Antioxidant genes of the emerald ash borer (Agrilus planipennis): Gene characterization and expression profiles

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A R T I C L E   I N F O

Article history:
Received 6 January 2011
Received in revised form 14 March 2011
Accepted 15 March 2011

Keywords:
Agrilus planipennis
Antioxidant
Reactive oxygen species
Superoxide dismutase
Catalase
Glutathione peroxidase

A B S T R A C T

Phytophagous insects frequently encounter reactive oxygen species (ROS) from exogenous and endogenous sources. To overcome the effect of ROS, insects have evolved a suite of antioxidant defense genes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). The emerald ash borer (Agrilus planipennis Fairmaire), an exotic invasive insect pest from Asia has killed millions of ash trees and continues to invade North America at a rapid pace. From an on-going expressed sequence tag (EST) project of A. planipennis larval tissues, we identified ESTs coding for a Cu–Zn SOD (ApSOD1), a CAT (ApCAT1) and a GPX (ApGPX1). A multiple sequence alignment of the derived A. planipennis sequences revealed high homology with other insect sequences at the amino acid level. Phylogenetic analysis of ApSOD1 grouped it with Cu–Zn SODs of other insect taxa. Quantitative real time PCR (qRT-PCR) analysis in different larval tissues (midgut, fat body, Malpighian tubule and cuticle) revealed high mRNA levels of ApCAT1 in the midgut. Interestingly, high mRNA levels for both ApSOD1 and ApGPX1 were observed in the Malpighian tubules. Assay of mRNA levels in developmental stages (larva, prepupa and adults) by qRT-PCR indicated high transcript levels of ApCAT1 and ApGPX1 in larval and prepupal stages with a decline in adults. On the other hand, the transcript levels of ApSOD1 were observed to be constitutive in all the developmental stages assayed. Results obtained reflect a plausible role of these A. planipennis antioxidant genes in quenching ROS from both diet (ash allelochemicals) as well as endogenous sources. These studies further help in understanding the adaptation/invasiveness of A. planipennis.

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1. Introduction

Aerobic organisms are continuously exposed to oxidative stress due to the by-products produced during oxygen metabolism and via the metabolism of the encountered toxins including allelochemicals and pesticides (Adamski et al., 2002; Barbehenn, 2002). These by-products include superoxide radical (•O2−), hydroxyl radical (•OH) and hydrogen peroxide (H2O2) collectively termed as reactive oxygen species (ROS). Adverse effects of ROS include damage to biologically important macromolecules (DNA, proteins and lipids) leading to programmed cell death (Hanham et al., 1983; Imlay et al., 1988; Felton and Summers, 1995; Halliwell and Gutteridge, 1999). However, in insects, ROS are also thought to be involved in innate immunity (Kumar et al., 2003; Hao et al., 2003).

Phytophagous insects have developed unique defense strategies via detoxification and antioxidant enzymes mainly to combat the deleterious effects of pro-oxidant rich diets obtained from their host plants (Felton and Summers, 1995; Krishnan and Kodrik, 2006; Mittapalli et al., 2007a). Among the antioxidant enzymes, the most studied group of enzymes are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). SOD catalyzes the dismutation of superoxide radical to stable hydrogen peroxide and oxygen; CAT catalyzes hydrogen peroxide to water and oxygen (Ahmad, 1992; Fridovich, 1987). On the other hand, APX also catalyzes the conversion of hydrogen peroxide to water but at low concentrations whereas GPX converts hydroperoxides to less reactive alcohols primarily in cell membranes (Ahmad et al., 1989). These antioxidant enzymes are collectively involved in scavenging the exogenous and endogenous ROS to prevent oxidative stress in insects (Krishnan and Kodrik, 2006; Mittapalli et al., 2007a; Yang et al., 2010).

