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The mRNA sequence polymorphisms of flowering key genes in bolting sensitive or tolerant sugar beet genotypes

MARYAM ALIMIRZAEE [1], ASGHAR MIRZAIE-ASL [1]*, MOHAMMAD REZA ABDOLLAHI [2], HASSAN EBRAHIMI KOLAEI [3], PARVIZ FASAHAT [4]

[1] Department of Biotechnology, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, I.R. Iran

[2] Department of Agronomy and Plant Breeding, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, I.R. Iran

[3] Sugar Beet Seed Department, Hamedan Agricultural and Natural Resources Research and Education Center, AREEO, Hamedan, Iran

[4] Sugar Beet Seed Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Abstract

One of the most important characteristics of sugar beet planting in temperate climates is the tolerance against early bolting. Understanding the genetic control of sugar beet flowering can help to develop the bolting-tolerant cultivars. In this study, the transcript sequences of 2 *VIN3* copies, *frigida*, *VRN1*, *EMF2*, *BvFT1*, and *BTC1* genes in 4 bolting tolerant and sensitive sugar beet genotypes were evaluated. Leaf samples were taken from plants in 2 growing stages, before and during cold exposure. The amplified fragments of both tolerant and sensitive genotypes were similar in length and the comparison of their transcript sequence showed polymorphism. In overall, 18 mutations comprising 1 mutation in *frigida* sequence, 14 single nucleotide polymorphisms (SNPs) in 2 copies of *BvVIN3* sequence, 2 SNPs in *BvFT1* sequence, and 1 insertion/deletion mutation for *BTC1* were characterized. The last one caused a frameshift in the encoded protein. Despite the key role of the above mentioned genes, results showed that SNPs identified in this study were not associated with bolting tolerance or sensitivity. There may be differences in the expression levels of these proteins, which necessitates further exploration.

Key words: sugar beet, bolting, single nucleotide polymorphism

Introduction

The transition to flowering is one of the major phase changes that a plant makes during its life cycle. In biennial roots and leaf crops such as sugar beet, the avoidance of flowering is of fundamental importance for high yields and good quality. Sugar beet (*Beta vulgaris*) is the second important sucrose-storing species in the world. Recent studies showed that sugar beet can be used as a renewable source of energy because of high dry matter production, which makes it a suitable source for methane production (Hoffmann and Kluge-Severin, 2011). Transition to the reproductive stage in sugar beet requires low temperatures (vernalization), followed by long-day conditions (Biancardi, 2005). After transitioning to the reproductive stage, the elongation of the main shoot occurs, which results in the reduction of sugar yield. In contrast, wild beet (*B. vulgaris* ssp. *maritima*) is an annual plant, which bolts without vernalization (Lexander, 1980). Genetically, the annual growth habit is under the control of a major dominant gene that has long been referred to as the bolting gene or *B* (Abegg, 1936). Plants that behave as a biennial carry the recessive alleles (*bb*), while the annual beets carry homozygous dominant (*BB*) or heterozygous (*Bb*) alleles. Apart from the major bolting locus *B*, using ethylmethanesulfonate (EMS) mutagenesis, 2 recent studies have identified 2 additional loci (*B2* and *B4*), which contribute to the annual bolting in wild beets (Abou-Elwafa et al., 2012; Büttner et al., 2010; Hohmann et al., 2005). At each of these loci, the homozygous recessive genotype bolted only after vernalization.

Pin and coworkers (2012) reported that the *B* locus encodes a pseudo-response regulator (PRR) gene named

* Corresponding author: Department of Biotechnology, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, I.R. Iran; email: a.mirzaie@basu.ac.ir

BOLTING TIME CONTROL 1 (*BTC1*), which has a homology to circadian clock gene PRR7 from *Arabidopsis thaliana* (Pin et al., 2012). In addition, they indicated that *BTC1* is a key regulator in beet life cycle (Pin et al., 2012). Downregulation of *BTC1/btc1* expression by RNA interference (RNAi) in both annual and biennial beets caused a continuous vegetative growth (Dally et al., 2014; Pin et al., 2012). In sugar beet, *BvFT1* and *BvFT2* (*FLOWERING LOCUS T* homologs) genes act antagonistically in the floral transition. *BvFT1* acts as a floral repressor, whereas *BvFT2* promotes the flowering and also downregulates *BvFT1* expression after vernalization (Pin et al., 2010). It has been shown that *BvFT1* repression was stably maintained after plants were moved to warm temperature, suggesting that *BvFT1* acts similar to *FLOWERING LOCUS C* (*FLC*) (Kim and Sung, 2014). Pin and coworkers (2012) suggested a model whereby *BvBTC1* acts upstream of *BvFT1* and *BvFT2*. Annual sugar beet carries a dominant *BTC1* allele, which promotes flowering under long days through blocking *FT1* and activating *FT2*. However, in biennials, the recessive *btc1* allele does not repress expression of the floral repressor *FT1*. Since *BvFT1* prevents *BvFT2* expression, plants stay in vegetative growth phase before an exposure to vernalization and during vernalization, therefore, *btc1* expression is increased and *BvFT1* expression is gradually decreased (Dally et al., 2014).

