In Silico Interactome Network Analysis and Phylogenetic Relationship of Potato Peroxidases and Catalases

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ABSTRACT

Peroxidases (POXs) and Catalases (CATs) are the main antioxidant enzymes involved in scavenging H$_2$O$_2$ in living cells. Different POXs and CATs may be capable of exhibiting interaction with the constituents of the plant cell. Whereas the activity or gene expression of POXs and CATs has been investigated in potato plants, their interactions with other proteins in this crop have not been investigated till now. Determining Protein-Protein Interaction (PPI) networks could be important in providing crucial insights into the regulation of plant defense responses to biotic and abiotic stresses. STRING analysis revealed interaction of cationic, suberization-associated anionic, and Class III peroxidases in potato with several enzymes involved in lignin biosynthesis and phenylpropanoid pathways, which was in accordance with close phylogenetic relationship of the three potato peroxidases investigated in this study. The CAT1 enzyme in potato interacted with several enzymes involved in ROS production. Phylogenetic analysis of the CAT1 and CAT2 genes in this plant species referred to their close relationship. Demonstrating how each isoform of these enzymes responds to environmental stimuli and how it interacts with other proteins at transcriptional, translational, and post-translational levels seems to be useful in designing novel and effective plant protection strategies against different stresses.

Keywords: Defense mechanisms, Interactome, Plant protection strategies, Protein-protein interaction.

INTRODUCTION

Resistance mechanisms, including numerous biochemical and cytomolecular changes, occur in plant defense against biotic and abiotic stresses. Defense signaling is complex and comprises mechanisms which distinguish a range of environmental stimuli. Accumulation of Reactive Oxygen Species (ROS; including Hydrogen peroxide: H$_2$O$_2$, Superoxide: O$_2^-$, and Hydroxyl radical: OH), known as oxidative burst, is one of the earliest plant defense responses to various biotic or abiotic stresses. Various types of ROS can enhance Hypersensitivity Response (HR) or play an important role as second messengers in resistance mechanisms leading to the upregulation of defense-related genes and interaction with other signaling molecules (Chen, et al. 2013). Previously, it had been assumed that various ROS accumulate sequentially from O$_2^-$ as the primary origin. Today, we know that different ROS can be produced independently by different sources, which seems reasonable because ROS accumulation must be under control of antioxidative systems to avoid toxicity (Hückelhoven and Kogel 2003). All aerobic organisms have developed antioxidant systems for controlling ROS accumulation and maintaining redox homeostasis, as well as to 'make use' of these highly reactive molecules in signal transduction, gene expression and cellular responses to biotic or abiotic stimuli.
(which is redox signaling). Uncontrolled ROS levels lead to cell death, which may enhance plant susceptibility to pathogens (Torres, et al. 2006). So, ROS accumulation and removal are controlled in plant-pathogen interactions.

Enzymatic antioxidants such as Peroxidase (POX) and Catalase (CAT) are responsible for scavenging H$_2$O$_2$ in living cells (Barna et al. 2002). Peroxidases (EC 1.11.1.7) are oxidoreductases that catalyze the reduction of hydrogen peroxide and the concomitant oxidation of various hydrogen donors, such as phenolic and non-phenolic substrates (Carpin, et al. 2001). Most of the functions recognized for plant peroxidases occur in cell walls. These functions can be divided into two major types. The first is the oxidative cross-linking of several aromatic molecules and structural non-enzymatic proteins such as extensins via using H$_2$O$_2$ as an electron acceptor. This reaction leads to lignification (Ros Barcelö et al. 1989) or production of suberin by the function of class III secretable plant peroxidases (Bernards 1999; Arrieta-Baez and Stark 2006) and also to cell wall reinforcement via formation of covalent bonds between polysaccharides such as pectin and hemicellulose (Fry 1986; Passardi, et al. 2005).

On the other hand, some POXs such as class III secretable POXs are involved in peroxidation by producing ROS including H$_2$O$_2$, O$_2^-$, and OH$^-$ (Bestwick, et al. 1999; Raggi et al. 2015). H$_2$O$_2$ and O$_2^-$ are used in a Fenton-type reaction to produce OH$^-$, which leads to non-enzymatic cleavage of cell wall polysaccharides (Passardi et al. 2005; Raggi et al. 2015). Therefore, the POXs are capable of playing opposite functions in cell expansion, by causing both wall thickening and loosening via negatively or positively regulating ROS accumulation, depending on growth conditions (Passardi et al. 2004).

