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Alginate oligosaccharides trigger multiple defense responses in tobacco and induce resistance to *Phytophthora infestans*

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Alginate oligosaccharides (AOSs), important plant immunity inducers, are widely used in agriculture because of their important role in the biological control of crop diseases. However, the mechanism by which AOSs induce plant resistance to pathogens is not clear. Here, we report AOS with a degree of polymerization of 2–5, which was obtained by a newly reported enzyme Aly2. AOS treatment exhibited high activity in enhancing resistance to *Phytophthora infestans* (*P. infestans*). AOS significantly induced reactive oxygen species (ROS) accumulation, calcium influx, stomata closure, and callose deposition. The salicylic acid (SA) synthesis-related gene and the defense-related genes were upregulated after AOS treatment. A transcriptome file generated from AOS-treated seedlings verified the SA pathway and suggested the presence of chitin elicitor receptor kinase (CERK). The subsequent results showed that AtCERK1 binds AOS tightly, suggesting that AtCERK1 is responsible for AOS recognition. This study laid a theoretical foundation for the broad application of AOS.

KEYWORDS

alginate oligosaccharides, immune inducer, defense response, salicylic acid, chitin elicitor receptor kinase

Introduction

During growth, plants are affected by many kinds of stresses, which can alter many plant processes. Plant diseases cause immense annual losses in crop yield, posing a great threat to food production. Approximately 3 billion kg of chemical reagents are used worldwide annually (Hernández et al., 2013), but approximately 1% of pesticides are effectively used on plants (Bernardes et al., 2015). The extensive use of agrochemicals has caused pernicious

pesticide residues and environmental and soil pollution (Qin et al., 2014; Rodriguez-Salus et al., 2016; Xue et al., 2006), decreased crop quality, and threatened human health (Al-Wabel et al., 2016; Duan et al., 2008; Lozowicka et al., 2015; Yadav et al., 2015). Therefore, new strategies are urgently required to improve plants' immunity and enhance their resistance to pathogens.

Plant immunity inducers are biological agents that can reduce the use of chemical pesticides while improving resistance to pathogens (Li et al., 2018a; Nisa et al., 2015). In addition to inducing the deposition of lignin, strengthening plant cell walls, and forming a physical barrier, such as callose that are deposited around the cell wall and plasmodesmata (Ton et al., 2009), to resist pathogen infection, plant immunity inducers also promote the production of endogenous substances such as pathogenesis-related proteins (PRs), reactive oxygen species (ROS), and salicylic acid (SA) (Cai et al., 2020; Jiang et al., 2023; Peng et al., 2020; Sticher et al., 1997). The non-expressor of PR (NPR) is a regulatory protein, and NPR1 plays a key role in this process (Durrant and Dong, 2004; Sun et al., 2018). PR-1A and NPR are the most abundant proteins produced by plants in response to pathogens via the SA pathway (Breen et al., 2017). When plant cells detect the presence of pathogens through recognition receptors on their surfaces, the phytohormone, such as the SA signaling pathway is activated, leading to the upregulation of the expression of the defense genes *PR-1a* and *NPR1*. This, in turn, induces resistance to pathogens.

Protein kinases on the cell surface are critical for transiting signals from the outside to the inside of the cell. From extracellular stimulation to the corresponding biological effect in cells, mitogen-activated protein kinase (MAPK) cascades must be activated. The MAPK pathway also affects plant resistance to pathogens by regulating stomatal closure. Oligosaccharides, such as mannan oligosaccharides (MOSs) and oligogalacturonic acid (OGA), also cause Ca^{2+} concentration changes and stomatal closure. The transient increase in Ca^{2+} and the MAPK cascade are important signaling pathways that stimulate the early defense response in plants (Lecourieux et al., 2006). NtMEK2 in the MAPK cascade pathway can be activated by multiple activators in tobacco and regulate phenylalanine lyase (PAL) expression, a key enzyme in SA synthesis (Yang et al., 2001). In addition, SA is an important hormone-signaling molecule that leads to plant systemic-acquired resistance.

Oligosaccharides are important plant immunity inducers that can be developed as a biostimulant. As a biological immunity inducer, AOS can be prepared through the enzymatic degradation of alginate and possesses advantages, such as low molecular weight, good water solubility, easy absorption, and non-polluting. AOS application in agriculture has become a popular research topic

(Peng et al., 2018). Increasing evidence confirms the function of AOS in enhancing plant stress resistance, growth, and development (Hien et al., 2000; Hu et al., 2004; Iwasaki and Matsubara, 2000; Ma et al., 2010; Natsume et al., 1994; Tang et al., 2011). Studies have shown that AOS can promote root development and elongation in Komatsu (Yonemoto et al., 1993), barley (Tomoda et al., 1994), rice (Zhang et al., 2014), and carrot (Xing et al., 2020; Xu et al., 2003); reduce the damage caused by salt stress (Liu et al., 2013; Tang et al., 2011); enhance the tolerance of cucumber to water stress (Li et al., 2018b); enhance drought resistance in wheat through the SA pathway (Liu et al., 2013); and enhance the resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 through the salicylic acid pathway (Zhang et al., 2019). However, relatively few studies have investigated the mechanism by which AOS induces plant resistance to pathogens, especially late blight of potato and tomato.

Oligosaccharides mimic the cell wall components of pathogens and are recognized by immune receptors/pattern recognition receptors on the plant cell surface to trigger pattern-triggered immunity (PTI), thereby enhancing plant resistance to disease (Jones and Dangl, 2006; Van Wees et al., 2008). Thus, lipopolysaccharide, chitin, OGA, and MOS (Zhang et al., 2019) can be recognized by plant receptors to stimulate PTI (Denoux et al., 2008; Hayafune et al., 2014; Yin et al., 2016). These elicitors are also known as pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) (Wiesel et al., 2014). Progress has been made in the studies of oligosaccharide receptors, such as chitin receptors (Espinoza et al., 2017; Liu et al., 2012) and OGA (Brutus et al., 2010). However, the receptor for AOS in plants has not been identified.

This study showed that AOS improved potato and tobacco resistance to *P. infestans*, and induced a series of defense responses in tobacco, including ROS accumulation, callose deposition, Ca^{2+} influx, and stomatal closure. Moreover, transcriptome sequencing was performed, and SA signal pathway-related genes were also detected. The receptor mutant *Arabidopsis thaliana* and enzyme-linked immunosorbent assays (ELISAs) were used to analyze the interaction between AOS, and the receptor was also analyzed. AOS can interact tightly with the cell surface receptor AtCERK1, while the binding ability was not significant between AOS and chitin elicitor binding protein (AtCEBiP), suggesting that AtCERK1 is the receptor of AOS in *Arabidopsis*. The results lay the foundation for the wide application of AOS as a new biopesticide.

