Defense responses in female gametophytes of *Saccharina japonica* (Phaeophyta) induced by flg22-derived peptides

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Abstract Plants and algae can detect the presence of bacteria via sensing of proteins or peptides of bacterial origin. Flg22, a fragment of bacterial flagellin, is one of these peptides and has been shown to be an effective elicitor in both plants and algae. Here, we investigated the elicitor activity of flg22-derived peptides in the brown alga, *Saccharina japonica*. By monitoring luminol-dependent fluorescence, we could observe that the release of H₂O₂ induced by flg22-derived peptides is maximal at 2 h after induction. The elicitor activity was depending on the length of the peptides in the order of flg22 > flg15 > flg14. Cytological observations regarding the presence of reactive oxygen species (ROS) after induction were consistent with quantitative measurements of H₂O₂ generation using a 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe. Addition of 1 µM each of flg22 and flg15 was sufficient to inhibit growth of female gametophytes. Furthermore, the elicitor activity of C-terminally shortened flg15-derived peptides suggests that flg15 apparently is the

smallest peptide with elicitor activity. Amino acid position D43 at the N-terminus of a flagellin was demonstrated to be involved in the elicitor activity. Finally, H₂O₂ was localized in the plasma membranes of female gametophytes by an NADPH oxidase inhibitor and electron-dense deposits of cerium perhydroxide by transmission electron microscope.

Keywords Defense response · flg22 derivative peptides · *Saccharina japonica* · Reactive oxygen species

Introduction

Like higher plants, algae have developed effective defense mechanisms to protect themselves against various pathogens. It is widely accepted that the initiation of defense responses largely depends on the successful recognition of the pathogens based on specific elicitors produced by the pathogens (Potin et al. 2002; Jones and Takemoto 2004; Zipfel 2009). Elicitors can be divided into endogenous and exogenous elicitors based on their origins (Weinberger and Friedlander 2000; Küpper et al. 2001, Küpper et al. 2006; Cosse et al. 2007). Endogenous elicitors originate from structural compounds of host, such as the oligosaccharides, which are released from the host cell walls during enzymatic degradation of wall components caused by the pathogens, as it has been described in the brown alga *Laminaria digitata* (Küpper et al. 2001) and in the red alga *Gracilaria conferta* (Weinberger et al. 1999). Exogenous elicitors, in contrast, originate from the pathogens and may consist of lipopolysaccharide (LPS) fragments from their outer membranes (Graham et al. 1977) of chitin fragments, derivatives of the cell walls (Sharp et al. 1984; Baureithel et al. 1994), or oligopeptides secreted by the bacteria (Wei et al. 1992; Nümberger et al. 1994).

Flagellin/flg22 is one of the best-studied exogenous elicitors, representing a pathogen-associated molecular pattern (PAMP)

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that can induce defense responses in higher plants (Medzhitov and Janeway 2002; Nürnberger et al. 2004; Sun et al. 2013; Koller and Bent 2014). Felix et al. (1999) were the first to report that a 22-amino-acid peptide called flg22, which corresponds to the highly conserved N-terminal region of flagellin, can induce immunity reaction in various plants such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), and *Arabidopsis thaliana*. Defense responses induced by flg22 include alkalization of the extracellular medium, generation of reactive oxygen species (ROS), expression of pathogenesis-related (PR) genes, callose deposition, and growth inhibition in *A. thaliana* and tomato (Felix et al. 1999; Gómez-Gómez et al. 1999). Furthermore, the aspartic acid (D) at position 43 of the flagellin was identified as an active site for the elicitor activity. Replacing this residue by valine (V) and alanine (A) in flg22 of *Pseudomonas syringae* pv. *tabaci* 6605 (flg22_{pta}D43V and flg22_{pta}D43A), abolished the elicitor activity (Naito et al. 2008). So far, it has been shown that flg15 is the smallest elicitor in tomato, having a much weaker activity than flg22 in *A. thaliana* (Felix et al. 1999; Meindl et al. 2000; Chinchilla et al. 2006). Shorter peptides have an even lower elicitor activity in various plants (Felix et al. 1999; Bauer et al. 2001). So far, flagellin and the respective recognition receptor FLAGELLINE SENSING 2 (FLS2) in *A. thaliana* are the best studied model for elicitor recognition in plants (Boller and Felix 2009). Although structure-activity relationships of flg22 have been studied over a long term, it is until 2013 to reveal the exact binding site of flg22 within the leucine-rich repeat (LRR) domains of FLS2 and BAK1 by crystallography (Sun et al. 2013).

