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RESEARCH ARTICLE

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A new polymorphism on chromosome 6 associated with bolting tendency in sugar beet

Chiara Broccanello¹, Piergiorgio Stevanato^{1*}, Filippo Biscarini², Dario Cantu³ and Massimo Saccomani¹

Abstract

Background: Premature flowering or bolting is an undesirable characteristic that causes severe sugar yield losses and interferes with harvesting. Vernalization is a prerequisite for the floral induction, achieved by exposure to low temperatures for 10–14 weeks. This process is also controlled by other environmental factors, such as long daylight photoperiods and a combination of genetic factors. The objective of this study was the identification of new genetic polymorphisms linked to bolting tendency in sugar beet.

Results: Two pollinators characterized by low and high bolting tendency were subjected to RAD-sequencing in order to detect discriminating SNPs between lines. 6,324 putative SNPs were identified. Of these, 192 were genotyped in a set of 19 pollinators, each comprising bolted and non-bolted individuals, for a total of 987 samples. Among the 192 candidate SNPs, the strongest overall association was found for SNP183 on chromosome 6 (p -value = 1.246×10^{-13}). The association between SNP183 and bolting tendency was then confirmed in an independent population of 730 plants from 11 breeding lines (p -value = 0.0061). SNP183 is located in the intron of *Bv_22330_orky*, a sugar beet homolog of a matrix metalloproteinase (MMP) gene that could be implied in flowering in *Arabidopsis thaliana*.

Conclusion: Our data support a significant association between an intronic SNP in the MMP gene located on chromosome 6 and the regulation of bolting tendency in sugar beet. The newly identified locus supports the polygenic nature of flowering control. The associated marker can be used to design SNP panels for the discrimination of bolters and non-bolters, to be used in sugar beet breeding programs for the development of improved germplasm with low bolting tendency.

Keywords: Bolting tendency, RAD sequencing, SNP association, Molecular breeding, *Beta vulgaris*

Background

For an effective genetic improvement of sugar beet (*Beta vulgaris* L.) it is critical to gain a better understanding of the biological processes behind the switch from vegetative growth to floral induction [1]. Premature flowering or bolting is an undesirable characteristic that causes severe sugar yield losses and interferes with harvesting [2]. Under field conditions, cultivated sugar beet is a biennial plant that requires two full growing seasons to switch from the vegetative phase to bolting. Vernalization is a prerequisite for the floral induction, achieved by exposure to low

temperatures for 10–14 weeks [3]. This process is also controlled by other environmental factors, such as long daylight photoperiods and a combination of genetic factors [4]. Sugar beet bolting tendency is known to be influenced genetically by the *B* locus, mapped on chromosome 2 [5–7]. Homozygous plants at the *B* locus (*BB*) initiate bolting under long day conditions whereas plants carrying recessive alleles in the homozygous state (*bb*) need vernalization for floral induction. Environmental and genetic factors strongly influence heterozygous plants (*Bb*) that show a more complex behaviour [5, 6, 8, 9]. *Bb* plants bolting without vernalization show a delay in bolting time compared to *BB* individuals [10]. The *B* locus was recently found to correspond to the *BOLTING TIME CONTROL 1 (BTC1)* gene. Biennial plants, which do not flower without a period of vernalization, carry a partial

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loss of function *BTC1* allele. A second locus (*B2*) mapped on chromosome 9 and acting epistatically with the *B* locus was also associated with bolting behaviour. *BvBBX19*, encoding a DOUBLE B-BOX TYPE ZINC FINGER protein B-box transcription factor was found to underlie the *B2* locus [11, 12].

Given the known complexity of floral regulation in model species it is likely that additional genes influence bolting behavior in sugar beet [2]. In *Arabidopsis thaliana*, *FLOWERING LOCUS C (FLC)*, *CONSTANS (CO)*, and *FLOWERING LOCUS T (FT)* are key genes controlling flowering. Similar genes also exist in sugar beet: *BvFL1* on chromosome 6 [13], *BvCOL1* on chromosome 2 [14], and *BvFT1* and *BvFT2* on chromosomes 9 and 4, respectively [15]. *BvFT1* and *BvFT2* are major regulators of bolting in beet [15] and act downstream of the *B* and *B2* locus genes *BTC1* and *BvBBX19* [12, 16]. The *FLC*-like gene *BvFL1* is a floral repressor. Its expression is down regulated during a prolonged cold period under long daylight condition [13]. Similarly, *CO*-like gene *BvCOL1* reinforces the late flowering phenotype [14]. The functional role of the *FLC*-like and *CO*-like genes suggests a partial evolutionary conservation in the regulation of floral transition between *Arabidopsis* and sugar beet [17].

