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Characterization of the proteasomeβ2 subunit gene and its mutant allele in the tephritid fruit fly pest, *Anastrepha suspensa*

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Abstract

In *Drosophila melanogaster* the β2 proteasome subunit gene, *Prosβ2*, was first identified as a dominant temperature sensitive mutant, DTS-7, that causes pupal lethality at 29 °C but allows survival to adulthood at 25 °C. To explore the use of proteasome mutations for a conditional lethal system in insect pests, we identified and isolated the β2 subunit gene of the 20S proteasome from the Caribbean fruit fly, *Anastrepha suspensa.* The caribfly ortholog *AsProsβ2* was isolated from pupal cDNA by 5′ and 3′ RACE. The AsProsβ2 protein has high amino acid sequence similarity to predicted insect Prosβ2 subunits and homologs from yeast and mammals, and it contains the well conserved amino acids that confer catalytic activity and substrate specificity. *AsProsβ2* is a single copy gene and its RNA accumulates throughout all developmental stages of the caribfly. For functional studies a point mutation, analogous to the *Prosβ2*^{1} mutation in *D. melanogaster*, was introduced into *AsProsβ2* to create an aberrant protein with a Gly170Arg substitution. Consistent with the DTS-7 mutation, transgenic insects carrying the mutant allele undergo normal metamorphosis at the permissive temperature (25 °C) but at the non-permissive temperature (29 °C) they exhibit effective pupal lethality. This is the first report of a functional characterization of a Prosβ2 cognate based on the creation of a dominant temperature-sensitive mutation. This type of temperature-dependent lethality could be used for biological control, where transgenic insects are reared to adulthood at 25 °C or lower and then released into the field where ambient temperatures averaging 29 °C or greater cause lethality in their progeny.

Keywords: proteasome subunits, ubiquitin-proteasome pathway, temperature-sensitive lethality, *Anastrepha suspensa*, biological control, AsProsβ2.

Introduction

In eukaryotic cells, regulated protein degradation during various biological processes such as cell-cycle progression, DNA repair and cell death, and the removal of abnormal or mis-folded proteins, is brought about by the ubiquitin-proteasome pathway. Proteins that are destined to be destroyed are hydrolyzed in the 26S proteasome (Ciechanover & Schwartz, 1998), a multi-subunit complex, comprised of a 20S core particle and 19S regulatory caps on one or either end (Yao & Cohen, 2002). The 20S catalytic core is cylindrical, consisting of four heptameric rings made from seven different α and seven distinct β subunits (Groll *et al*., 1997). Within the core the α subunits are not catalytically active but form antechambers to the central cavity formed by the β subunits. Studies in the yeast, *Saccharomyces cerevisiae*, have revealed that of the seven β subunits, only β1, β2, and β5 are active and exhibit a functional hierarchy with β5 being predominantly active, followed by β2 and β1 (Heinemeyer *et al*., 2004). The remaining four β subunits, although inactive, play multiple roles including structural and catalytic processing of other β subunits (Ramos *et al*., 2004).

Functional studies of individual proteasome subunits have been facilitated by mutational analysis, and conditional mutants have been especially useful since aberrant or truncated proteasome subunits often result in lethality (Neuburger *et al*., 2006). In *Drosophila melanogaster* the β2 proteasome subunit gene, *Prosβ2*, was isolated based on the chromosomal map position of its mutant allele, *Prosβ2*^{1} (Smyth & Belote, 1999), that was first identified as

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DTS-7 in a mutagenesis screen for dominant temperature-sensitive (DTS) phenotypes (Holden & Suzuki, 1973). The mutation results in a conserved amino acid residue substitution that causes early pupal lethality at the non-permissive temperature of 29 °C, but allows survival at permissive temperatures of 25 °C or below in heterozygotes. A similar DTS mutant phenotype is expressed by the β6 subunit mutation, *Pros26*^{1} (originally DTS-5), isolated in the same mutagenesis screen (Holden & Suzuki, 1973; Saville & Belote, 1993).