Materials and methods

Materials and growth conditions

Sodium alginate was degraded by Aly2 for 12 h, and then AOS with a degree of polymerization (DP) of 2–5 was obtained (Peng et al., 2018) by using a Superdex 30 Increase 10/300 GL column, the mobile phase was 0.20 M NH_4HCO_3 at a flow rate of 0.4 mL/min, and the eluted fractions were monitored at 232 nm using a UV detector. In this study, wild-type *Nicotiana benthamiana* and wild-type *A. thaliana* were preserved and propagated. The plants *Solanum*

Abbreviations: AOSs, alginate oligosaccharides; DAB, 3,3-diaminobenzidine; H_2O_2 , hydrogen peroxide; NBT, Nitroblue tetrazolium; NPR, non-expressor of pathogenesis-related; PAL, phenylalanine ammonia lyase; PR, pathogenesis-related protein; ROS, reactive oxygen species; SA, salicylic acid; *P. infestans*, *Phytophthora infestans*; CERK1, chitin elicitor receptor kinase 1; CEBiP, chitin elicitor binding protein.

tuberosum and *N. benthamiana* were grown at 22°C and 25°C, respectively, under 70% humidity with 16 h of light and 8 h of dark. *Arabidopsis thaliana* receptor mutants were cultured in an incubator (22°C, 16 h light/8 h dark). RNA extraction kits were purchased from Kangwei Reagent (Taizhou, China), reverse transcription kits were purchased from Hunan Aceri Bioengineering Co. (Changsha, China) and Novozymes (Beijing, China), and HRP-conjugated His₆ was purchased from Sigma Aldrich (St. Louis, MO, USA). Biotin hydrazide, 2-(N-morpholino)-ethanesulfonic acid (MES), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). *Escherichia coli* strains DH5 α and BL21 (DE3) and *Pichia pastoris* were preserved in our laboratory. *Phytophthora infestans* strain was grown in the dark at 18°C using rye A agar. The T-DNA insertion mutants SALK_007193C for At3g21630 (*AtCERK1*) and SALK_206271C for

At2g17120 (*AtCEBiP-LIKE1*) were obtained from EDITGENE Corporation (Guangzhou, China). All the other chemicals and reagents were of the highest quality. Primers were synthesized by Shanghai Shenggong Biotechnology Co. (Shanghai, China; Table 1).

Phytophthora infestans inoculation

Plates with *P. infestans* were flooded with 5 mL of ddH₂O and scraped to release sporangia. The suspension was poured into a clean Petri dish, placed on ice, and stored at 4°C for 3 h to release zoospores. Then, the sporangia were counted and adjusted to 30,000 sporangia per milliliter. The potato leaves were inoculated with *P. infestans* (originally isolated from the province of Heilongjiang, north of China) at a concentration of 4×10^5 sporangia mL⁻¹. Droplets of 20 μ L of *P. infestans* zoospores and sporangia

TABLE 1 Primers used for sequencing in the present study.

Sequencing primers	Primer sequence (5'–3')
<i>Nb-actin-F</i>	5'-TTGGCTTACATTGCTCTTG-3'
<i>Nb-actin-R</i>	5'-TCATTGATGGTTGGAACAG-3'
<i>P. infestans-O8-F</i>	5'-GAAAGGCATAGAAGGTAGA-3'
<i>P. infestans-O8-R</i>	5'-TAACCGACCAAGTAGTAAA-3'
qRT- <i>NbSOD-F</i>	5'-GCAGCAGTGAAGGTGTTAGC-3'
qRT- <i>NbSOD-R</i>	5'-GGATTGTAATGTGGTCCCG-3'
qRT- <i>NbCAT-F</i>	5'-CACTCACCTTACCTGTGCTG-3'
qRT- <i>NbCAT-R</i>	5'-GAACTTCATTCCATCACGG-3'
qRT- <i>NbAPX-F</i>	5'-CATCAGGCTATTGGAACCC-3'
qRT- <i>NbAPX-R</i>	5'-GCTCTGTCTTGTCTCTCTACC-3'
qRT- <i>NbRbohA-F</i>	5'-GAAGGCGGAGTTAAGGAGAT-3'
qRT- <i>NbRbohA-R</i>	5'-GAGCTCTATGAGCGCTGGAA-3'
qRT- <i>NbRbohB-F</i>	5'-GTGATGCTCGTTCGTCTT-3'
qRT- <i>NbRbohB-R</i>	5'-CTTTAGCCTCAGGGTGGTTG-3'
qRT- <i>NbICS-F</i>	5'-CAGTTGAAGAGCAGATAGAAG-3'
qRT- <i>NbICS-R</i>	5'-AAGTTCATTGAAGCACATT-3'
qRT- <i>NbPAL-F</i>	5'-CTCAAGTTGCGGCTATTG-3'
qRT- <i>NbPAL-R</i>	5'-CATTCTTGGTCTCTATGTG-3'
qRT- <i>NbPRIa-F</i>	5'-CGTTGAGATGTGGTCAATG-3'
qRT- <i>NbPRIa-R</i>	5'-CCTAGCACATCCAACACGAA-3'
qRT- <i>NbNPR1-F</i>	5'-GCACTTGAATCGGCTTAG-3'
qRT- <i>NbNPR1-R</i>	5'-TCTTCAGTTGACGCTCTT-3'
qRT- <i>AtCEBiP-LIKE1-F</i>	5'-GCTTGTTCCTCATCCGTCA-3'
qRT- <i>AtCEBiP-LIKE1-R</i>	5'-GCAAAATGGCATTCTGACATCC-3'
qRT- <i>AtCERK1-F</i>	5'-GGAATTCATATGAGGACTAGCTGTCCTTTAGC-3'
qRT- <i>AtCERK1-R</i>	5'-CCCAAGCTTAACAATTCACCAATACATT-3'

suspension were added to the leaves of wild-type *N. benthamiana*, wild *Arabidopsis*, and *Arabidopsis* receptor mutants after AOS (100 µg/mL) treatment for 24 h. The leaves were placed in a plastic dish with ddH₂O sprayed over them regularly. *Phytophthora infestans* infection was observed at 3 days post-inoculation (dpi), and *P. infestans* colonization was measured by quantitative real-time PCR. Briefly, total DNA was extracted from diseased leaves, including AOS-treated and untreated leaves, and then quantitative PCR was performed after DNA extraction by using the internal reference gene primers in Table 1 (*Nb-actin* and *P. infestans*-O8 primer pairs). The expression levels of the internal reference gene of tobacco and *P. infestans* were measured to evaluate *P. infestans* accumulation. The primer sequences for the experiment are presented in Table 1.

The optimal working concentration of AOS was determined by spraying 0, 25, 50, 100, or 200 µg/mL of gradient AOS aqueous solution on potato Désirée/Eshu 3 leaves for 24 h, followed by inoculation with *P. infestans*; the other conditions were the same as those described above. There were three biological replicates for each treated sample.

