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# Natural allelic variation in blueberry TERMINAL FLOWER 1

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## Abstract

A blueberry (*Vaccinium* sp.) germplasm collection was screened for allelic variation in *TERMINAL FLOWER 1* (*TFL1*) that could be used in breeding flowering or architecture traits. TFL1 has been found to repress the transition from vegetative to reproductive growth in diverse plant species, with mutations leading to altered flowering and form. The *VcTFL1* gene sequence was determined from the draft genome sequence of diploid *V. corymbosum* line W8520. *VcTFL1* is a member of a PEBP gene family and it could be distinguished from its family members by sequence comparison with PEBP family genes from other plants. High-resolution DNA melting analysis of 160 *Vaccinium* accessions detected *VcTFL1* exons that differed in sequence from the W8520 control. DNA sequence analysis confirmed the presence of single nucleotide polymorphisms (SNPs) and identified haplotypes of tetraploid accessions. A total of 18 SNP sites were detected in *VcTFL1* coding sequences of the *Vaccinium* germplasm screened. A SNP causing an alanine-to-valine change in exon 4 (A159V) was determined by multiple bioinformatic tools to be deleterious to *VcTFL1* function. A diploid *V. corymbosum* accession heterozygous for the *VcTFL1* mutation was identified as a candidate for breeding novel traits for blueberry.

**Keywords:** autotetraploid, ecotilling, germplasm collection, HRM, PEBP, *Vaccinium*

## Introduction

The detection of allelic variation in plant populations has benefited from advances in genomics. Genome and transcriptome sequence data, comparative genomics and bioinformatics have expedited the identification of candidate gene sequences. Natural variation in candidate genes can then be targeted in germplasm collections through screening approaches such as ecotype TILLING (Ecotilling; Till, 2014) or breeding with rare defective alleles (BRDA; Vanholme *et al.*, 2013). Through germplasm screens, alleles have been identified for drought resistance in rice (Yu *et al.*, 2012), improved oil quality in rapeseed (Wang *et al.*, 2010), early flowering in sugar beet (Frerichmann *et al.*, 2013) and modified lignin in poplar (Vanholme *et al.*, 2013). Our research interests involved examining a blueberry (*Vaccinium* sp.) germplasm collection for allelic variation in candidate genes controlling flowering and architecture.

The blueberry germplasm collection in the USDA-ARS National Clonal Germplasm Repository is composed primarily of *V. corymbosum*, but includes other *Vaccinium* species and hybrids (Ballington, 2001). The ploidy levels of these accessions range from diploid to hexaploid, with most being autotetraploid. Analysis with simple sequence repeat (SSR) markers found a high level of genetic diversity in *Vaccinium* accessions of the USDA-ARS collection (Boches *et al.*, 2006). The degree of allelic variation in specific genes among accessions of this germplasm collection is not known.

Genomic and bioinformatic resources have been developed for *V. corymbosum*, including expressed sequence tag (EST) libraries, EST-based molecular markers, genetic linkage maps and an online database to house blueberry genomic information (Rowland *et al.*, 2012; Die and

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Rowland, 2013). A draft genome sequence of the diploid *V. corymbosum* selection W8520 was generated by a combination of Roche 454 and Illumina sequencing (Bian *et al.*, 2014). Annotation of the genome assembly with RNA-Seq data identified approximately 60,000 gene models (Gupta *et al.*, 2015). The availability of the genome assembly, annotations and RNA Seq data through an Integrated Genome Browser platform facilitates the identification of blueberry orthologues of candidate genes.

*TERMINAL FLOWER 1* (*TFL1*) is a gene with the potential to affect both flowering time and plant architecture through its involvement in shoot meristem identity (Bradley *et al.*, 1997; McGarry and Ayre, 2012). *TFL1* is a member of a phosphatidyl ethanolamine-binding protein (PEBP) gene family and it has been found to repress the transition from vegetative to reproductive growth in shoot meristems of a variety of plants. In the perennial plants rose and woodland strawberry, *TFL1* mutations result in repetitive flowering (Iwata *et al.*, 2012). *TFL1* mutations in the annual plants tomato, soybean and cowpea cause a switch from indeterminate to determinate growth (Pnueli *et al.*, 1998; Tian *et al.*, 2010; Dhanasekar and Reddy, 2015). *TFL1* has served as a domestication gene for several crops and the traits conferred by *TFL1* variants may be of ornamental interest in blueberry. In this study, we identified *V. corymbosum* orthologues of *TFL1* and its family members, examined allelic variation of *VcTFL1* among accessions of a blueberry germplasm collection, and characterized a missense mutation likely to be deleterious to VcTFL1 function.

## Materials and methods

### Germplasm and DNA isolation

Young leaves of *Vaccinium* sp. were obtained from plants in the blueberry germplasm collection and breeding programme of the USDA-ARS (Corvallis, OR) and stored at −80°C until used. The accessions are listed in online Supplementary Table S1. To isolate genomic DNA, 50–100 mg of frozen leaf tissue was ground in 2 ml Eppendorf safe lock tubes using a bead mill (Tissuelyzer, Qiagen). DNA was extracted using a modified CTAB (cetyl trimethylammonium bromide) method (Porebski *et al.*, 1997). DNA samples were resuspended in TE (Tris-EDTA) buffer and quantified with a NanoDrop 800 spectrophotometer (Thermo Scientific).