Mutations such as *Prosβ2*^{1} and *Pros26*^{1} that allow temperature-dependent conditional lethality are, potentially, useful tools for the genetic manipulation of insect pest populations, especially those that inhabit tropical and sub-tropical environments. These include tephritid fruit flies such as the Mediterranean fruit fly, *Ceratitis capitata*, the Mexican fruit fly, *Anastrepha ludens*, and the Caribbean fruit fly, *Anastrepha suspensa*, that are major agricultural pests of more than 300 fruit and vegetable plant hosts world-wide (Metcalf, 1995). Since the *D. melanogaster Prosβ2*^{1} mutation affects a highly conserved amino acid, we explored the possibility of using analogous proteasome mutations to create strains for conditional lethal release in tephritid flies. Here we describe the isolation and characterization of the *Prosβ2* subunit from *A. suspensa* and the functional analysis of a mutated subunit, *AsProsβ2*^{1}, in transgenic flies. While Prosβ2 cognates have been identified in several insects based on sequence similarity, this is the first report of a functional characterization of a cognate based on the creation and ectopic expression of a dominant temperature-sensitive mutation.

Results

Isolation and identification of the AsProsβ2 *gene from Anastrepha suspensa*

The *AsProsβ2* gene (GenBank accession no. EU564727) was isolated from pupal cDNA by a 3′-RACE protocol using a poly(A) adaptor primer and a gene specific degenerate primer designed from the protein sequence motif YCCGAGT, which is highly conserved in eukaryotic *Prosβ2* cognates. Subsequently, the 5′ end of the transcript was amplified by 5′-RACE using a gene specific reverse primer and a 5′ adaptor primer. The full length *AsProsβ2* transcript is 1022 nt with a 98 nt 5′UTR, 81 nt 3′UTR and a 843 nt open reading frame. The *AsProsβ2* genomic sequence, isolated by PCR using gene specific primers in the 5′ and 3′UTR, contains three introns that are 57, 62 and 66 nts (Supporting Information Fig. S1). The first two introns in *AsProsβ2* are identical to *D. melanogaster Prosβ2* in their positions, but differ in size. In *D. melanogaster* the first intron is 69 nt and the second 256 nt, however the major difference in *AsProsβ2* is the presence of an additional third intron proximal to the 3′ terminus of the coding region.

The conceptual translation of *AsProsβ2* yields a 280 amino acid sequence that shares 75% identity to the *D. melanogaster* Prosβ2 subunit and 27% identity or less to the remaining six β-type subunits (Belote & Zhong, 2005), suggesting that it is indeed the *Prosβ2* ortholog. In addition, the AsProsβ2 protein has a conserved cleavage site for post-translational proteolytic processing at Gly39Thr40 in the propeptide sequence similar to the yeast *S. cerevisiae* protein, for which the proteasome subunit structure and function has been studied extensively (Groll *et al*., 1997). This cleavage presumably removes a propeptide of 39 residues and releases a mature protein with an N-terminal active site at Thr1 that also exists in the predicted Prosβ2 subunit of *D. melanogaster* (Smyth & Belote, 1999). This also has been shown to play a catalytic role in the active site of the mammalian and yeast proteasomes (Schmidtke *et al*., 1996). Other salient features of the AsProsβ2 protein are the presence of a conserved Glu53 residue that is critical for the trypsin-like activity of Prosβ2 subunits and a glycine preceding the active site at Thr1 (Heinemeyer *et al*., 2004). These amino acid residues are also observed in the predicted Prosβ2 proteins from *Aedes aegypti* (XP_001656819), *Anopheles gambiae* (XP_317882) and *Culex pipiens* (XP_001849640) that share more than 70% homology to AsProsβ2 (Fig. 1 and Supporting Information Table S1).

Copy number of AsProsβ2 *gene*

A single *AsProsβ2* gene copy per haploid genome was estimated by absolute quantification in a real-time PCR using a known single copy gene (*AsPros26*) as reference (Supporting Information Table S2). The copy concentration of *AsProsβ2* and *AsPros26* in a known quantity of genomic DNA was estimated from Ct values using standard curves. The *AsProsβ2* copy number was determined by dividing the copy concentration of *AsProsβ2* by *AsPros26.* This result was validated by southern blot hybridization of *A. suspensa* genomic DNA digested with the restriction enzymes *Age*I, *Nsi*I and *Eco*RI and probed with *AsProsβ2* cDNA (Fig. 2). Consistent with a single copy gene a single band is observed digesting with *Eco*RI, which does not cut within the gene, while two bands are observed with *Age*I and *Nsi*I that cut once within the hybridizing gene sequence.

