Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review

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REVIEW ARTICLE

Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review

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Abstract In agriculture, current control of pathogens relies mainly on chemical fertilizers and pesticides. However, alternative solutions are needed due to concerns for public health, environmental protection, and development of resistant pests. Chitosan is a nontoxic, biodegradable biopolymer showing antimicrobial and plant-immunity eliciting properties. Here, we review chitosan antimicrobial activities, modes of action, and the elicitation of plant defense responses. The major points are the following: (1) Chitosan exhibits various inhibitory efficiency against bacteria, fungi, and viruses; (2) the five main modes of action of chitosan are electrostatic interactions, plasma membrane damage mechanism, chitosan-DNA/RNA interactions, metal chelation capacity of chitosan, and deposition onto the microbial surface; (3) the elicitation of plant defense responses by chitosan may be related to various pathogenesis-related proteins, defense-related enzymes, and secondary metabolites accumulation, as well as the complex signal transduction network. The facing problems and strategies for antimicrobial mechanism research and agricultural application of chitosan are also discussed.

Keywords Chitosan · Plant diseases · Antimicrobial · Defense responses · Signal transduction · Agriculture

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Contents

1 Introduction

In agriculture, pathogens cause many important plant diseases and are responsible for losses in crop yield and quality in all parts of the world. Besides that, many pathogenic fungi can also produce kinds of harmful toxins and metabolites in the infection process, which is a great threat to the safety of

agricultural products. The end result of pathogens infection is a reduction in plant growth, lower yield, inferior product quality, and huge economic loss. Therefore, plant diseases need to be controlled to maintain the quality and safety of agricultural products. During the past 100 years, crop protection has relied heavily on chemical fertilizers and pesticides. However, the chemical pesticide is a double-blade sword. Excessive use of pesticides and fertilizers helps farmers raise productivity significantly, but it also harms biological diversity, natural and agricultural systems, and public health and leads to the development of resistant strains (Sun et al. 2012). As relatively recent, terms, genetic engineering, and genetic modification are ad hoc approaches that could improve plant traits, such as disease resistance and production of useful goods. However, in the face of public concerns about the safety of the genetically modified crops, alternative methods should be provided to solve the real problems in agricultural production. Therefore, it is important to develop environmentally friendly pesticides and techniques that can be used to reduce pesticide use while ensuring the healthy development of plants and sustainable agriculture. Natural products are an excellent alternative to synthetic pesticides as a means to reduce negative impacts to human health and the environment.

Chitosan, β-(1,4)-2-amino-2-deoxy-D-glucose, is a natural versatile biopolymer derived by partially deacetylation of chitin (Fig. 1), mainly as the structural component of the exoskeletons of crustaceans and insects, as well as in some fungal cell walls (Sanford 2003). In 1859, French C. Rouget reported finding chitosan after boiling chitin in potassium hydroxide. From then on, chitosan has attracted considerable interest in various fields due to its unique biological activities, such as biocompatibility (Hsu et al. 2011; Mi et al. 2002), biodegradability (Kim et al. 2011), nontoxicity (Shi et al. 2006), antimicrobial activity (Li et al. 2008; Rabea et al. 2009), antitumor activity (Toshkova et al. 2010), and immune-enhancing effect (Li et al. 2013c; Zaharoff et al. 2007). These properties make chitosan a promising candidate for medicine (Tan et al. 2013), food (Dutta et al. 2009; Qiu et al. 2014), cosmetic (Ray 2011), water treatment (Bhatnagar and Sillanpää 2009), and biomedical engineering industries (Silva et al. 2012; Upadhyaya et al. 2013), as well as for many agricultural uses (Cota-Arriola et al. 2013; El-Hadrami et al. 2010). In fact, a number of commercial applications of chitosan benefit from its antimicrobial activity. As a versatile material, chitosan exhibits proved antimicrobial activities against fungi, bacteria, and viruses and acts as an elicitor of plant defense mechanisms. With the wide-spectrum antimicrobial activities, chitosan has been utilized to reduce or prevent the spread of pathogens (Li et al. 2013a; Mansilla et al. 2013; Fig. 2) or to enhance plant innate immunity defenses (El-Ghaouth et al. 1994; Amborabé et al. 2008; Fondevilla and Rubiales 2012). The interplay of antimicrobial and eliciting properties makes chitosan a potential antimicrobial agent to control plant disease caused by pathogens. Furthermore, chitosan is an abundant and biodegradable biopolymer derived from chitin, which is the second large renewable resource after cellulose in the world. In addition, toxicity tests that reported the lethal dose for 50 % of test animals (LD_{50}) of chitosan in laboratory mice exceed 16 g/day/Kg body weight, which is very close to that of salt or sugar (Dodane and Vilivalam 1998; Singla and Chawla 2001). Therefore, the development of chitosan pesticide has potential social and economic benefits.

Based on the current state of research and progress in corresponding areas, this review is organized into sections discussing the antimicrobial properties of chitosan against plant pathogens (including fungi, bacteria, and viruses), the modes of action as antimicrobial compounds, and the ability to elicit natural plant defense responses.

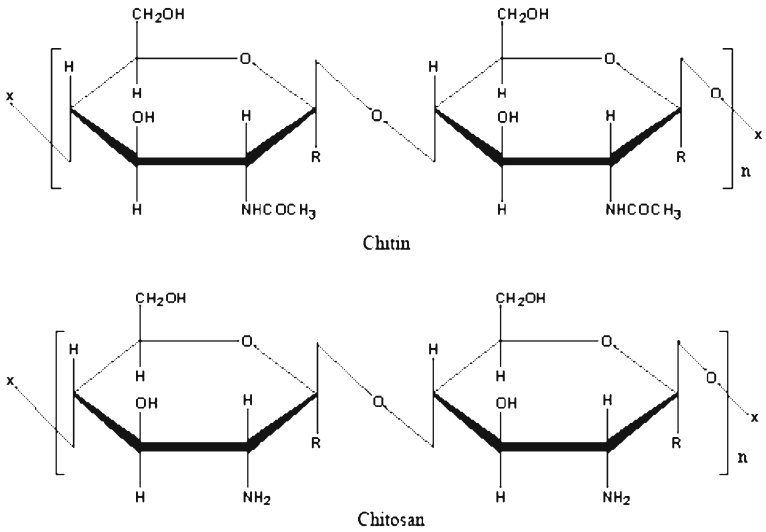

Fig. 1 The structure of chitin and chitosan. Chitin and chitosan are nitrogenous polysaccharides. The structure of the chitin molecule is similar to that of cellulose, but it is composed of the units of 2-acetylamino-2-deoxy-D-glucopyranose bound by a glycosidic bond. In contrast to chitin, chitosan amino groups are not mostly acetylated

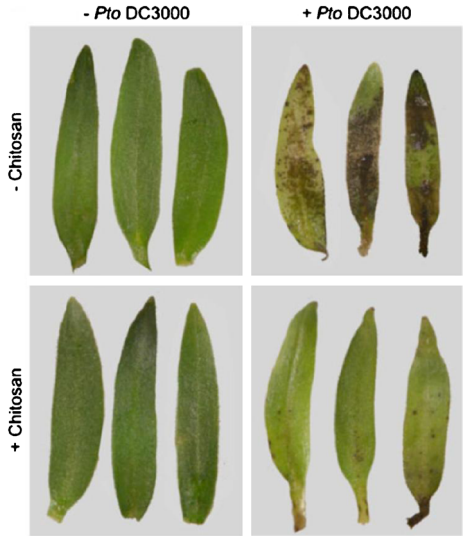

Fig. 2 Chitosan counteracted *Pto* DC3000 bacterial colonization in tomato seedlings. Disease phenotype of seedlings pretreated with 10 μg/mL chitosan (+*Chitosan*) or 0.001 % (*v/v*) acetic acid (−*Chitosan*) and then immersed in *Pto* DC3000 cell suspension (+*Pto DC3000*) or sterile distilled water containing 0.025 % (*v/v*) SILWET L-77 plus 10 mM $MgCl_2$ (−*Pto DC3000*). Photographs were taken at 7 days postinoculation. Tomato seedlings were healthy (−*Chitosan and* −*Pto DC3000*); it could induce the characteristic symptom of bacterial speck of tomato and make the seedling wilting and tawny (−*Chitosan and* + *Pto DC3000*); tomato seedlings remain healthy which showed that chitosan was harmless to plants (+*Chitosan and* −*Pto DC3000*); it significantly decreased bacterial damages in cotyledons which revealed that chitosan contributed to counteract bacterial growth in tomato seedlings (+*Chitosan and* + *Pto DC3000*)

2 Antimicrobial activities of chitosan

In 1979, the first study reported that chitosan showed a broad range of activities and a high inactivation rate against both Gram-positive and Gram-negative bacteria (Allan and Hadwiger 1979). Since then, many studies on the antimicrobial properties of chitosan and its derivatives have been reported (No et al. 2002; Xing et al. 2008; Lee and Je 2013). In modern agriculture, lots of plant pathogens have been found to be sensitive to chitosan (Manjunatha et al. 2008; Rabea et al. 2009; Li et al. 2013a, b, c). Although chitosan has been proved to be effective against bacteria, fungi, and viruses, it exhibits different inhibitory efficiencies against different microbial species.