In *A. thaliana*, *FLC* acts as a flowering time repressor under its upstream regulator *FRIGIDA* (*FRI*) (Johanson et al., 2000). *FLC* directly represses the expression of *FT* and *SOC1* (Helliwell et al., 2006; Searle et al., 2006). *FRI* mainly acts to upregulate *FLC* transcription (Michaels and Amasino, 2001). Before the cold exposure of winter, *FLC* chromatin is in an active state where through active histone marks, e.g., histone H3 Lys4 (H3K4) and histone H3 Lys36 (H3K36) methylation as well as histone H3 acetylation are present (He et al., 2003; Kim et al., 2005; Zhao et al., 2005). A prolonged exposure to low temperatures leads to an epigenetic downregulation of *FLC* (Amasino, 2010). In *Arabidopsis*, the vernalization-mediated repression of *FLC* is established by VERNALIZATION INSENSITIVE 3 (*VIN3*) gene (Sung and Amasino, 2004), VERNALIZATION 2 (*VRN2*) gene (Le Corre et al., 2002), a homolog of a Suppressor of Zeste 12 gene (a part of a chromatin-modifying complex in Drosophila), and VERNALIZATION 1 (VRN1), a plant-specific DNA-binding protein (Levy et al., 2002). During cold exposure, *VIN3* expression is upregulated (Sung and Amasino, 2004). *VIN3* contains a plant-specific plant homeodomain (PHD) finger, which is involved in the detection of histone modifications in eukaryotes (Musselman and Kutateladze, 2011). During and after vernalization, repressive histone modifications (i.e., H3K9me2 and H3K27me3) are substantially increased at the *FLC* chromatin locus (Bastow et al., 2004; Greb et al., 2007; Sung and Amasino, 2004; Sung et al., 2006). *VRN1* is required for the methylation of H3K9 and both *VIN3* and *VRN2* are required for H3K27 methylation at *FLC* chromatin loci by vernalization (Bastow et al., 2004; Kim and Sung, 2013; Sung and Amasino, 2004). It has been shown that *VRN1* expression was increased during cold treatment and it has been maintained at high level even when plants were returned to warm temperatures (Hemming et al., 2008; Trevaskis et al., 2006; Yan et al., 2003). Abou-Elwafa and coworkers (2012) identified and mapped 4 genes (*BvFLK*, *BvFVE*, *BvLD*, and *BvLDL1*) in sugar beet that are highly similar to their *Arabidopsis* counterparts in terms of exon–intron structure and domain organization.

Since the sugar reposition period is not precisely limited and root yield continues to increase as long as the plant does not bolt, increasing the growing season of sugar beet *via* autumn planting is a basic target to improve sugar beet productivity in temperate regions (Jaggard et al., 2009; Jaggard and Werker, 1999; Jung et al., 2007). The yielding potential of winter beet has been evaluated to be ~20% higher than that of spring beet (Hoffmann and Kluge-Severin, 2011), but sowing sugar beet in autumn increases the risk of bolting. Identification of flowering-time genes and their sequencing can be useful in the development of bolting-resistant varieties. In this study, mRNA sequences encoded by flowering-time genes including *BTC1*, *BvFT1*, *frigida*, *VRN1*, *VRN2* as well as two copies of *VIN3* of bolting tolerant and sensitive genotypes of sugar beet were evaluated.

