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Cloning and expression profiling polycomb gene *VERNALIZATION INSENSITIVE 3* in tomato

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Abstract

VERNALIZATION INSENSITIVE 3 (VIN3) is a chromatin remodelling protein that is induced by low temperatures and is required for the vernalization response in *Arabidopsis thaliana.* VIN3 is one of the polycomb group (PcG) proteins, which mediates epigenetic repression of *FLOWERING LOCUS C* (*FLC*) in *A. thaliana*. Here, we present cloning, characterization, and expression of a putative *SlVIN3* gene in tomato (*Solanum lycopersicum* L.) by isolating cDNA clones corresponding to *SlVIN3* gene using primers designed based on conserved sequences between *PcG* genes in *A. thaliana* and tomato. The *SlVIN3* cDNAs were cloned into a pBS plasmid and sequenced. Both 5' and 3' RACE were generated and sequenced. The flcDNA of 2 823 bp length for the *SlVIN3* gene was composed of 5'UTR (336 bp), ORF (2 217 bp), and 3'UTR (270 bp). The translated ORF encoded a polypeptide of 739 amino acids. Alignment of deduced amino acids indicates that there are highly conserved regions between tomato SlVIN3 predicted protein and plant *VIN3* gene family members. Both unrooted phylogenetic trees constructed using the maximum parsimony and maximum likelihood methods indicate that there is a close relationship between SlVIN3 predicted protein and VIN3 protein of *Vitis vinifera.* The expression of *SlVIN3* gene remained high during floral organ differentiation and growth and decreased when the fruit started to develop.

Additional key words: *FLC*, flowering, phylogenetic tree, RACE, *Solanum lycopersicum*.

Introduction

Tomato (*Solanum lycopersicum* L.) belongs to the family of *Solanaceae* which represent a unique portion of the family tree of plants. The exploration of tomato sequences will enable the comparative analysis for the discovery of distinct and common aspects of plant evolution (Knapp 2002). The tomato genome, sequenced by the International Solanaceae Genome Initiative, comprises approximately 950 Mb of DNA, more than 75 % of which is heterochromatin and largely devoid of genes. Recently, the genome has been published on the *Solanaceae Genomics Network* database (*SGN*) (http://solgenomics.net/tomato/). Of an estimated 950 Mb genome size, 760 Mb are assembled in 91 scaffolds that align to the 12 tomato chromosomes.

Plants have evolved a range of strategies to ensure that flowering occurs at the optimum time of the year for reproduction. In some plants, a vernalization requirement is a key component of the reproductive strategy (Chouard 1960). In *A. thaliana,* vernalization is associated with the repression of the floral repressor, FLOWERING LOCUS C (FLC; Michaels and Amasino 1999, Sheldon *et al.* 1999). A number of genes that are required for

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Abbreviations: BAC - bacterial artificial chromosomes; EST - expressed sequence tag; FLC - flowering locus C; FlcDNA - full length cDNA; FN3 - fibronectin type 3 domain; ORF - open reading frame; PcG - polycomb group; PHD - plant homeodomain; RT-qPCR - real time quantitative PCR; SGN - Solanaceae genomics network database; *SlVIN3* - *Solanum lycopersicum* vernalization insensitive 3; UTR – untranslated region; VID - VIN3-interacting domain; VIL - vernalization insensitive like; VIN 3 - VERNALIZATION INSENSITIVE 3; VRN - vernalization.
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epigenetic silencing *FLC* have been identified. Of these, VERNALIZATION INSENSITIVE 3 (VIN3), a chromatin remodelling protein, binds to the chromatin of *FLC* locus (Sung and Amasino 2004) and interacts with members of the polycomb group (PcG) complex containing the VERNALIZATION (VRN) protein which controls flowering *A. thaliana* after vernalization (Hennig and Derkacheva 2009). The VRN complex is one of PcG repressive complex2-like complexes (PRC2) which trimethylates histone H3 lysine 27 (H3K27me3), a repressive chromatin mark that increases at the *FLC* locus as result of vernalization (Schubert *et al.* 2006, Sung *et al.* 2006, Finnegan and Dennis 2007, Greb *et al.* 2007). *VIN3* exists as small gene family known as *VIN3-like* (*VIL*) (Sung *et al.* 2006, Greb *et al.* 2007). The *A. thaliana* genome encodes four genes that are members of the *VIN3* family, *VIL1*, *VIL2*, *VIL3*, and *VIL4* (Sung *et al.* 2006, Greb *et al.* 2007).