2.1 Against fungi

As a broad-spectrum fungicide, chitosan has been shown to be fungicidal against several fungal plant pathogens (Liu et al. 2001; Wiśniewska-Wrona et al. 2007; Rabea and Steurbaut 2010; Table 1). Chitosan can effectively inhibit the development of phytopathogenic fungi at different life-cycle stages. For instance, chitosan completely inhibited spore germination, germ tube elongation, and mycelial growth of *Alternaria kikuchiana* Tanaka and *Physalospora piricola* Nose at 5.0 g/L in vitro (Meng et al. 2010). In pear fruit, treatments with chitosan reduced the disease incidence and inhibited the lesion expansion caused by these two fungal pathogens (Meng et al. 2010). In commercial winegrapes, chitosan effectively inhibited growth of *Botrytis cinerea* in liquid culture and suppressed gray mold on detached grapevine leaves and bunch rot (Reglinski et al. 2010). Chitosan exhibited strong antifungal activity against *Rhizoctonia solani*, the rice sheath blight pathogen. Two types of acid-soluble chitosan (with different degrees of deacetylation) caused a 60–91 % inhibition in mycelial growth, 31–84 % inhibition of disease incidence, and 66–91 % inhibition in lesion length (Liu et al. 2012).

Chemical modifications as an approach are efficient in enhancing the biological activity against some economic plant pathogenic fungi and bacteria and widening their applications (Guo et al. 2006). Chitosan hydrochlorides, even at the lowest test concentration of 0.0025 %, inhibited growth of the *Candida* species significantly (Seyfarth et al. 2008). In the bioassay of *Fusarium oxysporum* and *Pythium debaryanum*, *N*-(benzyl) chitosan derivatives exhibited high inhibition percentage of spore germination at 1,000 mg/L (Rabea et al. 2009).

As evaluated by leakage of proteinaceous and other UV-absorbing material, there was no significant increase in leakage and any apparent symptoms of phytotoxicity when plants were grown in the presence of chitosan, even at a higher chitosan concentration (Kong et al. 2010), which showed that chitosan was harmless to plants.

2.2 Against bacteria

Chitosan and its derivatives inhibited the growth of a wide variety of bacterial plant pathogens (Liu et al. 2001; Wiśniewska-Wrona et al. 2007; Rabea and Steurbaut 2010; Badawy et al. 2014; Table 1). Based on the available evidences, bacteria appear to be generally less sensitive to the antimicrobial action of chitosan than fungi (Kong et al. 2010). In various microbial species, the antibacterial efficiency of chitosan against Gram-positive and Gram-negative bacteria is different, however, somewhat controversial. Several researchers have demonstrated that chitosan exhibited higher inhibition effects on Gram-positive bacteria than on Gram-negative bacteria (No et al. 2002; Tayel et al. 2010; Lee and Je 2013). Concerning the bacteria surface structure, Gram-positive bacteria tend to have a loose cell wall, while Gram-negative bacteria have an outer membrane structure in the cell wall. As a polymeric macromolecule, chitosan is unable to pass through the outer membrane of Gram-negative bacteria, since this membrane functions as an efficient outer permeability barrier against macromolecules (Helander et al. 2001). While in other studies, Gram-negative bacteria were more

Table 1 The minimum growth inhibitory concentrations (MIC) of native chitosan or its derivatives against fungal and bacterial plant pathogens

Microorganisms	Chitosan samples	MIC (ppm)
Fungi		
Botrytis cinerea	Chitosan	10
Drechstera sorokiana	Chitosan	10
Fusarium oxysporum	Chitosan	100
Micronectriella nivalis	Chitosan	10
Piricularia oryzae	Chitosan	5,000
Rhizoctonia solani	Chitosan	1,000
Trichophyton equinum	Chitosan	2,500
Bacteria		
Agrobacterium tumefaciens	*N*-(*o*,*o*-dichlorobenzyl) chitosan	500
Agrobacterium tumefaciens	Quaternary *N*-(benzyl) chitosan	500
Agrobacterium tumefaciens	*N*-(benzyl) chitosan	800
Clavibacter michganensis subsp. *michganensis*	Chitosan	1,000
Erwinia carotovora	Chitosan	200
Erwinia carotovora	*N*-(*o*,*o*-dichlorobenzyl) chitosan	480
Erwinia carotovora	Quaternary *N*-(benzyl) chitosan	600
Erwinia carotovora	*N*-(benzyl) chitosan	700
Erwinia carotovora	*N*-(α-methylcinnamyl) chitosan	1,025
Erwinia carotovora subsp. *carotovora*	Chitosan	5,000
Xanthomonas campestris	Chitosan	500

susceptible to chitosan (Park et al. 2004; Du et al. 2009). They suggested that hydrophilicity in Gram-negative bacteria is significantly higher than that in Gram-positive bacteria, making them more sensitive to chitosan (Chung et al. 2004). Moreover, the Gram-negative cell envelope contains an additional outer membrane composed by phospholipids and lipopolysaccharides, which face the external environment. The highly charged nature of lipopolysaccharides confers an overall negative charge to the Gram-negative cell wall. Therefore, Gram-negative bacteria with high electronegative charge will interact more effectively with the polycationic chitosan compared with Gram-positive bacteria. Besides microorganism species, diverse consequences may be due to various initial reaction material and conditions, such as pH, molecular weight, and degree of deacetylation of chitosan, etc. (Kong et al. 2010; Younes et al. 2014).

The in vitro antibacterial effect of chitosan and its ability in protection of watermelon seedlings from *Acidovorax citrulli* were evaluated. The disease index of watermelon seedlings planted in soil and the death rate of seedlings planted in perlite were significantly reduced by chitosan at 0.40 mg/mL compared with the pathogen control (Li et al. 2013b). Chitosan solution at 0.10 mg/mL markedly inhibited the growth of *Xanthomonas* pathogenic bacteria from different geographical origins. The surviving cell numbers in the chitosan solution decreased more than 3.86 $\log_{10}$CFU/mL compared with the control after 6 h of incubation regardless of the bacterial strain (Li et al. 2008). As shown in Fig. 2, pretreatment of tomato seedlings with 10 μg/mL chitosan before *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) inoculation significantly decreased bacterial damages in cotyledons compared with control (Mansilla et al. 2013). Not only does chitosan inhibit planktonic cell growth but also it affects the already established biofilms. Unexpectedly, log reductions were in some cases higher for biofilm than for planktonic cells, deserving further more detailed work (Orgaz et al. 2011).

2.3 Against viruses

Compared with the studies of antibacterial and antifungal activity of chitosan, relatively few research studies of its antiviral activity have been reported (Su et al. 2009). The antiviral activity of chitosan in animals, microorganisms, and plants has been reviewed (Chirkov 2002; Wang et al. 2012). Chitosan inhibited viral infections in animal cells and prevented the multiplication of bacteriophages in infected cultures of microorganisms (Chirkov et al. 2001; Chirkov 2002). In plants, chitosan induced resistance toward viral diseases and inhibited the systemic spreading of viruses and viroids so that most or all plants treated with chitosan did not develop systemic viral infection (Chirkov 2002; Rabea et al. 2003). Low-molecular chitosan inhibited the formation of local necroses induced by tobacco mosaic virus for 50–90 % (Davydova et al. 2011). Actually, the direct inhibitory effect of chitosan on viruses was mainly manifested in the inactivation

of viruses. Chitosan was effective in inhibiting coliphage infection and the replication of 1–97 A phage in *Bacillus thuringiensis* culture. When added to a phage suspension, chitosan decreased its titer. Electron microscopic observations showed that chitosan caused structural changes in phage particles and damaged their integrity (Chirkov 2002). Electron microscope photographs of tobacco mosaic virus suspension showed that the number of virus particles was notably decreased and most of them twisted together and bound into a bundle (Hu et al. 2009).