Histochemical staining of reactive oxygen species

Histochemical staining was performed using 3,3'-diaminobenzidinebutane (DAB) and nitroblue tetrazolium (NBT) to detect hydrogen peroxide (H₂O₂) and superoxide ion (O²⁻) accumulation, respectively, in the leaves of plants subjected to AOS and H₂O treatment. Plant tissues were placed in 1 mg/mL of DAB solution, vacuum-infiltrated for 30 min, washed three times with deionized water, and reacted with H₂O₂ for 12–24 h under light at 28°C. The excess dye solution was washed away by using a boiled solution (ethanol:lactic acid:glycerin = 3:1:1) at 100°C for 10 min, and the leaves were imaged. For NBT staining, *N. benthamiana* leaves were immersed in 1% (M/V) sodium azide solution, which increases the permeability of cells, vacuum-immersed for 30 min, and then transferred to 0.5 mg/mL of NBT solution, followed by vacuum infiltration for 30 min. O²⁻ reacted with NBT to form a deep blue insoluble complex. The boiled solution (ethanol:lactic acid:glycerin = 3:1:1) was also used to wash off the excess dye solution, after which the leaves were imaged. The strengths of H₂O₂ and O²⁻ were quantitatively analyzed by ImageJ software.

For aniline blue staining, 20 mL of lactic acid, 20 mL of phenol, 40 mL of 20% glycerol, and 20 mL of deionized water were mixed evenly; the volume was adjusted to 100 mL; and then anhydrous ethanol was added at a volume ratio of ~2:1. *Nicotiana benthamiana* leaves were put into the above solution under vacuum for 30 min and then treated at 60°C for 30 min. The 0.01% aniline blue solution was added after washing with deionized water, and the leaves were kept at room temperature overnight without light. Finally, the leaves were preserved in 50% glycerol and were observed and photographed under a fluorescence microscope.

Measurement of Ca²⁺ in guard cells and stomatal aperture measurement

Nicotiana benthamiana leaf epidermis strips were soaked in MES, pH 6.0 buffer under light for 3 h to open the stomata, and then a final concentration of 20 µmol/L of Fluo-3AM was added at 4°C for 2.5 h. The excess fluorescent dye was washed by using the MES buffer and then kept at room temperature for 1 h. The epidermis strips were treated with ddH₂O and 100 µg/mL of AOS for 24 h, and then fluorescence was observed using a confocal microscope. Fluo-3AM was used to analyze Ca²⁺ accumulation in the guard cells. Each treatment included an investigation of at least three epidermis strips, and the experiment was repeated three times. Images of the stomatal aperture were captured with an Olympus BX43 microscope (Olympus, Tokyo, Japan) using the cellSens Standard software, and the diameters of 50 randomly selected stomata were measured. Each assay was repeated three times.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tobacco leaves with TRIzol reagent (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. The cDNA was synthesized from 1 µg of total RNA using a FastKing gDNA Dispelling RT SuperMix kit (Tianjin, Beijing, China). Quantitative real-time PCR (qRT-PCR) was performed by using a Talent SYBR Green Kit (Tianjin, Beijing, China). Each reaction was conducted in triplicate and repeated three times. Bio-Rad CFX Manager software (Bio-Rad, California, USA) was used to analyze the data. The relative expression levels of the ROS-scavenging enzymes catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and ROS-generating-related genes, including the respiratory burst oxidase homolog genes (*RbohA* and *RbohB*), were measured by qRT-PCR after AOS treatment for 0, 2, 4, 8, 12, and 24 h, respectively. Primer sequences for the experiment are presented in Table 1.

Data analysis of RNA sequencing

The transcriptome was sequenced by Shanghai OE Biotech Co., Ltd. (Shanghai, China). RNA samples were taken from five- or six-leaf-stage leaves treated with H₂O (0 h) or 100 µg/mL of AOS for 24 h. Each sample was analyzed three times. The samples were selected depending on quality (RIN score ≥ 7). All differential gene expression data were based on the following criteria: an absolute log₂ ratio ≥ 1 and an FDR ≤ 0.001.

Expression and purification of AtCERK1 and AtCEBiP-LIKE1

The two PCR products of the *AtCERK1* gene and the *AtCEBiP-LIKE1* gene were individually cloned into the pET-

30a (+) vector and SacI vector. The proteins AtCERK1 (extracellular domain, At3g21630) and AtCEBiP-LIKE1 (At2g17120) were expressed by using BL21(DE3) and yeast with a His₆ tag at the C-terminus, respectively. For AtCERK1 expression, *E. coli* cells harboring the recombinant plasmid were initially cultured in LB broth. When the cell density reached an OD₆₀₀ of 0.8–1.0, the broth was supplemented with the inducer isopropyl 1-thio-β-D-galactopyranoside at a final concentration of 0.05 mM to initiate the expression of AtCERK1. AtCEBiP-LIKE1 was expressed in a similar manner, and yeast cells harboring AtCEBiP-LIKE1 were cultured in BMGY broth and then in BMMY broth. The broth was supplemented with 1% methanol to induce AtCEBiP-LIKE1 expression. Then, the proteins were purified by Ni²⁺ chelation chromatography according to the method provided by Peng et al. (2018).

Interaction analysis

Molecular interactions were analyzed by using biotin-labeled AOS and CERK1/CEBiP-LIKE1 based on the ELISA method. AOS (8 mg/mL) was biotinylated in 0.1 M of MES (Sigma-Aldrich) (pH 5.5) biotin LC-hydrazide solution (Deepa et al., 2002; Peng et al., 2021). EDC (1 mg) was added to the reaction mixtures and reacted overnight at room temperature, and each reaction mixture was desalted three times with PBS and centrifuged (4°C, 2,000 rpm, 2 min) to obtain the biotin-labeled AOS. Then, 50 μL of 1 mg/mL streptavidin was added to a 96-well plate and sealed at 4°C overnight, 1% BSA was added for 1 h, and biotin-labeled AOS was added at room temperature for 2 h. The binding reaction of chitin-binding proteins (AtCERK1 and AtCEBiP-LIKE1) was carried out at 4°C overnight, and the above process was avoided from light and washed with PBS. Then, the antibody was added for 30 min, the sample was washed with PBST, TMB was used to develop the color, and the absorbance was measured at 450 nm.

Results

AOS protected plants against *Phytophthora infestans* infection

The potato cultivar *Désirée* and *P. infestans* were used as materials to examine the activity of AOS with a DP of 2–5 (Figure 1A) against late blight. The potato leaves were inoculated with *P. infestans* after being sprayed with different concentrations of AOS for 24 h at 0, 25, 50, 100, and 200 μg/mL. Images were photographed at 4 dpi, and AOS could significantly enhance the resistance of potato to late blight. The infected area and disease index was gradually decreasing with increasing AOS concentration, but it was greater when the concentration was increased to 200 μg/mL (Figures 1B, C). We hence used 100 μg/mL of AOS in the next experiment.

AOS promoted hydrogen peroxide accumulation

To investigate whether AOS regulates ROS accumulation, DAB and NBT staining were applied to evaluate the H₂O₂ and O²⁻ levels in tobacco leaves that were detached from the water-spraying group and the 100-μg/mL AOS-spraying group. Figures 2A, B show that DAB staining was first strengthened and then weakened with the extension of time. DAB staining was the deepest after 24 h of spraying AOS, which indicated that the accumulation of H₂O₂ was elevated. Similarly, NBT staining was the deepest after 24 h of spraying AOS, suggesting that AOS could also promote O²⁻ accumulation in plants.