Due to the highly complex interactions between genotype and environment, initial progress in bolting resistance was obtained by selecting varieties specific for the climates where they would be grown [18]. Selection was based solely on phenotypic observations by discarding early bolting plants, which were considered dominant heterozygous or homozygous at the *B* locus.

The use of molecular markers can facilitate the detection of unfavorable alleles linked to the bolting tendency, allowing for earlier and more precise selection of non-bolters. Single Nucleotide Polymorphisms (SNPs) are ideal markers for this kind of work since they are spread throughout the genome and represent 90 % of sequence variation among plants [19]. SNP markers have already been applied in sugar beet breeding programs [20]. Additionally, technical progress and the cost reduction of next-generation sequencing (NGS) technology can facilitate the identification of a large number of SNPs in any genomic region of interest [21, 22]. Among NGS techniques, Restriction-site Associated DNA (RAD) sequencing allows the discovery of several thousands of genetic variants adjacent to restriction enzyme cleavage sites across a target genome [5].

In this paper we suggest the identification of a new putative locus involved in the genetic determination of bolting tendency in sugar beets. Two sugar beet pollinators, P1 and P2, characterized respectively by early- and late-bolting habit were subjected to RAD-SNP discovery. 192 SNPs were selected for further SNP association analysis. These SNPs were genotyped on a set of 19 pollinators, each

comprising bolted and non-bolted individuals, for a total of 987 samples. The association between SNP genotypes and bolting tendency was tested by fitting one SNP at a time in a logistic regression model. A SNP marker associated with bolting tendency was located on chromosome 6. This SNP was then tested in an independent sugar beet population. The novel associated polymorphism provides further indication of the polygenic nature of bolting tendency in sugar beet.

Results

SNP discovery

RAD sequencing of the two DNA bulks, including (respectively) 4 non-bolted P1 and four bolted P2 plants, produced 96,822,109 raw reads of which 81,031,436 (84 %) were of high quality (longer than 100 nt) with an average length of 103.26 nt. RAD paired end sequence assembly was created using the P1 reads. Sequences from the P2 bulk were aligned to reference assembly for P1 using Bowtie (parameter: bowtie -f -v1). The aligned reads revealed a total of 288,843 (~150× coverage) unique consensus RAD tags common between the two bulks. The SNP discovery pipeline highlighted a total of 6,324 SNPs. Contigs were aligned to the sugar beet reference genome (RefBeet-1.1; <http://bvseq.molgen.mpg.de>) to exclude SNPs with nearby flanking polymorphisms within 50 bp. A total of 192 polymorphic SNP between bulks, randomly distributed within and across all chromosomes, were selected for the SNP association analysis. The array of 192 SNPs used in this study along with their corresponding sequences are available as Additional file 1: Table S1.

SNP genotyping and association mapping

192 SNPs were genotyped on 987 samples from 19 pollinators each comprising both non-bolted and bolted individual plants. The relationship between SNP genotypes and bolting phenotypes was modeled with logistic regression. Among the 192 candidate SNPs, the only significant association was found for SNP183 on chromosome 6 ($P = 1.2 \cdot 10^{-13}$). Table 1 reports the analysis of deviance from the logistic regression model (see equation 1 in Methods section) for SNP183. From logistic regression, the probabilities for each plant, based on the population they belong to and their genotype at SNP183, of either showing or not bolting tendency were obtained. Figure 1 shows the distribution of such probabilities for the three genotypes at locus 183.

To obtain the NCBI Reference Sequence ID for SNP183, a 440 bp long segment centering on SNP183 was PCR amplified, sequenced by a Sanger sequencing platform (ABI 3730xl) and blasted on NCBI. The resulted NCBI ID was XM_010697593.1.