3 Antimicrobial mechanism of chitosan

Ever since the wide-spectrum antimicrobial activity of chitosan was discovered, great interests in this polymer and its derivatives have increased in recent years due to their unique properties. Undoubtedly, more and more research studies proved their potential use in agriculture, medical industry, food industry, and so on. As we all know, research of antimicrobial mechanisms is an absolutely necessary stage of the microbicide development. However, the exact mechanisms of the antimicrobial activities of chitosan and its derivatives are still unknown, which limit their further application to some extent. In the past decades, various mechanisms of action have been proposed to explain the antimicrobial activity of chitosan (Table 2). On the basis of present research studies, the antimicrobial mechanisms of chitosan and its derivatives can be summarized as follows.

3.1 Electrostatic interactions

Polycationic polymer chitosan has so many reactive amino groups in its structure that can be protonated, and thus the polymer will bear positive charge, while chitin as an *N*-acetylglucosamine polymer does not show any antimicrobial activity. Differences in the structures might account for their varying inhibition effects, which also suggest that the presence of amino groups is the base of the antimicrobial activity of chitosan. Because of the stable crystalline structure, chitosan is normally insoluble in water, but soluble in dilute aqueous acidic solutions below its pKa (~6.3), in which amine ($-NH_2$) groups in glucosamine units are converted into the soluble protonated form ($-NH_3^+$) (Madihally and Matthew 1999; Pillai et al. 2009; Silva et al. 2012). It was observed that dimethylaminoethyl-chitosan 90 prepared from 90 % deacetylated chitosan had more activity than dimethylaminoethyl-chitosan 50 prepared from 50 % deacetylated chitosan (Je and Kim 2006), which meant that the amino group (NH_3^+) as the active functional group was found to be essential to the antibacterial activity of chitosan (Chung and Chen 2008). However, a selection of three chitosan derivatives with increasing positive charge render the prevailing electrostatic explanations questionable, since chitosan-thioglycolic acid (slightly positive zeta potential) had superior effects compared with trimethyl chitosan (highly positive zeta potential) with all microbes tested (Geisberger et al. 2013). However, these observations did not repudiate electrostatic interactions of chitosan-pathogens totally and revealed that the antimicrobial action of water-soluble chitosans was dependent on the degree of deacetylation and the substituted group (Je and Kim 2006). It shed light on the modes of action of chitosan that is probably more complex, involving a series of molecules that may ultimately lead to a killing process.

The Gram-positive bacterial cell wall is made up of thick peptidoglycan layer that is rich in teichoic acids, which are negatively charged because of the presence of phosphate groups in the structure. While in Gram-negative bacteria, lipopolysaccharides impart a strongly negative charge to the bacterial surface. Also, there are similar negatively charged compounds (e.g., proteins and glycoproteins) in the fungal cell membrane and viral envelope. Thus, the positively charged chitosan molecules potentially interact with negatively charged pathogen surfaces, which is termed as electrostatic interactions, can destroy the cell structure, cause extensive cell surface alterations, and increase membrane permeability (Rabea et al. 2003; Chung et al. 2004; Liu et al. 2004), leading to the leakage of intracellular substances and ultimately resulting in impairment of pathogen vital activities (Helander et al. 2001; Zakrzewska et al. 2005; Je and Kim 2006).

To verify the possible involvement of teichoic acids of *Staphylococcus aureus* in chitosan's antimicrobial activity and to analyze their role in chitosan susceptibility, Raafat et al. (2008) tested *S. aureus* strain SA113 together with four mutants lacking one or more genes involved in teichoic acids biosynthesis. The minimum growth inhibitory concentration (MIC) of chitosan for wild-type *S. aureus* SA113 was 84.8 μg/mL. The *S. aureus* SA113*ΔtagO* deletion mutant, a completely lacked wall teichoic acids, was the most resistant of the strains to chitosan, with an MIC at 545.5 μg/mL (more than 5-fold higher than the wild type). The *S. aureus* SA113*ΔdltA* mutant, which lacked the D-alanine modification in teichoic acids, as a result of which the cells carried an increased negative surface charge, was almost 100 times more susceptible to the action of chitosan, with an MIC as low as 0.9 μg/mL. These data clearly indicated that teichoic acids played a major role in the chitosan-bacteria interaction since the lack of teichoic acids in *Staphylococcus* resulted in a less negatively charged cell wall and increased resistance to chitosan, and further substantiated the hypothesis that the polycationic nature of chitosan is a major factor contributing to its antimicrobial activity.

To clarify the possible role of phospholipids, the main composition of teichoic acids, involved in the antimicrobial

Table 2 Antimicrobial action of chitosan and 1,329 its derivatives

Chitosan sample	Microorganism	Mechanism	Reference
ε-Polylysine-chitosan	*Escherichia coli*, *Staphylococcus aureus*	Disrupted bacterial cell membranes with release of cellular cytoplasm	Liang et al. 2014
Gallic acid-*g*-chitosan	Foodborne pathogens	Increased the release of intracellular components; disrupted cell membranes	Lee and Je 2013
Chitosan	*Saccharomyce. cerevisiae* deletion mutants	Disrupted protein synthesis and membrane integrity	Galván et al. 2013
Chitosan	*Pseudomonas syringae*	Electrostatic interactions; caused morphological changes and damage in bacterial surfaces	Mansilla et al. 2013
Chitosan-thioglycolic acid	*Streptococcus sobrinus; Candida albicans; Neisseria subflava*	Affected cell wall integrity and intracellular ultrastructure	Geisberger et al. 2013
Chitosan-arginine	*Pseudomonas fluorescens; E. coli*	Increased membrane permeability resulted from chitosan-membrane interaction	Tang et al. 2010
Chitosan	*Beauveria bassiana; Pochonia chlamydosporia; F. oxysporum f.* sp. *radicis-lycopersici; Neurospora crassa* wild-type strain; *N. crassa* fatty acid desaturase mutant	Membrane fluidity determines sensitivity of filamentous fungi to chitosan	Palma-Guerrero et al. 2010
Chitosan	*Rhizopus stolonifer*	Induced K+efflux, inhibited H+-ATPase activity	García-Rincóna et al. 2010
Chitosan	*N. crassa*	Permeabilized the plasma membrane and killed cells in an energy-dependent manner	Palma-Guerrero et al. 2009
Chitosan nanoparticles	*E. coli; S. aureus*	Damaged cell membrane structures and putative bind to extracellular or intracellular targets	Xing et al. 2009b
Chitosan	*E. coli*; *S. aureus*	Electrostatic interactions, destroyed cell structures, induced the leakage of enzymes and nucleotides	Chung and Chen 2008
Chitosan microspheres	*E. coli*	Influenced the structure and permeability of membrane; caused cellular leakage	Kong et al. 2008
Chitosan	*S. simulans*; *S. aureus*	Electrostatic interactions; TA might represent a "target" for chitosan's action	Raafat et al. 2008
Chitosan	*Aspergillus fumigatus; B. cinerea Aspergillus. parasiticus; F. oxysporum; F. solani; Penicillium verrusosum* var. *verrucosum*	Had an affinity for plasma membrane lipids	Park et al. 2008
Chitosan	*E. coli*; *S. aureus*	Killed bacteria through cell membrane damage	Liu et al. 2004
Chitosan	*E. coli*; *P. aeruginosa*; *Salmonella typhimurium*	Disrupted the outer membrane of Gram-negative bacteria	Helander et al. 2001

action of chitosan, lecithin and Na_3PO_4 were used to simulate the effect of phospholipids and phosphate groups in the cytoplasmic membrane. Results showed that no matter whether treated with lecithin or phosphate groups, chitosan could inhibit the growth of *Escherichia coli* effectively. It meant that lecithin or phosphate groups did not influence the interaction between chitosan and *E. coli*. While in the case of *S. aureus*, the addition of lecithin or phosphate groups apparently influenced the inhibition rate (Xing et al. 2009b). Therefore, it is presumable that phospholipids might be a target molecule in the chitosan-pathogen interaction that occurred at the cell surface of *S. aureus*. The different effects of lecithin and phosphate groups on the antibacterial activity against *E. coli* and *S. aureus* have proved once again that the mechanisms of the antimicrobial activity of chitosan were different for Gram-positive and Gram-negative bacteria.