The mRNA levels of several important genes encoding the ROS-scavenging enzymes CAT, SOD, APX, and ROS-generating-related genes, including the respiratory burst oxidase homolog genes (*RbohA* and *RbohB*), were determined by qRT-PCR analysis and monitored before and after AOS treatment. After AOS treatment, there was a significant increase in the expression level of the ROS generation-related gene *RbohB* (Figure 2C). Conversely, the expression level of the *CAT* gene decreased significantly, while there was no significant change in the expression levels of the *SOD* and *APX* genes (Figure 2D). These results suggest that AOS may enhance hydrogen peroxide accumulation by inhibiting *CAT* gene expression and promoting *RbohB* gene expression.

AOS with a DP of 2–5 leads to Ca²⁺ influx

Ca²⁺ is an important secondary messenger that triggers plant defense response. Ca²⁺ usually stays at a low concentration in the plant cell cytoplasm, whereas biotic stresses, pathogen infection, and elicitor promote Ca²⁺ influx from the extracellular to the cytoplasm, thus leading to a rapid transient cytoplasmic Ca²⁺ increase (Zang et al., 2019). To investigate whether AOS leads to Ca²⁺ influx, the fluorescent-labeled Fluo-3AM was used to evaluate the cytoplasmic Ca²⁺ levels. ddH₂O and COS were used as negative and positive controls, respectively (Zhang et al., 2009; Iriti and Varoni, 2015). There was no obvious fluorescence in the ddH₂O-treated plant tissues, while the COS- and AOS-treated guard cells showed obvious fluorescence in the cells, indicating that AOS treatment significantly promoted Ca²⁺ influx in the guard cells. Moreover, AOS promoted stomatal closure, suggesting the prevention of pathogen infection (Figure 3).

To ascertain the ability of AOS to induce callose deposition in plants, wild-type *N. benthamiana* was subjected to 100 μg/mL of AOS spray treatment, while H₂O was used as a control. Subsequently, aniline blue staining was conducted after 24 h. Microscopic examination (Supplementary Figure S1A) of the stained samples revealed conspicuous callose deposition surrounding the veins of *N. benthamiana* treated with AOS compared to the control group. The fluorescence intensity (Supplementary Figure S1B) was quantified and found to be consistent with the observed phenotype. These results indicate

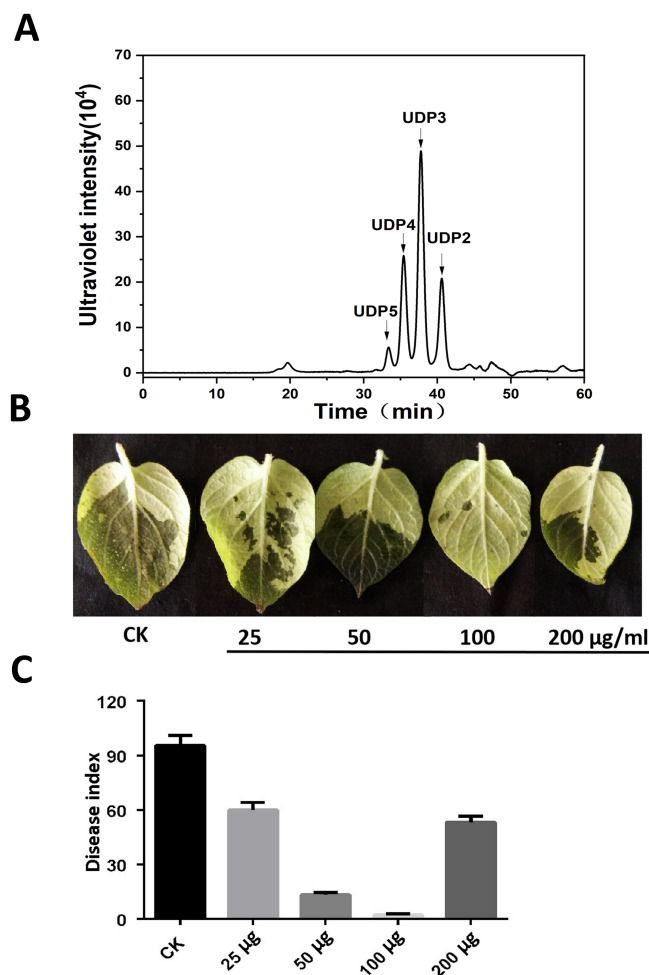


FIGURE 1

AOS treatment enhanced potato resistance against *Phytophthora infestans*. (A) Approximately 50 μg of AOS with a degree of polymerization (DP) of 2–5 was loaded on a Superdex 30 Increase 10/300 GL column. The elution positions of the unsaturated oligosaccharide product fractions with different degrees of polymerization are indicated by arrows: UDP2, unsaturated disaccharide; UDP3, unsaturated trisaccharide; UDP4, unsaturated tetrasaccharide; UDP5, unsaturated pentasaccharide. (B) Wild-type Désirée was inoculated with *P. infestans* after a gradient concentration (0, 25, 50, 100, and 200 $\mu\text{g/ml}$) of AOS treatment for 24 h, and the phenotype was observed at 3 dpi. (C) The disease index of *P. infestans* at 3 dpi. Error bars show the mean \pm SD of three replicates (at least 20 plants per replicate).

that AOS can effectively enhance callose deposition in plants and consequently improve their resistance against pathogen infections.

AOS activated the SA signaling pathway

Plants synthesize SA mainly by the isochlorogenic acid synthase (ICS) and phenylalanine ammonia lyase (PAL) pathways. Thus, we detected the gene expression level of the *ICS* and *PAL* genes by using qRT-PCR. The results showed that the *PAL* gene was upregulated by AOS, whereas the *ICS* gene basically remained unchanged (Figure 4A). These results suggested that AOS promotes SA synthesis by improving the *PAL* transcription level.

Pathogenesis-related (PR) proteins play an important role in plant defense. They can improve plant disease resistance by inhibiting pathogen reproduction and are mainly involved in plant-acquired systemic resistance. PR-1A and NPR are the key

factors in the SA pathway, involved in *N. benthamiana*'s resistance to pathogens or other pathogens, such as *Phytophthora* (Zang et al., 2019). The expression levels of genes related to these signaling pathways were examined by qRT-PCR in *N. benthamiana* leaves after AOS treatment for 24 h. The expression levels of the *PR1a* and *NPR1* genes, key genes in the SA pathway, were upregulated significantly (Figure 4B). This suggests that AOS can promote the expression of the *NPR1* and *PR1* proteins via the SA signaling pathway, enabling plants to acquire systemic resistance and enhance their resistance to late blight.