To study the sex- and stage-specific accumulation of *AsProsβ2* RNA a high stringency northern blot analysis was performed on total RNA isolated from various developmental stages. *AsProsβ2* RNA accumulates in the adult flies of both sexes and throughout all stages of development with relatively high levels in embryos, late third instar larvae and early pupae. The RNA level is relatively high in vitellogenic ovaries, but not in pre-vitellogenic ovaries suggesting that embryonic RNA is, to a large extent, provided maternally (Fig. 3).

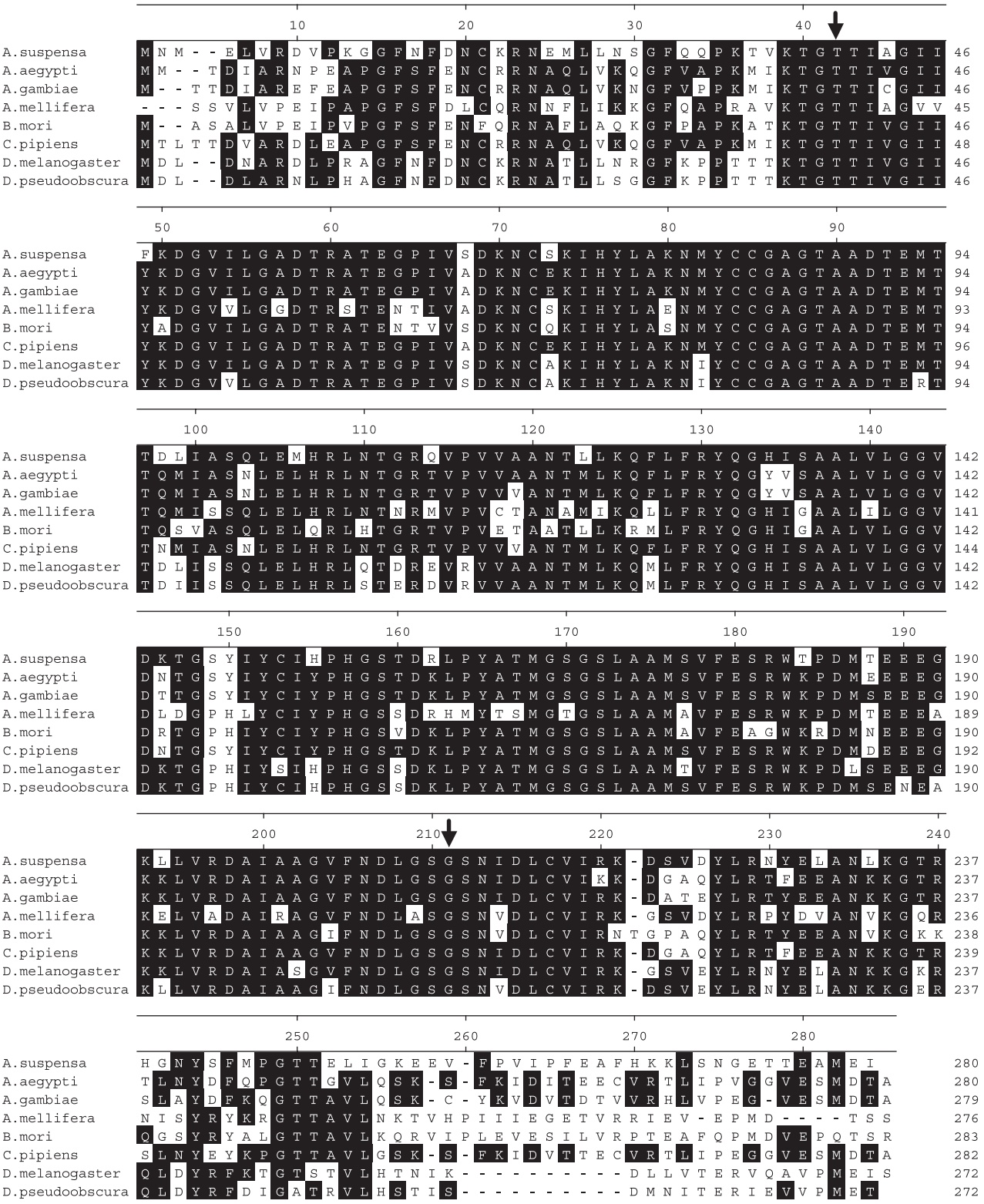

Figure 1. Multiple sequence alignment of Prosβ2 amino acid sequences from indicated insect species using CLUSTALW. Residue positions for each sequence are shown on the right side with consensus sequence positions indicated above the alignment. The active site Thr1 residue (consensus position 42) and the Gly170 residue (consensus position 211) substituted for Arg in AsProsβ2[1] are indicated by arrows.