Table 1 Analysis of deviance table for a logistic regression model with the effects of pollinator population (19 classes) and genotypes at SNP183 on chromosome 6

| | Df | Deviance | Residual Df | Residual Deviance | p-value |
|------------|----|----------|-------------|-------------------|----------------|
| NULL | | | 929 | 1286 | |
| Population | 18 | 173.01 | 911 | 1113 | 2.3 10^{-27} |
| SNP183 | 2 | 59.43 | 909 | 1053 | 1.2 10^{-13} |

SNP183 was mapped in the sequence of the single intron present in the *Bv_22330_orky* gene and it was not mapped in any gene known to be involved in bolting (Christian Jung, pers. comm.). As shown in Methods, SNP183 does not cosegregate with the *BTC1* locus on chromosome 2. In addition, though both on chromosome 6, SNP183 and *BvFL1* are on different (not anchored) scaffolds (*Bvchr6_un.sca007* and *Bvchr6.sca027*, respectively). Further studies are needed to clarify if SNP183 and *BvFL1* could co-segregate.

The frequency of the CC genotype was significantly increased in the bolting group (17 % vs. 5 %; $P = 4.4 \cdot 10^{-7}$), while the TT genotype was significantly higher in the non-bolting group (67 % vs. 49 %; $P = 1.8 \cdot 10^{-6}$) (Table 2). The two alleles of the SNP183 and the flanking sequences on each side of the SNP are reported in Additional file 1: Table S1. The sequences of the primers and TaqMan probes designed for the detection of the SNP183 are also given in Additional file 2: Table S2.

The location of SNP183 along the *Bv_22330_orky* gene sequence is shown in Fig. 2. The total length covered by the coding exons is 133 bp and 585 bp and the total length of the intron is 419 bp.

Bv_22330_orky encodes a putative Matrix Metalloproteinase (MMP) causing late flowering and early senescence in *Arabidopsis thaliana*. In sugar beet, four genes are annotated as MMPs gelatinase A based on the recently annotated genome [23]: *Bv5_099660_fneg*, *Bv1u_021120_ykma*, *Bv_22320_wuom* and *Bv_22330_orky*.

Five MMPs similar to *Bv_22330_orky* were found in *Arabidopsis thaliana* by BLASTP homology searches, as already reported in Golldack et al. [24]. We constructed a phylogenetic tree based on the NJ (neighbour-joining) method, using the full-length protein alignment (Fig. 3). Phylogenetic analysis shows the tight clustering, in a separate clade, of *Bv_22320_wuom* and *Bv_22330_orky* with 100 % bootstrap support.

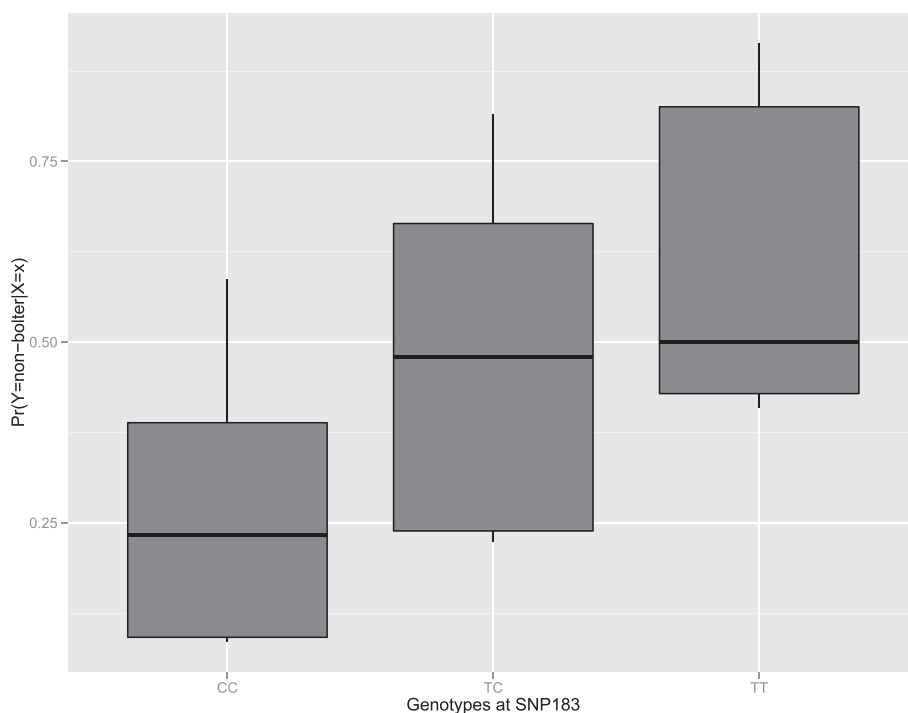
**Fig. 1** Boxplot of the distribution of probabilities of showing either high or low bolting tendency for the three genotypes at SNP locus 183 (CC, CT, TT) based on a logistic regression model