### Orthologue identification and primer design

The assembled draft genome sequence of the diploid *V. corymbosum* W8520 was available through GenSAS v3.0 (http://gensas2.bioinfo.wsu.edu/). A BLASTx search of the genomic database was conducted with *Solanum lycopersicum* SELF-PRUNING (GenBank AAC26161) as a query sequence. BLASTx analysis with TFL1 orthologues from other plant species (impatiens, apple, peach and Arabidopsis) identified the same *V. corymbosum* sequences. Coding regions with significant similarity to SlSP (*e*-value $<10^{-15}$) were used to query the SwissProt database (Bairoch and Apweiler, 2000) to confirm their identity. PEBP family protein sequences were compared by the neighbour-joining method of MEGA v6.06 (Tamura *et al.*, 2013), with a total of 100 bootstraps. The gene model of *VcTFL1* was predicted using AUGUSTUS (Stanke *et al.*, 2004), after training with *S. lycopersicum*. PCR primers to amplify exons of *VcTFL1* were designed using Primer3 (Rozen and Skaletsky, 2000) and are shown in online Supplementary Table S2.

### PCR and high-resolution melting (HRM) analysis

PCR and HRM were performed with a LightCycler 480 (Roche Diagnostics) in 96-well plates. PCR was conducted in a 20 µl volume containing 20 ng DNA, 10 × HRM master mix, 3.0 mM $MgCl_2$ and 0.8 µM of each primer. Reactions were denatured at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 s, step-down annealing temperature from 65–60°C at the rate of 0.2°C per cycle and extension at 72°C for 30 s. For exon four, 45 cycles of PCR was performed to allow the amplification curves to reach saturation point. The reference genome W8520 was included in each reaction at 12.5% of the total DNA (2.5 ng). Three technical replications of each sample–primer pair combination were conducted.

For HRM analysis, PCR products were denatured at 95°C for 1 min, cooled to 40°C for 1 min for re-annealing, and then heated from 70 to 95 °C at 0.02°C/s, while continuously measuring florescence with 25 data acquisitions/°C. DNA melting data were analysed with the LC480 Gene Scanning software (Roche Diagnostics) with settings for sensitivity and temperature shifting at 0.3 and 5, respectively. Difference plots were generated through subtraction of normalized and temperature shifted curves from the melting curve of the reference DNA (*V. corymbosum* W8520).

### Sequencing and haplotype calling

For sequencing, PCR products were fractionated in a 1% agarose gel and purified by EXOSAP PCR (Affymetrix) cleanup. The resulting DNA samples were sequenced in both directions by the Georgia Genomics Facility (Athens, GA) with an Applied Biosystems 3730xl DNA Analyser.

The sequence data were analysed and aligned for SNP discovery using GENEIOUS 8.1.7 software (Kearse *et al.*, 2012). The allelic dosage for heterozygous SNPs in

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autotetraploid individuals was predicted by using the 'Find Heterozygotes' tools available in GENEIOUS. For calling duplex heterozygotes (AAaa), peak similarity between primary and secondary peaks was set to be 70% and for calling simplex (Aaaa) or triplex (AAAa), peak similarity was set to 30%.

## *In silico SNP analysis*

For SNPs leading to non-synonymous amino acid substitutions, *in silico* analyses were conducted to predict, whether the amino acid change had an impact on protein function or stability. Functionally important regions of VcTFL1 were identified using ConSurf (Glaser *et al.*, 2003). The effect of mutations was evaluated using the computational tools PROVEAN (Choi *et al.*, 2012), SIFT (Ng and Henikoff, 2003), PredictSNP (Bendl *et al.*, 2014), Polyphen-1 and Polyphen-2 (Adzhubei *et al.*, 2010), Panther (Thomas *et al.*, 2003), MutPred (Li *et al.*, 2009) and MUpro (Cheng *et al.*, 2006). Default parameters of the software programs were used.

