**RESEARCH PAPER**

**Mining the genome of Arabidopsis thaliana as a basis for the identification of novel bioactive peptides involved in oxidative stress tolerance**

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**Abstract**

Although evidence has accumulated on the role of plant peptides in the response to external conditions, the number of peptide-encoding genes in the genome is still underestimated. Using tiling arrays, we identified 176 unannotated transcriptionally active regions (TARs) in Arabidopsis thaliana that were induced upon oxidative stress generated by the herbicide paraquat (PQ). These 176 TARs could be translated into 575 putative oxidative stress-induced peptides (OSIPs). A high-throughput functional assay was used in the eukaryotic model organism Saccharomyces cerevisiae allowing us to test for bioactive peptides that increase oxidative stress tolerance. In this way, we identified three OSIPs that, upon overexpression in yeast, resulted in a significant rise in tolerance to hydrogen peroxide (H₂O₂). For one of these peptides, the decapeptide OSIP108, exogenous application to H₂O₂-treated yeast also resulted in significantly increased survival. OSIP108 is contained within a pseudogene and is induced in A. thaliana leaves by both the reactive oxygen species-inducer PQ and the necrotrophic fungal pathogen Botrytis cinerea. Moreover, infiltration and overexpression of OSIP108 in A. thaliana leaves resulted in increased tolerance to treatment with PQ. In conclusion, the identification and characterization of OSIP108 confirms the validity of our high-throughput approach, based on tiling array analysis in A. thaliana and functional screening in yeast, to identify bioactive peptides.

**Key words:** Arabidopsis thaliana, bioactive peptide, hydrogen peroxide, paraquat, Saccharomyces cerevisiae, sORF, tiling array.

**Introduction**

Bioactive peptides have been identified from both plant and animal sources. In general, they are considered to be encrypted as inactive fragments in mature proteins. When released via enzymatic hydrolysis, they can exert various functional activities such as antimicrobial, antihypertensive, antithrombotic and antioxidative activity (Kim and Wijesekara, 2010; Sarmadi and Ismail, 2010). Very recently, Brand et al. (2012) developed a method to identify putative antimicrobial peptides encrypted in protein sequences from plants. Here, we have focused on bioactive peptides involved in oxidative stress tolerance, not encrypted in mature proteins but encoded by small open reading frames (sORFs) in the genome of Arabidopsis thaliana.

Oxidative stress is generated by the production of toxic compounds called reactive oxygen species (ROS). These are...
small, highly reactive molecules such as hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$) and hydroxyl (OH) radicals, containing one or more oxygen atoms. ROS production is induced in plants following various biotic and abiotic stress conditions, such as infection with phytopathogens (Govrin and Levine, 2000; Schouten et al., 2002; Heller and Tudażyński, 2011; O’Brien et al., 2012) and cold and heat stress (Lukatkin, 2002; Volkov et al., 2006), respectively. An important feature of the plant’s response to stress comprises the production of peptides with a signalling role, antimicrobial activity or antioxidant functions (Sels et al., 2008; Fukuda and Higashiyama, 2011; Katsir et al., 2011; Marshall et al., 2011; Matsubayashi, 2011; Dubreuil-Maurizi and Poinssot, 2012), some of which have also been implicated in oxidative stress tolerance, such as the A. thaliana peptides for late embryogenesis abundant 5 (AtLEA5; Mowla et al., 2006) and mitochondrial encoded ATP synthase (AtMtATP6; Zhang et al., 2008) and the unknown peptide encoded by At1g21520 (Luhua et al., 2008). AtLEA5 encodes a ‘late embryogenesis abundant’-like peptide that is expressed constitutively in roots and reproductive organs. In leaves, AtLEA5 transcripts are repressed when exposed to light but are abundant in the dark or under conditions of oxidative stress. Moreover, overexpression of AtLEA5 in A. thaliana confers tolerance to H$_2$O$_2$ (Mowla et al., 2006). AtMtATP6 is a component of the mitochondrial F$_{1}$F$_{0}$-ATPase, an enzyme involved in oxidative respiration. Overexpression of the corresponding gene in A. thaliana increases tolerance to paraquat (PQ), an oxidative stress-generating herbicide (Zhang et al., 2008). In recent years, it has become clear that genes encoding peptides are strongly underrepresented in the current genome annotation of several organisms, including A. thaliana. The main reason for this underrepresentation is the minimum length threshold of 330 nt that is imposed by gene annotation software to avoid false-positive predictions (Haas et al., 2005).

