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# *Tubulin* superfamily genes in *Tribolium castaneum* and the use of a *Tubulin* promoter to drive transgene expression

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ABSTRACT

The use of native promoters to drive transgene expression has facilitated overexpression studies in *Drosophila* and other insects. We identified 12 *Tubulin* family members from the genome sequence of the red flour beetle, *Tribolium castaneum*, and used the promoter from one of these to drive constitutive expression of a transgene. The activity of the *T. castaneum* α-*Tubulin1* (*TcαTub1*) putative promoter was pre-tested in conjunction with an eye-color gene, *T. castaneum vermilion* (*Tcv*), by transient expression in *Tcv*-deficient embryos. Such embryos showed complete rescue of larval eyespot pigmentation. We also examined the *TcαTub1* expression pattern in germline transformants using the enhanced green fluorescent protein (EGFP) reporter. Beetles transformed with this *piggyBac*-based reporter ubiquitously expressed EGFP at all stages.

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## 1. Introduction

The red flour beetle, *Tribolium castaneum*, is the first coleopteran to have had its genome sequenced (*Tribolium castaneum* Genome Project, Human Genome Sequencing Center, Baylor College of Medicine: http://www.hgsc.bcm.tmc.edu/projects/tribolium/). Understanding the functions of the estimated ~16,000 genes will require sophisticated methods of molecular dissection. One such technique involves the overexpression or misexpression of individual genes of interest to gain insight into their roles in development, metabolism, homeostasis or other physiological processes. Our goal in undertaking the present work was to develop native *Tribolium* promoters to enable such overexpression/misexpression studies.

Previously, we and our colleagues reported the use of transposon-based germline transformation in *T. castaneum* via injection of transformation constructs (Berghammer et al., 1999; Lorenzen et al., 2002a, 2003; Pavlopoulos et al., 2004), and through the use of helper/donor hybrids (Lorenzen et al., 2007). In each case *transposase* expression was driven by the *Drosophila melanogaster heat-shock protein 70* (*hsp70*) promoter. However, in a study comparing the frequency of *piggyBac* transposition in *Drosophila melanogaster* based on *transposase* expression driven by various native promoters, the α-*Tubulin 84B* (α*Tub84B*) promoter outperformed that of *hsp70* (Li et al., 2001). Therefore, while the heterologous *hsp70* promoter is functional in *Tribolium*, achieving optimal expression levels for efficient germline transformation, as well as a range of options for overexpression/misexpression studies will require the identification and testing of native *Tribolium* promoters.

To date only a few promoters have been cloned and characterized from *Tribolium*. These include *twist* (Handel et al., 2005), *hunchback* (Wolff et al., 1998), *hairy* (Eckert et al., 2004), and *tailless* (Schröder et al., 2000), all of which are involved in early embryonic development, and *Polyubiquitin* (Lorenzen et al., 2002a). Of these, only *Polyubiquitin* is constitutively active.

As part of an effort to identify additional native promoters for use in overexpression and misexpression studies we used a bioinformatic approach to identify a *Tribolium* ortholog of the ubiquitously and constitutively active *Drosophila* gene α*Tub84B*. Here we report the identification of 12 *Tubulin* family members from *Tribolium* and the cloning, characterization and use of a *Tubulin* promoter for transgene expression.

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## 2. Materials and methods

### 2.1. T. castaneum strains

The wild-type (GA-2) and eye-color-deficient *pearl* (*p*) and *vermilion*$^{white}$ ($v^w$) strains used in this study were described previously (Lorenzen et al., 2002a, b). The transgenic helper strain M59 is described by Lorenzen et al. (2007). Beetles were reared in yeast-fortified wheat flour under standard conditions (Beeman et al., 1986). Voucher specimens, No. 198, are deposited in the Kansas State University Museum of Entomological and Prairie Arthropod Research, Manhattan, Kansas.

### 2.2. Plasmids

p*TcPUb–Tcv* (Lorenzen et al., 2002a) is a transient-rescue plasmid containing 1.3 kb of *TcPUb* promoter sequence linked to the *Tcv*-coding sequence. pBac{3xP3-EGFP} (Horn and Wimmer, 2000) is a donor plasmid containing an eye specific, fluorescent reporter for *piggyBac*-mediated germline transformation. pBac{3xP3-*Tcv*} (Lorenzen et al., in progress) is a *Tcv*-marked *piggyBac* transformation vector in which the native *Tcv* promoter has been replaced by the eye-specific 3xP3 promoter.

### 2.3. Sequence analysis

The deduced amino-acid sequence of a constitutively active *D. melanogaster alpha-Tubulin*, *α-Tubulin 84B* (*αTub84B*), was used as query to identify putative *Tubulin* genes in the *T. castaneum* genome (TBlastN; Altschul et al., 1997). Corresponding *ab initio* gene predictions were identified (BlastN) in the official *T. castaneum* gene set (i.e. GLEAN gene predictions; Elsik et al., 2007) and compared to *Drosophila Tubulin* sequences (BlastP). *Tubulin*-specific expressed sequence tags (ESTs) were identified by using each GLEAN as query (BlastN) against a collection of 93,303 *Tribolium* ESTs (Park et al., 2008, unpublished data).

