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ORIGINAL PAPER

Genetic analyses of bolting in bulb onion (*Allium cepa* L.)

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Abstract
Key message **We present the first evidence for a QTL conditioning an adaptive trait in bulb onion, and the first linkage and population genetics analyses of candidate genes involved in photoperiod and vernalization physiology.**
Abstract Economic production of bulb onion (*Allium cepa* L.) requires adaptation to photoperiod and temperature such that a bulb is formed in the first year and a flowering umbel in the second. 'Bolting', or premature flowering before bulb maturation, is an undesirable trait strongly selected against by breeders during adaptation of germplasm. To identify genome regions associated with adaptive traits we conducted linkage mapping and population genetic analyses of candidate genes, and QTL analysis of bolting using a low-density linkage map. We performed tagged amplicon sequencing of ten candidate genes, including the *FT-like* gene family, in eight diverse populations to identify polymorphisms and seek evidence of differentiation. Low nucleotide diversity and negative estimates of Tajima's D were observed for most genes, consistent with purifying selection. Significant population differentiation was observed only in *AcFT2* and *AcSOC1*. Selective genotyping in a large 'Nasik Red × CUDH2150' F_2 family revealed genome regions on chromosomes 1, 3 and 6 associated (LOD > 3) with bolting. Validation genotyping of two F_2 families grown in two environments confirmed that a QTL on chromosome 1, which we designate *AcBlt1*, consistently conditions bolting susceptibility in this cross. The chromosome 3 region, which coincides with a functionally characterised acid invertase, was not associated with bolting in other environments, but showed significant association with bulb sucrose content in this and other mapping pedigrees. These putative QTL and candidate genes were placed on the onion map, enabling future comparative studies of adaptive traits.

Abbreviations

SD	Short-day
LD	Long-day
DH	Doubled-haploid
CAPS	Cleaved amplified polymorphic sequence
AMAL	Alien monosomic addition line

Introduction

Onion and shallot (*Allium cepa* L.) are staple functional foods central to most diets, grown from tropical regions to high latitudes. Economic production of dry bulb onion and shallot requires cultivars to be biennial. They must be adapted to local photoperiod and temperature such that a sufficiently large bulb is formed in the growing season without premature transition to flowering ('bolting'; Kamenetsky and Rabinowitch 2002). Bulb onions are broadly classified into 'short-day' (SD) and 'long-day'

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(LD) types depending on the length of day required to initiate bulb formation. In terms of flowering adaptation, all onions produce flowers in the spring. True shallot of species *A. cepa* is a horticultural type displaying a partial to complete tendency toward asexual reproduction. It is presumed that the shallot phenotype has been selected for practicality of production in tropical regions where florigenesis is compromised by want of vernalization and fungal disease pressure on floral structures (Rabinowitch and Kamenetsky 2002).

There is an extensive literature concerning the physiology of flowering in onion, reviewed most recently by Brewster (2008). Once onion plants reach a critical weight or leaf number, they can be induced to initiate inflorescences by exposure to low temperatures. The critical size and temperature requirements for this vernalization exhibit wide genetic variation. Depending on the conditions and genetic background, there may be 'competition' between bulb and inflorescence development (Van Kampen 1970), such that inflorescence development may be suppressed. It has been demonstrated that a vernalization period is all that is required to initiate flowering from onion bulbs, but that temperature and photoperiod affect the time taken for eventual inflorescence appearance and floret opening (Khokhar et al. 2007). De-vernalization in onion is commercially exploited by high-temperature (>25 °C) treatment of cold-stored onion 'sets' prior to transplanting in springtime.

The stringent requirement for adaptation of onion cultivars to environment for producing marketable crop places major constraints on breeding and exploitation of germplasm. A deeper understanding of the genetics of temperature and photoperiod adaptation could inform strategies for genetic resource exploitation and development, and provide insights into domestication and dissemination of onion over the past millennia. Most importantly, functional markers to major genes conditioning adaptation could enable more efficient introgression of desirable traits such as disease resistance from wide crosses, as well as enabling seed lot quality control.