The MEK2 (MAPK kinase)-SIPK/WIPK cascade, an *N. benthamiana* mitogen-activated protein kinase (MAPK) cascade, is an essential signaling pathway for plant immunity and is involved in the hypersensitive response (HR) accompanied by cell death. Figure 4B shows that *MEK2* genes were also upregulated significantly, suggesting that AOS may activate plant immunity through the MAPK cascade.

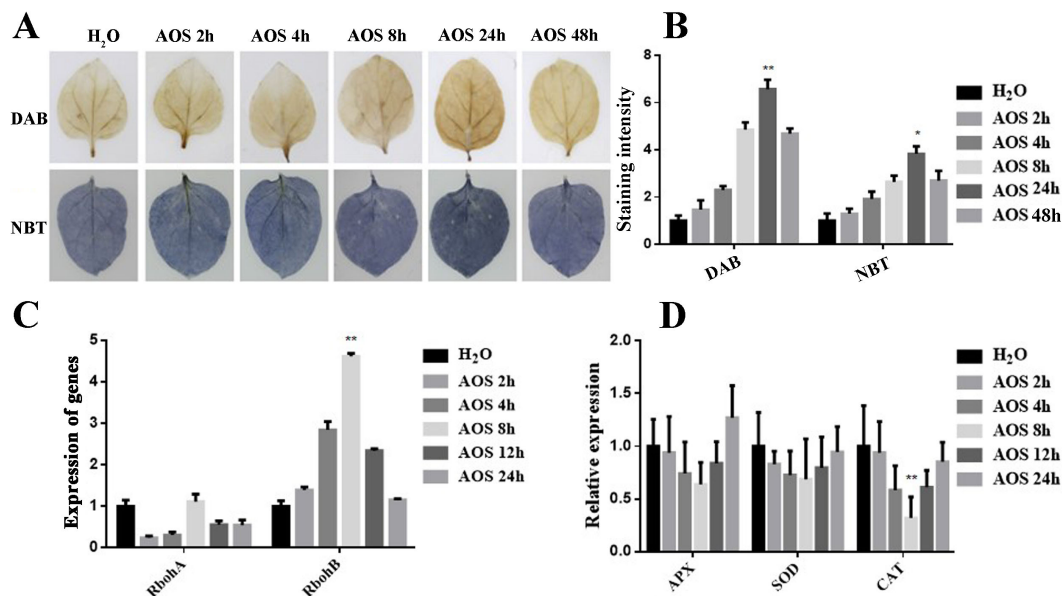


FIGURE 2
 AOS promoted hydrogen peroxide and superoxide anion accumulation. AOS promoted hydrogen peroxide accumulation in *Nicotiana benthamiana*. **(A)** Hydrogen peroxide (up) and superoxide accumulation (down) were measured in the leaves treated with 100 μg/mL of AOS at different (0, 2, 4, 8, 24, 48) hours post-treatment (hpt) ($n = 6$). **(B)** Quantification of hydrogen peroxide and superoxide levels in *N. benthamiana* treated with 100 μg/mL of AOS at 0, 2, 4, 8, 12, and 24 hpt. Data are shown as the mean ($n = 6$) ± SD. **(C)** qRT-PCR analysis of *RbohA* and *RbohB* expression at various time intervals. **(D)** CAT, SOD, and APX were detected using qRT-PCR at various time intervals. Data are shown as the mean ($n = 6$) ± SD. * indicates significant differences determined using the Student's *t*-test ($p < 0.05$), and ** indicates extremely significant differences determined using the Student's *t*-test ($p < 0.01$).

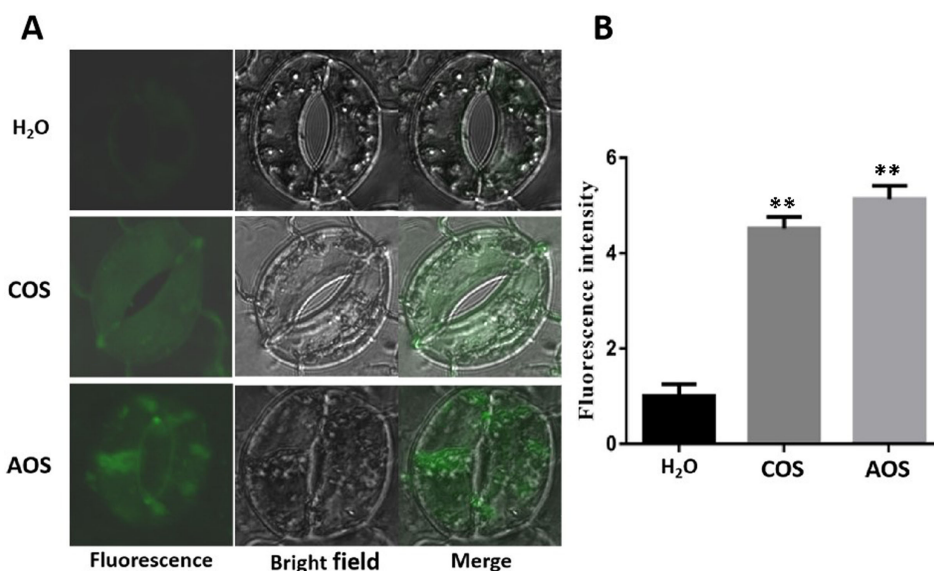


FIGURE 3
 AOS promotes intracellular Ca²⁺ accumulation in the guard cells and stomatal closure of *Nicotiana benthamiana*. The calcium-specific fluorescence probe Fluo-3AM was preincubated with epidermal peels at 4°C and then kept at room temperature for 1h. The fluorescence was observed by a laser confocal microscope after incubation with H₂O, COS (1,000 μg/mL), and AOS (100 μg/mL) for 3h. **(A)** Representative images (enlarged images). **(B)** Quantitative analysis of Ca²⁺ concentration by using the ZEN software. The experiments were repeated three times. Error bars indicate SEM. Statistics by the Student's *t*-test (** $p \leq 0.01$).

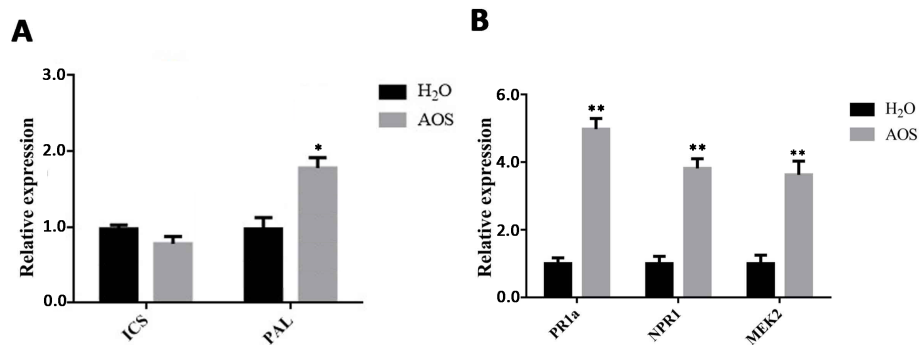


FIGURE 4

AOS promotes the accumulation of SA in *Nicotiana benthamiana*. (A) The SA biosynthesis-associated genes *ICS1* and *PAL* were measured at 24 hpt using qRT-PCR (means \pm SD, $n \geq 3$). (B) The SA signaling-associated genes *PR1a* and *NPR1* and the *MEK2* gene were measured at 24 hpt using qRT-PCR (means \pm SD, $n \geq 3$). * indicates extremely significant differences determined using the Student's *t*-test ($p < 0.05$); ** indicates extremely significant differences determined using the Student's *t*-test ($p < 0.01$).