*Nasonia vitripennis* and *Anopheles gambiae Tubulin* genes were identified using a similar approach. Each of the 12 *Tribolium* sequences (TBlastN) was used as query against NCBI organism-specific RefSeq databases to identify corresponding gene predictions from both *Nasonia* and *Anopheles*. In order to confirm gene prediction models ESTs were retrieved at the Human Genome Sequencing Center (HGSC), Baylor College of Medicine (http://www.hgsc.bcm.tmc.edu/projects/nasonia/) for *Nasonia* and VectorBase (http://agambiae.vectorbase.org/index.php) for *Anopheles*.

The sequences of genomic contigs (sequence gaps absent) and scaffolds (sequence gaps present) for *Tribolium* were obtained from the HGSC (http://www.hgsc.bcm.tmc.edu/projects/tribolium/). Putative promoters were identified via the Neural Network Promoter Prediction Program (Reese, 2001; www.fruitfly.org/seq_tools/promoter.html) using the default cut-off score of 0.80. DNA data analysis was performed using Vector NTI® (Invitrogen) sequence analysis software.

Phylogenies were determined based on sequence alignment (CLUSTAL W; Thompson et al., 1994) and a phylogenetic tree constructed using PAUP 4b.2 (Swofford, 2001) via the neighbor-joining method with 1000 bootstrapping. *Tribolium* (XP_966492, XP_966407, XP_966584, XP_974091, XP_967267, XP_969993, XP_967348, AAJJ01000888 [nts 14116–14056, 10062–9383 and 6849–6265], XP_968314, AAJJ01002303 [nts 1014–966, 915–429] and AAJJ01003022 [nts 4768–5392 and 8333–8524], AAJJ01000170 [nts 61822–60641] and genbank:XP_968261XP_968261); *Drosophila* (NP_524009, NP_524264, NP_524297, NP_476772, NP_651606, NP_523795, NP_523842, NP_524290, NP_476804 and NP_524290); *Nasonia* (XP_001601917, XP_001603341, XP_001606879, XP_001604160, XP_001607699, XP_001606870, XP_001605336, XP_001606290, XP_001603460, XP_001600854 [corrected using EV429872 and EV430552], XP_001602360 and XP_001600234); and *Anopheles* (XP_308639, XP_309723 [corrected using BX061199], XP_312152 [corrected using BM641018], XP_563022, XP_563022, XP_309765, XP_314483, XP_314718, XP_315005 and XP_317665).

### 2.4. Chimeric PCR

Chimeric PCR (Horton et al., 1989) was used to place the *T. castaneum α-Tubulin1* (*TcαTub1*) promoter upstream of the coding sequence of the *T. castaneum vermilion* (*Tcv*) gene (AY052392) thereby producing a chimeric gene, *TcαTub1–Tcv*. A proofreading polymerase (Ex Taq™ DNA polymerase; Takara Bio Inc., Seta, Japan) was used for all amplifications, under standard PCR conditions.

In the first step, the *TcαTub1* promoter was amplified by PCR from 8 ng of genomic DNA (GA-2) using the *TcαTub1*-specific forward primer TP1 (GACGTCTATGTATCTCCCGTGAAAC) and the chimeric primer TVR (AGGGTCTCAGTGGGCAACT**CAT**AAATTACACCACAAAAGAGC, underlined portion = *Tcv* with start codon in bold font, remainder = *TcαTub1*). To obtain a higher yield, a second, hemi-nested round of PCR was performed using the nested, *TcαTub1*-specific forward primer TP2 (GCGGAGTTTATCACACGTTTTCATATC) in conjunction with TVR.

In the second step, the *Tcv*-coding region was amplified by PCR from 1 ng of p*TcPUb–Tcv* (Lorenzen et al., 2002a) using the chimeric forward primer TVF (GCTCTTTTGTGGTGTAATTT**ATG**AGTTGCCCACTGAGACCCT, underlined portion = *Tcv* with start codon in bold font, remainder = *TcαTub1*) and the *Tcv*-specific reverse primer V4 (TTAATTTTATTCAATTGCCCCGTGCATTTAGTGTG).

To obtain a full-length, functional chimeric gene, tenfold dilutions of each of the PCR products from steps one and two were mixed and denatured at 95 °C for 5 min. This was followed by five cycles of denaturation (94 °C; 30 s), annealing (63 °C; 30 s) and extension (72 °C; 3 min). The chimeric gene was further amplified using the *TcαTub1*-specific forward primer TP2 and *Tcv*-specific reverse primer V4 (see above) by 20 cycles of denaturation (94 °C; 30 s), annealing (58 °C; 30 s) and extension (72 °C; 3 min).