Physiological and genetic studies have implicated numerous genes and pathways in control of flowering in *Arabidopsis* and other species (Matsoukas et al. 2012). Components of the photoperiodic floral induction pathway are conserved between dicots and monocots (e.g. cereals such as rice and barley): for example, the circadian clock is needed to measure day length and downstream components such as *Constans* (*CO*; known as *Heading date 1/HD1* in rice) play important roles in the photoperiodic flowering of *Arabidopsis* and rice (Tsuji et al. 2011). The vernalization pathway is less well-conserved and is believed to have evolved separately in grasses and dicots (Greenup et al. 2009). The photoperiodic and vernalization pathways converge on a small number of floral pathway integrators in all plants, including *Suppressor of overexpression of constans 1* (*SOC1*) (Hepworth et al. 2002), and *Flowering locus T* (*FT*) genes (*HD3a* and *Rice FT-LIKE1/RFT1* in rice and *FT1* in wheat and barley).

Although the extensive genetic and physiological studies of grasses are often assumed to generalise to all monocots, studies of adaptive trait genetics in other monocot clades are very limited. Onion is in the order Asparagales, which diverged 122 m years ago from the monocot grasses (Janssen and Bremer 2004). Therefore, the genetic mechanisms controlling cereal flowering may differ in the Asparagales. Although physiological and genomic studies of flowering are very limited in Asparagales, several recent studies have described transcriptional and functional studies in orchid genera (Chang et al. 2011; Ding et al. 2013; Liang et al. 2012; Xiang et al. 2012). In onion, a period of vernalization was found to be all that was required to initiate flowering from onion bulbs, but that temperature and photoperiod affected the time taken for eventual inflorescence appearance and floret opening (Khokhar et al. 2007). Taylor et al. (2010) hypothesised that components of the photoperiodic pathway might be involved in the induction of onion bulbing. While they found that circadian clock genes appear to have a conserved function based on their expression, whether they are involved in onion bulbing was not established. We recently reported functional characterisation of the *FT-like* gene family in onion (Lee et al. 2013), identifying three members with distinctive expression patterns associated with bulbing and floral initiation. This work indicated that two antagonistically acting *FT-like* genes (*AcFT1* and *AcFT4*) are involved in the photoperiodic induction of onion bulbing, while another *FT* (*AcFT2*) is involved in the vernalization responsive initiation of flowering (Lee et al. 2013).

Genetic analysis in numerous species has frequently revealed associations between allelic variation in conserved components of these pathways and integrators with flowering time. Natural variation in *FT-like* genes has been associated with flowering time in dicots such as sunflower and *Arabidopsis* (Blackman et al. 2010; Laurie et al. 2011), and monocot grasses such as ryegrass (Skøt et al. 2011) and rice (Kojima et al. 2002). Similar associations have been reported for components of the well-conserved photoperiodic pathway such as *CO/HD1* in rice (Takahashi et al. 2009).

In this study, we have taken a genetic approach as the first step in understanding the genetic architecture of premature bolting (flowering) in onion. The only reported genetic analysis of onion bolting is that of Hyun et al. (2009) who examined an F_2 family segregating for time to flowering using proteomic methods. This revealed that in the family examined, late flowering segregated as a dominant character, and although they identified proteomic differences among the parents they did not examine

Table 1 Onion populations used in this study

Population barcode number	NCBI BioSample accession	Label	Description	Type and latitude of origin	Source	Citation or link
1	SRS399772	CUDH2150	Doubled haploid	LD 42 N	Cornell University	Alan et al. (2004)
2	SRS399773	'Nasik Red'	Open-pollinated line	SD 20 N	USDA-ARS	PI 271311
3	SRS399774	Omani	Omani landrace	SD 23 N	EU Allium Genebank	HRIGRU 8158 http://ealldb.ipk-gatersleben.de/
4	SRS399775	GTM	'Criolla molonga' Guatemalan landrace	SD 14 N	EU Allium Genebank	HRIGRU 7293 http://ealldb.ipk-gatersleben.de/
5	SRS399776	ESP	'Colorada de conservar' Spanish landrace	LD 43 N	EU Allium Genebank	HRIGRU 9874 http://ealldb.ipk-gatersleben.de/
6	SRS399778	PRT	'Cebola valenciana' Portuguese landrace	LD 41 N	EU Allium Genebank	HRIGRU 11567 http://ealldb.ipk-gatersleben.de/
7	SRS399779	'Texas Grano 438'	Open-pollinated line	Origin SD 26 N, selected at 37 N	Seminis vegetables seeds, Plant and Food Research	McCallum et al. (2006a)
8	SRS399780	W202A	Inbred line	LD 43 N	University of Wisconsin	Goldman (1996)

