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# Transgene expression from the *Tribolium castaneum Polyubiquitin* promoter

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## Abstract

**The highly conserved Ubiquitin proteins are expressed from genes with strong, constitutively active promoters in many species, making these promoters attractive candidates for use in driving transgene expression. Here we report the cloning and characterization of the *Tribolium castaneum Polyubiquitin* (*TcPUb*) gene. We placed the *TcPUb* promoter upstream of the coding region of the *T. castaneum* eye-colour gene *Tc vermilion* (*Tcv*) and injected this construct into embryos from a *Tcv*-deficient strain. Transient expression of *Tcv* during embryogenesis resulted in complete rescue of the larval mutant phenotype. We then incorporated the *TcPUb-Tcv* chimera into a *piggyBac* donor. Resulting germline transformants were easily recognized by rescue of eye pigmentation, illustrating the potential of the *TcPUb* promoter for use in driving transgene expression.**

**Keywords: *Ubiquitin*, marker gene, tryptophan oxygenase, *Tribolium*, transformation.**

## Introduction

Transformation-based applications such as transposon tagging and enhancer trapping have facilitated the genetic dissection of model species. Several types of mobile elements have been tested in invertebrates, including small DNA elements, retro-elements and pantropic retroviruses (Atkinson *et al*., 2001). The *P* transposable element has been very useful in *Drosophila melanogaster*, but lacks function outside the genus (O'Brochta & Handler, 1988). *Mariner*, *hAT* and *piggyBac* transposons are more widely distributed or are less phylogenetically restricted in function, and thus have greater potential as broad-spectrum transgene vectors (Atkinson *et al*., 2001).

Eye-colour genes are ideal transformation markers, provided appropriate mutant strains are available as transgene recipients. In *D. melanogaster* eye-colour genes such as *rosy*, *white* and *vermilion* have been used as transformation markers in conjunction with the corresponding mutant strains (Rubin & Spradling, 1982; Klemenz *et al*., 1987; Fridell & Searles, 1991). In species lacking suitable eye-colour mutations, dominant selection based on green fluorescent protein has been a popular alternative (Handler, 2001).

Recently Berghammer *et al*. (1999) reported that the *Hermes* and *piggyBac* transposable elements could mediate germline transformation in the red flour beetle, *Tribolium castaneum*. They used elements marked with a gene encoding an enhanced green fluorescent protein (EGFP) driven by an artificial, eye-specific promoter (3xP3) based on Pax-6 binding sites and a minimal *D. melanogaster* hsp70 promoter (Horn & Wimmer, 2000). This system offers opportunities but also imposes limitations. While providing a convenient means for routine screening for transformants, the cell-autonomy of the fluorescent protein and the strict tissue-specificity of the promoter could result in reduced efficiency of detection, particularly in wild-type backgrounds where eye pigment can mask weak EGFP expression (M. D. Lorenzen, unpublished data). If transgene expression in the eye were diminished for any reason (e.g. position-effect variegation) the result could be failure to detect a true transformant. In addition to the potential for reduced efficiency of transformant selection, the convenience of the 3xP3-EGFP system is reduced by the technical demands of EGFP detection, which requires specialized fluorescence microscopy. Alternatively, if a constitutive promoter were used in conjunction with a cell-non-autonomous eye-colour marker, diminished transgene expression in the eye could be compensated by expression elsewhere. For example, *D. melanogaster rosy* mutants, mosaic for cells bearing a *rosy*-marked P-element have wild-type eye colour even in the absence of *rosy* gene function in the cells of the eye (Rubin & Spradling, 1982).

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In addition, the visible pigmentation of the rescue phenotype would allow the use of ordinary dissecting microscopes in transformant screens.

For these reasons, we decided to explore the use of a marker system designed for ubiquitous expression of a cell-non-autonomous eye-colour gene. We previously cloned *Tc vermilion* (*Tcv*), which is the *T. castaneum* ortholog of the *vermilion* (*tryptophan oxygenase*) gene of *D. melanogaster*, and identified a *Tcv*-deficient strain, *vermilion*$^{white}$ ($v^{w}$), which lacks pigmentation in the eyes and Malpighian tubules (Lorenzen *et al*., 2002). In the present study we turned our attention to the identification and use of a strong, constitutive promoter to ensure a high level of *vermilion* expression.

Ubiquitins are multipurpose proteins required in a wide range of cellular processes including protein degradation, modification of chromatin structure, apoptosis and DNA repair (for review see Jentsch, 1992). In *D. melanogaster* these vital proteins are found in all cell types during all stages of development (Lee *et al*., 1988). Moreover *Ubiquitin* genes have strong promoters that have been used to drive transgene expression in a variety of species (Lee *et al*., 1988; Garbarino *et al*., 1995; Schorpp *et al*., 1996). The *D. melanogaster Polyubiquitin* (*PUb*) promoter has been used to drive EGFP expression not only in *D. melanogaster* (Handler & Harrell II, 1999), but also in other dipterans including the Caribbean fruit fly, *Anastrepha suspensa* (Handler & Harrell II, 2001), and the sheep blowfly, *Lucilia cuprina* (Heinrich *et al*., 2002). In view of the strength and versatility of the *PUb* promoter in flies we elected to incorporate the *T. castaneum PUb* promoter into our marker system for transformant selection in beetles. Here we report the cloning and characterization of the *T. castaneum Polyubiquitin* (*TcPUb*) gene and the use of its promoter to drive expression of the *Tcv* eye-colour gene.