A similar approach was used to place the *TcαTub1* promoter upstream of the gene encoding enhanced green fluorescent protein (EGFP). The *TcαTub1* promoter was amplified from p*TcαTub1–Tcv* template DNA using the *TcαTub1*-specific primer **GGCGCGC**CACTGCAGTGAACGGTTATGA (flanking p*TcαTub1–Tcv* vector sequence underlined) in conjunction with the chimeric reverse primer TGR (TTAGCGACGTGTTCACTTTG**ACAAAAGAGCAGAAAAACGG**, underlined portion = *TcαTub1*, remainder = EGFP). The EGFP portion of the chimera was amplified from pBac{3xP3-EGFP} (Horn and Wimmer, 2000) using the chimeric forward primer TGF (CCGTTTTTCTGCTCTTTTGT**CAAAGTGAACACGTCGCTAA**, underlined portion = EGFP, remainder = *TcαTub1*) in conjunction with a reverse primer Vec2261 (CCACTCGGTGAATACTACGTGAAGG), located downstream of the SV40 polyA signal in pBac{3xP3-EGFP}. The full-length chimeric gene was amplified using an *αTub1*-specific forward primer and Vec2261.

In both cases, the resulting PCR product (i.e. chimeric gene) was cloned in pCR4-TOPO (Invitrogen) and purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI), yielding p*TcαTub1–Tcv* and p*TcαTub1*–EGFP, respectively.

### 2.5. Reporter construct

A *piggyBac*-based reporter construct, pBac{3xP3-Tcv, *TcαTub1*–EGFP}, was made by inserting an *Asc*I fragment generated from pTcαTub1-EGFP (see above) into the *Asc*I site of the *Tcv*-marked *piggyBac* transformation vector pBac{3xP3-*Tcv*} (Lorenzen et al., in progress). The transformation marker (3xP3-*Tcv*) and reporter (*TcαTub1*– EGFP) are in the same orientation. DNA was prepared for microinjection using the EndoFree® Plasmid Maxi Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol.

### 2.6. Microinjection and rearing

Preblastoderm $v^w$ embryos were injected with p*TcαTub1–Tcv* plasmid (400 ng/μl) using the method of Lorenzen et al. (2002a). Injected embryos were reared at 32 °C in an oxygenated, humidified chamber. Both late-stage embryos and first-instar larvae were screened for transient rescue of the $v^w$ mutant phenotype (lack of eyespot pigmentation).

The *TcαTub1*– EGFP reporter line was established by co-injection of the *piggyBac*-based donor plasmid, pBac{3xP3-*Tcv*, *TcαTub1*–EGFP} (see above), and the *piggyBac* transposase source, phspBac (Handler and Harrell, 1999), into preblastoderm M59 embryos (Lorenzen et al., 2007) homozygous for $v^w$. Final concentrations of donor and helper were 500 and 400 ng/μl, respectively. Injected embryos were reared as described above, and hatchlings transferred to yeast-fortified wheat flour and incubated under standard conditions (Beeman et al., 1986). $G_0$ adults were individually outcrossed to $v^w$ mates and the progeny screened for eye-pigment rescue.

