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Identification of chitosan oligosaccharides binding proteins from the plasma membrane of wheat leaf cell

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Abstract

Chitosan oligosaccharides (COS) have the ability to improve plant resistance to pests and diseases through activating plant immune system. However, it remains unclear whether stimulating reason of COS was associated with the plasma membrane proteins. Here, the interaction of COS with wheat leaf cell demonstrated that fluorescence-labeled COS were enriched on the cell surface and the interaction of COS with plasma membrane proteins was confirmed by quartz crystal microbalance (QCM) biosensor. What's more, HPLC and SDS-PAGE analysis showed that COS binding proteins exhibited more than three peaks and the molecular weight were 66 kDa to 97 kDa, where the COS binding proteins were fished out from wheat plasma membrane proteins by the COS affinity column. More importantly, LC-MS/MS analysis demonstrated that several candidates, including W5G2U8_WHEAT (a potential wall-associated receptor kinase protein), W5HY42_WHEAT and W5I0R4_WHEAT (potential G-type lectin S-receptor-like serine/threonine-protein kinase), have the potential to be COS receptor.

Keywords: Chitosan oligosaccharides (COS); COS binding proteins; Plasma membrane; Wheat leaf cell; Plant immune system

1. Introduction

Higher plants initiate various defense reactions when infected by myriads microbial pathogens. These defense reactions triggered by microbial elicitors play important roles in the basal resistance of plants to pathogens [1]. These microbial elicitors are named microbial-associated molecular patterns (MAMPs), which including flagellin, elongation factor Tu (EF-Tu), LPS, β-glucan, peptidoglycans, and fungal cell wall fragments (chitin/COS) [2]. Plants recognize those MAMPs by transmembrane pattern recognition receptors (PRRs) to elicit immune defense effectors such as phytoalexins and antimicrobial proteins accumulation to struggle with various pathogens [3-5]. Recently, the PRRs for several oligosaccharide elicitors have been found. WAK1 [6, 7] and GEBP [8, 9] as oligogalacturonide and β-Glucan receptors, respectively, have been identified. CEBiP (chitin-elicitor binding protein) and CERK1 (chitin-elicitor receptor kinase-1) were also identified as the cell-surface receptors of chitin, which play important roles in rice and *Arabidopsis* immunity [10-18].

COS are potent MAMPs, but it remains unclear that if they exert this biological function via PRRs-mediated recognition [19, 20]. Knowing that CEBiP has no specific binding ability to COS [11], the presence of new putative receptors for COS is hypothesized and very interesting to be explored [21]. Considering that all the known receptors of oligosaccharides are cell-surface membrane proteins, it is, therefore, critical pre-step in finding out the receptors of COS to identify if the binding sites are located in those plasma membrane proteins. In our previous research, it has shown that COS have binding sites on strawberry [22] and tobacco cell surfaces [23, 24], respectively. Although the chitosan binding proteins from Rubus and cabbage have been identified [25, 26], their function has not been reported. However, whether specific COS receptors exist on the plant plasma membrane is still unknown.

In this study, wheat was selected as the plant sample because of its economic effect. To determine whether COS exist the binding interaction with plasma membrane proteins in wheat protoplast plasma membrane surface, the fluorescence-labeled COS and the QCM biosensor were used to detect the interactions of COS with wheat leaf cell and plasma membrane proteins, respectively. Furthermore, the COS-Agrose-4FF affinity column to fish the binding proteins in wheat leaf cell surface was carried out. To further clarify whether those binding proteins were related with the plant defense immunity, the studies

based on LC-MS/MS combined with the proteins data bank to identify of those binding proteins was performed.

2. Materials and methods

2.1. Materials and reagents

COS with a degree of polymerization (DP) from 2 to 7 were obtained from Dalian GlycoBio Company, Ltd. (Dalian, China). The wheat seeds (*Triticum aestivum*, China wheat 895) were supplied by Northwest A&F University (Yangling, China). The 2-aminoacridine ketone (2-AMAC), $NaCNBH_3$, polyethylene glycol (PEG) 3350, sucrose, Tris, 2-(N-Morpholino) ethane sulfonic acid (MES), Triton X-100, EDTA, NaF, ascorbic acid, PVP-K 30, PMSF, DTT, cellulose (OnozukaR-10), and pectinase (Macerozyme R-10) were purchased from Sigma (St. Louis, US). Dextran T-500 was purchased from GE. Other reagents were purchased from Solarbio (Beijing, China) and Xinong (Guangxi, China) in analytical grade.