As an extension of classical microarray analysis performed on annotated gene-based arrays, tiling array technology focuses on the entire non-repetitive part of a genome, including intergenic regions and introns, and has often been used to identify previously undetected transcripts, including non-coding RNA (ncRNA) in A. thaliana (Yamada et al., 2003; Stolc et al., 2005; Hanada et al., 2007; Matsui et al., 2008). Using this technology, it was indeed shown that the annotation in A. thaliana is far from complete, as these studies identified many unannotated transcripts in non-stress and abiotic (drought, cold, and salt) stress conditions. Evidence is increasing that sORFs, including those occurring in ncRNA, can also encode bioactive peptides (Sousa et al., 2001; Narita et al., 2004; Castellana et al., 2008; Podkowski et al., 2009; Hanada et al., 2013). During the past decade, different approaches have been developed to isolate and identify novel plant bioactive peptides, including a combination of various chromatography techniques (Sagar et al., 2012) as well as the development of bioinformatics tools predicting peptides with specific bioactivities (Torrent et al., 2012). However, a weak point in such high-throughput approaches often is the proof of desired functionality of the predicted peptide sequences in vivo.

In this study, we used a combination of high-throughput identification with tiling array technology and functional screening in yeast to identify sORFs upregulated in A. thaliana during oxidative stress, and active in governing oxidative stress tolerance. The tiling array analysis resulted in 176 transcriptionally-active regions (TARs), which could be translated into 575 putative oxidative stress-induced peptides (OSIPs). To investigate the potential of these peptides in governing oxidative stress tolerance, they were overexpressed in yeast and the resulting transformants were screened for increased oxidative stress tolerance. It has been demonstrated that the lower eukaryote yeast is a potent model system for studying processes in higher eukaryotic organisms (Botstein et al., 1997). Moreover, the previously mentioned peptides AtLEA5 and AtMtATP6 were shown to increase oxidative stress tolerance in yeast upon overexpression (Mowla et al., 2006; Zhang et al., 2008). Overall, this study presents a strategy for the identification of novel bioactive peptides and resulted in the discovery of OSIP108, a decapeptide that increases oxidative stress tolerance in yeast and in planta when overexpressed and upon exogenous application.

**Materials and methods**

**Plant material, growth conditions, treatments, and yeast strains**

Seeds of A. thaliana wild-type ecotype Columbia-0 (Col-0) were obtained from the European A. thaliana Stock Centre (http://arabidopsis.info/). They were sown on ‘Zaai- en Stekgrond’ (DCM, Sint-Katelijne-Waver, Belgium) and grown in a growth chamber at 21 °C, 75% humidity and a 12 h day/night cycle with a light intensity of approximately 120 µmol m$^{-2}$s$^{-1}$.

Rosette leaves from soil-grown 4-week-old A. thaliana plants were sprayed with PQ (25 µM methyl 98% viologen dichloride hydrate; Sigma-Aldrich, St Louis, MO, USA) to obtain an equal and proportional distribution. Control plants were mock treated by spraying with H$_2$O. The treated plants were kept at 100% relative humidity for 48 h, and all aboveground plant material was then harvested in liquid nitrogen and stored at −80 °C until further analysis.

Spores from Botrytis cinerea B05-10 were harvested as described previously (Broekaert et al., 1990). Spores (2 × 10$^{7}$ ml$^{-1}$) were kept in 25% glycerol at −80 °C until further use. Plants were sprayed evenly with a spore suspension of 5 × 10$^{5}$ spores ml$^{-1}$ in 1/2 potato dextrose broth (PDB). Control plants were mock treated by spraying with 1/2 PDB. After inoculation, plants were kept at 100% relative humidity.

The yeast strain used was Saccharomyces cerevisiae strain BY4741 (Euroscarf, Germany), which was cultivated in yeast peptone dextrose (1% yeast extract, 2% peptone, 2% glucose). Yeast strains transformed with the pYES-DEST52 Gateway® vector (Invitrogen, Carlsbad, USA) were cultured in SC–ura (Glu) [comprising 0.8 g l$^{-1}$ of CSM–ura (complete amino acid supplement mixture minus uracil, Bio 101 Systems; 6.5 g l$^{-1}$ of yeast nitrogen base; 20 g l$^{-1}$ of glucose)]. Expression of the OSIPs was induced in SC–ura (Gal).