Materials and methods

Plant material

In this study 2 bolting sensitive (OT7112 and MS261) and 2 bolting tolerant (Vico and Posuda) sugar beet genotypes were used. Seeds were provided by the Sugar Beet Seed Institute and they were planted in July at the Re-

search Agricultural Farm in the suburbs of Hamedan, Iran. Seeds were planted on August 15th, 2013 and the first sampling was performed on September 24 before cold imposition and the second sampling was performed on January 4th, 2014 during cold imposition. Leaf samples were collected, immediately frozen in liquid N, and stored at −70°C for RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the sugar beet leaves using RNX plus solution (CinnaGen Company) according to the manufacturer's instructions. All RNA preparations were treated with RNase-free DNase to remove any genomic DNA contamination. The amounts of RNA were determined by the spectrophotometric method. cDNA fragments were synthesized from 6 µg of total RNA using oligo $(dT)_{12\text{-}18}$ primer and M-MuLV reverse transcriptase kit (CinnaGen Company).

Primer design

We used *Arabidopsis thaliana* genes that are involved in the flowering-time control as a reference to identify the homologous counterparts in sugar beet GenBank sequences in NCBI (Shojaei et al., 2017). Using Primer Blast software, 9 primer pairs were designed for *BvFT1*, *Bvfrigida*, *BvVIN3*, *BvVRN1*, *BvVRN2*, and *BTC1* genes from the identified homologs (Table 1) and were synthesized by Bioneer company (Seoul, Korea, Table 1).

RT-PCR conditions

For the mRNA sequence comparison of genes selected from both sensitive and tolerant genotypes, cDNA from samples collected before the cold exposure treatment were used as a template for *Bvfrigida* and *BvFT1* amplification and cDNA from samples collected during the cold exposure were used as a template for *BvVIN3*, *BvVRN1*, *BvVRN2*, and *BTC1* amplification, respectively. PCR was performed in 25 µl 1X PCR buffer containing 20ng cDNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer, and 1U of Pfu DNA polymerase. The cycle parameters in the PCR program were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 seconds, 56-61°C for 30 seconds, 72°C for 2 min followed by a final extension at 72°C for 7 min. About 5 µl of each PCR product was run on a 2% agarose gel in TBE buffer. Amplified fragments were sent for sequencing to the Bioneer Company in South Korea.

Sequencing PCR products

Sanger sequencing was performed at least twice for each individual primer. The sequencing results were explored in the Reference Sequence (RefSeq) database using BlastX software. Results were also analyzed by the SaqMan program of the Lasergene software. Single nucleotide polymorphisms (SNPs) and point mutations were identified by comparing the sequence chromatograms. mRNA sequences of flowering-time genes deposited in the NCBI database [BioProject: PRJNA268352] were used for the comparison and determination of SNPs. Protein secondary structures were evaluated through (PS)2v2: Protein Structure Prediction Server database (http://ps2.life.nctu.edu.tw).

Results

A comparison of the amplified mRNA fragments of some flowering genes from all genotypes indicated the differences among their nucleotide sequences. We observed heterozygous alleles in some genotypes, in both C and G nucleotides at position 196 in *BvFT1* mRNA (Table 2). The fragment amplified by VRN2 primer pair had 100% similarity to EMBRYONIC FLOWER 2 (EMF2) protein in the sugar beet. In this study, the focus was mostly given to the comparison of the coding regions of mRNA sequences in key flowering genes between the bolting sensitive and resistant genotypes. Using Lasergene SaqMan Pro software, the lengths of *EMF2* mRNA sequence, about 860 of 1443 bp including the coding sequence, in 4 genotypes and the length of polycomb group protein *EMF2* mRNA [GenBank: XM_010695441] were compared. No SNPs were observed among the tested genotypes. Either, there was no polymorphism detected in the 300 bp fragment of 1013 bp of *BvVRN1* mRNA sequence containing B3 domain among genotypes. The sequence was similar to *BvVRN1* mRNA.

Across 5 genes, namely VIN3-like protein1 mRNA (*BvVIN3-1*), VIN3-like protein2 mRNA (*BvVIN3-2*), *BvFRI*, *BvFT1*, and *BTC1*, a total of 18 SNPs were identified (Table 2); consisting of six SNPs in 722 bp fragment of *BvVIN3-1* and 8 SNPs in about 1800 bp sequence compared to *BvVIN3-2*. The results of the generated protein translation alignment to the DNA sequences showed that the changes in VIN3-like protein1 and VIN3-like protein2 mRNAs did not cause any changes in