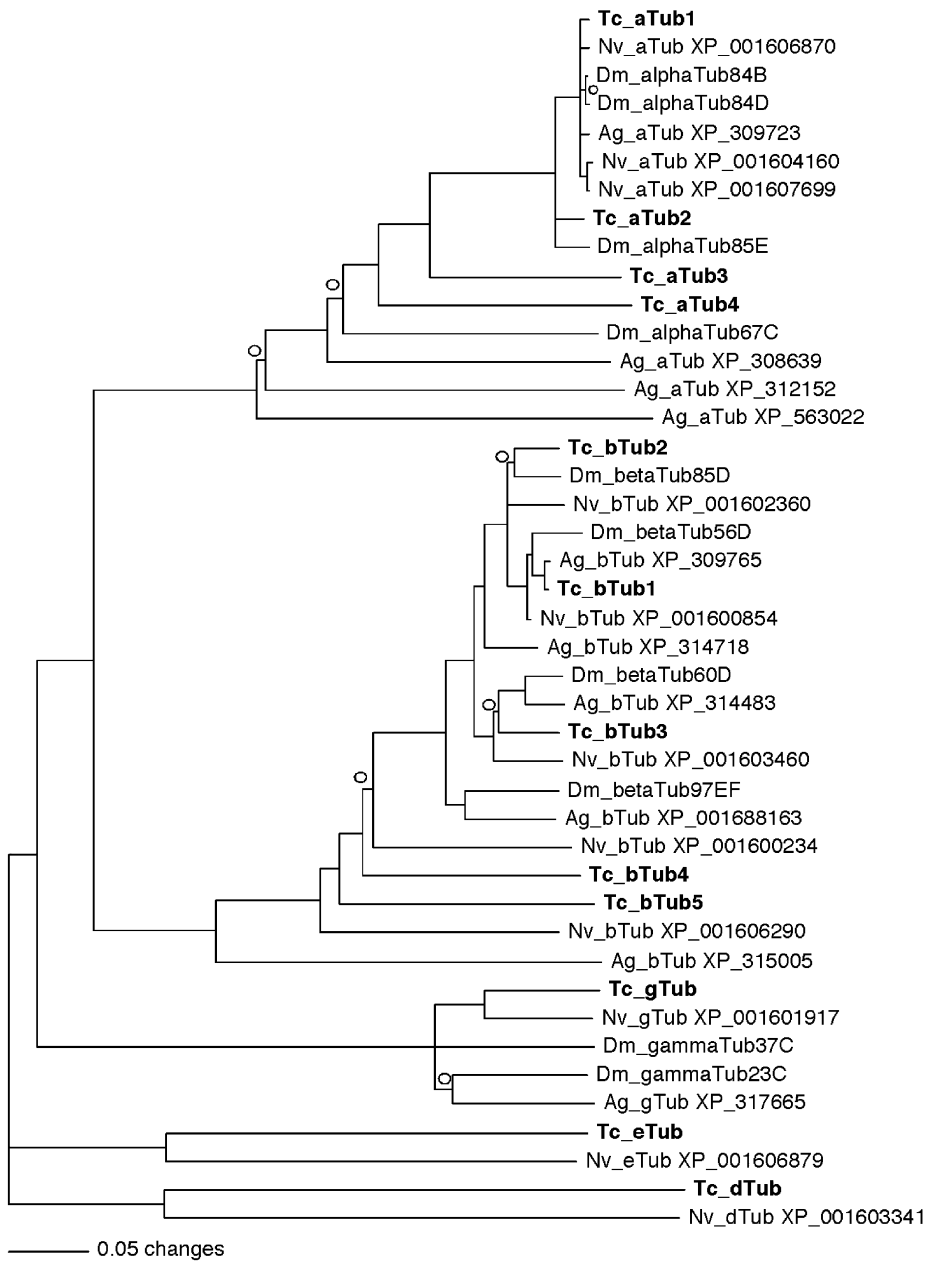


**Fig. 1.** Phylogenetic tree based on the deduced amino-acid sequences of *Tubulin* genes in *Tribolium castaneum* (*Tc*, bold font), *Drosophila melanogaster* (*Dm*), *Anopheles gambiae* (*Ag*), and *Nasonia vitripennis* (*Nv*). Each clade in the distance tree is supported by higher than 75% bootstrapping values in 1000, with the exception of the clades with small, open circles. See Materials and methods and Table 1 for GenBank accession numbers and cross-referencing of gene names.

**Table 1**
*Tubulin* family members in *Tribolium castaneum*

| Gene name | GenBank accession no.[a] | *Drosophila* cognate[b] | Identity (%)[c] | Map position[d] (cM) | ESTs |
|---|---|---|---|---|---|
| *Tc_αTub1* | XP_966492 | *αTub84B* (α1) | 99 | LG2; 1.8 | 155 |
| *Tc_αTub2* | XP_966407 | *αTub84B* (α1) | 97 | LG2; 1.8 | 10 |
| *Tc_αTub3* | XP_966584 | *αTub84D* (α3) | 77 | LG10; 47.1 | 48 |
| *Tc_αTub4* | XP_974091 | *αTub67C* (α4) | 60 | LG2; 47.9 | 1 |
| *Tc_bTub1* | XP_967267 | *βTub56D* (β1) | 96 | LG7; ~5.5 | 124 |
| *Tc_bTub2* | XP_969993 | *βTub85D* (β2) | 94 | LG7; 34.4 | 16 |
| *Tc_bTub3* | XP_967348 | *βTub60D* (β3) | 91 | LG7; ~5.5 | 8 |
| *Tc_bTub4* | AAJJ01000888 | *βTub97EF* (β4) | 72 | LG9; ~44.7 | 1 |
| *Tc_bTub5* | XP_968314 | *βTub97EF* (β4) | 71 | LG1; 41.0 | 25 |
| *Tc_gTub* | AAJJ01002303, AAJJ01003022 | *γTub23C* (γ1) | 81 | LG6; ~47 | 6 |
| *Tc_dTub* | AAJJ01000170 | *γTub23C* (γ1) | 25 | LG3; ~45 | 0 |
| *Tc_eTub* | XP_968261 | *βTub97EF* (β4) | 29 | LG2; 5.9 | 4 |

[a] Accession numbers for NCBI *Tribolium* RefSeq proteins, or genomic contigs if no model was available (see Materials and methods for contig coordinates).
[b] *Drosophila* ortholog or closest homolog (see Materials and methods for accession numbers).
[c] Percent identity is based on alignment of the deduced amino-acid sequence of the *Tribolium Tubulin* gene and the corresponding *Drosophila* cognate.
[d] Determined by BlastN of *Tubulin* genes against genome assembly, and association of sequence scaffolds with markers on the molecular recombination map (Lorenzen et al., 2005).