3.2 Membrane damage mechanism

A major function of the cell wall and cell membrane is to protect the interior substances so that they would not leak to the cell exterior. The electrostatic interaction, between the positively charged amino groups of chitosan and the negatively charged residues of macromolecules exposed at the microbial surfaces, changed the permeability of cell membranes and thereby caused the death of bacteria (Helander et al. 2001). Chitosan was found to react with both the cell wall and cell membrane, but not simultaneously, indicating that the inactivation of pathogens by chitosan occurs via a two-step sequential mechanism, i.e., an initial separation of the cell wall from its cell membrane, followed by destruction of the cell membrane (Chung and Chen 2008).

Light and electron microscope investigations revealed that growth inhibition of *F. oxysporum* as a response to chitosan was accompanied by marked cellular changes, which included hyphal swelling, increased vacuolation, retraction, and alteration of the plasma membrane, cytoplasm aggregation, and abnormal cell wall deposition (Benhamou 1992). In electron micrographs, the outer membrane of chitosan-treated *E. coli* was disrupted and covered by an additional tooth-like layer. In micrographs of chitosan-treated *S. aureus*, the membrane of dividing cells was disrupted in the constricting region with the loss of cell contents (Liu et al. 2004). Similar results were reported by lots of research studies with different chitosan derivatives and tested strains (Lee and Je 2013).

However, whether such remarkable modification is resulted from the direct effect of chitosan is unknown. This is because chitosan solution cannot be directly observed in electron micrographs, which makes it difficult to investigate the mode of action of chitosan on microbes. Our previous work gave a direct evidence for such interaction, which employed oleoyl-chitosan nanoparticles, combined with *E. coli* and *S. aureus* to explore the antibacterial interaction. Electron microscopy clearly demonstrated oleoyl-chitosan nanoparticles with intact spherical structure adhered to the surface of *E. coli* and *S. aureus* and efficiently permeabilized bacterial cell membranes (Xing et al. 2009a; Fig. 3). The morphological changes were observed more obviously as the contact time increased continuously (Xing et al. 2009a).

Besides morphological changes, detection and quantification of amino acids residues in membrane proteins reflected the

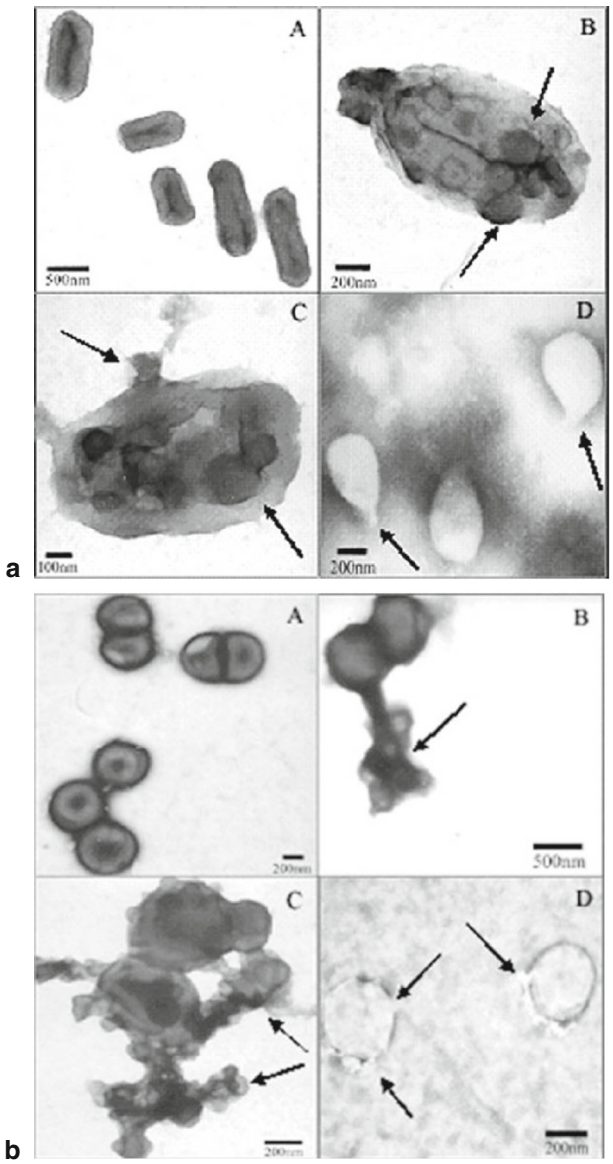

Fig. 3 Transmission electron microscope of *E. coli* (**a**) and *S. aureus* (**b**) cells treated with 300 mg/L oleoyl-chitosan nanoparticles for up to 30 min. **a** Untreated cell displayed a smooth and compact surface. **b** Some nanoparticles with intact spherical structure (*arrows pointed to*) adhering to the surface of cell after nanoparticles treated for 5 min. **c** Deep roughening and collapse of the cell surface was found after 15 min. **d** Apparent holes and loss of cell contents (*arrows pointed to*) were observed in lysed bacteria, surrounded by dark floccules instead of spherical nanoparticles after 30 min

integrity of cell membranes indirectly. When antibacterial agents interacted with cell membranes, the conformation of membrane proteins would be changed, and then Tyr residues located inside the membrane would be exposed to the surface (Ye et al. 2007). After treatment with oleoyl-chitosan nanoparticles, the fluorescence intensity of Tyr residues increased in *E. coli* and *S. aureus*, which indicated that chitosan influenced the structure of cell membranes by interacting with proteins on the cell membrane of the bacteria (Xing et al. 2009b). Accordingly, it is speculated that membrane proteins would be one of the target molecules on cell surfaces for chitosan's action.

The efflux of potassium ions was identified as an early response of the cell to the presence of some cationic compounds. A rapid efflux of potassium depended on the chitosan concentration was observed. In addition, there was an important inhibitory effect of chitosan on H^+-ATPase activity in the plasma membrane of *Rhizopus stolonifer*. The decrease in the H^+-ATPase's activity could provoke the accumulation of protons inside the cell, which would result in the inhibition of the chemiosotic driven transport that allows the H^+/K^+ exchange (García-Rincóna et al. 2010).

As we mentioned above, the plasma membrane protected the cell from harmful substances present in the external environment from entering into the interior. Why does the plasma membrane form a barrier to chitosan in some species but not in others? By imaging fluorescently labeled chitosan, a recent work shed new light on this question. It was observed that chitosan bound to the conidial surfaces of all species tested but only consistently permeabilizes the plasma membranes of chitosan-sensitive fungi. This suggested that the plasma membrane formed a barrier to chitosan in chitosan-resistant fungi but not in chitosan-sensitive fungi. Fatty acid analysis revealed that the plasma membranes of chitosan-sensitive fungi were shown to have more polyunsaturated fatty acids than chitosan-resistant fungi, suggesting that their permeabilization by chitosan may be dependent on membrane fluidity. Moreover, a fatty acid desaturase mutant of *Neurospora crassa* with reduced plasma membrane fluidity exhibited increased resistance to chitosan. These findings suggested a new strategy for antifungal therapy by increasing plasma membrane fluidity to make fungi more sensitive to fungicides such as chitosan (Palma-Guerrero et al. 2010).

Fluorescently labeled chitosan was found to be taken up and accumulated in bacteria and fungi by many researchers. Little is known, however, about its mode of endocytical internalization by fungal cells. A study focused on the internalization of chitosan by living cells made a number of novel findings (Palma-Guerrero et al. 2009). Sodium azide and low temperature (4 °C), two standard treatments to inhibit ATP production (Atkinson et al. 2002), prevented the endocytic marker FM4-64 uptake by chitosan-treated conidia indicating that chitosan-induced permeabilization of the plasma membrane was ATP-dependent but did not involve endocytosis.

3.3 Chitosan-DNA/RNA interactions

Chitosan with lower molecular weight is assumed to be able to pass through the bacterial cell wall (Sudarshan et al. 1992; Goy et al. 2009), destroy intracellular components from colloidal state to flocculation and degeneration, disrupt the normal physiological metabolic activity of bacteria, or directly interfere with genetic materials (Come et al. 2003; Issam et al. 2005), and then inhibit the reproduction of bacteria, resulting in the death of microorganisms ultimately. It is presumable that chitosan could bind with DNA and inhibit synthesis of messenger RNA (mRNA) through penetration toward the nuclei of the microorganisms and interfere with the synthesis of mRNA and proteins (Sudarshan et al. 1992; Rabea et al. 2003). Fluorescence micrographs evidenced that the fluorescein isothiocyanate labeled chitosan oligomers were observed at the inside of the cell. Permeated chitosan oligomers (molecular weight=8,000 and 5,000) were suggested to block the transcription from DNA to inhibit the growth of bacteria (Liu et al. 2001) and then disrupt the related protein synthesis.