2.2. Wheat protoplast preparation

Germinating seeds were treated with COS (50 ppm in distilled water) 2 times per day for two weeks. Seedlings with three leaves were harvested and conducted as described for protoplast preparation [27]. The second leaf of the seedlings was washed in sterilized water and cut before being treated with 5 mL mixed enzyme solution in the dark at 25 °C for three hours. After digestion, the protoplasts were shaken for 10 min and then centrifuged at 500 × g for 2 min. The process of wash and centrifugation was repeated 3 times for protoplasts collection. Finally, the protoplasts were re-suspended to a concentration of 1×10^5 cells/mL.

2.3. The fluorescent study of COS localization on protoplast

The 2-aminoacridine ketone-labeled COS (2-AMAC-COS) were synthesized as described [28]. In brief, an aliquot of 10 mg COS was added in 100 μL 1 M $NaCNBH_3$ in Tetrahydrofuran (THF) solution together with 3 μL 2-AMAC solution (0.1 M, in a 3:17 (v/v) mixture of AcOH and DMSO). The reaction mixture was incubated at 90 °C in the dark for 40 min, and then cooled down to -20 °C to stop the reaction.

The mixture was centrifuged at 12000 × g for 15 min. The precipitate was washed with THF, dried and stored at -80 ℃ in the dark.

The fluorescent microscope study was performed according to the procedure described previously [24]. In brief, protoplast suspension was treated with 300 μg/mL 2-AMAC, COS, or 2-AMAC-COS for 2 min, respectively. All samples were observed using the inverted fluorescent microscope (Leica DMI 4000B).

2.4. Purification of wheat plasma membrane

Purification of the plasma membrane was processed as described with some modifications [29-31]. In brief, the seedling tissue was homogenized and centrifuged (10,000 × g, 15 min). After that, the supernatant was withdrawn and ultra-centrifuged (100,000 × g, 40 min) to get the pellet. Then the pellet was resuspended and mixed with the aqueous two polymer phase solution (6.4% PEG3350 and 6.4% Dextran T-500). The upper phase was diluted before ultracentrifugation at 156,000 × g for 60 min to get the purified plasma membrane. All steps were performed at 4 ℃. The content of the membrane was detected by H^+-ATPase assay and the wavelength scanning of reaction system of H^+-ATPase activity was detected in 400 ~ 900 nm [32, 33].

2.5. Quartz crystal microbalance biosensor analysis

The experiment was performed on a QCM biosensor instrument (Attana). Firstly, the QCM system was equilibrated with the running buffer at 25 μL/min, followed by a flow of 200 μg/mL COS to make a COS Chip. After the base-line was balanced, the running buffer was then successively replaced by the OVA protein solution and plasma membrane protein solution. At last, the COS chip was regenerated with three injection of 1 M NaCl solution. All data were recorded and analyzed with the QCM-software.

2.6. COS affinity column preparation

Isolation of COS binding proteins was used by a COS-Agarose-4FF (4 Fast flow) column (1.5 × 3 cm). The column filler was prepared via the reaction of COS and N-hydroxysuccinimide (NHS) activated agarose. An aliquot of 100 mg COS was dissolved in 10 mL 0.1 M $NaHCO_3$-Na_2CO_3 buffer (pH8.3), then

added into 10 mL NHS-activated agarose. After the reaction mixture was shaken 50 rpm at 4 °C for 14 h, the product was washed with 20% ethyl alcohol then packed into a column.

2.7. Isolation of COS binding proteins from the wheat plasma membrane

Extraction of plasma membrane proteins was processed as described previously [29]. Membrane proteins were incubated with 0.5% Triton X-100 in 20 mM Tris-HCl buffer (TBS, pH 7.0) containing 1 mM $MgCl_2$, 0.1 M NaCl, 1 mM DTT, and 0.5 mM EDTA for 2 h at 0 °C. After ultracentrifugation (200,000 × g, 4 °C, 1h), the supernatant was collected for further purification. The column was pretreated with 1% BSA solution and successively washed with 0.17 M Glycine-HCl buffer (pH 2.3). After that, the column was equilibrated with a TBS buffer containing 0.005% Triton X-100. After the sample was loaded, the column was equilibrated with TBS buffer and eluted with 0.17 M Glycine-HCl buffer (pH 2.3). Eluted peak fraction was polled in tubes that was added with a 1/10 volume of 1 M Tris for immediate neutralization [12]. The collected fractions were further analyzed by SDS-PAGE and HPLC.

