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Abstract

Upon infection, the phenoloxidase system in arthropods is rapidly mobilized and constitutes a major defense system against invaders. The activation of the key enzymes prophenoloxidase (PPO) and their action in immunity through melanization and encapsulation of foreign bodies in hemolymph has been described in many insects. On the other hand, little is known about PPOs involvement in other essential functions related to insect development.

In this paper, we investigated the function of the two PPOs of the crop pest, *Spodoptera frugiperda* (PPO1 and PPO2). We show that PPOs are mainly expressed in hemocytes with the *PPO2* expressed at higher levels than the *PPO1*. In addition, these two genes are expressed in the same tissue and at the same stages of insect development. Through the generation of loss-of-function mutants by CRISPR/Cas9 method, we show that the presence of PPOs is essential for the normal development of the pupa and the survival of the insect.

Keywords

Phenoloxidase, CRISPR, melanization, development, *Spodoptera frugiperda*, Fall armyworm, Lepidoptera

1. Introduction

Like most organisms, insects must contend with micro- or macro-organisms of various nature and have evolved a sophisticated immune system (Lemaitre and Hoffmann, 2007). Even though an adaptive-like immunity essentially directed toward viruses has been reported (Tassetto et al., 2017), most of the molecular mechanisms described in insects belong to the so-called innate immunity. There are two main defense components, the cellular response (Jiravanichpaisal et al., 2006) and the humoral response (Ferrandon et al., 2007). The former is mediated by the hemocytes, circulating blood cells found in the hemocoel (Strand, 2008). Different types of hemocytes have been described in insects (Ribeiro and Brehélin, 2006), depending on their quiescent or activated state, the nature of the pathogens or the condition of the infection. Hemocytes may have multiple roles. Some of them are phagocytic cells that recognize the pathogens, phagocyte and destroy them. Other hemocytes are involved in the process of encapsulation against larger organisms such as nematodes (Ebrahimi et al., 2011) or eggs of parasitoids wasp (Anderl et al., 2016). They agglutinate around it and use the secretion of toxic compounds to kill the invader. Among such compounds, the prophenoloxidase (PPO) pathway is a particularly well known insect system (Nakhleh et al., 2017). Its final enzyme, prophenoloxidase, will be activated in reaction to a pathogen system by a cascade of serine proteases that will end up releasing the active form of the phenoloxidase (PO). This enzyme's main catalytic reaction is to convert phenolic compounds such as the amino acid tyrosine into quinones. This oxidative reaction releases reactive oxygen species (Nappi and Christensen, 2005) that will attack the pathogen, eventually killing it. Melanin is the product of this reaction and a blackening of the hemolymph is often observed due to the activation of the PPO pathway in a phenomenon called melanization (Cerenius and Söderhäll, 2004).

Beside their involvement in immunity within the hemolymph, POs have also been described for their role in other organs. In the gut of herbivorous insects, including the Lepidopteran *Bombyx mori* and *Helicoverpa armigera*, POs are excreted in the foregut and participate in the

detoxification of plant phenolic compounds (Wu et al., 2015). A role of POs in molting has also been described. It was originally assumed that POs were involved in the melanization of the cuticle during ecdysis but it is now clear that the tanning and sclerotization are due to the Laccase enzymes (Peng et al., 2020). However, POs were detected in the molting fluids (Zhang et al., 2014) of several insects. It has been demonstrated that PO activity during molting was essential for the successful ecdysis in Diptera (Bai et al., 2014) as well as in *Bombyx mori* (Wang et al., 2013a).

The current technological advancements in genetics and genomics, such as next-generation sequencing and CRISPR/Cas9 gene edition system, allow the tackling of this type of questions even in non-model organisms. Lepidoptera, for example, is an order of insects in which many studies on immunity or development have been historically performed (Jiang et al., 2010; Kanost et al., 2004). This has an enormous interest since some Lepidoptera species represent major agricultural pests of economic importance. Functional genomics in Lepidoptera has been hindered by unreliable RNAi strategies (Joga et al., 2016). In particular, a Lepidoptera specific nuclease could explain why Lepidoptera are so refractory to RNAi (Guan et al., 2018). Thus the attention turned to the promising technology of CRISPR/Cas9 (Jinek et al., 2012). Now a widely popular tool in functional genomics, CRISPR/Cas9 has been used in Lepidoptera first in *Bombyx mori* to successfully knock-down the Blos2 gene after co-injection of dsRNA and Cas9 in pre-blastoderm embryos (Wang et al., 2013b). It has since been applied many times in *Bombyx mori* (Baci et al., 2021), as well as in other non-model Lepidoptera (Jin et al., 2021; Wu et al., 2018; Zhu et al., 2020).

In the current study, we present the generation of knock-out mutants for both phenoloxidase enzymes of the fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera:Noctuidae). FAW, as a caterpillar, is a known pest of crops such as corn, cotton, sorghum and rice in the Americas where it is indigenous. But upon its first detection in West Africa in 2016 (Goergen et al., 2016), FAW has then invaded most of sub-Saharan Africa in 2017 (Cock et al., 2017; Jacobs et al., 2018; Nagoshi et al., 2017; Otim et al., 2018), India in 2018 (Ganiger et al., 2018; Sharanabasappa et al., 2018), South-East Asia in 2019 (Tay and Gordon, 2019; Wu et al.,

2019; Zhang et al., 2019) and is now a global menace. In our pest quarantine laboratory, we took advantage of the development of high-quality genomic assemblies and of a CRISPR mutagenesis platform, to generate null mutant FAW strains for PPO1 and/or PPO2, the two PPO genes found in FAW. We describe our strategy in order to obtain single and double mutants and we performed their genotypic and phenotypic characterization. We observed that the presence of both *S. frugiperda* PPOs is necessary for the melanization of the hemolymph but that *S. frugiperda* PPO can complement one another. Our main results show that single mutants are highly sensitive to their environment and that double mutants are lethal and present severe morphological defects at the pupal stage.

2. Materials and Methods

2.1. Insects

Corn-strain *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae were reared on a corn-based artificial diet (Poitout and Buès, 1970). They were maintained at 24°C+/−1°C with a photoperiod of 16h/8h (light/dark) and a relative humidity of 40%+/−5%. This strain corresponds to the published reference genome (Gouin et al., 2017).

2.2. RNA extraction and Quantitative Reverse transcriptase-PCR

For each developmental stage, a pool of five to ten insects were ground with 1 mL of Trizol (Life technologies) and one stainless steel bead (3 mm diameter) using the TissueLyzer 85210 Rotator (Qiagen) at 30 Hz for 3 min. The lysate was transferred to a new tube and left at room temperature for 5 min. After the addition of 200 µL of chloroform (Interchim), the preparation was homogenized, left at room temperature for 2 min and then centrifuged at 15,000 g and 4°C for 15 min. The aqueous phase was transferred in new eppendorf tubes and mixed to 400 µL of 70% ethanol. RNA purification was immediately performed with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Contaminating DNA was removed by the use of a Turbo DNA-free™ kit (Life Technologies). RNA yield and integrity were estimated by spectrophotometry using a Nanodrop ND-2000 and an electrophoresis on a 1% agarose gel, respectively.

Five µg of RNA was reverse-transcribed with Oligo(dT) primers (Promega) and the Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative PCR were performed in a final volume of 3 µL including 1x SensiFAST SYBR®No-ROX mix (Bioline), 0.5 µM of each primer (primers are listed in Table S1) and 1.25 µL of diluted cDNA corresponding to 13 ng of total RNA per well. The distribution in a 384-wells plate was realized by the use of an echo-525-liquid-handler (Labcyte). Amplification was performed in a LightCycler 480 System (Roche) under the following conditions: 95°C for 2 min, followed by 45 cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 15 s. Amplification specificity was checked by a final dissociation curve ranging from 65°C to 97°C. Primers efficiency, used in the

calculation of relative quantifications, was assessed by using serial dilutions of pooled cDNA samples. The LightCycler 480 software (Roche) was used to analyze amplification and dissociation curves and to determine crossing points using the Second Derivative Maximum method. Transcript abundance was determined using Elongation Factor 1α (genebank nr: KT218669) as a basis of comparison. Values are the mean of three independent experiments +/- SD.

2.3. DOPA and melanization assays

The presence of prophenoloxidase in hemolymph was evidenced by the method of Asada et al. (1993) modified as follows: 10 µL of 6th-instar larvae hemolymph were mixed to an equal volume of 95° ethanol by the use of a pipet and stand for 10 sec to activate the enzyme. Then, 180 µL of a solution of saturated L-DOPA (4 g/L) were added. The color development was recorded every 10 s, from the addition of L-DOPA up to 300 s, by taking photos with a camera Canon powershot G12. In a parallel experiment, the phenoloxidase activity was monitored by the measure of the change in absorbance at 490 nm as a function of time with a microplate reader (Infinite M200 TECAN).

After pricking, larvae were kept at 24°C with a piece of artificial corn-based medium and the point of injury was observed after an overnight period.

2.4. Establishment of KO mutants for PPO

The mutant strains PPO1$^{\Delta}$ and PPO2$^{\Delta}$ were established according to the protocol presented in Fig. S1. PPO1$^{\Delta}$,PPO2$^{\Delta}$ individuals (also called double mutants, DM) were obtained by creating a mutation on the *PPO1* gene in the PPO2$^{\Delta}$ strain (already carrying a homozygous KO mutation on the *PPO2* gene).

SgRNA target sequences were designed by CRISPOR (http://crispor.tefor.net/) (Concordet and Haeussler, 2018). *Streptococcus pyogenes*-Cas9-NLS-GFP-NLS protein (*Sp*-Cas9-GFP protein) and single guide RNA (sgRNAs, Table S2) were obtained commercially (Tacgene). The “CRISPR mixture” was prepared *in vitro* immediately prior to injection. Briefly, *Sp*-Cas9-GFP protein was mixed with one or two heat denatured sgRNAs in the presence of 300 mM

KCl and left at room temperature during 10 min to allow the formation of the complex. The concentrations of sgRNA and Cas9 proteins used were respectively 0.23 µg/µL and 4.5 µM for *PPO1* gene and 0.3 µg/µL and 6 µM for *PPO2* gene corresponding to a 1:5 sgRNA:Cas9 protein molar stoichiometry. The ready "CRISPR mixture" was injected into less than 4h-after oviposition old eggs using a Femtojet® Express (Eppendorf®) micromanipulator (Narishige®) and a Stereomicroscope Stemi 2000 (Zeiss®). Twenty minutes after the injection, the presence of a sufficient quantity of "CRISPR mixture" in each egg was checked by the observation of green fluorescence with a macrozoom fluorescent (Olympus®). Weakly fluorescent embryos were crushed to avoid rearing non-mutant individuals. Each egg clutch containing validated microinjected embryos was placed in a Petri dish with a piece of food and maintained at 24°C until hatching. Larvae were reared in normal conditions and G0 adults were individually mated with a wild-type moth to fix a hypothetical induced mutation. For each G1 brood, 5 to 10 2^{nd}-instar larvae were collected for genotyping. Pools of larvae were grounded using one stainless steel bead (5 mm diameter) and a TissueLyzer 85210 Rotator (Qiagen) and genomic DNA was extracted with the QIAamp® DNA Mini Kit (Qiagen) according to the manufacturer instructions. Target loci were amplified by PCR (primers are listed in Table S2) and mutant alleles were identified by comparison with the wild-type sequences using one of the following strategy: A) by digestion with a restriction enzyme cutting at the target locus and migration on 1% agarose gel, followed by sequencing when the restriction enzyme is unable to cut at the target locus (case of the $PPO1^{\Delta}$ strain), B) by migration on 1% agarose gel and validation by sequencing when the differences in the size of the PCR products are significant (case of the $PPO2^{\Delta}$ strain) or iii) by direct sequencing in the other cases (case of the PPO1 locus for the $PPO1^{\Delta}$,$PPO2^{\Delta}$ larvae). Positive G1 brood individuals mated each other to generate a G2 brood which produced homozygous mutants for the target locus. To identify these wanted individuals, the G2 6^{th}-instar larvae were individually genotyped. Five µL of freshly collected 6^{th}-instar larvae hemolymph were mixed with 15 µL of lysis buffer adapted from Biswas et al. (2014) [20 mmol/L tris (hydroxymethyl aminomethane (Tris)-HCl (pH 8), 1.5 mmol/L ethylenediaminetetraacetic

173 acid (EDTA) (pH 80), 1.4 mol/L NaCl and 200 µg/mL Proteinase K], incubated for 30 min at
174 65°C and then 2 min at 98°C. One µL of this mixture was used as template for the PCR and
175 one of the above strategies was applied to identify homozygous mutant individuals. When
176 homozygous mutant individuals were able to produce a living adult, they mated each other to
177 obtain the final homozygous mutant strain.

178 ## 2.5. Monitoring of development

179 Before initiating the developmental assay, the DM homozygous individuals present in a non-
180 homogeneous population had to be identified. This was performed phenotypically by
181 monitoring the blackening of the hemolymph. One drop of hemolymph was taken from 2nd-day
182 5th-instar larvae and homozygous DM larvae were identified if the hemolymph remained
183 colorless after a 2 h-incubation at room temperature (Fig. S2).
184 Thirty-six individuals each from wild type, PPO1$^{\Delta}$, PPO2$^{\Delta}$ strains and 42 individuals from
185 PPO1$^{\Delta}$,PPO2$^{\Delta}$ DM were reared for 20 days in individual boxes. Every day, some traits of their
186 development such as the survival, the metamorphosis and the size of the pupae were
187 observed. The survival of the insects was monitored by counting the dead individuals. Results
188 were analyzed with SPSS V18.0 (Statistical Package for the Social Sciences) using the non-
189 parametric Wilcoxon test with a 95% confidence interval. Five to six days after pupation (middle
190 of pupal stage), the pupae were photographed and measured using the macrozoom
191 fluorescent (Olympus®) and its software (CellSensEntry v1.18 [build 16686]). Results were
192 analyzed under R using the non-parametric U test of Mann-Whitney.