Our previous studies indicated that chitosan nanoparticles efficiently permeabilized bacterial cell membranes and adhered to the bacterial surface (Xing et al. 2009a) and then penetrate into the bacteria with the contact time increased (Xing et al. 2009b). As we discussed above, the phosphate group might be an extracellular target contributing to its interaction with the positively charged chitosan, ultimately resulting in impairment of vital bacterial activity. There are also phosphate groups in the main chain of nucleic acid (DNA/RNA). It is possible that the amino groups of chitosan that possess positive charges would attract the negatively charged phosphate groups of DNA/RNA. In vitro chitosan-DNA/RNA interaction obviously inhibited electrophoretic mobility of bacterial genomic DNA or total RNA on agarose gel. The brightness of bands weakened gradually as the concentration of chitosan nanoparticles increased, showing the aggravation of chitosan-DNA/RNA interactions. The possible reason might be that negative charges of DNA/RNA had been counteracted by chitosan so that they could not move in electric field accordingly. The gel-retardation experiment pointed out that DNA and RNA might be the intracellular targets of chitosan (Xing et al. 2009b).

In a recent work, about 4,600 nonessential gene deletion mutants of *S. cerevisiae* were employed to investigate the antifungal mechanism of low molecular weight chitosan. It was found that 31 % of the 107 mutants most sensitive to chitosan had deletions of genes related primarily to functions involving protein synthesis. As the chitosan concentration ranged from 0.35 to 1.25 mg/mL, the β-galactosidase activity was reduced from 32 to 13 % of no-chitosan controls, which could be the result from interference with transcription efficiency and other processes in addition to translation (Galván et al. 2013).

3.4 Metal chelation capacity of chitosan

In the cell wall of Gram-positive bacteria, peptidoglycan accounts for about 50–80 % of the cellular dry weight, as well as a large number of special ingredients like teichoic acids. Phosphate groups of teichoic acids are able to attract divalent metal cations (Lambert 2002), especially Mg^{2+} and Ca^{2+}, to maintain enzymatic functions and the stability of cytoplasmic membranes (Elsenhans et al. 1983). For Gram-negative bacteria, lipopolysaccharides not only increase the negative charge of the cell membrane but also have a strong affinity for cations such as Mg^{2+} and Ca^{2+}. The combination of metal ions and chelating agents, such as ethylenediaminetetraacetic acid (EDTA), released lipopolysaccharides and led to the collapse of the outer membrane (Vaara 1992). As a kind of complexing reagent, chitosan is able to chelate some essential nutrients, metal ions, and trace elements necessary for the growth of bacteria and fungi. When the pH is below 6.0, protonated NH^{+} groups of chitosan compete with divalent metal ions for phosphate groups in teichoic acids or lipopolysaccharide molecules. In the presence of chitosan, the cell wet weight of *P. syringae* pv. *tomato* DC3000 decreased 50 % compared with control. However, the addition of $MgCl_2$ rescued the values of the chitosan-treated group (Mansilla et al. 2013). The addition of Mg^{2+} or Ca^{2+} increased the concentration of positive charges in the system and weakened the bacteriostatic action that mainly depends on electrostatic forces. Therefore, the antibacterial activity of chitosan decreased obviously in a dose-dependent manner when Mg^{2+} and Ca^{2+} were added to the culture medium. It suggested that disruption of the barrier properties of the outer membrane is the first step for chitosan to exhibit antimicrobial effects.

3.5 Deposition onto the microbial surface

High molecular weight chitosan can deposit onto the bacterial surface and form a dense polymer film. Chitosan-treated cells exhibited altered outer membranes, the surface of which was covered by numerous vesicular structures and an additional layer of material, causing the cell envelope to appear considerably thickened (Helander et al. 2001). The thickened cell envelope prevents nutrients from entering the cell, as well as the extracellular transport of metabolite excretion. Similar to chitosan, chito-oligomers caused blockage of nutrient flow and were responsible for the growth inhibition and lysis of *E. coli*, which were evidenced by scanning electron microscopy (Vishu et al. 2005). The deposition of cationic oligomers on to the cell surface is more prominent than membrane disruption as in the case of Gram-positive bacteria, owing to stronger association of *O*-chains to the outer membrane structure (Vishu et al. 2005). Therefore, another possibility for the antimicrobial activity of chitosan is based on the formation of polymer film to damage the physiological metabolism process of the bacteria.

4 Elicitation of plant defense responses by chitosan

Nowadays, chitosan is considered to be a promising antimicrobial agent owing to its antibacterial, antifungal, and antiviral activities. This has led to the exploitation of its properties in various aspects of agriculture. Since the 1980s, the study of chitosan has been changed from a general sewage treatment agent to plant growth regulator, fruits and vegetables antistaling agent, soil conditioner, and seed coating agent, especially in the disease control in agricultural production. Lots of studies showed that chitosan is not only an antimicrobial agent but also an effective elicitor of plant systemic acquired resistance to pathogens (Table 3). Even applied on plants together with the biological control agents, chitosan enhanced the efficacy in the control of pathogens (Vallance et al. 2011; Abro et al. 2013). It is possible for chitosan as a new type of green pesticides to play an important role in agriculture owing to its nontoxic, biodegradable, and nonpollution characteristics.

4.1 Pathogenesis-related proteins

In many plant species, response to infection by plant pathogens or various abiotic stresses is accompanied by the synthesis of low molecular weight compounds, proteins, and peptides with antimicrobial activities, which are termed as pathogenesis-related proteins (Bol et al. 1990; Selitrennikoff 2001). These pathogenesis-related proteins were first detected by Van Loon and Van Kammen (1970), when they observed accumulation of various novel proteins in leaves of tobacco after tobacco mosaic virus infection. Since then, chitosan has been described as an elicitor to induce plants produce a wide range of pathogenesis-related proteins with antimicrobial activity to protect themselves from pathogen infection. Some of these pathogenesis-related proteins are hydrolytic enzymes that target cell walls, such as chitinase and β-1,3-glucanase, the markers of plant defense responses. Since there are specific hydrolytic enzymes but no corresponding substrate in plants, these enzymes may have been retained throughout evolution for the purpose of confronting challenges by insects and fungi (Hadwiger 2013).

Since insect exoskeletons and fungal cell walls contain chitin and/or β-D-glucans as major structural components, chitinase and β-1,3-glucanase are capable of catalyzing the hydrolysis of chitin and β-D-glucans, decomposing cell walls of fungi, thus preventing the growth of fungi on the plant (El-Ghaouth et al. 1992; Abbasi et al. 2009). Furthermore, chitinase and β-1,3-glucanase very often act synergistically

Table 3 Listing of some variable applications of chitosan as an elicitor of plant defense responses

Plant/crop	Disease/condition	Efficacy	Reference
Jute	Stem rot	Enhanced the activity of defense-related enzymes	Chatterjee et al. 2014
Rice	Leaf streak, leaf blight	Accumulated defense-related enzymes	Li et al. 2013a
Watermelon	Fruit blotch disease	Direct killing effect	Li et al. 2013b
Peach	Brown rot	Enhanced antioxidant and defense-related enzymes	Ma et al. 2013
Pine	Pitch canker	Upregulated the expression level of defense-related enzymes	Fitza et al. 2013
Camellia	Anthracnose	Accumulated H_2O_2, defense-related enzymes, and soluble protein	Li and Zhu 2013
Broccoli	Native microflora	Antimicrobial coating served as carriers for bioactive compounds	Alvarez et al. 2013
Sycamore	–	Enhanced the production of H_2O_2 and nitric oxide	Malerba et al. 2012
Rice	Sheath blight	Induced activity of defense-related enzymes	Liu et al. 2012
Safflower; sunflower	Salt stress	Induced the activity of antioxidant enzymes	Jabeen and Ahmad 2013
Tomato	–	Accumulated phosphatidic acid and nitric oxide	Raho et al. 2011
Hypericum perforatum	–	Produced xanthone-rich extracts with antifungal activity	Tocci et al. 2011
Apricot	Fruit rot	Direct inhibition activity	Lou et al. 2011
Radish	Cadmium stress	Promoted the uptake of nutrients, nitrogen, potassium and phosphorous, decreased cadmium concentration	Farouk et al. 2011
Barley	Mildew	Induced stomatal closure	Koers et al. 2011
Pear	Fungal pathogens in storage	Significantly increased defense-related enzymes activity	Meng et al. 2010
Grape	*Botrytis* bunch rot	Direct antifungal activity and induction of defense-related enzymes activities	Reglinski et al. 2010
Sweet cherry	Short shelf life	Maintained quality attributes and extended the postharvest life by inducing defense-related enzymes activities	Dang et al. 2010
Fresh-cut mangoes	Short shelf life	Combined effects of postharvest heat treatment and chitosan coating on quality and antimicrobial proprieties of fresh-cut mangoes	Djioua et al. 2010
Maize	Low-temperature stress	Increased the chilling tolerance of maize seedlings and induced higher activities of antioxidative enzymes	Guan et al. 2009
Pearl millet	Downy mildew	Elevated nitric oxide accumulation and activated early defense reactions	Manjunatha et al. 2009
Pearl millet	Downy mildew	Increased the level of the defense-related enzymes	Manjunatha et al. 2008
Tobacco	Tobacco necrosis virus	Elicited callose apposition and abscisic acid accumulation	Iriti et al. 2006