Table 1. List of primers used in this study

Primer	Accession no.	Primer sequences (5′–3′)	Annealing temperature
BvFT1 forward	HM448910	GTGAAGTATTCCTTAGCTTTCTAGC	56
BvFT1 reverse		GTTCATTGAAATGGAGAGGTGGA	
BvFRI forward	AVZW01032897	TACTCAGTCCGTTGGTGTGC	56
BvFRI reverse		ACGTTTTGCCAGAGTGTCCA	
BvVIN3-1 forward	AYZW01007287	CCAAGATATGTGTGCCGTCCT	58
BvVIN3-1 reverse		GCGGGACCTTGGGAAAATCT	
BvVIN3-2 forward	AYZW01053336	CGGTGCTGCAGAAATGCTAC	56
BvVIN3-2 reverse		CTGCTCTGAGAGGGATGCTG	
BvVIN3-2/2 forward		AGCCTTCTGATGTCGTTGAT	58
BVVIN3-2/2 reverse		TGCTGGGCTCTGAACTCTTT	
BvVRN1 forward	JP530325	CCCAAACAAACAAAGAAGA	
BvVRN1 reverse		TGATATTTCACAAGCAGTCG	61
BvVRN2 forward	JP493909 JP493908 JP493907 JP493912	TCCTGTTTTTGGCAACGCTC	
BvVRN2 reverse		CTTGGATTGAGGACCGGCAA	
BTC1-1 forward	HQ709093	AAAATGAAGATGGGCCCGGT	62
BTC1-1 reverse		ACACGAGGTCTTTGATCTGCT	
BTC1-2 forward		TGGATCCATGCAGGAACTTGAT	60
BTC1-2 reverse		ACACGAGGTCTTTGATCTGCT	

the amino acid sequences of proteins, which therefore can be considered as synonym mutations.

A comparison of 1250 bp segment of *BvFRI* mRNA sequence resulted in the identification of 1 SNP (Table 2). Protein translation demonstrated that the nucleotide substitution of guanine or adenine at position 614 of *BvFRI* mRNA causes a change to alanine or threonine. Substituting alanine with threonine and vice versa did not result in any changes in the secondary structure of protein.

A comparison of 630 bp sequence of *BvFT1* mRNA including the coding region allowed identifying 2 transversion mutations (Table 2). If the nucleotide at position 196 is guanine, it would be translated to a glutamate amino acid; while if a nucleotide in this position is replaced by cytosine, the translated amino acid would be glutamine. If the nucleotide at position 433 is replaced by guanine, it would be translated to serine and if the position is replaced by cytosine, it would be translated to threonine. Protein translation and secondary structure prediction was done in 4 forms: guanine$_{196}$- guanine$_{433}$ (G_{196}-G_{433}), guanine$_{196}$- cytosine$_{433}$ (G_{196}-C_{433}), cytosine$_{196}$- cytosine$_{433}$ (C_{196}-C_{433}), and cytosine$_{196}$- guanine$_{433}$ (C_{196}-G_{433}). Protein structure prediction showed a change in C_{196}-C_{433} when compared with other abovementioned forms (Fig. 1). To determine the effect of this mutation on bolting tolerance, *BvFT1* mRNA comparison was performed in further bolting-resistant genotypes including Monotana and Giada. SNPs observed in these genotypes were similar to the bolting-sensitive genotypes.

A comparison of 2225 bp sequence of *BTC1* mRNA identified 1 deletion with a frameshift change at the end of the related protein (Table 2). This deletion was identified in 7112 and 261 bolting-sensitive genotypes and in the bolting-resistant Monotana genotype.

Discussion

At the time of conducting this study, the sugar beet genome was released and the complete genomic sequence of double haploid sugar beet line KWS2320, as a reference genotype, was reported (Dohm et al., 2014).

Table 2. Identification of SNPs in selected flowering-time genes in both bolting sensitive and tolerant genotypes