Many different isozymes of POXs are present simultaneously in cell wall and other apoplastic spaces (Raggi, et al. 2015; Ros Barcelö, et al. 1989), cytoplasm (Ros Barcelö, et al. 1989), and vacuoles (Calderon, et al. 1992). These enzymes have been grouped in three different categories, including soluble, ionically bound, and covalently bound POXs, depending on the treatment necessary for their release from the plant cell wall (McDougall et al. 1995). This suggests that different POXs may be capable of exhibiting various interactions with the constituents of the plant cell or its extracellular matrix. Despite the probable importance of these interactions for the control of POX function, the current information in this case is scarce.

The CAT (EC 1.11.1.6) is a tetrameric heme protein with four polypeptide chains, which has α-helix and β-sheet domains. It is found in all aerobic living organisms and functions in converting H$_2$O$_2$ to H$_2$O and O$_2$ (Chelikani et al., 2004). This enzyme is also found in some anaerobic microorganisms such as Methanosarcina barkeri (Brioukhanov et al. 2006). It is a main enzymatic antioxidant, which protects the cells from harmful damages of oxidative burst. This enzyme has one of the highest turnover numbers among all enzymes, as one molecule of CAT can scavenge millions of H$_2$O$_2$ molecules in each second (Goodsell, 2016). Catalase is located in the peroxisomes of eukaryotic cells (Alberts et al., 2002.). Peroxisomes in plant cells are involved in photorespiration (the use of O$_2$ and production of CO$_2$) and symbiotic nitrogen fixation (the breaking apart of N$_2$ to reactive nitrogen atoms). Plant catalases can be classified into three classes based on the expression profiles of the CAT genes (Willekens, et al. 1994; Willekens et al. 1995). Class I of catalases includes isoforms which are highly expressed in leaves and involved in the removal of H$_2$O$_2$ produced during photorespiration. The class II of catalases includes the isoforms which are generally found in vascular tissues and its physiological role is unclear, so far (Dat et al. 2000; Feierabend 2005). The class III of catalases includes isoforms which are expressed in seeds and young seedlings and
believed to be involved in the removal of 
$\text{H}_2\text{O}_2$ during fatty acid degradation in 
glyoxysomes (Willekens, et al. 1994; 
Willekens et al. 1995).

Both CATs and POXs are involved in 
protecting living cells and tissues against 
toxic effects of ROS, including oxidative 
damage of DNA, proteins, and lipids 
(Halliwell and Gutteridge, 1989; Novo and 
Parola 2008). Whereas the activity or gene 
expression of POXs and CATs at different 
stress conditions have been investigated in 
potato plants, their interactions with other 
proteins in this crop plant is not investigated 
till now. Determining Protein-Protein 
Interaction (PPI) networks, which is also 
known as interactome (Braun et al. 2013), 
could be important in providing crucial 
insights into the regulation of plant 
developmental, physiological, pathological 
and defense-related processes against 
various biotic and abiotic stresses. 
Additionally, any interaction between plant 
proteins and pathogenic organism proteins 
will also increase understanding of the plant 
proteome and defense networks against 
pathogens.

In potato (Solanum tuberosum), various 
types of POX, such as cationic peroxidase 
(poxMM), suberization-associated anionic 
peroxidase, lignin-forming anionic 
peroxidase, phospholipid hydroperoxide 
glutathione peroxidase, ascorbate 
peroxidase, cell wall peroxidase, thioredoxin peroxidase, class III 
peroxidases and several isoenzymes of 
peroxidase are identified, so far (Espelie and 
Kolattukudy, 1985; Yu, et al. 2010). Three 
isozymes of CAT, including CAT1, CAT2, 
and CAT3 are reported in potato till know 
(Santos et al. 2006).

Interactome analysis methods could be 
classified into three groups, including in 
vitro, in vivo, and in silico approaches. 
Bioinformatics predictions, also referred to 
as in silico analysis, can suggest interactions 
that have avoided recognition by other 
methods or those of proteins that have not 
been investigated yet.