2.8. Reverse Phase HPLC separation of COS binding proteins

Chromatographic separation was carried out using an Agilent 1260 liquid chromatography equipped with an Agilent ZORBA × 300 SB-C18 HPLC column (4.6 × 250 mm, 5 μm). An aliquot of 50 μL (100 μg/mL) COS binding proteins solution was injected to the column and detected by Ultraviolet (λ = 280 nm), with linear gradient elution of 5% - 95% acetonitrile containing 0.1% TFA at a flow rate of 0.5 mL/min for 80 min.

2.9. LC-MS/MS analysis

The target band of the COS binding proteins was cut off from 12% SDS-PAGE gel and digested with trypsin. The digested sample was analyzed by LC-MS/MS (Orbitrap Fusion, Thermo Fisher scientific, USA). For the acquisition of MS data, the Mascot daemon and mascot server (V 2.5, Matrix science, Boston, USA) were used. According to the MS data, the candidates of COS binding protein were identified with the database of UniProt, Pubmed, and TMHMM.

3. Results

3.1. COS interacted with wheat protoplast surface

The interaction between COS and the wheat protoplast was studied by fluorescent microscopy utilizing 2-AMAC-COS. As shown in Fig. 1A, control exhibited no green fluorescence exists in protoplast surface. Noticeably, the 2-AMAC-COS showed a strong protoplast surface-binding pattern (Fig. 1B). The 2-AMAC-COS also appeared in the cytoplasm and nuclei with lower-intensity signals. Compared with 2-AMAC-COS, no specific signal was observed either on the surface, cytoplasm, or nuclei of the protoplast treated with 2-AMAC (Fig. 1C) or COS (Fig. 1D).

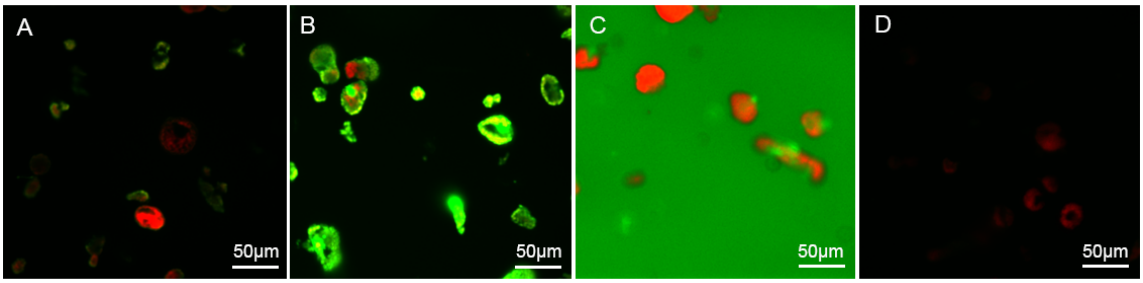

Fig. 1. 2-AMAC-COS were localized on the plasma membrane of *Triticum urartu*. Wheat protoplast was treated with (*A*) control (ultrapure water), or 300 μg/mL of (*B*) 2-AMAC-COS, (*C*) 2-AMAC, and (*D*) COS, respectively. 2-AMAC-COS and COS showed green signal under the fluorescent microscopy. All images were at the same magnification: 200 ×.

3.2. COS interacted with purified plasma membrane proteins

To identify potential COS binding proteins on the wheat plasma membrane, the interaction between COS and plasma membrane proteins was tested on the QCM biosensor with a COS chip, where the non-specific binding site was blocked by the OVA protein which produced maximal frequency shifts of 20 Hz. As can be seen in Fig. 2, the binding of plasma membrane proteins to the COS chip was recorded with maximal frequency shifts of 130 Hz, where the surface could be regenerated with three successive injection of 1 M NaCl solution.

