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Genome-wide identification of *Phytophthora sojae* SNARE genes and functional characterization of the conserved SNARE PsYKT6

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ABSTRACT

Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are central components of the machinery mediating membrane fusion and key factors for vesicular trafficking in all eukaryotic cells. Taking advantage of the available whole genome sequence of the oomycete plant pathogen *Phytophthora sojae*, 35 genes encoding putative SNARE proteins were identified in the genome of this organism. PsYKT6, one of the most conserved SNARE proteins, was functionally characterized by homology-dependent gene silencing. The phenotype analysis showed that PsYKT6 is important for proper asexual development, sexual reproduction, and pathogenesis on host soybean cultivars.

1. Introduction

Phytophthora species are destructive plant pathogens capable of attacking a wide range of agriculturally and ornamentally important plants (Erwin and Ribiero, 1996). To date, the genus comprises over eighty recognized species (Tyler, 2007). The typical representatives are *Phytophthora infestans*, which causes potato late blight and was responsible for the Irish potato famine in the 19th century (Erwin and Ribiero, 1996), *Phytophthora sojae*, the causal agent of stem and root rot in soybean, which is estimated to cause annual yield losses of up to $1–2 billion worldwide (Tyler, 2007), and *Phytophthora ramorum*, which infects trees and caused Sudden Oak Death in the USA (Rizzo et al., 2005). Although *Phytophthora* species have a filamentous growth morphology, similar to many fungi, they are not at all related to fungi. They are classified as oomycetes, a diverse group of eukaryotes within the kingdom Stramenopila that constitute a distinct major branch in the eukaryotic evolutionary tree (Baldauf, 2003; Tyler et al., 2006).

The thallus of *Phytophthora* consists of aseptate hyphae that readily grow in culture. The asexual propagules, called sporangia, are formed on branched sporangiophores that emerge from hyphae. Sporangia can germinate directly to produce hyphae or can differentiate into zoospores, vegetative propagules that lack a cell wall and have two flagella for swimming. Zoospores are typically triggered to form adhesive cysts, which in turn germinate to produce hyphae or a secondary zoospore. In the soilborne species *P. sojae* zoospores are the most important source of infection of roots, especially when the soil is flooded (Tyler, 2007). These zoospores swim chemotactically toward compounds released by the root surface where they encyst. Subsequently, these cysts germinate and the emerging hyphae penetrate the root directly (Tyler, 2007). Oospores are readily produced in susceptible soybean roots and can survive inhospitable environments such as freezing or dry conditions and can resist microbial degradation (Schmitthenner, 1985).

The success of *Phytophthora* as plant pathogen depends on its ability to overcome defense responses elicited in their hosts, as well as to gain nutrition and proliferate. During infection, *Phytophthora* secretes a variety of extracellular proteins such as elicitins, transglutaminases, and cellulose-binding proteins that contribute to pathogenicity (Kamoun, 2006). In addition, *Phytophthora* secretes inhibitory proteins produced during different stages of pathogenesis such as glucanase inhibitors GIP1 and GIP2 (Rose et al., 2002), serine protease inhibitors EPI1 and EPI10 (Tian et al., 2005), and cysteine protease inhibitors EPIC1 and EPIC2 (Kamoun, 2006). Effector molecules are another type of extracellular molecules, which constitute a key weapon used by *Phytophthora* pathogens to combat their hosts' defense systems. These effector molecules include RxLR-dEER effectors (Shan et al., 2004; Tyler et al., 2006; Qutob et al., 2009), the NEP1-like proteins (Qutob et al., 2006), and the crinkling and necrosis-inducing proteins (Haas et al.,

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2009). However, little is known about the mechanism and route for transport and secretion of these effector molecules.

In eukaryotic cells, intracellular vesicle trafficking is essential for many cellular processes including polarized growth and secretion of extracellular proteins. Eukaryotic cells contain membrane-enclosed organelles that communicate with each other through the exchange of trafficking vesicles and the membrane exocytic and endocytic pathway is mediated by a series of vesicular intermediates. Trafficking of cargo molecules through the secretory pathway relies on packaging and delivery of membrane vesicles. These vesicles, laden with cargo, carry integral membrane proteins that facilitate fusion with the target membrane. The membrane fusion process is highly conserved in all eukaryotes and the central components driving the membrane fusion events involved in vesicle delivery to target membranes are a set of integral membrane proteins dubbed as SNAREs, the abbreviation of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (Bonifacino and Glick, 2004). SNARE proteins form a superfamily of small proteins with 24 members in *Saccharomyces cerevisiae* (Burri and Lithgow, 2004), 21 members in *Aspergillus oryzae* (Kuratsu et al., 2007), 36 members in humans (Bock et al., 2001) and 64 members in *Arabidopsis thaliana* (Sanderfoot, 2007). SNAREs have a simple domain structure and posses one characteristic SNARE motif – an evolutionarily conserved stretch of 60–70 amino acids that are arranged in heptad repeats. At their C-terminal ends, most SNAREs have a single transmembrane domain that is connected to the SNARE motif by a short linker. Many SNAREs have independently folded domains that are positioned N-terminal to the SNARE motif and that vary between the subgroups of SNAREs (Jahn and Scheller, 2006). Functionally, SNAREs can be classified into v-SNAREs that are associated with transport vesicles and t-SNAREs that are associated with target compartments (Sollner et al., 1993). Specific membrane fusion between transport vesicles and target membranes ensures the accuracy of transport and is mediated by the SNARE complex that assembles into a tight cluster of four coiled-coil helices. The centre of the bundle contains 16 stacked layers of interacting side chains (Antonin et al., 2002). These layers are largely hydrophobic, except for a central layer or '0' layer that contains three highly conserved glutamine (Q) residues and one highly conserved arginine (R) residue. Based on the residues in the '0' layer in the four-helical SNARE bundles of the SNARE domain, SNAREs can be structurally divided into four groups, Qa-, Qb-, Qc-, and R-SNAREs (Fasshauer et al., 1998). Indeed, a phylogenetic analysis of SNARE sequences from *S. cerevisiae*, *A. thaliana* and mammals showed that these four SNARE subfamilies are highly conserved and diverged early in eukaryotic evolution (Bock et al., 2001).