The objectives of this study were to: (i) 
Investigate the interactome for each of the 
main peroxidases (including the cationic 
peroxidase poxMM, suberization-associated 
anionic peroxidase POX, and a member of 
Class III of POXs) in potato, (ii) Determine 
interaction networks for each of the three 
isozymes of CAT in this plant species, (iii) 
Predict interaction of these antioxidant 
proteins with each other via in silico 
analysis, and (iv) Determine phylogenetic 
relationships of the POX and CAT isozymes 
in potato for getting more insight about 
unknown functions and possible interactions 
of these important redox-related enzymes.

MATERIALS AND METHODS

Bioinformatic Analysis of Protein-
Protein Interaction Networks.

Interaction of CAT1, CAT2, CAT3, 
poxMM, suberization-associated peroxidase 
(POX) and a peroxidase belonging to Class 
III of POXs (CIIIPOX) with their predicted 
functional partners in potato was 
investigated based on bioinformatic analyses 
by the Search Tool for the Retrieval of 
Interacting Genes (STRING) software 
version 10 (Szklarczyk et al., 2015). 
Furthermore, the interactome of the above 
mentioned CATs and POXs with each other 
was analyzed using the same software.

Sequence Analysis and Construction of 
Phylogenetic Tree.

Sequences of the poxMM, CAT1 and CAT2 
genes of potato were obtained from NCBI 
and compared with other peroxidase and 
catalase genes in Arabidopsis thaliana, 
Solanum lycopersicum (tomato), and Oryza 
sativa (rice). Sequence alignments and 
editions were performed using the BioEdit 
Sequence Alignment Editor (Tom Hall, Ibis 
therapeutics, Carlsbad, CA, USA). Then, the 
phylogenetic trees were constructed using 
Treecon software (Van de Peer, and
Wachter, 1994). Bootstrap re-samplings with 1,000 trials were used for estimating the confidence degree in the clustering order of phylogenetic tree obtained from the sequences.

RESULTS

Bioinformatic Analysis of Protein-Protein Interactions

For detailed explanation of a protein’s function in basal resistance, knowledge about its interaction associates is a major prerequisite. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is one of the best sites to hold experimental, predicted and transferred protein-protein associations, together with interactions obtained through text mining. Using the STRING v10 database (http://STRING-db.org/), we investigated the known and predicted interactions among each of the poxMM, POX, CIIIPOX, CAT1, CAT2, and CAT3 with other proteins in potato. In addition, interaction of these proteins with each other was investigated. The protein networks consist of information concerning both nodes (proteins) and edges (interactions). Each node in the network shows a preview to 3D protein structure, and can be clicked to reveal a pop-up window with more information about the protein including its annotation, structure, and homology (Szklarczyk et al. 2015). Protein nodes that are enlarged indicate the availability of 3D protein structure information. Each edge in the obtained networks represents a known or predicted interaction, and leads to a pop-up window with information about the basic evidence and the interaction confidence scores. Colored lines between the proteins indicate different types of interaction. According to the STRING analysis, cationic peroxidase (poxMM) in potato had interaction with glycosyltransferase, aminotransferases, cinnamyl alcohol dehydrogenase, alcohol dehydrogenase and the isozymes of phenylalanine ammonia-lyase (Figure 1-a). However, no information about co-expression of the poxMM with other genes of this network was found in database.

Suberization-associated anionic Peroxidase (POX) interaction analysis based on databases (Figure 1-b) revealed the association of this protein with the same proteins that were found in the interactome of poxMM. In addition, the same proteins were found in the interactome of Class III Peroxidase (CIIIPOX) in potato (Figure 1-c). Interaction analyses data obtained for all three types of potato peroxidases tested in this study were based on databases and no evidence of neighborhood, gene fusion, and coexpression was observed in the interactome of these proteins (Figure 1).

Bioinformatic analysis revealed that the CAT1 in potato had interaction with Peroxisomal Targeting Signal (PTS) 1 receptor, Glycolate Oxidases (GOX), (s)-2-hydroxy-acid oxidases, Superoxide Dismutase (SOD), and trehalose-6-phosphate synthase (Figure 2). The STRING analyses revealed co-expression of the CAT1 with putative homologs of other genes in the obtained network, except for the PTS (Figure 2).