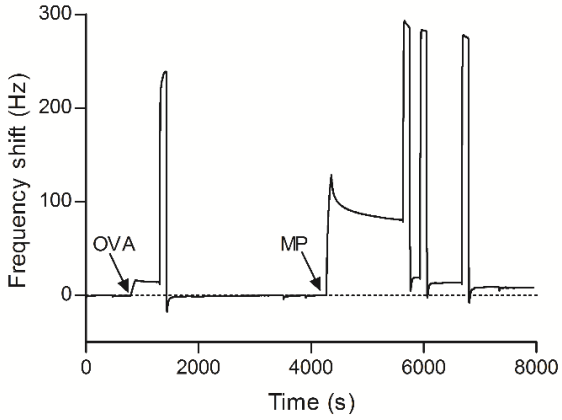

Fig. 2. Interaction of COS and plasma membrane proteins of *Triticum urartu*. The binding of the plasma membrane proteins of *Triticum urartu* to the polystyrene chip which was modified by the COS injected, then the proteins were injected and the frequency shift was increased and eluted the over proteins by NaCl solution during the interaction. The sample was injected into the instrument as arrows indicated.

3.3. Isolation of putative COS-binding proteins

The plasma membrane was extracted from the wheat leaf. The purity of the plasma membrane was determined by H^+-ATPase assay with the optimal wavelength in 820 nm (Supplementary material, Fig. S1A). The contents of the plasma membrane, mitochondria, and tonoplast were 83.25%, 13.13%, and 5.78%, respectively (Supplementary material, Fig. S1B). The membrane proteins were loaded to a COS-Agrose-4FF affinity column (20mm × 60mm) to separate potential COS binding factors (Supplementary material, Fig. S2). The eluted fraction showed single peak on the chromatographic profile and single band between 66 kDa and 97 kDa on SDS- PAGE (Fig. 3).

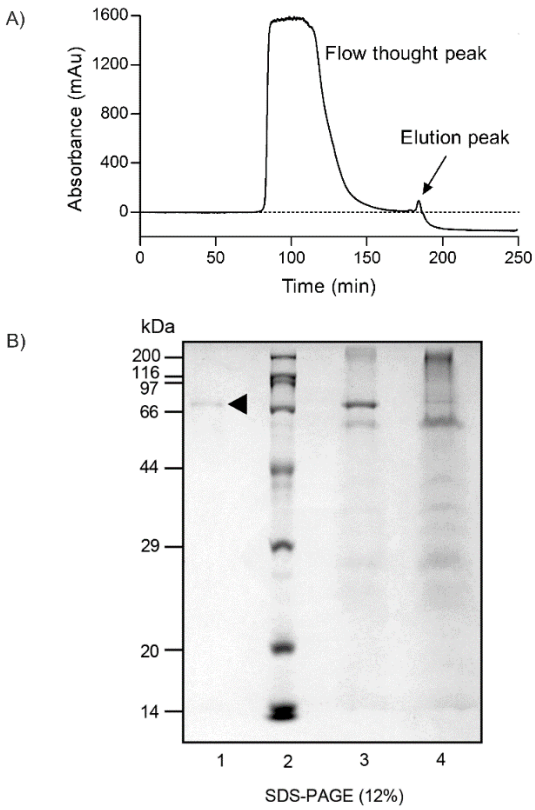

Fig. 3. (*A*) The elution of the COS-binding protein. COS-binding proteins were isolated by a COS-Agrose-4FF affinity column. When the baseline was balanced by TBS buffer, the plasma membrane proteins were added to the affinity column after washed by TBS to except the unbinding proteins and eluted by Gly-HCl buffer. Arrow was the elution peak by Gly-HCl buffer (pH 2.3). (*B*) The SDS-PAGE analysis of the COS binding proteins. Coomassie blue staining of the 12% SDS-PAGE gel loaded with samples from COS-Agrose-4FF affinity column. Line 1, the sample eluted from the COS-Agrose-4FF affinity column; Line 2, marker; Line 3, the loading sample; Line 4, flow through. Arrow indicated the potential COS-binding proteins.

3.4. HPLC analysis of COS-binding protein

To further analyze the purified sample from the COS-Agrose-4FF column, the eluted sample was separated by using ZORBA × 300 SB-C18 HPLC column. There was no peak detected in the blank control (Fig.4A). Three peaks appeared in the chromatographic profile of the sample with a retention time of 48 min, 54 min, and 61 min, respectively (Fig. 4B).

