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IDENTIFICATION, ISOLATION AND *in silico* CHARACTERIZATION OF *Fragaria vesca* HOMOLOGUE OF *TEMPRANILLO* GENE

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ABSTRACT

The woodland strawberry, *Fragaria vesca* belongs to the Rosaceae family, one of the most economically important family of fruit tree crops, normally with long juvenility period. Therefore, knowledge about the molecular basis of flowering time regulation and photoperiodic responses in these species is essential for breeding goals. Shortening the juvenility period has been described as an interesting breeding goal for most of the fruit trees, which could be achieved through genetic engineering. *TEMPRANILLO* (*TEM*) belongs to the *RAV1* (Related to ABI3/VP1) transcription factors family, which is known as a flowering repressor, containing AP2 and a B3 DNA-binding domains. In this study, we identify and isolate *F. vesca* homologue of *Arabidopsis TEM (AtTEM)*. The full-length cDNA of *FvTEM* consisting of a coding sequence of 1152 bp, predicted to encode 383 amino acids was obtained, cloned and further analyzed *in-silico*. Successful cloning was confirmed using colony PCR and sequencing. Bioinformatic analysis showed that the promoter region of *FvFT1* contains CAACA and CACCTG motifs which could be recognised as binding sites for *FvTEM* and directly represses *FvFT1* expression. *In-silico* expression analysis in *F. vesca* EST database found 21 ESTs with identities more than 50%. Phylogenetic analysis using neighbor joining method revealed that *FvTEM* is homologous with *AtTEM.*

Keywords: *TEMPRANILLO;* flowering regulation; *in-silico* analysis; floral repressor; BLAST.

INTRODUCTION

In angiosperms, flowering time regulation is a crucial process for efficacious plant reproduction. The initiation of flowering is regulated by the combination of internal and external signals, including photoperiod, temperature, plant age, and gibberellic acid [1,2]. In the recent years, orthologous/ homologous sequences of key genes of *Arabidopsis thaliana* (L.), which are involved in flower induction and development, including *LEAFY (LFY), APETELA1 (AP1), AGAMOUS (AG), TERMINAL FLOWER (TFL1), BpMADS4,* and *SERRATED LEAVES* and *EARLY FLOWERING (SEF)*

have been identified and evaluated for their expression [3-8].

The Rosaceae family is one of the most important plant families which contain many economically valuable fruit crops including Apple, Peach, Pear, Plums, Strawberry, etc. The long juvenile period of fruit trees makes their breeding costly and time-consuming. The diploid strawberry, *Fragaria vesca* shares some features which make it a prosperous model plant for genomic and photoperiodic research in this family. These features including the presence of different accessions with different photoperiodic responses, the short seed to seed cycle, easy transformation and regeneration protocols and accessible whole genome sequencing draft [9-12]. In the last decade, studies have been done to discover the molecular basis of flowering time regulation in *Fragaria vesca*, which proposed some genes which involve in floral induction and repression including *FvFT1 (FLOWERING LOCUS T), FvSOC1 (SUPPRESSOR OF THE OVEREXPRESSION OF CONSTANS1), FvTFL1 (TERMINAL FLOWER1)* and *FvCO (CONSTANS)* [13-19]. While, there is no information about the presence and the role of TEM homologue in flowering process in strawberry, the role of TEM has been studied well in *Arabidopsis* [20-23].

RAV1 is a plant specific transcription factors family. Two DNA binding proteins, TEM1 and TEM2 from *A. thaliana*, which belong to this family, contain two individual amino acid sequence domains which are only found in higher plant species. The N-terminal regions of *RAV1* and *RAV2* are homologous to the AP2 domain existing in this transcription factors family, while the C-terminal region exhibits homology to the highly conserved C-terminal domain, designated B3, which belong to VP1/ABI3 transcription factors. The AP2 and B3-like domains bind independently to the CAACA and CACCTG motifs, respectively, and together organize a high binding specificity. It has been suggested that the AP2 and B3-like domains of *RAV1* are linked by a highly flexible structure enabling the two domains to bind to the CAACA and CACCTG motifs in various positions and directions [24,25]. *TEM* gene plays an important role in various processes in plants, including floral induction and flowering time regulation, trichome initiation and formation, GA biosynthesis and distribution and many biological process during plant development [21,25]. In *Arabidopsis*, *TEM1* and *TEM2* negatively control flower induction by controlling and integrating both the photoperiod and GA signaling pathways [20,21].

