*A WD-repeat gene from peach (*Prunus persica *L.) is a functional ortholog of* Arabidopsis thaliana TRANSPARENT TESTA GLABRA1

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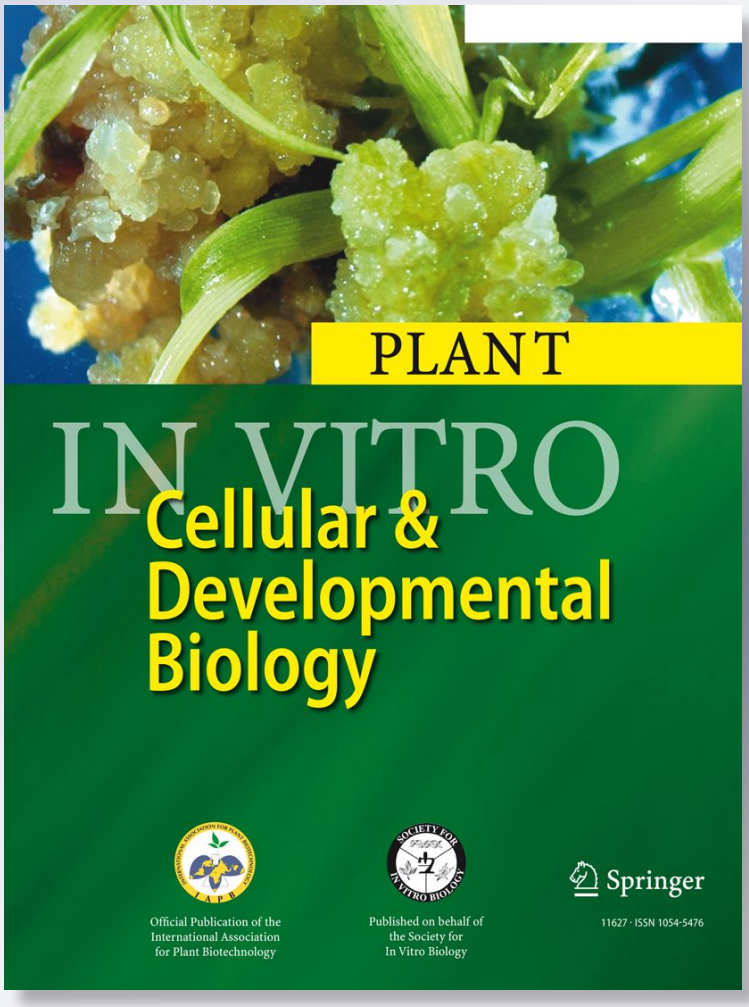

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DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

A WD-repeat gene from peach (*Prunus persica* L.) is a functional ortholog of *Arabidopsis thaliana TRANSPARENT TESTA GLABRA1*

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Abstract We have cloned a WD-repeat gene from peach. The cloned gene is more than 3 kb and contains signature domains characteristic of WD-repeat genes. Because of its high homology with AtTTG1, we hypothesized that this gene could be a TTG1 ortholog in peach. Functional studies were carried out by complementing the trichome minus *Arabidopsis ttg1-1* mutant with the putative peach TTG1 homolog. Successful restoration of normal trichomes was achieved in the resulting transgenics. We further tested the possibility that this gene was the candidate gene differentiating peach and nectarine. Sequence analysis indicated no difference in the full-length TTG1 and 1,600 bp of its promoter between peach and nectarine.

Keywords *Arabidopsis* · Fruit fuzz · Mutant complementation · Peach · Trichome · TTG1

Introduction

Most plants exhibit some form of hairy growth, which is associated with the presence of trichomes. In some species, such as peach and kiwi fruits, trichomes also cover the fruit and are often referred to as fuzz. These fruit fuzz are similar to trichomes in other plant species, including *Arabidopsis,* in structure and perhaps function. The underlying genetic mechanism that controls the fruit fuzz development is largely unknown. However, molecular studies leading to trichome development in *Arabidopsis* have identified a number of genes, such as *TTG1* (TRANSPARENT TESTA GLABRA 1), *GL1* (GLABRA 1), and *GL3* (GLABRA3), which are involved in this pathway (Walker *et al*. 1999; Zhang *et al*. 2003; Schellmann and Hulskamp 2005; Schellmann *et al*. 2007; Zhao *et al*. 2008). In addition, fiber development in cotton seems to follow the same pathway as trichomes in *Arabidopsis* and some of the trichome ortholog genes have been studied in cotton (Humphries *et al*. 2005; Guan *et al*. 2008). Recently, a WD-repeat gene in apple was also shown to be a functional homolog of AtTTG1 (Brueggemann *et al*. 2010).