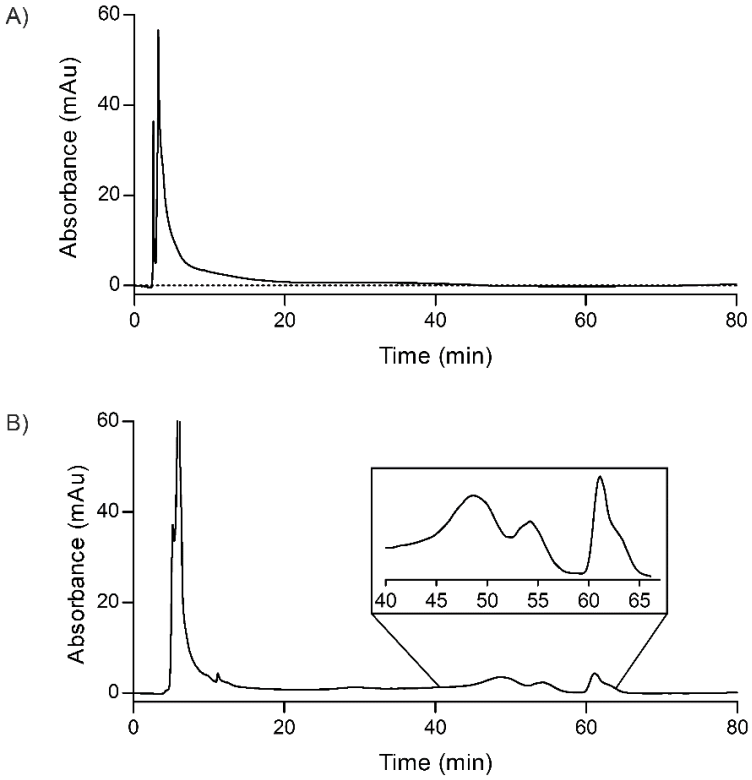

Fig. 4. (*A*) Elution buffer for COS-Agrose-4FF column. (*B*) Eluted sample from COS-Agrose-4FF column were loaded on HPLC. The sample was eluted under gradient conditions (flow rate, 0.5 mL/min): 5% acetonitrile to 95% acetonitrile in 80 min.

3.5. Identification of COS binding proteins by LC-MS/MS analysis

To identify the proteins in the purified sample from the affinity column, the sample was separated by the SDS-PAGE, where the single band around 66-97 kDa was further cut out for the LC-MS/MS analysis. The MS data was analyzed with Triticum database of UniProt and Pubmed. Transmembrane domain prediction with TMHMM server provided 23 possible wheat plasma proteins listed as COS binding candidates (Table 1).

Table 1 The chitosan oligosaccharides binding proteins candidates.

Entry Name	Putative function	Peptide Quantity	Protein Mw (kDa)
W5G2U8_WHEAT	Wall-associated receptor kinase	757	83.6
W5HY42_WHEAT	G-type lectin S-receptor-like serine/threonine-protein kinase	802	90.4

W5I0R4_WHEAT	G-type lectin S-receptor-like serine/threonine-protein kinase	841	93.3
W5HDV2_WHEAT	TIM-barrel signal transduction protein	746	79.3
M8AN05_TRIUA	LRR receptor-like serine/threonine-protein kinase	> 700	unknown
T1MSJ2_TRIUA	Putative LRR receptor-like serine/threonine-protein kinase	1072	117.9
M7YAV8_TRIUA	Putative metal-nicotianamine transporter YSL1	809	88.7
W5AGW0_WHEAT	ABC transporter C family member 3	1284	142.4
W5DQ60_WHEAT	Putative ABC transporter	1161	130.4
W5EY65_WHEAT	Pleiotropic drug resistance protein 2	901	101.8
W5HVL0_WHEAT	Putative ABC transporter C family member	1287	≈142
T1MCL6_TRIUA	Putative pentatricopeptide repeat (PPR)-containing protein	1156	125.9
M7ZZN1_TRIUA	Phospholipid-transporting ATPase (EC 3.6.3.1)	1617	182.4
W5HXW4_WHEAT	Phospholipase D Z	516	57.8
W5DHS9_WHEAT	Predicted: protein Zinc-Induced Facilitator	263	28.8
W5DZI0_WHEAT	Auxin-induced protein 5NG4	373	40.1
M8AUX6_TRIUA	ATP synthase subunit alpha	568	61.8
W5EPY2_WHEAT	thylakoid formation 1, chloroplastic-like protein	212	24.4
M7ZEH1_TRIUA	Pectin methylesterase inhibitor	180	19.4
W5ECV7_WHEAT	B12D superfamily protein	97	11.0
M8AQ64_TRIUA	Putative lipid-transfer protein DIR1	147	16.1
T1LTA7_TRIUA	Uncharacterized protein	144	16.1
W5EMG7_WHEAT	Uncharacterized protein	97	10.8