Proteins obtained in the interaction network of CAT1 were also observed in the STRING results for CAT2, except SOD. Co-expression of CAT2 with all genes in its network was predicted as observed for their putative homologs in other species (Figure 3).

Interaction network of CAT3 in potato consisted of the same proteins, which were observed in the interactome of CAT2 (Figure 4). In the interactome of each catalase investigated, the evidence of coexpression of the gene encoding the main protein of the network (CAT1, CAT2, or CAT3) with the genes associated with other proteins in each network, except the gene of peroxisomal targeting signal 1 receptor, was observed.
Interaction and Relationships of Potato Antioxidants

Figure 1. Interaction of cationic peroxidase 1 (a) (poxMM, Identifier: PGSC0003DMT400047150), suberization-associated anionic Peroxidase (b) (POX, PGSC0003DMT400057522), and Class III Peroxidase (c) (CIIIPOX, PGSC0003DMT400063828) with their functional partners in Solanum tuberosum based on bioinformatic analysis using STRING software (version 10).
Figure 2. Interaction of Catalase 1 (CAT1, Identifier: PGSC0003DMT4000075611) protein with its functional partners in *Solanum tuberosum* based on bioinformatic analysis using STRING software (version 10).

No interactions were observed among the CAT1, CAT2, CAT3, poxMM, POX, and CIIIPOX in *S. tuberosum* (Figure 5-a). Whereas, association analysis of Peroxidases (POXs) in this plant species revealed the presence of interactions among 15 out of 132 POXs (Figure 5-b). As is shown in Figure 5-b, most of the interactions in this network were based on text mining. Association of glutathione peroxidases (169 and 170 aa), thioredoxin peroxidases (162, 267, and 272 aa), and an ascorbate peroxidase (287 aa) was also observed based on the data obtained by various experiments, which are shown as pink lines. In addition, coexpression might occur in the interaction of the gene encoding thioredoxin peroxidase 1 (169 aa) with the genes associated with each of the ascorbate peroxidase (287 aa), phospholipid hydroperoxide glutathione peroxidase (169 aa), glutathione peroxidase (170 aa), and thioredoxin peroxidases (267 and 272 aa). Coexpression of the thylakoid-bound ascorbate peroxidase with thioredoxin peroxidases was also observed in Figure 5-c.

Comparative Molecular Phylogeny of the CAT and POX Genes in Potato and other Plants.

To better understand POX and CAT sequence variance and similarities among potato genes and other plants, a phylogenetic tree was constructed using multiple
Figure 2. Interaction of Catalase 1 (CAT1, Identifier: PGSC0003DMT400075611) protein with its functional partners in *Solanum tuberosum* based on bioinformatic analysis using STRING software (version 10).

Figure 4. Interaction of Catalase 3 (CAT3, Identifier: PGSC0003DMT400025653) protein with its functional partners in *Solanum tuberosum* based on bioinformatic analysis using STRING software (version 10).
sequence alignment. Phylogenetic tree revealed that the cationic peroxidase of Potato (poxMM) and suberization-Associated Peroxidase (POPA) were clustered in a group together with the TMP1 and TAP1 genes, which are anionic peroxidases in tomato. Other Tomato Peroxidases (TPX1, TPX2, and pox3) were grouped together in the next cluster and the rice Cationic Peroxidase (POC1), which was used as an out group, was clearly separated from other peroxidase genes analyzed (Figure 6a).

Interestingly, the CAT1 and CAT2 genes in potato clustered together with high bootstrap value of 92 percent and showed high similarity with isoforms of CAT1 in tomato. The catalase genes of Arabidopsis had high similarity with the tomato CAT2 and the CAT of a japonica cultivar of rice (Figure 6-b).
**DISCUSSION**

Proteins control all biological systems in a cell, and as many proteins perform their roles independently, the vast majority of proteins interact with others for proper biological activities. Investigating protein-protein interactions by bioinformatics or via various *in vitro* and *in vivo* methods (reviewed by Rao et al. 2014) is critical to understand protein functions in plant defense responses.