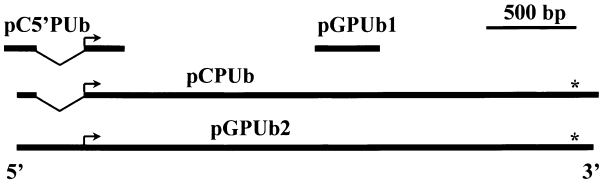


**Figure 1.** *TcPUb* gene structure. The location of the original *TcPUb* genomic clone (pGPUb1), as well as the *TcPUb* cDNA clones (pC5′PUb and pCPUb) and the *TcPUb* genomic clone (pGPUb2) are shown in relationship to the *TcPUb* locus. Arrows indicate the position of the translational start site, while asterisks mark the location of the stop codon. The location and size of the single intron is represented by v-brackets on cDNA clones. GenBank accession numbers are as follows: AF506020 (pGPUb1), AF506021 (pC5′PUb), AF506022 (pCPUb) and AF506023 (pGPUb2).

## Results

### *Homology-based cloning of* TcPUb

Analysis of *TcPUb* genomic and cDNA clones is shown in Figs 1 and 2. PCR with degenerate primers based on the highly conserved Ubiquitin orthologs amplified a 336 bp fragment from *T. castaneum* genomic DNA (pGPUb1). Analysis of the sequence from this fragment revealed the partial coding sequences of two contiguous Ubiquitin monomers in head-to-tail orientation. Primers for 5′ RACE were designed from the sequence of pGPUb1. The 5′-clone (pC5′PUb) provided an additional 302 bp of sequence, including an apparent translational start site and 110 bp of 5′ UTR. Primers designed from the 5′ UTR were used with vector primers to amplify a cDNA containing the entire coding sequence. This cDNA (pCPUb) is 2229 bp in length and includes 68 bp of 5′ UTR, 2058 bp of open reading

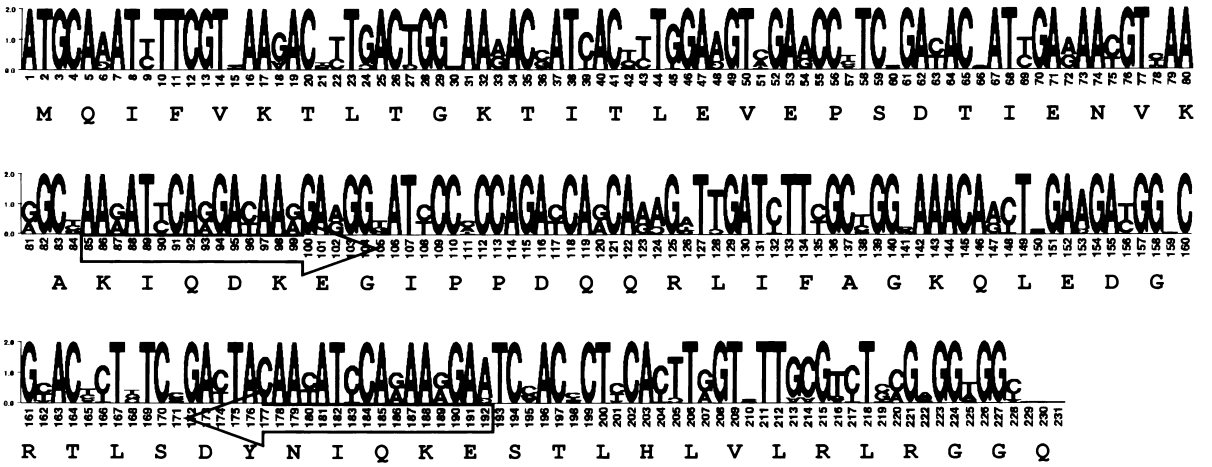


**Figure 2.** Sequence logo of monomer-encoding units from *Tribolium castaneum Polyubiquitin*. The nine monomer-encoding units of *Tc Polyubiquitin* (pCPUb) were aligned to one another by their first nucleotide and a sequence logo was generated using *GENIO*/logo (http://genio.informatik.uni.stuttgart.de/GENIO/logo/logo.cgi) (Schneider & Stephens, 1990). The height of each nucleotide is proportional to its frequency at that position in each monomer. Nucleotides are ranked, with the most frequent on top. Arrows indicate the locations of the degenerate primers.

frame (ORF), 88 bp of 3′ UTR and a poly (A) tail. Alignment (Pearson & Lipman, 1988) of mammalian and insect Ubiquitin proteins showed that the conceptual translation of pCPUb is more than 99% identical to the *D. melanogaster* and human Ubiquitin proteins (data not shown).

Conceptual translation of pCPUb predicts a 685 amino acid polypeptide consisting of nine nearly identical direct repeats, each encoded by a unique DNA sequence (see Fig. 2). Two of the nine monomers differ from the 76-amino acid consensus sequence, each by a single residue. A leucine-to-serine substitution occurs at position 43 in the sixth monomer, while an additional, C-terminal glutamine is appended to the last (ninth) monomer. Wiborg *et al*. (1985) reported that the human *Polyubiquitin* gene also encodes an additional C-terminal residue, namely valine. With the exception of these two amino acid residues, the polypeptides predicted from the *T. castaneum* and human *Polyubiquitin* genes are identical, both consisting of nine tandem copies of identical Ubiquitin monomers. The sequence identity between the polypeptide monomers encoded by *T. castaneum PUb* and *Polyubiquitin* from *D. melanogaster* and *Homo sapiens*, as well as the tandem copy number conservation between beetle and human, indicates that we isolated the *T. castaneum Polyubiquitin* ortholog.