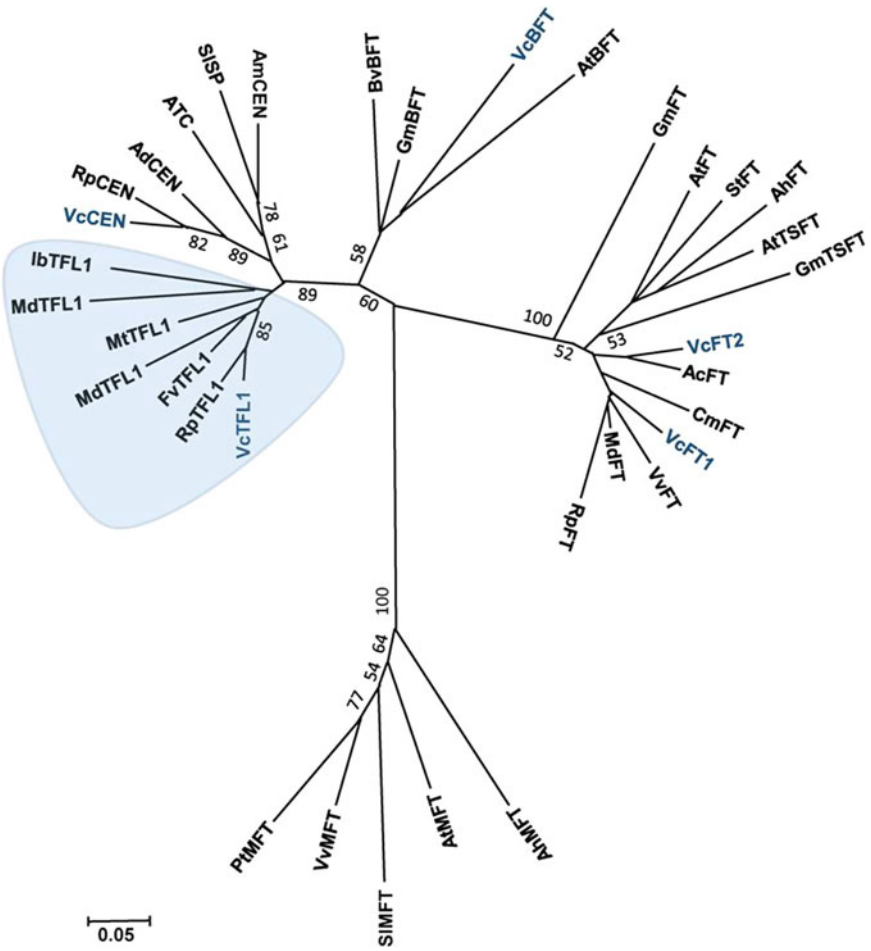


**Fig. 1.** Phylogenetic tree of PEBP family members. The NJ method of MEGA v6.06 was used with following protein sequences: Md*TFL1* (*Malus domesticus* TFL1: ACD69429.1), MtTFL1 (*Medicago trunculata TFL1*: XP_003625808.1), AtTFL1 (*Arabidopsis thaliana TFL1*), IbTFL1 (*Impatiens balsamina TFL1*: CAI61981.1), RpTFL1 (*Rhododendron pulchrum* TFL1), FvTFL1 (*Fragaria vesca* TFL1-like: NP_001267006.1), Rp*CEN* (*Rhododendron pulchrum CEN*: BAL70256.1), AdCEN (*Actinidia deliciosa* CEN: AGK89940.1), ATC (*Arabidopsis thaliana CEN*: BAA75932.1), SlSP (*Solanum tuberosum* Self-Pruning: NP_001233974.1), Am*CEN* (*Antirrhinum majus* CEN: Q41261.1), BvBFT (Beta vulgaris BFT: ADM92613.1), GmBFT (*Glycine max* BFT: NP_001236597.1), AtBFT (*Arabidopsi thaliana* BFT: NP_201010.1),GmFT (*Glycine max* FT: NP_001240185), AtFT (*Arabidopsis thaliana* FT: NP_176726.1), StFT (*Solanum tuberosum* FT: NP_001274897.1), AhFT (*Arachis hypogea* FT: AFP33416.1), AtTSFT (*Arabidopsis thaliana* TSFT: NP_193770.1), GmTSFT (*Glycine max* TSFT: NP_001241104.1), AcFT (*Actinidia chinensis* FT: AGK89939.1), CmFT (*Chrysanthemum x morifolium* FT-like, BAJ14266.2), VvFT (*Vitis vinifera* FT: NP_001267907.1), MdFT (*Malus domesticus* FT: ACV92037.1), RpFT (*Rhododendron pulchrum*: BAL70257.1), AhMFT (*Arachis hypogea* MFT: AFP33419.1), AtMFT (*Arabidopsis thaliana* MFT: NP_173250.1), SlMFT (*S. olanum lycopersicum* MFT: XP_004235817.1), VvMFT (*Vitis vinifera* MFT: XP_003634198.1), PtMFT (*Populus tricocarpa* MFT: XP_002321507.1).

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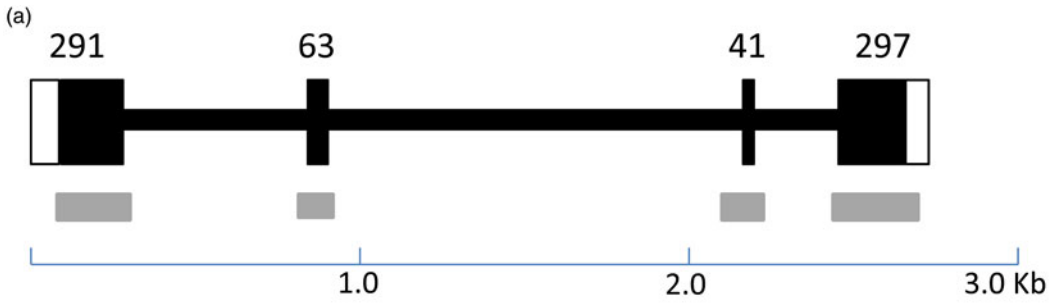
## Results

### *Identification of VcTFL1*

At the time of analysis, the draft blueberry genome sequence was assembled on 13,787 scaffolds. Five scaffolds contained sequences with significant similarity (*e*-value $<10^{-17}$) to the tomato *TFL1* orthologue *SlSP*. Query of the SwissProt database with the five sequences identified them as members of the PEBP gene family. A comparison of the predicted amino acid sequence of the *V. corymbosum* sequences with PEBP family members of other plant