Gene	Reference gene*	mRNA length (bp)	Nucleotide number	Modified amino acid	Nucleotide type for position mutation in each genotype					
					7112	261	Vico	Pusoda	Monotana	Giada
BvVIN3-1	XM_010689861	2949	2107	no	Y (C or T)	T	W (A or T)	R (A or G)	–	–
BvVIN3-1	XM_010689861	2949	2344	no	Y (C or T)	C	Y (C or T)	Y (C or T)	–	–
BvVIN3-1	XM_010689861	2949	2362	no	R (A or G)	A	R (A or G)	R (A or G)	–	–
BvVIN3-1	XM_010689861	2949	2395	no	C	C	Y (C or T)	Y (C or T)	–	–
BvVIN3-1	XM_010689861	2949	2518	no	C	C	Y (C or T)	T	–	–
BvVIN3-1	XM_010689861	2949	2572	no	G	G	A	A	–	–
BvVIN3-2	XM_010690174	2704	608	no	T	T	W (A or T)	T	–	–
BvVIN3-2	XM_010690174	2704	847	no	R (A or G)	G	G	R (A or G)	–	–
BvVIN3-2	XM_010690174	2704	1264	no	A	R (A or G)	G	R (A or G)	–	–
BvVIN3-2	XM_010690174	2704	1548	no	T	T	Y (C or T)	T	–	–
BvVIN3-2	XM_010690174	2704	1798	no	Y (C or T)	T	T	Y (C or T)	–	–
BvVIN3-2	XM_010690174	2704	1864	no	Y (C or T)	C	C	Y (C or T)	–	–
BvVIN3-2	XM_010690174	2704	2042	no	C	M (A or C)	C	C	–	–
BvVIN3-2	XM_010690174	2704	2164	no	T	A	A	A	–	–
BVFT1	XM_010692083	902	196	yes	G	S (C or G)	S (C or G)	S (C or G)	G	S (C or G)
BVFT1	XM_010692083	902	433	yes	G	G	S (C or G)	S (C or G)	G	G
BvFRI	XM_010680700	2303	614	yes	R (G or A)	G	R	G	–	–
BTC1	HQ709093	2367	2222	yes	–	–	T	–	–	–

*sequences have been used as a reference for the comparison and determination of SNPs

We deposited *Bvfrigida* mRNA sequence with the accession number KJ755196.1 and *BvVIN3* mRNA sequence with the accession number KJ755197.1 in the GenBank database (unpublished data) before the sugar beet genome release. The sugar beet genome sequence provided a better comparison of flowering key gene sequences. After sugar beet genome release, we noted that the fragment amplified by VRN2 primer pair had 100% similarity to EMBRYONIC FLOWER 2 protein in the sugar beet. This protein contains VEFS-box domain, which also exists in VRN2, FIS2, and Su(z)12 proteins (Birve et al., 2001; Gendall et al., 2001; Köhler and Grossniklaus, 2002; Luo et al., 1999; Yoshida et al., 2001). *EMF2* mRNA is expressed throughout the life cycle of *Arabidopsis* without any significant changes during its growth (Köhler and Grossniklaus, 2002).

In the sugar beet genome database at NCBI, 3 *BvVIN3* sequences have been deposited. *VIN3-like 1* is located on chromosome 8 while 2 *VIN3-like 2* genes are located on chromosomes 1 and 8. These 2 *VIN3-like 2* mRNAs have only 17% coverage with 68% identity in the nucleotide blast result. VIN3 is a PHD motif containing protein. It has been shown that *VIN3* mRNA levels directly correlate with the vernalization response (Sung and Amasino, 2004). *VIN3* is necessary for both histone H3 Lys 9 and histone H Lys 27 methylation at the *FLC* chromatin locus. Kim and Sung (2010) introduced a point mutation in the PHD finger motif of VIN3-LIKE 2 (VIL2) in *Arabidopsis*. This mutation resulted in the substitution of 1 cysteine residue, which is important for finger structure formation with alanine preventing VIL2 from binding to modified histone peptides (Kim and Sung, 2010). PHD is vital for finger binding specificity (Kim and Sung, 2010; Li et al., 2007). In the present study, 2 copies of *VIN3* mRNA in sugar beet were investigated and 12 SNPs (Table 2) were identified, but none of them resulted in any changes in the specific domain or the conserved regions of protein sequences.

The dominant *FRI* gene in *Arabidopsis* upregulates the *FLC* expression and as a consequence delays the flowering. Mutations occurring naturally in *FRI* locus cause early flowering without vernalization in most *Arabidopsis* accessions (Johanson et al., 2000; Strange et al., 2011). Although the mutation in the *FRI* gene in *Arabidopsis* has a major contribution to flowering-time variations, the comparison of 1250 bp of *BvFRI* mRNA sequence among 4 sugar beet genotypes identified a single point mutation [*BvFRI* (A_{614}) or (G_{614})]. However, this SNP was not related to bolting as 2 nucleotide types (G or A) for this position mutation were observed in both sensitive and tolerant genotypes (Table 2). In all genotypes, GCG or ACG code is translated to alanine or threonine amino acid, respectively. Substitution in the aforementioned amino acids did not create any changes in the protein secondary structure. Although *FRI* influences bolting in *Arabidopsis* (Le Corre et al., 2002), it appears that mutants identified in this gene, especially in our study, have no association with bolting tolerance in sugar beet.