193

3. Results

3.1. PPO1 & PPO2 in *S. frugiperda* genome

Two genes encoding the phenoloxidase enzyme (*PPO1* and *PPO2*) have been identified in the *Spodoptera frugiperda* genome (Gouin et al., 2017; Nam et al., 2020). A phylogenetic analysis with 91 amino acid sequences of hemocyanin domain-containing proteins such as phenoloxidases (PPO), hexamerins (Hexa), arylphorins (Aryl), methionine-rich storage proteins (MrSp), sex specific storage proteins (Ssp) and larval serum proteins (Lsp) from lepidopteran, dipteran and hymenopteran (File S1) was performed. It showed that *Spodoptera frugiperda* PPO1 belongs to the same clade A than *Drosophila melanogaster* PPO2 and PPO3 and at least one PPO from each insect species (Fig. S3)(Lu et al., 2014) while *S. frugiperda* PPO2 belongs to clade B along with other lepidopteran specific PPO. *D. melanogaster* PPO1 belongs to clade C, a dipteran specific PPO clade. We manually curated these two genes thanks to the BIPAA platform (https://bipaa.genouest.org/sp/spodoptera_frugiperda_pub/jbrowse/) to retrieve the exon-intron structure of those genes based on available reference transcriptome (Fig. 1)(Legeai et al., 2014). This step was essential in order to design our mutational strategy based on targeting the desired exon of both genes to induce indels that would cause frame-shifts resulting in the production of truncated and non-functional proteins. In this study, exon 3 and exon 2 were targeted for the generation of PPO1 and PPO2 mutants, respectively (see Materials and Methods). It is noteworthy that the target site for PPO1 sgRNA contains a restriction site for PciI (ACATG), which was used for the genotyping of the PPO1 mutants. To obtain a double mutant (DM), exon 1 of *PPO1* was targeted in a PPO2 mutant background.

We verified that these annotated genes were indeed transcribed in *S. frugiperda* caterpillars by performing a series of RT-qPCR for both *PPO* at different times of development (Fig. 2A). We noticed that at all stages investigated, *PPO2* is generally expressed at higher levels than *PPO1*. Gradually increasing expression levels during the developmental time-course was observed for both *PPO* with a maximum expression occurring during the 6^{th}-instar, the last

larval stage before pupation. The two FAW *PPOs* are constitutively expressed since these levels of expression were observed without any microbial challenges. We also investigated tissue-specific expression of *PPO* based on published RNAseq expression data for *S. frugiperda* (Huot et al., 2019; Orsucci et al., 2020) (Fig. 2B). We could confirm that (1) expression levels in the whole animals are generally low for both *PPOs* and mostly come from hemocytes, and (2) *PPO2* showed generally slightly higher expression levels in all tissues and stages than *PPO1*.

3.2. Generation of PPO mutants

To determine the function of the two PPO in *S. frugiperda* development, we generated knock-out single mutants for each PPO ($PPO1^{\Delta}$ and $PPO2^{\Delta}$) and one DM ($PPO1^{\Delta}$,$PPO2^{\Delta}$) using the CRISPR/Cas9-based genome editing method (Fig. S1) (for details see Materials and Methods). Briefly, eggs from our laboratory strain were individually injected at an early stage of development with a mix containing a Cas9-GFP fusion protein and sgRNA(s) (Table S2) corresponding to the target exon (see above). In order to maximize the probability of generating heritable mutations, we made the following choices. First, we injected a ribonucleic complex assembled *in vitro* into very young eggs (less than 4h-old), which allowed having a CRISPR system immediately functional and helped to the production of mutation in the germinal cells. Second, we used a high dose of “CRISPR mix” to favor the mutation rate to the survival of injected eggs and a GFP-Cas9 protein to identify and eliminate eggs, which have received too low a dose. In our conditions, we obtained between 1.7% and 6.3% of survival after injection (Table S3).

G0 moths were crossed individually with the parental strain and genotyping was performed in the progeny (G1) of each cross in order to identify desired mutations. Mutant genotypes were characterized by analyzing the size of PCR products on agarose gels and/or by Sanger sequencing using PCR primers (Table S2) around the presumptive mutation site. G1 moths from one selected brood were mated with each other to produce homozygous mutant individuals in the G2 population, which can then be crossed to form a stable mutant

homozygous line in G3. The results of genotyping of homozygous mutant insects are presented in Fig. S4. For PPO1$^{\Delta}$ line, the sequencing of the PCR product showed that 4 nucleotides (ACAT) were missing in the sequence of the mutant compared to the wild-type sequence (Fig. S4A-1). In addition, as mentioned in the "PPO1 & PPO2 in *Spodoptera frugiperda* genome" section, a restriction site for the enzyme PciI (ACATG) was present at the CRISPR target site and appeared to be modified in the mutant line. As expected, the digestion of the PCR product with PciI showed two bands for wild-type larvae (Fig. S4A-2, lanes 1 and 2) whereas only one band was visible for individuals from the mutant line (Fig. S4A-2, lanes 3 and 4) or when the PciI treatment was omitted (Fig. S4A-2, lane 5). In the case of PPO2$^{\Delta}$, CRISPR induced a (-23+3 nucleotides) deletion in the sequence of mutant insects (Fig. S4B-1). Fig. S4B-2 presents the agarose gel showing the difference in size of the PCR products from mutant (Lanes 6, 8 and 10) and non-mutant (Lanes 7 and 9) or wild-type individuals (Lane 11). Finally, PPO1$^{\Delta}$,PPO2$^{\Delta}$ DM were more difficult to identify. Indeed, there was only one nucleotide less in the *PPO1* mutant sequence compared to that of the wild type and thus this mutation could only be found by sequencing (Fig. S4C).

This genome editing strategy was very efficient since for all PPO mutants, a single injection session was necessary to obtain a frameshift mutation at the desired locus (Table S3). For the PPO1$^{\Delta}$ and PPO2$^{\Delta}$ mutants, stable homozygous single mutant lines were obtained. On the other hand, because of their sensibility, DM individuals were reared as PPO1$^{\Delta}$ heterozygous in a PPO2$^{\Delta}$ background and homozygous DM individuals were identified by the use of our hemolymph blackening assay (Fig. S2) and isolated before each experimentation.

3.3. Melanization defects in PPO mutants

Once homozygous mutants were established, we analyzed the phenoloxidase activity in the hemolymph of these larvae and compared it to that of wild type larvae. We used a classical colorimetric assay derived from Asada et al. (1993) in which larval hemolymph was mixed with ethanol to activate the PPO enzymes. Then, the colorless L-DOPA, a substrate of phenoloxidase, was added and its conversion to the reddish dopachrome, indicating the

presence of phenoloxidase in the tested samples, was followed at different times (for details see Materials and Methods). The results presented in Fig. 3A shows the development of colors in all samples but PPO1$^{\Delta}$,PPO2$^{\Delta}$ DM. However, there were differences in both the kinetic and the type of color produced in the different positive samples. First, the coloring in PPO single mutants seemed to appear more slowly and was less intense than in wild-type hemolymph, which might indicate that each of the KO mutations have indeed affected the amount of PPO present in these individuals. Second, while a reddish color developed in PPO1$^{\Delta}$, a more grayish one was observed in PPO2$^{\Delta}$ (clearly visible at times 10 s and 40 s). Moreover, in accordance with the difference in expression levels observed between the two enzymes (Fig. 2), we noticed that the reaction was more reduced in PPO2$^{\Delta}$ than in PPO1$^{\Delta}$. The DM, on the other hand, had completely lost the phenoloxidase activity reflecting the expected total absence of PPO in its hemolymph. At a more advanced time of the reaction (240 s), the hemolymph of wild-type individuals turned black due to melanization and this phenomenon, completely absent in DM, was also observed in single mutants.

To investigate more deeply the differences between mutant lines, the phenoloxidase activity was also measured spectrophotometrically at 490 nm. Fig. 3B shows that while the enzyme activity in PPO1$^{\Delta}$ followed closely the one of wild-type larvae, that of PPO2$^{\Delta}$ increased more slowly. After 50 s, the activity was just half of that of wild-type. However, after about 4 min, PO activity appeared to be at the maximum for all mutant as well as wild-type lines but DM line.

When insects are wounded, a melanization dot appears at the site of injury. In the course of our DOPA assay, we prickled wild-type caterpillars and single mutants as well as DM larvae close to the second pair of prolegs. These larvae were kept and the healing process was observed. Fig. S5 shows the presence of a black dot at the wounding site in single PPO mutants, PPO1$^{\Delta}$ and PPO2$^{\Delta}$, similar to that found in wild-type larvae. Once again, the PPO DM did not respond like the single mutants since no dot of melanization was detected. Note that although DM did not present phenoloxidase activity, these individuals still managed to heal.

3.4. Effects of PPO mutations on development

Beyond immunity, it has been documented that prophenoloxidases have a broader role in insect physiology (Lu et al., 2014). Thus, in order to assess the effect of PPO mutation, we followed different life history traits such as pupal size, pupation process from 6th-instar to adult stage or survival. Our results show that in our experimental conditions, the size of pupae was drastically reduced in DM whereas single PPO mutant individuals were only slightly smaller than wild type ones although statistically significant (Fig. 4A). In addition, the pupation was affected. Indeed, we observed that pupae resulting from DM individuals showed major morphological defects (Fig. 4B) and this phenotype was present in a large majority (more than 80%) of DM pupae, while PPO1$^{\Delta}$ and PPO2$^{\Delta}$ single mutants did not show obvious defaults of morphology. On the other hand, PPO2$^{\Delta}$ insects did not have a significantly affected survival compared to wild type (TL_{50} = 20 days, p-value = 0.412), whereas a statistically significant increased mortality in PPO1$^{\Delta}$ individuals was observed (TL_{50} = 16 days, p-value < 0.001) (Fig. 5). This survival defect was more dramatic in DM (TL_{50} = 12 days, p-value < 0.001) since none of them were able to develop into adults (Fig. S6) suggesting an essential role of PPO in the completion of development in *S. frugiperda*. However, even though some adults emerged in both single mutant lines, PPO1$^{\Delta}$ and PPO2$^{\Delta}$, these two lines showed high sensitivity to their environment and were rapidly lost after only a few generations. Taken together, these results show that PPOs seem involved in the size of pupae and that the activity of at least one of the two PPOs is essential for a correct formation of pupae in *Spodoptera frugiperda*, suggesting an important role of PPO during metamorphosis.

4. Discussion

Prophenoloxidase (PPO) is a crucial protein of insect immunity (Kanost and Gorman, 2008; Kanost et al., 2004). It is mainly produced by circulating hemocytes (Ashida and Brey, 1995), activated by a complex molecular cascade involving pathogen receptors such as peptidoglycan recognition proteins (Park et al., 2007; Takehana et al., 2002) or c-type lectins (Yu et al., 1999), and serine-proteases (Veillard et al., 2016; Wang et al., 2020), and controlled

by serine protease inhibitors (Ligoxygakis et al., 2002; Tang et al., 2008; Wang et al., 2020). Activated PPOs oxidize phenolic compounds to produce melanin (Fig. 3C)(Nappi et al., 2009), which is observed in the melanization of the hemolymph and around pathogens. The by-products of this reaction are also reactive compounds that destroy bacteria (Zhao et al., 2007). In *Drosophila*, PPO mutants show an increased susceptibility to pathogens (Binggeli et al., 2014). In addition, PPOs have also been shown to play a role in wound healing and clotting (Karlsson et al., 2004; Lai et al., 2002), which suggests additional roles of PPO beyond immunity (Lu et al., 2014). As a matter of fact, generation of PPO mutants (Asada et al., 1999) as well as PPO knock-down insects (Bai et al., 2014; Wang et al., 2013a) demonstrated the role of these enzymes in development.

PPOs have been described in arthropods and other invertebrates (Smith and Söderhäll, 1991). Among insects, three major clades of PPOs can be observed (Fig. S3)(Lu et al., 2014). Clade A contains PPOs common to all insect selected species *Diptera*, *Hymenoptera* and *Lepidoptera*. *Spodoptera frugiperda* PPO1 belongs to this clade together with the dipteran *Drosophila melanogaster* PPO2 and PPO3 as well as *Anopheles gambiae* PPO1 and PPO6 and therefore, these orthologous PPOs are likely to have similar functions. On the other hand, PPOs in clades B and C are specific to *Diptera* and *Lepidoptera*, respectively, and thus might represent PPO with specific activities, which may indicate that *D. melanogaster* PPO1 and *S. frugiperda* PPO2 might be involved in different biological processes.

Hemocytes are the main source of PPO in insects. Our transcriptional studies clearly showed that both PPO are mainly expressed in the hemocytes of *S. frugiperda*. The specific type of hemocyte producing PPO seems to be diverse in different insect clades *i.e.* crystal cells in *Drosophila* (Rizki et al., 1985) and oenocytoids in *Bombyx mori* and *S. littoralis* (Liu et al., 2013; Ribeiro and Brehélin, 2006). The expression of PPOs has also been described in other tissues such as head and fat body in *Ostrinia furnacalis* (Zhang et al., 2016), head, thorax, abdomen and malpighian tubules in *A. gambiae* (Müller et al., 1999) and, to a lesser extent, gut (Müller et al., 1999; Zhang et al., 2016). Recently, the expression of PPO has been detected in the hindgut in Lepidoptera, *Drosophila* and other insects (Shao et al., 2012) where PPO would be

involved in the regulation of intestinal microbiota and melanization of the feces. In *S. frugiperda*, we observed a very weak expression in the midgut compared to the strong expression in hemocytes (Fig. 2). Feces of our laboratory insects are not black and their color seems to be due to the insect diet. Actually, feces are brown-yellow or green (Fig. S6) when insects are fed on corn-based artificial diet or leaves, respectively, suggesting weak to absent PPO activity in the hindgut. As noted in the introduction however, PPOs have also been found in the molting fluids surrounding the cuticle during ecdysis in *Bombyx mori* (Zhang et al., 2014). In some cases of infection, the ecdysis is not realized properly and a phenotype of melanization appears, especially at the larval-pupal transition (Zhang et al., 2014) when most PPO is present. The defects we observe in our study are thus reminiscent of the phenomenon described in *B. mori* and show that PPO in molting fluids is essential to protect the pupae during ecdysis in Lepidoptera and probably in other insects as well (Zhang et al. 2014).