Interestingly, the induction of VIN3 also involves changes in active histone marks at *VIN3* chromatin, including histone H3 acetylation, histone H4 acetylation, and histone H3 Lys 4 trimethylation (H3K4me3) (Finnegan *et al.* 2005, Bond *et al.* 2009a). Finnegan *et al.* (2011) found that the quantitative induction of VIN3 is regulated by the components of PRC2 which trimethylates histone H3 lysine 27 (H3K27me3) in *VIN3* chromatin. This observation defines a new role for PcG proteins in regulating the rate of gene induction. *FLC* gene family members are also regulated during vernalization to mediate a proper vernalization response. The FLC and VIN3 families cooperated during the course of evolution to ensure a proper vernalization response through epigenetic changes (Kim and Sung, 2013).

The VIN3 family member, VIL1, promotes flowering under short day *via* the repression of *FLOWERING LOCUS M* (*FLM*), a floral repressor gene, through chromatin modifications (Ratcliffe *et al.* 2003, Werner *et al.* 2005, Sung *et al.* 2006) and participates in both photoperiod and vernalization pathways in *A. thaliana* by regulating the expression of the floral repressors *FLC* and *FLM* (Sung *et al.* 2006). The other VIN3 family member, VIL2, binds preferentially to dimethylated histone H3 lysine 9 (H3K9me2) *in vitro* and is necessary for the establishment and/or maintenance of H3K9me2 at *MADS AFFECTING FLOWERING 5* (*MAF5*) chromatin under short day conditions. Furthermore, *VIL2* co-precipitates with a component of PRC2 and is required for the establishment and/or maintenance of trimethylated histone H3 lysine 27 (H3K27me3) at *MAF5* chromatin. Thus, the repression of *MAF5* by VIL2 provides a mechanism to promote flowering in non-inductive photoperiods which contributes to the facultative nature of the *A. thaliana* photoperiodic response (Kim and Sung 2010).

All VIN3 family members characterized so far monitor certain environmental factors, such as temperature and photoperiod, suggesting that the VIN3 family of proteins may have evolved to serve as gateway between environmental stimuli and developmental programs through chromatin-based mechanisms. VIN3 is also induced in response to a nicotinamide treatment (Bond *et al.* 2009a) and to hypoxic conditions. The mechanisms that regulate the induction of VIN3 by low oxygen and vernalization are different (Bond *et al.* 2009b).

Full length (fl) cDNA clones are fundamental resources for investigations of gene functions as well as for detection of intron-exon structures in genomes. The general aim of this work was to identify a putative PcG gene, *SlVIN3*, from tomato by cloning and sequencing its flcDNA of mRNA transcripts in tomato tissues. *SlVIN3* gene has an epigenetic role in flowering and floral organs development and as far as we know, this is the first gene of DNA polymerases to be sequenced and characterized from *Solanaceae*.

Materials and methods

Tomatoes (*Solanum lycopersicum* L.) cv. Castlerock (*Pacific Seed Company,* Walnut Creek, USA) were grown in a greenhouse under standard cultural practices. Various tissues were collected from plants at different stages, then quickly frozen in liquid nitrogen, and stored at -80 °C.

Arabidopsis thaliana L. VIN3 protein available in the *Arabidopsis information resource* database (http://www.arabidopsis.org/) under acc. No. AT5G57380.1 was compared to the tomato SGN database. A considerable similarity was found between *A. thaliana* VIN3 protein and two bacterial artificial chromosomes (BACs; C07HBa0325D07.2 and C07SLm0031B19.1) and two tomato expressed sequence tags (ESTs; SGN-U569309 and SGN-U569308). Predicted tomato *SlVIN3* gene was constructed based on the high similarity found between VIN3 protein in *A. thaliana* and the mentioned BACs and ESTs using the *VectorNTI v. 11* software (*Invitrogen*, Carlsbad, USA). Forward and reverse primers were designed for *SlVIN3* gene from predicted exon regions and used to amplify the cDNA clones (Table 1).