### 2.7. Microscopy

EGFP expression was observed using a Leica MZ FLIII fluorescence stereomicroscope (Leica Microsystems Inc., Wetzlar, Germany) equipped with a GFP Plus filter set (excitation filter: 480/40 nm, barrier filter: 510 nm). Photography was performed with a DXM1200F digital camera (Nikon Inc., Melville, NY).

## 3. Results and discussion

### 3.1. Identification of the T. castaneum Tubulin family genes

We used a bioinformatic approach to detect members of the *Tubulin* superfamily in the *T. castaneum* genome sequence. This approach identified 12 putative members which cluster into five groups; *alpha-*, *beta-*, *gamma-*, *delta-* and *epsilon-Tubulins* (Fig. 1 and Table 1).

Analysis of the deduced amino-acid sequences revealed a *Tribolium alpha-Tubulin*, *αTub1*, possessing a high degree of sequence conservation with the query, *αTub*84B. In fact, a high degree of conservation was observed throughout the *alpha-Tubulin* group, particularly for *αTub1* and *2*. However, clear one-to-one orthologies could not be established. Moreover, since *αTub1* shares a similar degree of conservation with both *αTub84B* and another *Drosophila Tubulin*, *αTub84D*, we cannot conclude orthology based on sequence comparison.

Orthology between members of the *beta-Tubulin* subfamily are somewhat clearer (Fig. 1). Nonetheless some *Tribolium beta-Tubulin* genes (e.g. *βTub4* and *βTub5*) encode proteins that are more similar to each other, than to cognates in flies, mosquitoes or wasps, suggesting recent duplication. A similar situation is observed in the *gamma-Tubulin* subfamily in *Drosophila*. While *gamma-Tubulin* is a single-copy gene in *Tribolium*, *Nasonia* and *Anopheles*, there are two copies in the *Drosophila* genome, suggesting a recent duplication in flies. Alternatively, a common ancestor could have possessed two copies, with one copy being lost independently in several lineages.

Interestingly, two single-copy *Tribolium* genes, *delta-* and *epsilon-Tubulin*, each have a one-to-one ortholog in the *Nasonia vitripennis* genome, but *delta-* and *epsilon-Tubulins* are both absent from the genome sequences of *D. melanogaster* and *Anopheles gambiae*. Each *Tribolium Tubulin* gene is represented in the EST database, with the exception of *delta-Tubulin*. However, since two of these (*αTub1* and *βTub1*) are far more highly represented in the EST libraries than the others, they probably have higher levels of expression (Table 1).

We were interested in the *Tribolium* ortholog of *Drosophila αTub84B* since the *αTub84B* promoter has been characterized and used to drive transposase in helper constructs in *Drosophila* (Li et al., 2001). However, both *αTub1* and *2* share a high degree of sequence identity with *αTub84B*, (99% and 97%, respectively). Since *αTub1* has the highest identity with *αTub84B*, strong support for orthology via phylogenetic analysis (Fig. 1) and appears to have a high level of expression (Table 1), the promoter region of *αTub1* was chosen for further study.

### 3.2. Tubulin gene structure

Gene structure was determined by comparison of cDNA and genomic sequences (Fig. 2). Although clear one-to-one orthologies could not be established between the *Tribolium* and *Drosophila α-Tubulins*, members of this subfamily do have common structural features. In particular, the first intron in three of the four *α-Tubulins* in both *Tribolium* and *Drosophila* is positioned immediately downstream of the start codon. This degree of conservation suggests the possibility of functional constraints. O'Donnell et al. (1994) demonstrated that specific sequences within the first intron of *αTub84B* are required for optimal gene expression, as well as protection against position effects. It is possible that the observed conservation of gene structure is a result of analogous regulatory regions in each of these genes.

A high degree of structural conservation is also seen among the *β-Tubulins*, specifically the three for which one-to-one orthologies were established between *Tribolium* and *Drosophila*. Identical intron/exon structure (i.e. splice-site conservation) is observed between *TcβTub1* and *βTub56D*, while *TcβTub2* and *βTub85D* have the same number of exons but lack splice-site conservation. *TcβTub3* and *βTub60D* share three conserved splice sites, but the *Drosophila* gene has an additional exon. Another commonality appears to be the presence of alternative first exons in the *β-Tubulin1* genes. *Drosophila βTub85D* and other testis-specific *beta-Tubulins* are known to have alternative first exons (Dobner et al., 1987), and sequence analysis of *TcβTub1* revealed the presence of putative alternative exons which would generate in-frame transcripts. However, no such transcripts were detected in our EST database.