BvFT1 is known to be a flowering suppressor and its expression is downregulated by the vernalization (Pin et al., 2010). Frerichmann and coworkers (2013) identified a single point mutation in exon 4 of *BvFT1* by EcoTILLING method in fodder beet, leaf beet, garden beet, and sugar beet (Frerichmann et al., 2013). However, they did not detect any haplotype variation effect of *BvFT1* on the bolting rate (Frerichmann et al., 2013). In our study, 2 SNPs were identified in nucleotide positions 196 and 433 when compared with *BvFT1* mRNA deposited in the GenBank database. The mutation at the nucleotide position 196 caused substitution of glutamine with glutamate amino acid and the mutation in nucleotide position 433 caused substitution of threonine with serine. The protein structure prediction showed a change in alpha helix in positions C_{196}-C_{433} (Fig. 1). It was reported that *BvFT1* downregulation after vernalization remained constant even after returning the plant to warm temperatures, indicating that *BvFT1* acted similar to *FLC* (Pin et al., 2010). Considering the key role of *BvFT1* in sugar beet, which is similar to *FLC* in *Arabidopsis*, it is plausible that the response to bolting among sugar beet genotypes originates in different FT proteins. However, due to the occurrence and position of mutations in both tolerant and sensitive genotypes it seems that mutation positioning has no relation to the bolting resistance.

One-nucleotide ins/del mutation was identified in *BTC1* mRNA sequence. In Vico genotype at there was T at position 2222 present, but a lack of nucleotide was observed in other genotypes. *BTC1* encodes a protein that contains a response regulator receiver (REC) and a CONSTANS, CO-like, and TOC1 (CCT) domain (Dally et al., 2014; Pin et al., 2012). In annuals, *BTC1* represses *BvFT1* and activates *BvFT2* to promote the bolting and flowering (Dally et al., 2014; Pin et al., 2012).

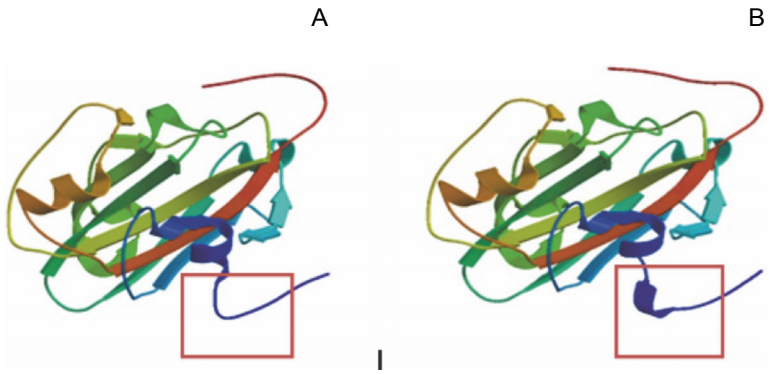

Fig. 1. Protein structure prediction of *BvFT1* SNPs: A) in C_{196}-C_{433} form, B) in (G_{196}-G_{433}), (G_{196}-C_{433}), and (C_{196}- G_{433}); the red box indicates the difference

Beets carrying the dominant *BTC1* allele display an annual growth habit, while the biennial beets have recessive *btc1* allele. Pin and coworkers (2012) surveyed *BTC1* allele in haploid annual and biennial beets. *BTC1* allele in annual and biennial beets differed by 11 non-synonymous SNPs and ~28 kbp insertion in 5′UTR region, which was present only in biennial beets (Pin et al., 2012). In 3 biennial haploid beets, 2 transition mutations were identified in exons no. 7 and 8, respectively (Pin et al., 2012). Frerichmann and coworkers (2013) identified 2 point mutations in noncoding regions of *BTC1* by EcoTILLING (Frerichmann et al., 2013). Although *BTC1* is known as a bolting promoter without proceeding to the vernalization requirement (Pin et al., 2012), in our study, 1 deletion that caused a frameshift change at the end of the protein was identified in bolting-sensitive genotypes 7112 and 261 and in the bolting-resistant Monotana genotype. For *BvFT1* and *BTC1* no significant difference was observed among their fragments in bolting sensitive or resistant genotypes.

Despite the key role of the studied genes, results have shown that the identified SNPs were not associated with the bolting tolerance or sensitivity. There may be differences in the expression levels of these proteins, but it requires further exploration.

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