9-cm-diameter plastic pot filled with sterilized soil mix and grown in
141 growth room at 23 ˚C with 16 h of light per day. Cucumber plants were sprayed with Oligo-
142 Mix or control solution once every week (2 ml per plant, 4 times altogether) until sampling.
143 After one week of the last treatment, the fresh weight of the root and above-ground tissues are
144 measured.
145
146 *2.5. Measurement of chlorophyll contents*
147 Chlorophyll was extracted according to the method described in Shibata et al., (2016). One
148 g of second true leaves of cucumber was soaked in 50 ml methanol overnight. The absorbance
149 intensity of the solution was quantified by spectrophotometer (Multiskan GO, Thermo Fisher
150 Scientific, Waltham, MA, USA), and the concentration of chlorophyll a and b was calculated

151 according to the method of Porra and Scheer (1989) as follows; Chlorophyll a (μg/ml) = 16.29
152 x A^{665} – 8.54 x A^{652}; Chlorophyll b (μg/ml) = 30.66 x A^{652} – 13.58 x A^{665}.
153
154 *2.6. MAP kinase activation assay*
155 Activation of MAP kinases in Arabidopsis was detected as previously reported (Kato et al.,
156 2022). Cucumber seedlings were treated with Oligo-Mix or control solution (by infiltration
157 with a needleless syringe) for 15 or 30 min and frozen in liquid nitrogen. Proteins were
158 extracted in extraction buffer (50 mM HEPES-KOH pH 7.4, 5 mM EDTA, 0.5 mM EGTA, 50
159 mM ß-glycerophosphate, 10 mM NaF, 10 mM Na_3VO_4, and 2 mM DTT). Phosphorylated
160 MAP kinases were detected by western blot using Phospho-p44/42 MAPK (Erk1/2; Thr-
161 202/Tyr-204) rabbit monoclonal antibodies #9101 (Cell Signaling Technology). Blots were
162 stained with PageBlue Protein Staining Solution (Thermo Fisher Scientific) to verify equal
163 loading.
164
165 *2.7. Staining of callose deposition*
166 Staining of callose deposition was performed as previously reported (Shibata et al., 2016).
167 Cucumber seedlings were treated with Oligo-Mix or control solution by infiltration with
168 needleless syringe, and seedlings were fixed 24 h after treatment in fixation solution (1% [v/v]
169 glutaraldehyde, 5 mM citric acid, and 90 mM Na_2HPO_4, pH 7.4) overnight. Fixed seedlings
170 were heated in boiled water for 5 minutes, decolorized in ethanol, and stained in aniline blue
171 stain (0.1% [w/v] aniline blue in 67 mM phosphate buffer, pH 12.0) to detect callose deposition.
172 The fluorescence spots of deposited callose were detected by a fluorescence microscope (BX51,
173 Olympus, Tokyo, Japan) using an excitation wavelength of 365 nm. The number of callose
174 spots per area was quantified using ImageJ software (Schneider et al., 2012).
175
176 *2.8. Pathogens and inoculation*
177 Powdery mildew of cucumber, *Podosphaera xanthii* strain SP23, was isolated from cucumber
178 grown in a greenhouse in Togo field, Nagoya University, and maintained by inoculating
179 healthy cucumbers every 5-10 days. The species of powdery mildew was determined based on
180 the sequencing of ITS (Internal transcribed spacer) region using the method previously
181 described (Ashida et al., 2023). Conidia newly formed on cucumber leaves were suspended in
182 water and adjusted to a concentration of $1x10^5$ spores/ml. The 5-leaf-stage cucumber plants
183 were inoculated by spraying 0.5 ml spore suspension per plant at 24 h after the treatment with

184 control solution or Oligo-Mix. One week later, spot symptoms were observed on the leaf
185 surface, and number and type (spored, yellowish, or cell death) of colony were counted. To
186 measure conidial formation, cut leaves with colonies (1.5 × 1.5 cm) were scrubbed with 2 ml
187 of distilled water using a cotton bud, then filtered with a stainless filter disc (mesh size 100
188 μm). Subsequently, the number of conidia was quantified with a hemocytometer (Erma, Tokyo,
189 Japan).
190 Anthracnose pathogen, *Colletotrichum orbiculare* strain T-104 (MAFF240422, which was
191 isolated by Dr. Yasumori, Kyoto University in 1951) was provided by Prof. Yoshitaka Takano
192 (Kyoto University, Japan). *C. orbiculare* was grown on potato dextrose agar (PDA) in 23˚C
193 for 7 days, and conidia formed on the colony were suspended in water and adjusted to a
194 concentration of 1×10^5 spores/ml for the inoculation. Cucumber was spray-inoculated with 0.5
195 ml of spore suspension per plant. The symptoms observed on the leave were photographed 7
196 days after the inoculation.
197
198 *2.9. Detection of anthracnose lesions on cucumber leaves*
199 Briefly, the area affected by anthracnose on cucumber leaves was calculated as the
200 percentage of the affected area divided by the total leaf area. The calculation of the total leave
201 area was done using the custom-made MATLAB script (OAM_240209_leave_seg) to isolate
202 all pixels in an image that belonged to a leaf according to a median intensity threshold. To
203 identify specific infected areas on individual leaves, we trained a modified pixel flow-driven
204 CNN architecture (Stringer et al., 2021) to identify each pixel on a leaf that could be classified
205 as affected with more than 0.9 probability. The CNN model was trained using the preliminary
206 segmentations from the MATLAB script based on median thresholding, which generated
207 single-leaf masks depicting the affected areas. The preliminary masks were manually corrected
208 using custom-made software to remove incorrectly classified pixels, and the final corrected
209 images were split into training (70%), and testing (30%) data sets for deep learning model
210 training. The CNN architecture was modified to obtain a binary lesion/no-lesion single-pixel
211 classification (semantic segmentation) as direct output. The CNN model "Takemoto_1" and
212 the accessory code for whole leaf segmentation, and to obtain the final semantic segmentation
213 from the CNN, as well as the original image dataset with labels and a toy dataset to test the
214 segmentation code, are freely available from the GitHub repository of the Miranda Lab:
215 https://github.com/MirandaLab/Deep_Leaf_Segmentation. Leaf segmentations were
216 performed on a Dual Intel Xeon Silver 4216 (2.1GHz,3.2GHz Turbo, 16C,9.6GT/s 2UPI,

217 22MB Cache, HT (100W) equipped with an Nvidia Quadro RTX5000, 16GB, 4DP,
218 VirtualLink (XX20T) outfitted with 64GB 4x16GB DDR4 2933MHz RDIMM ECC Memory
219 and a 2TB primary NVMe SSD. Differences between control and treatments were evaluated
220 using the Kolmogorov-Smirnov tests *kstest2* MATLAB function, with significance set at $p <$
221 0.05.
222
223 *2.10. Trypan blue staining and microscopy*
224 To visualize the pathogen hyphae and conidiophores, or HR-like cell death of plant cells,
225 the infected leaves were stained with trypan blue as described previously (Takemoto et al.,
226 2003). Small pieces of leaves were cleared in methanol in 1.5 ml tube for more than 24 h
227 (methanol was changed once) until the chlorophyll (green color) was removed. Then methanol
228 was replaced with lactophenol trypan blue stain (10 ml lactic acid, 10 ml glycerol, 10 g phenol,
229 10 mg trypan blue, 10 ml H_2O) and heated in boiled water for 3 mins. After cooling down to
230 room temperature, the stain was replaced with 1 g/ml chloral hydrate, and gently shaken
231 overnight. Pathogen hyphae and plant cells were monitored under a light microscope (BX51,
232 Olympus, Japan).
233
234 *2.11. RNA-seq and data analysis*
235 The four-weeks old cucumber plants were sprayed with Oligo-Mix or control solution and
236 total RNA was isolated at 24 h after treatment. RNA extraction was performed using the
237 RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Evaluation of RNA quality, construction
238 of library and sequencing were performed principally as previously described (Kuroyanagi et
239 al., 2022, Bulasag et al., 2023). Libraries were constructed using KAPA mRNA Capture Kit
240 (Roche Diagnostics, Tokyo, Japan) and MGIEasy RNA Directional Library Prep Set (MGI,
241 Shenzhen, China), and sequenced on DNBSEQ-G400RS (MGI) with 150 bp paired-end
242 protocol. The RNA-seq reads were filtered using trim-galore v.0.6.6 (Martin, 2011,
243 bioinformatics.babraham.ac.uk) and mapped to the cucumber genome (genome assembly
244 Cucumber_9930_V3, NCBI RefSeq sequence GCA_000004075.3) using HISAT2 v.2.2.1
245 (Kim et al., 2019) and abundance inferred via StringTie v.2.1.7 (Kovaka et al., 2019).
246 Significant differential expression was determined using DESeq2 v.1.32.0 (Love et al., 2014).
247 All software used during RNA-seq analysis was run with default settings. RNA-seq data for
248 cucumber reported in this work are available in GenBank under the accession numbers
249 DRA019928.
250 SRplot (https://www.bioinformatics.com.cn/en), a free online data analysis and

visualization platform, was used to draw the graphs.