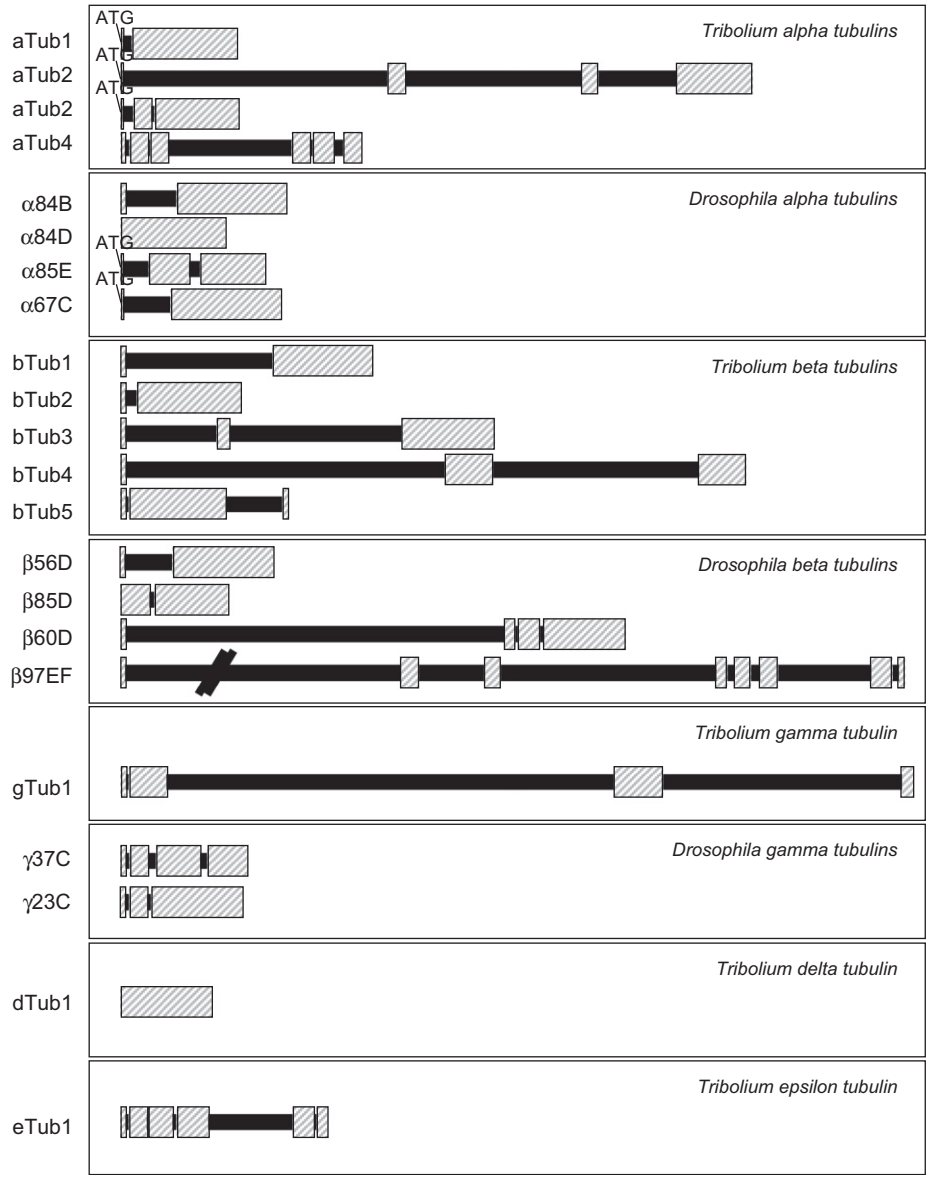


**Fig. 2.** Schematic representation of the genomic structures of the *T. castaneum* and *D. melanogaster Tubulin* superfamily members. All genes are drawn to scale with coding exons depicted as hatched boxes and introns as black lines. Genes with an intron immediately following the start codon are indicated by "ATG". 5′- and 3′- untranslated regions are not represented.

### 3.3. Identification of a constitutively active Tubulin promoter

To identify a constitutively active *Tubulin*, an EST database containing all available EST data (Park et al., 2008) was queried (BLASTN). This analysis revealed the presence of two highly abundant *Tubulins*, *α-Tubulin1* (*TcαTub1*) and *β-Tubulin1* (*TcβTub1*) (Table 1). *TcαTub1*, encoded by the most highly represented *Tubulin* in the EST libraries, shows a high degree of sequence identity with *Drosophila* αTub84B. The latter is known to be expressed from a constitutively active promoter (Theurkauf et al., 1986; O'Donnell et al., 1994). Therefore *TcαTub1* was selected for further analysis.

Three putative *TcαTub1* promoters (Fig. 3) were identified within a 600-bp region (upstream of the longest EST) using the Neural Network Promoter Prediction Program (Reese, 2001). The first is located from −323 to −274 (score = 0.85), the second from −235 to −186 (score = 0.80) and the third from −51 to −2 (score = 0.92). Of these, two have consensus TATA boxes (−313 to −308 and −43 to −38); while only one has potential CAAT boxes (−134 to −131 and −91 to −88). Both 5′ RACE and bulk EST data indicate the third promoter generally drives *TcαTub1* expression. In 92 of the 106 ESTs possessing 5′UTR sequence, the 5′-most base is located near +1 (+1 = 5′-most base of RACE product).