segregation of these with bolting phenotype. Physiological and genomic studies in onion have been complicated by a lack of freely available reference lines, combined with very limited genomics resources. Recent transcriptome sequencing and mapping studies based on homozygous doubled haploid (DH) lines and RNASEQ have greatly expanded scope for reproducible and comparative approaches (Baldwin et al. 2012b; Duangjit et al. 2013) as well as enabling global searches for candidate genes. We recently functionally characterised members of the *FT-like* gene family in onion (Lee et al. 2013). In the current study, we sought to place these and other candidate genes on the onion genetic map and to search for QTL conditioning bolting in a wide onion cross (Baldwin et al. 2012b). This revealed that a QTL located on onion chromosome one plays a major role in conditioning bolting in this cross.

Materials and methods

Plant materials

DH onion lines were provided by Cornell University (Alan et al. 2004). Diverse onion genotypes from SD and LD origins were obtained from the EU *Allium* Gene Bank at Warwick University (http://ealldb.ipk-gatersleben.de/; Table 1). Seed lots were sown in spring either directly as seed or as transplants in Jiffy 7 plugs (Jiffy Corp, Lorain, OH, USA). Crop management followed standard commercial practices.

F_2 families ('2262' and 'G2320'; Baldwin et al. 2012b) were derived by self-pollinating individual F_1 plants from the cross between the doubled haploid (DH) onion line 'CUDH2150' and the heterozygous landrace 'Nasik Red'. Families were increased further by cage-pollinating clonal topsets from the original F_1 umbels. These families were sown in spring in 2008/9 (sowing date 25 Sept 2008) and 2011/12 (sowing date 9 Sept 2011) seasons at Lat 42° S near Christchurch, New Zealand and in 2011/12 (sowing date 29 August 2011) at Lat 37° S near Pukekohe, New Zealand. A plant was scored as bolting if it exhibited a clearly elongated flower scape and expanded umbel at harvest in early March. Bulb carbohydrate analysis of these populations has been described previously (Revanna et al. 2013).

DNA extraction and molecular marker analysis

DNA was isolated from F_2 plants of the CUDH2150 × Nasik Red family '2262' in the 2008/9 season from fresh leaf material or freeze-dried bulb tissue of bolting and non-bolting plants as described previously (Baldwin et al. 2012b; McCallum et al. 2006a). For validation in the 2012 season, template DNA was sampled directly from bulbs by stabbing fleshy scales with a 200 µl pipette tip (Axygen). The tip was then placed into a 50 µl volume solution containing 1 × buffer Gold and 1 µL prepGEM reagent (prepGEM™ Tissue kit; ZyGEM Corp, Hamilton, New Zealand). This was incubated for 15 min at

Table 2 Primer pairs used for amplicon re-sequencing of flowering candidate genes

Primer set	Forward primer	Reverse primer
ACR036	GCGAAAACTGAAGGACTGAAA	TTAAAGGAAGGTTCGCCAAA
ACR043	CATTTTCTGAGACGCATTGCT	CGGCAAAATTTGAGAGGAAA
ACR045	GGCGTATACCTCTAAGGGTTATGT	GAGAAACAATCGAACTAAACAGCA
ACR047	TCATTTTGTTTCTTCTGCTTCC	CCCATCATCTCCATTTGCTT
ACR048	CATGGCAAGAGAAAGTGACC	TCATCATCAACTTCAACCCAAA
ACR049	ACAATGTTGCTGCTGCTTGA	GGATGGTGACTGATATACCAGAAG
ACR051	AGGGGCGTTATTTGTGAGTA	CCCTGGTGCATACACAGTTT
ACR054	GCAAAAGGCGGTTGTACATT	AAGGTTGCTCATCCAGTGCT
ACR055	TTGTGATTGTCCGAAGTAGCC	TCCGCAGTTTCATTCTTCAA
ACR056	GCAGAGCAGACACAGCAAAG	CCACATGCTAGATTTCGATCC