### *Gene structure and promoter analysis*

To determine if the gene structure of *TcPUb* is similar to that of *D. melanogaster PUb*, primers from the 5′ and 3′ UTRs of *TcPUb* were used to amplify a genomic fragment (pGPUb2, see Fig. 1). Gene structure was determined by comparison of cDNA and genomic sequences (Fig. 1). Both *D. melanogaster* (Lee *et al*., 1988) and *T. castaneum Polyubiquitin* genes have a single intron located immediately upstream of the translational start site (11 bp upstream in *D. melanogaster* and coincident with the translational start site in *T. castaneum*). The introns of *T. castaneum* and *D. melanogaster* are 305 bp and 758 bp, respectively.

To identify the *TcPUb* promoter we first probed a *T. castaneum* genomic BAC library with pGPUb1. The screen revealed both strongly and weakly hybridizing clones in equal numbers. The latter contained a 450 bp Ubiquitin-fusion gene (*TcUb-CEP52*; data not shown). A sequencing primer from the 5′ UTR of *TcPUb* was used to obtain an upstream genomic sequence from one of the strongly hybridizing clones (bTcPUb). This sequence was analysed for the presence of promoter elements (Fig. 3). A consensus TATA box is located at –104 and another *c*. 1 kb upstream, while a third is found within the 5′ UTR. Four TATA-like sequences (AAATAA) and five putative CAAT boxes are also present.

### Tc Polyubiquitin *is highly expressed*

Northern analysis showed that a *TcPUb* fragment (pCPUb) hybridized to a 2.5 kb transcript (pupal mRNA, Fig. 4), which corresponds to the length of the *TcPUb* cDNA (2229 bp), assuming a 270 bp poly (A) tail. Upon longer exposure (3.5 h) a 650 bp transcript was revealed, consistent with the size predicted for the Ubiquitin fusion species (data not shown). Subjective comparisons to other RNAs hybridized from the same sample (eye-colour genes *Tcv* and *Tc white*) suggest that *TcPUb* is an abundant transcript in *T. castaneum* pupae.

### TcPUb-Tcv *rescues the* $v^w$ *mutant phenotype*

Although *T. castaneum* larvae lack compound eyes, pigmented eyespots are visible in wild-type larvae and late-stage embryos (Fig. 5A). We previously reported that mutants exhibiting white-eyed adult phenotypes lack larval eyespot pigmentation (Lorenzen *et al*., 2002). In addition, we demonstrated that the white-eye mutation *vermilion*$^{white}$ ($v^w$), formerly known as *white* (Eddleman & Bell, 1963), carries a null mutation in *Tcv* (Lorenzen *et al*., 2002). We decided to use larval eyespot pigmentation to assess the ability of the *TcPUb* promoter to drive *Tcv* expression in $v^w$ larvae.

To determine whether the *TcPUb* promoter could drive *Tcv* expression, a chimeric transient-rescue construct (pTcPUb-Tcv) was injected into *Tcv*-deficient ($v^w$), preblastoderm embryos. After pTcPUb-Tcv injection, 197 of 228 late-stage $v^w$ embryos and newly hatched larvae showed eyespot rescue (Fig. 5C). The effect was transient, because eye-colour failed to develop during pupation. Eyespot rescue has not been seen with injection of other DNAs (> 1000 hatchlings examined), or by mock injections with buffer alone (> 1000 hatchlings examined). The mutant eyespot phenotype was also rescued by a smaller (2 kb) transient-rescue construct (pmTcPUb-Tcv), which employed only the proximal 210 bp of the *TcPUb* promoter (Fig. 5D). These results demonstrate that the *TcPUb* promoter can drive *Tcv* expression, and indicate that the *TcPUb-Tcv* chimera can serve as a scorable marker for transformant selection when introduced into the $v^w$ background.

### Tcv *rescue is an efficient indicator of germline transformation*

To compare the efficiency and sensitivity of the 3xP3-EGFP marker system to that of mutant eye-colour rescue, the larger (3 kb) *TcPUb-Tcv* chimera was incorporated into the pB-3xP3-EGFP (Horn & Wimmer, 2000) *piggyBac* vector. Approximately 700 preblastoderm $v^w$ embryos were injected with a mixture of *piggyBac* helper and the doubly marked donor. Transient expression of *Tcv* was detected as rescued larval eyespot pigmentation in approximately 50% of the late-stage embryos and newly emerging larvae (data not shown). Only 5% of eyespot-rescued $G_0$ larvae survived to the adult stage (three out of sixty-two), compared to 92% survival of the non-rescued larvae (sixty-six out of seventy-two). Each of the sixty-nine $G_0$ adults (three rescued and sixty-six non-rescued) were individually backcrossed to homozygous $v^w$ beetles. All three transiently rescued $G_0$