3. Results and discussion

3.1. Effect of Oligo-Mix treatment on the growth of cucumber

To elucidate the effect of the Oligo-Mix (mixture of cello-oligosaccharide COS, xylo-oligosaccharide XOS, and chitin-oligosaccharide CHOS) on cucumber growth, newly germinated cucumber seeds were spray-treated with Oligo-Mix or control solution, and root number and length were measured 2 days after the treatment. The number of roots per seed and length of longest root were significantly increased (almost 2 times), indicating that Oligo-Mix can promote the growth of cucumber roots (Fig. 1 A). Those seedlings were transplanted to the pot and sprayed with Oligo-Mix weekly (once a week). One week after the last spray (sprayed 4 times and 5 weeks after transplant), the growth of cucumber plants was evaluated. The fresh weight of roots and above-ground tissue was significantly increased by Oligo-Mix treatment (Fig. 1 B). Previously, we have reported the positive effects of Oligo-Mix on root and plant growth of tomato (Sreynich et al., 2023). Additionally, leaves of cucumber treated with Oligo-Mix exhibited a visibly darker green compared to those in the control group. To evaluate the effect of Oligo-Mix on chlorophyll contents, cucumber leaves were immersed with methanol to extract and calculated chlorophylls according to the method reported by Porra and Scheer (1989). Chlorophyll a in leaves treated with Oligo-Mix was approx. 405 μg/ml, whereas control leaves contained 256 μg/ml. Similarly, chlorophyll b contents was 143 μg/ml in leaves treated with Oligo-Mix, while at 89 μg/ml for control leaves. These results suggest that Oligo-Mix can effectively enhance photosynthetic capacity, probably leading to improved plant growth.

3.2 Induction of MAP kinase phosphorylation and callose deposition of cucumber by Oligo-Mix treatment

Activation of MAP kinase cascade is a common initial response of plants to induce disease resistance (Asai et al., 2002). Cucumber cotyledons or Arabidopsis seedlings were treated with Oligo-Mix and phosphorylated MAP kinases were detected using antibody against phospho-MAP kinases (Fig. 2A). Within 15 min after Oligo-Mix treatment, rapid activation of cucumber and Arabidopsis MAP kinases was detected. In Arabidopsis, AtMPK6 (approx. 44 kDa) is the major MAP kinase activated by Oligo-Mix, and relatively minor activation of AtMPK3 and AtMPK4 (approx. 39 kDa and 37 kDa, respectively) were also detected. Similarly, in cucumber, a major activated MAP kinase (approx. 46 kDa) and weak activation of two MAP kinases were

284 also detected within 15 min after Oligo-Mix treatment, suggesting that analogous activation of
285 similar MAP kinases is induced by Oligo-Mix treatment in Arabidopsis and cucumber.
286 Callose deposition is another typical plant defense response to reinforce plant cell walls
287 during pathogen attack (Ellinger and Voigt, 2014). After treatment of cucumber cotyledons
288 with Oligo-Mix for 24 h, a significant increase in callose depositions was detected (Fig. 2B).
289 These results suggested that Oligo-Mix treatment can promote the defense responses of
290 cucumber.
291
292 *3.3 Effect of Oligo-Mix* treatment on disease resistance of cucumber against powdery mildew
293 and anthracnose pathogens
294 Two types of plant pathogens were employed to examine the effect of Oligo-Mix on plant
295 disease resistance. Cucumber powdery mildew fungus, *Podosphaera xanthii*, is an obligate
296 parasitic pathogen of Cucurbitaceae. Cucumber plants were sprayed with control solution or
297 Oligo-Mix, and inoculated with spore suspension of *P. xanthii* 24 h after the treatment.
298 Although the number of lesions developed on leaves was not affected by Oligo-Mix treatment,
299 the ratio of yellowish or necrotic lesions was significantly increased for leaves treated with
300 Oligo-Mix (Fig. 3). Microscopic observation revealed the successful penetration, formation of
301 haustoria, and massive production of conidiophores and conidia on control leaves, while HR
302 (hypersensitive response)-like cell death was often found for the leaves treated with Oligo-Mix
303 (Fig. 4). Consistently, Oligo-Mix treatment significantly reduced the spore formation on the
304 leaves (Fig. 4). These results indicate that the Oligo-Mix treatment is effective in reducing the
305 amount of spore formation of powdery mildew, thus presumed to be effective in suppressing
306 the secondary spread of the disease.
307 *Colletotrichum orbiculare*, a cucurbit anthracnose fungus, is a hemibiotrophic pathogen that
308 infects cucumber, melon, watermelon, and other cucurbitaceous plants. As the experiment for
309 the powdery mildew, cucumber leaves treated with control solution or Oligo-Mix were
310 inoculated with spore suspension of *C. orbiculare* 24 h after the treatment. Yellow primary
311 lesions appeared on inoculated leaves about 4 days after inoculation and were obvious by 7
312 days. Image analysis to quantify the percentage of infected and yellowed areas (see details in
313 Material and methods section) indicated that Oligo-Mix treatment reduced the severity of the
314 anthracnose disease (Fig. 5). In control leaves, necrotic lesions and outgrowth of intracellular
315 biotrophic hyphae from necrotic area were observed (Fig. 6). Formation of acervuli and setae
316 (asexual fruiting body), and extensive growth of hyphae on leaf surface were also observed in
317 control leaves, indicating the successful hemibiotrophic infection of anthracnose pathogen. For

leaves treated with Oligo-Mix, in contrast, only immature acervuli and setae were found and necrotic lesions with no further extension of pathogen hyphae were observed (Fig. 6). These observations indicated that pre-treatment of Oligo-Mix can reduce the infection of anthracnose pathogen.

3.4 Expression profile of cucumber genes in response to Oligo-Mix treatment

Four-weeks old cucumber leaves were treated with the control solution or Oligo-Mix, and expression of cucumber genes at 24 h after treatment was investigated by RNA-seq analysis. The list of significantly up-regulated genes (based on the average TPM score) in cucumber leaf is shown as Table S1. Oligo-Mix treatment upregulated 123 genes (FC > 2, $p<0.05$, average TPM>1) and downregulated 46 genes (FC < 0.5, $p<0.05$, average TPM>1). The upregulated genes include some genes predicted to be involved in cell reinforcement and expansion (Fig. 7A, Table S1). Exordium-like 1 (CsaV3_3G047580) encodes extracellular protein EXO mediates leaf cell expansion (Schröder et al., 2009). Xyloglucan endotransglucosylases/hydrolases (CsaV3_5G031640 and CsaV3_6G038030) are known as enzymes for the modification of cell wall structure by cleaving and re-joining xyloglucan in primary cell walls (Eklöf et al., 2010). Arabinogalactans (CsaV3_7G003940, CsaV3_4G009610 and CsaV3_7G028250) are a class of glycoproteins anchored to the plasma membrane, presumed to be involved in cellulose synthesis and deposition during plant cell wall biogenesis (Lin et al., 2022). Expression of genes for stress responses were also upregulated such as ERF/AP2 family transcription factors (e.g. CsaV3_3G016760, CsaV3_3G018600 and CsaV3_5G005890, Imano et al., 2022) and chaperon HSP70 (CsaV3_5G026520) (Fig. 7B, Table S1). Oligo-Mix treatment enhanced genes for plant disease resistance, including Avr9/Cf-9 rapidly elicited protein-like (CsaV3_4G025220, Rowland et al., 2005), F-box family protein (CsaV3_6G048900), CCR4-associated factor 1 homolog (CsaV3_6G038490, Liang et al., 2009), PAR1 protein (CsaV3_3G017150, Herbers et al., 1995, Takemoto et al., 2003) and thaumatin-like pathogenesis-related protein 5 (PR-5, CsaV3_3G045940, de Jesús-Pires et al., 2020).

The activation of genes associated with stress tolerance and disease resistance often comes at a trade-off with the plant growth, such as photosynthesis (Karasov et al., 2017). We assessed the effect of Oligo-Mix treatment on highly expressed genes, however, Oligo-Mix treatment did not decrease the expression of genes involved in photosynthesis, but rather increased the expression of chlorophyll a-b binding protein, although this effect was not statistically significant (Table S2). These results indicate that Oligo-Mix treatment promotes the activation