Although various techniques have been considered to accelerate flowering and fruiting of seedlings in the juvenile phase but none of these is fully satisfying yet. Utilization of genetic engineering for shortening juvenile period can be carried out through two different approaches *i.e.,* overexpression of flower promoting transcription factors and silencing of flower inhibitors. Among these, *TEMPRANILLO,* as a flowering repressor transcription factor, can be a beneficial tool for this purpose. The aim of this study was to identify, isolate and *in-silico* characterize the wild strawberry homologue of *AtTEM* gene.

MATERIALS AND METHODS

Gene Identification

To aid the design of primers the protein sequences of *Arabidopsis TEM1* (AT1G01030) was obtained from the TAIR database (*http://www.arabidopsis.org/*) and used as a query in tBLASTn program from the National Center for Biotechnology

Information (NCBI) GenBank database to identify other sequences with high sequence homologies in *F. vesca* (taxid:57918) nucleotide collection (nr/nt) database. Predicted *FvTEM* sequence (XM_004297092.2) with highest identity score in the blast result was used to design the primer pairs. The forward and reverse primers were ATGGACATAACTAGCAGCACAACAG and AAAATGTTACAAAGCTCCAATTATCCTT, respectively.

Gene Isolation and Cloning of FvTEM

Total RNA was extracted from the leaves of the diploid strawberry, *F. vesca* (L.) Hawaii-4 using the pine tree method [26] and treated with RNase-Free DNase (Fermentas, Germany) according to the manufacturer's recommendations. The quantity of RNA was measured by nanodrop (NanoDrop Technologies, Wilmington, DE, USA), and the RNA was electrophoresed on a 1% agarose gel for quality confirmation. 1µg total RNA was used to synthetize the first strand cDNA using *MMLV* reverse transcriptase (Fermentas) and oligo dT. The *AtTEM* homolog of *F. vesca* was amplified using above mentioned primers with the following PCR program: an initial denaturation at 94 C for 5 min, denaturation at 94 C for 30s, annealing 58 C for 30s, and extension at 72 C for 30s for 35 cycles.

The PCR amplified fragment of strawberry homolog of *AtTEM*, was cloned into the pTG19-T cloning vector (Vivantis, Malaysia) by TA cloning method according to the manufacturer's recommendations, and transformed into DH5alpha *E. coli* electrocompetent cell using electroporation (BioRad Gene Pulser). To confirm that the recombinant DNA contains the right fragment, colony-PCR was done using the same primer pair and PCR conditions used for gene isolation. Double-stranded plasmid DNA isolated by the miniprep alkaline lysis method [27]. The DNA sequence of positive clones was verified by sequencing the PCR amplified fragment. Sequencing results was analyzed using the software Chromas version 2.6.5. Results of DNA sequencing then blasted using BLASTn program in NCBI and confirmed that the isolated fragment was *FvTEM*.

***In silico* Analysis**

To do *in-silico* expression analysis, the deduced protein sequence was used as a query to be blasted using tBLASTn tool of NCBI website in EST database while *F. vesca* (taxid:57918) used as the favored organism. EST's with identity score more than 50% percent was selected.

Amino Acid Sequence Comparisons and Phylogenetic Analysis

The deduced amino acid sequences of *FvTEM* was aligned with other RAV sub-family class I members and identity scores were calculated using *CLUSTALW* alignment tool of *MEGA7* software. Evolutionary relationships of RAV sub-family members were inferred using the neighbor joining method and visualized using *MEGA7*. Bootstrap values were derived from 500 replicate runs. The numbers at each node represent the bootstrap support (percentage).