### 3.4. TcαTub1–Tcv rescues the $v^w$ mutant phenotype

Previously, we used a transient expression assay (Lorenzen et al., 2002a) to pre-test the *Tribolium Polyubiquitin* promoter. This system assesses the ability of a putative promoter region to drive expression of an eye-pigment gene, *T. castaneum vermilion* (*Tcv*), manifested by rescue of the mutant white-eyed phenotype seen in *Tcv*-deficient ($v^w$) recipient embryos (Lorenzen et al., 2002a, b).

To determine whether the *TcαTub1* promoter could drive *Tcv* expression, a chimeric, transient-rescue construct (p*TcαTub1–Tcv*) was injected into preblastoderm $v^w$ embryos. This construct included 447 bases of *TcαTub1* upstream sequence, as well as 96 bp of *TcαTub1* 5′UTR (−487 to +56). Nine of 42 newly hatched

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-360                        -340          -320             -300
CACTGCAGTGAACGGTTATGATGGTGCAACGACCGGGACCTCAGCTGTATAAAAAATCTGAAAATAATTG

           -280                    -260        -240
ATAATGGTCGAAGACTAAGGAAAAATAATTCGAAAATTAAAGGCAAAGTATGAAAATGCTGGAAATAACA

-220                        -200          -180              -160
ATCGGGAAACACCGAATTTTTGGTCACAGGAACACGAATTTTTTTTTGAATATTGATTTGAAAAGAGACA

           -140                          -120      -100
TCGAGAACAAGGATAACAATCTTCCGTTAGAGACCGTTGGTACCGACATGGCGGGGAGCCAATCAGAAGT

-80                         -60             -40
                                                          -20
CTGGAAATCGGGCGTTGGTTGCTGACGTCAACAAGCTTATAAATAGCAACGACAGTTGAAAATCGAATCA

          +1                 +20                  +40
AAGTCGTTTGGAAAAAGCCAGAGCTTGTATTTCCGAAGCGTACTCCCGTTTTTCTGCTCTTTTGTGGTGT

+60                +80
AATTTGTAAAACTCAACTACCAAAATGgtaagttatcccatttctaatgctagtgtacgttttgaatgca

taatttcgccaaattgttcgatttttgccggtaaaacgtgacgctgttggccgccattttggttggacgc

cggttctggaataatttcctgattttccgttttccttccagCGTGAATGTATCTCAGTTCATGTCGGCCA
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**Fig. 3.** Nucleotide sequence of the predicted *T. castaneum* α-*Tubulin1* promoter. The three potential promoters predicted by the Neural Network Promoter Prediction Program are underlined and in boldface type. +1 indicates the start of the longest α*Tub1* transcript detected (ES552063). The start codon is boxed and the nucleotide sequence of the first intron is shown in lowercase italics. (AAJJ01000552; nts 33200–32571).