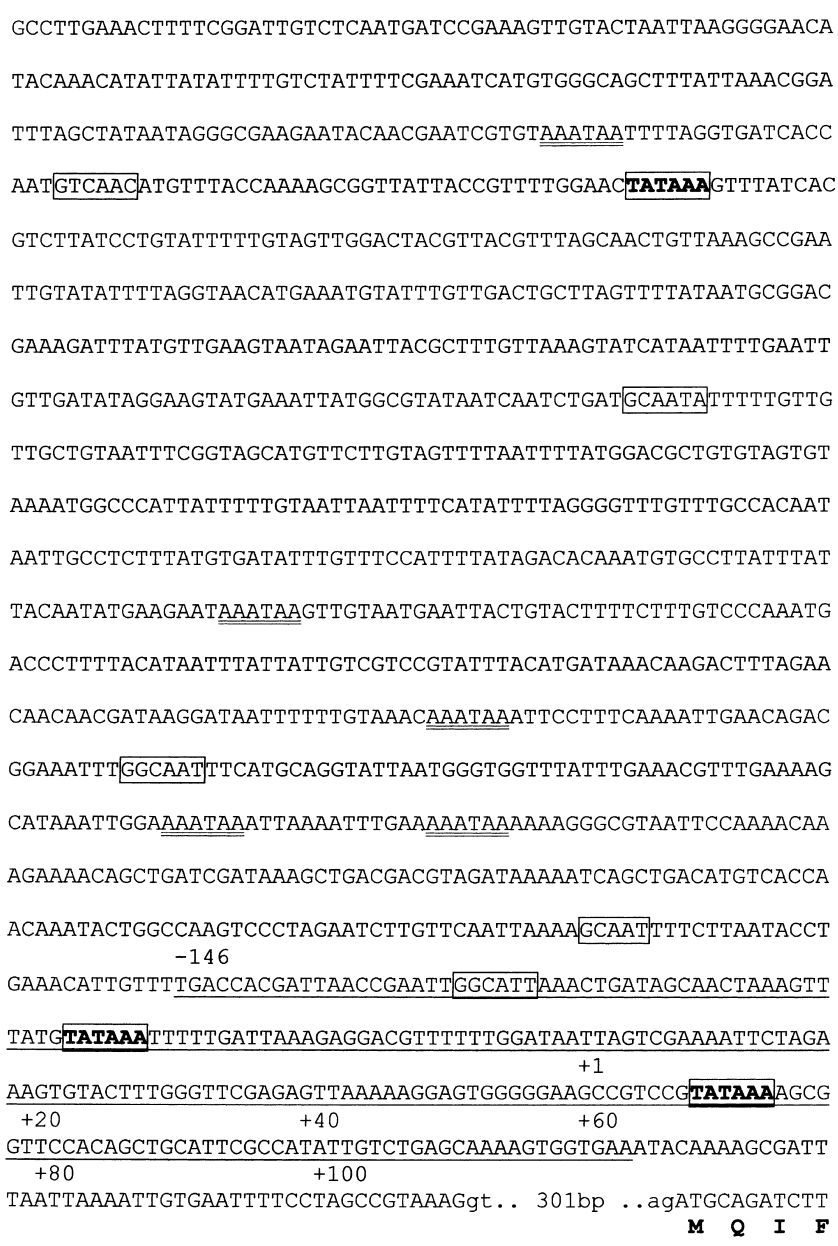


**Figure 3.** *Tribolium castaneum Polyubiquitin* promoter sequence. Genomic sequence was derived from a BAC clone. The single *TcPUb* intron is located in the 5′ UTR immediately upstream of the start codon (splice junctions are indicated in lowercase font). +1 indicates the start of the longest *TcPUb* transcript detected. All 1285 nt shown upstream of position +62 (i.e. from –1222 through +63) were included in the larger chimeric construct (pTcPUb-Tcv). The smaller construct (pmTcPUb-Tcv) included only the 210 nt of underlined promoter sequence. The three putative TATA motifs are boxed and in boldface type, CAAT motifs are boxed, and TATA-like sequences are double-underlined. The GenBank accession number for BAC subclone pGPUb3 (containing the *TcPUb* promoter sequence) is AF506024.

adults produced germline-transformed $G_1$ progeny, whereas none of the sixty-six non-rescued $G_0$ adults produced any transformed progeny. The three rescued lines were designated V3, V27 and V29. In addition to our primary screen for eye-colour rescue (i.e. *Tcv* expression), we also screened at least 100 $G_1$ progeny from each of the sixty-nine lines for EGFP expression. Seven fluorescent individuals were found among the 6900 $G_1$ progeny screened, all from the V29 line. EGFP expression was not detected in any of the remaining sixty-eight lines, including the V3 and V27 lines that previously produced *Tcv*-rescued progeny. Upon closer inspection at high magnification, a small number of fluorescent ommatidia were detected in V3 progeny, but these individuals lacked sufficient EGFP expression to have been identified as putative transformants by standard methods. EGFP expression was not seen in any V27 progeny (Table 1). A comparison of Tcv and EGFP marker expression in transformants is shown in Fig. 6.

*Tcv*-positive $G_1$ adults were individually outcrossed to homozygous $v^w$ beetles. *Tcv*-positive and -negative $G_2$ progeny segregated in nearly equal numbers, consistent with a single transgene insertion or multiple insertions into a single site (Table 2). These insertions appear to be stable because each line has displayed a consistent, line-specific phenotype for over twenty generations. In addition, insertion of the *piggyBac* element into the *T. castaneum* genome has

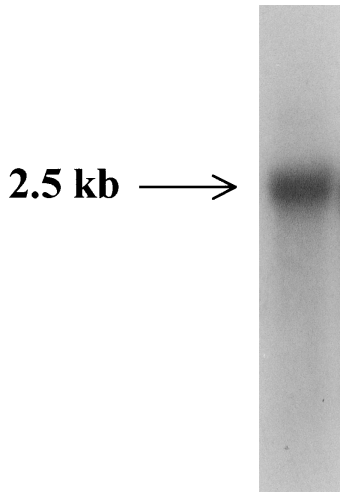


**Figure 4.** *TcPUb* expression. This Northern blot reveals a single 2.5 kb transcript in *Tribolium castaneum* pupal mRNA using a *TcPUb*-specific probe (pCPb) (15 min exposure).

been confirmed by cloning and sequencing *piggyBac* junctions from these, as well as other *T. castaneum* transformants (unpublished observations).