The first indication of PAMP-induced defense responses in algae was described for the red alga *Chondrus crispus* which can be induced by its pathogen *Acrochaete operculata*. The cell-free extract of *A. operculata* triggered an oxidative burst in *C. crispus* (Bouarab et al. 1999). Additionally, liposaccharides and lipoteichoic acids isolated from the cell envelope of bacteria were found to modify gene expression of *C. crispus*, indicating that PAMP elicitors are present in these cell-free extracts (Weinberger 2007). Similarly, the red alga *G. conferta* reacts on the presence oligosaccharides with the production of ROS, which controls the growth of epiphytic bacteria (Weinberger and Friedlander 2000). LPS extracted from the Gram-negative bacterium *Salmonella abortus equi* was shown to be an efficient elicitor triggering an oxidative burst in *L. digitata* (Küpper et al. 2006). Also unicellular algae, such as diatoms, may react on the presence of bacteria not only with hypersensitive cell death (Canter and Jaworski 1979), but also with the release of defensive enzymes. For example, the diatom *Chaetoceros didymus* releases proteases which confer resistance to the lytic bacterium *Kordia algicida* (Paul et al. 2013). In striking contrast to the intensive investigations of PAMPs and induced defense responses in higher plants, knowledge on respective elicitors and immunity mechanisms in marine algae is still very poor. Recently,

Wang et al. (2012, 2013) found that flg22 can induce oxidative bursts and hypersensitive responses (HR) in both female gametophytes and sporophytes of *Saccharina japonica*, indicating that algae and plants may share similar mechanisms for recognizing pathogens.

Typically, early defense responses following the recognition of an elicitor include oxidative bursts, such as the generation of ROS including O₂ ·, H₂O₂, and ·OH. Plasma membranes and cell walls have been identified as the source of H₂O₂ during PAMP-triggered immunity responses. A membrane-located, diphenyleneiodonium chloride (DPI)-sensitive NADPH oxidase can be assembled in the plasma membranes after receptor-based recognition of pathogens or elicitors, catalyzing an electron transfer from NADPH to O₂ resulting in O₂ · and H₂O₂ (Cosse et al. 2007). While, cell wall-located pH-dependent and salicylhydroxamic acid (SHAM)- and sodium azide (NaN₃)-sensitive peroxidases can be activated due to an increase of pH caused by the cationic efflux after pathogens invading plants (Bestwick et al. 1997; Song and She 2010). These enzymes reduce O₂ rapidly to O₂ · and H₂O₂ (Song and She 2010). The sources of H₂O₂ can be distinguished by pharmacological investigations and electron microscopy, demonstrating the release of H₂O₂ in the marine red algae *G. conferta* and *Gracilaria chilensis* (Weinberger et al. 2005). However, so far, there is no report yet on the source of H₂O₂ induced by flg22 in gametophytes of the very distantly related brown alga *S. japonica*.

According to our previous study, flg22 is an effective elicitor for *S. japonica* (Wang et al. 2012; Wang et al. 2013); thus, the question raised here is whether flg22-derived peptides can also trigger defense responses in *S. japonica*. Therefore, we investigated the elicitor activity of flg22-derived peptides, as well as the active site of flg22 on female gametophytes of *S. japonica*. We also studied the subcellular origin of H₂O₂ induced by flg22.