For reverse trascription (RT) PCR, cDNA was synthesized, using *SuperScript® III* reverse transcriptase (*Invitrogen*), from total RNA extracted from various tissues with the *TRI* reagent (*Sigma*, St. Louis, USA) and oligo(dT) primers by the Chomczynski and Mackey (1995) method. Synthesized cDNA was used as template for a PCR reaction to amplify tomato *SlVIN3* cDNAs with designed primers using a 2× PCR ready mix (*KAPA*$_2$, Wilmington, USA), 0.4 μmol of each primer, and 3 mm^3 of cDNA as template with the following program: an initial denaturation at 94 °C for 5 min, then 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and an extension at 72 °C for 2 min, and after that

a final extension at 72 °C for 7 min. The cDNA clone was reamplified using *Platinum*® *Pfx* DNA polymerase (*Invitrogen*) which produces PCR products with blunt ends for cloning. The obtained blunt ended fragments were purified by a gel extraction kit (*Omega Bio-tek*, Norcross, USA) according to the manufacturer's instruction.

The cloning vector *pBluescript II SK* (+) (pBS) from *Stratagene* (La Jolla, USA) was prepared from bacterial cells using a *QIAprep* spin miniprep kit (*Qiagen*, Valencia, USA) according to the manufacturer's protocol. The vector was digested using an *Eco*RV restriction enzyme (*Promega*, Madison, USA) to produce blunt ends and then it was collected from the gel. The purified cDNA clones were ligated into the linearized plasmids with T4 DNA ligase (*Promega*). The recombinant plasmids were transformed into *Escherichia coli* strain DH5α cells prepared to be competent by the calcium chloride method (Sambrook and Russel 2001). Transformation was performed by the heat shock protocol (Froger and Hall 2007). The cells were spread over Lurrie brot (LB) plates (*Display Systems Biotech*, Vista, USA) containing 100 μg cm^{-3} ampicillin, 3 mg cm^{-3} X-gal, and 2.4 mg cm^{-3} (IPTG), and incubated at 37 °C overnight. The recombinant plasmids were prepared from positive clones and used as templates in a sequencing reaction.

Rapid amplification of cDNA ends (RACE) was performed to determine the 5' and 3' noncoding regions of tomato *PcG* gene, *SlVIN3*, by using a *SMART RACE* cDNA amplification kit (*Clontech*, Mountain View, USA). Total RNA from tomato flower tissues was used as template to synthesize the first-strand cDNAs for 5' and 3' ends following the manufacturer's instruction. The *SlVIN3*-specific primers were designed based on the *SlVIN3* contig sequence which was constructed by overlapping sequenced ESTs (*SlVIN3* – a specific primer for 5' end amplification, 5'-CTCCTGACGGCTCCAAG ATTGCAGC-3', and *SlVIN3* – a specific primer for 3' end amplification, 5'-GCTGCACCACGGTCGCCTT TGCTAG-3').

The obtained ESTs and generated 5' and 3' RACE fragments were used as templates in a sequencing reaction by the dideoxynucleotide method (Sanger *et al.* 1977). Sequencing results were assembled using *Contig Express Vector NTI*. The open reading frame (ORF) prediction and translation were done using a *Vector NTI ORF Finder* tool. Deduced amino acids were subjected to the *BLASTp* program in the http://www.phytozome.net/ database to obtain homologous protein sequences within sequenced plant species. VIN3 protein sequences of plant species were aligned by *ClustalW 1.83* multiple sequence alignments (Thompson *et al.* 1997) using the *BioEdit* software. The phylogenetic analysis was done using two methods, maximum parsimony (Eck and Dayhoff 1966, Fitch 1971) and maximum likelihood (Felsenstein and Churchill 1996), with the help of the *Phylip-3.68* package. *Selaginella moellendorffii* was used as outgroup. Domains were predicted in the deduced SlVIN3 protein using the *SMART* database from the website http://smart.embl-heidelberg.de/ (Schultz *et al.* 1998, Letunic *et al.* 2012).