Table 2 Genotype frequencies of SNP183 on bolting and non bolting individuals

| | Bolting individuals (n = 436) | | Non bolting individuals (n = 495) | | χ^2 | | <i>p</i> -value | |
|--------|-------------------------------|----|-----------------------------------|----|----------|-------|-----------------|--|
| | n | % | n | % | n | % | | |
| SNP183 | | | | | | | | |
| TT | 214 | 49 | 332 | 67 | 22.8 | 1.8 | 10^{-6} | |
| TC | 150 | 34 | 138 | 28 | 0.5 | 0.479 | | |
| CC | 72 | 17 | 25 | 5 | 25.5 | 4.4 | 10^{-7} | |

Testing SNP183 in an independent sugar beet population

The SNP183 was genotyped in 730 individual plants from 11 breeding lines. A TaqMan assay was developed to discriminate rapidly and reliably between the C and T alleles at SNP183 locus. The frequency of the dominant C allele was 66 % in the bolting group and 46 % in the non-bolting group. Based on these results, individual plants carrying the C allele associated to high bolting tendency were discarded from the breeding program. The association between SNP genotype and bolting behavior was tested with a logistic regression model and was mildly significant ($P = 0.0062$).

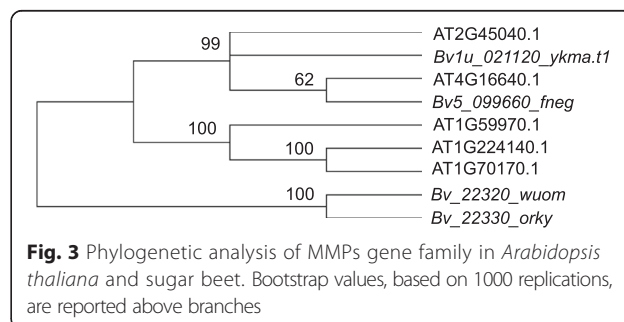
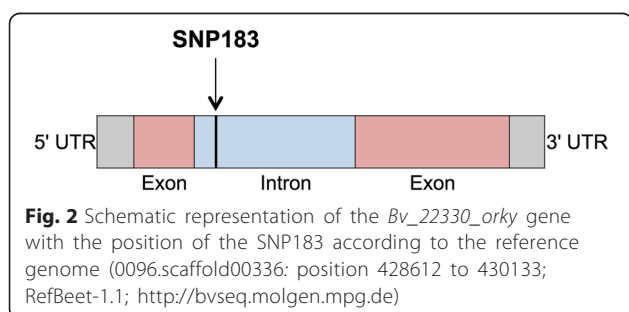
Discussion

This study revealed a significant association between the polymorphism SNP183 on chromosome 6 and bolting tendency in sugar beet. The association was first detected in a population of 19 pollinators, where SNP discovery and association studies were carried out. Later, the association was tested in an independent population of 11 breeding lines. In both cases, the association between SNP183 genotypes and bolting behavior was significant. This suggests the presence of a new putative locus for bolting control on chromosome 6 of the sugar beet genome, which has not been reported, yet. This marker can be used in marker-assisted selection (MAS) programs to select for bolting resistance in sugar beets. MAS approaches to the reduction of bolting tendency are highly desirable in sugar beet breeding, since they are more efficient, faster, and often more reliable and less expensive than phenotypic selection, and allow to breed for complex traits like resistance to bolting. Bolting tendency is a complex trait controlled by

environmental and developmental cues and multiple genetic loci [25]. The intricate network of regulatory pathways reflects complexity of the flowering process, with the vernalization, photoperiod, autonomous and gibberellic acid pathways and the circadian clock all contributing to the control of flowering [1, 26]. Given this complexity, multivariate statistical approaches to combine different sources of information are recommended for breeding applications to reduce bolting tendency in sugar beet. Previous attempts to model genomic predictions for binary traits in sugar beet have been reported [20, 27], and could be applied to the likewise binomially distributed bolting behavior. SNP183 can therefore potentially be used to design a SNP panel which includes polymorphisms from genomic associated with bolting tendency in sugar beet and that can differentiate bolters from non-bolters.

SNP183 was mapped to the intron sequence of the sugar beet gene *Bv_22330_orky*. While this gene may play a role in bolting control, which has not been previously reported in sugar beet, the SNP183 may actually be in linkage disequilibrium with neighbouring genes associated to bolting tendency. Besides being a marker linked to a gene involved in bolting behaviour, SNP183 -though less likely- could actually have a biological role itself: it can be a silent informative mutation that modifies splicing, if located in the donor/acceptor splice site; or it could affect the micro RNA binding.