(b)

```
SlSP        -------MASKMCEPLVIGRVIGEVVDYFCPSVKMSVVYNNNKHVYNGHEFFPSSVTSKP
AtTFL1      ----MENMGTRVIEPLIMGRVVGDVLDFFTPTTKMNVSYN-KKQVSNGHELFPSSVSSKP
IbTFL1      MYMRKRSSSSSSTEPLAVGRVIGDVIDPFVLSVKMLVTYN-NKQVYNGYELFPSQITSKP
MtTFL1      -------MARMSQEPLIVGRVIGEVLDSFTTSMKMTVSYN-KKQVFNGHEFFPSTINTKP
VcTFL1      -------MARMSAEPLIVGRVIGDVLDPFTPTVKMLATYN-NKQVSNGHEFYPSQVTTKP
FvTFL1      -------MAR-MSEPLAVGRVIGDVLDSFTPTTKMIVSYN-SKLVCNGHELFPSAVTAKP
MdTFL1      -------MAR-VPEPLVVGRVIGDVLDSFTPTTHMSVTYN-AKLVCNGLELFPSVVTAKP
                     .    *** :***:*:*:* *  : :* . **  : * ** *::** :.:**
SlSP        RVEVHGGDLRSFFTLIMIDPDVPGPSDPYLREHLHWIVTDIPGTTDCSFGREVVGYEMPR
AtTFL1      RVEIHGGDLRSFFTLVMIDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVVSYELPR
IbTFL1      RVEVHGGDLRTFYTLVMTDPDVPGPSDPYLKEHLHWMVTDIPGTTDATFGKEMVKYEMPM
MtTFL1      KVEIDGGDMRSFYTLVMTDPDVPGPSDPYLREHLHWIVTDIPGTTDATFGKEVVSYEIPK
VcTFL1      RVEIHGGDMRTFFTLVMTDPDVPGPSDPYLREHLHWIVTDIPGTTDATFGREVVSYEIPR
FvTFL1      RVEIQGGDMRSFFTLVMTDPDVPGPSDPYLKEHLHWIVTDIPGTTDATFGREVVSYEMPR
MdTFL1      RVEIQGGELRSFFTLVMTDPDCPGPSDPYLREHLHWIVTDIPGTTDAAFGREALSYEMPR
            :**:.**::*:*:**:* *** ******:*:*****:**:******.:**:* : **:*
SlSP        PNIGIHRFVFLLFKQKKRQTISSAPVSRDQFSSRKFSEENELGSPVAAVFFNCQRETAAR
AtTFL1      PSIGIHRFVFVLFRQKQRRVIFPNIPSRDHFNTRKFAVEYDLGLPVAAVFFNAQRETAAR
IbTFL1      PNIGIHRFVFVLFKQRCRQSVQ-PPSSREHFNTRRFAADNDLGQPVAAVFFNAQRETAAR
MtTFL1      PNIGIHRFVFVLFKQKNRESVTASPSSRDYFNTRNFASQNDLGLPVAAVYFNAQRETAAR
VcTFL1      PNIGIHRFVFVLFKQKSRQAIS-PPSSRDHFTTRCFAEENDLGLPVAAVFFNAQRETAAR
FvTFL1      PNIGIHRFVFVLFMQKRRQSVN-PPSSRDHFNTRTFAAENDLGVPVAAVYFNAQRETAAR
MdTFL1      PNIGIHRFVFVLFKQKRRQSIN-IPSSRDCFSTRSFAAENDLGLPVAAVYFNAQRESAAR
            *.********:** *: *. :     **: *.:* *: : :** *****:**.***:***
SlSP        RR-
AtTFL1      KR-
IbTFL1      CRR
MtTFL1      RR-
VcTFL1      RR-
FvTFL1      RR-
MdTFL1      RR-
             *
```

**Fig. 2.** *VcTFL1* gene model (a) and sequence comparison of predicted protein (b). Exons are indicated by boxes with their size shown above (in bp); white boxes indicate untranslated regions. Horizontal grey lines indicate the location of PCR amplicons used in HRM analysis. The triangles point to amino acids altered by SNPs. The boxed sequences are a conserved region in which an amino acid change potentially affects VcTFL1 function.

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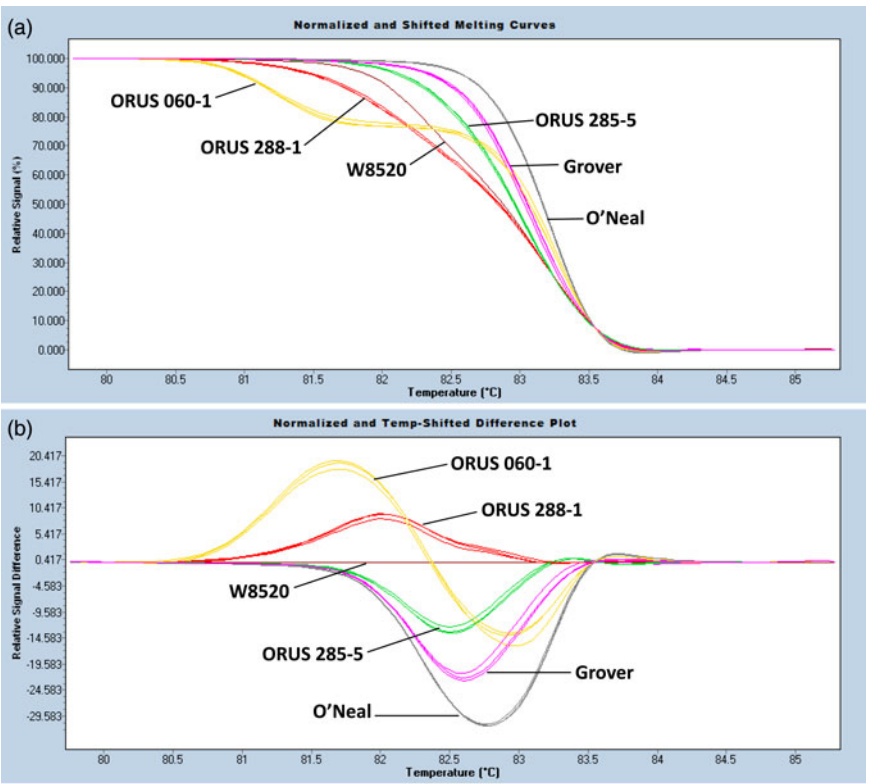
species found that one candidate sequence grouped with the TFL1 orthologue clade, while the others grouped in FLOWERING LOCUS T (FT), BROTHER OF FT, or CENTRORADIALIS clades (Fig. 1). The *V. corymbosum* gene that was similar in sequence to other *TFL1* orthologues was designated *VcTFL1* (GenBank KX834412).