Materials and methods

Female gametophytes of *Saccharina japonica* were cultivated as a suspension culture. The gametophytes were grown in illuminated incubators (GXZ-280A, Jiangnan, China) at 10 °C with a 12:12-h (L/D) photoperiod at 25 μmol photons m⁻² s⁻¹. The culture medium consisted of sterilized seawater provided with 0.2 mM KNO₃ and 0.02 mM KH₂PO₄ and was replaced once a week.

Flg22-derived peptides

The peptides flg22 (QRLSTGSRINSKDDAAGLQIA), flg22D43A (QRLSTGSRINSKADAAGLQIA), flg15 (RINSKDDAAGLQIA), flg14 (INSKDDAAGLQIA), flg15-Δ1 (RINSKDDAAGLQI), flg15-Δ2

(RINSKDDAAGLQ), flg15- Δ 3 (RINSKDDAAGL), flg15- Δ 4 (RINSKDDAAG), flg15- Δ 5 (RINSKDDAA), flg15- Δ 6 (RINSKDDA), flg15- Δ 7 (RINSKDD), and flg15- Δ 8 (RINSKD) were synthesized by Sangon Biotech (Shanghai) Co. Ltd. Peptides were dissolved to 4 mM in sterilized seawater and diluted in a solution containing 0.1 % bovine serum albumin (BSA).

Incubation experiments

The density of the fresh female gametophytes was generally 5 mg mL⁻¹. In order to facilitate the following experiments, the female gametophytes were broken into pieces in a crusher (JYD-P11S81, Joyoung, China) for 20 s then subcultured for 3–5 days at 10 °C with a 12:12-h (L/D) photoperiod at 25 μ mol photons m⁻² s⁻¹. Short-time incubation with different peptide concentrations was performed in an illumination incubator under the subculture conditions described above. Control experiments of sterilized seawater or 0.1 % BSA in sterilized seawater were employed. All experiments had at least three replicates.

H₂O₂ detection and quantification

H₂O₂ was monitored by measuring luminol-dependent luminescence ascribable to ferricyanide-catalyzed oxidation according to a previous method described by Takai et al. (2008) and Wang et al. (2013). Twenty microliters of incubated gametophytes was mixed with 150 μ L of 10 mM potassium phosphate buffer (pH 7.8), 20 μ L of 14 mM potassium ferricyanide, and 10 μ L of 1.1 mM luminol solution; then, the samples were placed in a luminometer (GloMax 20/20, Promega, USA) and analyzed for chemiluminescence immediately.

Cytological detection of the ROS

The gametophytes were incubated for 2 h with different concentrations of the different peptides and then were washed with sterilized seawater twice to remove the peptides, followed by re-suspension for 5 min in sterilized seawater containing 50 μ M 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma, USA). Subsequently, the gametophytes were rinsed with sterilized seawater twice to remove the residual DCFH-DA, and they were placed onto glass slides for observations by using a fluorescence microscope (BX51, Olympus, Japan) at an excitation wavelength of 380–490 nm.

Growth inhibition assay

One milliliter of the female gametophytes was added to 100 mL sterilized seawater containing 1 μ M of flg22, flg15, flg14, and flg22D43A, respectively. Controls were sterilized

seawater and 0.1 % BSA in sterilized seawater. Gametophytes were grown at 10 °C with a 24-h photoperiod at 50 μ mol photons m⁻² s⁻¹. Sterilized seawater medium was provided with 0.2 mM KNO₃, 0.02 mM KH₂PO₄, and 1 μ M of the four respective peptides and refreshed every week. The gametophytes were briefly blotted dry, and the fresh weight was measured after 40 days.