In the proposed model for trichome development in *Arabidopsis*, TTG1, GL3, and GL1 form a complex protein that acts as a transcription factor and activates downstream genes including *TTG2* and *GL2*. TTG2 encodes a WRKY transcription factor, and TTG2 controls the expression of *GL2* (Ishida *et al*. 2007). GL2 is a member of the class IV homeodomain-leucine zipper transcription factors that induces the expression of further downstream genes necessary for trichome development. Fuzz development in peach may be controlled by a pathway similar to trichome development, and classical genetic studies have lent support to this view (Wen *et al*. 1995). In this study, we cloned the *TTG1* homolog from peach, a gene that controls the trichome development in *Arabidopsis* and other plant species. We then compared full-length *PpTTG1* and its promoter sequences among different cultivars of peach and nectarine to determine if differences existed between sequences in peach and nectarine.

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Materials and Methods

Plant materials and genomic DNA isolation. Three cultivars of peaches ("Redhaven," "Harrow Beauty," and "Bounty") and nectarines ("Fantasia," "Ruby Gold," and "Early Sungrand") from the University of Guelph, Vineland Research Station, Ontario, Canada, were selected for the current study. Genomic DNA was isolated from 1 g of frozen leaves using the CTAB extraction method (Bielenberg *et al*. 2004).

RNA extraction, cDNA synthesis, and cloning full-length peach TTG1 ortholog by RACE and genome walking. Fruit skin was peeled from peach at the early stages of growth (2 cm size, about 45 d after full bloom) and immediately frozen in liquid nitrogen. Total RNA was extracted using the CTAB method (Gasic *et al*. 2004). cDNA was synthesized from total RNA using StrataScript™ Reverse Transcriptase (Stratagene, La Jolla, CA), as per the manufacturer's instructions. PCR primers were designed based on conserved regions in *TTG1* orthologs in *Arabidopsis*, cotton, and apple. Amplified fragments from cDNA samples were excised from the agarose gel and purified with QIAquick gel extraction kit (Qiagen, Mississauga, ON, Canada). The eluted PCR product was cloned using the TOPO-TA cloning kit (Invitrogen, Burlington, ON, Canada).

The TOPO-TA clones containing the PCR products were transformed into TOP10 competent cells (Invitrogen, Burlington, ON, Canada) using heat-shock method. Plasmid DNA was extracted from three positive colonies for each PCR product using the QIAprep mini kit protocol (Qiagen, Mississauga, ON, Canada) as outlined by the manufacturer. Sequencing was performed on a Beckman CEQ2000XL Sequencer (Beckman, Mississauga, ON, Canada) or an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). TOPO 3′ and 5′ primers were used for sequence initiation. The full-length putative *TTG1* ortholog and 1,600 bp of its promoter were cloned from peach and nectarine using a Genome Walker™ Universal kit (Clontech, Mountain View, CA) and 3′ RACE (Ambion, Applied Biosystems, Streetsville, ON, Canada).

Peach TTG1-pBI121 vector construction. A full-length coding sequence of peach *TTG1* was amplified using primers that had restriction sites designed into their 5′ ends. An *Xba*I restriction site was introduced to the 5′ end of the *TTG1* forward primer (5′-AAGAAACAAGAAAAG GAAATGGAGAAC-3′), and for *TTG1* reverse primer (5′-GCTGACTCGCCAAGTGATCT-3′), a *Sac*I restriction site was added to the 5′ end. The cDNA fragment amplified with these primers was cloned in TOPO-TA vector, and its integrity was confirmed by sequencing. The TOPO-*TTG1* vector was digested with *Xba*I and *Sac*I, run on a 1% (*w*/*v*) agarose gel and the fragment corresponding to *TTG1* was purified using QIAquick gel extraction kit (Qiagen). For cloning of the peach *TTG1* ortholog, a pBI121 vector plasmid was digested with *Xba*I and *Sac*I and ligated with the similarly digested *TTG1* fragment. The resultant *TTG1*-pBI121 vector was then used to transform *Agrobacterium tumefaciens* EHA105 strain (Pérez-Clemente *et al*. 2005) using the freeze–thaw method (Weigel and Glazebrook 2006).