## Discussion

Transposition frequency is limited by vector size (Handler & Harrell II, 1999). Our dual-marker *piggyBac* element exhibited a transformation frequency of approximately 4% (three out of sixty-nine surviving $G_0$ adults) based on *Tcv* selection, and 1.5% (one out of sixty-nine surviving $G_0$ adults) when based on EGFP selection. Similar transformation frequencies have been reported with *piggyBac* in *Ceratitis capitata* (Handler *et al*., 1998), *D. Melanogaster* (Handler & Harrell II, 1999) and *Bombyx mori* (Tamura *et al*., 2000). The transformation frequency obtained with the dual-marker *piggyBac* vector is, however, far lower than the ~60% reported for the single-marker pB-3xP3-EGFP vector (Berghammer *et al*., 1999). The unusually high transformation frequency obtained in that work might indicate that *T. castaneum* embryos are highly permissive for *piggyBac* insertion, but that our dual-marker element exceeded the upper limit to the amount of DNA that can be efficiently transposed by *piggyBac*.

Our goal is to use the *TcPUb-Tcv* chimera as a scorable marker for transformant selection in various transposon-based germline transformation assays, therefore minimizing its size is of considerable importance. The chimeric transient-rescue construct pTcPUb-Tcv includes 1200 bp of upstream *TcPUb* sequence, possibly more than the minimum required for high promoter activity. When 1 kb (all but the proximal CAAT and TATA boxes) were removed from the 1.2 kb promoter region of pTcPUb-Tcv (see Fig. 3), the resulting construct (pmTcPUb-Tcv) still rescued the mutant

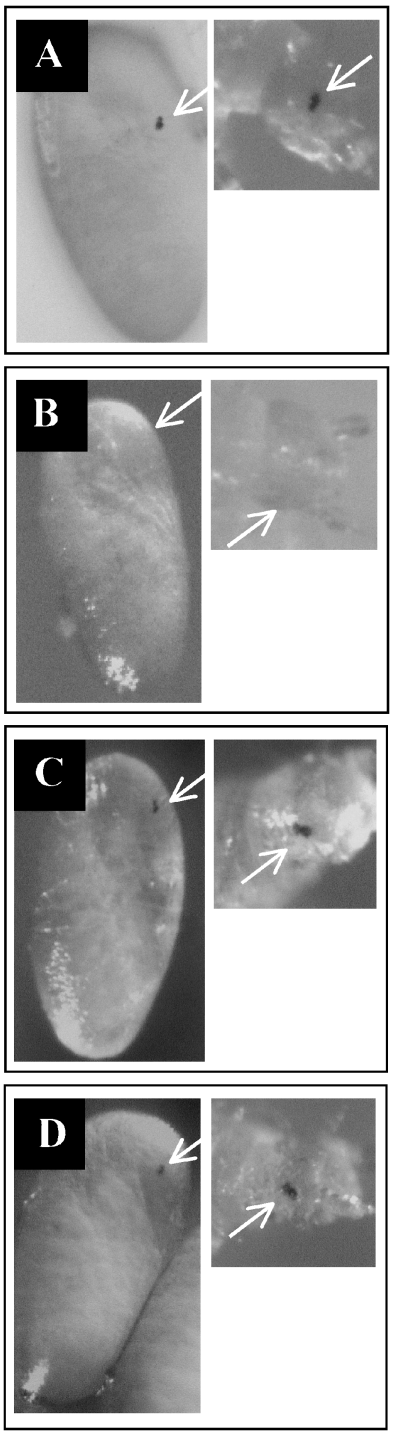


**Figure 5.** Transient expression of *Tcv* in late-stage $v^w$ embryos and first-instar $v^w$ larvae. (A–D) Lateral views of late-stage embryos and first-instar larvae illustrate the effect of *Tcv* expression from transient-rescue constructs. (A) Late-stage GA-1 embryo and first-instar larva showing wild-type eyespot pigmentation. (B) Late-stage $v^w$ embryo and first-instar larva showing absence of eyespot pigmentation. (C) Late-stage $v^w$ embryo and first-instar $v^w$ larva previously injected with the pTcPUb-Tcv construct. (D) Late-stage $v^w$ embryo and first-instar $v^w$ larva previously injected with the smaller pmTcPUb-Tcv construct. Arrows indicate location of eyespots on the dorsolateral head. Note rescue of eyespot pigmentation in (C) and (D).

**Table 1.** $G_0$ individuals producing $G_1$ progeny with eye-colour rescue or EGFP expression

| $G_0$ line | No. of $G_1$s screened | *Eye-colour phenotype | *EGFP expression | $G_1$ progeny with eye-colour |
|---|---|---|---|---|
| V3 male | 476 | Red | Weak | 50 (10%) |
| V27 male | 228 | Light red | None | 25 (11%) |
| V29 male | 177 | Black | Strong | 9 (5%) |

*Eye-colour phenotype and EGFP expression observed in transgenic $G_1$ progeny from V3, V27 and V29 $G_0$ crosses ($v^{w*}/v^{w} \times v^{w}/v^{w}$).