Bv_22330_orky was found to code for a matrix metalloproteinase (MMP). MMPs are a family of zinc and calcium dependent proteases and are divided into three subfamilies: gelatinases, collagenases and stromelysins [28]. Human MMPs play important roles in many physiological



processes such as embryogenesis and organ morphogenesis. The unregulated MMPs activity is involved in the development of cancer, and neurodegenerative, cardiovascular and autoimmune disorders [29]. The diversity of functions inside mammalian MMPs derives from tandem duplication events and exon shuffling which took place during evolution. Most of the actual MMPs derive from a single gene cluster, conserved from amphibians to mammals. Plant MMPs are secreted during growth, development and stress response and play an important role in the degradation of extracellular matrix [30]. In *Arabidopsis*, MMPs is a family of proteins that could be implied in flowering [24] and, as it was found also in cucumber, are involved in the apoptosis [31]. In tobacco, they are expressed during senescence and the response to pathogens [32]. In sugar beet, we found two tandem-duplicated MMP genes with 69 % sequence similarity at DNA level. The gene duplication event, in *Bv_22330_orky*, led to the loss of the first 220 bp. This is also found in rice, where in duplicated blocks, DNA segment loss occurred with high frequency [33]. Tandem duplications are the most important events that generate new members of family proteins during evolution, generating novelty that may be selected in response to environmental changes [34].

Today, molecular markers are used to evaluate sugar beet germplasm only for the presence of annual bolters [3]. Several polymorphisms in *BTC1* are able to discriminate between the annual or biennial habit of sugar beet [16]. However, these markers do not differentiate among biennial beets characterized by either high or low bolting tendency after exposure to a period of cold temperatures, suggesting that other (modifying) genes (and/or yet undiscovered polymorphisms in *BTC1*) affect bolting tendency in cultivated biennial sugar beets. Therefore, a next challenge is the discovery of additional DNA polymorphisms associated with this trait. As a first specimen of such polymorphism, SNP183 on chromosome 6 can be used -together with other- polymorphisms as a tool to improve selection efficiency and accelerate the development of novel sugar beet varieties displaying low-bolting tendency.

Conclusions

Our study provides indication for the association of a DNA polymorphism on chromosome 6 with bolting tendency in sugar beet. The results support the polygenic nature of flowering control in sugar beet confirming the importance of previously reported QTLs. The SNP183, together with other associated polymorphisms, could assist breeding programs aimed at developing germplasm with low bolting tendency. Further studies on this gene will provide new insights into genetic mechanisms of bolting, which are needed to breed for bolting resistance in sugar beet.

Methods

Plant material

The plant material used in this study was provided by the Department of Agronomy, Food, Natural Resources, Animals, and Environment, University of Padova (DAFNAE, Università degli Studi di Padova, Italy). For SNP discovery, two sugar beet pollinators, P1 and P2, characterized respectively by early- and late-bolting habit, were subjected to RAD-sequencing. The majority of P1 plants started to bolt 5 weeks from sowing while P2 plants started to bolt much later (at 15 weeks) after vernalization and in long-daylight conditions. Both P1 and P2 pollinators carrying the allele for biennial habit at the *BTC1* locus in the homozygous state [16].

For SNP association analysis, 19 sugar beet pollinators segregating for bolting tendency were evaluated. Approximately 1000 seeds per pollinator were sown early (February 22, 2013) in a randomized block design at the Experimental Farm of the University of Padova. As expected, several plants for each pollinator died due to cold stress during the early seedling stage. The surviving plants were inspected every week for onset of bolting until June 30, 2013. Every week plants showing stem elongation were scored as bolting individuals while plants that did not show stem elongation were classified as non-bolting individuals [7]. A leaf sample was collected from each plant. Plants were divided into a group of non-bolted individuals and a group of bolted individuals for a total of 987 samples (Table 3).

SNP discovery

High-quality genomic DNA, from the parental lines (P1 and P2) used for discovery of markers, was extracted from leaf tissue following the procedure described by Stevanato et al. [35]. DNA samples were quantified on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, USA). RAD sequencing was performed on two DNA bulks containing respectively 4 non-bolted P1 and 4 bolted P2 plants. All steps, including library preparation, were carried out by Floragenex (Eugene, OR) following the protocol described by Baird et al. [22] and Stevanato et al. [35]. Sequencing was performed on an Illumina HiSeq2000 platform. Raw sequences were trimmed to remove low quality reads, resulting from base-duplication calling, and those that lacked a correct barcode. The reads obtained were compared between the two bulks and the monomorphic sequences were removed. Only sequences with one nucleotide variation between the high and low bolting tendencies and mapped to the reference genome (version RefBeet-1.1; <http://bvseq.molgen.mpg.de>) were retained.