352 of cell growth and disease stress tolerance while maintaining the expression levels of genes
353 required for photosynthesis.
354
355 *3.5 Effect of Oligo-Mix treatment on powdery mildew resistance in greenhouse*
356 The effect of Oligo-Mix treatment on disease incidence in cucumbers grown in greenhouses
357 was investigated. Two-week-old cucumber seedlings grown in a controlled growth chamber
358 were transferred to pots and grown in a greenhouse. Cucumber plants were sprayed with
359 control solution or Oligo-Mix once every week (6 times altogether) and the number of naturally
360 occurring colonies of powdery mildew on leaves was counted. Although no statistically
361 significant differences were found because some plants did not show natural disease symptoms
362 in both treatments, but the Oligo-Mix treatment tended to reduce the incidence of powdery
363 mildew on cucumbers grown in the greenhouse (Fig. 8), suggesting the potential effectiveness
364 of the practical use of Oligo-Mix in cucumber cultivation.
365
366 *3.6 Conclusion remarks*
367 The study suggests that treatment with Oligo-Mix, a mixture of the DAMPs COS and XOS
368 and the MAMP CHOS, promotes both growth and disease resistance in cucumbers. Against
369 the rational trade-off theory, why Oligo-Mix did not suppress the growth of plants while
370 improving disease resistance? First, Oligo-Mix promoted root growth, which is expected to
371 have the effect of allowing plants to grow basically robust and underpin the energy that is spent
372 in the immune response. Second, the RNA-seq data indicated that the induced genes included
373 some transcriptional regulators (Table S1). Increased amounts of various transcriptional
374 regulators are expected to allow plants to respond rapidly and robustly to the next
375 environmental stresses. Such effect is called priming, and the disease resistance induced by the
376 Oligo-Mix may be at the level of priming (Conrath et al., 2002). Induction of priming is
377 presumed to be less energetic loss than actual resistance to a pathogen that exhibits a resistance
378 effect (e.g., accumulation of antimicrobial substances). In fact, hypersensitive cell death was
379 not induced by Oligo-Mix treatment alone but was induced after inoculation with the powdery
380 mildew (Fig. 4).
381 A mixture of substances promoting growth and weakly activating disease resistance would
382 be ideal combination as agricultural materials. Biostimulant materials, however, require further
383 research and study to accumulate experiences for their effective use in agriculture, since
384 different crops (and different cultivars) often have variable sensitivities to such substances.
385

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Figure legends

Fig. 1. Oligo-Mix treatment can promote the growth of cucumber.

(A) Effect of Oligo-Mix treatment on seed germination of cucumber. Cucumber seeds were placed on laboratory tissue soaked with distilled water in Petri dishes, and germinated seeds were sprayed with control solution or Oligo-Mix (containing 20 µg/ml cello-oligosaccharide [COS], 40 µgl/m1 xylo-oligosaccharide [XOS], and 20 µg/ml chitin-oligosaccharide [CHOS]). The number of roots and root length (longest root) were measured 2 days after spray. **(B)** Effect of Oligo-Mix treatment on growth of cucumber. Cucumber plants (28 days old) were sprayed with control solution or Oligo-Mix every week (4 times), and fresh weight of above-ground tissue (left) and root (right) were measured 7 days after the last treatment. (middle) Chlorophyll contents in cucumber leaves were quantified according to the method described in Porra and Scheer (1989). Asterisks indicate a significant difference from control as assessed by a two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.

Fig. 2. Oligo-Mix treatment induces activation of MAP kinase and callose deposition of cucumber.

(A) Cucumber or Arabidopsis seedlings were treated with control solution (Cont.) or Oligo-Mix (containing 20 µg/ml cello-oligosaccharide, 40 µg/m1 xylo-oligosaccharide and 20 µg/ml chitin-oligosaccharide). The phosphorylation of MAP kinases was determined by western blot using phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr-204) antibody. **(B)** Cucumber seedlings

420 were treated with control solution (Cont.) or Oligo-Mix and callose deposition was visualized
421 by aniline blue staining. The fluorescence spots were counted 24 h after treatment under a
422 fluorescence microscope (n = 12). Bar = 200 μm.
423
424 **Fig. 3.** Oligo-Mix induces disease resistance against powdery mildew (*Podosphaera xanthii*)
425 on cucumber. Cucumber plants (4-weeks-old) were treated with control solution (Cont.) or
426 Oligo-Mix (containing 20 μg/ml cello-oligosaccharide, 40 μg/m1 xylo-oligosaccharide and 20
427 μg/ml chitin-oligosaccharide) and spray-inoculated with powdery mildew spore suspension
428 (1×10^5 spores/ml, 0.5 ml/plant) at 24 h after treatment. The number of powdery mildew colony
429 types (spored, yellowish , or cell death) per plant was counted one week after the inoculation
430 (n = 7). Asterisks indicate a significant difference from the control as assessed by a two-tailed
431 Student's *t*-test. * $p < 0.05$. n.s., not significant.
432
433 **Fig. 4.** Pretreatment of Oligo-Mix can promote the resistance of cucumber against powdery
434 mildew pathogen *Podosphaera xanthii*. Cucumber plants (4-weeks-old) were treated with
435 control solution (Cont.) or Oligo-Mix (containing 20 μg/ml cello-oligosaccharide, 40 μg/m1
436 xylo-oligosaccharide and 20 μg/ml chitin-oligosaccharide) and spray-inoculated with powdery
437 mildew spore suspension (1×10^5 spores/ml, 0.5 ml/plant) at 24 h after treatment. Inoculated
438 leaves were stained with lacto-phenol trypan blue 8 days after inoculation. **(A)** Extensive
439 hyphal growth and conidiophores formation were observed on cucumber leaves 8 days after
440 the inoculation, while induction of cell death was detected for the leaves pretreated with Oligo-
441 Mix. Bars = 200 μm. **(B)** Fully developed conidiophores (c) and haustoria (h) were observed
442 on the leaves of control plants. Local cell death (arrowheads) at the sites of
443 penetration/haustoria formation of *P. xanthii* was detected on cucumber leaves pretreated with
444 Oligo-Mix. Bars = 50 μm. **(C)** Number of conidia formed on leaves (with symptoms) was
445 counted 7 days after the inoculation (n = 16). Asterisks indicate a significant difference from
446 the control as assessed by a two-tailed Student's *t*-test. ** $p < 0.01$.
447
448 **Fig. 5.** Oligo-Mix induces disease resistance of cucumber against anthracnose disease by
449 *Colletotrichum orbiculare*. **(A)** Cucumber plants (4-weeks-old) were treated with control
450 solution (Cont.) or Oligo-Mix (containing 20 μg/ml cello-oligosaccharide, 40 μg/m1 xylo-
451 oligosaccharide and 20 μg/ml chitin-oligosaccharide) and spray-inoculated with spore
452 suspension of *C. orbiculare* (1×10^5 spores/ml, 0.5 ml/plant) at 24 h after treatment.
453 Photographed 7 days after inoculation. **(B)** The diseased area of leaves was detected using an

454 image analysis algorithm (See the method section) (n = 10). Asterisks indicate a significant
455 difference from the control as assessed by a two-tailed Student's *t*-test. * $p < 0.05$.
456
457 **Fig. 6.** Microscopic observation of anthracnose infection on cucumber with or without Oligo-
458 Mix treatment. Cucumber plants were treated with control solution (Cont.) or Oligo-Mix (20
459 µg/ml COS, 40 µg/ml XOS, 20 µg/ml CHOS) and conidial suspension of *Colletotrichum*
460 *orbiculare* ($1x10^5$ spores/ml, 0.5 ml/plant) were spray-inoculated. Inoculated leaves were
461 stained with lacto-phenol trypan blue 8 days after the inoculation. In control cucumber leaves
462 (left), intracellular biotrophic hyphae (white arrowheads) growing out from necrotic lesions
463 (black arrowheads) and developing acervulus (a) with multiple setae (s) were often observed.
464 In Oligo-Mix treated leaves, biotrophic hyphae were hardly detected and fewer and less-
465 developed acervulus were observed. Bars = 50 µm.
466
467 **Fig. 7.** Expression profiles of representative cucumber genes upregulated by treatment with
468 Oligo-Mix. Gene expression (TPM value) was determined by RNA-seq analysis of cucumber
469 leaves treated with control solution (Cont.) or Oligo-Mix (20 µg/ml COS, 40 µg/ml XOS, 20
470 µg/ml CHOS) for 24 h. **(A)** Genes related to cell wall reinforcement. **(B)** Genes related to the
471 responses to abiotic stresses. **(C)** Genes related to plant defense. Data marked with asterisks
472 are significantly upregulated from control as determined by two-tailed Student's *t*-test ** p
473 <0.01, * $p < 0.05$. See Table S1 for the list of upregulated genes by Oligo-Mix treatment.
474
475 **Fig. 8.** Effect of Oligo-Mix treatment on the appearance of powdery mildew on cucumber
476 grown in greenhouse. Cucmber plant was sprayed control solution (Cont.) or Oligo-Mix (20
477 µg/ml COS, 40 µg/ml XOS, 20 µg/ml CHOS) once every week (6 times altogether) and
478 naturally allowed to get infected by powdery mildew. The number of colonies per plant was
479 counted at 6 weeks after first treatment. Data are shown as mean ± SD (n = 14).
480
481 **References**

482 A. Ashida, M. Camagna, I. Sato, S. Chiba, A. Tanaka, D. Takemoto, Draft genome sequences
483 of *Neofusicoccum parvum* strains isolated from mango and rice-paper plant (*Tetrapanax*
484 *papyrifer*), Microbiol. Resour. Announc. 12 (2023) e0025923,
485 https://doi.org/10.1128/mra.00259-23.
486
487 A.S. Bulasag, M. Camagna, T. Kuroyanagi, A. Ashida, K. Ito, A. Tanaka, I. Sato, S. Chiba,
488 M. Ojika, D. Takemoto, *Botrytis cinerea* tolerates phytoalexins produced by Solanaceae and

Fabaceae plants through an efflux transporter BcatrB and metabolizing enzymes. Front. Plant Sci. 14 (2023) 1177060, https://doi.org/10.3389/fpls.2023.1177060.