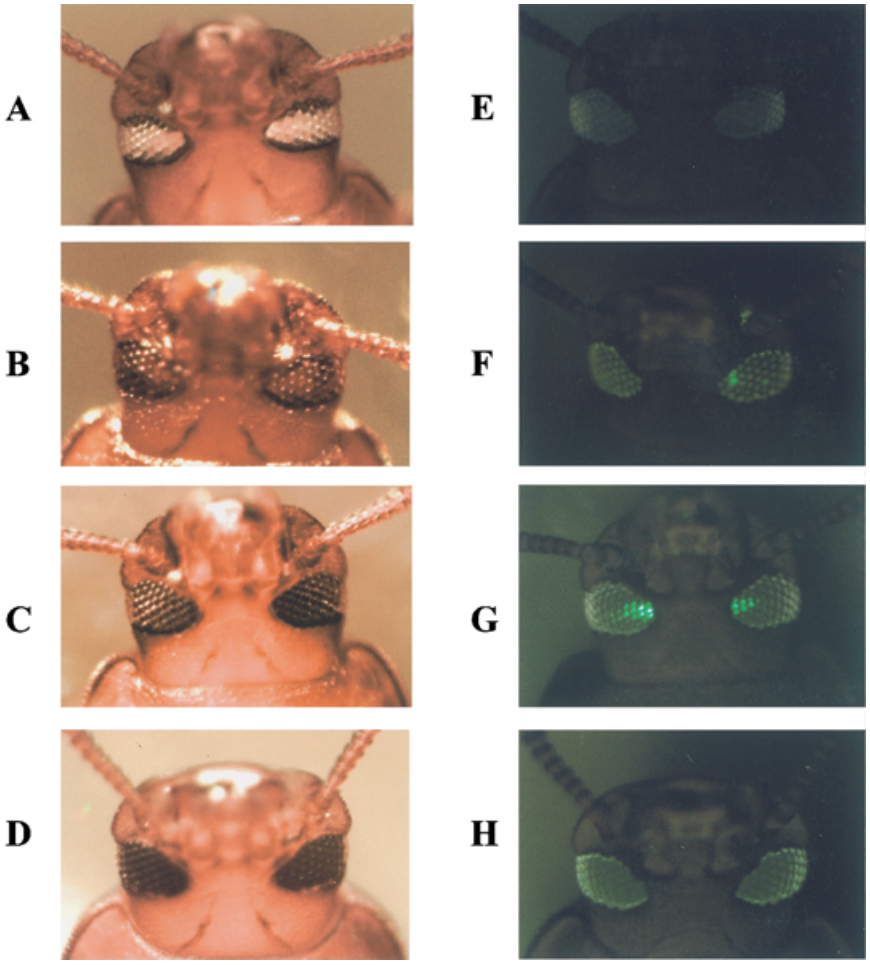


**Figure 6.** Comparison of adult eye-colour phenotypes: *Tcv* expression vs. EGFP expression. (A–D) Eye pigment as seen in white light. (E–H) Same individuals in UV light. (A and E) Non-transformed, homozygous $v^{w}$ beetle. (B and F) Typical heterozygous V3 adult. (C and G) Typical heterozygous V29 adult. (D and H) Wild-type (GA-1) adult. Note fluorescent ommatidia in F and G.

eyespot phenotype to a degree equal to that of the larger pTcPUb-Tcv construct (Fig. 5D).

Although the doubly-marked *piggyBac* element showed a reduction in transposition frequency, it allowed direct comparison between *Tcv* and EGFP markers. Neglecting any possibility of transcriptional interference between the tandem markers in the donor plasmid, and ignoring any potential masking of green fluorescence by the rescued eye pigmentation, it appears that transformant selection based on *Tcv* expression is more sensitive than that based on EGFP expression. As a rule homozygous transformants have darker eye pigmentation than heterozygotes, yet little difference is seen in EFGP fluorescence. One possible reason for the weaker expression of EGFP is position-effect variegation (PEV), which can silence genes inserted into or near a heterochromatic region (Levis *et al.*, 1985). Position effects have been reported in several insect species (e.g. Hazelrigg *et al.*, 1984; Coates *et al.*, 1998), and a cell-autonomous marker such as EGFP might be more susceptible to the effect of PEV than a cell-non-autonomous marker such as Vermilion. Another advantage of this system is that transient expression of *Tcv* in the $G_0$ parent can be used to preselect $G_0$s that are more likely to produce transformed progeny. In this case 100% of the $G_0$s that transiently expressed *Tcv* gave rise to transformants. A potential disadvantage of *Tcv*-based selection is that Vermilion proteins might be phylogenetically restricted in function. However, the *D. melanogaster vermilion* gene has