On the contrary to other insects (Zhang et al., 2016), we did not detect any tissue specificity of one PPO compared to the other suggesting that their activity in *S. frugiperda* might be functionally redundant even though *PPO2* is clearly expressed at a higher level than *PPO1*. The results obtained with the DOPA assay also seem to indicate that the activity of one PPO can complement, to some extent, the loss-of-function of the other (Fig. 3). Indeed, *PPO1* has a lower expression level in hemolymph, thus PPO1$^{\Delta}$ has a weaker phenotype than PPO2$^{\Delta}$. In the melanin biosynthesis pathway, several steps can be achieved non-enzymatically except the first oxidation reaction of L-DOPA (Sugumaran, 2002) and subsequent steps will be achieved faster by enzymatic reactions (Fig. 3). In fact, we observed in our DOPA assay that the production of the red color, characteristic of dopachrome, appeared earlier in PPO1$^{\Delta}$ compared to PPO2$^{\Delta}$ suggesting either that the two PPOs would have the same function but their amounts would be different in the hemolymph of our insect, or that PPO2 would be the main PPO responsible for the conversion of DOPA to dopachrome in *S. frugiperda*. In *Drosophila*, Bingelli et al. (2014) reported that PPO1 is involved in the rapid delivery of PO activity while PPO2 would be a storage of PPO released if/when necessary. The role of a third

PPO in *Drosophila*, DmPPO3, has been investigated by CRISPR/Cas9 mutagenesis (Dudzic et al., 2015) and showed that DmPPO1 and DmPPO2 are the main source of hemolymph melanization and double mutants for both but not DmPPO3 reduce life expectancy of flies. Instead DmPPO3 seems specifically expressed in lamellocytes and contributes to capsule melanization (Dudzic et al., 2015). Thus, as suggested by the phylogenetic analysis (Fig. S3), DmPPO3 activity is specific to Diptera. On the other hand, the final black melanization color in DOPA assay appears incomplete in PPO1$^{\Delta}$ compared to PPO2$^{\Delta}$. Taken together, these data suggest that PPO1 and PPO2 both contribute to phenoloxidase activity in the *S. frugiperda* hemolymph. So, in *S. frugiperda*, the two PPOs could complement each other but they would not do it with the same efficiency. It has been reported that the two PPOs from *Manduca sexta* (Jiang et al., 1997), which are homologous in sequence to both *Bombyx mori* PPOs and the two *S. frugiperda* PPOs, form a heterodimer (Li et al., 2009) and are produced mainly in oenocytoids. Therefore, it would be very interesting to use the mutant lines to test in the future whether PPO homodimers are present in the mutants with only one functional PPO. Our results in *S. frugiperda* are in accordance with other Lepidoptera works, with the presence of two different PPOs, expressed mainly in hemocytes and showing individual phenoloxidase activity. The DM phenotypes (DOPA assay, hemolymph darkening and wound-healing) show that no other PO activity is present in *S. frugiperda* hemolymph since at least one PPO is required for the melanization process. This latter is independent of the melanin synthesis in the cuticle, which is known to involve a phenoloxidase-like activity encoded by the laccase genes (Arakane et al., 2005) in a process known as sclerotization. In our study, changes in cuticle pigmentation have not been observed neither at larval nor at pupal stage in any of the single or double PPO mutants, while in the same time, the lack of melanization around wounds was clearly visible in DM (Fig. S5). This confirms that PPO activity is not involved in cuticle pigmentation in *Spodoptera*, despite some reported studies (Cotter et al., 2008).

In *S. frugiperda*, both *PPO*s are expressed all along the developmental stages studied, with *PPO2* always more expressed than *PPO1*. Of note, there is a peak of *PPO*s expression at the last larval stage just before pupation (Fig. 2A). This could explain why we observed major

411 defects in the survival and development of larvae and pupae of double mutants (Fig. 4 and 5).
412 In fact, DM pupae were statistically smaller than wild-type as well as single mutants, and in
413 addition, most of the pupae showed major developmental defects and adults did not emerge
414 showing that KO of both PPOs in *S. frugiperda* is lethal at pupal stage. A similar result was
415 reported in *B. mori* (Wang et al., 2013a). Using the RNAi technology, these authors showed
416 the involvement of PPO in metamorphosis since after injection of *PPO1* dsRNA, an incomplete
417 pupation and the failure of emergence of adults were observed. In *Diptera*, the situation seems
418 to be more complex. In fact, Asada et al. (1999) produced PPO double mutants in *D.*
419 *melanogaster* by genetic crosses of Mox^{GM95} and $Dox\text{-}3^{KD95}$, PPO single mutants obtained from
420 natural populations. These double mutants were not viable. In *A. subalbatus*, injection of *PPO3*
421 dsRNA induced high pupal mortality and deformed adults that died following emergence (Tsao
422 et al., 2009). In *Bactrocera dorsalis*, the *PPO1* gene silencing by RNAi in larvae resulted in low
423 pupation rate and abnormal phenotype during the larval-pupal transition (Bai et al., 2014). On
424 the contrary, Binggeli et al. (2014) and Dudzic et al. (2015) showed that no such lethality
425 occurred in unchallenged *Drosophila* with complete loss-of-function for all PPOs. No
426 hypothesis has been raised to explain this discrepancy.
427 While still understudied, the role of PPO in wing development and cuticle formation at pupal
428 stages has been reported (Tsao et al., 2010; Tsao et al., 2009). In Lepidoptera, expression in
429 wing discs has also been reported in *B. mori* (Diao et al., 2012). Therefore, it is clearly likely
430 that *S. frugiperda* PPOs have a role in development as well as in immunity and that these
431 functions will require further investigations.

432 5. Conclusion

433 In this article, we describe the identification of two PPOs in the *Spodoptera frugiperda* genome
434 and their expression in hemocytes. We used CRISPR technology to produce mutant lines for
435 each PPO, as well as DM individuals. We show that these mutants are loss-of-function for
436 phenoloxidase activity. We also show that the presence of only one PPO is sufficient but
437 necessary for the phenoloxidase activity and the melanization of the hemolymph. Finally, we

also observed that both PPOs are needed to the survival of *S. frugiperda* larvae and that major developmental defects can be observed in double mutants. It would be interesting to study further the potential role of PPOs in the development of *S. frugiperda* larvae since interfering with this system could open new strategies of biocontrol against this pest.

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Authors Contributions

M. E., B. D. and N. N. conceived the experiments and wrote the manuscript. M. E., P.-A. G., L. L. and M. F. performed the experiments, established and maintained the mutant lines. S. P. realized statistical analysis of data.

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Competing Interests

The authors declare no competing interests.

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Figures legends

Fig. 1: Fall armyworm PPOs

Schematic and coordinates of *PPO1* and *PPO2* in the FAW genome. Exons are represented by boxes. Exon 3 and exon 2 were targeted for the generation of PPO1 and PPO2 mutants, respectively (see Materials and Methods). It is interesting to note that the target site for PPO1 contains a restriction site for PciI (ACATG) that will be used for the genotyping of PPO1 mutants.

Fig. 2: Fall armyworm PPOs expression profiles.

(A) qPCR measurement of *PPO1* and *PPO2* expression at different time-points during the life cycle of *Spodoptera frugiperda*, (B) Heatmap of expression of *PPO1* and *PPO2* from previously published experiments (Huot et al., 2019; Orsucci et al., 2020).

Fig. 3: Effects of mutations on phenoloxidase activity in hemocyte lysate.

Phenoloxidase activity was measured by the use of a DOPA assay described in Materials and Methods. Briefly, 10 µL of 6th-instar larvae hemolymph were mixed to an equal volume of 95° ethanol and stand for 10 sec to activate the enzyme. Then, 180 µL of a solution of saturated L-DOPA (4 g/L) were added. Phenoloxidase activity was analyzed both with a qualitative (A) as well as quantitative (B) approach. It is worth noting that a reddish color develops in PPO1$^{\Delta}$ while a greyish one is observed in PPO2$^{\Delta}$. (C) Simplified melanin biosynthesis pathway.

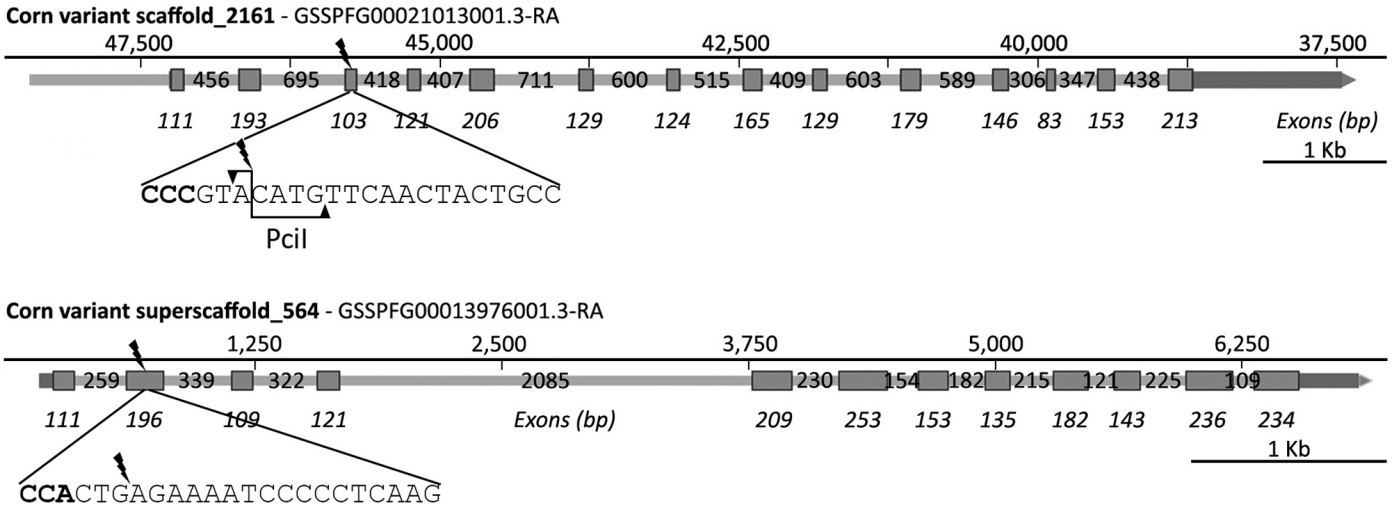
Fig. 4: Effects of mutations on development of *S. frugiperda*.

Thirty-six individuals from each wild type, PPO1$^{\Delta}$, PPO2$^{\Delta}$ strains and 42 individuals from PPO1$^{\Delta}$,PPO2$^{\Delta}$ DM were reared for 20 days in individual boxes. Every day, the size of the pupae (A) and the metamorphosis (B) were observed. Five to six days after pupation (middle of pupal stage), the pupae were photographed and measured using a macrozoom (Olympus®). Results were analyzed under R using the non-parametric U test of Mann-Whitney.

Fig. 5: Survival of PPO mutants.

L6 instar larvae from each insect lines wild-type (n=36), single (n=36) and double mutants (n=42) were followed from Day1 L6 instar up to adult stage. Dead larvae or pupae were counted every day. Results were analyzed with using the non-parametric Wilcoxon test with a 95% confidence interval. Stage duration of individual were not identical between the different lines and are presented in **Fig. S6**.

Fig. 1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45,000
42,500
40,000
37,500
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111 193 103 121 206 129 124 165 129 179 146 83 153 213
Exons (bp)
1 Kb
CCCGTACATGTTCAACTACTGCC
PciI
Corn variant superscaffold_564 - GSSPFG00013976001.3-RA
1,250
2,500
3,750
5,000
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Fig. 2

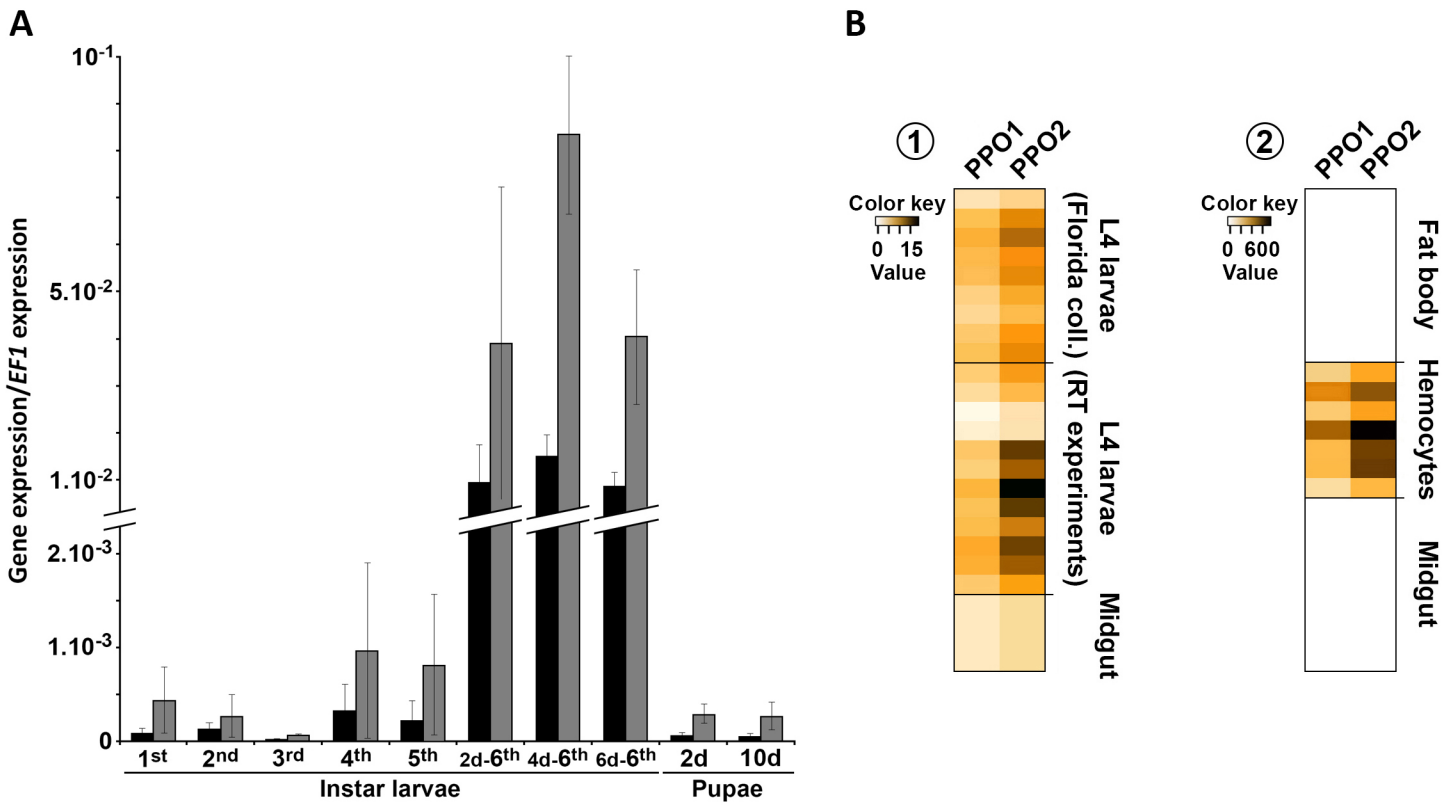

A
Gene expression/EF1 expression
10-1
5.10-2
1.10-2
2.10-3
1.10-3
0
1st
2nd
3rd
4th
5th
2d-6th
4d-6th
6d-6th
2d
10d
Instar larvae
Pupae
B
①
PPO1
PPO2
Color key
0 15
Value
L4 larvae (Florida coll.)
L4 larvae (RT experiments)
Midgut
②
PPO1
PPO2
Color key
0 600
Value
Fat body
Hemocytes
Midgut