75 °C with a 5 min hold at 95 °C. An aliquot of the solution was then diluted in half with TE buffer (10 mM Tris and 0.1 mM EDTA) and the remainder stored at −20 °C. SSR, CAPS and HRM marker analyses were carried out as described previously (Baldwin et al. 2012b) but the HRM amplification conditions were modified to include a touchdown PCR. The modified conditions were: 95 °C for 15 min followed by seven cycles of 94 °C for 30 s, 62 °C for 30 s (reducing by 1 °C per cycle), and 72 °C for 15 s. Another 45 cycles were carried out at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 15 s. Then, a final hold temperature of 25 °C for 2 min.

Parallel-tagged sequencing

Template genomic DNA was prepared from the homozygous doubled-haploid reference 'CUDH2150' and from pooled DNA bulks of at least 25 plants from seven diverse populations adapted to short- to long-day environments (Table 1). Long-range PCR was performed according to the Expand Long Template PCR System (Roche Applied Science) protocol with Expand Long Template buffer 1 and primers (Table 2). Genomic DNA (100 ng) was amplified in a volume of 50 µl by touchdown PCR using the following conditions: 94 °C, 2 min, followed by 8 cycles of 94 °C for 10 s, 65 °C for 30 s (reducing 1 °C per cycle), and 68 °C for 2 min. Another 40 cycles were carried out at 94 °C for 15 s, 57 °C for 30 s and 68 °C for 3 min (increasing by 20 s per cycle), with a final extension at 68 °C for 7 min. PCR products were purified with AMPure XP (Agencourt), and then subjected to Fragmentase treatment to generate sizes in the range of 300–600 bp according to the NEBNext dsDNA Fragmentase (NEB) protocol. The fragmented PCR products were purified as previously, and blunt end repair, adapter ligation and fill-in were carried out according to Meyer et al. (2007) using NEBNext End Repair Module (NEB) and bar-coding adapters from Meyer et al. (2008). Fragmented PCR products were barcoded by genotype (Table 1) then quantified on a Victor3 plate reader (Perkin Elmer) with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen). Pooling, dephosphorylation and restriction digestion were carried out according to Meyer et al. (2008). Normalised tagged fragmented PCR products were pooled by amplicon into two libraries for 454 sequencing on separate 1/16 regions of a GS-FLX plate.

Sequence reads were separated into populations based on barcodes using the *sff_extract* tool (https://github.com/JoseBlanca/seq_crumbs.git). A reference assembly was generated for the CUDH2150 homozygous control using Newbler 2.7 (Roche) with default settings. Reference assemblies from CUDH2150 were submitted to Genbank as Accessions KC677631–KC677640 (Table 3). Gene models were obtained by mapping reference cDNA sequences onto these using gmap (Wu and Watanabe 2005). Experimental data was deposited as NCBI SRA study SRP018027.

Reads from the seven heterozygous populations were mapped onto the CUDH2150 assemblies using BWA_SW (Li and Durbin 2010; http://www.ncbi.nlm.nih.gov/pubmed/19451168). SNPs were called using samtools mpileup (Li et al. 2009), patterns of nucleotide diversity were determined using PoPoolation (Kofler et al. 2011a) and allele frequencies were compared among genes and populations using PoPoolation2 (Kofler et al. 2011b). A reproducible workflow containing scripts and all settings for read mapping, analysis and visualisation can be accessed at GitHub in the repository https://github.com/cfljam/onion_PTS_workflow. Variant effects were determined using snpEff (Cingolani et al. 2012). Primer sets for indel, HRM or CAPS markers were designed to variants of interest as described previously (Baldwin et al. 2012b).