The emerald ash borer (Agrilus planipennis Fairmaire), an invasive wood-boring beetle in the family Buprestidae has reached
high impact status by killing millions of North American (NA) ash trees (Fraxinus spp.) since its discovery in southeast Michigan in 2002 (Poland and McCullough, 2006). At the current rate of invasion A. planipennis has the potential to decimate NA ash with impacts reminiscent to those of Dutch elm disease and chestnut blight (Herms et al., 2004). However, the damage caused by this beetle to its native host (Manchurian ash, Fraxinus mandshurica) is insignificant, perhaps a virtue of their co-evolutionary history (Rebek et al., 2008). The unique phloem chemistry of Manchurian ash (phenylethanoids and hydroxycoumarins) may contribute to its resistance to A. planipennis (Eyles et al., 2007). The major damage to ash is caused by the larvae as they feed on the phloem forming typical S-shaped galleries and eventually killing the tree. Adults emerge in late spring or early summer leaving a D-shaped exit hole and feed on foliage (Poland and McCullough, 2006).

Despite the high impact status of A. planipennis as an invasive pest, there is a paucity of knowledge available at the molecular level. Therefore, we have undertaken a functional genomics approach and developed an expressed sequence tag (EST) database for A. planipennis larval midgut and fat body using 454 pyrosequencing (Mittapalli et al., 2010). From this transcriptomic database we identified the sequences of candidate antioxidant genes including a Cu–Zn SOD, a CAT and a GPX, and have designated them as “ApSOD1”, “ApCAT1” and “ApGPX1”. We report their molecular characterization and expression analysis (via quantitative real time PCR, qRT-PCR) in different tissues (midgut, fat body, Malphigian tubules and cuticle) and developmental stages (1st-4th instars, prepupa and adult) of A. planipennis.

2. Materials and method

2.1. Sequence retrieval, annotation and alignment

The sequences of antioxidant gene were retrieved from the EST database reported per Mittapalli et al. (2010). From the annotated sequences of A. planipennis EST database we recovered a full length EST of a Cu–Zn SOD (ApSOD1) and partial ESTs of a CAT (ApCAT1) and a GPX (ApGPX1). Further identification and annotation of these antioxidant genes were performed by searching against the non-redundant database using the BlastX algorithm at National Center for Biotechnology Information, (NCBI, Bethesda, MD, USA) [http://www.ncbi.nlm.nih.gov/]. Multiple sequence alignment was performed using an online tool MUSCLE provided by a web service, phylogeny.fr. (www.phylogeny.fr). The protein secondary structure of ApSOD1 was predicted using the PSSPRED protein structure prediction server at http://bioinf.cs.ucl.ac.uk/psipred/ (Jones, 1999). For phylogenetic analyses, an unrooted neighbour joining tree was constructed with 1000 bootstrap replicates and excluding positions with gap using MEGA version 5 (Tamura et al., 2007).

2.2. Insect material and dissections

A. planipennis larvae were collected from two naturally infested sites of green ash trees at Michigan and Ohio, USA. Larvae of different development stages (1st-4th instar larvae, prepupa and adults) were collected and maintained in moist conditions on ice till dissections. For tissues, 3rd and 4th instars (7–8 larvae) were dissected in ice cold 1X phosphate buffer saline as described by Vasanthakumar et al. (2008) with few modifications in the dissection technique. Larvae were pinned on a paraffin wax platform at the head and last abdominal segment to perform dissections more efficiently. Tissues including midgut, fat body, Malphigian tubules and cuticle were collected separately in 1.5 ml centrifuge tubes containing pre-chilled Trizol reagent and stored at −80 °C until RNA isolation.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from tissues and developmental stages including 1st-4th instars, prepupa and adults using Trizol reagent following the manufacturer’s protocol (including DNase treatment). The total RNA obtained was resuspended in 40 μl of DEPC-treated water and the concentration was measured using a Nanodrop (Thermo scientific Nanodrop 2000). About 1–2 μg of total RNA was used for first strand cDNA synthesis using the Superscript II first strand cDNA synthesis kit (Invitrogen). The resultant cDNA was diluted to 20 ng/μl for further use in gene expression analysis using qRT-PCR as per Mittapalli et al. (2010).