Fig. 3

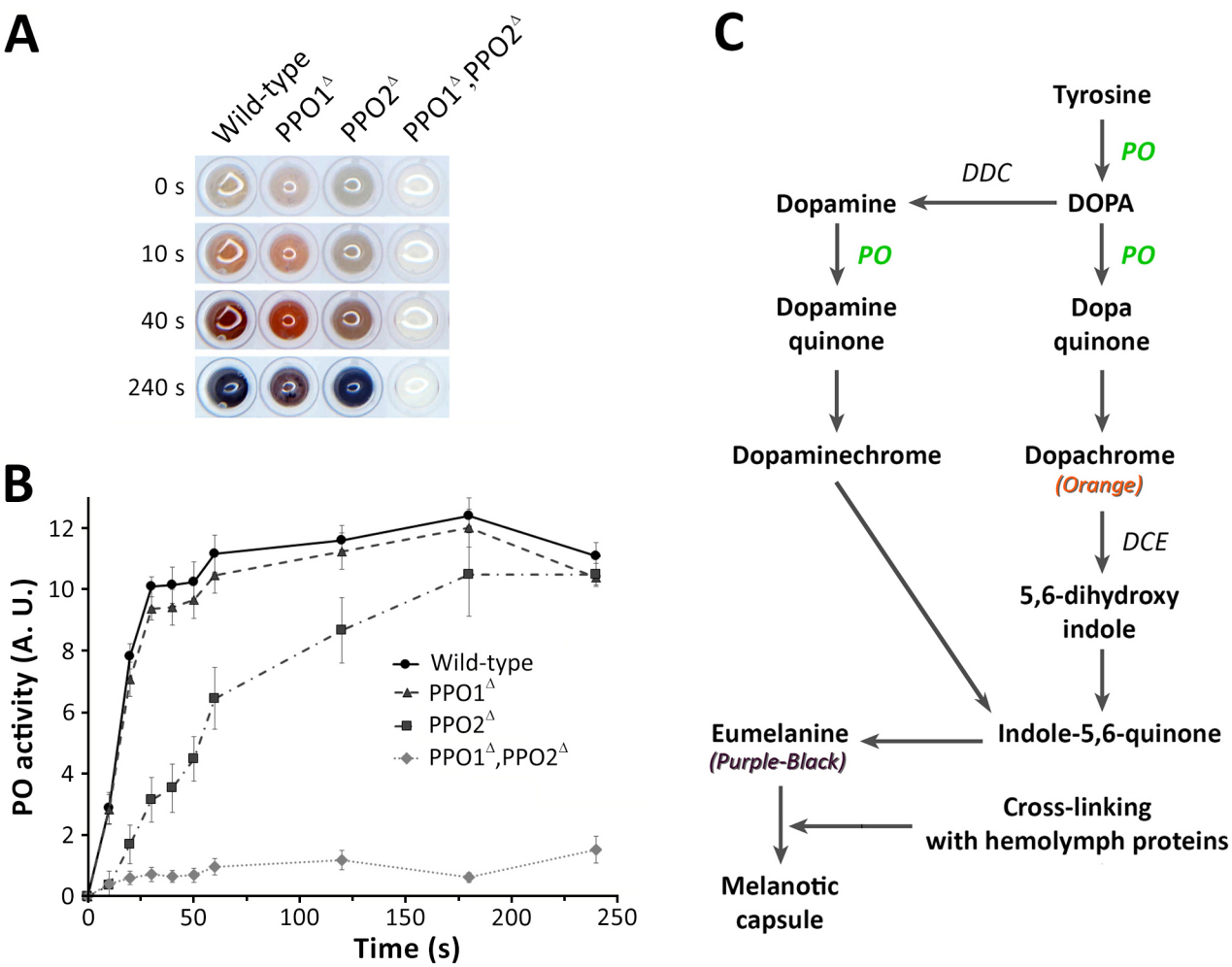

A
Wild-type
PPO1Δ
PPO2Δ
PPO1Δ,PPO2Δ
0 s
10 s
40 s
240 s
B
PO activity (A. U.)
Time (s)
Wild-type
PPO1Δ
PPO2Δ
PPO1Δ,PPO2Δ
C
Tyrosine
PO
DDC
Dopamine
DOPA
PO
PO
Dopamine quinone
Dopa quinone
Dopaminechrome
Dopachrome
(Orange)
DCE
5,6-dihydroxy indole
Indole-5,6-quinone
Eumelanine
(Purple-Black)
Cross-linking with hemolymph proteins
Melanotic capsule

Fig. 4

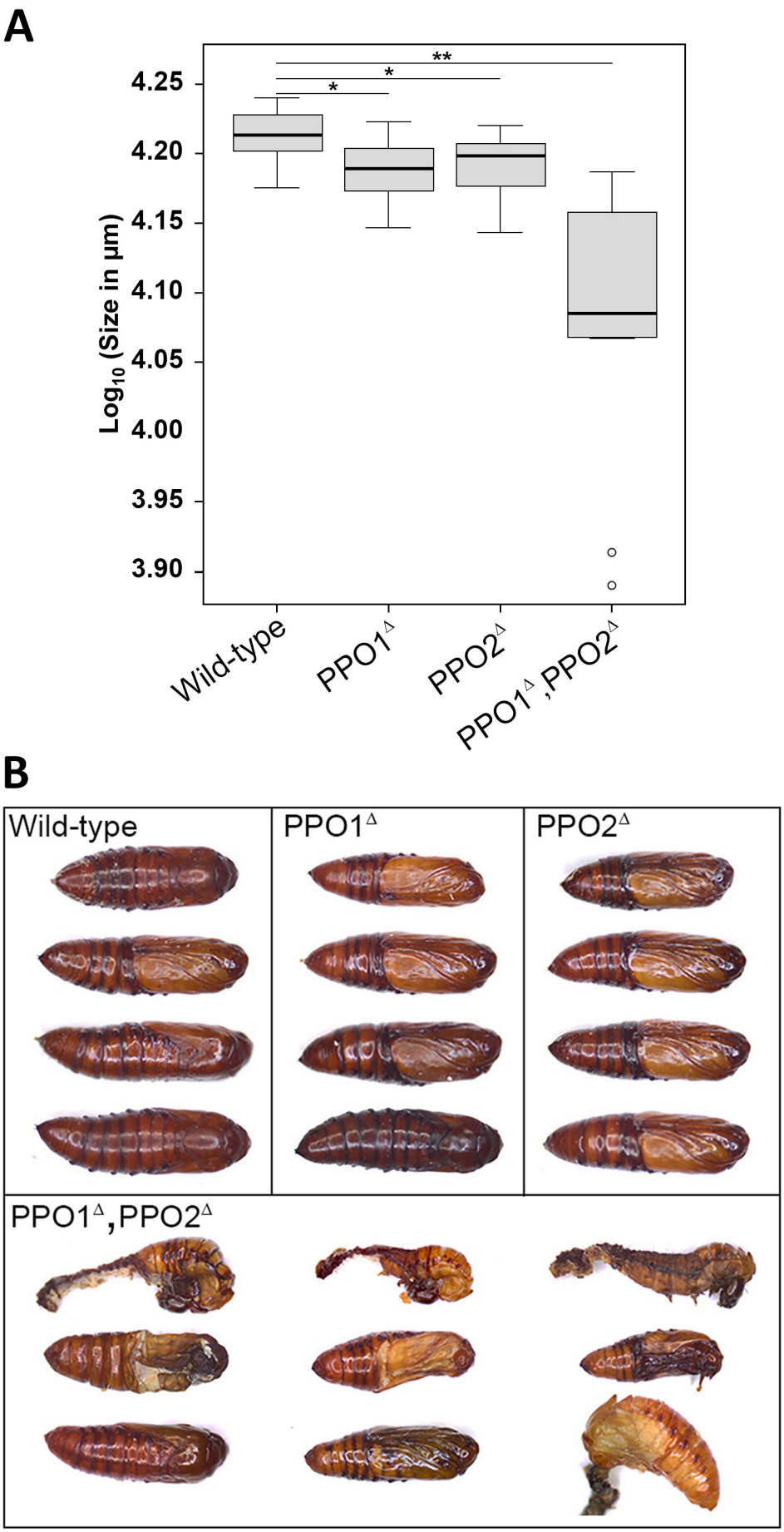
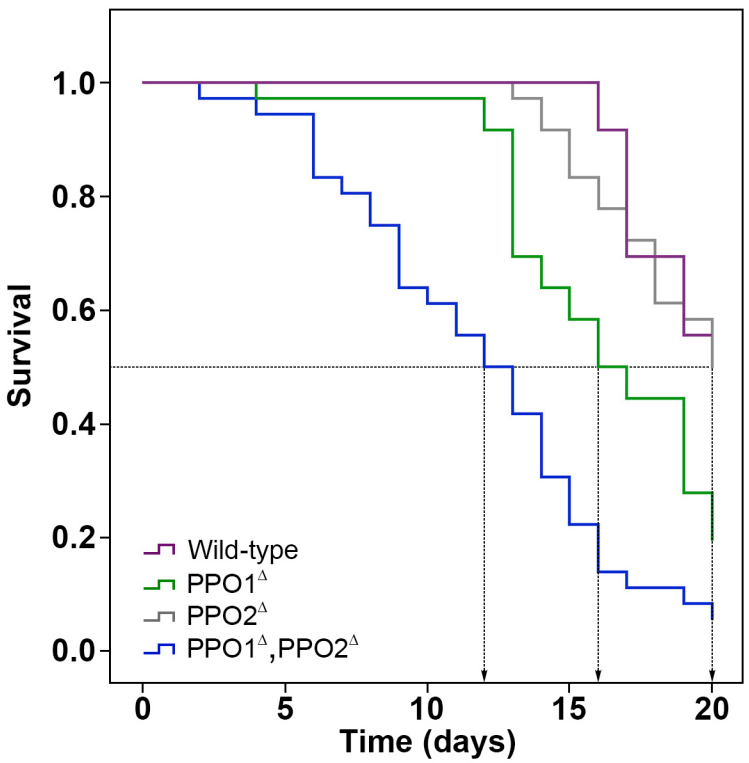

A
**
*
*
4.25
4.20
4.15
4.10
4.05
4.00
3.95
3.90
Log10 (Size in µm)
Wild-type
PPO1Δ
PPO2Δ
PPO1Δ,PPO2Δ
B
Wild-type
PPO1Δ
PPO2Δ
PPO1Δ,PPO2Δ

Fig. 5


Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
Time (days)
Wild-type
PPO1Δ
PPO2Δ
PPO1Δ,PPO2Δ

List of Supplementary Data

Table S1 - Primers sequences and genes.

Fig. S1 - Experimental strategy for the generation of KO mutant lines in *Spodoptera frugiperda*. For details, see Materials and Methods.

Table S2 - Primers for CRISPR /Cas9 technology.

Fig. S2 – Natural *ex vivo* blackening of hemolymph. Larvae were bled and their hemolymph was left at room temperature for 2 hours.

Fig. S3 - Phylogenetic analysis of *S. frugiperda* PPOs. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 91 amino acid sequences of hemocyanin domain-containing proteins such as phenoloxidases (PPO), hexamerins (Hexa), arylphorins (Aryl), methionine-rich storage proteins (MrSp), sex specific storage proteins (Ssp) and larval serum proteins (Lsp). Evolutionary analyses were conducted in MEGA 7 (Kumar et al., 2016). Branches colors: green, Lepidopteran (Bm: *Bombyx mori*, Dp: *Danaus plexippus*, Hm: *Heliconius melpomene*, Ms: *Manduca sexta*, Px: *Papillio xuthus*, Sf: *Spodoptera frugiperda*), blue, Dipteran (Aa: *Aedes aegypti*, *Anopheles gambiae*, Dm: *Drosophila melanogaster*) and orange, Hymenopteran (Am: *Apis mellifera*, Nv: *Nasonia vitripennis*). Sequences used in this analysis are listed in **File S1**. *H. melpomene* sequences were retrieved from http://metazoa.ensembl.org/ Heliconius_melpomene/Tools/Blast.

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- Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425. doi:10.1093/oxfordjournals.molbev.a040454.
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File S1 - Deduced amino acid sequences of PPO from different insects used to construct the phylogenetic tree presented in **Fig. S3**.

Fig. S4 - Genotyping of mutations. (A) PPO1$^{\Delta}$, (B) PPO2$^{\Delta}$ and (C) PPO1$^{\Delta}$,PPO2$^{\Delta}$. (1) Chromatograms showing the mutation, (2) Agarose gels. Lightning indicates the targeted sites for mutagenesis. Note that PPO1 contains a restriction site for PciI.

Table S3 - PPO CRISPR statistics.

Fig. S5 - Effect of mutation on wound healing of pupae.

Fig. S6 – Duration of development of wild-type, PPO1$^{\Delta}$, PPO2$^{\Delta}$ and PPO1$^{\Delta}$,PPO2$^{\Delta}$ lines from Day1 L6 larvae to adults.

Fig. S7 – Feces of *Spodoptera frugiperda* larvae fed on (A) corn-based artificial diet or (B) leaves.

Table S1: Primers sequences and genes.