J. Claverie, S. Balacey, C. Lemaître-Guillier, D. Brulé, A. Chiltz, L. Granet, E. Noirot, X. Daire, B. Darblade, M.C. Héloir, B. Poinssot, The cell wall-derived xyloglucan is a new DAMP triggering plant immunity in *Vitis vinifera* and *Arabidopsis thaliana*, Front. Plant Sci. 9 (2018) 1725, https://doi.org/10.3389/fpls.2018.01725.

U. Conrath, C.M. Pieterse, B. Mauch-Mani, Priming in plant-pathogen interactions, Trends Plant Sci. 7 (2002) 210–216, https://doi.org/10.1016/s1360-1385(02)02244-6.

D.J. Cosgrove, Growth of the plant cell wall, Nat. Rev. Mol. Cell Biol. 6 (2005) 850–861, https://doi.org/10.1038/nrm1746.

C. de Jesús-Pires, J.R.C. Ferreira-Neto, J. Pacifico Bezerra-Neto, E.A. Kido, R.L. de Oliveira Silva, V. Pandolfi, A.C. Wanderley-Nogueira, E. Binneck, A.F. da Costa, G. Pio-Ribeiro, G. Pereira-Andrade, I.M. Sittolin, F. Freire-Filho, A.M. Benko-Iseppon, Plant thaumatin-like proteins: Function, evolution and biotechnological applications, Curr. Protein Pept. Sci. 21 (2020) 36–51, https://doi.org/10.2174/1389203720666190318164905.

J.F. Dean, K.C. Gross, J.D. Anderson, Ethylene biosynthesis-inducing xylanase : III. Product characterization, Plant Physiol. 96 (1991) 571–576, https://doi.org/10.1104/pp.96.2.571.

L. Derevnina, Y.F. Dagdas, J.C. de la Concepcion, A. Bialas, R. Kellner, B. Petre, E. Domazakis, J. Du, C.H. Wu, X. Lin, C. Aguilera-Galvez, N. Cruz-Mireles, V.G. Vleeshouwers, S. Kamoun, Nine things to know about elicitins, New Phytol. 212 (2016) 888–895, https://doi.org/10.1111/nph.14137.

J.M. Eklöf, H. Brumer, The *XTH* gene family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling, Plant Physiol. 153 (2010) 456–466, https://doi.org/10.1104/pp.110.156844.

D. Ellinger, C.A. Voigt, Callose biosynthesis in Arabidopsis with a focus on pathogen response: what we have learned within the last decade, Ann. Bot. 114 (2014) 1349–1358, https://doi.org/10.1093/aob/mcu120.

G. Felix, M. Regenass, T. Boller, Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: Induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state, Plant J. 4 (1993) 307–316, https://doi.org/10.1046/j.1365-313X.1993.04020307.x.

J. Fliegmann, G. Felix, Immunity: Flagellin seen from all sides, Nat. Plants 2 (2016) 16136, https://doi.org/10.1038/nplants.2016.136.

L. Gómez-Gómez, G. Felix, T. Boller, A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*, Plant J. 18 (1999) 277–284, https://doi.org/10.1046/j.1365-313x.1999.00451.x.

538 N.A.R. Gow, M.D. Lenardon, Architecture of the dynamic fungal cell wall, Nat. Rev.
539 Microbiol. 21 (2023) 248–259, https://doi.org/10.1038/s41579-022-00796-9.
540
541 A.A. Gust, R. Biswas, H.D. Lenz, T. Rauhut, S. Ranf, B. Kemmerling, F. Götz, E. Glawischnig,
542 J. Lee, G. Felix, T. Nürnberger, Bacteria-derived peptidoglycans constitute pathogen-
543 associated molecular patterns triggering innate immunity in Arabidopsis, J. Biol. Chem. 282
544 (2007) 32338–32348, https://doi.org/10.1074/jbc.M704886200.
545
546 T. Hander, Á.D. Fernández-Fernández, R.P. Kumpf, P. Willems, H. Schatowitz, D. Rombaut,
547 A. Staes, J. Nolf, R. Pottie, P. Yao, A. Gonçalves, B. Pavie, T. Boller, K. Gevaert, F Van
548 Breusegem, S. Bartels, S. Stael, Damage on plants activates Ca_2^+-dependent metacaspases for
549 release of immunomodulatory peptides, Science 363 (2019) eaar7486,
550 https://doi.org/10.1126/science.aar7486.
551
552 K. Herbert, G. Mönke, R. Badur, U. Sonnewald, A simplified procedure for the subtractive
553 cDNA cloning of photoassimilate-responding genes: isolation of cDNAs encoding a new
554 class of pathogenesis-related proteins, Plant Mol. Biol. 29 (1995) 1027–1038,
555 https://doi.org/10.1007/BF00014975.
556
557 S. Imano, M. Fushimi, M. Camagna, A. Tsuyama-Koike, H. Mori, A. Ashida, A. Tanaka, I.
558 Sato, S. Chiba, K. Kawakita, M. Ojika, D. Takemoto, AP2/ERF transcription factor NbERF-
559 IX-33 is involved in the regulation of phytoalexin production for the resistance of *Nicotiana*
560 *benthamiana* to *Phytophthora infestans*, Front. Plant Sci. 12 (2022) 821574,
561 https://doi.org/10.3389/fpls.2021.821574.
562
563 T.L. Karasov, E. Chae, J.J. Herman, J. Bergelson, Mechanisms to mitigate the trade-off
564 between growth and defense, Plant Cell 29 (2017) 666–680,
565 https://doi.org/10.1105/tpc.16.00931.
566
567 S. Kamoun, M. Young, C.B. Glascock, B.M. Tyler, Extracellular protein elicitors from
568 *Phytophthora*: host-specificity and induction of resistance to bacterial and fungal
569 phytopathogens, Mol. Plant Microbe Interact. 6 (1993) 15–25, https://doi.org/10.1094/MPMI-
570 6-015.
571
572 H. Kato, K. Nemoto, M. Shimizu, A. Abe, S. Asai, N. Ishihama, S. Matsuoka, T. Daimon, M.
573 Ojika, K. Kawakita, K. Onai, K. Shirasu, M. Yoshida, M. Ishiura, D. Takemoto, Y. Takano, R.
574 Terauchi, Recognition of pathogen-derived sphingolipids in Arabidopsis, Science 376 (2022)
575 857–860, https://doi.org/10.1126/science.abn0650.
576
577 D. Kim, J.M. Paggi, C. Park, C. Bennett, S.L. Salzberg, Graph-based genome alignment and
578 genotyping with HISAT2 and HISAT-genotype, Nat. Biotechnol. 37 (2019) 907–915,
579 https://doi.org/10.1038/s41587-019-0201-4.
580
581 S. Kovaka, A.V. Zimin, G.M. Pertea, R. Razaghi, S.L. Salzberg, M. Pertea, Transcriptome
582 assembly from long-read RNA-seq alignments with StringTie2, Genome Biol. 20 (2019) 278,
583 https://doi.org/10.1186/s13059-019-1910-1.
584
585 T. Kuroyanagi, A.S. Bulasag, K. Fukushima, A. Ashida, T. Suzuki, A. Tanaka, M. Camagna,
586 I. Sato, S. Chiba, M. Ojika, D. Takemoto, *Botrytis cinerea* identifies host plants via the