**Isolation of total RNA**

RNA was isolated by the guanidinium isothiocyanate method (TRizol reagent; Invitrogen) followed by purification using an RNeasy Mini kit (Qiagen, Valencia, USA). First, ~100 mg of plant material was crushed in liquid nitrogen, 1 ml of TRizol reagent was added, and samples were mixed and incubated for 5 min at room temperature. Following centrifugation (20 000g, 10 min, 4 °C) the supernatant was transferred to a fresh tube and 200 µl of chloroform was added. The thoroughly mixed samples were incubated for 2 min at room temperature and the upper aqueous phase was transferred to a fresh tube after centrifugation (20 000g, 15 min, 4 °C). An equal
volume of 70% ethanol was added after which the samples were thoroughly shaken and applied to a Qiagen column. The procedure was continued according to the manufacturer’s instructions.

Target preparation and tiling array hybridization
Preparation and labelling of mRNA and hybridization on GeneChip® Arabidopsis Tiling 1.0R Arrays (Affymetrix, Santa Clara, USA) were performed by the VIB Nucleomics Core Facility (http://www.nucleomics.be). In summary, RNA was converted to double-stranded cDNA in a reverse transcription reaction with a poly(dT) primer. Subsequently, the sample was converted and amplified to antisense cRNA and labelled with biotin in an in vitro transcription reaction according to the Affymetrix One Cycle Target Labeling Kit. cRNA was subsequently random primed to generate ds cDNA according to the Affymetrix WT Double-Stranded cDNA Synthesis Kit, and then fragmented and labelled with biotin in a terminal labelling reaction according to the Affymetrix WT Double-Stranded DNA Terminal Labeling Kit. Two independent biological replicates were used for each condition. Scanning and image processing were performed by the Nucleomics Core Facility using Affymetrix equipment and software.

Identification of short unannotated and PQ-induced genomic regions
Background correction, log₂ transformation, and inter-slide normalization were performed with the Affymetrix Tiling Analysis Software version 1.1, Build 2. As the tiling arrays were based on the A. thaliana genome sequence of the 2007 version of the TAIR database, the corresponding TAIR7 genome annotation was used for analysis of the tiling array data. All steps of our strategy are illustrated in Supplementary Fig. S1 at JXB online. In a first step, the average log₂ intensities of biological replicates were calculated and a threshold value of 8.8 was chosen to identify transcriptionally active probes. This threshold was determined by comparing the log₂ intensity distribution of genomic regions with known expression status (expressed and not expressed). Probes with log₂ intensities below the threshold that were surrounded by transcriptionally active probes were considered active and vice versa to remove the effect of probes with poor hybridization properties. Secondly, groups of 4–13 successive transcriptionally active probes were combined into short TARs. Selection of even smaller regions would lead to a high number of false positives and to the identification of very small genes that are difficult to amplify in later stages, while all active regions of 14–15 successive probes were already annotated (TAIR7), supporting our choice of size limits. In a third step, TARs were withheld if the number of transcriptionally active probes was smaller in the control than in the PQ treatment and if the average log₂ intensity of all probes in the smaller of the two regions was at least 1.4 higher in the PQ treatment than in the control. Both rules were based on the tiling array results for genomic regions that are known to be induced upon PQ treatment. The former rule was added because of the frequent occurrence of probes having zero intensity in one of the four hybridizations. In small regions, these zero values can have a substantial influence on the average log₂ intensity leading to false-positive calls. The threshold for upregulation by PQ was refined by comparing quantitative reverse transcriptase-PCR (qRT-PCR) and tiling array results for 30 selected TARs. All upregulations of 1.4 or more (corresponding to 2.6-fold or more) in the tiling arrays could be confirmed by qRTPCR supporting our choice of threshold (data not shown). Finally, each selected region was compared with the A. thaliana TAIR7 genome annotation and only regions that were located in intergenic regions, introns, or pseudogenes were withheld.

Selection of ORFs
Using the TAIR7 genome sequence, the corresponding DNA sequences of all selected regions were retrieved. On each side of the active region an extra sequence of 50 bp was selected to minimize the chance of missing the start or stop codon. Each sequence was translated in the six possible reading frames and peptide sequences that were located entirely in the outer 50 bp or were shorter than 10 aa were discarded. The latter decision was made to avoid too many false positives.