Gene	Primer	Sequence
PPO1	q-ppo1-F	GGGCAACATGATGGAGTCTT
	q-ppo1-R	GGGGTGAGCTTGACCTTGTA
PPO2	q-ppo2-F	ACTGCCCAGAGATGCTGACT
	q-ppo2-R	TGGCACATTCTTGGTGTCAT
EF1α	q-EF1α-F	GACGTATACAAAATCGGTGGTATT
	q-EF1α-R	GATTTGATGGATTTAGGGTTGTCT

Fig. S1

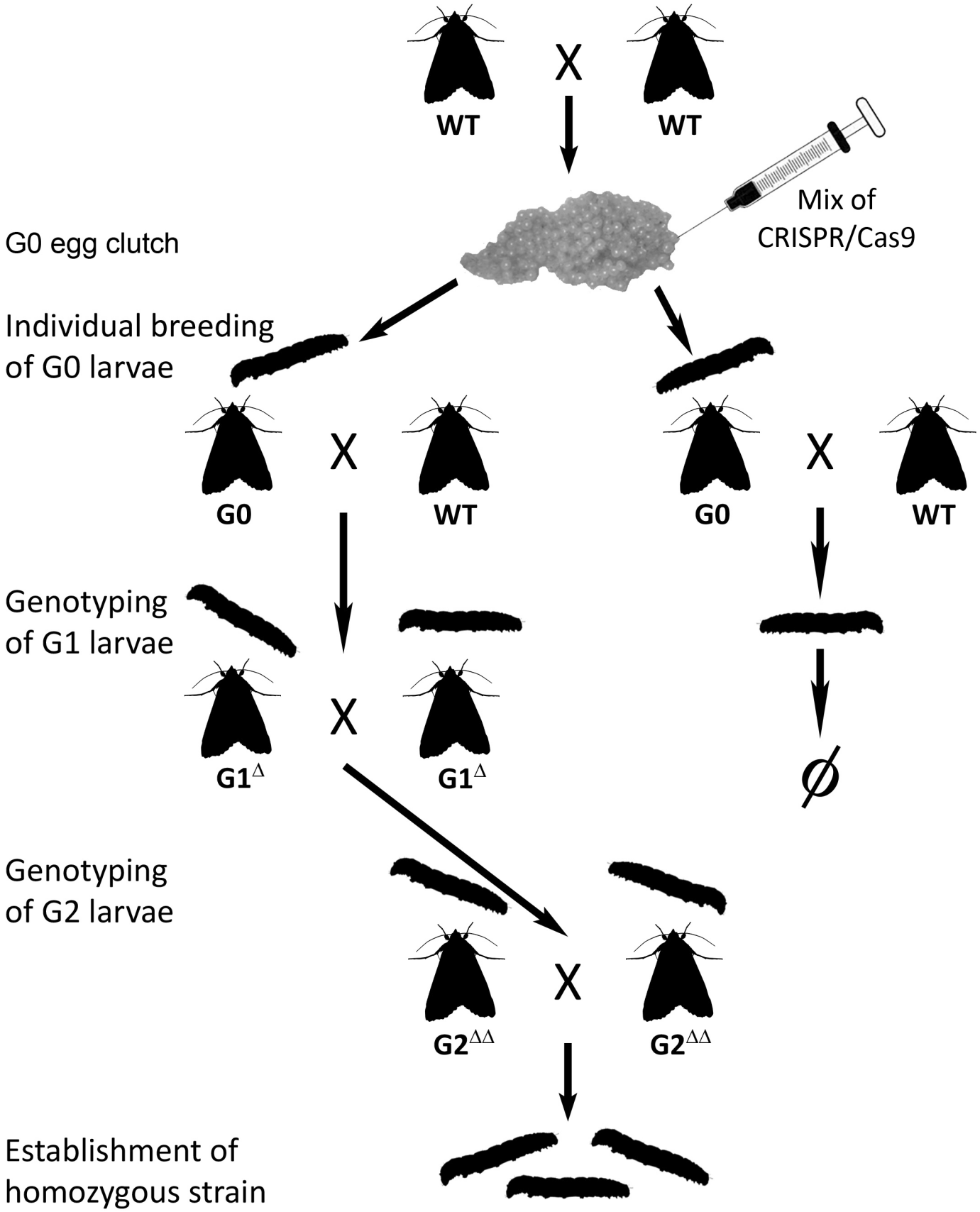

WT
X
WT
Mix of
CRISPR/Cas9
G0 egg clutch
Individual breeding
of G0 larvae
G0
X
WT
G0
X
WT
Genotyping
of G1 larvae
G1Δ
X
G1Δ
Ø
Genotyping
of G2 larvae
G2ΔΔ
X
G2ΔΔ
Establishment of
homozygous strain

Table S2: Primers for CRISPR /Cas9 technology.

Line	Target	Primer	Function	Sequence
PPO1$^{\Delta}$	Sf-PPO1 exon 3	sg1353	gRNA	GGCAGTAGTTGAACATGTACGGG
		g-Sf-PPO1E3-F	genotyping primer	AGTACCTAATACCCTTATGTTCCAG
		g-Sf-PPO1E3-R	genotyping primer	CACACACAGTTGCTCCATGC
PPO2$^{\Delta}$	Sf-PPO2 exon 2	sg1075	gRNA	GTCTTGAGGGGGATTTTCTCAGTGG
		g-Sf-PPO2E2-F	genotyping primer	TTGGGTGTCATTTGTCTTTCC
		g-Sf-PPO2E2-R	genotyping primer	GGTGTTTTGGCAGGAAGAGT
PPO1$^{\Delta}$, PPO2$^{\Delta}$	Sf-PPO1 exon 1	sg1352	gRNA	GCCGTGCTTCATGCAGAAGGGGG
		g-Sf-PPO1E1-F	genotyping primer	AGGCAATTGACTCACACCTT
		g-Sf-PPO1E1-R	genotyping primer	ACTCACGTAGTAGTGTTCAGGG

Fig. S2

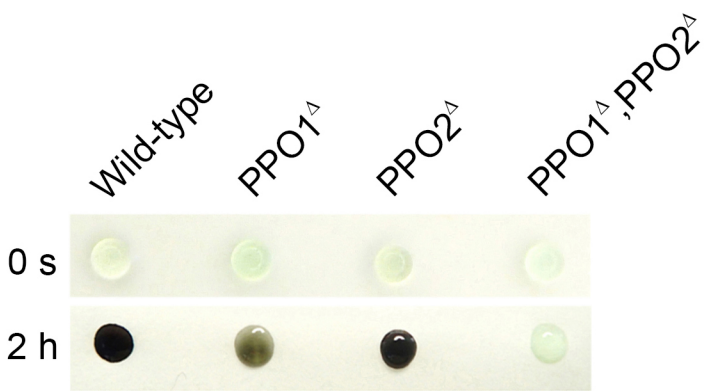
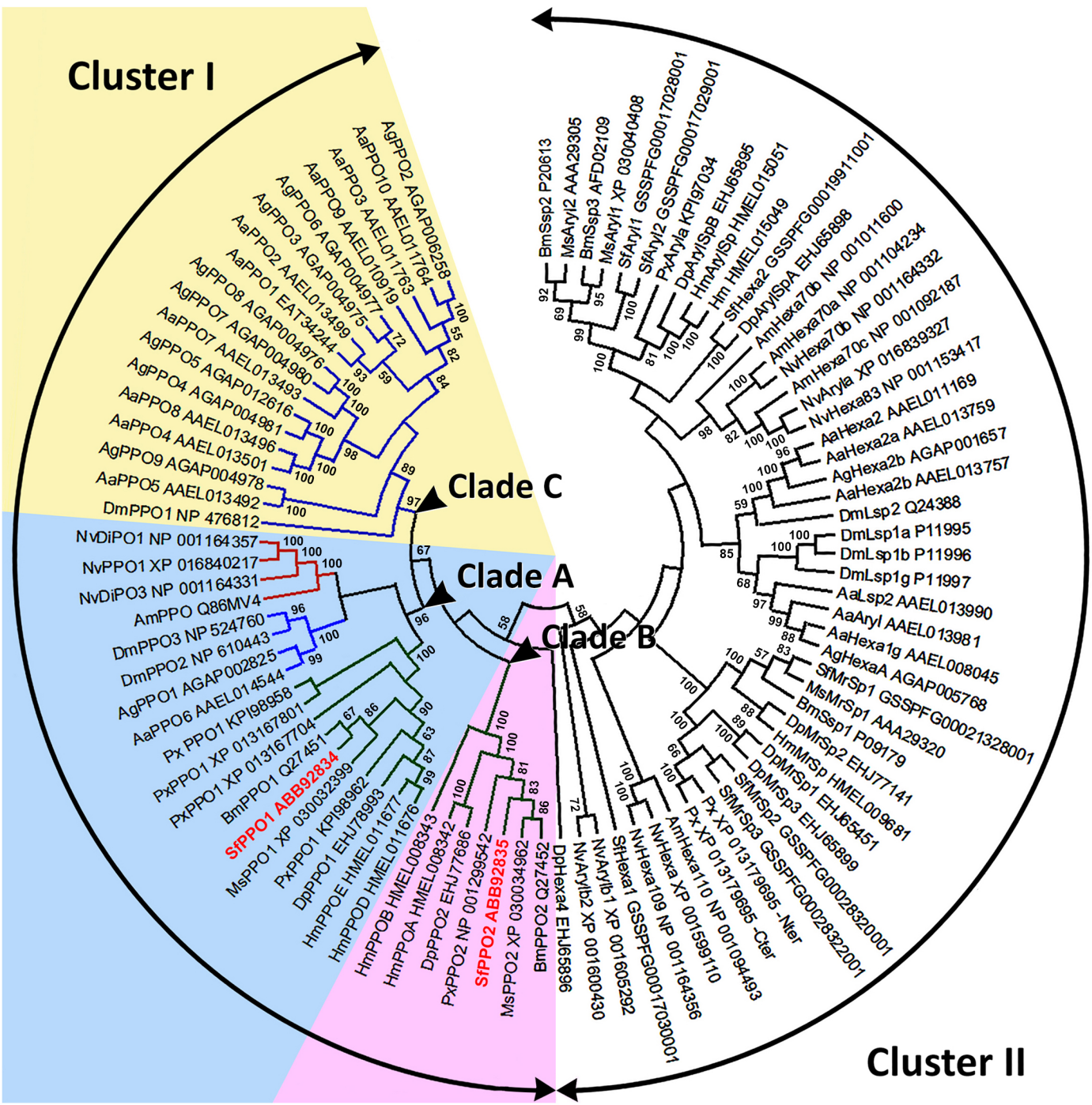


Fig. S3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Clade C
Clade A
Clade B
Cluster II
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AaPPO3 AAEL011763
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AgPPO6 AGAP004977
AgPPO3 AGAP004975
AaPPO2 AAEL013499
AaPPO1 EAT34244
AgPPO8 AGAP004976
AgPPO7 AGAP004980
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AgPPO5 AGAP012616
AgPPO4 AGAP004981
AaPPO8 AAEL013496
AaPPO4 AAEL013501
AgPPO9 AGAP004978
AaPPO5 AAEL013492
DmPPO1 NP 476812
NvDiPO1 NP 001164357
NvPPO1 XP 016840217
NvDiPO3 NP 001164331
AmPPO Q86MV4
DmPPO3 NP 524760
DmPPO2 NP 610443
AgPPO1 AGAP002825
AaPPO6 AAEL014544
Px PPO1 KPI98958
PxPPO1 XP 013167801
PxPPO1 XP 013167704
BmPPO1 Q27451
SfPPO1 ABB92834
MsPPO1 XP 030032399
PxPPO1 KPI98962
DpPPO1 EHJ78993
HmPPOE HMEL011677
HmPPOD HMEL011676
HmPPOB HMEL008343
HmPPOA HMEL008342
DpPPO2 EHJ77686
PxPPO2 NP 001299542
SfPPO2 ABB92835
MsPPO2 XP 030034962
BmPPO2 Q27452
DpHexa4 EHJ65896
NvArylb2 XP 001600430
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NvHexa109 NP 001164356
AmHexa110 XP 001599110
Px XP 013179695 -Nter
SfMrSp3 GSSPFG00028320001
DpMrSp3 EHJ65899
DpMrSp1 EHJ65451
HmMrSp HMEL009681
DpMrSp2 EHJ77141
BmSsp1 P09179
MsMrSp1 AAA29320
SfMrSp1 GSSPFG00021328001
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AgHexa1g AAEL008045
AaAryl AAEL013981
AaLsp2 AAEL013990
DmLsp1g P11997
DmLsp1b P11996
DmLsp1a P11995
DmLsp2 Q24388
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AaHexa2 AAEL011169
NvHexa83 NP 001153417
NvAryla XP 016839327
AmHexa70c NP 001092187
AmHexa70b NP 001164332
AmHexa70a NP 001104234
DpArylSpA EHJ65898
Hm HMEL015049
HmArylSp HMEL015051
DpArylSpB EHJ65895
PxAryla KPI97034
SfAryl2 GSSPFG00017029001
SfAryl1 GSSPFG00017028001
MsAryl1 XP 030040408
BmSsp3 AFD02109
MsAryl2 AAA29305
BmSsp2 P20613

File S1

>*Sf*PPO1 [GSSPFG00021013001]
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PDTKGLNIPTFAETFPDKFMDPKVFRKAREVSNVVTSGVRMPVTIPVNYTANDSEPEQRVAYFREDIG
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QQRR

>*Sf*PPO2 [GSSPFG00013976001]
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IDDTCQRHKESQYVRPYTRSELENPGVQVTSIAVETAGGQPNTLNTFWMSSDVDLSKGLDFSDRGAVY
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VVEPNPRNPPSTV

>*Aa*PPO1 [EAT34244]
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IART

>*Aa*PPO2 [EAT34245]
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VNLHHWHWHLVYPGDGPDAVVRKDRRGELFYYMHQQLIARYNVERFCARLARVRPLNNLRVPIPEGYF
PKIIRSLTNRAFPPRPQNQILSDVNRVDDQVVFTITDLENWEQRISDSIDAGFVMGMNGQRIPLTEQN
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DNMFHRHKNTLPPYTASQLNYNGVQIQSLGVQLNRANAPANVLLTYWQRSQVDLATGLDFGPQGNVFA
SFTHLQHAPFTFRLMVNNSGARRRGTCRIFIGPKTDERNIPLTYQEQRILMIELDKFNVTLNPGANSI
VRRSEQSSVTIPYERTFRSVAMSNQPTGAEQFRFCNCGWPSHLLIPKGTPEGMTFDLFAMISDFTGDT
VNQEFDENVDCNDSHSFCGLRDRLYPDRRPMGYPFDRPATGATRTLPDFISSNSNMALANVQIRFTNT
VIART

>*Aa*PPO3 [EAT36126]
MAESKSFQALLQRPLEPSFMPKDDGKTSLIIPDEYLSDRYRPLAGDLQSRFTSGAEVEIPVRNVGIPD
LAFAEVIDRRGAFSLFVEKHRDIAGKLIDVFVKVPDASSLVGIATYARDRLNPYLFQYALSVAIQHRP
DTKDVPIPSILDLFPDQFVDPSTFPKLREEGSIVNQAQRTAIDIKPNFTASDREPEQRMAYFREDIGV
NMHHWHWHLVYPAGAPPEIAKKDRRGELFYYMHSQIISRYNVDRFCNRLGQLQPLTNLQAPIPEAYFP
KMVRSLNNRAYPARHDNQVMRDLNRVDSDTVITVNELQRWRDRIYQAIDQGFVVDSSGKNIPLDEVKG
IDILGDLVEASTLSVNRRLYGSLHNFGHDFLAYIHDPEYRYLEDFGVMGDVTTAMRDPVFYRWHGMID
GLFRRFLETLNPYTSTQLAYNGVKVNSITARVNRANAPMNILLTYWQRSQVDLAAGLDFGPQGNVFAT
FTHLQNAPFTYEIKVTNSSGSLKRGTARIFLAPKVDERNTSLQFREQRRYFIELDKFGVNLKPGLNTL
TRRSDQSSVTIPYERSFRRIGTSETPTDARQLAEFRFCGCGWPQHMLLPKGAPEGVQFDLFIMISNFD
DDSVGQEFDETVDCNDAHSFCGIRDKLYPDKRTMGFPFDRRTEANVATLTDFMRPYSNMAATNVQIKF
TNTIISRT