Our analysis of the available genome sequences of *P. sojae*, *P. ramorum* and *P. infestans*, revealed the presence of 35, 34 and 37 putative SNARE proteins respectively. We chose *PsYKT6*, one of the *P. sojae* SNARE-coding genes, for a further functional characterization because it encodes an evolutionarily conserved SNARE that is involved in multiple intracellular transport steps (Kweon et al., 2003). Using gene silencing we demonstrate that PsYKT6 plays critical roles in both asexual development and sexual reproduction, and is essential for pathogenesis of *P. sojae* on host soybean cultivars.

2. Materials and methods

2.1. Gene identification and domain analysis

A set of SNARE genes was previously identified by Sanderfoot (2007). Amino acid sequences of 24 *S. cerevisiae*, 21 *A. oryzae* SNARE proteins and 64 *A. thaliana* SNARE proteins were obtained from the *Saccharomyces* genome database (www.yeastgenome.org/), the DNA Data Bank of Japan (www.ddbj.nig.ac.jp/searches-e.html) and The Arabidopsis Information Resource (www.arabidopsis.org), respectively (Uemura et al., 2004; Sanderfoot, 2007). These proteins were used to iteratively search through the *P. sojae* (genome.jgi-psf.org/Physo1_1/Physo1_1.home.html), *P. ramorum* (genome.jgi-psf.org/Phyra1_1/Phyra1_1.home.html), and *P. infestans* (www.broad.mit.edu/annotation/genome/phytophthora_infestans) genome databases using BLASTp, tBLASTn, and related searches (including keyword searches of autoannotations when possible) to assure that all possible SNARE-encoding genes were identified. When available, EST information was used to confirm exon structure; in other cases the best possible gene model was chosen based upon homology with a related genome sequence (either an internal paralog or a homolog in another organism). In one case (PITG_05324) an assembly error was taken into account based on available ESTs and trace files (www.ncbi.nlm.nih.gov/Traces/home/). In order to identify gene family members each predicted SNARE protein was iteratively compared to the non-redundant protein database at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Gene fragments and pseudogenes represent cases where the most similar sequence (both protein and nucleotide) is from a related gene in the same genome, but the encoded gene is a truncated protein or a protein lacking characteristic SNARE domains, like the *P. ramorum* pseudogene *Pr47858*. All manual modifications and the *Phytophthora* protein, DNA and coding sequences used in this study are summarized in Supplementary Table S1.

Conserved domain searches were performed using SMART (smart.embl-heidelberg.de/) and InterProScan (www.ebi.ac.uk/Tools/InterProScan/). Prediction of transmembrane domains was performed using SOSUI (bp.nuap.nagoya-u.ac.jp/sosui/) and HMMTOP (www.enzim.hu/hmmtop/). Multiple sequence alignments were performed using EBI ClustalW2 and default parameters (www.ebi.ac.uk/Tools/clustalw2/index.html). The Ykt6 homologs in other eukaryotes were retrieved from GenBank by BLASTp using the protein sequence of PsYKT6. The phylogenetic tree was constructed based on protein sequences and using Mega4 with the neighbor-joining method, 1000 replicates, and pairwise-deletion option (Tamura et al., 2007).

2.2. P. sojae culture conditions and harvesting of tissues

P. sojae strain P6497, provided by Brett Tyler (Virginia Bioinformatics Institute, Blacksburg, VA) and all transgenic isolates in this study were routinely grown on lima bean agar (LBA) media at 25 °C in the dark. Growth characteristics of the transformants were analyzed on LBA medium or minimal medium supplemented with 2.4 g/l sucrose, pectin, or xylan, The minimal medium is composed of 0.27 g asparagine, 0.15 g KH_2PO_4, 0.15 g K_2HPO_4, 0.10 g $MgSO_4{\cdot}7H_2O$, 10 mg cholesterol (dissolved in ether), 10 mg ascorbic acid, 2 mg thiamine–HCl, 4.4 mg $ZnSO_4{\cdot}7H_2O$ 1.0 mg $FeSO_4{\cdot}7H_2O$, and 0.07 mg $MnCl_2{\cdot}4H_2O$ in 1 l of distilled water. To obtain axenically prepared mycelium, agar plugs containing hyphal tips were inoculated in 30 ml of sterile clarified 10% V8 broth in 90-mm Petri dishes. Stationary mycelial cultures were incubated at 25 °C in the dark for 3 days. Sporulating hyphae were prepared by repeatedly washing 2-day old mycelium incubated in 10% V8 broth with sterile distilled water (SDW) and incubating the washed hyphae in the dark at 25 °C for 4–8 h until sporangia developed. Zoospores were filtered through Miracloth (Calbiochem) and collected by centrifugation at 2000*g* for 2 min. Cysts were obtained by vortexing a zoospore suspension for 30 s and subsequent centrifugation at 2000*g*. Cysts were germinated in clarified 5% V8 broth for 2 h. All of the collected samples were immediately frozen in liquid nitrogen and then stored at −70 °C prior to RNA extraction.