Construction of a mini library in yeast
ORFs were amplified via PCR with specific primers for each selected ORF (Primer3 software; Rozen and Skaletsky, 2000) (Supplementary Table S1 at JXB online). For transformation using Gateway® technology and efficient translation in yeast, the sequence 5’-CACAAAAA-3’ was added to each forward primer. The pool of full coding sequences of a selection of 138 potential OSIP genes was cloned into the pENTR™ plasmid (Invitrogen) using the pENTR™/D-TOPO® cloning system according to the manufacturer’s instructions and the presence of each ORF in the resulting pool of Escherichia coli transformants was confirmed by PCR. The inserts were transferred to the pYES-DEST52 Gateway® vector behind a galactose-inducible promoter and used for transformation to S. cerevisiae BY4741 using the Gietz protocol (Gietz et al., 1995). The presence of each ORF in the pool of yeast transformants was confirmed by PCR.

Oxidative stress assays in yeast
Oxidative stress tolerance of individual yeast transformants was examined by determination of the minimal inhibitory concentration (MIC) for H₂O₂, using a two-fold dilution series (0–20 mM) in SC–ura (Gal) inoculated with 1/200 of an overnight culture (2 × 10⁶ cells ml⁻¹) in SC–ura (Glu). The resistance of yeast transformants against oxidative stress induced by H₂O₂ was additionally assayed by spotting 15 μl of 240 mM H₂O₂ on SC–ura (Glu) and SC–ura (Glu) agar, inoculated with 1/200 of an overnight yeast culture as described previously (Aerts et al., 2006). After 48 h of incubation at 30 °C, the diameters of the halos on the SC–ura (Gal) and SC–ura (Glu) plates were measured and the relative difference in halo diameter for each transformant was determined as: relative difference=(diameter_wt−diameter_orf)/diameter_wt.

Survival of wild-type yeast in PBS upon H₂O₂ treatment in the presence of 200 μM OSIP in 1% dimethyl sulfoxide (DMSO) was assessed as described previously (Aerts et al., 2006). Control treatment consisted of 1% DMSO. In short, an overnight culture of S. cerevisiae BY4741 was washed in PBS and diluted to an optical density at 600 nm of 0.05. The cells were incubated with 200 μM of OSIP108 at 30 °C and afterwards treated with different concentrations of H₂O₂ (1.25, 2.5 and 5 mM). Survival was determined by plating out the cells 3 h after H₂O₂ treatment.

Peptide synthesis
OSIP108 and the [C3A]OSIP108 mutant were synthesized using standard fmoc chemistry on a microwave automatic synthesizer (Liberty, CEM) as described previously (Skjærbaek et al., 1997). The mass and purity of the synthetic peptides were confirmed by mass spectrometry and reversed-phased high-performance liquid chromatography (data not shown). The peptides had a purity of ≥95%.

In vitro H₂O₂-scavenging activity assay
Peroxidase activity was tested using an Amplex® Red Hydrogen Peroxide/Peroxidase activity assay (Invitrogen). To this end, 100 μM of OSIP108, glutathione and [C3A]OSIP108 were co-incubated with 0.75 and 1.25 mM H₂O₂, 0.5 mM ml⁻¹ of horseradish peroxidase, and 50 μM Amplex red reagent. After 30 min of incubation at room temperature, the fluorescence was measured (560–590 nm) (Synergy™MX, Biotek).
Experiments were statistically analysed using Statistica® (Release 7; Statsoft, Tulsa, USA). The data of exogenous application of OSIP108 and its effect on PQ tolerance in planta were analysed by analysis of variance (ANOVA). The Tukey HSD test was applied for multiple comparisons of group means. The data of the oxidative stress-protective effect of the yeasts overexpressing the different OSIPs were analysed using non-parametric Kruskal–Wallis ANOVA by ranks, because of the smaller sample size. When the Kruskal–Wallis statistic was significant, comparisons of treatments versus control were calculated as described by Siegel and Castellan (1988). The effect of exogenous application of OSIP108 to yeast, the expression analysis of OSIP108, and the PQ treatment of OSIP-overexpressing plants were analysed using Student’s t-test.

Results

Identification of OSIP genes in A. thaliana using tiling arrays

Tiling array analysis (Supplementary Fig. S1) was performed on mRNA extracted from leaves treated with PQ and H2O (control), resulting in the identification of 92 844 and 86 272 TARs, respectively (GEO GSE49001). A total of 176 short unannotated TARs were induced by PQ treatment compared with the control treatment (Supplementary Table S2 at JXB online and Fig. S1). The bulk of these TARs were intergenic, although 70 and 18 OSIP-encoding regions were located in pseudogenes and introns of annotated genes, respectively. The DNA sequences of the selected TARs were translated in all reading frames, and ORFs of at least 30 bp were retained, resulting in a list of 575 potential OSIPs (Supplementary Fig. S1 and Table S3 at JXB online), ordered based on the induction level of the corresponding genes in the tiling array (Fig. 1A).