2.4. Expression analysis using quantitative real-time PCR (qRT-PCR)

First strand cDNA was used as the template for measuring mRNA levels via qRT-PCR. Reaction mixture consisted of 2 μl cDNA (20 ng/μl), 1 μl of 5 pmol/μl forward primer, 1 μl of 5 pmol/μl reverse primer, 5 μl of SYBR green (Bio-Rad) and 1 μl of nuclelease free water making the total reaction volume to 10 μl. Concentration of the template and primers were standardised based on the Ct values and melting curves. Target gene was amplified at the following conditions: 95 °C for 3 min and then, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Primer pairs were designed using the Beacon primer designer 7.0 software (BioRad) with optimal parameters (Supplementary Table 1). Quantitation of target mRNA levels in all the samples (tissues and developmental stages) was analysed by relative standard curve method as described in the ABI Prism 7700 user bulletin (User Bulletin #2: ABI Prism 7700 Sequence Detection System (http://www3.appliedbiosystems.com). A. planipennis specific TEF-1α was used as the internal control which was found to be consistent among the samples assayed (Rajarapu et al., unpublished).

2.5. Statistical analysis

Tissues were considered as treatments, and the statistical model included both treatments and an interaction between the treatments. Relative expression values (REV) obtained from standard curve method were analysed by PROC MIXED Analysis of Variance (ANOVA) with a significance level (α) of 0.05 using SAS (SAS Institute Inc. SAS/STAT User’s Guide, Version 9.1). For each treatment there were two biological replicates nested with two technical replicates each. Biological replicates were included as random effects in the analysis and technical replicates were included to count for pipetting errors.

3. Results

3.1. Gene characterization of antioxidant genes

Among the annotated antioxidant genes, we obtained the full length sequence for ApSOD1 with an open reading frame consisting of 154 amino acids (465 bp; Fig. 1A). A similarity search of the retrieved ApSOD1 against the non redundant nucleotide (nr) database at NCBI using BlastX, revealed 81% similarity to a cytoplasmic Cu–Zn SOD of Tribolium castaneum (XP_9682841.2; e–68), 77% similarity with Gryllotula orientalis Cu–Zn SOD (AAV738091.3; e–63) and 76% identity with Campo notus floridanus Cu–Zn SOD (ENF700851.5; e–62) at the amino acid level. A similar search for ApCAT1 showed 71% identity with catalase of T. castaneum (NP_001153712.1; 8e–107), 78% similarity with two different catalase isoforms of Anopheles gambiae (ABLO93761.1; 8e–107; AAR903271.1; 8e–107) at the amino acid level. Similarity search of ApGPX1 showed 62% similarity with Hydra sps. (AD156239.1; 2e–07), 56% similarity with GPX of Aedes
The deduced amino acid sequence of ApSOD1 revealed all the important characteristics of a typical Cu–Zn SOD protein including Cu binding site, Zn binding site and two signature motifs (Fig. 1A). The Cu binding site has three histidine residues at H46, H48 and H120. Three amino acid residues including histidine (H63), histidine (H80) and aspartate (D83) interact with the Zn ion at the zinc binding site. The two signature motifs were present at the N terminal and C terminal end spanning 9 and 12 amino acids respectively (Fig. 1A). The deduced amino acid sequence of ApSOD1 was subjected to secondary structure analysis using the PSIPRED software. In total the derived secondary structure yielded 9 alpha helices and 10 coils. The two signature motifs were located within the alpha helices of the deduced protein structure (Fig. 1B).

3.2. Multiple sequence alignment

A partial multiple sequence alignment of the deduced amino acid sequences of ApSOD1, ApCAT1 and ApGPX1 with other insect sequences revealed a high level of homology among the taxa (Fig. 2). The percentile of similarity (i.e. identical residues) was 53.3% for ApSOD1, 63.3% for ApCAT1 and 34.7% for ApGPX1 among the sequences compared.