Subcellular localization of H₂O₂

The cytochemical method for localization of H₂O₂ was based on the generation of insoluble cerium peroxide according to Bestwick et al. (1997). After inoculation with flg22 for 2 h, the female gametophytes were incubated in culture medium containing 5 mM cerium chloride and 50 mM 3-(morpholino) propanesulfonic acid (Mops) buffer (pH 7.2) for 1 h. The treated gametophytes were then fixed in 3 % (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4 °C for 6 h and post-fixed in 2 % osmium tetroxide in the same buffer. Samples were dehydrated in a graded ethanol series and embedded in Araldite. The control was treated with sterilized seawater, while the positive control was treated with 5 mM H₂O₂ for 10 min. All the experiments were designed three replicates. According to our previous studies, 0.1 % BSA has no effect on the production of H₂O₂; therefore, control experiments with 0.1 % BSA were not included in this and the following experiments.

Effects of DPI, SHAM, and NaN₃ on H₂O₂ accumulation

Fragmented filamentous gametophytes at a density of 5 mg mL⁻¹ were inoculated with 200 μ M flg22 and 60 μ M DPI, 50 μ M SHAM, or 50 μ M NaN₃ for 2 h. For two control experiments, one algal suspension was treated with sterilized seawater for 2 h; the other was algal suspension treated with 200 μ M flg22 for 2 h. All experiments had at least three replicates.

Results

Quantitative detection of H₂O₂ in female gametophytes induced by flg22 and its derived peptides

The release of H₂O₂ was investigated by using a luminol-dependent assay. Within the range of 0–200 μ M of flagellin peptides, H₂O₂ release was related to the concentrations of flg22 and flg15, respectively (Fig. 1). The response was saturated at 260 μ M. The concentration of flg22 required to induce half maximal activity (EC₅₀) is 90 μ M, while the EC₅₀ value of flg15 is 110 μ M (Fig. 1). Moreover, the concentrations required to induce the same H₂O₂ production were slightly lower for flg22 compared to flg15; however, there was no

Fig. 1 Release of H_2O_2 in female gametophytes of *S. japonica* by different concentrations of flg22, flg15, flg14, and flg22D43A after 2 h of incubation. Values are means $\pm$ SD ($n=6$)

significant difference in elicitor activity induction between flg22 and flg15, while flg14 and flg22D43A did not show any elicitor activity (Fig. 1). For flg14, the production of H_2O_2 increased in a concentration-dependent manner within the range of 400 to 1000 μM and the EC_{50} was about 720 μM (Fig. S1). There was no H_2O_2 detected in female gametophytes induced by flg22D43A (Fig. S1), which implied that aspartic acid at position 43 also played a crucial role for flg22 as an effective elicitor in *S. japonica*.

Figure 2 shows the release of H_2O_2 by female gametophytes of *S. japonica* induced by flg22, flg15, flg14, and flg22D43A at concentration of 90 μM . The production of H_2O_2 induced by flg22 and flg15 increased and reached maximum values after 2 h, respectively (Fig. 2). After that time, the H_2O_2 production declined gradually and returned to the initial level after 6 h (Fig. 2). No measurable H_2O_2 was detected after induction by flg14 and flg22D43A within 6 h (Fig. 2).

Cytological detection of ROS in female gametophytes induced by flg22, flg15, flg14, and flg22D43A

The fluorescent probe DCFH-DA was utilized for cytological detection of ROS via fluorescence microscopy in the female

gametophytes of *S. japonica* after induction by flg22, flg15, flg14, and flg22D43A. Compared to the controls (Fig. 3a, b), weak green DCF fluorescence was observed in female gametophytes after induction with flg22 and flg15 at the concentrations of 20 μM . The fluorescence intensity increased with the increasing concentrations in the range of 20–200 μM . ROS-based fluorescence induced by flg22 and flg15 at a concentration of 80 μM is shown in Fig. 3c, d. In flg14-induced female gametophytes, the fluorescence was observed at a minimum concentration of 600 μM (Fig. 3e) and the fluorescent intensity increased within the range from 600 to 1000 μM . For flg22D43A, no ROS was detected even at a concentration of 1000 μM (Fig. 3f). The cytological observations were consistent with the results of luminol fluorescence detection.