Several potential OSIPs increase the oxidative stress tolerance of yeast when overexpressed

To screen for OSIPs that effectively improve oxidative stress tolerance, we selected 138 OSIP-encoding ORFs that were at least 8-fold (log₂ ratio=3) induced by PQ (Fig. 1A). These ORFs were cloned behind a galactose-inducible promoter and overexpressed in yeast. As PQ induces oxidative stress in yeast during respiration, which occurs upon cultivation in glycerol but not in galactose, we screened for yeast transformants with increased tolerance against H₂O₂ in galactose-containing medium. As positive controls, we included AtLEA5 and AtMtATP6. The MIC for H₂O₂ of 500 individual yeast transformants was determined and we identified four yeast transformants characterized by a 2-fold increase in H₂O₂ tolerance (MIC=5 mM H₂O₂), compared with the other 496 yeast transformants (MIC=2.5 mM H₂O₂) (data not shown). A similar increase in MIC value was observed for yeast transformants overexpressing AtLEA5 or AtMtATP6. Upon sequencing of these four H₂O₂-tolerant yeast transformants, we identified two containing OSIP108, one containing OSIP152, and one containing OSIP163 (Fig. 1A). The log₂ ratios of OSIP108, OSIP163, and OSIP152 upon PQ treatment in the tiling array were 5.42, 5.07, and 4.74, respectively (Fig. 1B). To validate these expression data in A. thaliana, the expression levels of the three selected OSIPs were assessed using qRT-PCR analysis under the same conditions and at the same time point as used in the tiling array analysis. The log₂ ratios of OSIP108, OSIP163, and OSIP152 were 5.32, 5.68, and 5.01, respectively (Fig. 1B), which are in line with...
the normalized log₂ ratios from the tiling arrays, thereby confirming the observed induction after treatment with PQ.

Next, we assessed the tolerance to H₂O₂ of the three selected yeast transformants and the two positive control transformants in a halo test by spotting 240 mM H₂O₂ on to agar plates inoculated with the specific yeast transformants. The diameters of the halos on galactose-containing medium and glucose-containing medium were measured and the relative difference was determined to quantify the effect of the OSIP on oxidative stress tolerance (Fig. 2). The relative difference for OSIP108-overexpressing yeast (0.078 ± 0.019) was significantly lower than yeast transformants overexpressing negative controls (0.350 ± 0.029), which consisted of four yeast OSIP transformants that previously showed no altered H₂O₂ MIC value. These data indicated that OSIP108 can protect yeast cells against the lethal effect of the oxidative stress agent H₂O₂. The yeast transformants expressing OSIP152 (0.405 ± 0.041), OSIP163 (0.267 ± 0.067), AtMtATP6 (0.273 ± 0.057), or AtLEA5 (0.400 ± 0.064) did not show significantly smaller halos compared with the negative controls. These data indicated a significant 4.5-fold reduction in halo diameter for OSIP108-overexpressing yeast transformants compared with control transformants, and hence increased oxidative stress tolerance upon overexpression of OSIP108 in yeast.

Fig. 1. Expression levels of 575 potential OSIPs of A. thaliana. (A) The OSIPs are ranked in the graph based on their log₂ ratio, derived from the tiling array analysis. In total, 138 ORFs with a log₂ ratio >3 (represented by the baseline on the graph) were used for overexpression in yeast. (B) Expression levels of OSIP108, OSIP152, and OSIP163 were further quantified using qRT-PCR. Log₂ ratios determined via tiling array analysis and qRT-PCR are indicated. Values are means ±SD of three biological replicates.

Exogenous application of OSIP108 to yeast increases survival upon H₂O₂ treatment

Based on the data obtained above, further experiments on oxidative stress tolerance of yeast and plants focused on the effect of OSIP108, a decapeptide with the amino acid sequence MLCVLQGLRE. In the first instance, the tolerance to H₂O₂-induced oxidative stress was assessed in yeast co-incubated with the chemically synthesized OSIP108 peptide. Survival of yeast cells was determined upon treatment with 1.25, 2.5, and 5 mM H₂O₂, and simultaneous application of 200 µM OSIP108. OSIP108 significantly increased (15%) the survival of H₂O₂-treated yeast compared with the mock treatment (1% DMSO) (Fig. 3).