A very good software for data integration and analysis of protein-protein interaction networks (in terms of methods, resources, and graphical display) is the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (Szklarczyk, et al., 2011). This database combines and analyzes several data points to predict functional linkages and
physical interactions between proteins. It takes extensive advantage of genome context methods (gene neighboring, gene split/fusion, and phylogenetic profile), clusters of orthologous genes, coexpression data, experimental and predicted knowledge obtained from biological databases, and the literature to assign a probabilistic confidence score to each functional association (Braun et al. 2013). Primarily, the STRING database was focused on prokaryotes, but now it manages more than 1,000 sequences not only from prokaryotes but also for eukaryotes such as fungi, animals, and plant species including potato.

According to the STRING analysis, cationic peroxidase (poxMM) in potato had interaction with several genes/proteins involved in lignin biosynthesis and phenylpropanoid pathways, such as cinnamyl alcohol dehydrogenase, alcohol dehydrogenase, and the isozymes of phenylalanine ammonia-lyase. In addition, the poxMM had association with glycosyltransferase, which strongly controls phenylpropanoid pathway (Aksamit-Stachurska et al. 2008.), and also with aminotransferases, which are involved in phenolics production (Ma, et al. 2013; Tohge, et al. 2013). However, any information about co-expression of the poxMM with other genes of this network was not found in the database, which could be the subject of future researches in this plant species.

The same proteins that were found in the interaction network of poxMM were also observed in the interactomes of suberization-associated anionic Peroxidase (POX) and Class III Peroxidase (CIIPPOX) in potato. This data indicated similar functional properties and close relationship of the three potato peroxidases investigated in this study. In the phylogenetic tree, the poxMM showed high similarity to the TAPI of tomato that is involved in suberization. So, the poxMM might be involved in suberization or other phenolics production in potato cells that should be investigated in future researches.

Plants contain multiple forms of catalase, which may reveal various functions of this antioxidative enzyme (Feierabend, 2005). Higher plants generally have three main isoforms of CAT including CAT1, CAT2, and CAT3 (Feierabend, 2005; Heinz and Gerhardt 2002). The CAT1 in potato had interaction with superoxide dismutase (SOD) as a potent scavenger of $O_2^-$. Both CAT1 and CAT2 had association with various isozymes of Glycolate Oxidase (GOX) as alternative sources for $H_2O_2$ production in peroxisomes, which led to callose deposition (Rojas et al., 2012.) that is a main defense response against several phytopathogens (Hukkanen, et al. 2007; Noorbakhsh and Taheri, 2016). Interaction of both the CAT1 and CAT2 with peroxisomal targeting signal 1 receptor, $(s)$-2- hydroxy-acid oxidase, and trehalose-6-phosphate synthase was also observed in the STRING analysis. All peroxisomal proteins, such as GOX, are synthesized in the cytoplasm and then must be directed to the peroxisome. The first step in this process is binding the protein to a receptor, which directs the complex to the peroxisome. The receptors bind to a region of the peroxisomal protein that is called Peroxisomal Targeting Signal (PTS). The $(s)$-2- hydroxy-acid oxidase, found in the interaction network of both CAT1 and CAT2, is involved in phenolics production pathway (Khadem and Marles, 2010). Trehalose-6-Phosphate Synthase (TPS), which was associated with both CAT isozymes in potato, is an important enzyme in the trehalose biosynthetic pathway. Trehalose contents, which play a key function in metabolic regulation and plant resistance to biotic and abiotic stresses, are modulated by the TPS (Xie et al. 2015). So, detailed investigations on the role of TPS in potato defense against various destructive fungal and Oomycete pathogens such as Alternaria spp., Rhizoctonia solani, Phytophthora infestans and its association with in planta levels of ROS, enzymatic and non-enzymatic antioxidants could be an interesting subject of future investigations. The CAT1 of potato...
is classified in class I of catalases, which are involved in removing H$_2$O$_2$ from plant cells, whereas potato CAT2 belongs to Class II of catalases, with a role in stress protection and lignin formation (Almeida et al. 2005). Presence of the same proteins in the interaction networks obtained for CAT2 and CAT3 in potato suggested that the isozymes of CAT might have similar functions. It is known that CAT3 is a powerful H$_2$O$_2$ scavenger during degradation of fatty acids in glyoxysomes (Willekens, et al. 1994; Willekens, et al. 1995). Therefore, CAT2 in potato might be involved in this process, which needs to be investigated experimentally. Bioinformatic analyses in the present work revealed no interaction among different CATs and POXs in potato. Interaction analysis of POXs in potato revealed that 15 out of 132 of these antioxidant enzymes interacted with each other with a complex network. Most of these interactions were obtained via the STRING software using textmining, and some of them by the experimental and coexpression data. So, proving all of the observed interactions among potato POXs using valid in vitro and in vivo experiments seems to be necessary.