Complementation of Arabidopsis ttg1-1 mutant with peach TTG1. Arabidopsis ttg1-1 mutants were planted in 10-cm diameter pots at a density of 10–15 plants per pot and grown in short-d conditions (120 μmol m^{-2} s^{-1} light intensity, 9-h day length, and 23°C). The first inflorescence was removed immediately after emergence and plants were moved to long-d light conditions (120 μmol m^{-2} s^{-1} light intensity, 16-h day length, and 23°C) to induce more inflorescences. Transformation of *Arabidopsis* was performed by the floral dip method (Clough and Bent 1998). To increase transformation efficiency, floral dip was repeated after 1 wk. Seeds were harvested from mature siliques approximately 3 wk after transformation. The seeds were cleaned and screened *in vitro* on MS (Murashige and Skoog 1962) selection medium containing 50 μg ml^{-1} kanamycin.

Promoter analysis using PLANTCARE database. Genome walking was used to clone 1.6 kb of the *TTG1* promoter from peach and nectarine. ClustalW was used to make sequence alignments, and the PLANTCARE database was used to identify the possible motifs present in the promoter region of *TTG1* (Lescot *et al*. 2002).

Results

Structural analysis of TTG1. Sequencing of amplified products using primers designed from *TTG1* homologous genes in other species revealed a full-length *TTG1* from peach. The deduced amino acid sequence of full-length peach *TTG1*-like protein was compared with available *TTG1* orthologs in the NCBI GenBank (Fig. 1). The deduced protein was 95% identical to apple (AAF27919), 80% to cotton (AAM95645), and 80% to *Arabidopsis* TTG1 (NP_197840), respectively. This suggests that the cloned sequence is likely a peach TTG1 ortholog. The deduced amino acid sequence had 342 amino acids and was similar to the WD-repeat protein family as characterized by four WD repeats—the minimum number of repeats in these proteins (Smith 2008). Phylogenetic analysis of peach TTG1 also suggests that this gene is closely related to homologs in other dicot species (Fig. 2). This gene is less