Candidate gene mapping

Candidate genes that were not segregating in the F_2 population '2262' were tested on the 'W202A × Texas Grano 438' mapping population (McCallum et al. 2006a, b) or on sets of *A. fistulosum–A. cepa* alien monosomic addition

Table 3 Primer pairs used and nucleotide diversity of genomic loci from parallel-tagged sequencing of candidate genes

Gene Description	Primer Set	Amplicon size bp	CUDH2150 reference assembly Genbank accession	Reference cDNA Genbank accession	S	Tajimas D	Tajimas Pi
AcFT1	ACR048	3,360	KC677631	KC485348	10	−2.41	2.01
AcFT2	ACR043	586	KC677640	KC485349	18	−2.78	2.44
AcFT3	ACR051	2,250	KC677632	KC485350	10	−2.52	1.57
AcFT4	ACR045	2,079	KC677636	KC485351	2	−1.79	0.47
AcFT5	ACR049	1,437	KC677638	KC485352	7	−1.96	2.82
SOC1-like	ACR055	1,983	KC677637	CF449085	3	−2.21	0.61
AcCO	ACR036	1,928	KC677633	JR851107	25	−1.58	3.36
Constans-like	ACR056	2,796	KC677635	GAAO01015443.1	7	−0.32	4.85
Indeterminate-like	ACR047	853	KC677634	GAAO01013966.1	23	−2.81	1.49
VIN3-like	ACR054	933	KC677639	GAAO01012585.1	4	−2.13	0.62

D and Pi were calculated on CDS only

S number of segregating sites, *Pi* average nucleotide differences per kb

Table 4 Genetic map locations of flowering candidate gene sequences in onion

Flowering-like gene	Marker(s)	Marker type	Reference sequence	Chromosome	Map position (cM)	Closest marker	Population
AcFT1	ACP670	CAPS-Hpy188i	KC677631	1	40	ACM050	W202A × Texas Grano
AcFT2	ACP691	CAPS-HpaII	JR854848	5	92	ACP929	Nasik Red × CUDH2150 (2262)
AcFT3	ACP675 and ACP676	CAPS-AluI and RsaI	KC677632	2	0	ACM066	W202A × Texas Grano
AcFT4	ACP868	PCR-dominant	KC677636	6			AMAL
AcFT5	ACP686	CAPS-EcoRV	KC677638	6	74	ACM115	W202A × Texas Grano
AcFT6 (AcFTL)	APCP996	HRM	KC485353	3	96		Nasik Red × CUDH2150 (2262)
AcCO (AcCOL)	ACPA16	HRM	KC677633	4			AMAL
Constans-like	ACI046 and ACP681	InDel and CAPS-AluI	KC677635	6	42	ACM121	W202A × Texas Grano
SOC1-like	ACPA09	HRM	KC677637	1			AMAL
VIN3-like	ACP689	CAPS-RsaI	KC677639	5	111	ACM065	Nasik Red × CUDH2150 (2262)
Indeterminate-like	ACP102	HRM	CF436030	3			AMAL

The naming used by (Taylor et al. 2010) is given in brackets where appropriate. With exception of ACP868, which was dominant, all are co-dominant markers. Where the marker type was CAPS the restriction enzyme used is listed. Gene markers were mapped in the 'Nasik Red × CUDH2150' population, or alternatively in 'W202A × Texas Grano 438' (McCallum et al. 2006a), or assigned to physical chromosomes using AMAL (Shigyo et al. 1996). Map positions based on linkage mapping in Joinmap (Van Ooijen 2006)

lines (AMALs) developed by Shigyo and colleagues (1996). Linkage maps were produced using JoinMap v4 (Van Ooijen 2006) as described previously (Baldwin et al. 2012b).

QTL analysis

A subset of 43 non-bolting and 50 bolting progeny from the F_2 population '2262' from season 2008/9 was tested with molecular markers for both flowering candidate genes and random markers at regular intervals across the genetic map reported previously (Baldwin et al. 2012b) (Table 4). Single marker and interval analysis using a binary model were used to identify genome regions associated with bolting for the 'Nasik Red × CUDH2150' mapping populations using R/QTL (Broman et al. 2003). Significantly associated markers were then tested in 2011/12 on large samples of bolting and non-bolting progeny from populations '2262' and 'G2320' grown at Lincoln and Pukekohe.