RESULTS AND DISCUSSION

Gene Identification

To find the homologous gene of *Arabidopsis TEM* in *F. ragaria vesca*, the protein sequence of *AtTEM1* (NP_173927.1) blasted using tBLASTn against *F. ragaria vesca* (taxid:57918) as the favored organism. The results of tBLASTn showed that predicted AP2/ERF and B3 domain-

containing transcription repressor TEM1-like (XM-004297092.2) had higher sequence homology with overall sequence identity and E-value of 65% and $1e^{-143}$, respectively. So we considered it as *FvTEM* and used for designing the primer.

Gene Isolation and Cloning of *FvTEM*

The primers designed to amplify the complete CDS of the predicted *FvTEM* gene. The result of RNA extraction on the agarose gel is shown in (Fig. 1a) and as expected, RT-PCR using designed primers resulted in a 1158 bp fragment (Fig. 1b). The amplified fragment was successfully cloned into pTG19-T cloning vector and transformed to *DH5alpha E. coli*. Transformation was confirmed using colony PCR (Fig. 1c) and DNA sequencing. Results of DNA sequencing then blasted using BLASTn program in NCBI, results showed that the isolated fragment had 0, 99% and 99%, E-value, Identity and Query cover with *FvTEM*, respectively. Therefore, we concluded that the amplified fragment was *FvTEM*.

***In silico* Analysis**

In-silico expression analysis showed that *FvTEM* expressed in diploid strawberry. Blast *FvTEM* against *F. vesca* (taxid:57918) using TBLASTN 2.8.0 in EST database found 21 ESTs with identity more than 50% (Table 1).

It has been found that both AP2 and B3 domains of *TEM* are needed for its inhibition of FT expression in *Arabidopsis*. The CAACA and CACCTG sequences previously have been recognized as the DNA recognition sites for *RAV1* family. The AP2 and B3 domains of *RAV1* genes recognize the CAACA and CACCTG motifs, respectively [28]. Analysis of the *FvFT1* promoter sequence revealed that it contains CAACA and CACCTG motifs that could be targeted for binding by *FvTEM*. Additionally, a putative *CONSTANS* binding site and a CCAAT box are also present in the promoter region of *FvFT1* (Fig. 2). CCAAT box is a common cis-acting element in promoter and enhancer regions; has been proposed as a putative binding site for the complex formed by *CO* and the CCAAT box binding proteins involved in *FT* activation [29]. The presence of these motifs in the promoter region of *FvFT1* gene suggested that *TEM* can directly repress *FvFT1*. Therefore, it is supposed that a competing mechanism might exist for regulation of *FvFT1* by *FvCO* and *FvTEM*. Previous studies have found complementary changes in TEM and FT during early development in *Arabidopsis* and *Antirrhinum* [20,21,22]. Based on the above information, it is suggested that the photoperiod pathway is active during juvenile phase and the *FT* activation by *CO* during juvenility is inhibited by the repression of *FT* by *TEM*.

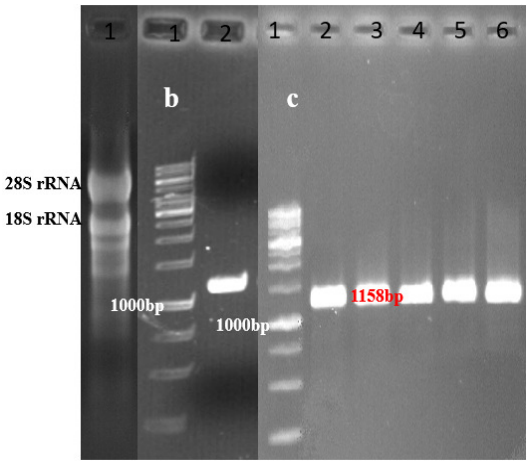

Fig. 1. Electrophoresis on 1% agarose gel: (a) RNA extraction using Pine Tree Method, (b) RT-PCR of *FvTEM*, (c) Colony PCR with *FvTEM* primers