The expression of *SlVIN3* was detected by real time quantitative PCR (RT-qPCR). First-strand cDNA was synthesized from total RNA extracted from closed floral buds, flowers, fruits, and leaves and was assayed for the *SlVIN3* gene expression using gene specific primers (forward: 5'-ACGGTCACAGATGAGGGAAA-3' and reverse: 5'-CCTTTGACTGGTTTGCCTGT-3'). An *Actin 2/7* gene was used as constitutively expressed gene control (forward: 5′-GGACTCTGGTGATGGTGTTAG-3′ and reverse: 5′-CCGTTCAGCAGTAGTGGTG-3′). The reactions were carried out in triplicate. Each reaction mix consisted of a 2× *Hot Start SYBR* ready mix (*Qiagen*), forward and reverse primers (0.125 μM each), 3.5 mm^3 of first strand cDNA, and H_2O to 20 mm^3. QRT-PCR reaction conditions were as follows: 50 °C for 2 min, 95 ºC for 15 min, 40 cycles (95º C for 15 s, 60 ºC for 30 s). Cycles to threshold (Ct) were used to measure a gene expression (Livak and Schmittgen 2001). Normalization of Ct values (ΔCt) was obtained by subtraction of *actin 2/7* gene Ct values of each replicate. ΔCt values were calibrated using the *SlVIN3* gene expression in leaf tissues as calibrator to obtain ΔΔCt and then $2^{-\Delta\Delta Ct}$ was calculated.

Table 1. Five primer pairs used to amplify cDNA clones.

Primer name	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
SlVIN3 01	GAAATGCAGCCTTCATCAGAGAGTGGCC	CTTTGACAATGCTATCTTCC
SlVIN3 02	ACATGGTGCCCCTGAAATGC	TAATATGTCTACACGCCTGG
SlVIN3 03	GAGCCGTCAGGAGATTTTGC	CTTGTGGTCCATCCTCCTTCCCCG
SlVIN3 04	TTTTGGAACTGCATCCATGG	CAAAGTTGCTGAACAGCAGG
SlVIN3 05	CTCATTTGGCCCTGCTGTTCAGC	CTTGTGGTGTAGCTCTTAAGC

Results

The *SlVIN3* cDNA contig was obtained by overlapping five ESTs. The lengths of ESTs varied between 652 and 1 760 bp with an average length of 1 173 bp. The generated 5'RACE fragment was 749 bp in length and

that of 3'RACE was 683 bp. Both the 5' and 3'RACE fragments were sequenced and assembled with obtained ESTs to construct *SlVIN3* flcDNA contig. The computer analysis using the *BLASTn* algorithm confirmed that the obtained sequence corresponded to *SlVIN3* gene. The constructed *SlVIN3* flcDNA was located on tomato chromosome 7 in a genomic region about 11 523 bp. Four exons were determined using the flcDNA and *BLASTn* searches in the *SGN* database (Fig. 1). The constructed *SlVIN3* flcDNA comprised 2 823 bp, containing 336 bp in the 5' untranslated region (5'UTR), 2 217 bp in ORF, and 270 bp in 3'UTR without a poly (A) tail. The ORF encoded a polypeptide of 739 amino acids spanning from a nucleotide position (np) 337, where the first ATG codon was located, to np 2 554, adjacent to a termination codon (TAA). The flcDNA and deduced amino acid sequences have been submitted to the *NCBI* GenBank as acc. Nos. JQ669017 and AFD98847, respectively.

The 2 217 bp ORF from *SlVIN3* flcDNA encoded

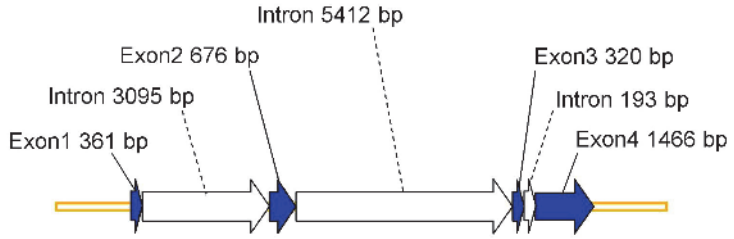

Fig 1. Predicted tomato *SlVIN3* gene construction based on high similarity found between obtained *SlVIN3* flcDNA and corresponding *SGN* BACs using the *VectorNTI* software. Four exons (the *blue regions*) were determined by their presence within flcDNA using the *BLASTn* search in the *SGN* database. Sizes of the exon/intron and UTRs are illustrated.