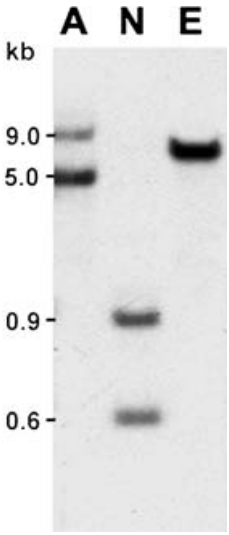

Figure 2. Southern blot analysis of the *AsProsβ2* gene. *Anastrepha suspensa* genomic DNA was digested with restriction enzymes *Age*I (A), *Nsi*I (N) and *Eco*RI (E). Size designations (to the left) were determined by ethidium bromide-labeled DNA markers.

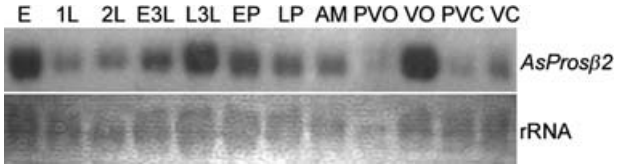

Figure 3. Developmental profile of *AsProsβ2* RNA accumulation by northern blot hybridization analysis. *AsProsβ2* RNA is shown in the upper panel and methylene blue stained rRNA as a loading control in the lower panel. Abbreviations are as follows: E, embryo (20–21 h post oviposition); 1L, 1st instar larva; 2L, 2nd instar larva; E3L, early 3rd instar larva; L3L, late 3rd instar larva; EP, early pupa; LP, late pupa; AM, adult male; PVO, pre-vitellogenic ovary; VO, vitellogenic ovary; PVC, pre-vitellogenic carcass; and VC, vitellogenic carcass.

Analysis of the mutant AsProsβ2^1 *gene in transgenic flies*

To functionally identify the *AsProsβ2* gene and assess the potential use of an aberrant *AsProsβ2*^{1} mutation for population control, the *AsProsβ2* cDNA sequence was mutated *in vitro* at position +723 resulting in a codon change from GGG to AGG, creating the amino acid substitution Gly170Arg (in the mature protein), which is analogous to the Drosophila Gly170Arg substitution in *Prosβ2*^{1} (Smyth & Belote, 1999). Transgenic mutant strains were created by introducing the mutated gene with 5′ (1125 nt) and 3′ (368 nt) proximal sequences into a *piggyBac* transformation vector to create pB[PUbDsRed.T3-AsProsβ2^1], which was then used to transform wild type *A. suspensa*. Injection of ~400 caribfly embryos yielded nine transgenic G1 individuals, from which five stable independent strains were created with each line having a single transgene integration (Fig. 4).

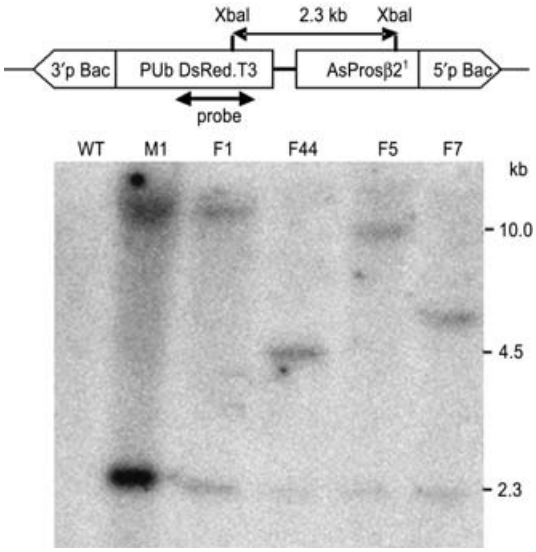

Figure 4. Schematic diagram of the *piggyBac* transformation vector pB[PUbDsRed.T3-AsProsβ2^1] used to transform wild-type *Anastrepha suspensa* (not to scale). Below is a genomic Southern DNA hybridization blot showing an internal 2.3 kb fragment and a single vector arm DNA fragment from each transformed line digested with *Xba*I and probed with ^{32}P-labeled DsRed.T3. Region of probe hybridization is shown in the schematic.