Fig. 2. *FvFT1* nucleotide sequence and *FvTEM* putative binding sites. The start codon shown by red font. Putative binding sites for AP2 and B3 domains of *FvTEM* in promoter region of *FvFT1* are highlighted in yellow and green respectively. The putative binding site of *CO* and the CCAAT-box binding protein are highlighted in pink and grey respectively

Amino Acid Sequence Comparisons and Phylogenetic Analysis

It has been shown that *TEM* plays a role as a regulator of juvenility in *Arabidopsis*, to determine whether *TEM* might has this role beyond *Arabidopsis*, we investigated its role in diploid strawberry *in-silico*. The complete RNA coding sequence of *FvTEM* cDNA was obtained, consisting of a coding sequence of 1152 bp. *FvTEM* have no intron region and predicted to encode 383 amino acids. This protein contained the AP2 and B3 domains which shows that it's a member of the RAV class I protein family (Fig. 3a).

In order to identify the evolutionary relationships among the RAV1 family members, phylogenetic tree was constructed using the deduced amino acid sequence by *Neighbor–Joining* (N–J) method. Phylogenetic analysis revealed that *FvTEM* is homologous to RAV-like sub-family class I DNA binding proteins from other organisms. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Fig. 3b). Multiple sequence Alignment of FvTEM protein with the *Arabidopsis TEM* proteins discovered sequence homology covering the length of the coding sequence with overall sequence identities of 62% and 61% with *AtTEM1and AtTEM2*, respectively (Fig. 4).

To obtain information about the widespread of *TEM* in the plant kingdom, we isolated the full-length cDNA indicating strawberry TEM orthologue (*FvTEM*) which contained AP2 and B3 domains and at the amino acid level shared 62% and 61% identity to *Arabidopsis TEM1* and *TEM2* proteins, respectively. The protein sequence alignment using the deduced amino acid sequences of *FvTEM* and some RAV1 proteins from other plant species showed that *FvTEM* contains two highly conserved domains, the AP2 domain, and the B3 domain which are the characteristic features for these proteins (Fig. 5).