The gene model of *VcTFL1* revealed that the 2758 bp sequence was comprised of four exons that encode a protein of 174 amino acids, typical of *TFL1* orthologues (Fig. 2a). Sequence comparison of VcTFL1 (Fig. 2b) found that it shared a high percentage of identical amino acids with orthologues from *Fragaria vesca* (83.1%), *Medicago truncatula* (80.9%), *Malus domestica* (79.7%), *S. lycopersicum* (75.7 %), *Arabidopsis thaliana* (76.9%) and *Impatiens balsamina* (77.5%). The *VcTFL1* sequence was used to design primers that amplified coding sequences (Fig. 2a, online Supplementary Table S2).

## *Screening for allelic variation in VcTFL1*

A collection of 160 blueberry accessions obtained from the USDA-ARS (Corvallis, OR) included 135 *V. corymbosum* accessions, 15 *V. corymbosum* × *V. darrowii* hybrids, one *V. corymbosum* × *V. angustifolium* hybrid, and nine accessions of other *Vaccinium* species, including *V. darrowii, V. virgatum, V. fuscatum, V. simulatum* and *V. angustifolium* (online Supplementary Table S1). *TFL1* exons from these accessions were examined individually for allelic variation by HRM analysis. Figure 3 shows an example of variation found in exon 1 of five accessions. Relative to exon 1 of the reference genotype W8520, accessions ORUS 060-1 and ORUS 288-1 had a lower melting temperature and accessions ORUS 285–5, Grover, and O'Neal had a higher melting temperature (Fig. 3a). HRM could distinguish the ORUS 285–5, Grover, and O'Neal accessions, although they all varied from W8520 at the same nucleotide




(c)

| SNP (nt) | O'Neal | Grover | ORUS 285-5 | ORUS 288-1 | ORUS 060-1 |
|---|---|---|---|---|---|
| 30 | | | | | CCCA |
| 104 | GGGG | AAAA | AAAG | AAGG | AAAG |
| 144 | | | | | TTAA |
| 156 | | | | | AATT |
| 171 | | | | | AATT |
| 195 | | | | CCTT | |

**Fig. 3.** HRM and sequencing analysis of *VcTFL1* exon 1 of five blueberry accessions. (a) Normalized and temperature-shifted melting curves. (b) HRM difference plots. (c) Haplotypes determined from sequence chromatograms. HRM was conducted with three technical replicates.

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**Table 1.** SNPs identified in *VcTFL1* exons and their effect on protein composition and function

| SNP location (nt) | Variation (W8520/ accession) | Amino acid change | Accessions with SNP (no.) | Effect on protein function[a] |
|---|---|---|---|---|
| 27 | C/A | S | | – |
| 104 | A/G | N | 125 | Neutral |
| 117 | T/C | S | | – |
| 144 | A/T | S | | – |
| 149 | C/T | N | 6 | Neutral |
| 156 | T/A | S | | – |
| 170 | A/C | S | | – |
| 171 | A/T | S | | – |
| 195 | C/T | S | | – |
| 199 | C/T | S | | – |
| 234 | T/C | S | | – |
| 261 | C/T | S | | – |
| 282 | C/T | S | | – |
| 420 | C/T | S | | – |
| 471 | C/T | S | | – |
| 474 | G/A | S | | – |
| 475 | C/T | N | 1 | Deleterious |
| 504 | C/A | S | | – |

S, synonymous; N, non-synonymous.
[a]Predicted by PROVEAN analysis.

position (nt 104; Fig. 3b). GENIOUS analysis of secondary peaks of chromatograms at SNP104 identified these autotetraploid accessions as nulliplex, simplex and quadraplex haplotypes (Fig. 3c). The melting curves of exon 1 of ORUS 288–1 and ORUS 060–1 are more complex because of the presence two and five SNPs, respectively.

### *Allelic variants and potential effects on VcTFL1 function*

Analysis of the four *VcTFL1* exons by HRM and sequencing identified 18 SNP positions among the 160 accessions (Table 1). Most of these SNPs resulted in synonymous amino acid substitutions, but three SNPs caused non-synonymous changes (Fig. 2b). *In silico* analysis of the effect of non-synonymous changes predicted that SNPs at nucleotides 104 and 149 would have no impact on protein function, but the SNP at nucleotide 475 would be deleterious.

SNP475 causes a missense mutation from alanine to valine. Amino acids that are critical to VcTFL1 structure and function were identified by ConSurf analysis (Fig. 4). The alanine at amino acid 159 was predicted to be a buried residue that is conserved in TFL1 orthologues. The consequence of the A159 V substitution was further assessed by the software programs MuPro, MuPred and PredictSNP, a combination of tools including SIFT, Polyphen and Panther. In general, the A159 V substitution was predicted to be deleterious to *TFL1* function (Table 2). A diploid accession (DE596) was found to be heterozygous for this SNP, which can potentially be used to obtain a novel ornamental phenotype through breeding.