**Table 2.** Segregation of the *Tcv* marker gene in $G_1$ test crosses

| $G_1$ cross | Replicates | Phenotypic class | No. of $G_2$ beetles | Total | $\chi^2$ |
|---|---|---|---|---|---|
| V3 | 1 | Pigmented eyes | 59 | 110 | 0.58 |
| | | White eyes | 51 | | |
| | 2 | Pigmented eyes | 44 | 100 | 1.44 |
| | | White eyes | 56 | | |
| | 3 | Pigmented eyes | 89 | 175 | 0.05 |
| | | White eyes | 86 | | |
| V27 | 1 | Pigmented eyes | 86 | 161 | 0.75 |
| | | White eyes | 75 | | |
| | 2 | Pigmented eyes | 43 | 90 | 0.18 |
| | | White eyes | 47 | | |
| | 3 | Pigmented eyes | 29 | 64 | 0.56 |
| | | White eyes | 35 | | |
| V29 | 1 | Pigmented eyes | 29 | 51 | 0.96 |
| | | White eyes | 22 | | |
| | 2 | Pigmented eyes | 30 | 53 | 0.92 |
| | | White eyes | 23 | | |
| | 3 | Pigmented eyes | 35 | 71 | 0.01 |
| | | White eyes | 36 | | |

$G_2$ progeny from test crosses between individual *Tcv* rescued $G_1$ progeny with homozygous $v^w$ beetles ($v^{w*}/v^w \times v^w/v^w$). Phenotypic distributions are consistent with 1 : 1 segregation of a single *Tcv* transgene as evaluated by $\chi^2$, d.f. = 1.

been shown to rescue a *tryptophan oxygenase*-deficient mutation in the house fly, *Musca domestica* (White *et al.*, 1996), and the *Anopheles gambiae vermilion* gene has been shown to rescue a *vermilion* mutation in *D. melanogaster* (Besansky *et al.*, 1997). This raises the possibility that *Tcv* could also serve as a transformation marker in other families of beetles, provided the appropriate mutant strains were available.

## Experimental procedures

### T. castaneum *strains*

Beetles were reared in yeast-fortified wheat flour under standard conditions (Beeman *et al.*, 1986). The wild-type (GA-1 and GA-2) and *Tcv*-deficient (*vermilion*$^{white}$) strains used in this study have been described previously (Lorenzen *et al.*, 2002).

### *Degenerate PCR*

Ubiquitin proteins are very highly conserved over their entire length (> 99% identity between insect and primate). Thus, regions of relatively low codon degeneracy (see Fig. 2) were targeted for the design of *Ubiquitin*-specific oligonucleotides. PCR was performed using 6 ng of GA-1 genomic DNA with primers (UBIQ-F) AARATHCARGAYAARGARGG and (UBIQ-R) YTCYTTYTGDATRTTRTARTC (all primer sequences are shown 5′ to 3′). The PCR product was ligated into pCRII-TOPO (Invitrogen, Carlsbad, CA) and the insert of the resulting clone (pGPUb1) sequenced.

### *Isolation of cDNA and genomic clones*

Approximately 3.6 ng of purified DNA from a *T. castaneum* embryonic cDNA library (Shippy *et al.*, 2000) was used as a template for rapid amplification of cDNA ends (RACE) using *TcPUb*-specific primers in conjunction with pCMVSPORT4 vector primers. To obtain a cDNA containing the 5′ UTR of *TcPUb* a degenerate *Ubiquitin*-specific, reverse primer (UBIQ-R, see above) was used in conjunction with the SP6 vector primer. The insert of the resulting clone (pC5′PUb) was sequenced, and nested, forward primers were designed from the sequence of the 5′ UTR for use in 3′ RACE. First-round PCR was performed with 3.6 ng of DNA from the cDNA library as a template, using GST-F (GTATAAAAGCGGTTCCACAG) and M13 (–20) with DyNAzyme™ EXT PCR Kit (Finnzymes, MJ Research). A second round of PCR was performed with the nested primers UB5-F (TGTCTGAGCAAAAGTGGTG) and T7, using 1 μl of the first-round product as a template. The second-round PCR product was ligated into pCRII-TOPO (Invitrogen) and the resulting clone (pCPUb) sequenced. The corresponding genomic fragment was amplified using UB5-F with a 3′ UTR primer, UB3-R (TCGTAATTTCAGGAAACTTGTAAC), and the resulting clone (pGPUb2) was sequenced.

A GA-2 *T. castaneum* bacterial artificial chromosome (BAC) library constructed in pBACe3.6 (a gift from the Exelixis Pharmaceutical Co., South San Francisco) was screened for genomic clones of *TcPUb*. The *TcPUb* degenerate PCR product, pGPUb1, was radiolabelled by random priming (Prime-It, Stratagene, La Jolla, CA). After prehybridization for 3 h at 65 °C in 5× Denhardt's buffer (0.5% SDS, 5× SSC, and 20 μg/ml herring sperm DNA) the filter was hybridized overnight at 65 °C with the $^{32}$P-labelled probe in fresh hybridization buffer and washed in 2× SSC, 0.1% SDS at 65 °C.

### *Northern analysis*

Northern analysis of *TcPUb* was performed as previously described by Shippy *et al.* (2000), but using pupal rather than embryonic mRNA. The *TcPUb* cDNA (pCPUb) was used as probe.