in the chitin-glucan degradation of fungal cell walls. Not unexpectedly, increased resistance could be achieved in plants simultaneously expressing high levels of both enzymes (Dumas-Gaudot et al. 1996). Many reports revealed that chitosan was able to induce resistance in the host by increasing chitinase and β-1,3-glucanase activities in cucumbers, pears, and peaches (El-Ghaouth et al. 1994; Meng et al. 2010; Ma et al. 2013). More interestingly, El-Ghaouth et al. (1992) found that chitosan only induced chitinase activity in wounded strawberry fruit but not in intact fruit and suggested that the nonporous strawberry cuticle might have physically separated chitosan from the tissue and, therefore, prevented chitosan from inducing chitinase (Romanazzi et al. 2009).

Chitosan-mediated induction resulted in the rapid activation of a subset of genes called pathogenesis-related genes, generally regarded as the genes that functionally develop disease resistance. Chitosan appeared to employ multiple modes to increase pathogenesis-related gene function, including activating cell surface or membrane receptors and internal effects on the plant's DNA conformation that influenced gene transcription in turn (Hadwiger 1999). In oat leaves, chitosan strongly activated the expression of general defense response genes, such as pathogenesis-related 10 (Hoat et al. 2013). In rice seedlings, chitosan triggered a set of defense responses, including the transcriptional upregulation of defense-related genes (β-1,3-glucanase and chitinase) and accumulation of pathogenesis-related protein 1. Furthermore, chitosans of low molecular weight were more effective at inducing the described defense responses than those of higher molecular weight (Lin et al. 2005).

4.2 Defense-related enzymes

As an exogenous elicitor, chitosan can induce resistance in the host by increasing the activities of several defense-related enzymes, such as phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase, catalase, and superoxide dismutase activity.

Phenylalanine ammonia-lyase is an enzyme that catalyzes the biotransformation of L-phenylalanine to ammonia and trans-cinnamic acid (MacDonald and D'Cunha 2007). As the key enzyme of phenyl propanoid pathway, phenylalanine ammonia-lyase is induced in host tissues following pathogen infection of plant tissues and by abiotic elicitor treatments, such as chitosan (Khan et al. 2003). Phenylalanine ammonia-lyase activity in the skin of table grape berries sprayed with 1.0 % chitosan was 2-fold higher than that in the untreated control. Both preharvest and postharvest chitosan treatments significantly reduced the incidence of gray mold and were effective to control decay of table grapes (Romanazzi et al. 2002). Similar induced activity of phenylalanine ammonia-lyase was also reported to increase in response to elicitation with chitosan in rice and wheat (Li et al. 2013a).

Peroxidase is widely distributed in higher plants and contributes to the oxidization of phenolic and enodiolic cosubstrates to quinones and generates hydrogen peroxide (Borsani et al. 2001). While the exact mechanisms have yet to be elucidated, peroxidase is known to play a part in increasing plants' defenses against pathogens (Karthikeyan et al. 2005). Chitosan treatment significantly increased peroxidase activity in flesh around wound of pear fruit (Meng et al. 2010). Peroxidase activity in the peach treated with 5 g/L chitosan reached the peak at 24 h, and it was almost 3-fold as that in control fruit. Moreover, peroxidase gene expression in chitosan-treated fruit maintained relatively higher than that in control fruit (Ma et al. 2013).

Polyphenol oxidase, catalyzing the phenolic substances to synthesize lignin, is ubiquitous among angiosperms and assumed to be involved in plant defense by promoting the formation of lignin that contributes to the reinforcement of the cell wall structure preventing the penetration of pathogen (Chen et al. 2000; Li and Steffens 2002; Li and Zhu 2013). Chitosan significantly increased polyphenol oxidase activity in rice seedlings following inoculation of two rice pathogens (*Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*) (Li et al. 2013a). When injected into date palm roots at three concentrations (0.1, 0.5, and 1 mg/mL), chitosan elicited peroxidase expression activity, particularly at the concentration of 1 mg/mL, and increased the level of phenolic compounds (El-Hassni et al. 2004). Plant phenolics have been in the center of a myriad of discoveries related to plant defenses to different pathogens (Nicholson and Hammerschmidt 1992; Treutter 2006).

Catalase, which is involved in the degradation of H_2O_2 into H_2O and O_2, is the major H_2O_2-scavenging enzyme in all aerobic organisms. Accumulating evidence indicated that catalase played an important role in plant defense, aging, and senescence (Yang and Poovaiah 2002). The increase of catalase activity was detected both in the chilling-sensitive and chilling-tolerant maize seedlings after priming with chitosan at three concentrations (0.25, 0.50, and 0.75 %, *w/v*) (Guan et al. 2009). It suggested that seed priming with chitosan might accelerate their germination speed and improve their tolerance to stress conditions. Similar increase of chitosan-induced catalase activity in peach suggested that chitosan exhibited antioxidant capability (Ma et al. 2013), as enhancement of catalase is helpful to eliminate free radicals (Chen 2008). Thus, it was speculated that chitosan might delay repining and senescence of plant by regulating antioxidant enzyme.

4.3 Defense-related secondary metabolites accumulation

Secondary metabolites are not directly involved in growth or reproduction, but they are often involved with plant defense. Elicitation is a tool extensively used for enhancing secondary metabolite yields. Chitosan is an example of elicitors inducing defense-related secondary metabolites accumulation in plant tissue.

4.3.1 Phytoalexins

Phytoalexins are antifungal and antioxidative compounds synthesized by plants in response to a pathogen challenge or induced by treatment with elicitors such as chitosan. In a narrow sense, phytoalexins tend to fall into several classes including terpenoids, isoflavonoid, and alkaloids; however, researchers often find it convenient to extend the definition to include all phytochemicals that are part of the plant's defensive arsenal.

Hadwiger and Beckman (1980) demonstrated that chitosan at concentration as low as 0.9 μg/mL elicited phytoalexin induction and inhibited germination of macroconidia. When chitosan was applied to pea pod tissue with or prior to *Fusarium solani*, the tissue was protected from infection. Similar to the inhibitory effect, phytoalexin production was affected by molecular weight and degree of acetylation of chitosan. The highest phytoalexin production was achieved in grapevine leaves within 48 h of incubation with chitosan at 200 μg/mL with a molecular weight of 1,500 and a degree of acetylation of 20 % (Aziz et al. 2006). It was observed that pretreating cottonseeds with chitosan markedly increased cotton resistance to vascular wilt caused by *F. oxysporum* f. sp. *vasinfectum*. All chitosan derivates tested significantly stimulated phytoalexin (gossypol) production in roots more than stems, which greatly increased with a maximum of 1.16 mg/5 g in chitosan-treated fresh root tissue (Awadalla and Mahmoud 2005).

Therefore, chitosan can be extensively used for inducing phytoalexin accumulation in plant tissue and enhancing secondary metabolite yields (Komaraiah et al. 2003; Eilenberg et al. 2010). *Ruta graveolens* L. accumulated

various types of secondary metabolites, such as coumarins and alkaloids; both of them could be regarded as phytoalexin and defense tools for plants against pathogenic fungi. Chitosan induced a severalfold increase in the concentrations of coumarins and fluoroquinolone alkaloids. Such a dramatic increase suggested that chitosan might be participating in the natural resistance mechanisms of *Ruta graveolens*. The application of chitosan as elicitors may be considered a promising prospect in the biotechnological production of biologically active phytoalexins and other secondary metabolites (Orlita et al. 2008).