OSIP108 has in vitro H₂O₂ scavenging activity

As it is known that thiol groups, e.g. from the cysteine present in OSIP108, are effective ROS scavengers, we examined the H₂O₂ scavenging activity of native OSIP108 and its analogue [C3A]OSIP108, in which cysteine on position three was replaced by alanine, with an Amplex® Red Hydrogen Peroxide/Peroxidase Assay kit. This test is based on the formation of a fluorescent product, resorufin, upon reaction of the Amplex Red reagent with H₂O₂ in the presence of a
peroxidase. We tested whether resorufin was less prone to peroxidation by horseradish peroxidase in the presence of OSIP108, due to H₂O₂ scavenging. A significant decrease in fluorescence was observed upon addition of either 100 µM OSIP108 or glutathione, an antioxidant tripeptide, compared with the mock treatment (1% DMSO) (Fig. 4). These results showed that the H₂O₂ scavenging activity of OSIP108 was similar to that of glutathione. Addition of [C3A]OSIP108 did not result in significantly decreased fluorescence.

OSIP108 is induced by PQ and B. cinerea in A. thaliana leaves

The coding sequence of OSIP108 is contained within the pseudogene At3g61185, which has been suggested to encode a potential defensin-like peptide within its C-terminal sequence (Silverstein et al., 2007). The sequence of OSIP108 is, however, not in frame with the defensin-like peptide (Fig. 5). The upstream region of the At3g61185 gene in A. thaliana comprises the genes At3g61180 and At3g61182 encoding a RING/U-box superfamily protein and a low-molecular-weight cysteine-rich protein (LCR54), respectively (Fig. 5). In order to gain more insight into the role of OSIP108 in planta, a more detailed expression analysis by qRT-PCR of OSIP108 in A. thaliana was performed at different time points after PQ treatment (Fig. 6). Significant induction of OSIP108 was detected at 48 h (21-fold), 72 h (7.2-fold), and 96 h (18-fold) after treatment. As oxidative stress is often linked with biotic stress, expression of OSIP108 was additionally analysed in A. thaliana leaves at different time points after inoculation with the necrotrophic pathogen B. cinerea, reported to induce ROS in plant tissues upon infection (Govrin and Levine, 2000; Heller and Tuzdynski, 2011) (Fig. 6). Again, a comparable significant induction of OSIP108 was detected at 24 h (11-fold), 48 h (26-fold), and 72 h (20-fold) after B. cinerea inoculation.

Exogenous application of OSIP108 to A. thaliana leaves increases PQ tolerance

To determine whether OSIP108 also boosts oxidative stress tolerance in plants, leaves of A. thaliana were syringe
Infiltrated with OSIP108. Subsequently, the plants were treated with PQ and the diameters of the resulting lesions were measured (Fig. 7). Two d after PQ application, the average lesion diameter of plants pre-treated with OSIP108 (2.92 ± 1.93 mm, \(P < 0.01\)) was significantly lower than the average lesion diameter of the control plants (5.87 ± 2.79 mm). Interestingly, the cysteine at position 3 seemed to be crucial in this oxidative stress-protectant activity, as no difference in lesion diameter was observed between control and [C3A] OSIP108 (6.00 ± 2.22 mm) treatment.

**Overexpression of OSIP108 increases PQ tolerance in A. thaliana**

In order to prove the bioactivity of OSIP108 in planta, this peptide was constitutively overexpressed in *A. thaliana*. The homozygous T3 overexpression line 35S::OSIP108 (175 364.8 ± 1.65 fold change) and wild-type plants were sprayed with 40 μM PQ. At 7 d post-application, ASSESSE software was used to determine the percentage of total chlorotic leaf area. Compared with wild-type plants (47.43 ± 9.08%), the percentage of chlorotic leaf area was three times less in 35S::OSIP108 plants (14.99 ± 4.39%) (Fig. 8). As such, it could be shown that overexpression of OSIP108 in *A. thaliana* resulted in a significantly higher tolerance to PQ (\(P < 0.01\)). These data confirmed the ability of OSIP108 to increase oxidative stress tolerance not only in yeast but also in plants.