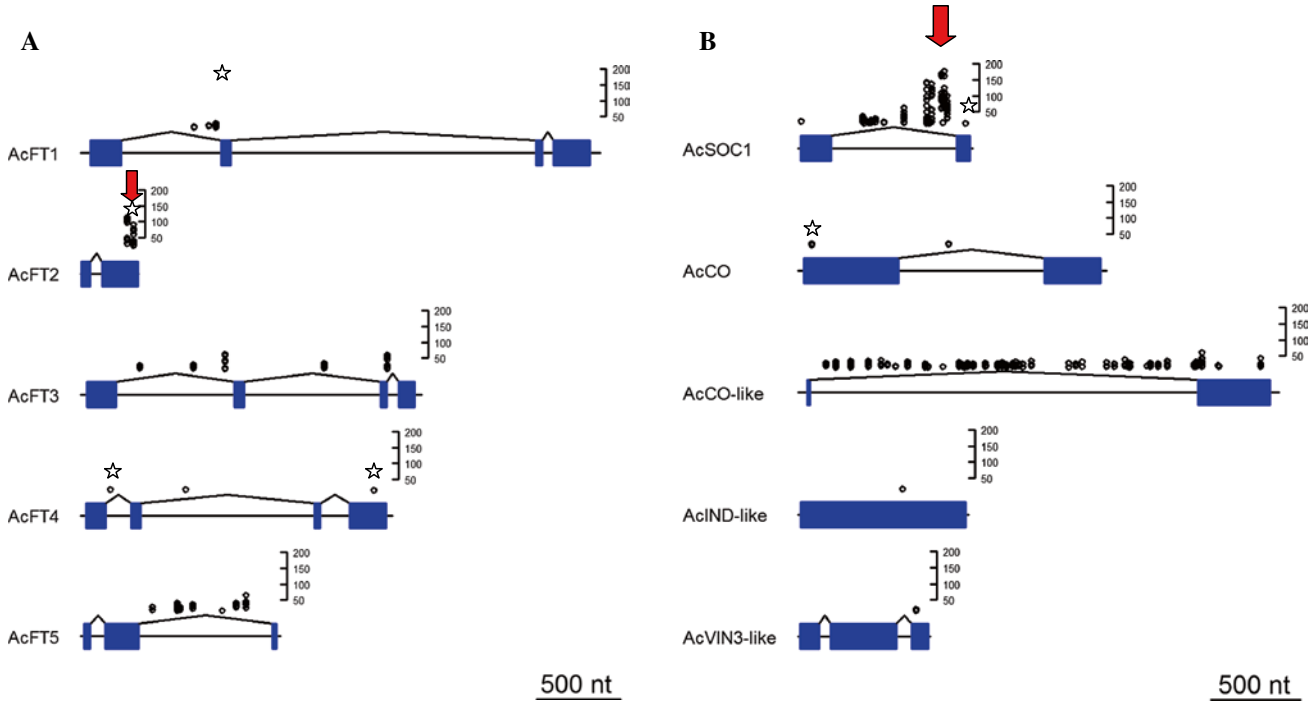

Fig. 1 Distribution of SNPs in flowering-related genes that exhibit significant allele frequency differences among seven open-pollinated SD and LD onion populations. **a** FT-like gene family. **b** Other candidate genes. Scales denote $\log_{10}$ of *P* value for Fishers exact test of allele frequency equivalence. Points denote pair-wise comparisons with $\log_{10}$ of *P* greater than Bonferrroni-corrected 1 % significance level (13.97). *Arrows* denote regions exhibiting strong differentiation among SD and LD populations. *Stars* denote non-synonymous polymorphisms

Results

Candidate gene identification and re-sequencing

During bioinformatics searches of existing onion EST data for analysis of the *FT-like* gene family in onion (Lee et al. 2013), we identified other putative candidates by BLASTX matches to *Arabidopsis* proteins associated with flowering regulation (Srikanth and Schmid 2011). To identify polymorphisms to enable genetic mapping of *FT-like* genes, and to identify possible differentiation among populations, we surveyed sequence variation in 5 *FT-like* and 5 other candidate gene loci using amplicon re-sequencing on the 454 platform. We were unable to develop long-range PCR primers for the other *FT-like* gene we denoted *AcFT6* (Lee et al. 2013), which is synonymous with that denoted *AcFTL* by Taylor et al. (2010). The non-FT candidates we surveyed included the *CONSTANS*(*CO*)*-like* gene described by Taylor et al. (2010) and four others with similarity to *CO*, *SOC1*, *VIN3* and *indeterminate*. PCR products of 800–3,000 bp were amplified from the homozygous reference line 'CUDH2150' and seven other lines of diverse short-day and long-day origin. Relatively low nucleotide diversity and negative estimates of Tajima's D were observed in the coding regions of all except *Constans-like* (Table 3). These observations are consistent with expectations for genes under purifying selection. Comparison of the distribution of significant (Bonferroni corrected $P < 0.01$) pair-wise differences in allele frequency among the seven non-DH SD and LD populations showed only two regions exhibiting strong evidence of population differentiation, in the *FT-like* gene *AcFT2* and the *SOC1* homolog *AcSOC1* (Fig. 1). Notably, these two regions include non-synonymous variants (*AcFT2* C411T P/S; *AcSOC1* C1376A $T > K$), which were rare in all genes. Non-synonymous variants were also observed in *AcFT4* (T127C $L > P$; C1847T $P > L$) and *AcCO* (C54A $L > I$), which also exhibited significant differences in allele frequency among some populations (Fig. 1).