## Discussion

The *TFL1* orthologue of *V. corymbosum* was identified and allelic variation in this gene was discovered in a *Vaccinium* germplasm collection. Among 160 blueberry accessions, 18 SNP sites were detected, one of which was predicted by bioinformatic analyses to be deleterious to *VcTFL1* function. The predominant allele among the accessions analysed

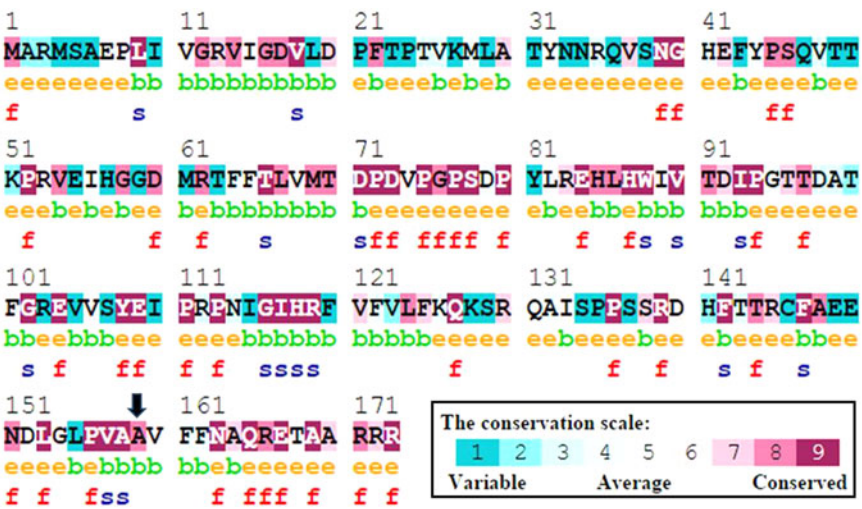


**Fig. 4.** Consurf analysis of VcTFL1 amino acid conservation and functionality. Black arrow shows the alanine changed by SNP475. E, exposed residue; b, buried residue; f, predicted functional residue; s, predicted structural residue.

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**Table 2.** *In silico* analysis of the amino acid change (A159 V) on protein function/stability

| Software | % Confidence/ score[a] | Prediction |
|---|---|---|
| PredictSNP | 55% | Deleterious |
| SIFT | 45% | Deleterious |
| PolyPhen-1 | 67% | Neutral |
| PolyPhen-2 | 65% | Deleterious |
| Panther | 57% | Deleterious |
| MutPred | 88.2% | Deleterious |
| MuPro | 0.75 (SVM) | Increase in protein stability |
| | −0.57 (neutral network) | Decrease in protein stability |
| PROVEAN | −2.994 | Deleterious |

[a]software tools SIFT, PolyPhen and Panther are integrated in PredictSNP, which transformed the score of each to a confidence level of 0–100%.

was similar to the *VcTFL1* present in the sequenced genome of diploid *V. corymbosum* line W8520, with other alleles having polymorphisms leading to synonymous amino acid changes or non-synonymous changes with no effect on VcTFL1 function. The detection of a genotype with a potentially deleterious *VcTFL1* mutation among 160 blueberry accessions is indicative of the heterogeneity within this germplasm collection, as well as the utility of Ecotilling for SNP identification.

The identification of useful alleles for crop improvement in screens of germplasm collections of this size has been reported in other plant species. The size of an Ecotilling population needed to identify functional polymorphisms depends on factors such as ploidy, breeding habit, and heterogeneity. Ecotilling of 117 accessions of three *Brassica* species identified a SNP in *fatty acid elongase1* leading to the loss of *FAE1* function (Wang *et al.*, 2010). In maize, screening of 175 inbred breeding lines found a SNP in *isopentenyl transferase 2* that was associated with higher kernel weight (Weng *et al.*, 2013). SNPs affecting seed weight were also detected in eight chickpea (*Cicer arietinum*) transcription factor genes in an analysis of 192 accessions (Bajaj *et al.*, 2016).

The blueberry germplasm collection that was examined is composed primarily of autotetraploid genotypes, which complicated SNP identification because of the presence to two nearly identical subgenomes. HRM analysis was able to discriminate between blueberry accessions with different haplotypes at the same SNP position. Haplotype variants could also be identified in candidate genes of autotetraploid potato genotypes by HRM analysis (De Koeyer *et al.*, 2010). In autotetraploid alfalfa, a combination of next-generation sequencing (NGS) and HRM was used to discover thousands of SNPs (Han *et al.*, 2011). HRM was also found to be an efficient approach for SNP genotyping and mapping in alfalfa (Han *et al.*, 2012).

A diploid *V. corymbosum* accession, DE596, was identified with an A159 V variation in VcTFL1. The alanine at position 159 is in a highly conserved region of exon 4. Exon 4 has been shown to be critical for normal *TFL1* function (Ahn *et al.*, 2006). In soybean, a missense mutation in *TFL1* exon 4 (R168W) caused a switch from indeterminate to determinate growth habit (Tian *et al.*, 2010). A similar change in growth habit was due to a missense mutation (P139H) in exon 4 of the cowpea *TFL1* orthologue (Dhanasekar and Reddy, 2015).

Germplasm database records indicate that *V. corymbosum* DE596 has growth and flowering phenology typical of blueberry, which is to be expected for an accession with a heterozygous *TFL1* mutation. This accession can potentially be used in a breeding programme to develop an ornamental blueberry with continuous flowering (Iwata *et al.*, 2012). *TFL1* is a target for breeding remontancy in cultivated strawberry (Koskela *et al.*, 2016). A homozygous *TFL1* mutation may also result in an ornamental blueberry with more compact form due to a change in the balance of vegetative and reproductive meristems (McGarry and Ayre, 2012). Obtaining homozygosity of *tfl1* will require breeding or doubled haploid technology, similar to an approach used to improve potato with a loss-of-function allele (Muth *et al.*, 2008).