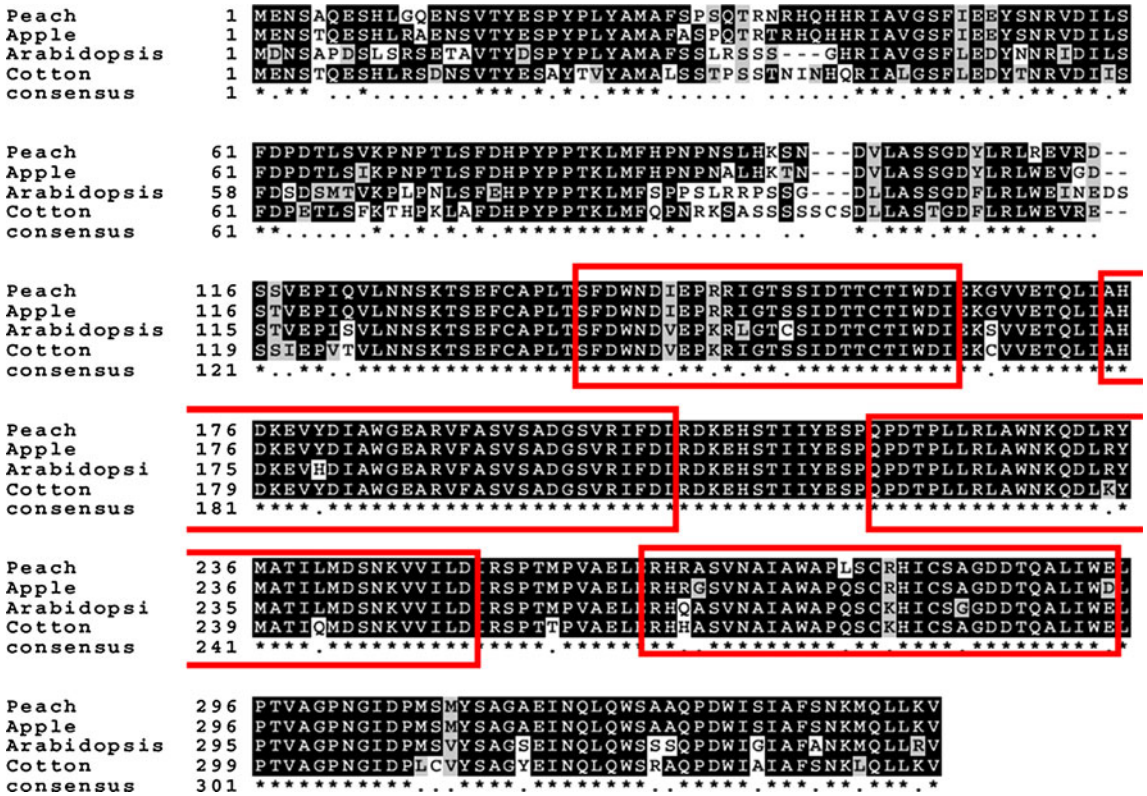

Figure 1. The deduced amino acid sequence comparison between peach PpTTG1 and its orthologs from *Malus x domestica*, *Arabidopsis*, and cotton (*G. hirsutum)*. *Dark shading* indicates positions where all four sequences were identical, and *lighter shading* represents positions where there is a lower conservation. The positions of four WD repeats were determined by BMERC Protein Structure Prediction Server (http://bmerc.bu.edu/projects/wdrepeat/) and were indicated by *open boxes*.

closely related to other WD-repeat genes from the monocot maize. The DNA sequence of peach *TTG1* ortholog isolated from "Redhaven" cultivar is deposited in the NCBI GenBank as FJ771043.

Complementation of Arabidopsis ttg1-1 mutant by the peach TTG1-ortholog. To test the functional similarity of the peach *TTG1* ortholog to *Arabidopsis TTG1*, the peach gene was transformed into the *Arabidopsis ttg1-1* mutant using *Agrobacterium*-mediated transformation. Transgenic *Arabidopsis* containing the peach *TTG1* ortholog (Fig. 3*a*) produced trichomes similar to wild-type *Arabidopsis* plants (Fig. 3*c*, *d* and *e*). The incorporation of peach *TTG1* into *Arabidopsis ttg1-1* was confirmed by PCR on genomic DNA extracted from the transgenic plants (Fig. 3*f*). Thus, putative peach *TTG1* was able to complement *Arabidopsis ttg1-1* mutants and in the first generation after transformation, *Arabidopsis* produced trichomes (Fig. 3*d*) similar to the wild type (Fig. 3*e*). From this point forward, we will refer to peach *TTG1* as *PpTTG1*.

Cloning the full-length PpTTG1 from both genomic and cDNA in peach. The peach *PpTTG1* open reading frame contained 1,026 nucleotides and was not interrupted by

Figure 2. Phylogenetic relationship of PpTTG1 with its orthologs in other species including petunia, *arabidopsis*, apple, cotton, *Perilla fruitescence*, *Brassica rapa*, maize, and grape, with GenBank accession numbers: PhAN11 (AAC18914), AtTTG1 (CAB45372), MdTTG1 (ADI58760.1), GhTTG1(AAM95641), PfWD (BAB58883), BrTTG1(ABQ10570), ZmPAC1 (AAM76742), and VvWD (XP_002270777), respectively. The present tree was designed using neighbor-joining analysis and 100 bootstrap replicates.