SNP genotyping and association mapping

A set of 192 randomly distributed SNPs was selected for SNP association analysis. These SNPs were tested on a

Table 3 Sugar beet pollinators used for SNP association analysis

| Name | Total number of individuals (n) | Number of bolting individuals (n) | Number of non-bolting individuals (n) |
|-------|---------------------------------|-----------------------------------|---------------------------------------|
| 101 | 20 | 10 | 10 |
| 102 | 20 | 10 | 10 |
| 103 | 20 | 10 | 10 |
| 104 | 88 | 13 | 75 |
| 105 | 90 | 15 | 75 |
| 106 | 88 | 29 | 59 |
| 107 | 47 | 10 | 37 |
| 108 | 94 | 29 | 65 |
| 109 | 20 | 10 | 10 |
| 110 | 95 | 65 | 30 |
| 111 | 20 | 10 | 10 |
| 112 | 20 | 10 | 10 |
| 113 | 94 | 64 | 30 |
| 114 | 96 | 66 | 30 |
| 115 | 20 | 10 | 10 |
| 116 | 20 | 10 | 10 |
| 117 | 20 | 10 | 10 |
| 118 | 95 | 64 | 31 |
| 119 | 20 | 10 | 10 |
| Total | 987 | 455 | 532 |

set of 19 pollinators, each comprising bolted and non-bolted individuals, for a total of 987 samples. Genotyping was performed using the Quant Studio 12 K Flex Real-Time PCR System and Open Array technology (Life Technologies, CA, USA). The PCR reaction was prepared using 2.5 μ l of genomic DNA, at a concentration of 10 ng μ l⁻¹, added to 2.5 μ l of TaqMan OpenArray Genotyping Master Mix in a 384 well-plate. Samples from 384 well plate were loaded in the Open Array plate using the AccuFill system. The association between SNP genotypes and bolting tendency was tested by fitting one SNP at a time in a logistic regression model. A logit link function was used in a generalised linear model of the following form:

$$\begin{aligned} \text{logit}(p(x_i)) &= \log\left(\frac{p(x_i)}{1-p(x_i)}\right) \\ &= \mu + \text{population}_k + z_{ij}\text{SNP}_j \end{aligned} \quad (1)$$

where $\text{logit}(p(x_i))$ is the log-odds of the probability p for plant i of having either high or low bolting tendency; μ is the overall trait mean, population_k and SNP_j are the fixed effects of plant population k (19 classes) and SNP locus j , with z_{ij} an indicator variable for the genotype of plant i at locus j (0, 1 and 2 for AA, AB and BB).

Testing the detected association in an independent sugar beet population

The detected SNP-bolting association was tested in an independent annual beet population. The SNP183 was genotyped in 730 individual plants from 11 breeding lines. A TaqMan assay was developed to discriminate rapidly and reliably between the C and T alleles at SNP183 locus. All 730 plants were subjected to long photoperiod (16 h light / 8 h darkness) and 20.8 % of the plants started to bolt from two weeks after sowing (bolting group), while 79.2 % of plants did not show bolting behavior (non-bolting group). The association between SNP183 and bolting in the validation population was tested with the same logistic regression model used in the discovery population (see Equation (1)).

Phylogenetic analysis

Amino acid sequences were aligned with ClustalW [36] and phylogenetic tree was constructed using the neighbour-joining method as implemented in the software Mega version 6 [37, 38], with 1,000 bootstrap replicates.

Availability of supporting data

All supporting data are included as additional files.

Additional files

Additional file 1: Table S1. Information on 192 SNPs used in the study. (XLSX 37 kb)

Additional file 2: Table S2. Sequences of the designed primers and TaqMan probes for detection of the SNP183. (DOC 28 kb)

Abbreviations

SNP: Single Nucleotide Polymorphism; RAD: Restriction-site Associated DNA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CB and PS collected the data and wrote the manuscript. FB and DC helped with bioinformatics, data analysis and revision of the text. MS provided critical revision of the manuscript. All authors approved final version for publication.

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