DNA sequencing was used in this study to confirm HRM results and determine haplotypes. With the advantages offered in scale and throughput, NGS will likely be the most efficient means to discover allelic variation. Sets of genes known to be involved in a trait, a biochemical pathway, or a regulatory cascade can be targeted by sequence capture methods such as SureSelect (Gnirke *et al.*, 2009) or NimbleGen (Kiss *et al.*, 2008). For example, Uitdewilligen *et al.* (2013) used SureSelect to develop sequencing libraries of 807 target genes from 84 autotetraploid potato genotypes. This resulted in the detection of allelic variants associated with tuber flesh colour and plant maturity. The identification of allelic variation in candidate genes can provide functional or 'perfect' markers for breeding new traits (Moose and Mumm, 2008).

## Supplementary Material

The supplementary material for this article can be found at https://doi.org/10.1017/S1479262116000435

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## References

Adzhubei IA, Schmidt S, Peshkin L, Ramensky V, Gerasimova A, Bork P, Kondrashov AS and Sunyaev SR (2010) A method and server for predicting damaging missense mutations. *Nature Methods* 7: 248–249.

Ahn JH, Miller D, Winter VJ, Banfield MJ, Lee JH, Yoo SY, Henz SR, Brady RL and Weigel D (2006) A divergent external loop confers antagonistic activity on floral regulators *FT* and *TFL*1. *EMBO Journal* 25: 605–614.

Bairoch A and Apweiler R (2000) The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. *Nucleic Acids Research* 28: 45–48.

Bajaj D, Srivastava R, Nath M, Tripathi S, Bharadwaj C, Upadhyaya HD, Tyagi AK and Parida SK (2016) EcoTILLING-based association mapping efficiently delineates functionally relevant natural allelic variants of candidate genes governing agronomic traits in chickpea. *Frontiers in Plant Science* 7: 450. DOI: 10.3389/fpls.2016.00450.

Ballington JR (2001) Collection, utilization, and preservation of genetic resources in *Vaccinium*. *HortScience* 36: 213–220.

Bendl J, Stourac J, Salanda O, Pavelka A, Wieben ED, Zendulka J, Brezovsky J and Damborsky J (2014) PredictSNP: robust and accurate consensus classifier for prediction of disease-related mutations. *PLoS Computational Biology* 10: e1003440.

Bian Y, Ballington J, Raja A, Brouwer C, Reid R, Burke M, Wang X, Rowland LJ, Bassil N and Brown A (2014) Patterns of simple sequence repeats in cultivated blueberries (*Vaccinium* section *Cyanococcus* spp.) and their use in revealing genetic diversity and population structure. *Molecular Breeding* 34: 675–689.

Boches P, Bassil NV and Rowland L (2006) Genetic diversity in the highbush blueberry evaluated with microsatellite markers. *Journal of the American Society for Horticultural Science* 131: 674–686.

Bradley D, Ratcliffe O, Vincent C, Carpenter R and Coen E (1997) Inflorescence commitment and architecture in *Arabidopsis*. *Science* 275: 80–83.

Cheng J, Randall A and Baldi P (2006) Prediction of protein stability changes for single-site mutations using support vector machines. *Proteins: Structure, Function, and Bioinformatics* 62: 1125–1132.

Choi Y, Sims GE, Murphy S, Miller JR and Chan AP (2012) Predicting the functional effect of amino acid substitutions and indels. *PLoS ONE* 7: e46688.

De Koeyer D, Douglass K, Murphy A, Whitney S, Nolan L, Song Y and De Jong W (2010) Application of high-resolution DNA melting for genotyping and variant scanning of diploid and autotetraploid potato. *Molecular Breeding* 25: 67–90.

Dhanasekar P and Reddy KS (2015) A novel mutation in *TFL*1 homolog affecting determinacy in cowpea (*Vigna unguiculata*). *Molecular Genetics and Genomics* 290: 55–65.

Die JV and Rowland LJ (2013) Advent of genomics in blueberry. *Molecular Breeding* 32: 493–504.

Frerichmann SL, Kirchhoff M, Müller AE, Scheidig AJ, Jung C and Kopisch-Obuch FJ (2013) EcoTILLING in *Beta vulgaris* reveals polymorphisms in the *FLC*-like gene *BvFL*1 that are associated with annuality and winter hardiness. *BMC Plant Biology* 13: 52. DOI: 10.1186/1471-2229-13-52.

Glaser F, Pupko T, Paz I, Bell RE, Bechor-Shental D, Martz E and Ben-Tal N (2003) ConSurf: identification of functional regions in proteins by surface-mapping of phylogenetic information. *Bioinformatics* 19: 163–164.

Gnirke A, Melnikov A, Maguire J, Rogov P, LeProust EM, Brockman W, Fennell T, Giannoukos G, Fisher S, Russ C and Gabriel S (2009) Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nature Biotechnology* 27: 182–189.

Gupta V, Estrada AD, Blakley I, Reid R, Patel K, Meyer MD, Andersen SU, Brown AF, Lila MA and Loraine AE (2015) RNA-Seq analysis and annotation of a draft blueberry genome assembly identifies candidate genes involved in fruit ripening, biosynthesis of bioactive compounds, and stage-specific alternative splicing. *GigaScience* 4: 5. DOI 10.1186/s13742-015-0046-9.