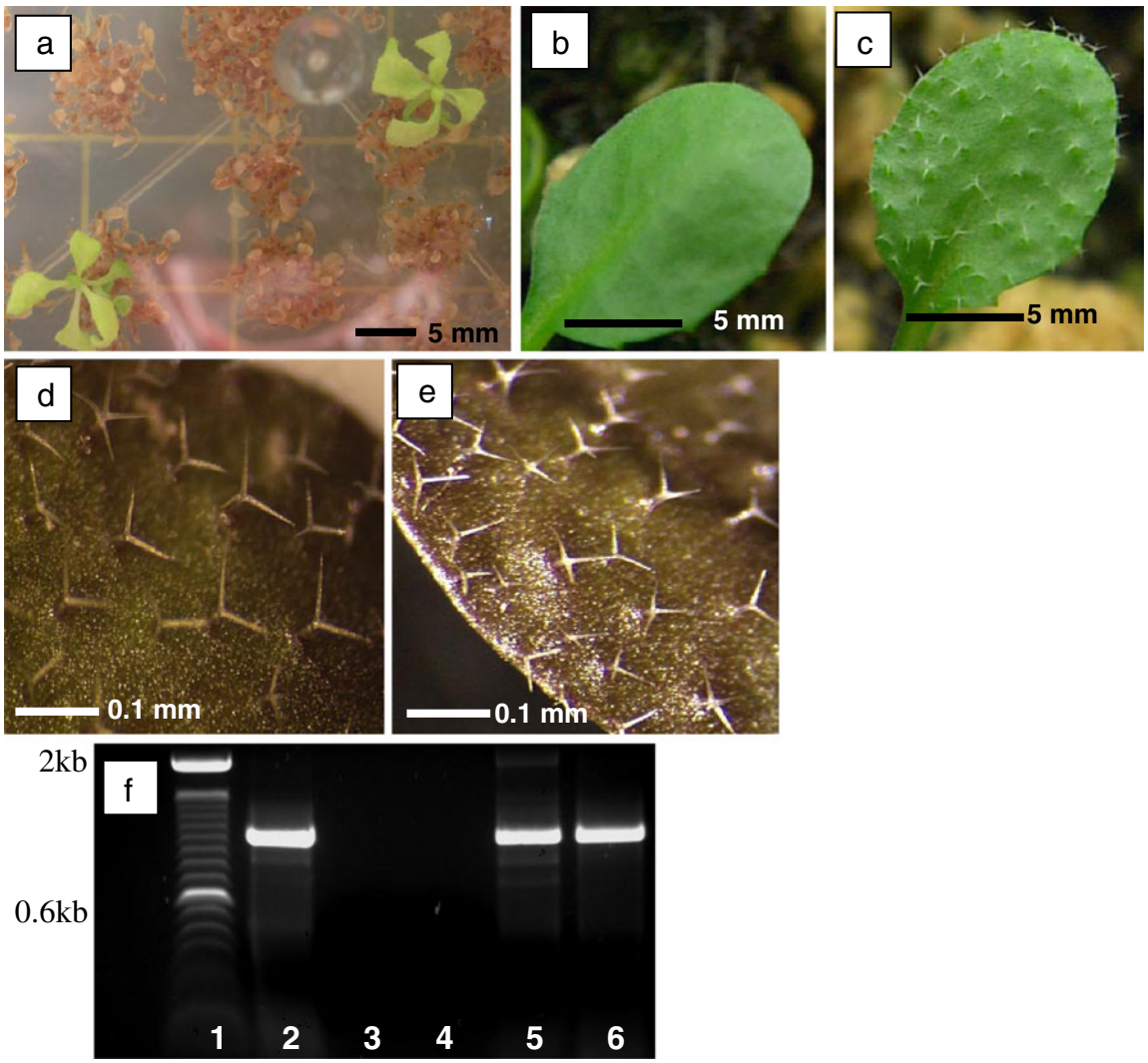

Figure 3. Complementation of *Arabidopsis ttg1-1* mutant with peach *TTG1*. *a*, Transformed *Arabidopsis* plants. *b*, The *ttg1-1* mutant without trichomes. *c*, The mutant with the inserted peach *TTG1*. Trichomes in transgenic plants (*d*) were similar to the wild-type plants (*e*). *f*, Gel electrophoresis of PCR products from two transgenic *Arabidopsis ttg1-1* mutants harboring the *PpTTG1*, using peach TTG1 primers (*lane 1*, 100 bp ladder; *lane 2*, peach cDNA; *lane 3*, wild-type *Arabidopsis*; *lane 4*, *Arabidopsis ttg1-1* mutant; *lanes 5 and 6*, transgenic *Arabidopsis ttg1-1* mutants, harboring the *PpTTG1*).

an intron. However, a 1,000 bp intron was present at the 3′ end, right after the stop codon for this gene. The sequence was confirmed in three peach cultivars, using both cDNA and genomic sequences. Comparison of these sequences revealed that no significant differences either at the coding, intron or promoter regions between peach cultivars. Sequences were identical, except that seven nucleotides (–TTTGAGG–) were missing from the 3′ end of the coding sequence in three clones. These seven nucleotides were located from 1,038 to 1,045 bp in the *PpTTG1* gene. Other polymorphisms were not detected in the coding sequence of *PpTTG1*. The promoter region of *TTG1* was sequenced up to 1,600 bp upstream of the start codon using "Redhaven" peach genomic DNA.