**Discussion**

Peptides have been shown to play crucial roles in plant defence responses and development. Whereas some peptides have been reported to have a signalling role in the plant and are often referred to as peptide hormones, others exhibit direct antimicrobial activity (Sel et al., 2008; Fukuda and Higashiya, 2011; Katsir et al., 2011; Marshall et al., 2011; Matsubayashi, 2011). The latter group generally consists of pathogenesis-related peptides such as the cysteine-rich plant defensins, thionins, knottins, hevein-like peptides, snakins, lipid transfer proteins, and cyclotides (Sel et al., 2008; Hammami et al., 2009; Marshall et al., 2011). Like most signalling peptides, they contain a signal sequence, resulting in secretion of the peptide. A first group of secreted peptide hormones are initially synthesized as pre-propeptides and then undergo major post-translational modifications and proteolytic processing, resulting in the formation of a small peptide (Matsubayashi, 2011). This type of peptide signal has been implicated in both plant defence and development, including for instance the 18 aa tomato systemin, which is processed from a 200 aa precursor (Constabel et al., 1995; Farrokhi et al., 2008) and CLAVATA3, which is translated as a 96 aa peptide and is post-translationally processed to a 13 aa peptide (Katsir et al., 2011). A second group of secreted peptide hormones are cysteine-rich peptides (Marshall et al., 2011; Matsubayashi, 2011) such as RALF and SCR/SP11. Some peptide hormones have been reported to be encoded by sORFs and have no signal sequence. Two examples of this class are ENOD40 and POLARIS. The ENOD40 gene is found in several plant species, in which it has a role in new organ initiation. ENOD40 contains two sORFs, one encoding ENOD40A with a similar size as OSIP108 and also containing a cysteine residue, and the second encoding a 27 aa peptide (Sousa et al., 2001). The POLARIS gene encodes three putative peptides of 8, 9, and 36 aa, respectively. It was demonstrated that the 36 aa peptide is implicated in root growth.
Fig. 7. Exogenous application of OSIP108 to A. thaliana leaves increases their tolerance to PQ. OSIP108 (100 µM) was used for syringe infiltration of leaves from A. thaliana, followed by application of a 5 µl drop of PQ (100 µM). The lesion diameters of plants pre-treated with OSIP108 or [C3A]OSIP108 and mock-treated plants (treated with 1% DMSO only) were measured 2 d post-application. Each bar represents the mean (±SEM) of at least 13 plants, and this figure is a representative of two independent experiments. Different letters indicate significant differences (P<0.01) according to an ANOVA Tukey HSD test.

Fig. 8. 35S::OSIP108 overexpression plants are more tolerant to PQ treatment than wild-type plants. A 35S::OSIP108 overexpression line and wild-type plants were evenly sprayed with 40 µM PQ. At 7 d post-application, ASSESS software was used to determine the percentage of chlorotic leaf area. Each bar represents the mean (±SEM) of six sets of four plants and this figure is a representative of two independent experiments. Significant differences are indicated by an asterisk (P<0.01, Student’s t-test).

and this peptide is proposed to play a role in auxin, ethylene, and cytokinin crosstalk (Casson et al., 2002; Liu et al., 2010), although it cannot be excluded yet that the shorter peptide forms are also active. However, the discovery of new sORFs encoding peptides and the elucidation of their bioactivity is challenging. To this end, we adopted a novel approach combining tiling arrays with a functional characterization assay in yeast.

Using this genome-wide approach based on tiling array analysis, we identified 176 previously unannotated TARs in the A. thaliana genome that were upregulated upon PQ treatment. Translation of these TARs gave rise to 575 sORFs that potentially encoded OSIPs, with variable length (from 10 to >100 aa) and without sequence homology. In recent years, tiling arrays on A. thaliana plants subjected to different treatments have revealed the presence of many unannotated transcripts, most of them occurring in intergenic regions and pseudogenes (Thibaud-Nissen et al., 2006; Hanada et al., 2007; Matsui et al., 2008). Our study indicated that OSIP108 is also located in a pseudogene (At3g61185). However, the functional significance of most of these transcripts identified in tiling arrays, often regarded as ncRNA, is largely unknown (Guttman and Rin, 2012; Jin et al., 2013). It cannot be ruled out that ncRNAs contain sORFs that encode peptides (Kageyama et al., 2011), but determination as to whether a transcript is non-coding is challenging. Here, a high-throughput functional assay was used to identify OSIPs that significantly increased oxidative stress tolerance in the eukaryotic model yeast S. cerevisiae upon overexpression. The use of yeast to characterize plant proteins has been proven previously, for example for AtLEA5, AtMtATP6, and the defensin AhPDF1.1 (Mirozou et al., 2006; Mowla et al., 2006; Zhang et al., 2008). Via this approach, OSIP108 was identified, and this bioactive peptide was proven to increase oxidative stress tolerance in both S. cerevisiae and A. thaliana, when applied exogenously or overexpressed.