587 recognition of antifungal capsidiol to induce expression of a specific detoxification gene,
588 PNAS Nexus 1 (2022) pgac274, https://doi.org/10.1093/pnasnexus/pgac274.
589
590 A. Lebeda, E. Křístková, B. Sedláková, M.D. Coffey, J.D. McCreight, Gaps and perspectives
591 of pathotype and race determination in *Golovinomyces cichoracearum* and *Podosphaera*
592 *xanthii*, Mycoscience 52 (2011) 159–164, https://doi.org/10.1007/S10267-010-0098-8.
593
594 W. Liang, C. Li, F. Liu, H. Jiang, S. Li, J. Sun, X. Wu, C. Li, The Arabidopsis homologs of
595 CCR4-associated factor 1 show mRNA deadenylation activity and play a role in plant defence
596 responses, Cell Res. 19 (2009) 307–316, https://doi.org/10.1038/cr.2008.317.
597
598 S. Lin, Y. Miao, H. Huang, Y. Zhang, L. Huang, J. Cao, Arabinogalactan proteins: Focus on
599 the role in cellulose synthesis and deposition during plant cell wall biogenesis, Int. J. Mol. Sci.
600 23 (2022) 6578, https://doi.org/10.3390/ijms23126578.
601
602 M.I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-
603 seq data with DESeq2, Genome Biol. 15 (2014) 550, https://doi.org/10.1186/s13059-014-
604 0550-8.
605
606 M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads,
607 EMBnet J. 17 (2011) 10–12, https://doi.org/10.14806/ej.17.1.200.
608
609 H. Matsuo, Y. Ishiga, Y. Kubo, Y. Yoshioka, *Colletotrichum orbiculare* strains distributed in
610 Japan: race identification and evaluation of virulence to cucurbits, Breed Sci. 72 (2022) 306–
611 315, https://doi.org/10.1270/jsbbs.22011.
612
613 G. Pearce, D. Strydom, S. Johnson, C.A. Ryan, A polypeptide from tomato leaves induces
614 wound-inducible proteinase inhibitor proteins, Science 253 (1991) 895–897,
615 https://doi.org/10.1126/science.253.5022.895.
616
617 R.J. Porra, H. Scheer, Towards a more accurate future for chlorophyll a and b determinations:
618 the inaccuracies of Daniel Arnon's assay, Photosynth Res. 140 (1989) 215–219,
619 https://doi.org/10.1007/s11120-018-0579-8.
620
621 S. Pring, H. Kato, S. Imano, M. Camagna, A. Tanaka, H. Kimoto, P. Chen, A. Shrotri, H.
622 Kobayashi, A. Fukuoka, M. Saito, T. Suzuki, R. Terauchi, I. Sato, S. Chiba, D. Takemoto,
623 Induction of plant disease resistance by mixed oligosaccharide elicitors prepared from plant
624 cell wall and crustacean shells, Physiol. Plant. 175 (2023) e14052,
625 https://doi.org/10.1111/ppl.14052.
626
627 M. Ron, A. Avni, The receptor for the fungal elicitor ethylene-inducing xylanase is a member
628 of a resistance-like gene family in tomato, Plant Cell 16 (2004) 1604–1615,
629 https://doi.org/10.1105/tpc.022475.
630
631 O. Rowland, A.A. Ludwig, C.J. Merrick, F. Baillieul, F.E. Tracy, W.E. Durrant, L. Fritz-Laylin,
632 V. Nekrasov, K. Sjölander, H. Yoshioka, J.D.G. Jones, Functional analysis of Avr9/Cf-9
633 rapidly elicited genes identifies a protein kinase, ACIK1, that is essential for full Cf-9-
634 dependent disease resistance in tomato, Plant Cell 17 (2005) 295–310,
635 https://doi.org/10.1105/tpc.104.026013.
636

C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9 (2012) 671–675, https://doi.org/10.1038/nmeth.2089.

F. Schröder, J. Lisso, P. Lange, C. Müssig, The extracellular EXO protein mediates cell expansion in Arabidopsis leaves, BMC Plant Biol. 9 (2009) 20, https://doi.org/10.1186/1471-2229-9-20.

J.K. Sharp, B. Valent, P. Albersheim, Purification and partial characterization of a beta-glucan fragment that elicits phytoalexin accumulation in soybean, J. Biol. Chem. 259 (1984) 11312–11320, https://doi.org/10.1016/S0021-9258(18)90864-1.

Y. Shibata, M. Ojika, A. Sugiyama, K Yazaki, D.A. Jones, K. Kawakita, D. Takemoto, The full-size ABCG transporters Nb-ABCG1 and Nb-ABCG2 function in pre- and postinvasion defense against *Phytophthora infestans* in *Nicotiana benthamiana*, Plant Cell 28 (2016) 1163–1181, https://doi.org/10.1105/tpc.15.00721.

C.A. Souza, S. Li, A.Z. Lin, F. Boutrot, G. Grossmann, C. Zipfel, S.C. Somerville, Cellulose-derived oligomers act as damage-associated molecular patterns and trigger defense-like responses, Plant Physiol. 173 (2017) 2383–2398, https://doi.org/10.1104/pp.16.01680.

C. Stringer, T. Wang, M. Michaelos, M. Pachitariu, Cellpose: a generalist algorithm for cellular segmentation, Nat. Methods 18(2021) 100–106, https://doi.org/10.1038/s41592-020-01018-x.

D. Takemoto, A.R. Hardham, D.A. Jones, Differences in cell death induction by *Phytophthora* Elicitins are determined by signal components downstream of MAP kinase kinase in different species of *Nicotiana* and cultivars of *Brassica rapa* and *Raphanus sativus*, Plant Physiol. 138 (2005) 1491–1504, https://doi.org/10.1104/pp.104.058388.

D. Takemoto, D.A. Jones, A.R. Hardham, GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of Arabidopsis by oomycete pathogens, Plant J. 33 (2003) 775–792, https://doi.org/10.1046/j.1365-313x.2003.01673.x.

D. Takemoto, H. Yoshioka, N. Doke, K. Kawakita, Disease stress-inducible genes of tobacco: expression profile of elicitor-responsive genes isolated by subtractive hybridization, Physiol. Plant. 118 (2003) 545–553, https://doi.org/10.1034/j.1399-3054.2003.00145.x.

A. Vielba-Fernández, Á. Polonio, L. Ruiz-Jiménez, A. de Vicente, A. Pérez-García, D. Fernández-Ortuño, Fungicide resistance in powdery mildew fungi, Microorganisms 8 (2020) 1431, https://doi.org/10.3390/microorganisms8091431.

L. Wang, E. Einig, M. Almeida-Trapp, M. Albert, J. Fliegmann, A. Mithöfer, H. Kalbacher, G. Felix, The systemin receptor SYR1 enhances resistance of tomato against herbivorous insects, Nat. Plants 4 (2018) 152–156, https://doi.org/10.1038/s41477-018-0106-0.

Y. Yamaguchi, A. Huffaker, A.C. Bryan, F.E. Tax, C.A. Ryan, PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis, Plant Cell 22 (2010) 508–522, https://doi.org/10.1105/tpc.109.068874.

Y. Yamauchi, A. Matsuda, N. Matsuura, M. Mizutani, Y. Sugimoto, Transcriptome analysis of *Arabidopsis thaliana* treated with green leaf volatiles: possible role of green leaf volatiles

as self-made damage-associated molecular patterns, J. Pestic. Sci. 43 (2018) 207–213, https://doi.org/10.1584/jpestics.D18-020.

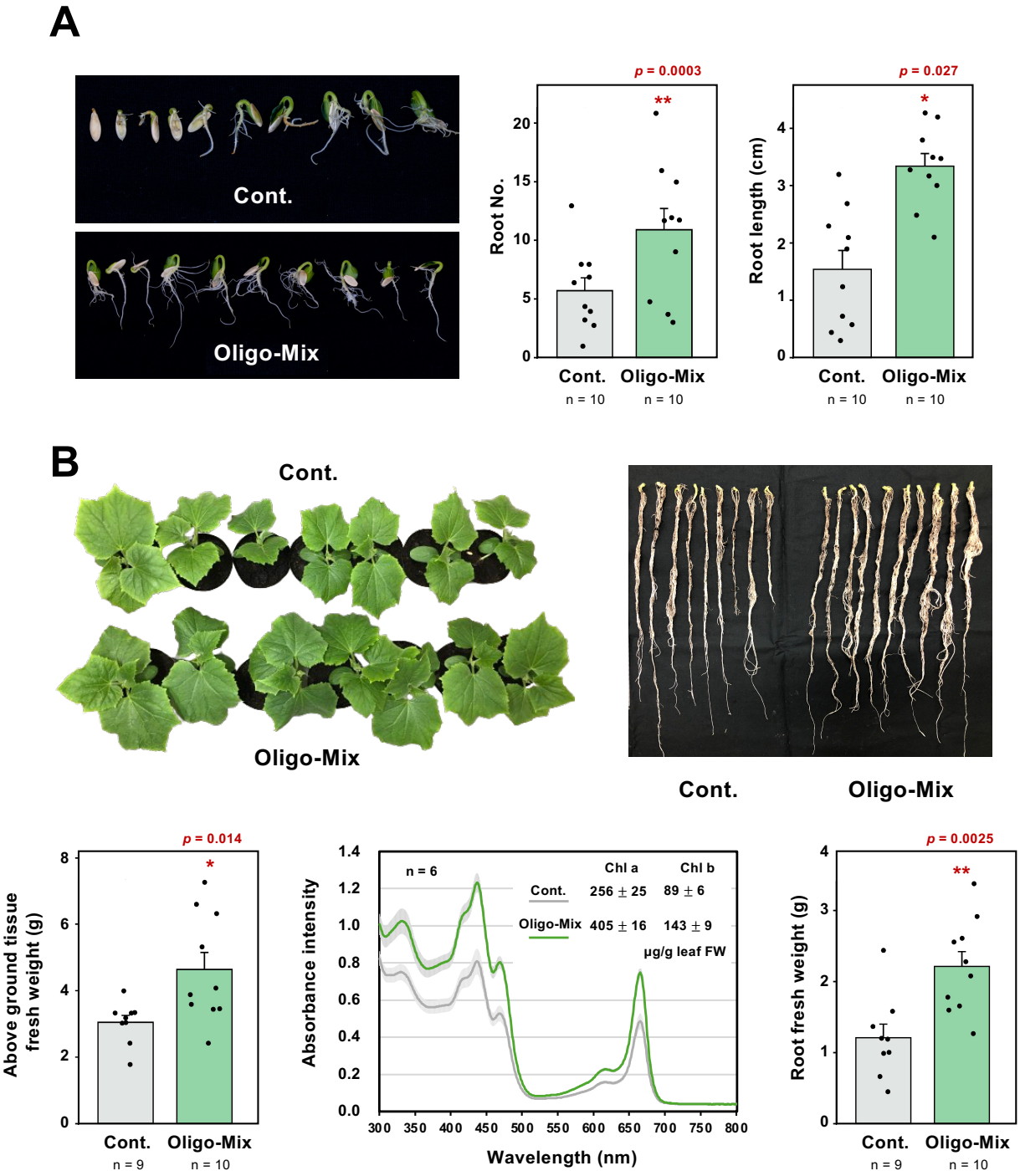

Fig. 1. Oligo-Mix treatment can promote the growth of cucumber.
(A) Effect of Oligo-Mix treatment on seed germination of cucumber. Cucumber seeds were placed on laboratory tissue soaked with distilled water in Petri dishes, and germinated seeds were sprayed with control solution or Oligo-Mix (containing 20 µg/ml cello-oligosaccharide [COS], 40 µg/m1 xylo-oligosaccharide [XOS], and 20 µg/ml chitin-oligosaccharide [CHOS]). The number of roots and root length (longest root) were measured 2 days after spray. **(B)** Effect of Oligo-Mix treatment on growth of cucumber. Cucumber plants (28 days old) were sprayed with control solution or Oligo-Mix every week (4 times), and fresh weight of above-ground tissue (left) and root (right) were measured 7 days after the last treatment. (middle) Chlorophyll contents in cucumber leaves were quantified according to the method described in Porra and Scheer (1989). Asterisks indicate a significant difference from control as assessed by a two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.