Quantitative differences in gene expression in *Nicotiana benthamiana* after AOS treatment

Transcriptome sequencing analysis was performed on the leaves treated with AOS to reveal the role of AOS in inducing plant resistance. The leaves were harvested after 24 h of 100 $\mu\text{g}/\text{mL}$ AOS treatment. The differentially expressed genes (DEGs) after AOS and H₂O treatment (AOS0H) were analyzed. The results revealed 2,595 DEGs in the AOS24H group vs. the AOS0H group, of which 1,219 genes were upregulated and 1,376 genes were downregulated (Figure 5A).

Most of the AOS-regulated genes at 24 hpt were annotated with a wide range of Gene Ontology (GO) terms in the molecular functions (Figure 5B). Molecular function results suggested receptor activity and receptor regulator activity, and the transcriptome DEG showed that the *CERK1* gene expression level was upregulated, consistent with the results in Supplementary Figure S2B. The enriched GO terms (Table 2) of the biological process category included positive regulation of defense response, chitinase activity, and signal transduction. Within the KEGG classification, plant hormone signal transduction genes showed the greatest changes in expression (Figure 5C), and 155 DEGs were upregulated (Figure 5D). These results further verified that resistance to *P. infestans* was induced by the SA signaling pathway. Plant chitinases are described as pathogen-associated proteins because they are induced in response to invasion by plant pathogens. The genes related to the chitin catabolic process and chitinase (*Niben101Scf01789g03003*, *Niben101Scf02041g00002*, and *Niben101Scf02171g00007*) were also significantly upregulated (Table 2). Based on the above results, we speculate that the receptors in the plants of AOS may be related to chitin elicitor receptor proteins.

To determine the AOS receptor protein in plants, several *Arabidopsis* receptor loss mutants, including the T-DNA insertion mutants for *At3g21630* (*AtCERK1*) and *At2g17120* (*AtCEBiP-LIKE1*), were inoculated with *P. infestans*. There was no significant difference in mortality in the *Arabidopsis* mutant group after AOS or ddH₂O treatment, while AOS improved the resistance to *P. infestans* in the Columbia wild-type *Arabidopsis* group (Supplementary Figure S2A).

The levels of the *CEBiP-LIKE1* gene and the *AtCERK1* gene were significantly increased after AOS treatment for 24 h (Supplementary Figure S2B).

To analyze the interaction between AOS and the receptor proteins, the receptors *AtCERK1* (extracellular domain, *At3g21630*) and *AtCEBiP-LIKE1* (*At2g17120*) were expressed and purified. SDS-PAGE showed that the molecular weights of *AtCERK1* (with amino acids ranging from 26 to 230) with a His₆ tag at the C-terminus and *AtCEBiP-LIKE1* (with a secretory peptide at the N-terminus and a His₆ tag at the C-terminus) were approximately 23 kDa (Figure 6A) and 47 kDa (Figure 6B), respectively, which are consistent with the theoretical molecular weights.

Then, ELISA was used to analyze the interaction between the biotin-labeled AOS and the different concentrations of *AtCEBiP-LIKE1*, as well as between the biotin-labeled AOS and the different concentrations of *AtCERK1*. The OD₄₅₀ absorption value was greatest (0.60) when the *AtCERK1* concentration was 1 ng/ μL but was only 0.056 when the *AtCEBiP-LIKE1* concentration was 1 ng/ μL . The data suggested that AOS binds tightly to *AtCERK1*, while AOS cannot bind tightly to *AtCEBiP-LIKE1*. Furthermore, COS was used as a control and had a binding affinity to *AtCERK1* and *AtCEBiP-LIKE1*, but the affinity was lower than that of AOS (Figures 7A, B). For further confirmation of AOS combined with *CERK1*, different concentrations of *AtCERK1* and *AtCEBiP-LIKE1* and a mix of *AtCERK1* and *AtCEBiP-LIKE1* were used to check the affinity to AOS. The results (Figure 7C) showed that with the increase of *CERK1* concentration, the absorption value was higher to a certain extent; however, the concentration of *AtCEBiP-LIKE1* had no significant effect on the absorption value. Identical molar values of *AtCERK1* and *AtCEBiP-LIKE1* were used to bind AOS, and the absorption value was lower when only *AtCERK1* was used. The above results confirmed that AOS binds tightly to *AtCERK1*, indicating that *AtCERK1* is the main plant receptor of AOS and is involved in the resistance induced by AOS. Moreover, the ELISA process and a schematic diagram of the interaction between AOS and *AtCERK1/AtCEBiP-LIKE1* are shown in Figure 7D.