Motifs at the promoter sequence of PpTTG1. Analysis of the 1,600 bp promoter sequence using the PLANTCARE database (Lescot *et al.* 2002) revealed potential elements in the upstream region of peach *TTG1* including 14 light-responsive elements (Table 1). Other elements within this region were responsive to fungal elicitors and MeJA and a 5′UTR pyrimidine-rich sequences that were mainly CT repeats responsible for controlling the expression of the downstream genes (Daraselia *et al.* 1996).

TTG1 comparison in peach and nectarine cultivars. TTG1 sequence comparison was also revealed that there is no differences between peach and nectarine cultivars (data not shown).

Discussion

In this study, a WD-repeat gene homolog to TTG1 in *Arabidopsis* was cloned from peach that was able to complement *ttg1-1* mutant *Arabidopsis,* confirming it as a functional ortolog of this gene in peach. We hypothesized that peach fuzz-a type of trichome specifically expressed on peach fruits was similar to the trichome of *Arabidopsis* and hence investigated this gene was involved in fuzz development.

The *PpTTG1* had the highest homology to apple *TTG1,* which was not surprising as they both belong to the *Rosaceae* family. The 342 amino acid protein was also highly homologous to the TTG1 protein of cotton and *Arabidopsis* (more than 80%). The differences in the sequences between *Pp*TTG1 and *Arabidopsis* TTG1 were in the N terminus of these genes, which did not include the WD repeats. It appeared that the location of *TTG1* intron is conserved between peach and *Arabidopsis*, as in both species, it is located at the 3′ end of this gene. Therefore, similar to other WD-repeat genes (including *Arabidopsis TTG1*), peach *TTG1* may also be involved in a number of regulatory roles. The peach TTG1-like protein had four WD repeats that are required to form a functional β-propeller

Table 1. *cis*-Acting elements detected by the PLANTCARE promoter database (Lescot et al. 2002) in the 1,600 bp peach *TTG1* promoter

Site name	Organism	Sequence	Function
5UTR Py-rich stretch	*Lycopersicon esculentum*	TTTCTCTCTCTCTC	High transcription levels
ABRE	*Arabidopsis thaliana*	CACGTG	Abscisic acid responsiveness
ACE	*Petroselinum crispum*	GACACGTATG	Light responsiveness
AE-box	*A. thaliana*	AGAAACAT	Light responsiveness
ARE	*Zea mays*	TGGTTT	Essential for the anaerobic induction
as-2-box	*Nicotiana tabacum*	GATAatGATG	Shoot-specific expression and light responsiveness
Box II	*Petroselinum hortense*	TCCACGTGGC	Light responsive element
BOX4	*P. crispum*	ATTAAT	Light responsiveness
BOX-W1	*P. crispum*	TTGACC	Fungal elicitor
CAAT-box	Various species	CAAAT	Common *cis*-acting element in promoter and enhancer regions
CAT-box	*A. thaliana*	GCCACT	Meristem expression
CATT-motif	*Z. mays*	GCATTC	Light responsive element
CGTCA-motif	*Hordeum vulgare*	CGTCA	MeJA-responsiveness
Circadian	*L. esculentum*	CAANNNNATC	Circadian control
GA motif	*Helianthus annuus*	AAAGATGA	Light responsiveness
GAG-motif	*Spinacia oleracea*	AGAGATG	Light responsiveness
GATA-motif	*A. thaliana*	AAGATAAGATT	Light responsiveness
G-BOX	*Pisum sativum*	CACGTG	Light responsiveness
GCN4_motif	*Oryza sativa*	TGAGTCA	Endosperm expression
GT1-motif	*A. thaliana*	GGTTAA	Light responsive element
I-box	*Flaveria trinervia*	GATATGG	Light responsive element
MNF1	*Z. mays*	GTGCCC	Light responsive element
O2-site	*Z. mays*	GATGACATGA	Zein metabolism regulation
Skn-1_motif	*O. sativa*	GTCAT	Endosperm expression
Sp1	*Z. mays*	CC(G/A)CCC	Light responsive element
TATA-box	Various species	TATAAA	Core promoter element around −30 of transcription start
TATC-box	*O. sativa*	TATCCCA	Gibberellin responsiveness
TCA-element	*Brassica oleracea*	CAGAAAAGGA	Salicylic acid responsiveness
TC-rich repeats	*N. tabacum*	ATTCTCTAAC	Defense and stress responsiveness
TCT-motif	*A. thaliana*	TCTTAC	Light responsive element
TGACG-motif	*H. vulgare*	TGACG	MeJA-responsiveness
TGA-element	*B. oleracea*	AACGAC	Auxin-responsive element
W box	*A. thaliana*	TTGACC	