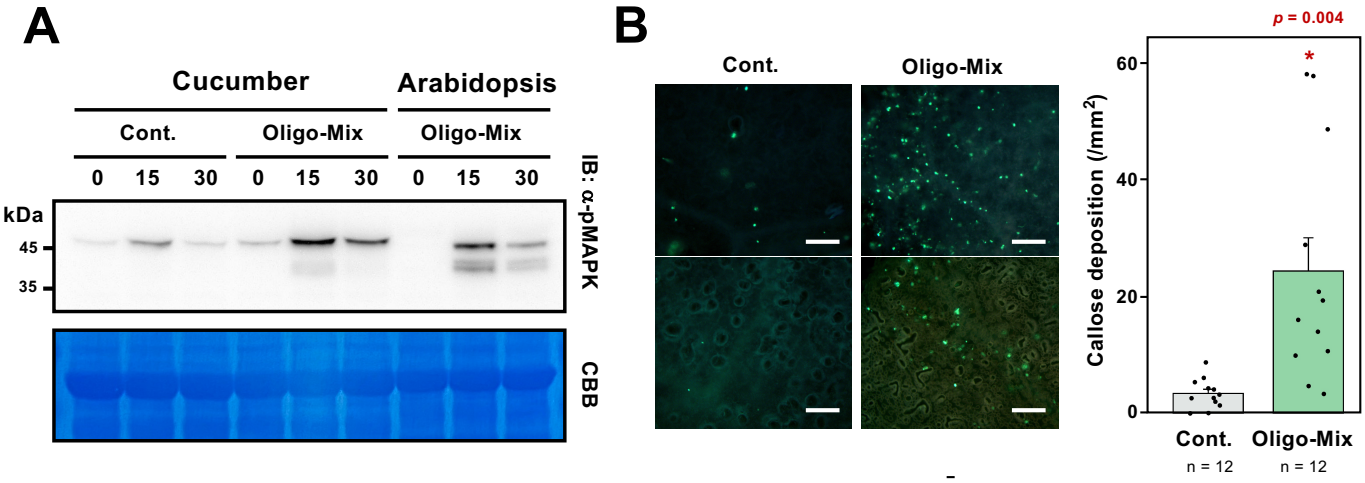

Fig. 2. Oligo-Mix treatment induces activation of MAP kinase and callose deposition of cucumber.

(A) Cucumber or Arabidopsis seedlings were treated with control solution (Cont.) or Oligo-Mix (containing 20 µg/ml cello-oligosaccharide, 40 µg/m1 xylo-oligosaccharide and 20 µg/ml chitin-oligosaccharide). The phosphorylation of MAP kinases was determined by western blot using phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr-204) antibody. **(B)** Cucumber seedlings were treated with control solution (Cont.) or Oligo-Mix and callose deposition was visualized by aniline blue staining. The fluorescence spots were counted 24 h after treatment under a fluorescence microscope (n = 12). Bar = 200 µm.

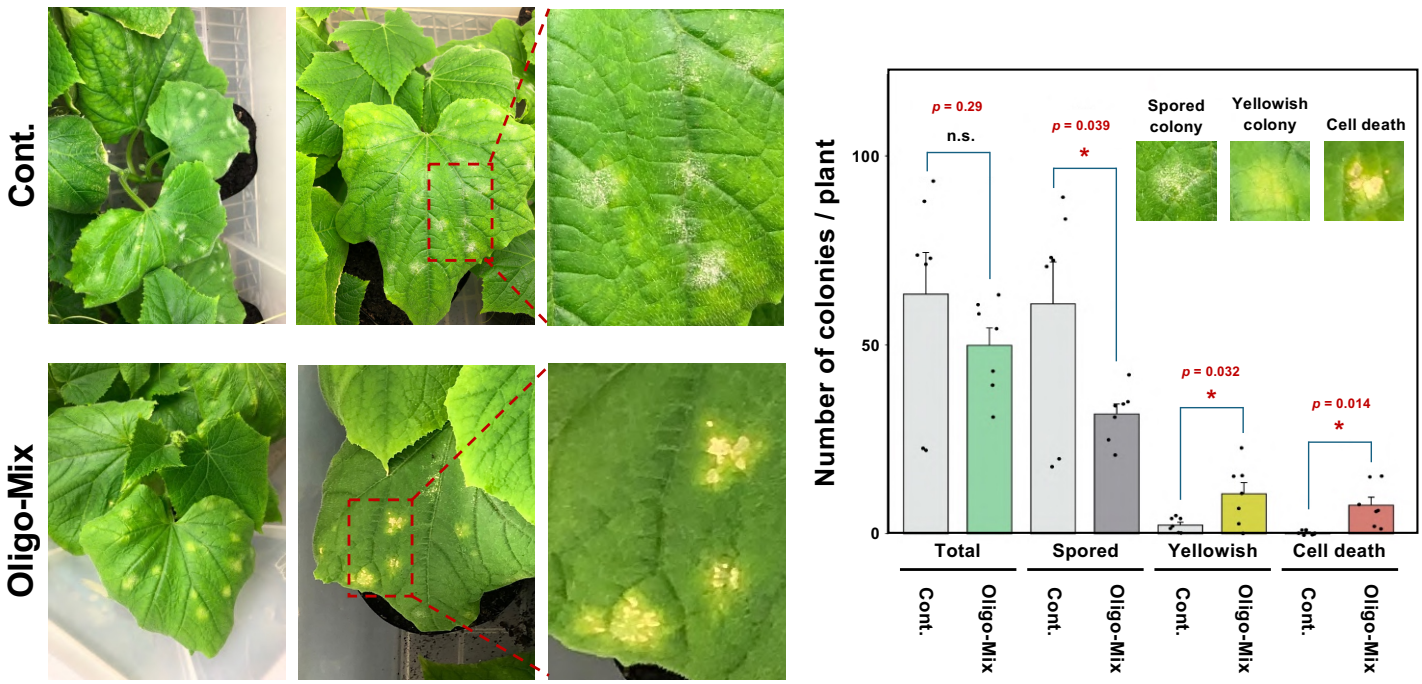

Fig. 3. Oligo-Mix induces disease resistance against powdery mildew (*Podosphaera xanthii*) on cucumber. Cucumber plants (4-weeks-old) were treated with control solution (Cont.) or Oligo-Mix (containing 20 µg/ml cello-oligosaccharide, 40 µg/m1 xylo-oligosaccharide and 20 µg/ml chitin-oligosaccharide) and spray-inoculated with powdery mildew spore suspension (1×10^5 spores/ml, 0.5 ml/plant) at 24 h after treatment. The number of powdery mildew colony types (spored, yellowish , or cell death) per plant was counted one week after the inoculation (n = 7). Asterisks indicate a significant difference from the control as assessed by a two-tailed Student's *t*-test. * $p < 0.05$. n.s., not significant.