Phylogenetic analyses revealed the relationship among various types of peroxidases in potato compared to those of tomato. The fact that suberization–Associated Peroxidase (POPA) in potato was closely related to the anionic peroxidases in tomato, including TMP1 and TAP1, suggested the possibility of anionic nature for the POPA, which needs further investigations.

Our data obtained via phylogenetic analysis of the potato CAT1 and CAT2 genes were in accordance with a previous report by (Frugoli et al. 1998) referring to their close phylogenetic relationship, whereas, (Santos et al. 2006) reported that potato CAT1 and CAT2 genes were not phylogenetically closely related. The CAT1 belongs to the class I of catalases and is associated with photorespiration, but CAT2 belongs to the class II of catalases and is associated with

\[ \text{CONCLUSIONS} \]

This study provides information on phylogenetic structure and protein-protein interaction network to understand the critical function of peroxidases and catalases as the main antioxidants of potato, which are involved in plant responses to various biotic and abiotic stresses. Findings obtained in this work present the opportunity for further \textit{in vitro}, \textit{in vivo}, and \textit{in silico} assays, which can provide a clearer demonstration about cytomolecular and physiological roles of peroxidases and catalases in potato. These proteins could be involved not only in the ROS homeostasis, but also possibly in other yet unknown signal transduction pathways during development of different tissues, and through interaction of the host plant with various phytopathogens having different lifestyles. Identification of protein-protein interaction networks could be essential in providing critical insights into the regulation of plant defense responses against different environmental stresses. Additionally, any
interaction between plant proteins and pathogenic organism proteins will also increase understanding of the plant proteome and defense networks against phytopathogens. Therefore, demonstrating how each isoform of these antioxidant enzymes responds to various environmental stimuli and how it interacts with other proteins at transcriptional, translational, and post-translational levels seems to be useful in designing novel and effective plant protection strategies against harmful agents. The information obtained in this study about POXs and CATs interactome networks and phylogenetic analysis will help researchers in the field of oxidative burst and of biotic and abiotic stresses to design and test new hypotheses about the mechanisms of ROS scavenging and their critical roles in plant defense mechanisms in a more holistic manner.

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آنالیس شبکه برهمکنش و ارتباط فیلوژنتیکی پراکسیدازها و کاتالازهای سیب زمینی

ب. طاهری

چکیده

پراکسیدازها (POX) و کاتالازها (CAT) از آنزیم‌های آنتی اکسیدان عمدده دخیل در اندام H2O2 در سلول‌های زنده می‌باشند. انواع مختلف آنزیم‌های CAT و POX برهمکنش با اجزای سازنده سلول گیاهی باشند. با این که فعالیت آنزیم‌یا توسط این آنزیم‌ها و کاتالاز‌ها در گیاهان سبب زمینی مورد بررسی قرار گرفته، برهمکنش آنها با سایر پروتئین‌ها در این گیاه زراعی تاکنون ارزیابی نشده است. تعیین شکل‌های شکوه‌ی برهمکنش پروتئین-پروتئین در توانایی ایجاد دیدگاه های مهمی در مورد نحوه تنظیم پاسخ‌های دفاعی گیاه به تنش‌های ناشی از عوامل زنده و غیرزنده حائز اهمیت بوده. آنالیز STRING نشان داد که پراکسیدازهای کاتیوئی، آنیونی مرتبط با تولید سورین و گروه سوم پراکسیدازها در سبب زمینی با آنزیم‌های متعددی که در بیوسنتر لیگنی و مسیر فیل پروپانوئید به دلایلی ارتباط بورد. آن‌انی از این آنزیم‌ها به فعالیت در سطح تنسیت و در طراحی استراتژی‌یا بهبود حفاظت گیاهان می‌باشد.