To assess the temperature-sensitive effect of the *AsProsβ2*^{1} mutation, survival was tested in three strains homozygous for the mutant transgene and reared at the permissive and non-permissive temperatures of 25 °C and 29 °C, respectively. In *D. melanogaster,* heterozygous mutants carrying a single copy each of the mutant and wild-type *Prosβ2* alleles metamorphose to adults at 25 °C, but at 29 °C pupation is abnormal with underdeveloped imaginal discs and a lack of adult abdominal structures (Smyth & Belote, 1999). Fully penetrant lethality in Drosophila mutants is dose dependent and relies on a ratio of at least one dominant mutant allele to counteract each wild type allele. We therefore reasoned that at a minimum, a homozygous dominant-acting mutant transgene would be necessary to effectively negate the function of the two wild-type resident genes. In independent experiments performed in triplicate transgenic larvae from homozygous strains M1, F44, and F1 developed into normal appearing pupae at similar frequencies at both 25 °C and 29 °C, with slightly lower viability compared to the non-transgenic wild type strain (Fig. 5A). In contrast, the number of larvae surviving to adulthood was significantly lower at 29 °C for all three transgenic lines. At 25 °C, approximately 54 to 61% of the transgenic larvae reached adulthood, compared to 83% of wild type, but at 29 °C 74% of wild type larvae survived compared to complete lethality in strain F44, 3% survival in strain M1 and 16% in strain F1 (Fig. 5B). All three strains exhibited complete lethality in at least one independent experiment.