3.3. Phylogenetic analysis

In order to reveal the phylogenetic relationship of ApSOD1 with other insect sequences and/or the types of SODs, an unrooted neighbour joining tree was constructed with the amino acid sequences of different types of SODs. The dendrogram clearly grouped the SOD sequences into two major clades (i) Cu–Zn SODs and (ii) Mn SODs. ApSOD1 was included within the Cu–Zn SOD clade (Fig. 3). Specifically, ApSOD1 was more closely related to the Diptera Cu–Zn SODs, compared to the Lepidopteran Cu–Zn SODs.

3.4. Transcript levels in larval tissues

The transcript levels of ApSOD1, ApCAT1, and ApGPX1 were determined in different tissues (midgut, fat body, Malpighian tubule and cuticle) of 3rd and 4th instar larvae using qRT-PCR. All the gene assayed showed a tissue specific expression pattern, wherein ApSOD1 showed high mRNA levels in the Malpighian tubules with minimal expression levels in fat body and midgut. High mRNA levels of ApCAT1 were observed in the midgut tissue with low levels in all the other tissues assayed. ApGPX1 showed high transcript levels in fat body and Malpighian tubules with comparatively low mRNA levels in the midgut and cuticle (Fig. 4). All the genes analysed showed the least transcript levels in the...
cuticle sample. Therefore, the cuticle sample was taken as the calibrator (1×) (Pfaffl, 2001) to calculate the relative fold change among the tissues. ApCAT1 showed a fold difference of >10,000× (p < 0.05) in the midgut compared to the cuticle. A fold change of 55.17× (p < 0.05) was observed for ApSOD1 in the Malpighian tubules compared to the cuticle. ApGPX1 showed a fold change of 25× (p < 0.05) in the Malpighian tubules compared to the cuticle.

3.5. Transcript levels during development

qRT-PCR analysis of ApSOD1, ApCAT1 and ApGPX1 was also performed in the developmental stages including the four larval instars (1st–4th), prepupa and adults. Both ApCAT1 and ApGPX1 displayed a differential gene expression pattern among the developmental stages assayed, whereas ApSOD1 showed a constitutive expression pattern throughout the developmental stages assayed. High mRNA levels of ApCAT1 were observed in the 1st and 3rd instars, and prepupal stages with a sudden drop in adults. ApGPX1 transcript levels were found to be high in the 4th instar and prepupal stage compared to other developmental stages (Fig. 5). Relative fold change of transcript levels was calculated by taking the adult sample as the calibrator (1×) (Pfaffl, 2001). Significant (p < 0.05) fold change of >10,000 was observed in all stages for ApCAT1 when compared to the transcript levels in the adult sample. ApGPX1 showed a significant (p < 0.05) fold change in 4th (3.3×) and prepupal (4.85×) instars when compared to the adult stage. ApSOD1 had no significant variation among the developmental stages assayed.
Fig. 5. Temporal expression patterns in developmental stages of Agrius planipennis including the larval instars (1st–4th), prepupa (PP) and adult via quantitative real time PCR. Relative expression values (REV) were calculated for all genes by normalizing the mRNA levels with A. planipennis translation elongation factor 1-α (TEF-1α). Error bars represent the pooled standard error for two biological replicates (each with two technical replicates).

4. Discussion

Phytophagous insects have developed a suite of antioxidant defense response in order to deal with the dietary allelochemicals biosynthesized by their host plants. This feature has enabled them to exploit even some of the most noxious and/or naïve ecological niches. A. planipennis seems to fit well with the latter, evident from its rampant invasion of NA ash. Resistance of Manchurian ash to A. planipennis may result from its unique phloem chemistry with high constitutive levels of phenolics compared to the NA ash species (Eyles et al., 2007). Phenolic compounds have the potential to generate ROS and in turn could create oxidative stress within insect tissues (Krishnan and Kodrik, 2006). However, to date little is known on the molecular driven antioxidant defense strategies within Coleoptera. To our knowledge this is the first report on the antioxidant genes of A. planipennis.