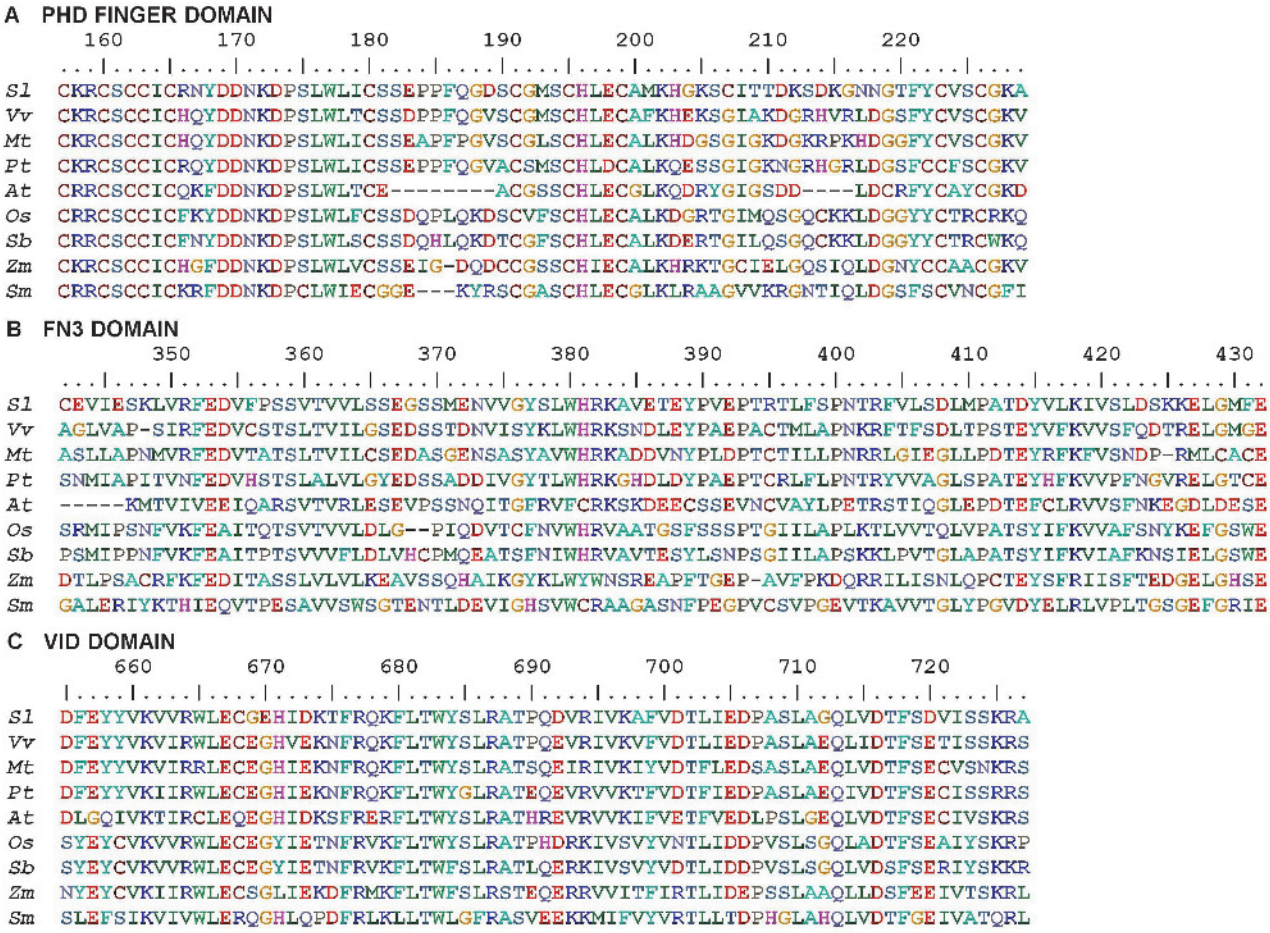

Fig 2. Multiple sequence alignments of conserved domain sequences for a SlVIN3 predicted protein and 12 protein sequences of VIN3 hypothetical gene family members. The alignment was done using the *ClustalW 1.83* by *BioEdit* software. Conserved domains identified in the sequence of the SlVIN3 protein using *SMART*. *A* - PHD finger domain from amino acid 161 to 227, *B* - FN3 domain from amino acid 343 to 428, *C* - VID domain from amino acid 653 to 726.