>*Aa*PPO4 [EAT34239]
MFAMASNSTKFHGLLQRPYEPMFLPKSNGQVYFDLPDNYLTDRYRPLGQSLQNRFGSNVQTRIPVQNI
TAPDITFAQVIPRRGGFSVFNQRHRQVAGRLIEMFLNQNDADTLCAVASYCRDRLNGPLFQYALSVAL
QHRTDTAEIPVPSFLELFPDRFIEASAFPQLQEEGRIVNQGDRMAIDIPVNYTASDRELEQRMAYWRE
DIGVNLHHWHWHLVYPAEGPDVVVRKDRRGELFYYMHQQTMARYNIERFCNGLSRVRPFAQLREPIPD
GYYPKITQSSNNRSYPGRGRNQLLNDLNRVEDQVIVSITDMETWTGRIFEAIDSGSASTSDGRQIPLD
DKSGIDVLGNMVENSSLSPNVNFYGRLHNEGHNMLAYVHDPDNSFLESFGVVGGNTTAMRDPVFYRWH
QHIDDIFQRHKRRFKPYTKDDLSFSDVEVDSFNVQLNRAGAKNNILLTFWQRSQVDLGAGLDFGPEGN
VFATFTHIQHAPFTYRIEIKNESRTPKRGTVRLFMGPRTDEKGNNIPFGDQRRWMIELDKFTVNLNPG
ENNVVRRSEQSSVTIPYERTFRNIAMSNQPESDQFRFCNCGWPSHMLLPKGTPQGQQYDLFVMVSNFN
NDTVNQEYNENTPCDDSHSFCGLRDRMYPDARNMGFPFDRVTPSNVSSLKEFVKPYKNMATTPIQIRF
TNTVIARS

>*Aa*PPO5 [EAT34242]
MTDWKTGFMCLMTRPREPLFYPKYGGQTFMVLPEHFFTDRYRPLAPIINDRFGSAPNHVVVRSIDPPD
FTYAMEIPRNGNYNQFSPKHRNIAGKLVQDFMNAPDPEILLSMASYGRDRLNPLLFQYALTVALLHRK
DTQDVPVLSLLETFPKRFVDPLLFPQLEEEGFVVDQPQRVAIEIPMVYTASDELLEQRLAYFREDIGI
NLHHWHWHIIYPMEGPIEIVRKDRRGELFYYMHRQILVRYIAERFCHLMPAPTSLDNLREPIPEAYFP
KLLNSALNRTYSGRHENAVMTHVNRPEDEDSVATVLEIENWITRFKEAITHGYVVGEDGQRIPLEDST
GIDLLGNVMENSLLSPNIPHYGNIHSMLHSLIGFVHDPDNKYLEGVGVIGDIATSIRDPIFYRLHTFV
DDLFEDYKKKLAPYTTKELGYPGVTIEDVSVQDVNGKAAVNRLLTFWQRSQVDLGVGLDFGPSGHVLA
TFTHLQHAPFVYRINVMNDAQRVRRGTVRIFLAPIYNGSGEPLSFNQQRRYVIELDKFTVTLTPGMNN
IVRRSEDSSVTIPFERTFRRQDISTMPGTESYRFCNCGWPDHMLIPQGSPHGLPYDLFVMVSDFKQDA
VNVDFDENLDCNDSHSYCGLRDKFYPDRRAMGFPFDRTVPKEITHMEAFTGSFSNMKRTVVEIRFTNT
TISKV

>*Aa*PPO6 [EAT33194]
MNYKKNLLLLYDRPREPIFMGKGKSVFDVPDNYLTDRYRPIGPEIQNRFGELAEERIPVRSIALPDLR
IPMSLGRQEQFSLFIPRHRKIAARLIDIFMGMRNIEELQSCAVFARDRINPYLFNYALSVALLHRRDT
KNLDLPSVVEVFPDKYVDSRVFEQIREEATVVPEGMRMPIVIPKDFTASDLDEEHRLWYFREDIGVNL
HHWHWHLVYPFEASNRAIVDKDRRGELFYYMHSQLIARYNFERFCNRLQRVKRLNNLREPIAEGYFPK
LDSLVASRTWPGRVDNAVIKDLNRELDQIKQDVSDLERWIDRIYEAVHQGYVVDESGNRIFLDEEKGI
DILGNIIESSILSPNRQLYGDMHNVGHVFLSYTHDPDHRHLESFGVMGDVATAMRDPVFYRWHSFIDD
IFQEHKIKLPAYTKSQLTYEGISVTGIIVQSEGAPVNTLHTYWQQSDVDLSRGMDFVPRGNVFARFTH
LQHAPFQYVIQIDNTSDAQRMGFVRIFMAPKNDERGQPMLFRDQRLFMVEMDKFLVALRPGANRIRRR
SNESTVTIPFERTFRNLDDKRPETGTPEEEAFNFCGCGWPAHMLVPKGLPEGFPADLFVMVSNYEDDR
VVQDLVGTCNDAASYCGVRDRLYPDRKAMGFPFDRLARTGVDRLSNFVTPNMAIQSVNVIHIDKTVPR
T

>*Aa*PPO7 [EAT34241]

MISNQVKFSGHFYNPHVPIFVPKENGRVFYEVPERFLSDRYRPLGSNLMNRFGTNAGVRIPVRDIDPP
NIEFANVVSRRGDFSLFNRSHREAANQLIQLFMQQPDADTLNAVSAFVRDRLNAPLFQYALSVALHHR
TDTADVTVPSILHTFPDRFIDPATFPRMREEGRIVSQGDRMAIDIPMNFTALDREPEQRLAYFREDIG
VNLHHWHWHLVYPGVGPREIVNKDRRGELFYYFHGQMVARYHVERFCNGLGPLAPLDNLREPIREPYY
PKILRSANNRTHPARYPGMVLMDVNRSADQINITVAEMEQQLSRIIRSIDDGFVMSSNGERISLDNPG
GIDVLGNILESSILSPNTDFYGDVHNNGHIMISYIHDPDGTYLESFGVMGDVSTAMRDPIFYRWHLFI
DQICRRHKNRFSPYSYPELSYQGITLLKFETRLQRRGSPPNLWLTFWQRSQVDLGTGLDFGPEGNLFA
TFTHLQHAPFLYQLQVINSDNRNRRGTVRIYLAPRYDEKGARLTFEQQRLHTIELDSFRVRLNPGVNN
IMRRSEQSNVTIPYERTFRNISQSNESGNEQFRFCNCGWPSHMLIPKGNADGVTYDLFAMISNFSGDA
VDVDYDENQNCDDSYSFCGLRNRRYPDARNMGYPFDRRVPPGVNSFADFVAPYQNMTVTTVALRRTSR
TSGDPYACEQLLSLLG

>*Aa*PPO8 [EAT34240]
MASNSAKFHGLLQRPYEPLFMPKSNGQLYFDLPDNYLTDRYRAIGQSLQTRFSTNISTRVPLQNITPP
DISFAQVVPRRGGFSVFNTRDRKAAGQLIELFLNQSNPDALFAVAAYSRDRLNGPLFQYALSVALQHR
PDTANIPIPSFLELFPDRFIEASTFPRLQEEGRIVNQGDRMAVDIPINNTASDLEPEQKVAYWREDIG
VNLHHWHWHLLYPSEGPDRVVRKDRRGEIFYYMHQQIQGRYNIERFCNGLPRVKSLAQFRDPIPEGYY
PKITQSSNNRTYPGRSRNQVLHDLRRVEDQVEVTIAEMEVWTGRIFEAIDSGFAMSANGQRIPLDNKE
GIDVLANMVENSSLSPNADFYGRLHIEGHQMIGYIHDPDNSFLEGFGVIADNTTSMRDPAFFRWHQHV
DDIFERHKRKFRPYTASDLSNPNIEVNSIDISLNRAGASNNVLLTFWQRSQVDLGIGLDFGPDGNAFV
TFTHIQHAPFSYQIKITNNSRTPKRGTVRIFLGPTGDQNGKSIPIQQKRRWMLELDKFAVNLNPGVNN
VVRRSEQSNLTIPYERTFRNVAASTQPESDQFRFCNCGWPSHMLIPKGTPQGTRFDLFVMVSNYNDDV
ISPGFDENIPCDDAHTFCGLRDRLYPDARSMGFPFDRLLGPSVNSLEDFAKPYKNMALNQVLIRFTNT
VIARS

>*Aa*PPO9 [EAT37047]
MSSSADVLALLQRPLEPTFYPKDNGKVVLEIPDEYLTDRYRPIGDELQKRFTDNVDIRIPVRNVGTPD
ISFAQVIDRRASFSLFIEKHREIASKLIELFIQQPDIPSLIGVASFCRDRLNVFLFQYALAVAVQHRQ
DTNSVNLPSILELFPDQFVDPAVFPKLREEGKVVSQANRMVIELPQNFTASDREDEQRLAYFREDIGV
NMHHWHWHLVYPTSGPREIIDKDRRGELFFYMHQQIIHRYNVERFCNRLGKVRSMHNFREPIPEAYFP
KMVRTSNSRPYASRPKNFTIKDIDRDVEGLKFTINEMEQWRDRIYTAIDAGFVVDATGKNVPLDEKKG
IDILSNIIEASDLSINPKLYGNLHISGHIALAFCHDPDNRYLEEYGIMGDVATAQRDPTFYKWHSFID
DVLVRHKDTFDPYTAKDLSLPGVSISSLSVSLNRPKAPPNVLLTYWQRAQVDLASGLDFGPDGSVFAS
FTHLQHAPFAYNFEVINSNRAPVRGTARIFIAPKTDDRNTPLKYSEWRGYVLEMDKFVVMLNPGSNRI
TRRSDQSSVTVAFDRTFRQIKTDKDLPDKTELEKFRFCGCGWPDHMLLPKGTPEGMRFDLFVMISNYA
DEDIGQNIDMTSPARCDDSHSFCGLRDQKYPDGRSMGFPFDRKSPSSVQTVQDYVAPYPNMRTTDMTI
KFTNTVIART

>*Aa*PPO10 [EAT36127]
MTEANNLLALLQRPLEPTFLPKDDGKTVIDVPDDFLTDRYRPIGAELQSRFSSDAEQRIPIRSTAPPD
LSFADGIDRRGPFSLFIPKHREAAATLITLFMNQPDVQTLMSVATYSRDRVNPVLFQYALAVAIQHRP
DTKDVNIPSIVSLFPDQFVDPAVFPKLREEGSVVQRANRMVIDIPPNYTASDREEEQRLAYFREDIGV
NMHHWHWHLVYPGDGPDSVVRKDRRGELFYYMHSQLIARYNIERFCNRLARVKNLSNLREPIPEAYFP
KMIRSSNARSYPGRHANITLQDVNRVDNNTNVQVNDLERWRDRILEAIDQGFVLDTQGNRITLDETRG
IDILGDIVEASSLSPNQRLYGSLHNMGHNVIAYIHDPDYRYLEDYGVMGDVTTAMRDPIFYRWHGLID
QIFRRFKDSLTPYTSSDLTFPGVTVNSVGVQLNGANTPPNVLLTYWQRSQVNLASGLDFGPEGNVFAS
FTHLQHAPFAFRVEINNDTGAVRRGTLRIWLAPKVDERGTPLTFQEQRTYFVEMDTSTVTLNPGVNTI
VRRSDQSSVTIPYERTFRAVGNTTQPSDPNALAQFRFCGCGWPQHMLIPKGAAGDGVEFDIFAMVTDY
TMDAVDQTSDTLGPCDDAHSFCGLRDKLYPDRRAMGFPFDRKVEASVATLQDFVTPNSNMRTNSLRVR
YTNKLIARA

>*Ag*PPO1 [XP 312089]

MPFDKKHLLLLLDRPREPVFMGKGRVVFDVPDNYLTDRYRPIGSEIQNRFGENAEERIPVRSIALPDL
RIPMSLGRQEQFSLFIPRHRKIAARLIDIFMGMRNVEDLQSCAVFARDRINPYLFNYALSVALLHRKD
THDLDLPTIIEVFPDKYVDSKVFSQIREEATVVPEGMRMPIVIPKDYTASDLDEEHRLWYFREDIGVN
LHHWHWHLVYPFDASNRAIVDKDRRGELFYYMHQQLVARYNFERFSNRLQRVKRLNNLREPISEGYFP
KLDSLVASRAWPGRVDASVLKDLNREADQIKQDVADLERWIDRIYEAIHQGFVVDESGNRIPLDEQKG
IDHLGNIIESSILSPNRQLYGDMHNMGHVFISYAHDPDHRHLESFGVMGDVATAMRDPVFYRWHSYID
DIFQEHKNKLPPYTRSQLTFDGISITGITVQPEDGPPNTFQTFWQQSDVDLSRGMDFVPRGNVFARFT
HLQHSPFVTTIMIENDSDAQRMAFVRVFLAPKNDERGTPMVFRDQRLFMIELDKFLVALRPGANRIRR
RSKESTVTIPFERTFRNLDQNRPEADTPQEAEFNFCGCGWPAHMLIPKGLPEGLPADLFIMVSNYEED
RVVQDLVGTCNDAASYCGVRDRLYPDRKAMGYPFDRAARSGVDSLANFLTPNMAVQSITVVHNDRTVN
RTG

>*Ag*PPO2 [EAA10758]
MTDANNILALLQRPLEPTFLPKDDGKTVIDLPDEFLTDRYRAIGADLQSRFSNDAEQRIPVRSVPMPD
LSFANGIDRRGAFSLFAPKHRDAAGALINLFLQQPDFATLMSVATYCRDRLNPVLFQYSLAVAVQHRE
DTKDVNIPSIVSLFPDQFVDPAVFPKLREEGAAVQQENRMVIDIPPNYTASDREDEQRMAYFREDIGV
NMHHWHWHLVYPGDGPDEVVRKDRRGELFFYMHSQLIARYNADRFCAKLKKVRNLTNYREPIVEGYYP
KMIRSSNNRSYPARAANTTLQDVDRVDNGTTVSVNDLERWRDRIHEAIDQGFVLDKSGNRIMLDEQRG
IDILGDVVEASSLTPNAQLYGSLHNMGHNVIAYVHDPDYRYLEDYGVMGDVTTAMRDPIFYRWHGMID
GIFRRHKELLTPYTAEQLGNPGVTVNSVGVQLSRPNTPANVLLTYWQRSQVDLAAGLDFGPKGNVFAS
FTHLQHAPFSFRVEVNNESGAVRKGTLRIWLAPKSDERGTALTFREQRRYFIEMDTSTVTLNPGMNTI
VRRSDQSSVTIPYERTFRAIGTKSAPTDKDALAQFRFCGCGWPQHMLVPKGSPEGVQFDLFAMVTDFE
QDSVAQELDPNAPCSDAHSFCGLRDKKYPDRRAMGYPFDRRTADTVATLADFVTPNSNMKTATVQVKF
NNTVIART