Growth inhibition of female gametophytes by flg22 and flg22-derived peptides

After culturing the female gametophytes of *S. japonica* in the presence of flg22, flg15, flg14, and flg22D43A for 40 days, both flg22 and flg15 significantly induced growth inhibition of the algae at a concentration of 1 μM . The fresh weights of flg22- and flg15-challenged female gametophytes were less than one half of the control (Fig. 4). Flg14 resulted only in a weak inhibition of growth (Fig. 4), while flg22D43A did not affect growth of *S. japonica*.

Quantitative detection of H_2O_2 in female gametophytes induced by flg15 and flg15-derived peptides

At low peptide concentrations (20–200 μM), all of the flg15-derived peptides failed to induce the release of H_2O_2 . At higher concentrations (200–1000 μM), we found that H_2O_2 production induced by flg15- Δ 1, flg15- Δ 2, and flg15- Δ 3 increased with the increasing concentrations, reaching similar H_2O_2 production as with flg15 (Fig. 5). There was no significant difference in elicitor activities among the peptides of flg15- Δ 1, flg15- Δ 2, and flg15- Δ 3 (Fig. 5). The release of H_2O_2 induced by flg15- Δ 4 showed a similar trend to that of flg15- Δ 1, flg15- Δ 2, and flg15- Δ 3 within a concentration

Fig. 2 Release of H_2O_2 in female gametophytes of *S. japonica* induced by 90 μM flg22, flg15, flg14, and flg22D43A at different time courses. Values are means $\pm$ SD ($n=6$).

Fig. 3 Fluorescence images of ROS production in female gametophytes of *S. japonica* after 2 h of incubation and induced by flg22 derived peptides. **a** Control experiment treated with sterilized seawater. **b** Control experiment treated with 0.1 % BSA + sterilized seawater. **c**

flg22, 80 μ M. **d** flg15, 80 μ M. **e** flg14, 600 μ M. **f** flg22D43A, 1000 μ M. The green fluorescent spots indicate ROS production in cells of female gametophytes. Bars 10 μ m

range of 200–1000 μ M; however, flg15- Δ 4 showed a comparatively lower elicitor activity (Fig. 5). No production of H_2O_2 was detected after incubation with flg15- Δ 5, flg15- Δ 6, flg15- Δ 7, and flg15- Δ 8 (Fig. 5). These findings indicate that peptides lacking five or more residues at the C terminus (from flg15- Δ 5 to flg15- Δ 8) might not be detected by *S. japonica*. However, flg15- Δ 5, flg15- Δ 6, and flg15- Δ 7, but not flg15- Δ 8, did suppress the release of H_2O_2 induced by

flg15, respectively (Fig. 6), indicating an antagonistic effect of these three peptides.

Cytological detection of ROS in the female gametophytes induced by flg15-derived peptides

Cytological detection of ROS using DCFH-DA showed that the elicitor activity of all flg15-derived peptides declined with a reduced length. However, a ROS production similar to flg15 induction can be induced by flg15- Δ 1, flg15- Δ 2, and flg15- Δ 3 at higher concentrations (1000 μ M) (Figs. S2g, S3g, and S4g). Interestingly, we could not detect any significant differences in elicitor activity among these three peptides. While for ROS production, flg15- Δ 4 had to be applied in very high concentrations (Fig. S5). We could not detect DCF fluorescence in female gametophytes when challenged with flg15- Δ 5, flg15- Δ 6, flg15- Δ 7, and flg15- Δ 8 (Fig. S6). The cytological observations were consistent with the results of luminol fluorescence detection.