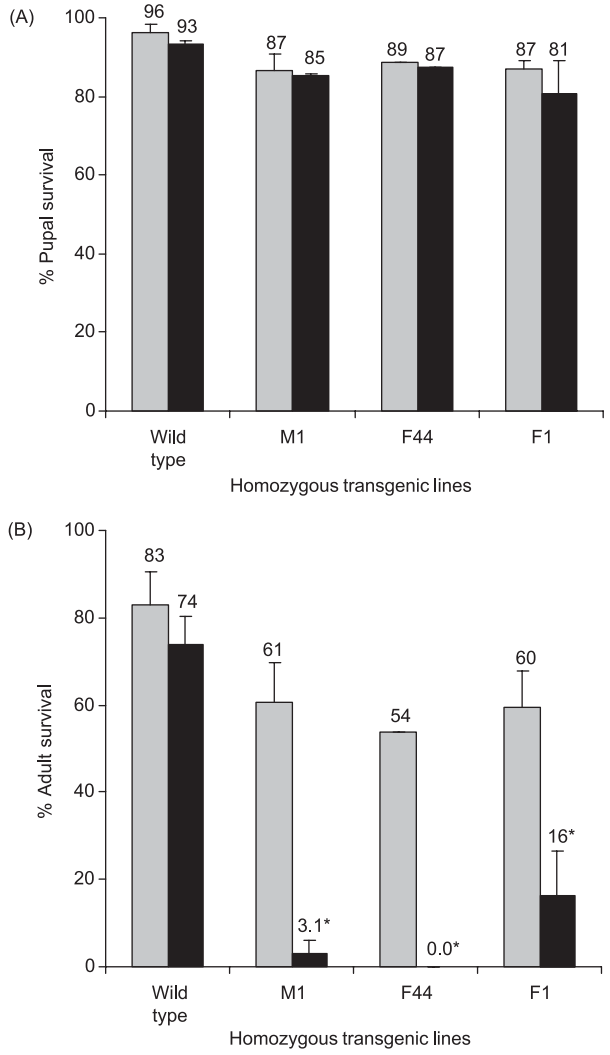

Figure 5. Temperature-sensitive lethality in *Anastrepha suspensa* transformed with the *AsProsβ2*^{1} allele. Wild type non-transgenic and homozygous transgenic embryos collected at room temperature (23 °C ± 2 °C) were transferred to 25 °C (gray bars) or 29 °C (black bars) within 24 h of larval hatching and reared until adult emergence. Histograms represent the mean survival percentage (± S.E.) to pupal stage (A) or adult stage (B) calculated from the total number of first instar larvae tested (wild type: n = 350 at 25 °C and n = 353 at 29 °C; M1: n = 199 at 25 °C and n = 191 at 29 °C; F44: n = 97 at 25 °C and n = 103 at 29 °C; F1: n = 300 at 25 °C and n = 291 at 29 °C). Three independent experiments were performed for each line except line F44. Statistical significance as measured by one way ANOVA using the program SIGMASTAT is represented as asterisks ($P < 0.001$).

To test the dosage effect of *AsProsβ2*^{1} on lethality, pupal survival at 25 °C and 29 °C was tested in hemizygous lines having a single mutant transgene allele and two wild-type *AsProsβ2* alleles. This is expected to result in approximately 50% lethality for each line hemizygous for the transgene. At 25 °C pupal survival to adulthood was comparable between wild type and hemizygous lines ranging from 53% to 73%. However, at 29 °C pupal survival in all three transgenic strains decreased to between 20% and 39% compared to 61% in the wild type (data not shown). It is presumed that the presence of a single mutant allele, versus two alleles in homozygotes, impairs normal pupal development although to a lesser degree.

Discussion

We have identified and isolated the *AsProsβ2* gene from *A. suspensa* and studied a mutant form of the gene, *AsProsβ2*^{1}, for its ability to create a temperature-sensitive dominant-negative phenotype. The AsProsβ2 amino acid sequence shares 60% identity to the bovine and 50% identity to the yeast (PUP1) proteasome β2 subunits, with high similarity to amino acid residues conferring structural significance in the yeast and bovine 20S proteasomes (Groll *et al*., 1997; Unno *et al*., 2002). In both yeast and bovine proteasomes the catalytic subunits β1, β2 and β5 specifically hydrolyze acidic, basic and hydrophobic amino acids, respectively. Consistent with the residues conferring trypsin specificity to the β2 subunit in yeast, the AsProsβ2 protein sequence contains the critical residues Cys31, His35, Gly45, Ala49 and Glu53 of the S1 substrate specificity pocket (Groll *et al*., 1997). While Ser20 present in yeast is replaced by Ala20 in AsProsβ2, this substitution is also common to the functional bovine (Unno *et al*., 2002) and human β2 subunits (Hirano *et al*., 2005) suggesting that the residue substitution does not alter subunit activity. The Ala20 substitution also appears in *D. melanogaster* Prosβ2 and all other predicted insect Prosβ2 proteins compared in this study except for Prosβ2 in *Apis mellifera*.

The ubiquitous accumulation of *AsProsβ2* RNA during all stages of caribfly development signifies its role as a housekeeping gene and is consistent with the accumulation pattern of *D. melanogaster Prosβ2* RNA observed in embryonic, larval, pupal and adult male and female stages (Lyne *et al*., 2007). In addition to the constitutively expressed Prosβ2, *D. melanogaster* also contains two male-specific isoforms, Prosβ2R1 and Prosβ2R2 that share 62% and 35% amino acid identity with the non-sex-specific Prosβ2 (Ma *et al*., 2002). Since the presence of these isoforms in the caribfly is uncertain, high stringency was maintained for the northern analysis to avoid cross-hybridization of the *AsProsβ2* probe to potential isoforms. The fact that the southern hybridization pattern is consistent with a single copy gene without spurious bands supports our conclusion that stringency was high enough for sequence specific hybridization.

Functional analysis of the *AsProsβ2*^{1} mutation, analogous to *Prosβ2*^{1} in *D. melanogaster*, shows a similar dominant temperature-sensitive lethal effect during *A. suspensa* pupal development. It is presumed that substitution of glycine170 to arginine causes instability between subunits that disrupts catalytic function at elevated temperatures (Neuburger *et al*., 2006). Thus, at 29 °C or above the temperature-labile mutant subunit acts as an antimorph to

poison the multi-subunit 20S proteasome complex, rendering it non-functional. This is manifested by consistent lethality during pupal development at 29 °C, yielding low level or no survival in the three homozygous transgenic strains tested. Given that transgene expression is typically suppressed by well-established genomic position effects (Sarkar *et al*., 2006), variations in lethality between strains were not unexpected. However, we presume that *AsProsβ2*^{1}-induced lethality could be enhanced by increasing the expression of the mutation, or dosage of mutant to wild-type alleles.