>*Ag*PPO3 [EAA10503]
MSDNKKLLALLQRPLEPTFYPKDNGKTVVDLPENFLTERYRPIGASLQSRFGNDADTRIPVRNVSPPA
ISFAEAVPRRGGFSLFNPKHRQIAGDLINLFMNQPDVETLMSVAAYSRDRLNPILFQYALSVAIQHRP
DTKDLNIPSFLELFPDSFVDPSVFPKLREEGAIVQAENRMTIDIPMNYTASDREDEQRLAYFREDIGV
NLHHWHWHLVYPGEGPDRVVNKDRRGELFYYMHQQLIARYNVERFCNRLARVRPLTNLREPLPEGYFP
KIIRSLNNRAFPPRPQNTVLRDINRVDDSVTFTVSDLERSESRIAESIDGGYVVGPGGNRIPLDERTG
IDVLGNIMEPSALSVNSQFYGNYHGNLHNIIAYSHDPDNRFLEGYGVVGEFQTAMRDPSFYRLHAQVD
NMFHRYKRTLQPYNANQLNYNGIQIQSLGVQLNRANAPANVLLTYWQRSQVDLATGLDFGPEGNVFAS
FTHLQHAPFTFRLTVNNTSGATRRGTCRIFIGPKVDERNTGLTMDEQRLLMIELDKFTVNLNPGTNNI
VRRSEQSSVTIPYERTFRQVALSNINEPSTEQFRFCNCGWPHHLLIPKGTPEGMQFDLFAMISNYADD
TVNQEFDENVNCNDSHSFCGLRDQLYPDRRPMGYPFDRRMPTAVRSLTDFTRGNTNMATSQVQIRFTN
TVIART

>*Ag*PPO4 [EAA10495]
MTDINTRFRGLLQRPYEPTFVPKNNGQLYFDVPDSYLTDHYRPFGAALQNRFGTNAQTRIPLPNITAP
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TDTRDVEIPSFLELFPDRYVDPAVFPQLREEGTLVDQGDRRAIEIPMNFTASDRVDEQRLAYWREDIG
VNLHHWHWHLVYPARGPDRIVRKDRRGELFYYMHQQTMARYNIERFANGMPRVVAFRNFREAIPEAYF
PKITRSSDGRSYPARHPNETLSDLKRVEDGVIVSIADMELWTTRIFEAIDNGFAQSSSNQRVPLDNDS
GIDLLGNIVEASTLSVNPQYYGDLHNNGHNILGYIHDPDNSFLEGFGVVGDNTTAMRDPVFYRWHQHI
DDIFVRHKQRLPAYTGQELAFNDVAVDSFEIQLNKANAPVNILLTFWQRSQVNLGTGLDFGPEGNLFA
TFTHIQHAPYSYRIRVNNRAGDTRRGTVRIFFGPKTNERGQTLPFREQRRLMVELDKFTVTLNAGANT
IVRRSDQSSVSIPYERTFRNVAASSLTQNEAFQFCNCGWPNHMLLPKGSPDGIEYDFFVMVSDFAQDR
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IARA

>*Ag*PPO5 [EAA03423]

MTDNNTLFRGLMQKPYEPTFVPKVDGKLYYDLPDAYLTDQYRPYGPSIQNRFGINAETRLPFPNISPP
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DDTRDVEIPSFLELFPDRFVDPAVFPQLREESNLLDRGSRRAIDIPSNYTASDRVDEQRVAYWREDIG
LSLHHWHWHLVYPSTGPDRVVRKDRRGELFYHMHQQTIARYNIERFANGLARTLSFSQLRESIPEAYF
PKIVRSSDGRAFSCRYPNQVMKDVNRVEDESTIRLADMDVSIKRIFEAIDNGYAQATNGDRVPLDNEK
GIDLIGDLLEASTNSINFNYYGDYHQNGHVMLGYIHDPDNSYLEGVGVMGDLTTTMRDPLFYRWHQHI
DDIFVRHKQRLPAYTSSELSFNDITVDSFDVQLNKANAPKNVLLTFWQRSQFDLGTGIDFVPEGNLFV
TFTHIQHAPFSYRIQATNNGGSMRRGTVRLFLGPKVNDRGQILPFRDQRRHMVELDKFTVNLRPGQNS
IVRRSDESNLTIPYERTFRNIAASSQPGMEVFQFCNCGWPSHMLLPKGSASGLEYDFFVMISNYNQDR
VEEFNENDNCNDAHMFCGLRDRRYPDARSMGYPFDRFTPNSVGSLQEFARPYRNMATTPVSIRFTNTV
IART

>*Ag*PPO6 [EAA44338]
MADNTKLLALLQRPLEPTFYPKDDGKTVVDLPENYLTDRYKPIGQSLQTRFSSEADTRIAVRATTLPD
IRFAEELPRRGDFSLFIPKHRKIAGDLIKLFLDQPDVDTLMSVSSYARDRLNPVLYQYAMAVAIQHRP
DTKNLNIPSFFDLFPDSFVDPTVIPKLREEGAVVNNQRDRITIDIAMNYTASDREDEQRLAYFREDIG
VNLHHWHWHLVYPGEGPNNVVNKDRRGELFYYMHQQLIARYNVDRFCNRLSRVRPLTSLREPLPEGYF
PKIVRSLTNRGFPARPQNTILRDLNRIEDDVVLSITDIELWGSRIAESIDGGYVVAPGGNRIPLDEQT
GIDVLGNIIEPSALSVNSQYYGNYHGHMHNLISFSHDPENRFLEGYGVVGEFQTAMRDPAFYRLHAQV
DNMFHRYKRTLQPYNANQIGYAGVQIQSFGVQLNRANAPANVLLTYWQRSQIDLGTGLDFGPQGNVFA
SFTHLQHAPFTYRFAVNNTTGAARRGTCRIFIAPKTDERNTPLTMDEQRLLMIELDKFRVNLTPGVNN
IVRRSEQSSVTIPYERTFRPMALSNINRPETEQFRFCNCGWPHHLLLPKGTAEGMKFDLFLMISNFAD
DTVNQEFNE

>*Ag*PPO7 [EAA10424]
MATNIRDKFRGLLQHPYEPLFLPKSDGQLFYDLPEKFFTDRYRPIGQNLINRFSAAAAAPAGTSADTP
TMNRVSLNNIPDPDIKFAEAVPRRGAFSLFIPEHRVIAGRLIKLFLDQPDADTLGDVAAYARDRLNGP
LFQYALASALLHRSDTSDVPVPSFLHLFPDQFIDPAAFPQIREEGRAVLQPNRMSIDIPLNYTASDRV
TEQRLAYFREDIGVNLHHWHWHLVYPAEGPERVVRKDRRGELFYYMHQQMIARYQVERYSQGLGRVTP
LDNLRTPIPEPYYPKILRSANNRTYPARYANMTLEDVIRPNDGLRVIISEVERQLQRIIAAIDEGVVV
APSGQRTQLDNFRGIDILGNIVESSTISVNRQLYGDTHNSGHVLLSLVHDPREDFLESFGVMGDVTTA
MRDPVFYRWHTFVDSIFQRHKQRFAPYGPAELRNPGVNLLSLETELDRRDSVKNTLLTFWQRSQFDLG
AGIDFGAEGSVFVTFTHLQHAAFNYRLQVAYSGTAKPATLRIFLAPKRNERGQSLTFEEQRRLAIEMD
TFRVNLTPGINNIIRRSANSSVTIPYERTFRNVANTNIGDANFRFCGCGWPSHMLVPKGDQFGVEYDL
FAMLSDHEQDRVNPLFDERTDCNDAHSFCGLRDRTYPDARNMGFPLDRRVANTVRSFQDFVAPYQNMR
VATITIRFTNTVVERT

>*Ag*PPO8 [EAA44337]
MATLTQKFHGLLQHPLEPLFLPKNDGTLFYDLPERFLTSRYSPIGQNLANRFGPNSPASSQVSNDTGV
PPTVVTIKDLDELPDLTFATWIKRRDSFSLFNPEHRKAAGKLTKLFLDQPNADRLVDVAAYARDRLNA
PLFQYALSVALLHRPDTKSVSVPSLLHLFPDQFIDPAAQVRMMEEGSIVLDENRMPIPIPMNYTATDA
EPEQRMAFFREDIGVNLHHWHWHLVYPASGPPDVVRKDRRGELFYYMHQQLLARYQIDRYAQGLGRIE
PLANLREPVREAYYPKLLRTSNNRTFCPRYPGMTISDVARSADRLEVRIADIESWLPRVLEAIDAGFA
VSDDGVRVPLDETRGIDVLGNILERSAISINRNLYGDVHNMGHVLLAFIHDPRGTYLESSGVMGGVAT
AMRDPIFYRWHKFIDNIFLRNKARLAPYTMAELSNSNVTLEALETQLDRAGGAVNSFVTFWQRSQVDL
RAGIDFSAAGSAFVSFTHLQCAPFVYRLRINSTARSNRQDTVRIFLLPRQNEQGRPLSFEDRRLLAIE
LDSFRVNLRPGMNNIVRQSSNSSVTIPFERTFGNVEQANAGNAQSRFCGCGWPAHMLLPKGNANGVEF
DLFAMVSRFEDDNANVNYDENAGCDDSYAFCGLRDRVYPSRRAMGFPFDRRASNGVRSVADFVAPYKN
MRLATVTLRFMNTIIDRPTN

>*Ag*PPO9 [EAA10430]
MSDLQTGFLCLMTRPTEPLFYPKYNGEVFMDLPPEYLPERYQRIAGSIKTHHGSTAKHHISIKPVELP
DFGYTARVPRHGEFNLFNPAQRQVAGRLVGDLLSQPDPQAMLSVAAYARDRLNPTLFQYALAVALVHR

KDTGNVPVPSFLEMFPTRFVDPALFPKLVEEGFVVQQGERVAIEVPPSFSASEADPEQRLAYFREDIG
VNLHHWHWHLVYPQEGPLEVVDKDRRGELFYYMHRQTVARYNVERFCNRLPAVKPLKNLREPIPEAYF
PKLLNSALNRTYPGRHANMVLSHVNRPDDDAVATILELESSLGRIKEAIQSGFAMAADGTRVPLDPKK
GIDILGNIMENSILSVNVPYYGNYHSLGHVLIGFIHDPDNLYLEGHGVMGDFTTAMRDPTFYRFHGHV
DDVFDMHKQKLSPYKAHELSFPGVSISDATVQITSGKAARNRLLTFWQRTQVDLGTGLDFGPQGNVLA
TFTHIQHAPFAYQIMVQNETAEQKKGTVRIFLAPIYDANGEQLLLSQQRRYMLEMDKFVVKLHPGDNR
IIRRSDQSSVTIPYERTFRRVDASNMPGTESFRFCNCGWPDHMLLPKGHPDGQPFDLFIMISDYKDDA
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IISRT

>*Am*PPO [Q86MV4]
MASDKSGILYLFDRPSEPVYVPKGDNKVAFDIPPDYLPDRYRSVATQVFNRFGDDTESKLPVKAITLP
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PDTKDLPVPPLTEVFPDKYMDSGIFSRAREEANVVPEGARVPIEIPRDYTASDLDVEHRVAYWREDIG
INLHHWHWHLVYPFEGDIRIVNKDRRGELFYYMHQQIMARYNCERLCNRLGRVKRFINWHEPIPEAYF
PKLDSLVASRTWPFRPSGTVLKDINRQVDELNFDIQDLERWRDRIYEAIHTGSVINTRGERIQLTEKN
GIDVLGNIMEASILSPNQNVYGDLHNFGHVAISYIHDPDHRYLESFGVMGDSATAMRDPIFYRWHAFV
DDVFQEHKNTLPQYTVQQLDFPGIEIADIKLTTNQQRNILNTFWTKSDVDLSRGLDFTPRGAVLARFT
HLNHADFSYTIVINNRNNTSMKGTVRIFIGPKEDERGLPFTFREQKNLMIELDKFPITLQPGKNTIEQ
KSTKSSVTIPFERTFRNLDENRPIGGDSLERFDFCGCGWPQHMLIPKGNKEGFAMELFVMVSDYKDDR
VEQNEPIGCKDASSYCGLRDRKYPDARAMGYPFDRQPRAGVETLAQFLTGNMAVTEVTVRFSDTIVPR
SRSGSISNTLTFM

>*Bm*PPO1 [Q27451]
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NLDLPMELPYNEQFSLFVPKHRKLAGRLIDIFMGMRDVEDLQSVCSYCQLRINPYMFNYCLSVAILHR
PDTKGLSIPTFAESFPDKFMDPKVFRQAREVSSVVPSGARMPIVIPSNYTASDTEPEQRVAYFREDIG
INLHHWHWHLVYPFDAADRAIVNKDRRGELFYYMHQQIIARYNVERMCNNLSRVRRYNNFRAAIEEGY
FPKLDSTVASRAWPPRFAGTTIRDLDRPVDQIRSDVSELETWRDRFLQAIENMSVMLPNGRQLPLDEE
TGIDVLGNLMESSIISRNRPYYGDLHNMGHVFISYSHDPDHRHLEQFGVMGDSATAMRDPVFYRWHAY
IDDIFHLYKYKLTPYGNDRLDFPNIRVSSVSIEGGGTPNTLNTLWEQSTVDLGRGMDFTPRGSVLARF
THLQHDEYNYVIEVNNTGGSSVMGMFRIFIAPTVDESGKPFSFDEQRKLMIELDKFSQGVKPGNNTIR
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RGQQG

>*Bm*PPO2 [Q27452]
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MHRRDTRKVRVKNFAEVFPSKFLDSQVFTQARETAAVIPPDVPRIPIIIPRDYTATDLEEEHRLAYWR
EDIGINLHHYHWHLVYPFTANDLSIVAKDRRGELFFYMHQQVIARFNCERLCNSLKRVKKFSNWREPI
PEAYFPKLDSLTSSRGWPPRQSGMQWQDLNRAAEGLFVTIDEMERWRRNVEEAIATGTVRLPNGQTRP
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IDDVFQKHKESAYVRPYTRSELENPGVQVRSVSVETPGGQPNTLNTFWMLSDVNLSRGLDFSDNGPVY
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IVRQSTESSITIPFEQTFRDLSAQGNDPRRDELATFNYCGCGWPQHMLVPKGTEAGMPFQLFVMLSNY
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VTEPNPRNPPMSV

>*Dm*PPO1 [NP 476812]
MTNTDLKALELMFQRPLEPAFTTRDSGKTVLELPDSFYTDRYRNDTEEVGNRFSKDVDLKIPIQELSN
VPSLEFTKKIGLKNQFSLFNNRHREIASELITLFMSAPNLRQFVSLSVYTKDRVNPVLFQYAYAVAVA
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VFNDRVIDKA

>*Dm*PPO2 [NP 610443]
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NLHHWHWHLVYPFEASDRSIVAKDRRGELFYYMHQQVIARYNAERFSNNLARVLPFNNLRDPIAEGYF
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QRPN