Pharmacological results in *S. japonica* challenged by flg22

The release of H_2O_2 induced by flg22 was approximately twice that of the seawater control (Fig. 7). DPI, a specific inhibitor of the NADPH oxidase, strongly inhibited the release of H_2O_2 in flg22-induced *S. japonica* at a concentration of 60 μ M (Fig. 7). H_2O_2 production was increased after inhibition of the peroxidase activity by addition of 50 μ M of either NaN_3 or SHAM (Fig. 7). These results imply that in *S. japonica*, H_2O_2 generation is by a membrane-located NADPH oxidase, but not by cell wall-bound peroxidases.

Fig. 4 Growth inhibition of cultured clones of female gametophytes of *S. japonica* by four various peptides. Values are means $\pm$ SD ($n=3$). Statistically significant differences (ANOVA, Tukey) are indicated by lowercase letter superscripts ($P<0.05$)

Fig. 5 Release of H_2O_2 in female gametophytes of *S. japonica* induced by various concentrations of flg15 and flg15 derived peptides. Values are means $\pm$ SD ($n=6$)

Subcellular localization of H_2O_2 in *S. japonica* challenged by flg22

In order to localize H_2O_2 production intracellularly, H_2O_2 -dependent cerium peroxide precipitates were observed by electron microscopy in the cell wall after incubation of female gametophytes of *S. japonica* with both cerium chloride and H_2O_2 (Fig. 8b). Visible electron-dense precipitates appeared in the plasma membranes (Fig. 8c–e) when female gametophytes were incubated in the presence of both cerium chloride and flg22. Precipitates of cerium peroxide were not observed in the control with sterilized seawater (Fig. 8a).

Fig. 6 Release of H_2O_2 in female gametophytes of *S. japonica* co incubated with flg15 and flg15 derived peptides at a concentration of 90 μ M, showing antagonistic elicitor effects of flg15 Δ 5, flg15 Δ 6, and flg15 Δ 7. Values are means $\pm$ SD ($n=6$). Statistically significant differences (ANOVA, Tukey) are indicated by lowercase letter superscripts ($P<0.05$)

Discussion

In this study, we investigated the structure-elicitor activity of flg22- and flg15-derived peptides in female *S. japonica* gametophytes by using a luminol-dependent assay and a DCFH-DA fluorescent probe. Because flg22 has been described as an effective elicitor in both female gametophytes (Wang et al. 2013) and sporophytes of *S. japonica* (Wang et al. 2012), this paper focuses on the structure-elicitor relationship of flg22- and flg15-derived peptides. The relation between the structure and the elicitor activity of flg22- and flg15-derived peptides has been intensively studied in various plants, especially in tomato and *A. thaliana*. The elicitor activity of flg22, flg15, and flg14 in tomato and in *A. thaliana* shows some similarity but also exhibits respective characteristic differences. While flg22 shows the full elicitor activity in both species, flg15 exhibits a reduced elicitor activity in tomato and almost no

Fig. 7 Release of H_2O_2 in flg22 induced female gametophytes of *S. japonica* by DPI, SHAM, and NaN_3 . The concentrations of DPI, SHAM, and NaN_3 were 60, 50, and 50 μ M, respectively. Values are means $\pm$ SD ($n=3$). Statistically significant differences (ANOVA, Tukey) are indicated by lowercase letter superscripts ($P<0.05$)

Fig. 8 Subcellular localization of H_2O_2 in *S. japonica* by electron microscopy. **a** Cell of *S. japonica* (control). **b** Cell of *S. japonica* incubated in 5 mM H_2O_2 for 10 min (positive control). **c–e** Cells of *S. japonica* incubated with flg22 for 2 h. Dense cerium peroxide precipitates (arrows) were observed on cell walls (**b**) and plasma membranes (**c–e**). CW cell wall, PM plasma membranes. Bars=1 μ m. GraphPad Prism was used to create the artwork