4.3.2 Lignin

Lignin is closely associated with cellulose and hemicellulose in hardening and strengthening of plant cell wall (Rajan et al. 2005). Lignification renders the cell wall more resistant to mechanical pressure during penetration by fungal appressoria as well as more water resistant and thus less accessible to cell wall degrading enzymes. Thus, it forms a barrier offering protection against microbial and chemical degradation. In the plant-pathogen interaction, the lignification of infected plant cell walls is a mechanism for disease resistance and provides plants with effective protection against pathogens. The synthesis of precursors of lignin and phenolic acids having antimicrobial activity in wheat seeds was stimulated by chitosan treatment. Chitosan also inhibited fungal transmission to the primary roots of germinating seedlings. Results suggested that chitosan controlled seed-borne *Fusarium graminearum* infection and increased the resistance in seedlings by stimulating the accumulation of phenolics and lignin (Bhaskara Reddy et al. 1999). Treatment of wounded wheat leaves with a partially acetylated chitosan hydrolysate elicited lignification at wound margins and invoked significant increases in phenylalanine ammonia-lyase, peroxidase expression, and catalase activities (Mitchell et al. 1994).

4.3.3 Suberization

Suberization is another common mechanism of cell wall for disease resistance in plants. Suberization is a tissue-specific process, whereby cell walls become impregnated with a poly(-phenolic) matrix coincident with the deposition of a poly(aliphatic) matrix between the plasmalemma and carbohydrate cell wall (Bernards et al. 1999). As a biogenic elicitor, chitosan locally and systemically stimulated wound healing in potato tuber tissues by increasing the number of wound periderm layers, accelerating the development of cork cambium (phellogen), and inducing proteinase inhibitors (Ozeretskovskaia et al. 2009).

4.3.4 Phenolic compounds

Phenylalanine ammonia-lyase is the key enzyme in the phenylpropanoid pathway and is involved in the synthesis of phenolic compounds, which are associated with the expression of disease resistance (Treutter 2006). Since chitosan produced elevated phenylalanine ammonia-lyase activity in plant, the levels of total phenolic content may also increase following chitosan treatments.

Increase in phenylalanine ammonia-lyase activity on chitosan treatment and subsequent augmentation of total phenolic contents has been previously reported in soybean leaves. Application of chitosan led to elevated activity of phenylalanine ammonia-lyase in soybean leaf tissues but markedly declined at 48 h. It was observed the total phenolic content was elevated at 60 h in chitosan-treated plants, showing a positive correlation between enzyme activity and total phenolic content (Romanazzi et al. 2002). In Eurasian traditional medicine Greek oregano, 200 and 500 ppm chitosan oligosaccharide treatments promoted plant height growth, whereas 50 and 200 ppm chitosan oligosaccharide upregulated the content of polyphenols significantly (38 and 29 %, respectively) (Yin et al. 2012). Chitosan also increased total phenolics in date palm seedlings of two cultivars, Jihel (JHL, susceptible) and Bousthami noire (BSTN, resistant). The highest phenolic levels were recorded at a chitosan concentration of 1 mg/mL 30 days after incubation, when they were about three times higher than in the control roots (Nicholson and Hammerschmidt 1992). As the major phenolic compound in sweet basil, rosmarinic acid has been reported to have various bioactive properties such as antioxidant, antimicrobial, and anti-inflammatory activities. The total amount of phenolic compounds significantly increased after chitosan treatments, especially rosmarinic acid that increased 2.5 times by 0.1 % chitosan treatment. Therefore, due to the significant induction of phenolic compounds, the corresponding antioxidant activity increased at least 3.5-fold (Kim et al. 2005).

Chlorogenic acid, another phenolic compound, is an important biosynthetic intermediate, for example in lignin biosynthesis. Studies showed that chlorogenic acid displayed antibacterial and antifungal activity against certain microorganisms (Sung and Lee 2010; Hemaiswarya et al. 2011; Atanasova-Penichon et al. 2012). In ginseng callus cultures, accumulation of phenolic compounds was increased 3-fold within 12 h after 1 % chitosan treatment. HPLC analysis revealed significantly higher levels of chlorogenic acid. Enhanced activity of phenylalanine ammonia-lyase and peroxidase and enhanced levels of phenolic compounds, for example, chlorogenic acid, all point to an enhanced defense response in ginseng rusty roots (Rahman and Punja 2005).

4.3.5 Callose

Callose exists in the cell walls of a wide variety of higher plants. It plays important roles during many processes in plant development and in response to numerous biotic and abiotic stresses, such as wounding and pathogens infection. Callose-containing cell-wall appositions, called papillae, are effective barriers that are induced at the sites of attack during the relatively early stages of pathogen invasion (Luna et al. 2011). Chitosan was known to have eliciting activities leading to callose formation in host plants in response to microbial infections (Iriti and Faoro 2008; El-Hadrami et al. 2010; Jabeen and Ahmad 2013). After treatments with 0.1 % chitosan, tobacco plants significantly reduced tobacco necrosis virus-induced necrotic lesions and enhanced inducible defenses, which was associated with a network of callose deposits, micro-oxidative bursts, and micro-hypersensitive responses (Bol et al. 1990). In fact, chitosan induced callose deposition at pathogen entry points during the initial hours of pathogen inoculation (Iriti et al. 2006). The elicited callose apposition in plant tissues exerted a determinant role in limiting microbial spread in the early phase of pathogen infection (Iriti and Faoro 2008).

4.4 Signal transduction

During the long-term coevolution, plants and pathogens have evolved an intricate relationship. Pathogens have developed an array of offensive strategies to parasitize plants, and in turn, plants have evolved a complex multilayered defense system to prevent infection (Nurnberger et al. 2004; Chisholm et al. 2006). Based on the mechanisms mentioned above and other literatures, chitosan can behave like a general elicitor, exhibiting a wide variety of defense responses to pathogens infestation, including increases in chitinase and β-1,3-glucanase, defense-related enzymes, phytoalexins, and secondary metabolites by expressing related responsive genes and defense genes. There appears to be multiple modes by which chitosan can increase these gene expressions and functions, including activating cell surface or membrane receptors and internal effects on the plant's DNA conformation that influence gene transcription in turn (Hoat et al. 2013). It is attractive to elucidate the role of chitosan in plant immunity regulation.

4.4.1 Extracellular signal perception of chitosan

The first step in the elicitor-induced transduction pathway is the recognition of the signaling molecule by a specific receptor (Benhamou 1996). In the dicotyledonous model plant *Arabidopsis*, chitin elicitor receptor kinase 1 (CERK1), a LysM receptor kinase, has been shown to play a critical role in fungal microbe-associated molecular pattern perception (Miya et al. 2007). Petutschnig et al. (2010) suggested that CERK1 was not only required for chitin but also for chitosan perception. However, a recent work showed that defense response genes were upregulated by chitosan, both in wild-type and in the chitin-insensitive *cerk1* mutant, indicating that chitosan is perceived through a CERK1-independent pathway (Povero et al. 2011).

A lectin specific for glucosamine oligomers has been purified by chitosan affinity chromatography from cultured cells of *Rubus*. Sodium dodecyl sulfate-polyacrylamide gel electroploresis (SDS-PAGE) showed that the lectin appeared as a membrane-bound protein of molecular weight 67 kDa with two apparent binding sites, i.e., the tetrasaccharide and the hexasaccharide, but did not exhibit any affinity for the cellotetraose, *N*-acetylchitotetraose, and maltotetraose. Considering its affinity for chitosan, the lectin may be a receptor for chitosan-derived oligomers with elicitor activity, which ultimately trigger plant defense reactions (Liénart et al. 1991).

Unfortunately, research papers about the binding protein or receptor of chitosan are few. To our knowledge, the lectin is the only receptor discovered that is likely to bind to chitosan. However, whether there are binding proteins for chitosan on other plants remains to be further studied.

4.4.2 Intracellular signal perception of chitosan

Besides the signal perception via cell surface or membrane receptors, many researchers (Hadwiger et al. 1989; Hadwiger 1999; Dumas-Gaudot et al. 1996) demonstrated that chitosan exhibited internal effects on the plant's DNA conformation and regulated at the chromatin level directly since chitosan entered most regions of the cell. The highly positively charged chitosan possessing a strong affinity for the negative charged phosphates of the DNA backbone, especially the minor groove of DNA (Liu et al. 2005), may compete with histone proteins containing lower densities of positive charges (Isaac et al. 2009). Chitosan treatments to the pea endocarp tissue resulted in subtle DNA fragmentation of the pea DNA within 2.5 h, indicating that it can affect DNA in vivo (Hadwiger et al. 1997). As a pathogenesis-related gene elicitor, chitosan may alter chromatin via competition with basic nuclear proteins for DNA attachment sites, potentially displacing H2A/H2B histones (Hadwiger 2008).