Like glutathione, OSIP108 is also able to scavenge ROS, as OSIP108 inhibited the formation of resorufin to the same extent as glutathione. The activity of glutathione results from the presence of the highly reactive nucleophilic thiol group in the cysteine residue (Deneke, 2000). Similarly, we demonstrated that the oxidative stress-protectant activity of OSIP108 upon infiltration in planta was completely abolished by replacement of the cysteine by alanine. However, having a cysteine in the peptide sequence was not enough to induce oxidative stress tolerance, as several of the OSIPs (Supplementary Table S3) contained a cysteine but did not exhibit this protectant activity.

We further observed that OSIP108 is induced not only by PQ but also by the necrotrophic fungal pathogen B. cinerea in A. thaliana leaves, which is not surprising as ROS are also reportedly implicated in the defence response against pathogens. Additionally, we compared our results with those of other tiling array studies in A. thaliana using the AtTAX browser (Zeller et al., 2009), and the gene encoding OSIP108 seemed to be transcriptionally active under various conditions, including abiiotic stress by cold or osmotic stress treatment.

More research is necessary to characterize the role of OSIP108 in planta. Unfortunately, so far we have been unable to detect the OSIP108 peptide in PQ-treated A. thaliana using both liquid chromatography/mass spectrometry and western blotting (data not shown). This is, however, also the case for the previously mentioned peptides POLARIS and ENOD40 for which in planta existence could only be proven in an indirect way. ENOD40 was assumed to be translated based on translational fusions and in vitro translation (Sousa et al., 2001; Rohrig et al., 2002; Podkowski et al., 2009). However,
Medicago truncatula ENOD40 could not be detected in roots using antibodies (Sousa et al., 2001) and soybean ENOD40A-specific antibodies were not able to detect the peptide in soybean nodules. Nevertheless, these antibodies recognized several compounds with high molecular masses, which might suggest that the peptide forms large aggregates (Rohrig et al., 2002). The assumption of translation of the POLARIS gene is based on the observation that the pls mutant phenotype could be partially rescued by the ORF encoding the 36 aa peptide (Casson et al., 2002). Very recently, Hanada et al. (2013) showed that 10% of 473 selected sORFs, identified in intergenic regions of A. thaliana, resulted in phenotypic altered morphogenic-related effects when overexpressed in A. thaliana. Moreover, recent findings in Drosophila (Kondo et al., 2010), human cells (Slavoff et al., 2013), and plants (Castellana et al., 2008; Yang et al., 2011) suggest that sORFs can be translated into peptides. For example, evidence of translation (Castellana et al., 2008) was found for one of the sORFs from Hanada et al. (2013), whose overexpression resulted in an altered phenotype.

In conclusion, the identification and characterization of OSIP108 confirms the validity of our high-throughput approach, based on tiling arrays and functional screening in yeast, to identify bioactive peptides in A. thaliana. Our results on the protective activity of OSIP108 in A. thaliana against the ROS-inducing agent PQ indicates its potential to apply OSIP108 as a molecular trait to generate crops with higher resistance to oxidative stress conditions (Apel and Hirt, 2004). The protective role of OSIP108 might also be interesting for potential applications outside plant research, as oxidative stress has been implicated in the progression of several human diseases, including Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (Barnham et al., 2002). The damage caused by ROS to lipids, proteins, and nucleic acids plays an important role in the pathogenic events of these neurodegenerative diseases (Jellinger, 2009). Several peptides have been shown to increase tolerance against oxidative stress in this respect. For example, overexpression of the peptide DSEP/dermcidin, initially purified from the secretions of oxidatively stressed neural cell lines, increased the tolerance of human neural cells to oxidative stress (Cunningham et al., 2002). In this context, preliminary data from our group have shown recently that the observed oxidative stress-protectant activity of OSIP108 in yeast and plants can be further extrapolated to mammalian cellular models for oxidative stress-related diseases (P. Spincemaille, unpublished results), indicating the potential of this newly discovered plant peptide in medical treatments.

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References


Hanada K, Zhang X, Borevitz JO, Li WH, Shiu SH. 2007. A large number of novel coding small open reading frames in the intergenic regions of the *Arabidopsis thaliana* genome


peptides resembling antimicrobial peptides have been under-predicted in plants. *The Plant Journal* 51, 262–280.