elicitor activity in *A. thaliana* (Felix et al. 1999). This indicates that *A. thaliana* recognizes peptides with longer domain lengths than tomato plants (Bauer et al. 2001). In our study on the brown alga *S. japonica*, the EC_{50} values for flg22, flg15, and flg14 were determined to be 90, 110, and 720 μ M, respectively. The elicitor activity of the peptides was reduced with shorter lengths (flg22 > flg15 > flg14), which is similar to respective studies in tomato, indicating a similar mechanism (Felix et al. 1999). Felix et al. found that the concentrations required for half-maximal alkalization of flg22, flg15, and flg14 were 0.03, 0.1, and 1 nM, respectively. For the C-terminally truncated peptides, the elicitor activity of flg15- Δ 2 and flg15- Δ 3 decreased significantly in tomato (Felix et al. 1999). Peptides lacking four to eight amino acid residues at the C-terminus of flg15, such as flg15- Δ 4, flg15- Δ 5, flg15- Δ 6, flg15- Δ 7, and flg15- Δ 8, have no elicitor activity but do have antagonistic effects on elicited defense response, while flg15- Δ 9 is completely inactive in tomato (Felix et al. 1999). While for *A. thaliana*, the flg15- Δ 2 acts as a weak antagonist (Bauer et al. 2001). Our results showed that the elicitor activity of flg15- Δ 1, flg15- Δ 2, and flg15- Δ 3 decreased significantly in *S. japonica* compared to flg15 and the activity was reduced stronger for flg15- Δ 4. Flg15- Δ 5, flg15- Δ 6, flg15- Δ 7, and flg15- Δ 8 showed no elicitor activity. Interestingly, flg15- Δ 5 to flg15- Δ 7 had no elicitor activity but acted as competitive and specific antagonists of flg15 in *S. japonica*, which is similar to the flg15- Δ 4 to flg15- Δ 7 peptides in tomato. This may be explained by competition between the antagonistic peptides and the elicitor-active peptides for the same binding site on the hypothetical flagellin

receptor. Regarding receptor activation by flg15, Felix et al. (1999) proposed a mechanism involving the binding of the N-terminal part and the activation of signaling by the C-terminal part as two functionally separate steps. This kind of mechanism for recognizing elicitors meanwhile has been found in plant cells, in particular for the yeast-derived glycopeptide (Basse et al. 1992), the wound hormone system (Meindl et al. 1998), and flg22 (Meindl et al. 2000). Therefore, according to our results, structure-elicitor activity of flg22- and flg15-derived peptides is more similar to the tomato response system.

The elicitation-active site of flagellin/flg22 was intensively investigated in *A. thaliana*. Sun et al. (2006) identified defense-eliciting structural elements by dissecting the entire flagellin protein of *Xanthomonas campestris* pv *campestris* (Xcc). Domain replacement and site-directed mutagenesis together showed that the amino acid at position 43 is crucial for the elicitation activity. Replacements of D43 inevitably caused the loss of elicitation ability of Xcc B305 flagellin, and importantly, the reciprocal V43D substitution converted non-eliciting Xcc B186 flagellin to an eliciting flagellin. D43 also plays an essential role in *A. thaliana*. Naito et al. (2008) found that the replacement of D43 with valine (V) and alanine (A) in *P. syringae* pv. *tabaci* flagellin and its flg22 resulted in the reduced abilities of flagellins to induce cell death and growth inhibition in *A. thaliana*, whereas the abilities of flg22 D43V and flg22 D43A were abolished. These results indicate that D43 is important for elicitor activity in *P. syringae* pv. *tabaci*. Here, we observed a similar phenomenon in *S. japonica*. Even at a concentration of 1000 μ M, flg22D43A failed to induce the production

of H_2O_2 , implying that D43 is also the elicitation-active site in *S. japonica*. Combining our data of the elicitor activity of flg22 and flg15 as well as functional site of D43, it appears that *S. japonica* may possess similar flagellin/flg22 recognition systems and signal transduction pathways as found in tomato.