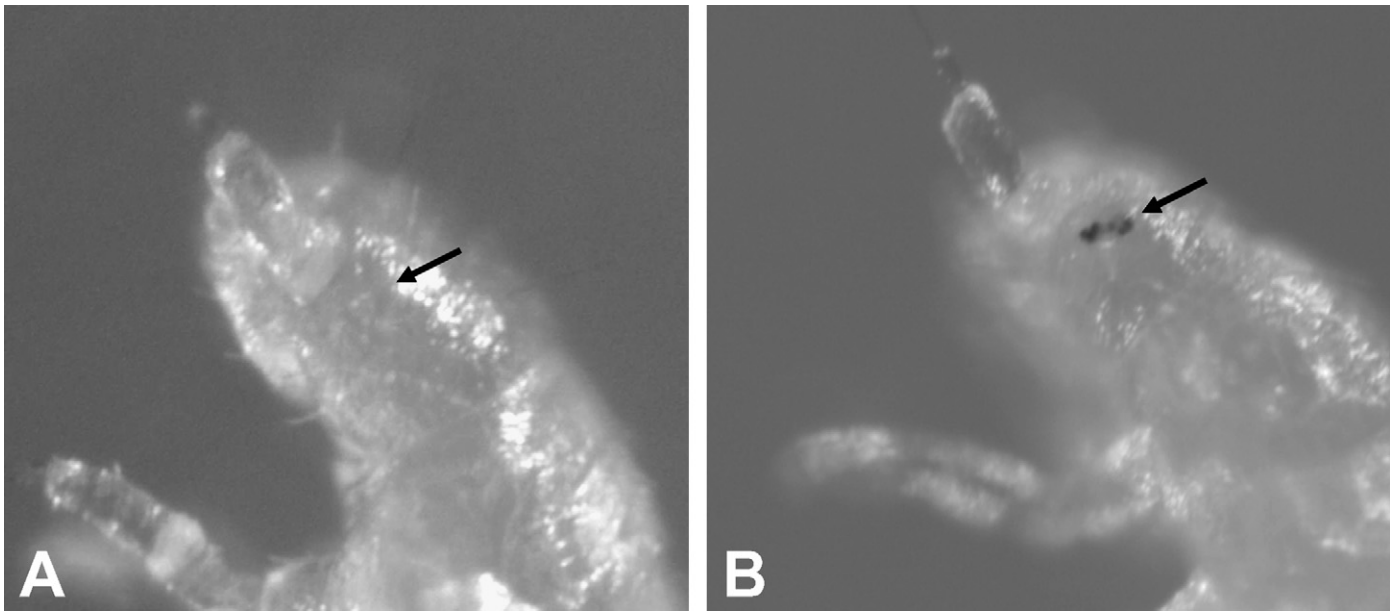


**Fig. 4.** Transient expression of *Tcv* in first-instar $v^w$ larvae. Lateral views of first-instar $v^w$ larvae illustrate the effect of *Tcv* expression from the p*Tc*α*Tub1*–*Tcv* construct. (A) First-instar $v^w$ larvae (uninjected). (B) First-instar $v^w$ larvae previously injected with the p*Tc*α*Tub1*–*Tcv* construct. Arrow indicates location of larval eye. Note rescue of mutant phenotype in (B).

larvae showed rescue of eye-spot pigmentation (Fig. 4). Rescue was never seen in control larvae, either mock-injected or injected with buffer alone. These results demonstrate that this 543-bp region is sufficient to drive *Tcv* expression.

### 3.5. *Tc*α*Tub1–EGFP is ubiquitously expressed in germline transformants*

We then examined the tissue-specificity of the *Tc*α*Tub1* promoter by placing it upstream of the EGFP gene. In this case, rather than assaying for transient expression from a plasmid, we used a *piggyBac*-based construct to generate germline transformants. This construct also carried an eye-specific marker, 3xP3-*Tcv*, to aid in selection of transformed progeny. A transformed line (αT-EGFP) was established, and the spatial and temporal patterns of EGFP expression were examined in embryos, larvae and adults. Ubiquitous expression of EGFP was seen in all stages based on gross inspection of whole insects (Fig. 5). Although we were able to examine only a single αT-EGFP insertion line, the ubiquitous expression pattern observed is consistent with expectation, based on the known properties of other α*Tub* promoters. In addition, we have examined thousands of insertion lines for enhancer effects using an EGFP reporter not carrying the α*Tub1* promoter (manuscript in preparation), and have never detected the global pattern of reporter expression seen in the αT-EGFP line.

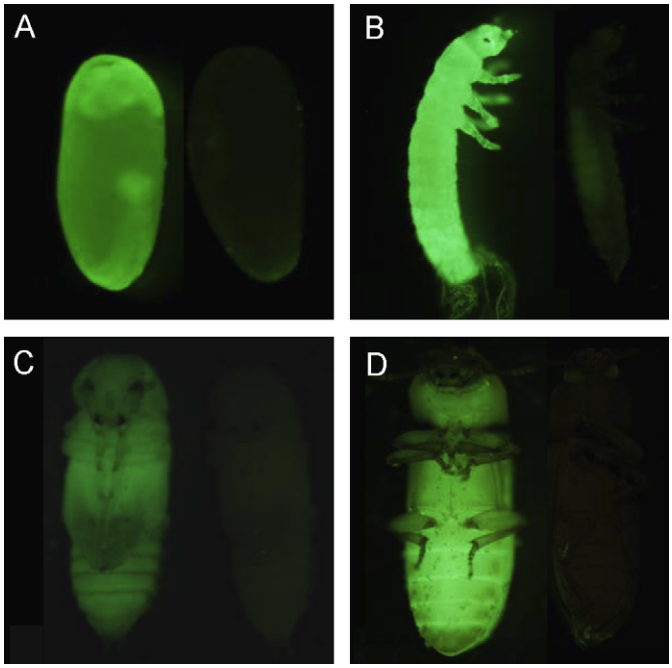


**Fig. 5.** Expression pattern of the *Tcαtub1*–EGFP reporter in the αT-EGFP transgenic strain. Embryos (A), larvae (B), pupae (C) and adults (D) of transformed and non-transformed strains. Note that transformants possess eye pigment due to the presence of the *Tcv* marker gene, while non-transformed (*pearl*) individuals lack eye pigmentation.

Finally, we have cloned the insertion junction and found it to be located in an intron of an *adipokinetic hormone receptor* gene (unpublished observations). EGFP expression in the αT-EGFP line is ubiquitous, but in adults is particularly strong in the midgut epithelium, especially in the structures known as "crypts" or "papillae" that appear as eversions into the hemolymph. There is also strong expression in the hindgut epithelium, testes and testis accessory glands. None of the aforementioned tissues are known to show significant expression of the *adipokinetic hormone receptor* gene, and in particular, we have shown that the *Tribolium adipokinetic hormone receptor* gene is not expressed in gut (unpublished observations).

These observations make it highly unlikely that significant position effects are affecting the observed expression pattern in the αT-EGFP line, or that nonspecific enhancer sequences are mimicking the pattern expected for *alpha-Tubulin*. Further studies will be required to determine tissue- and cell-specific EGFP expression patterns at higher resolution.

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