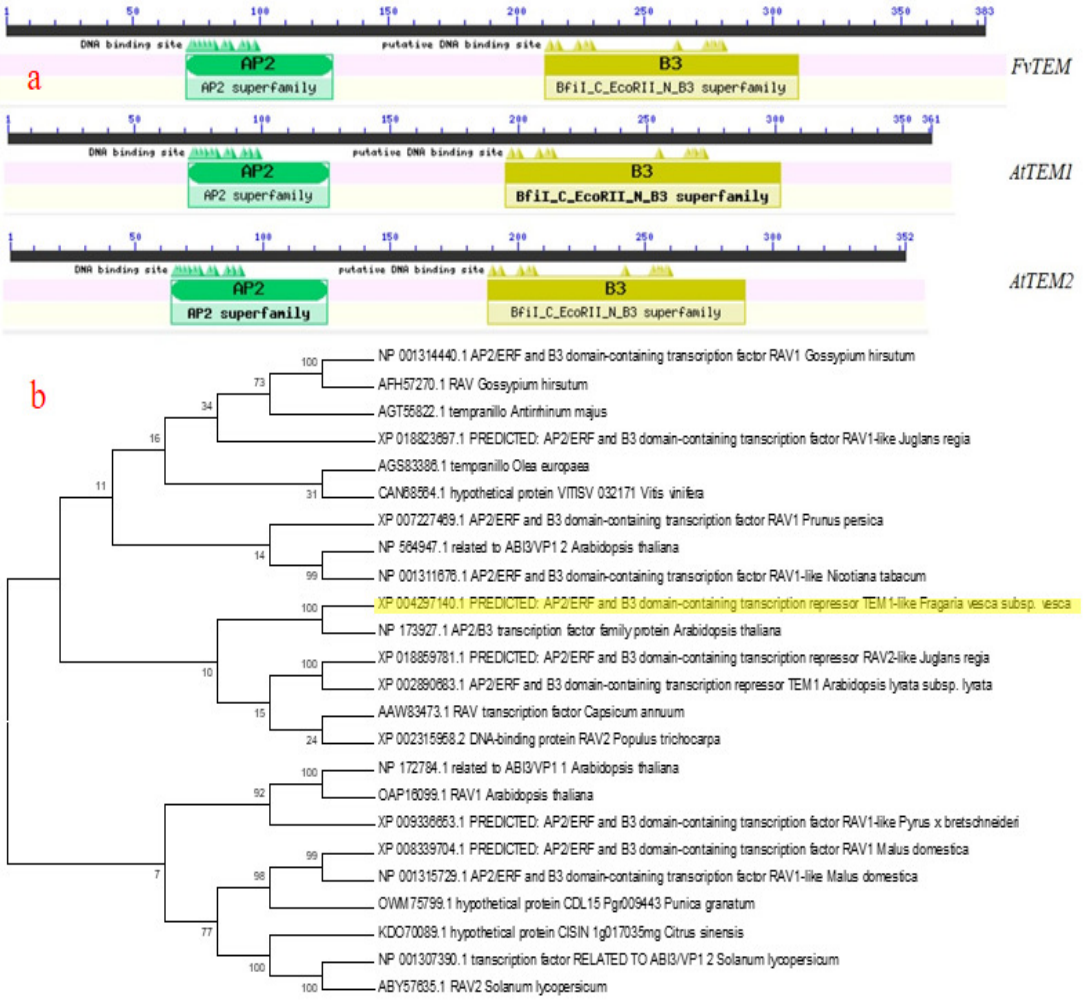

Fig. 3. Phylogenetic Relationship between *FvTEM* and RAV sub-family class I members. a) Comparison of protein domain structure in *FvTEM*, *AtTEM1* and *AtTEM2*. b) Phylogenetic analysis of *FvTEM* deduced amino acid sequences and other RAV sub-family class I members homologs. The evolutionary history was inferred using the *Neighbor-Joining* method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analyses were conducted in *MEGA7*. Accession numbers are given next to the species name

Table 1. *In silico* analysis revealed expression of FvTEM gene in *Fragaria vesca* EST database

EST GenBank no.	Identity	E value	Score	EST GenBank no.	Identity	E value	Score
EX672271.1	100	5.19E-149	420	EX675708.1	55.357	7.27E-04	40
EX660801.1	100	1.31E-111	323	EX662534.1	55.172	1.72E-12	66.2
EX668865.1	100	4.73E-88	265	EX679787.1	54.386	5.19E-11	61.2
EX659247.1	100	4.18E-68	211	DY671470.1	53.571	5.86E-11	62
EX666818.1	100	1.33E-45	154	EX681054.1	53.571	6.77E-11	62.4
DV440647.1	86.111	7.26E-29	112	EX659681.1	53.571	9.22E-11	62
EX674390.1	64.865	1.55E-06	48.5	EX664389.1	53.571	1.13E-09	58.9
EX679522.1	63.889	3.46E-07	49.3	EX680923.1	53.571	1.15E-09	58.5
EX664923.1	59.524	4.46E-07	50.1	EX676181.1	53.571	1.24E-09	58.5
EX666174.1	55.556	2.57E-04	42.4	EX674305.1	51.786	4.94E-10	59.7
DY673274.1	55.357	6.17E-11	62				

Fig. 4. Amino acid sequence comparisons of *FvTEM* and related proteins in *Arabidopsis thaliana*. Alignment of deduced amino acid sequences of *FvTEM* with *AtTEM1* and *AtTEM2*