This type of temperature-dependent lethality could be used for biological control, where transgenic insects are reared to adulthood at 25 °C or lower and then released into the field where ambient temperatures average 29 °C or greater. The expectation is that the progeny of the released flies (preferably males only) would die as pupae resulting in a suppressed population. We have established that, similar to the Drosophila DTS mutation, the lethal effect is dose dependent such that released flies would require at least two pairs of duplicated lethal transgenes (e.g. a homozygous tandem transgene duplication) so that their progeny would inherit at least one pair of transgenes. Tests with the Drosophila *Prosβ2*^{1} and *Pros26*^{1} proteasome mutations indicate that the frequency and timing of lethality can be advanced to early larval stages by increased mutated gene product or the use of the two mutations in heteroallelic combination. Thus the prospect of improving DTS conditional lethality for practical application appears to be quite feasible. However, this DTS system has the potential for use in its current state for other tropical pests such as mosquitoes. The Prosβ2 orthologs from *Ae. aegypti*, *An. gambiae* and *C. pipiens* have high amino acid sequence identity to the Drosophila gene, and DTS mutations resulting in conditional pupal lethality could actually be more advantageous for these species in which adults are most harmful.

Experimental Procedures

Fruit fly rearing

Wild type and transgenic *Anatrepha suspensa* were maintained at 25 °C with larvae fed a wheat germ-yeast-glucose diet. Pupae were maintained in humidified vermiculite and adults were provided a yeast-sucrose diet. For temperature sensitivity assays approximately 50 larvae per rearing dish were maintained in ECHOtherm incubators (Torrey Pines Scientific, San Marcos, CA, USA) at 25 °C or 29 °C until adult emergence. Except for one transgenic strain (F44) and initial tests, experiments were done in triplicate with 50 to 100 larvae per experiment.

Southern and northern hybridization analysis

Southern blot hybridizations were performed on digested genomic DNA isolated from wild-type and transgenic flies as described (Handler & Harrell, 2001). Northern blot hybridizations were performed on approximately 5 μg total RNA isolated from indicated *A. suspensa* tissues and developmental stages resolved on a 1% denaturing formaldehyde agarose gel that was capillary blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH, USA). High stringency hybridization was performed at 68 °C in solutions containing 7% SDS, 1% BSA and 1mM EDTA. Hybridizations used ^{32}P-labeled *AsProsβ2* complete cDNA or DsRed.T3 as probe.

Isolation and analysis of the Anastrepha suspensa Prosβ2 *gene and proximal sequences*

Total RNA extracted from *A. suspensa* pupae using Trizol (Invitrogen, Carlsbad, CA, USA) was used to synthesize full length cDNA using the SMART cDNA synthesis kit (BD Biosciences, San Jose, CA, USA). *AsProsβ2* cDNA was isolated from pupal cDNA by performing 3′-RACE using the manufacturer's CDS 3′ primer and a degenerate forward primer (5′-TACTGCTGTGGTGCYGGIAC-3′) designed from highly conserved amino acid sequences from known Prosβ2 cognates. Subsequently, the full length 5′ end was amplified by 5′-RACE using the gene specific reverse primer (5′-GCTTCCAAGGGGATAACAGGGAA-3′) and SMART IV primer from the kit. To isolate 5′ and 3′ genomic sequences a standard inverse PCR protocol was followed. DNA amplifications were performed with Expand Long Template DNA polymerase (Roche Applied Science, Indianapolis, IN, USA) using the following cycling conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 68 °C for 2 min with a final extension at 68 °C for 7 min. Products were cloned into the TOPO TA vector pCR 2.1 (Invitrogen) and sequenced with vector primers using BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled into a contig using the SeqMan program, translation of DNA to amino acid sequence was performed in SeqBuilder and alignment of amino acids was done by ClustalW using MegAlign from DNAstar (Madison, WI, USA).