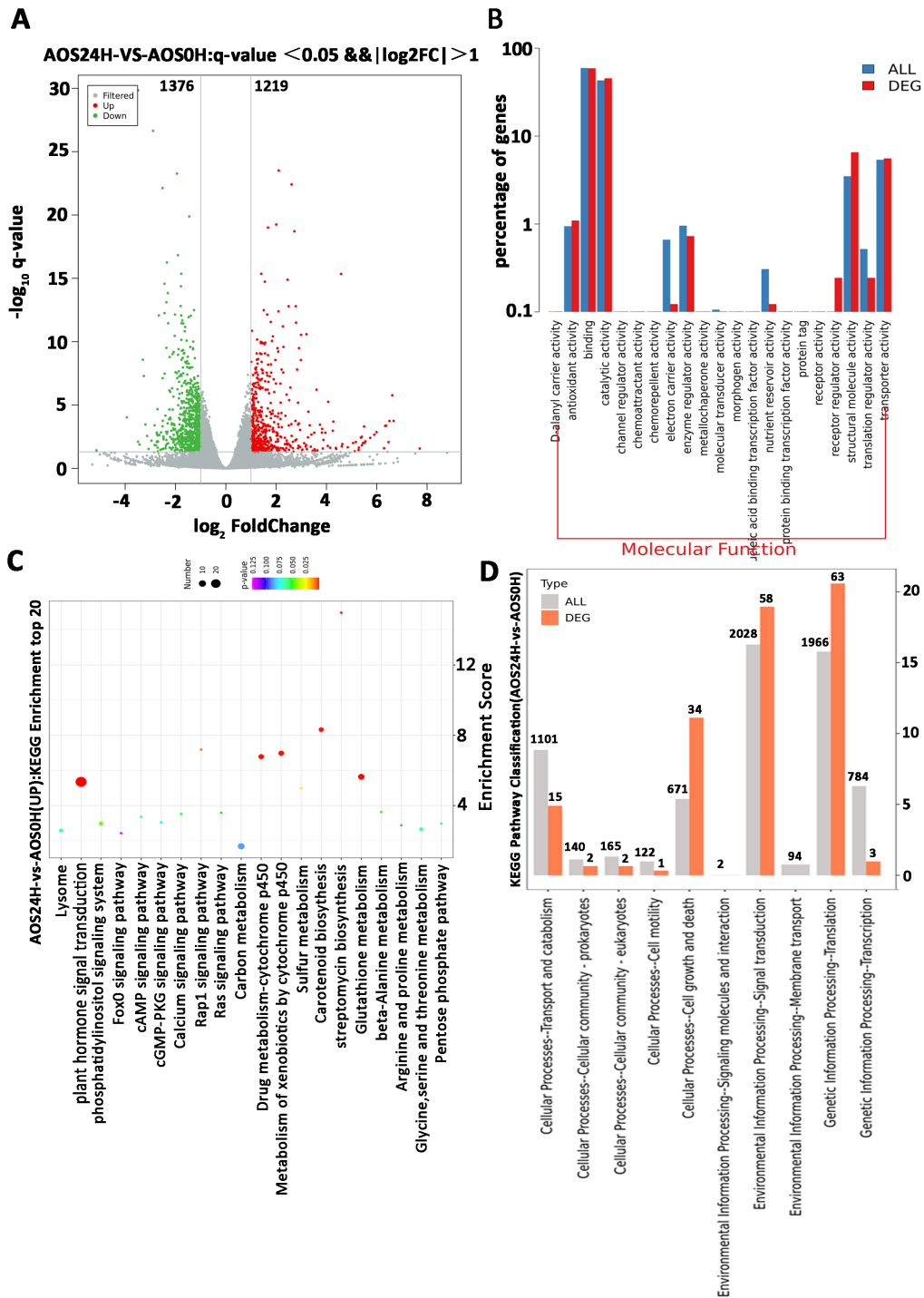
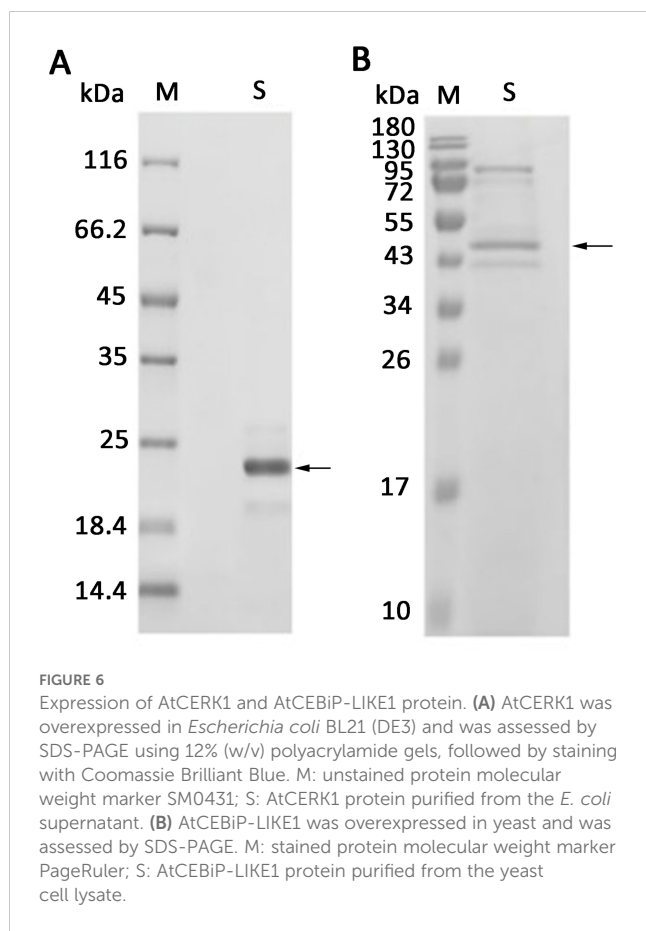


FIGURE 5

A parametric transcriptome analysis after AOS treatment in *Nicotiana benthamiana*. (A) Volcano plot showing the fold change and adjusted *p*-value of the normalized read counts of the transcriptome sequencing data. The criteria of $\log_2|$ (fold change) ≥ 1 and $\text{padj} \leq 0.05$ were used to identify the DEGs. The green dots indicate the downregulated DEGs, and the red dots indicate the upregulated DEGs. (B) Comparative plots of the distribution of DEGs and all genes at the GO level 2. The horizontal axis is the name of the entry, and the vertical axis indicates the number of genes corresponding to the entry and their percentages. (C) The characteristic KEGG pathways with significant enrichment of DEGs after treatment with AOS. (D) Partial KEGG pathway classification. The abscissa axis is the ratio of DEGs in a pathway: all DEGs in the KEGG level 2 pathway (%); the ordinate axis is the name of the pathway. The numbers above the column represent the quantity of DEGs in the pathway.



Discussion

Since the 1960s, a large number of studies on oligosaccharide elicitors have been reported, with researchers concluding that oligosaccharides have certain biological activities, such as stimulating systemic responses and regulating plant growth and development, reproduction, and immunity (Jia et al., 2016; Salachna et al., 2018). Currently, the role of oligosaccharides in immunity has been intensively studied, and a variety of oligosaccharide products have been widely used. Oligosaccharides, such as COS, have been widely reported as PAMPs (Jia et al., 2016; Kim and Rajapakse, 2005). However, few studies have focused on the elicitor activities of AOS on plant immunity. The immune elicitor AOS against *P. infestans* was explored for the first time in this study.

AOSs (UDP2-UDP5) with specific structural characteristics have been prepared by using a new alginate lyase, Aly2 (Peng et al., 2018). The results of this study demonstrated that AOS improved plant resistance to *P. infestans* (Figure 1) and triggered various defense and resistance responses in tobacco, including increased ROS bursts (Figure 2), callose deposits (Supplementary Figure S1), intracellular Ca^{2+} , stomatal closure (Figure 3), and defense-related gene expression (Figure 4). Moreover, transcriptome sequencing analysis revealed that AOS treatment upregulated the expression level of the genes of the phytohormone signaling pathway and the chitin biosynthesis pathway, three of which were related to chitinase (Figure 5; Table 2), and these genes can promote plant resistance to pathogens. Chitinase is an extracellular complex of enzymes that degrade chitin and has the application value of hydrolyzing the cell wall of the pathogen fungi to inhibit growth.

TABLE 2 Gene Ontology (GO) enrichment of upregulated genes.