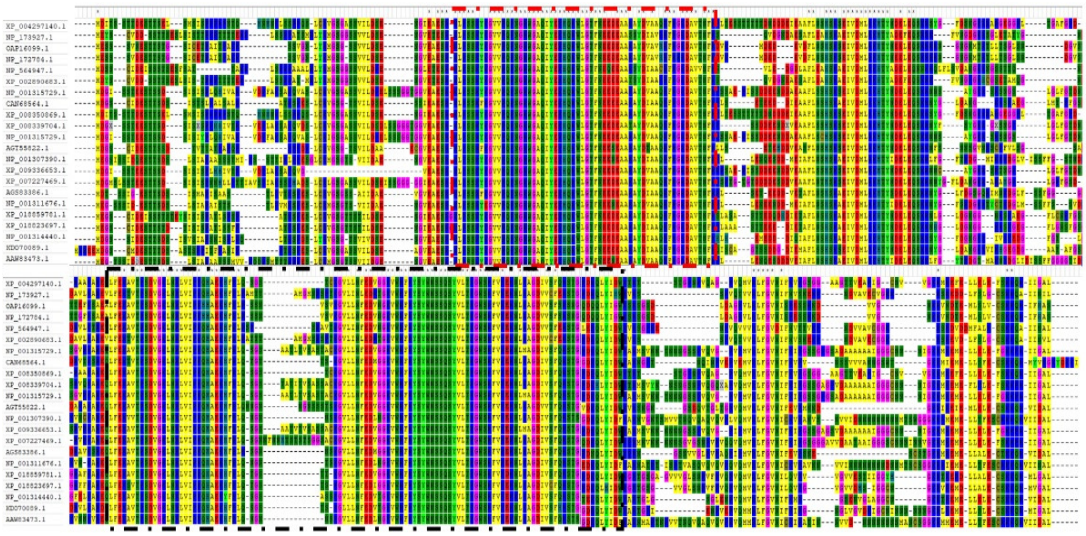

Fig. 5. Multiple Sequence Alignment of *FvTEM* deduced amino acid sequences and other RAV sub-family class I members homologs using *CLUSTALW* as visualized by MEGA7. *Fragaria vesca TEM1*-like (XP_004297140.1); *Arabidopsis thaliana TEM1* (NP_173927.1); *Arabidopsis thaliana RAV1-like* (OAP16099.1); *Arabidopsis_thaliana RAV1* (NP_172784.1); *Arabidopsis thaliana RAV2* (NP_564947.1); *Arabidopsis lyrata TEM1*(XP_002890683.1); *Malus domestica TEM1* (NP_001315729.1); *Vitis vinifera RAV1* (CAN68564.1); *Malus domestica TEM2* (XP_008350869.1); *Malus domestica RAV1-like* (XP_008339704.1); *Malus domestica RAV1* (NP_001315729.1); *Antirrhinum majus TEM* (AGT55822.1); *Solanum lycopersicum RAV2* (NP_001307390.1); *Pyrus x bretschneideri RAV1*-like (XP_009336653.1); *Prunus persica RAV1* (XP_007227469.1); *Olea europaea TEM* (AGS83386.1); *Nicotiana tabacum RAV1*-like (NP_001311676.1); *Juglans regia RAV2-like* (XP_018859781.1); *Juglans regia RAV1-like* (XP_018823697.1); *Gossypium hirsutum RAV1* (NP_001314440.1); *Citrus sinensis RAV1* (KDO70089.1); *Capsicum annuum RAV1* (AAW83473.1). The Red dotted box delimits the AP2 domain, the black dotted box delimits the B3 domain

CONCLUSION

In the present study new members of *RAV1* transcription factors family have been identified and isolated, which seem to be involved in flowering time regulation. It also performs the role in juvenility phase and transition to adult phase and also plays a role in some other developmental process like GA biosynthesis and distribution. *In-silico* analysis showed that *FvTEM* is homologous to *Arabidopsis TEM1* and could repress the *FT* expression and regulate the flowering time in competition with *CO*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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