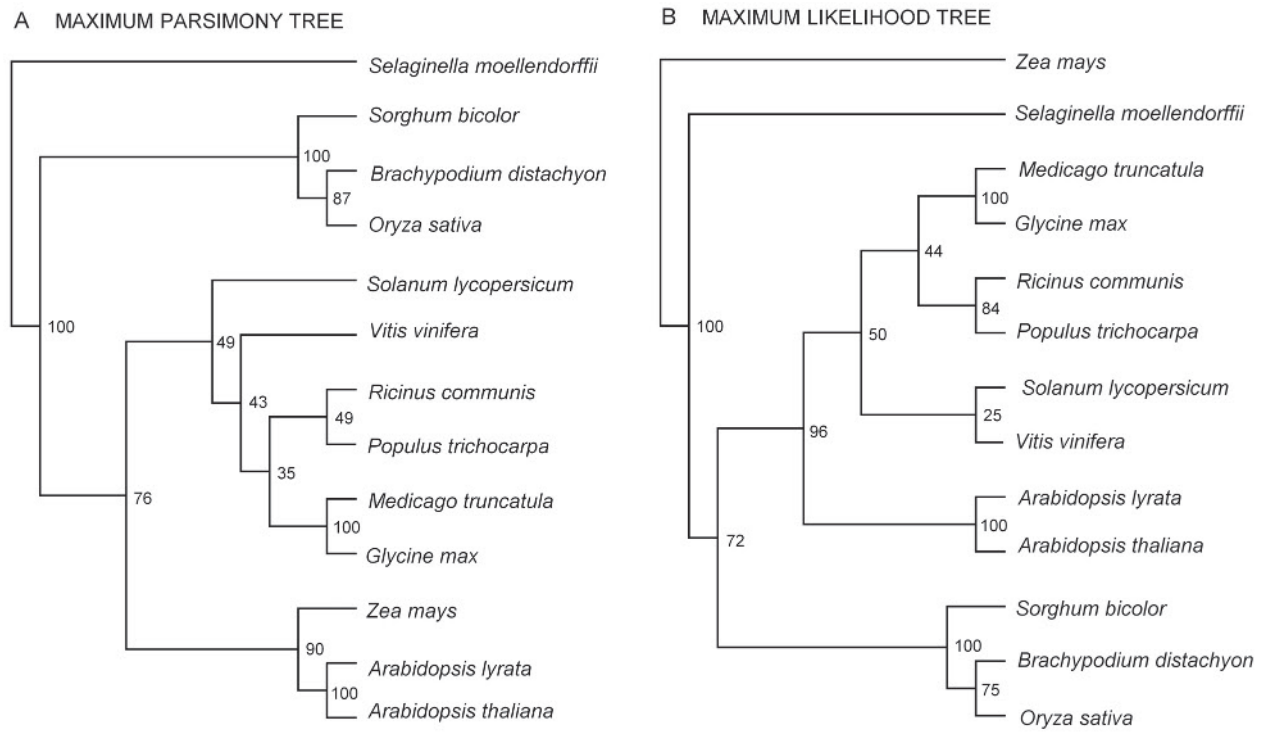

Fig 3. Unrooted phylogenetic trees of 12 hypothetical VIN3 protein sequences with the tomato SlVIN3 predicted protein sequence. *S. moellendorffii* was used as outgroup. Numbers on nodes are bootstrap percentages supporting given partitioning. *A* – A tree constructed using the maximum parsimony method, *B* – a tree constructed using the maximum likelihood method.