fold (Yu *et al*. 2000). These WD repeats co-ordinate the formation of a multi-protein complex with other proteins including bHLH transcription factors, which are involved in various plant developmental processes such as trichome development, anthocyanin biosynthesis and root hair differentiation (Walker *et al*. 1999; Zhang *et al*. 2003). A nuclear localization signal was not found, suggesting that transport to the nucleus probably occurs through the formation of complexes with other proteins (including GL3 and GL1 in the case of trichome development) (Cokol *et al. 2000).*

Complementation of the Arabidopsis ttg1-1 mutant with peach TTG1. Transformation of *Arabidopsis ttg1-1* mutants, which have glabrous vegetative organs due to lack of trichome, with the *PpTTG1* resulted in the restoration of trichome development. This demonstrates that even though the two genes were not exactly the same, they were able to function similarly, suggesting that the essential elements needed for trichome development are present in *PpTTG1*. Similar results were obtained when 2 *TTG1* homologues from cotton were introduced into the *Arabidopsis ttg1-1* mutant (Humphries *et al*. 2005). Genes in the WD repeat family from other species, including apple, petunia *AN11*, and maize *PAC1*, are also able to complement the *Arabidopsis ttg1-1* mutant (Payne *et al*. 2000; Carey *et al*. 2004; Brueggemann *et al*. 2010).

Sequence comparison of TTG1 in peach and nectarine. To further confirm the peach *TTG1* sequence, we amplified both the coding sequence and promoter region from three different peach cultivars. Sequence analysis of *TTG1* in all three cultivars revealed that there were no significant differences either in the coding region or in the promoter region, 1,600 bp 5′ to the start site. Within the *TTG1* coding region, two isoforms of *TTG1* mRNAs were detected between the cultivars. The first isoform had a predicted protein product of 342; the other had 343 amino acids. The difference occurred at the 3′ end of the coding sequence with seven nucleotides (–TTTGAGG–) missing in the shorter isoform. These missing stretches of nucleotides code for a valine and a stop codon in the longer isoform of *TTG1*. There was another stop codon in the second isoform that completed this isoform with the addition of two different amino acids, including alanine and serine instead of valine. These two isoforms appear to be due to the availability of two intron/exon junctions close to each other within the 3′ end of the *TTG1* coding sequence. In fact, the occurrence of such short-distance tandem splicing sites is common in all organisms (Hiller and Platzer 2008). Multiple copies of this gene could be located as tandem repeats in this locus. Analysis of a first draft of peach genome revealed that *TTG1* was a single-copy gene without any tandem repeats. In the promoter region, a small difference in the number of CT repeats was observed 1,026 bp upstream of the start codon. The number of CT repeats was not consistent even in different clones from a single cultivar. This suggests that the differences may be due to sequencing errors in the repeated regions (Kieleczawa 2006). Since the *TTG1* 5′ end was amplified from the genomic DNA using genome-walking method, there is no information about the transcription initiation site in *TTG1* to determine the number of nucleotides that belong to *TTG1* 5′UTR or its promoter. Although there was no difference in the 1,600 bp of *TTG1* promoter that was sequenced in our study, more distal regions of this gene may contain regulatory elements or possible differences, which account for variations in gene expression in specific cell types or certain times (Lodish *et al*. 2004).

In conclusion, we have cloned a functional ortholog of *Arabidopsis TTG1*. Since there was no difference between peach and nectarine *TTG1* at the coding and promoter region, it is unlikely that *TTG1* is the candidate gene in peach and nectarine differentiation. Perhaps analysis of other genes that may have a role in trichome development, such as, *GL3*, *GL1*, *GL2*, and *TTG2*, as other candidates for the G locus, might reveal additional information. Trichome spacing in *Arabidopsis* is under the control of *CPC* and *TRY* that are expressed in trichome cells and transported to adjacent epidermal cells. Identification and analysis of their homologous genes in peach could be an attractive possibility to unravel the complete genetic mechanisms underlying the development of peach fuzz.

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