Growth-inhibiting effects of flg22 were first reported by Gómez-Gómez et al. (1999) in *A. thaliana*. They found that 0.01 μM of flg22 can inhibit the growth of *A. thaliana* seedlings after 7 days. This growth inhibition was positively correlated with strength of the elicitor activity. Growth inhibition phenomena have become typical features of defense responses of *A. thaliana*. Female gametophytes of *S. japonica* also show growth inhibition effects induced both by flg22 and by flg15, correlating with the elicitor activities. The elicitor activities of flg22, flg15, and flg14 decline in this order, and the growth-inhibiting effects followed the same pattern. We thus conclude that growth inhibition is a typical feature of the defense response in *S. japonica*.

In our experiments, the production of H_2O_2 was inhibited by DPI at a concentration of 60 μM (Fig. 7), which was about 200 times higher than the concentration required for *G. conferta* (Weinberger et al. 2005). The difference between *S. japonica* and *G. conferta* might be caused by the thicker cell walls of *S. japonica*. Similar to *G. conferta* (Weinberger et al. 2005), SHAM, and NaN_3 , inhibitors of peroxidases (O'Brien et al. 2000; Weinberger et al. 2005), could increase the production of H_2O_2 in *S. japonica* (Fig. 7). Peroxidases have at least two functions: they reduce O_2 into $\text{O}_2 \cdot$ and H_2O_2 , and they can scavenge H_2O_2 . Therefore, in our study, the increase of H_2O_2 was correlated with the decreased capacity of peroxidases to scavenge H_2O_2 , because of inhibition of the peroxidases by SHAM and NaN_3 (Weinberger et al. 2005). From our results, we could infer that H_2O_2 in *S. japonica* was generated by a membrane-located NADPH oxidase, which was supported by transmission electron microscopy. By using the $\text{Ce-H}_2\text{O}_2$ electron staining technique, electron-dense deposits of cerium perhydroxide were observed in the plasma membranes (Fig. 8c–e) in female gametophytes challenged with flg22, which was similar to the results of *G. conferta* (Weinberger et al. 2005). Contrary to our results, in *G. chilensis*, H_2O_2 accumulation was inhibited by SHAM and NaN_3 and was not affected by DPI with electron-dense deposits in cell walls instead of the plasma membranes (Weinberger et al. 2005). This may be caused by different mechanisms for the production of H_2O_2 between *S. japonica* and *G. chilensis*. We thus conclude that the source of H_2O_2 in female gametophytes of *S. japonica* is located in the plasma membranes.

In conclusion, our study showed that flg15 is the smallest epitope of flagellin recognized by female gametophytes of *S. japonica* and that D43 at the N-terminus of the flagellin is essential for elicitor activity. The characteristic elicitor activities of flg22 and its derived peptides in the female gametophytes were similar to those described for tomato plants. This

indicates that plants and algae share some very conservative features in elicitor recognition and subsequent defense reactions. This is surprising regarding the large phylogenetic distance between land plants and brown algae and considering the evolution of brown algae by secondary endosymbiosis and, thus, the involvement of a secondary host cell different to the primary host cell of land plants. The results of pharmacological investigations and of transmission electron microscopy revealed that the production of H_2O_2 induced by flg22 in *S. japonica* was related to NADPH oxidase-like enzymes, which are located on the plasma membranes. Our work here lays the foundation for future structure-function research on pathogen recognition by *S. japonica*, including the identification of flagellin/flg22 elicitor activity of pathogenic bacteria, elicitation-active site, and receptors.

Acknowledgments This study was sponsored by the National Natural Science Foundation of China (No. 41576158) granted to G.G. Wang; Science and Technology Development Project of Qingdao, China, 12 1 4 1 (4) jch; the Shandong Agriculture Breeding Engineering Biological Resources Innovation of Research Project; and Student Research Training Program (SRTP) of Ocean University of China (No. 201210423048).

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