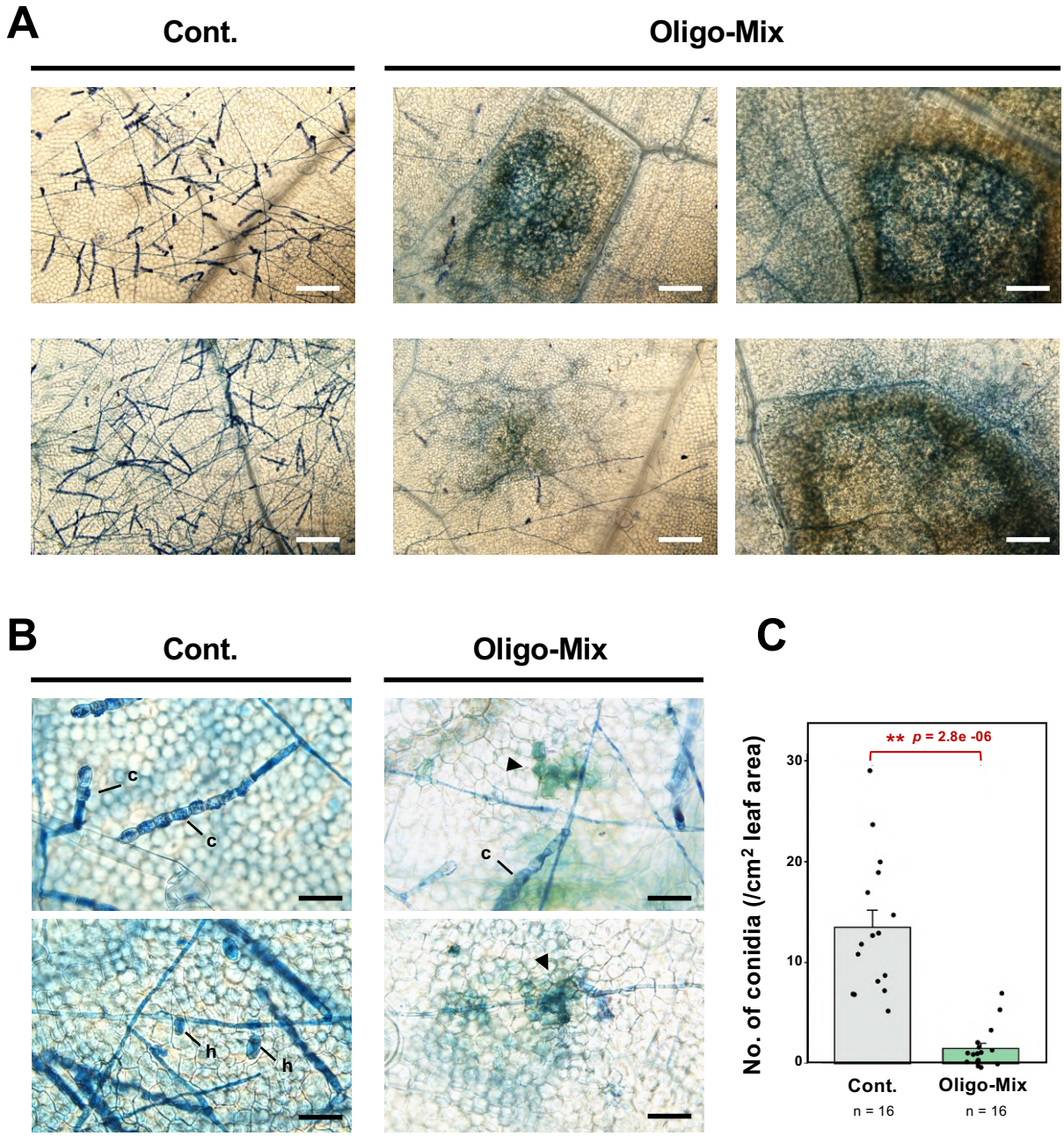

Fig. 4. Pretreatment of Oligo-Mix can promote the resistance of cucumber against powdery mildew pathogen *Podosphaera xanthii*. Cucumber plants (4-weeks-old) were treated with control solution (Cont.) or Oligo-Mix (containing 20 µg/ml cello-oligosaccharide, 40 µg/m1 xylo-oligosaccharide and 20 µg/ml chitin-oligosaccharide) and spray-inoculated with powdery mildew spore suspension (1×10^5 spores/ml, 0.5 ml/plant) at 24 h after treatment. Inoculated leaves were stained with lacto-phenol trypan blue 8 days after inoculation. **(A)** Extensive hyphal growth and conidiophores formation were observed on cucumber leaves 8 days after the inoculation, while induction of cell death was detected for the leaves pretreated with Oligo-Mix. Bars = 200 µm. **(B)** Fully developed conidiophores (c) and haustoria (h) were observed on the leaves of control plants. Local cell death (arrowheads) at the sites of penetration/haustoria formation of *P. xanthii* was detected on cucumber leaves pretreated with Oligo-Mix. Bars = 50 µm. **(C)** Number of conidia formed on leaves (with symptoms) was counted 7 days after the inoculation (n = 16). Asterisks indicate a significant difference from the control as assessed by a two-tailed Student's *t*-test. ** $p < 0.01$.

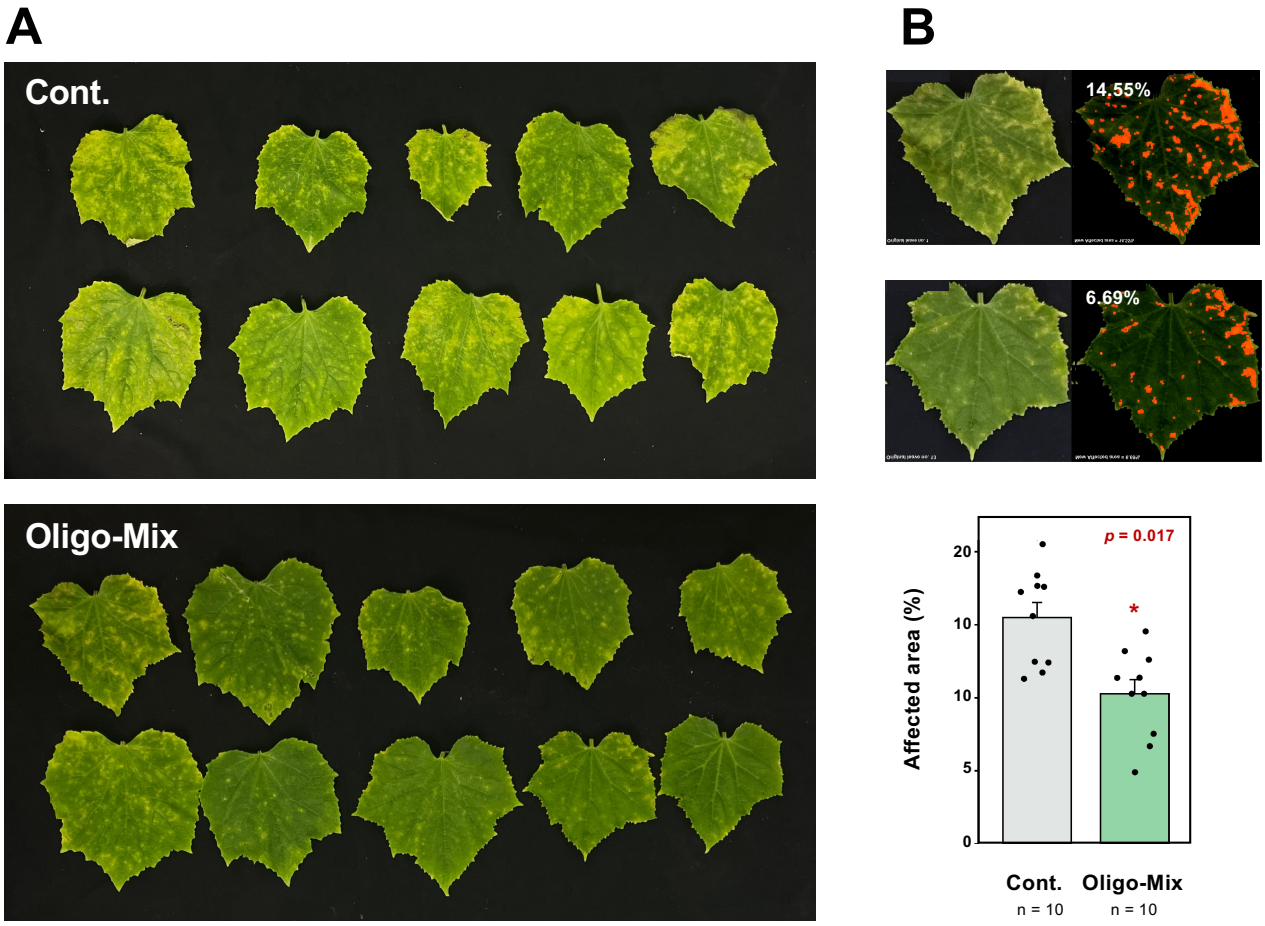

Fig. 5. Oligo-Mix induces disease resistance of cucumber against anthracnose disease by *Colletotrichum orbiculare*. **(A)** Cucumber plants (4-weeks-old) were treated with control solution (Cont.) or Oligo-Mix (containing 20 µg/ml cello-oligosaccharide, 40 µg/m1 xylo-oligosaccharide and 20 µg/ml chitin-oligosaccharide) and spray-inoculated with spore suspension of *C. orbiculare* (1×10^5 spores/ml, 0.5 ml/plant) at 24 h after treatment. Photographed 7 days after inoculation. **(B)** The diseased area of leaves was detected using an image analysis algorithm (See the method section) (n = 10). Asterisks indicate a significant difference from the control as assessed by a two-tailed Student's *t*-test. * $p < 0.05$.