4.4.3 Signal transduction of chitosan with other signal molecules

When the extracellular signaling molecule chitosan activates the specific receptor on the cell membrane or located intracellular, one or more second messengers transmit the signal into the cell and create a series of physiological responses. In the

above process termed as signal transduction, a single signal can be amplified and develop a complex signaling networks. According to published literatures, reactive oxygen species (ROS), Ca^{2+}, nitric oxide (NO), ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) all involved in chitosan-mediated signal pathway.

The oxidative burst, a rapid and transient production of huge amounts of ROS, is one of the earliest responses to microbial pathogen attack (Wojtaszek 1997) and has been shown to occur upon chitosan elicitation (Luna et al. 2011). The production of ROS included hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radicals (OH^-), and so on. As the important signals mediating defense gene activation, ROS are centrally involved in the induction of plant disease resistance responses. H_2O_2 served as a signal of oxidative stress and activation of signaling cascades as a result of the early response of the plant to biotic stress (Mejía-Teniente et al. 2013). In sycamore cultured cells, 0.01 % chitosan induced an accumulation of H_2O_2 reaching about 50 nmol/g fresh weight after 24 h (Jabeen and Ahmad 2013). In *Arabidopsis* cell suspension cultures, chitosan induced the accumulation of H_2O_2 within 1 h. The addition of ascorbic acid (a H_2O_2 scavenger) blocked the formation of the brown coloration (chemical interactions took place in the presence of H_2O_2) confirming that chitosan induced H_2O_2 accumulation in the *Arabidopsis* cell cultures (Ndimba et al. 2003). Similar results were obtained in chitosan-treated sweet peppers and tomatoes (Orozco-Cardenas and Ryan 1999; Mejía-Teniente et al. 2013).

Calcium metabolism is intimately related to ROS signaling. Increase in cytosolic Ca^{2+} is also one of the fastest responses upon pathogen infection, and the use of specific inhibitors showed that Ca^{2+} influx was required for ROS production after elicitation (Blume et al. 2000; Grant et al. 2000). It was demonstrated that the polycationic nature of chitosan might lead to membrane disturbance through its interaction with negatively charged membrane phospholipids (Shibuya and Minami 2001). According to published reports, treatments that disrupt plasma membrane integrity are often accompanied by alterations of cell Ca^{2+} signaling (Pizzo et al. 2002). In suspension-cultured cells of *Glycine max*, synthesis of callose started within 20 min of treatment with chitosan and parallels over hours of the accumulation of 1,3-linked glucose in the wall. However, chitosan-induced callose formation was not possible without the presence of external Ca^{2+} and partly recovered upon restoration of 15 μM Ca^{2+} (Köhle et al. 1985). In *Arabidopsis*, chitosan induced transient elevations in the concentration of free cytosolic Ca^{2+} and stomatal closure in guard cells (Klüsener et al. 2002).

NO, another second messenger recently established in plants, is involved in the plant defense response of a growing list of plant-pathogen interactions (Klüsener et al. 2002; Lamattina et al. 2003; Neill et al. 2003). Chitosan treatment showed downy mildew disease protection of 79.8 % over the untreated control and elevated NO accumulation in pearl millet seedlings beginning from 2 h postinoculation. However, the degree of protection was reduced after NO scavenger c-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt] or NO synthase inhibitor L-NAME (*N*-nitro-L-arginine methyl ester hydrochloride) treatment; this indicated the possible involvement of NO in chitosan-induced resistance (Hadwiger 2013). In tomato cells, chitosan induced a rapid NO production, as well as the formation of phosphatidic acid by activating both phospholipase D and phospholipase C/diacylglycerol kinase. Pretreatment with NO scavenger c-PTIO inhibited the activation of either phospholipase-mediated signaling pathway. This indicated that NO was required for phosphatidic acid generation via both the phospholipase D and phospholipase C/diacylglycerol kinase pathway during plant defense response in chitosan-elicited cells (Tocci et al. 2011).

Phytohormones are not only instrumental in regulating developmental processes in plants but also play important roles for the plant's responses to biotic and abiotic stresses (Halim et al. 2006). For example, disease resistance in *Arabidopsis* is regulated by multiple signal transduction pathways in which SA, JA, and ET function as key signaling molecules in mediating or orchestrating biotic/abiotic stress responses. SA is involved in the systemic acquired resistance in which a pathogenic attack on one part of the plant induces resistance in other parts, whereas JA and ET are central signaling molecules in the induced systemic resistance. JA, the terminal product of the octadecanoid pathway, has been proposed to be part of a signal transduction pathway that regulates the induction of defense-response genes in plants against pathogen invasion. In rice, chitosan caused a rapid increase in the endogenous JA level within 3 min. Furthermore, the rise in JA level by chitosan was again significantly higher upon wounding, and reached a peak at 60 min versus 30 min in wounded leaves, suggesting that this observed increase is a specific response to applied chitosan (Rakwal et al. 2002). An oilseed rape cDNA microarray containing 8,095 expressed sequence tags was used to analyze the *Brassica napus* gene expression changes elicited by oligochitosan. Transcript levels for 136 genes were induced 2-fold or more in oligochitosan-treated seedlings compared with control seedlings. Results of semiquantification RT-PCR showed that an important JA synthase gene, a JA-mediated defense required for kinase gene, an ET receptor gene, and two ET responsive element binding protein genes were induced by oligochitosan, suggesting that oligochitosan activated the plant self-defense through JA/ET signaling pathway (Yin et al. 2006).

Recently, in a series of plant pathosystems, it has been shown that the intensity and speed of callose deposition are regulated by ABA. ABA, also called abscisin II and dormin, is now known to be the case only in a small number of plants. ABA-mediated signaling transduction also plays an important role in plant responses to environmental stress and plant pathogens (Seo and Koshiba 2002). Chitosan treatment reduced tobacco necrosis virus lesion area per leaf by 95.2 % in respect to untreated controls. Furthermore, chitosan application elicited both callose apposition and ABA accumulation in leaf tissues, at 12 and 24 h after treatment, respectively. Besides, treatment with the ABA inhibitor nordihydroguaiaretic acid, before chitosan application, reduced both callose deposition and plant resistance to the virus, thus indicating the involvement of ABA in chitosan-mediated processes. It was indicated that the increase of ABA synthesis induced by chitosan played an important role in enhancing callose deposition (Iriti and Faoro 2009).

Based on the above analysis, chitosan activated the plant self-defense through different signaling pathways or involved in signal transduction as a regulatory molecules. Despite extensive research, the mechanisms of how chitosan acted upon plant immunity regulation have not been elucidated clearly. It is believed that the mode of action of chitosan is probably more complex than assumed above, involving a series of events, which need to study further in the future.

5 Conclusion and future perspectives

During the last 150 years, ever since the discovery of chitosan, considerable progresses have been made in understanding and exploiting its new properties as well as new applications. As reviewed in this article, the versatile chitosan, naturally occurring compound, possessing broad-spectrum antimicrobial effects and plant innate immunity elicited activities, has potential in agriculture with regard to controlling plant diseases. Its application may counteract the wide use of chemical pesticides, in part at least. The polysaccharide chitosans represent a renewable source of natural biodegradable polymers and meet with the emergence of more and more food safe problems.

Though much work has been done, there are still many unclear points in the mechanisms of chitosan that inhibited the growth of pathogens and induced the plant immunity. Appropriate chemical modification could significantly enhance its antimicrobial activities, improve the physical and chemical properties, and make it more suitable for field applications. In the case of antimicrobial mode of action, future work should aim at clarifying the actual target molecule on the cell surface or other intracellular targets. It would have potential values to construct gene mutant strains for further study on the antimicrobial mechanisms. Combined transcriptome and proteome analysis of key defense genes and proteins will enhance our understanding of the complicated chitosan-mediated signal pathway and enable better biotechnological applications in plant disease control. A wider comprehensive knowledge of the mechanism of action of chitosan in pathogens and plants will increase the chance of its successful application to control disease spread in plants. We also suggest comprehensive cooperation among global chemists, microbiologists, phytophysiologists, and agronomists to better exploit chitosan's antimicrobial properties, plant innate immunity elicited activity, and biotechnological potential for agricultural sustainable development.

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