The tissue specific transcriptomic study of A. planipennis revealed a number of antioxidant genes along with other candidate defense genes (Mittapalli et al., 2010). In this study the identified antioxidant genes (ApSOD1, ApCAT1 and ApGPX1) shared homology with similar genes from other insect and non-insect species. Specifically, the derived secondary structure of ApSOD1 revealed several conserved regions of Cu-Zn SOD and the phylogenetic analysis clearly corroborates with previous studies on insect SODs (Yamamoto et al., 2007).

In general, phytophagous insects overcome oxidative stress during feeding with an up regulation of antioxidant enzymes like SOD, CAT, GPX (Krishnan and Kodrik, 2006). Metabolically active tissues like the midgut and fat body provide a platform for major physiological functions which are crucial for insect survival and adaptation (Mittapalli et al., 2007a, 2010). We observed tissue specific upregulation of antioxidant genes in the midgut, fat body and Malpighian tubules which is in agreement with other studies (Mittapalli et al., 2007a; Munks et al., 2005). Among the antioxidant genes assayed, high mRNA levels of ApCAT1, were observed in the midgut compared to ApSOD1 and ApGPX1. This observation probably suggests H2O2 as one of the primary ROS encountered during A. planipennis–ash interaction. Putative hypothesis supporting these results include: (i) H2O2 might be one of the primary defense responses of the host plant against insect attack as observed in previous studies (Bi and Felton, 1995); (ii) H2O2 is the by product of lipoygenase pathway and is also produced by polyphenol oxidases upon herbivory in plants (Felton et al., 1989; Kanofsky and Axelrod, 1986). However, these hypotheses with relevance to A. planipennis–ash interaction needs to be validated.

An upregulation of ApSOD1 transcripts in the Malpighian tubules is intriguing. Recently, it was shown that Malpighian tubules play an important role in detoxification and elimination of toxins (Beyenbach et al., 2010). Although speculative at this point, the function of ApSOD1 within the Malpighian tubules of A. planipennis is to be determined in future studies. Other tissues that revealed high mRNA levels of ApSOD1 include the midgut, which is a major interface for digestion and detoxification. Oxidation of dietary phenolics within this vital tissue could lead to the formation of superoxide radicals and therefore be the probable source for ApSOD1 in the midgut of A. planipennis as observed in other studies (Krishnan and Kodrik, 2006; Krishnan et al., 2007). While the high transcript levels of ApGPX1 in the Malpighian tubules of A. planipennis is intriguing but unknown at this time. The up-regulation of ApGPX1 in the fat body suggests their probable role in protecting the latter tissue from oxidative damage (Law and Wells, 1989).

High mRNA levels of ApCAT1 in larval and prepupal stages further supports its role in quenching H2O2 derived from the diet and endogenous sources. Negligible ApCAT1 mRNA levels in adult beetles might be due to (a) the difference in the secondary metabolites of ash phloem versus foliage; (b) an alternative catalase gene in dealing with the foliage specific secondary metabolites. However, further studies are required to substantiate these hypotheses both in the insect as well as the host.

The constitutive expression of ApSOD1 during developmental stages indicates its continuous participation in removal of ROS generated from endogenous sources. Higher levels of ApGPX1 in late instars (4th and prepupa) might suggest the defense against lipid peroxides stemming from tissue differentiation (Kostaropoulos et al., 1996; Mittapalli et al., 2007b). Future research on enzymatic activity and knockout studies (RNAi mediated) of these antioxidant genes may shed light on the function of these genes within A. planipennis.

Acknowledgements

The research was supported by a grant from the USDA APHIS Accelerated Emerald Ash Borer Research Program, and by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Amy Stone, The Ohio State University Extension, assisted with collection of A. planipennis larvae. Help provided by Loren Rivera-Vega (Department of Entomology, The Ohio State University/OARDC) with sample collection is appreciated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2011.03.017.

References