GO accession	Description (term)	DEG number	Category
GO:0004568	Chitinase activity	3	Molecular_function
GO:0006032	Chitin catabolic process	3	Biological_process
GO:0035556	Intracellular signal transduction	7	Biological_process
GO:0008152	Metabolic process	67	Biological_process
GO:0009725	Response to hormone	4	Biological_process
GO:0005388	Calcium-transporting ATPase activity	2	Molecular_function
GO:0070588	Calcium ion transmembrane transport	2	Biological_process
GO:0005047	Signal recognition particle binding	1	Molecular_function
GO:0008061	Chitin binding	1	Molecular_function
GO:0019901	Protein kinase binding	2	Molecular_function
GO:0009966	Regulation of signal transduction	1	Biological_process
GO:0007165	Signal transduction	10	Biological_process
GO:0004871	Signal transducer activity	3	Molecular_function
GO:0006952	Defense response	2	Biological_process

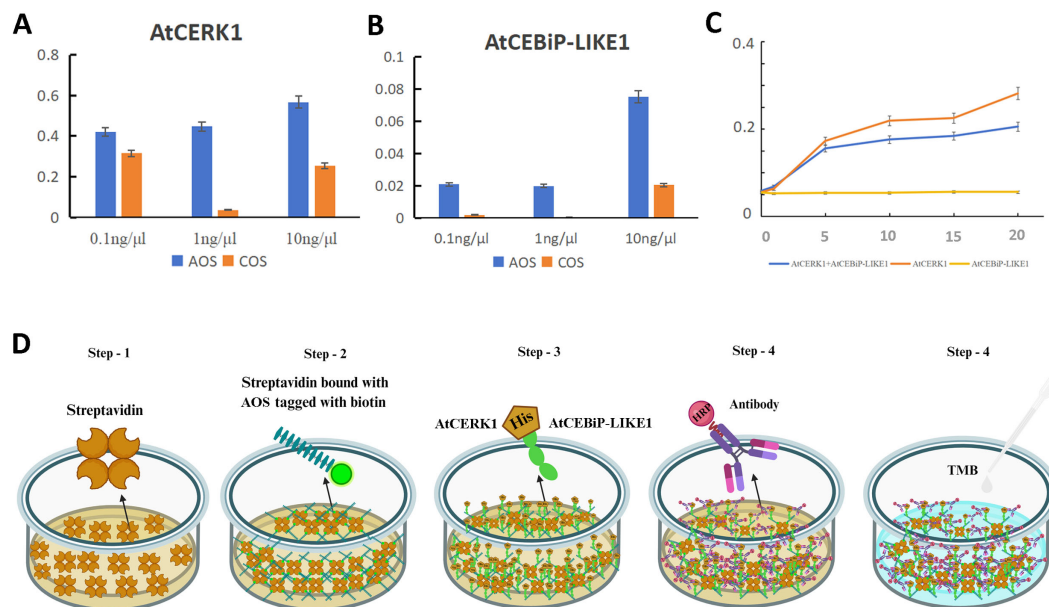


FIGURE 7

AtCERK1 is a key receptor kinase when AOS induces resistance to pathogens. Absorbance values at 450 nm for AOS and 0.1, 1.0, and 10 ng/ μ L of AtCERK1 (A) and AtCEBiP-LIKE1 (B). COS was used as a control. (C) Association curve of the different concentrations of AtCERK1 and AtCEBiP-LIKE1. (D) Schematic representation of the binding assay of AOS with AtCERK1 or AtCEBiP-LIKE1. Step 1: streptavidin plate incubation; step 2: addition of biotin-labeled AOS; step 3: addition of AtCERK1/AtCEBiP-LIKE1 protein with a His tag; step 4: addition of HRP-labeled anti-His antibody; step 5: TMB chromatography at 450 nm. A high reading with a dark yellow color indicates a high degree of binding.

Chitinase can degrade chitin-producing N-acetylglucosamine oligomers or monomers, and the above oligomers can bind to the plant surface receptor CERK1 and stimulate plant disease resistance signals. Hence, the elicitor AOS identified in this study can be regarded as a novel PAMP.

Plants produce signal molecules when pathogens are recognized by cell surface receptors, and then the infection signal is transmitted to the cell through the signaling pathway, where it can cause local or systemic resistance (Baccelli et al., 2017). The phytohormone SA plays important roles in regulating disease resistance. In this study, the SA synthesis key gene *PAL* was upregulated (Figure 4A), and qRT-PCR revealed that the expression of the related marker genes *PR1A* and *NPR1* in the SA pathway increased (Figure 4B).

Many elicitors (Boller and Felix, 2009), including the elongation factor (Zipfel et al., 2006), flagellin (Denoux et al., 2008), chitin (Liu et al., 2016; Shinya et al., 2015), and other oligosaccharides (e.g., OGA) (Benedetti et al., 2015), can induce plant defense responses and improve plant resistance to pathogens. These elicitors are PAMPs, which can interact with the plant receptors to activate the PTI.

CERK1 is a plasma membrane protein that contains three LysM motifs in its extracellular domain and an intracellular Ser/Thr kinase domain with autophosphorylation/myelin basic protein kinase activity. It plays a key role in plants, detecting fungal microbe-related molecular patterns. Currently, it is regarded as a

key receptor for plant immunity and symbiosis (Miya et al., 2007; Yang et al., 2022). CEBiP is a membrane glycoprotein with LysM motifs that functions as a cell surface receptor for chitin elicitors in rice (Liu et al., 2016) and plays an important role in the recognition of chitin. The AtCERK1 and AtCEBiP-LIKE1 proteins were expressed in this study (Figure 6), and the results showed that AtCERK1 binds AOS tightly, suggesting that AtCERK1 interacts with AOS, but this is not the case for AtCEBiP-LIKE1 and AOS (Figure 7). These results are consistent with those of Tomonori Shinya, who reported that AtCERK1 alone is sufficient for AOS detection (Shinya et al., 2012).

Conclusion

We demonstrated that the elicitor AOS could induce plant resistance to late blight for the first time. The elicitor AOS activates the SA pathway and a series of defense responses to improve its resistance to pathogens. Moreover, AtCERK1 which binds to AOS is first reported here. We speculate that AOS is recognized by the receptor kinase CERK1 and transmits the signal to cells via its kinase activity and induces a series of defense responses, but the detailed signal pathway should be studied in-depth in the future. This study lays the theoretical foundation for AOS's wide plant nosotropic applications.

Data availability statement

The Transcriptome Sequence data are deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1097808. The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

CP: Conceptualization, Data curation, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. WX: Writing – review & editing, Formal analysis, Investigation, Methodology, Software. XPW: Formal analysis, Investigation, Methodology, Data curation, Writing – review & editing. FM: Data curation, Investigation, Software, Writing – review & editing. YZ: Writing – review & editing, Writing – original draft, Data curation, Methodology. QW: Data curation, Investigation, Conceptualization, Project administration, Resources, Writing – review & editing. XKW: Resources, Validation, Writing – review & editing. RL: Data curation, Formal analysis, Methodology, Writing – review & editing. XD: Supervision, Writing – review & editing. CZ: Conceptualization, Supervision, Validation, Visualization, Writing – review & editing. LP: Funding acquisition, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2025.1506873/full#supplementary-material>

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