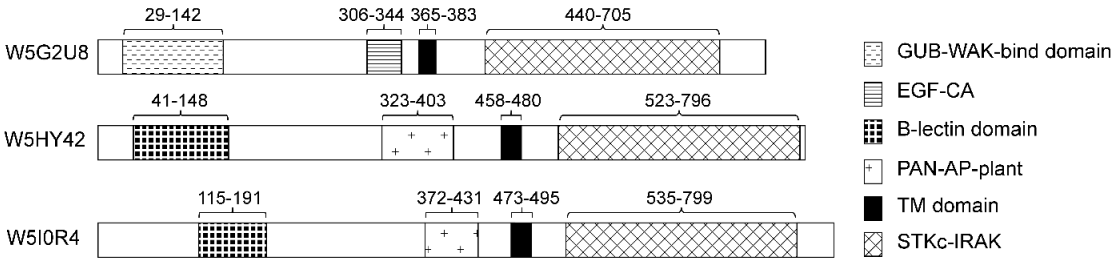

Fig. 5. Cartoon of the COS-binding candidates on the wheat membrane. Comparative cartoon of our interest proteins. The transmembrane domain separates the extracellular domain from the STKc-IRAK (serine/threonine kinase domain (cytoplasm)).

Fig. 6. Alignment of the deduced amino acid sequences of the W5G2U8 and five WAK proteins. Identical residues conserved among all six proteins are displayed in white type (black boxes). Residues identically among five proteins and their similar counterparts are in pink boxes. Identical residues among three to four members and their similar counterparts are displayed in blue boxes. Gaps introduced to improve the alignment are indicated by dots. Multiple sequence alignment was done using DNAMAN.