739 amino acids. The predicted SlVIN3 protein had a molecular mass of 81.54 kDa with an estimated isoelectric point of 5.44. Domain prediction using *SMART* indicates that tomato SlVIN3 protein included three conserved domains: a plant homeodomain (PHD) finger domain from amino acid 161 to 227, a fibronectin type 3 (FN3) domain from amino acid 343 to 428, and a VIN3-interacting (VID) domain from 653 to 726. These domains are conserved in homologous proteins of other plant species as shown by multiple sequence alignments (Fig. 2).

A protein homology search, which was carried out with the *BLASTp* program, shows that there was a high homology between sequences of the tomato SlVIN3 predicted protein and VIN3 protein of *Vitis vinifera* which revealed the highest score value (897 bits). The second highest score value (832 bits) was observed with VIN3 of *Glycine max*. Dicot species revealed a higher sequence identity in comparison with monocot species. The sequence identity for VIN3 proteins of dicot species were between 42 and 61 %, whereas the monocot species sequence identity was less than 40 %. Unexpectedly, VIL1 (VRN5) protein of *A. thaliana* revealed a high score value (582 bits) in comparison with VIN3 proteins of *Arabidopsis halleri* subsp. *gemmifera, A. thaliana*, and *A. lyrata* subsp. *lyrata* (343, 340, and 338 bits, respectively). VIL1 (VRN5) protein of *Theobroma cacao* also revealed a high score value (855 bits) in comparison with its VIN3 protein (359 bits).

To better understand the relationship of SlVIN3 protein of tomato and VIN3 proteins of other plant species, a phylogenetic analysis was performed using protein sequences of a hypothetical gene family reported in the phytozome database (code #26881026). Protein sequences for 12 members which are homologous to *SlVIN3* gene were chosen for the phylogenetic analysis. Phytozome acc. numbers for analyzed protein sequences are as follows: *Arabidopsis lyrata* (491730), *Arabidopsis thaliana* (AT5G57380), *Brachypodium distachyon* (Bradi3g04140), *Glycine max* (Glyma13g00920), *Medicago truncatula* (Medtr8g129220.1), *Oryza sativa* (LOC_Os02g05840), *Populus trichocarpa* (POPTR_0006s18340), *Ricinus communis* (29763.t000010), *Selaginella moellendorffii* (442479), *Sorghum bicolor* (Sb04g003700_118), *Vitis vinifera* (GSVIVG01029220001), and *Zea mays* (GRMZM2G021471).

Both the constructed phylogenetic trees (Fig 3) confirmed the close relationships between *Arabidopsis* species. Cereals and related grass species, with the exception of *Z. mays*, also grouped together and revealed close relationships in both the phylogenetic trees. All dicot accessions were grouped together in the same cluster in both the phylogenetic trees. The tomato SlVIN3 predicted protein appeared to be the closest to VIN3 of *V. vinifera*. Despite some deviations between the two trees, SlVIN3 and VvVIN3 were clustered together in the same clade.

The comparative or ΔΔCT method was used to compare the expression of *SlVIN3* gene in tomato tissues using leaf tissue as calibrator. *SlVIN3* was found to be expressed in closed floral buds, flowers, unripe fruits, and leaves, but the relative expression varied. The calculated $2^{-\Delta\Delta Ct}$ values showed the highest transcript abundance in flowers, moderate in closed floral buds, and the lowest in unripe fruits.

Discussion

Since the completion of sequencing the *A. thaliana* genome in 2000, it has become clear that information about the genome of a particular plant species can have significant benefits in promoting plant molecular genetics in general. The present study focused on the identification and characterization of tomato gene *VIN3*, belonging to the PcG gene family, using the sequence homology to the orthologous gene in *A. thaliana*. Schubert *et al.* (2005) discussed the epigenetic role of this gene in flowering and floral organs development in *A. thaliana*. As far as we know, this is the first gene from the VIN3 gene family to be sequenced and characterized from *Solanaceae*. We obtained the complete sequence for flcDNA for the tomato *SlVIN3* gene. The available ESTs sequences at the *SGN* database covered just 69.22 % of the total length that was cloned and presented in this study. The exon/intron regions were determined, the ORF was predicted and the 5' and 3'UTR for flcDNA were determined. *SlVIN3* flcDNA had four exons and encoded 739 amino acids. Likewise, the *A. thaliana VIN3* has four exons, however, it encodes a shorter protein with 600 amino acids (Sung and Amasino 2004).