### *Construction of* TcPUb-Tcv *chimera*

The *TcPUb* promoter was linked to the *Tc vermilion* coding region via chimeric PCR (Horton *et al.*, 1989). All amplifications were performed using the DyNAzyme™ EXT PCR Kit (Finnzymes). In the first step, the *TcPUb* promoter was amplified by PCR from 1 ng of BAC DNA (bTcPUb, see above) using the *TcPUb*-specific forward primer UbF (GCCTTGAAACTTTTCGGATTGTCTC, bases –1222 through –1198, Fig. 3) and the chimeric primer UbVer (TGACTCGATTGGATCGTTGACTTCACCACTTTTGCTCAGACAATATGGC). The 5′ end (first 21 bases, underlined) of the latter primer are complementary to bases –29 through –49 of *Tcv* (Fig. 2 in Lorenzen *et al.*, 2002) while the 3′ end (last 28 bases) are complementary to bases +63 through +36 of the *TcPUb* 5′ UTR (Fig. 3). In both *TcPUb* and *Tcv*, +1 refers to the 5′ most nucleotide of the longest transcript.

In the second step, the *Tcv* coding region was amplified by PCR from 1 ng of BAC DNA (bTcv in Lorenzen *et al.*, 2002) using the chimeric forward primer VerUb (GCCATATTGTCTGAGCAAAAGTGGTGAAGTCAACGATCCAATCGAGTCA) and the *Tcv*-specific reverse primer TcvR (TTAATTTTATTCAATTGCCCCGTGCATTTAGTGTG). The 5′ end (first 28 bases, underlined) of the former primer match bases +36 through +63 of the *TcPUb* 5′ UTR (see Fig. 3) and the 3′ end (last 21 bases) match bases –49 through –29 of *Tcv* (Fig. 2 in Lorenzen *et al.*, 2002). The latter primer is complementary to bases +1607 to +1572 of *Tcv* (Fig. 2 in Lorenzen

*et al*., 2002). PCR conditions for steps 1 and 2 were thirty cycles of 94 °C denaturation (30 s), 62 °C annealing (30 s), 72 °C extension (3 min).

In the third step, a mixture of the above two PCR products (1 μl each) was denatured at 95 °C for 5 min and cycled five times (annealing temp 62 °C) in the absence of primers to produce a chimeric substrate. An additional twenty cycles were performed in the presence of the *TcPUb*-specific forward primer UbF and the *Tcv*-specific reverse primer TcvR. PCR conditions were twenty cycles of 94 °C denaturation (30 s), 62 °C annealing (30 s), 72 °C extension (3 min).

### *Plasmids*

The chimeric *TcPUb-Tcv* fragment (~3 kb) was ligated into pCRII-TOPO (Invitrogen). A smaller expression construct was produced using the above clone as a template for DyNAzyme™ EXT PCR (Finnzymes) using a nested *TcPUb* forward primer (TGACCACGATTAACCGAATTGG, see Fig. 3) in conjunction with TcvR. The ~2 kb product was ligated into pCRII-TOPO (Invitrogen) to create the smaller pmTcPUb-Tcv construct. The pTcPUb-Tcv or pmTcPUb-Tcv plasmids were dissolved in buffer (see below) and injected into 0–4 h $v^w$ embryos for assessment of transient rescue of eye colour.

The *piggyBac* transposase helper plasmid, phsp-pBac, has been previously described (Handler & Harrell II, 1999). The doubly marked *piggyBac* donor plasmid, pB-3xP3-EGFP-TcPUb-Tcv, was created in two steps. First the *TcPUb-Tcv* chimera was cloned as a 3 kb *Not*I-*Bam*HI fragment from pTcPUb-Tcv (see above) into *Not*I- and *Bam*HI-digested pSLfa1180fa (Horn & Wimmer, 2000). The *TcPUb-Tcv* chimera was then cloned as a 3.1 kb *Asc*I-*Fse*I fragment from the pSLfa1180fa shuttle vector into *Asc*I- and *Fse*I-digested pB3xP3-EGFPaf (Horn & Wimmer, 2000). Plasmids were purified using the Wizard™ Maxipreps DNA Purification System (Promega).

### *Microinjection and rearing*

*T. castaneum* eggs were collected from $v^w$ beetles ≤ 2 h after oviposition and injections were completed within 2 h of collection. Eggs were injected (≈ 100 pL/embryo) though the chorion with plasmid DNA in injection buffer (5 mM KCl, 1 mM $KPO_4$ and 1% v/v green food colouring, pH 6.8). Plasmids used for injection included either pTcPUb-Tcv or pmTcPUb-Tcv (500 ng/μl) for transient expression assays, or a mixture of helper (phsp-pBac; 400 ng/μl) and donor (pB3xP3-EGFP-TcPUb-Tcv; 500 ng/μl) plasmids for transformation assays. After injection, embryos were held for 1 week in a humidified, oxygenated chamber (Billups-Rothenberg, Del Mar, CA) at room temperature. Late-stage embryos and first-instar larvae were scored for the presence or absence of larval eyespot pigmentation and reared under standard conditions. Reciprocal, virgin crosses were made between individual $G_0$ beetles and mates from the $v^w$ stock. $G_1$ offspring were examined for eye-colour rescue and EGFP expression. Single $G_1$ transformants were again backcrossed to $v^w$ stock for segregation analysis.

### *DNA sequencing and analysis*

BAC DNA templates were sequenced using the ThermoSequenase kit (Amersham Life Sciences, Cleveland). PCR products were cloned using the pCRII-TOPO kit (Invitrogen) and sequenced with the aid of an ABI 373 A DNA sequencer (DNA Sequencing Core Facility, College of Veterinary Medicine, Kansas State University) or an ABI 3700 DNA sequencer (Sequencing and Genotyping Facility, Plant Pathology, Kansas State University). Data were analysed using the MacVector sequence analysis program (Eastman Kodak Company, New Haven, CT).

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