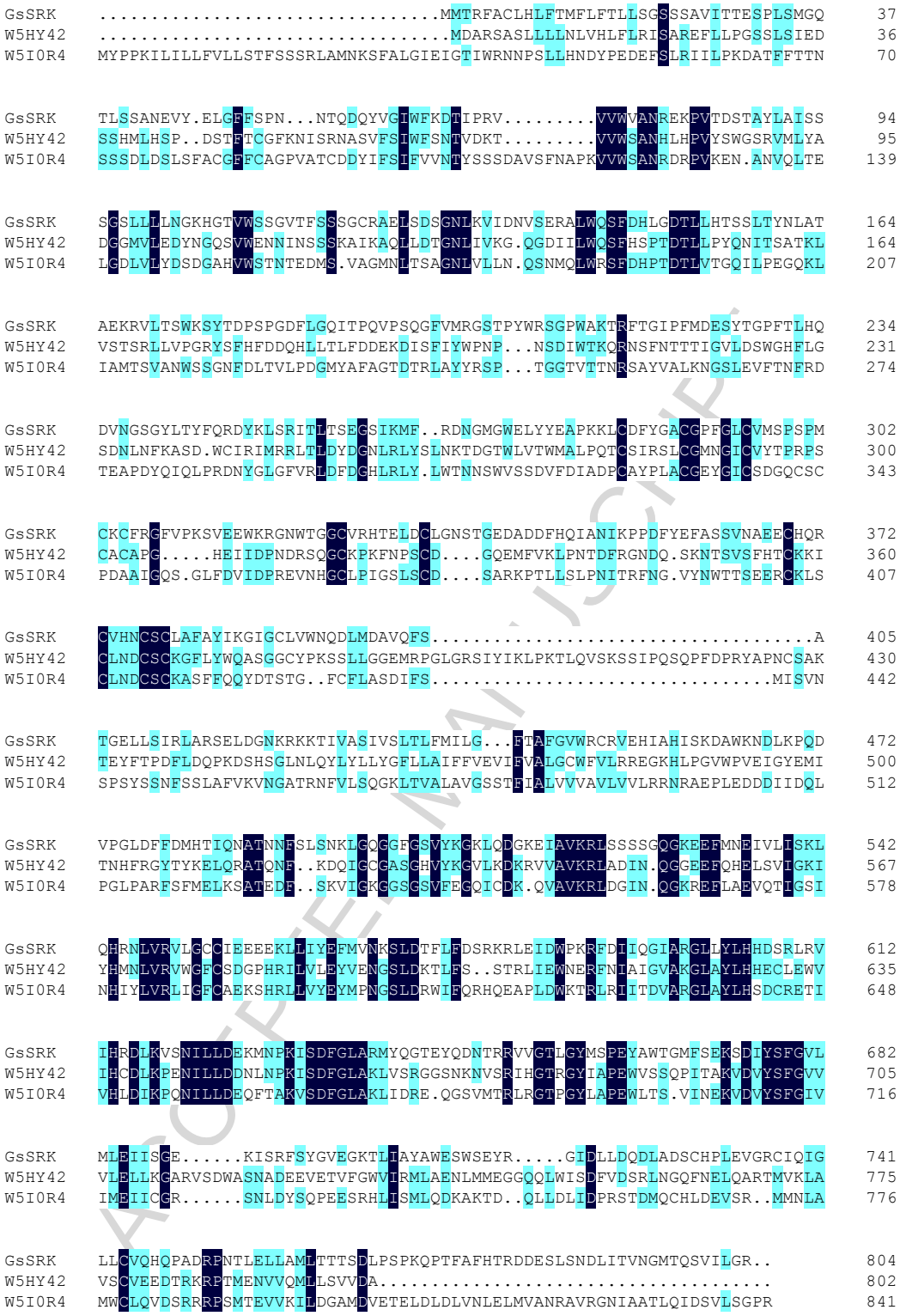

Fig. 7. Alignment of the deduced amino acid sequences of the W5HY42, W5I0R4, and GsSRK protein. Identical residues conserved among all three members are displayed in black boxes. Similar residues among at least two family members are in blue boxes. Gaps introduced to improve the alignment are indicated by dots. Multiple sequence alignment was done using DNAMAN.

These proteins were ranked by the possibility of involvement in COS recognition. Top three in the list, W5G2U8_WHEAT, W5HY42_WHEAT, and W5I0R4_WHEAT have the potential domain of kinase. The W5G2U8_WHEAT has GUB-WAK-bind domain, EGF-CA, and STKc-IRAK. The W5HY42_WHEAT and W5I0R4_WHEAT have B-lectin domain, PAN-AP-plant, and STKc-IRAK (Fig. 5). The highest homology of sequence of the W5G2U8_WHEAT was 64.55% compared with the Wall-associated receptor kinases (WAK1-5) in *A. thaliana* (Fig. 6). The highest homology of W5HY42_WHEAT and W5I0R4_WHEAT was 32.97% compared with G-type lectin S-receptor-like serine/threonine-protein kinases (GsSRK) based on studies of their homologs in *A. thaliana* (Fig. 7). Considering the proteins showed a molecular weight between 66 kDa and 97 kDa on SDS-PAGE, there were four proteins, W5G2U8_WHEAT, W5HDV2_WHEAT, M7YAV8_TRIUA, and M8AUX6_TRIUA within this range.

4. Discussion

Oligosaccharides are potent PAMP which could induce plant disease resistance by regulating immunity in plant. COS were important representative among these oligosaccharides, which have been shown to elicit defense responses to a broad spectrum of pathogens in many plant species, including tobacco, rapeseed, rice, and grapevine [34-36]. Furthermore, COS have been developed as bio-pesticides in several countries and widely used in several crops, such as wheat, apple, kiwi fruit, and cucumber [20]. However, mechanism of COS induced plant immunity remains unclear, especially in the first step of COS signal recognition [21].

Thus, the discovery of the receptor for COS has been attempted in the past decades, but no great progress has been achieved. Although the specific binding of COS to tobacco and rapeseed was verified by the use of fluorescent probes [37], no further study was made on the nature of the bound substances. To date, only three COS-binding proteins have been purified by chitosan affinity chromatography from Rubus, tobacco and *A. thaliana*, but the three proteins have not been identified [21, 38].

Notably, a series of LysM domain proteins such as OsCEBiP, AtCERK1 and AtLYK5 were identified as receptors for chitin and chitin oligosaccharides in rice and *A. thaliana*, respectively [11, 39, 40]. However, some papers reported that the LysM domain proteins had no binding effect on COS [15, 39], resulting from they contain different N-acetyl moiety with chitin and chitin oligosaccharides, which are

important for recognition of LysM domain protein [39, 41, 42]. To the best of our knowledge, the receptors or binding proteins for COS in plants have not been identified.