VIN3 gene codes a chromatin remodelling protein that controls flowering and enables plants to flower after vernalization by interacting with other PcG proteins (Sung and Amasino 2004). Previous studies of VIN3-like proteins focused on their roles in vernalization of *A. thaliana* and wheat (Sung *et al.* 2006 Fu *et al.* 2007). VIN3 proteins contain three highly conserved elements. The first is PHD, a zinc-finger motif with a conserved Cys4-His-Cys3 (C4HC3) arrangement (Sung and Amasino 2004, Fu *et al.,* 2007). PHD domain is associated with a chromatin-mediated transcriptional regulation (Sung and Amasino 2004) and serves as reader motif for modified histones (Mellor 2006, Sanchez and Zhou 2011). With the exception of VIL4, all members of the VIN3 family contain PHD (Wysocka *et al.* 2006). The second element is FN3 which is involved in protein-protein interactions (Potts and Campbell 1994, Pankov and Yamada 2002, Sung and Amasino 2004). The third is VID which is conserved among all members of this family (Fu *et al.* 2007) and is unique to the plant kingdom (Sung *et al.* 2006, Greb *et al.* 2007). Our domain prediction revealed that the tomato SlVIN3 predicted protein contains all these three conserved domains.

A homology analysis for the SlVIN3 predicted protein revealed a high homology with VIN3 protein of *V. vinifera* and other proteins of the VIN3 family of dicot species. Less homology was observed with the VIN3 family proteins of monocot species. Some dicots and monocots require vernalization to stimulate flowering (Michaels 2009), but the key genes controlling the response to vernalization differ between them (Dennis and Peacock 2009). On the other hand, tomato SlVIN3 was found to be more similar to VIL1 of *A. thaliana* and VIL1 of *Theobroma cacao* than to VIN3 proteins of these species.

The constructed phylogenetic trees confirmed a close relationship between *Arabidopsis* species. Cereals and related grasses, with the exception of *Z. mays*, also show close relationships in both the phylogenetic trees. The tomato SlVIN3 predicted protein appeared to be closer to VIN3 of *V. vinifera* and clustering together in one clade in both the trees. It is noteworthy that *VIN3* genes can be found in various plant species including rice which does not have a vernalization response. Therefore, the *VIN3*-related genes from various plant species can be more broadly involved in relaying environmental signals to developmental programs (Sung *et al.* 2007).

VIN3 gene was found to be expressed in shoot and root apices and displays the same pattern of expression as the floral repressor, FLC (Sung and Amasino 2004). We demonstrated clear differences in the expression of *SlVIN3* gene in tomato tissues in reproductive organs. As expected, the *SlVIN3* gene expression was abundant in flower tissues, very weak in unripe fruit tissues, and intermediate in closed floral bud tissues. This result indicates that the *SlVIN3* gene expression remained high during floral organ differentiation and growth, but decreased when the fruit started to develop. Our observation reflected the specific role of *SlVIN3* gene in promoting flowering. In contrast with our result, the expression of rice *VIN3* gene (*LC2*) is high in leaves, medium in seedlings, roots, and old flowers, and low in stems and young flowers (Zhao *et al.* 2010). The rather low *VIN3* expression in flower tissues may be due to the fact that rice plant does not require vernalization and flowers preferentially under short days, unlike *A. thaliana* and temperate cereals (wheat and barley) which reveales a strong response of flowering to vernalization (Oliver *et al.* 2009). The high expression of rice *LC2* gene in leaf tissues reflected its role in rice leaf architecture (Zhao *et al.* 2010). Its expression is abolished when plants are exposed to high temperature (Sung and Amasino 2004). The constitutively repressed *VIN3* in the absence of cold is caused by multiple repressive components which include PRC2, Like Heterochromatin Protein 1 (LHP1), and a transposable element (Kim *et al.* 2010). For tomato, further studies are needed to identify other *PcG* genes and to clarify the epigenetic mechanisms which regulate flowering and will hopefully help in understanding the vernalization pathway in *Solanaceae*.

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