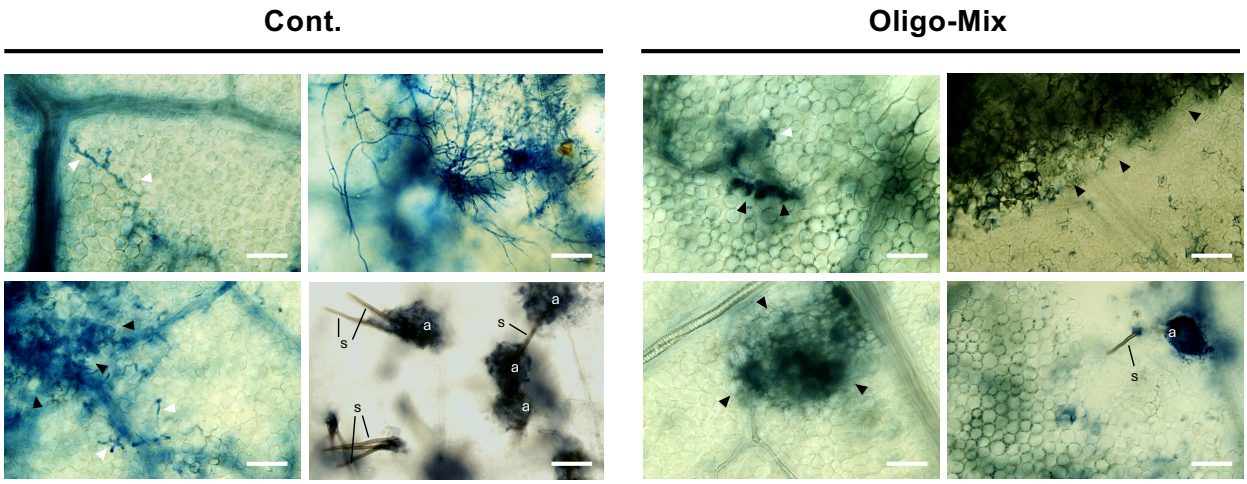

Fig. 6. Microscopic observation of anthracnose infection on cucumber with or without Oligo-Mix treatment. Cucumber plants were treated with control solution (Cont.) or Oligo-Mix (20 µg/ml COS, 40 µg/ml XOS, 20 µg/ml CHOS) and conidial suspension of *Colletotrichum orbiculare* ($1x10^5$ spores/ml, 0.5 ml/plant) were spray-inoculated. Inoculated leaves were stained with lacto-phenol trypan blue 8 days after the inoculation. In control cucumber leaves (left), intracellular biotrophic hyphae (white arrowheads) growing out from necrotic lesions (black arrowheads) and developing acervulus (a) with multiple setae (s) were often observed. In Oligo-Mix treated leaves, biotrophic hyphae were hardly detected and fewer and less-developed acervulus were observed. Bars = 50 µm.

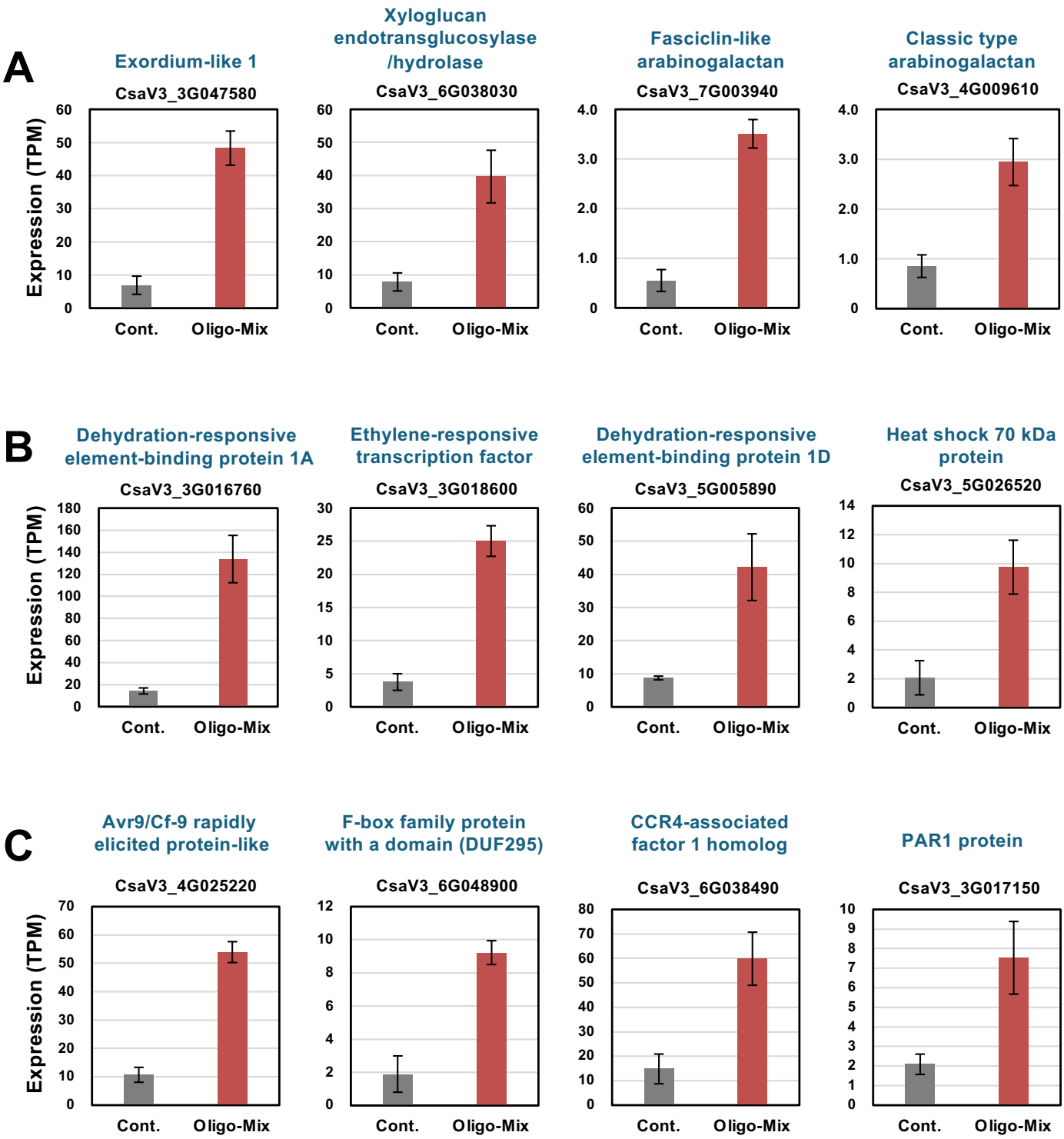

Fig. 7. Expression profiles of representative cucumber genes upregulated by treatment with Oligo-Mix. Gene expression (TPM value) was determined by RNA-seq analysis of cucumber leaves treated with control solution (Cont.) or Oligo-Mix (20 µg/ml COS, 40 µg/ml XOS, 20 µg/ml CHOS) for 24 h. **(A)** Genes related to cell wall reinforcement. **(B)** Genes related to the responses to abiotic stresses. **(C)** Genes related to plant defense. Data marked with asterisks are significantly upregulated from control as determined by two-tailed Student's *t*-test ** $p < 0.01$, * $p < 0.05$. See Table S1 for the list of upregulated genes by Oligo-Mix treatment.

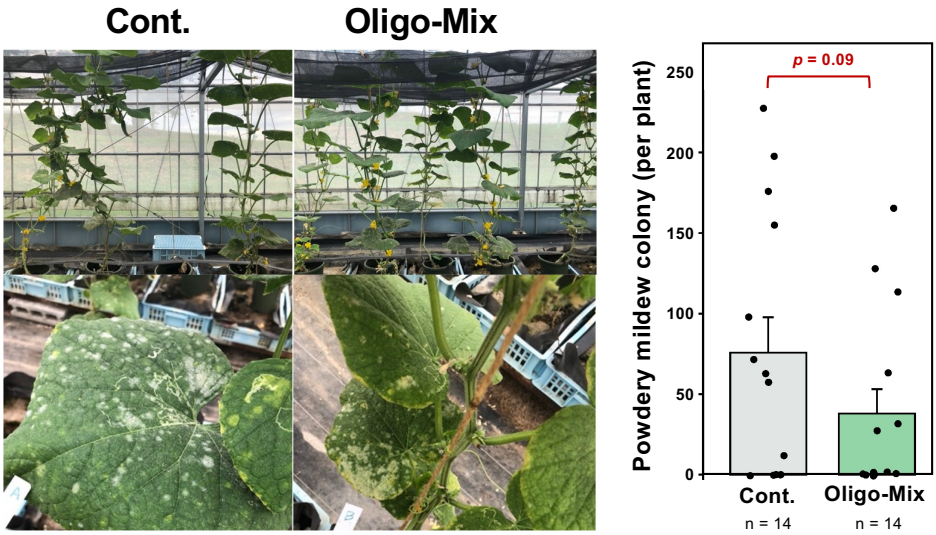

Fig. 8. Effect of Oligo-Mix treatment on the appearance of powdery mildew on cucumber grown in greenhouse. Cucmber plant was sprayed control solution (Cont.) or Oligo-Mix (20 μg/ml COS, 40 μg/ml XOS, 20 μg/ml CHOS) once every week (6 times altogether) and naturally allowed to get infected by powdery mildew. The number of colonies per plant was counted at 6 weeks after first treatment. Data are shown as mean ± SD (n = 14).