In our study, wheat was selected as the plant sample to determine whether COS exist the binding interaction with plasma membrane proteins in wheat protoplast plasma membrane surface. Firstly, the fluorescent signals of COS were enriched on the plasma membrane of wheat (Fig. 1B) by using 2-AMAC-COS. This result was consistent with previous studies using strawberry and tobacco as the subject for COS treatment [22-24]. Moreover, 2-AMAC-COS also showed a dot-staining pattern in the cytosol, which suggested that COS also had special binding targets inside the cell. However, it should be noted that the signal in the cytosol had lower intensity than that on the plasma membrane, which demonstrated that the binding sites of COS are mainly located on the cell surface. In addition, the maximal frequency shifts of 130 Hz in QCM showed high responses between the COS and plasma membrane proteins to imply the interaction of COS with plasma membrane proteins of wheat. These results indicated that the COS binding sites exist on the protoplast surface, while it is possible that COS also crossed the membrane into the cytoplasm. Moreover, those COS binding sites very much depend on those plasma membrane proteins of wheat.

The results of SDS-PAGE and HPLC suggested that COS bound to more than one membrane protein with a similar molecular weight 66-97 kDa, which is may possibly result from the different of isoelectric point. LC-MS/MS analysis helped to identify 23 membrane protein candidates involved in the COS-plant interaction (Table 1), where the top three of them are especially interesting (Fig. 5). W5G2U8_WHEAT was found as a wheat homolog of Wall-associated receptor kinases (WAKs) in *A. thaliana* (Fig. 6). Until now five WAKs have been identified in *A. thaliana* [43-45]. WAK1 has been identified as a receptor reacting with pectin, as well as its oligosaccharide α-1-4 oligo-galacturonic acid [6, 45]. The GUB-WAK-bind domain which contains rich cysteine, is part of the extracellular domain of this serine/threonine kinase, can bind pectin in cell walls [45-47]. On the other hand, the STYKc domain is part of the phosphorylated kinases and might relate with immune signal transduction (Fig. 5). WAK1 receptor recognizes oligogalacturonic acids to induce resistance response [7, 44, 46, 48, 49] and WAK3 expression pattern is related to lesions [50]. Those observations raise the possibility that W5G2U8_WHEAT functionalizes in COS binding and plant resistance response.

W5HY42_WHEAT and W5I0R4_WHEAT were both identified as putative G-type lectin S-receptor-like serine/threonine-protein kinases (GsSRK), which showed a homology to GsSRK in *A. thaliana* (Fig. 7). They are all belonged to Lectin-like protein which related to immune stress responses in plants [5, 51-53]. Furthermore, GsSRK has a B-lectin domain which is usually related to mannan-oligosaccharides binding region [54-57]. It thus suggests that B-lectin domain of GsSRK can also bind COS. STKc-IRAK, the intracellular part of the GsSRK, contains serine/threonine catalyzed region. It has the function of protein phosphorylation, which related with signal transduction. Moreover, GsSRK has shown a function related to plant tolerance to stress [58]. Those observations raise the possibility that W5HY42_WHEAT and W5I0R4_WHEAT functionalize in COS binding and plant resistance response.

5. Conclusion

In conclusion, we confirmed that COS could interact with plasma membrane proteins in wheat protoplast plasma membrane surface. COS-Agrose-4FF affinity column was used to separate putative COS binding proteins from wheat plasma membrane proteins. SDS-PAGE demonstrated that the molecular weight of the COS binding proteins was between 66 kDa and 97 kDa, where W5G2U8 (a potential WAK1 receptor protein), W5HY42 and W5I0R4 (potential GsSRK receptor proteins) were identified. This study showed that COS binding proteins existed on the plasma membrane surface of the wheat cell. They are associating with the function of oligosaccharide which could recognize and stimulate the defense immunity of the plant. Based on the finding, our research suggests COS as MAMPs recognize potential PRRs including W5G2U8, W5HY42, and W5I0R4. Moreover, the plant immune defense is stimulated by immune signals transferred to cells via interactions between COS and COS binding proteins. Our study is a major step forward to identify the COS binding proteins from wheat cell, which will greatly contribute to a better understanding of the plant immune mechanism of COS.

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Highlight

Several COS binding proteins were preliminarily identified in the wheat cell surface.

COS binding proteins have the function of kinase receptor.

The COS affinity column was firstly adopted to purify COS-binding proteins.