Han Y, Kang Y, Torres-Jerez I, Cheung F, Town CD, Zhao PX, Udvardi MK and Monteros MJ (2011) Genome-wide SNP discovery in tetraploid alfalfa using 454 sequencing and high resolution melting analysis. *BMC Genomics* 12: 350.

Han Y, Khu DM and Monteros MJ (2012) High-resolution melting analysis for SNP genotyping and mapping in tetraploid alfalfa (*Medicago sativa* L.). *Molecular Breeding* 29: 489–501.

Iwata H, Gaston A, Remay A, Thouroude T, Jeauffre J, Kawamura K, Oyant LH, Araki T, Denoyes B and Foucher F (2012) The *TFL*1 homologue *KSN* is a regulator of continuous flowering in rose and strawberry. *Plant Journal* 69: 116–125.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C and Thierer T (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649.

Kiss MM, Ortoleva-Donnelly L, Reginald Beer N, Warner J, Bailey CG, Colston BW, Rothberg JM, Link DR and Leamon JH (2008) High-throughput quantitative polymerase chain reaction in picoliter droplets. *Analytical Chemistry* 80: 8975–8981.

Koskela EA, Sønsteby A, Flachowsky H, Heide OM, Hanke MV, Elomaa P and Hytönen T (2016) TERMINAL FLOWER1 is a breeding target for a novel everbearing trait and tailored flowering responses in cultivated strawberry (Fragaria × ananassa Duch.). *Plant Biotechnology Journal* 14: 1852–1861.

Li B, Krishnan VG, Mort ME, Xin F, Kamati KK, Cooper DN, Mooney SD and Radivojac P (2009) Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics* 25: 2744–2750.

McGarry RC and Ayre BG (2012) Manipulating plant architecture with members of the CETS gene family. *Plant Science* 188: 71–81.

Moose SP and Mumm RH (2008) Molecular plant breeding as the foundation for 21st century crop improvement. *Plant Physiology* 147: 969–977.

Muth J, Hartje S, Twyman RM, Hofferbert HR, Tacke E and Prüfer D (2008) Precision breeding for novel starch variants in potato. *Plant Biotechnology Journal* 6: 576–584.

Ng PC and Henikoff S (2003) SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Research* 31: 3812–3214.

Pnueli L, Carmel-Goren L, Hareven D, Gutfinger T, Alvarez J, Ganal M, Zamir D and Lifschitz E (1998) The SELF-PRUNING gene of tomato regulates vegetative to reproductive switching of sympodial meristems and is the ortholog of CEN and *TFL*1. *Development* 125: 1979–1989.

Porebski S, Bailey LG and Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high poly-

saccharide and polyphenol components. *Plant Molecular Biology Reporter* 15: 8–15.

Rowland LJ, Alkharouf N, Darwish O, Ogden EL, Polashock JJ, Bassil NV and Main D (2012) Generation and analysis of blueberry transcriptome sequences from leaves, developing fruit, and flower buds from cold acclimation through deacclimation. *BMC Plant Biology* 12: 46.

Rozen S and Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S and Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Totowa, NJ: Humana Press, pp. 365–386.

Stanke M, Steinkamp R, Waack S and Morgenstern B (2004) AUGUSTUS: a web server for gene finding in eukaryotes. *Nucleic Acids Research* 32: W309–W312.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular and Biological Evolution* 30: 2725–2729.

Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, Diemer K, Muruganujan A and Narechania A (2003) PANTHER: a library of protein families and subfamilies indexed by function. *Genome Research* 13: 2129–2141.

Tian Z, Wang X, Lee R, Li Y, Specht JE, Nelson RL, McClean PE, Qiu L and Ma J (2010) Artificial selection for determinate growth habit in soybean. *Proceeding of the National Academy of Science of the United States of America* 107: 8563–8568.

Till BJ (2014) Mining genetic resources via Ecotilling. In: Tuberosa R, Graner A, Frison E (eds) *Genomics of Plant Genetic Resources*. Netherlands: Springer, pp. 349–365.

Uitdewilligen JG, Wolters AM, Bjorn B, Borm TJ, Visser RG and van Eck HJ (2013) A next-generation sequencing method for genotyping-by-sequencing of highly heterozygous autotetraploid potato. *PLoS ONE* 8: e62355.

Vanholme B, Cesarino I, Goeminne G, Kim H, Marroni F, Acker R, Vanholme R, Morreel K, Ivens B, Pinosio S and Morgante M (2013) Breeding with rare defective alleles (BRDA): a natural *Populus nigra* HCT mutant with modified lignin as a case study. *New Phytologist* 198: 765–776.

Wang N, Shi L, Tian F, Ning H, Wu X, Long Y and Meng J (2010) Assessment of *FAE*1 polymorphisms in three *Brassica* species using EcoTILLING and their association with differences in seed erucic acid contents. *BMC Plant Biology* 10: 137. DOI: 10.1186/1471-2229-10-137.

Weng J, Li B, Liu C, Yang X, Wang H, Hao Z, Li M, Zhang D, Ci X, Li X and Zhang S (2013) A non-synonymous SNP within the *isopentenyl transferase* 2 locus is associated with kernel weight in Chinese maize inbreds (*Zea mays* L.). *BMC Plant Biology* 13: 1.

Yu S, Liao F, Wang F, Wen W, Li J, Mei H and Luo L (2012) Identification of rice transcription factors associated with drought tolerance using the ecotilling method. *PLoS ONE* 7: e30765.