>*Dm*PPO3 [NP 524760]
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DTKDLELPAFAQTFPDRFIDSKMLRSMREESFVVERSAARLPVVSSVKYTASDLDVEHRLWYFREDLG
VNLHHWHWHLVYPIEAPDRSIVDKDRRGELFYYMHQQIIARYNAERLSNHMARVQPFNNLDEPIAEGY
FPKMDSLVASRAYPPRFDNTRLSDVDRPNNQLRVGIDDMKRWRERIYEAIHQGYVLDTNNEKIVLDDA
KGIDILGNIIEASDLTPNSTLYGDFHNMGHILIAYSHDPTNKHLEYAGVMGDASTAMRDPIFYKWHAF
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THLQHHEFSYTIKVENSSEATRYGYVRIFLAPKLDDRNAPMLLEQQRLMMVELDKFVVTMPPGSHTIT
RNSTESSVTIPFERTFRNLDKLEELQNFLCGCGWPQHMLIPKGRPEGLRFELFVMVSNYEEDKVDQTV
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ARS

>*Dp*PPO1 [EHJ78993]
MSNAKENLLLFFDRPTEPCFMQKGEEKATFDLPANYYPDKYKSASAALANRFGSESNRRIPVRNIALP
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THLQHDEFNYLIEVNNTSGAGVMGTVRLFMAPVVDENGTPLTFDEQRKLMMELDKFTHAIPSGSSTIR
RSSTQSSVTIPYERTFRSQGSRPGDPGSVEAAEFDFCGCGWPHHLLLPKGTTRGYPVVLFCMISNWNN
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R

>*Dp*PPO2 [EHJ77686]
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HRKDTKNVPLQNFAETFPSKFVDSKFFSQARESAALAKQGAPRVPIIIPRDFTANDLDIEHRLAYWRE
DIGINLHHWHWHLVYPFSATKREIVAKDRRGELFFYMHQQVIARYNTERLANQLARAKKFSDFTEPTP

EPYYPKLDSLTSSRSYPPRQANMRWSDLNRPVDGLVVTIADMNRWKRNLEEAIATGMVKLPNGSTQPL
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ARFTHLNYRPFRYVIDVDNTGSARRTTVRIFIAPKFDERGLPWILSDQRKMFIEMDRFVVPLNAGKNV
ITRESTESSLTIPFEQTFRDLSSQGSDPRREDLASFNFCGCGWPQHMLVPRGTESGMLFDFFVMLSNY
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NTVEINPRNAKN

>*Hm*PPO_A [HMEL008342]
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PRFNKAKRLQHDQEFSILLPAHQEMADEVIDELLAVPENQLQDFLSTCIFARVNLNPQLFNYCYSVAL
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PEAYFPKLDSLTTSRGWPPRQAYMRWQDLNRPDDGLVVTLADMNRWRRNIEEAISTGLVRLPNGGTQP
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>*Hm*PPO_B [HMEL008343]
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DIFQSFKESPHVPQYTEYDLSNPGVQIRGVRVENDYGDINILKTFWMQSDVDIAGGLDFSERASVYTR
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VRLSTKSSVTIPFEKTFRNLTSPPDLSAAATNYCGCGWPQHMLVPRGTETGTKYDLFVMLSNYDMDSV
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PLNPHF

>*Hm*PPO_D [HMEL011676]
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DSQVASRAWPPRFAFSDIRDLDRPVEQIRSDVSQLELWRDHFIEAVNNMAILLPNGRTMPLDEQTGID
VLGNLMESSIISRNRSYYGDMHNMGHVFISYAHDPDHRFLELFGVMGDSATAMRDPVFYRWHAYIDDL
FNLYKTKLPPYGRDRLDFPGIRVSAVSVQSQGPLGRGSNQLHTQWEQSTVELSRGLDFMPRGSVNARF
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RNSRLSSVTIPYERTFRNQNSRPGDSGSVEAADFDFCGCGWPHHMLIPKGTAEGYPMVLFCMVTNWNE
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QR

>*Hm*PPO_E [HMEL011677]
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QQ

>*Nv*PPO1 [XP 016840217]
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QPSTL

>*Nv*DiphenolOxidase1 [NP 001164357]
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ANSNAQSVVRF

>*Nv*DiphenolOxidase3 [NP 001164331]
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>*Px*PPO1 [KPI98962]
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QQG

>*Px*PPO2 [NP 001299542]
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TEPNPRNVQN

>*Px*PPO1 [XP 013167801]
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RVRGGQ

>*Px*PPO1 [XP 013167704]
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ARFTHLQHEEFNYVIEVNNTSGNYAMGTVRIFMAPVFDDNGNRMTFDVQRKLMIELDKFTHGLKQGVN
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EIRDRKQ

>*Px*PPO1 [KPI98958]
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THLQHDEFDYVLEVDNSSGKKAMGTVRIFLAPITDEKGNKLAFNDQRKLMIELDKFSQDLAPGKNTIR
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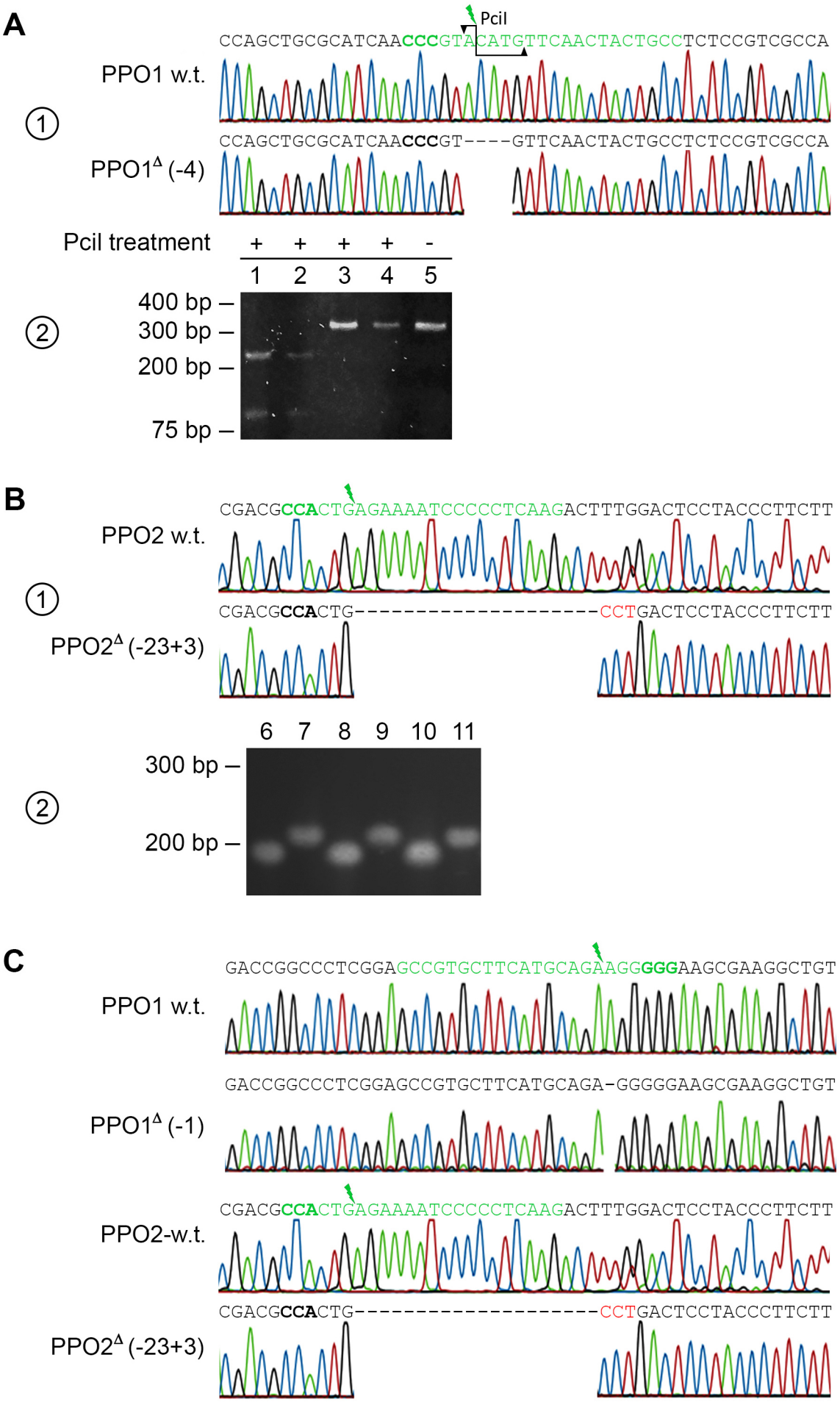
VRGAK

Fig. S4


A
PciI
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6 7 8 9 10 11
300 bp –
200 bp –
②
C
GACCGGCCCTCGGAGCCGTGCTTCATGCAGAAGGGGGAAGCGAAGGCTGT
PPO1 w.t.
GACCGGCCCTCGGAGCCGTGCTTCATGCAGA-GGGGGAAGCGAAGGCTGT
PPO1Δ (-1)
CGACGCCACTGAGAAAATCCCCCTCAAGACTTTGGACTCCTACCCTTCTT
PPO2-w.t.
CGACGCCACTG----------CCTGACTCCTACCCTTCTT
PPO2Δ (-23+3)

Table S3: PPO CRISPR statistics.

Mutant line	Target gene	sgRNA	Injected embryo	Hatched G0 larvae (%)	G0 founder	Mutated lines /Sequenced lines
PPO1$^{\Delta}$	PPO1	sg1353	344	6 (1.7 %)	6	2/4 (50 %)
PPO2$^{\Delta}$	PPO2	sg1075	540	34 (6.3 %)	8	1/2 (50 %)
PPO1$^{\Delta}$,PPO2$^{\Delta}$	PPO1	sg1353 + sg1352	648	37 (6.0 %)	18	1/8 (13 %)

Fig. S5

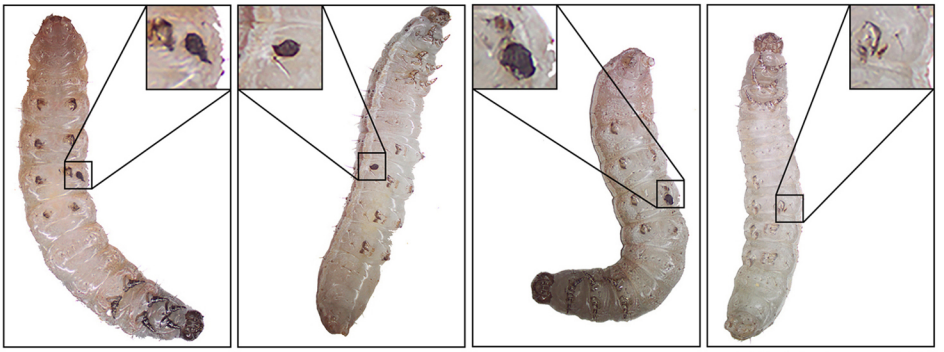

Fig. S6

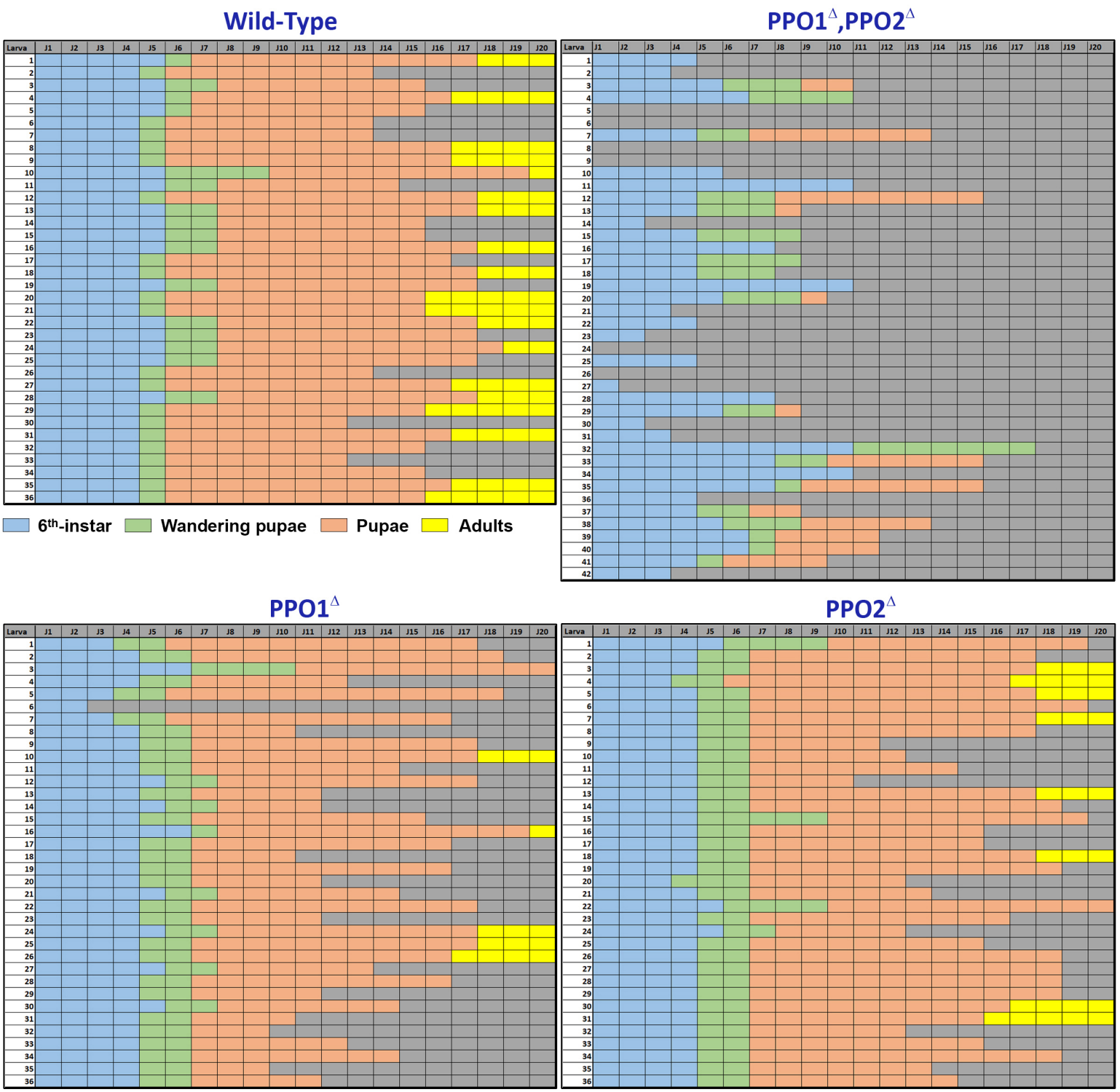
Wild-Type
PPO1Δ,PPO2Δ
PPO1Δ
PPO2Δ
6th-instar
Wandering pupae
Pupae
Adults

Fig. S7