Quantitative real-time PCR

Copy number of *AsProsβ2* gene per haploid genome was estimated by absolute quantification in a real-time PCR (Lee *et al*., 2008) using the single copy gene, *AsPros26*, as reference (Nirmala and Handler unpublished). *AsProsβ2* was amplified using primers TaqDTS7F (5′-ATTGTTGGTACGCGATGCTA-3′) and TaqDTS7R (5′-CCTTCAGATTGGCCAACT-3′) and *AsPros26* was amplified using primers QDTS5F (5′-CAATCGTTCCCTGAATATCAAGTG-3′) and QDTS5R (5′-GCAATCACAACATAATCCTCACC-3′). Reactions were performed with the iQ SYBR Green Supermix in a Chromo4^{TM} real-time PCR detector (BioRad, Hercules, CA, USA). PCR cycling included an initial denaturation at 95 °C for 3 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 10 s and 72 °C for 30 s followed by a melting curve to analyze amplicon specificity. All reactions were performed in triplicate.

AsProsβ2^{1} *mutant gene creation*

A single nucleotide missense mutation at nt +723 that results in a Gly170Arg substitution was incorporated in the primer AsDTS7M that was used to amplify the *AsProsβ2* cDNA template (mutation site corresponds to +842 in the genomic sequence; see Fig. S1). A 601 nt 3′ terminal sequence containing the mutation and the 3′ UTR was amplified using primers AsDTS7M (5′-GGTTCCAGGTCTAACATCGATTTATGTGT-3′) and TDTS7R (5′-TACCGCGGGTCTTCGTCTAGCTCAT-3′) and the 1022 nt full length *AsProsβ2* gene was amplified using primers AsDTS7F

(5′-GACAATTTTGTTGGTCATC-3′) and AsDTS7R (5′-GGATAAAACAATTCTTTAAAAACT-3′). The two fragments having a 300 nt overlap were assembled under the following cycling conditions: 10 ng of each DNA fragment with dNTPs and Expand Long Template polymerase (Roche) were incubated for 10 cycles at 95 °C for 30 s and 68 °C for 2 min. An aliquot of this amplification was used as template in a second gene amplification using standard procedures with primers AsDTS7F and TDTS7R to amplify the 1317 nt fragment containing the *AsProsβ2*^{1} mutation. The amplified fragment was cloned into pCR2.1 and sequenced to verify incorporation of the missense mutation.

Plasmid construction and Anastrepha suspensa *transformation*

To create the pB(PUbDsRed.T3-AsProsβ2^{1}) *piggyBac* transformation vector, a 1125 nt upstream 5′ putative regulatory sequence and 368 nt 3′ sequence from *AsProsβ2* were cloned into the analogous positions relative to the *AsProsβ2*^{1} mutant gene. The entire *AsProsβ2*^{1} cassette was subcloned into pB(XLPUbDsRed.T3) in opposite orientation to the PUb-DsRed.T3 reporter gene to create the pB(PUbRed.T3-AsProsβ2^{1}) vector. A vector and phspBac helper plasmid mixture was injected into *A. suspensa* embryos as described previously (Handler & Harrell, 2001). G1 offspring were selected by DsRed epifluorescence using a Leica MZ FLIII microscope with the HQ Texas Red filter set (Chroma, Rockingham, UT, USA). Independent homozygous strains were established by single pair inbreeding for successive generations with testing by segregation analysis of transformants outcrossed to wild type flies.

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Supplementary information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Genomic DNA sequence and conceptual translation of the *AsProsβ2* gene. Nucleotide positions shown on the left are relative to the transcription initiation site (A at +1 in bold). Amino acid positions shown on the right are relative to the N-terminal active site at Thr1 (boxed and in bold). Nucleotide sequence exons are shown in upper case and introns in lower case, the G mutated to create the Gly170Arg substitution in AsProsβ2^{1} is at +842 (bold), and a polyadenylation signal is at +1176 (bold and underlined).

Table S1. Amino acid percent identity and divergence between indicated insect Prosβ2 cognates determined by ClustalW multiple sequence alignment.

Table S2. Estimation of *AsProsβ2* gene copy number by absolute quantification. Critical threshold (Ct) values (± S.D.) are from experiments done in triplicate.