PCR-based markers designed to selected variants using Galaxy-based tools (Baldwin et al. 2012b) were either placed on the onion genetic map or assigned to physical chromosomes where mapping was not possible using AMAL (Table 4). The fixation of several candidate loci in the inbred line 'W202A' enabled mapping of several variants in the 'W202A × Texas Grano 438' mapping population. Candidate genes tested were present on chromosomes 1–6 with chromosome 6 containing three candidates including two *FT-like* genes (*AcFT4* and *AcFT5*). Using molecular markers all six onion *FT-like* genes were assigned to the chromosomes of onion along with other major candidates.

Map position could not be assigned for *AcFT4* along chromosome 6 because none of the markers were polymorphic in the populations tested. Few of the candidates could be mapped in the 'Nasik Red × CUDH2150' F_2 population.

Segregation of bolting phenotype in mapping populations

All genetic analysis in this study was based on phenotypic evaluations performed on field-grown plants sown in spring, in a temperate intermediate-day environment, to permit parallel analysis of bulb phenotypes (Baldwin et al. 2012b). Bolting was scored simply and conservatively based on whether a plant exhibited an elongated flower scape and umbel at harvest. During development of the 'Nasik Red × CUDH2150' populations, extremely vigorous F_1 plants were obtained which exhibited a strong tendency (i.e., over 50 %) to bolt in spring-sown field trials at lat. 42° S. The vigour is consistent with the findings of Hyde et al. (2012) who reported excellent combining ability of DH lines from this background. By contrast, bolting of both parents in such trials in this environment was very rare. Since it is a SD variety, 'Nasik Red' is poorly adapted to growth at this latitude and forms relatively small bulbs in spring plantings. This absence of visible bolting in this mal-adapted line may reflect consequence of 'competition' between bulbing and inflorescence development (Van Kampen 1970). During initial propagation in 2008/9 of the 'Nasik Red × CUDH2150' population, a bolting frequency of ~10 % was observed in family '2262', and samples were retained to permit genetic analysis (Table 5). During the 2011/12 validation trials of the two families, observed bolting frequencies were 12–30 % (Table 5). Mean daily temperatures at Lincoln were slightly cooler in early summer 2011 (mid November to mid-December) than in 2008 (Supplementary Table 3; Supplementary Fig. 3).

Molecular marker and QTL analysis

Markers from the low-density map, developed using non-bolting progeny from the '2262' family (Baldwin et al. 2012b), as well as candidate gene markers, were used to conduct selective genotyping of bolting progeny from the 2008/9 trial. Putative associations with bolting (LOD > 3) were identified on chromosomes 1, 3 and 6 (Fig. 2; Table 6). Those genotypes homozygous for the 'Nasik Red' allele for the CAPS marker ACP267 on chromosome 1 tended to bolt (23/27). This association was validated by testing F_2 lines from the 2011/2012 season that had been grown in two different environments. The association was significant ($P < 0.05$) and the marker was then tested in an F_2 population 'G2320' of the same pedigree, again revealing a significant association (Table 7). We denote this locus *AcBlt1*. The candidate genes present on chromosome 1 include *AcFT1* and *AcSOC1*. These could not be mapped in the 'Nasik Red × DH2150' background and comparative mapping with the alternative population 'W202A × Texas Grano438' (McCallum et al. 2006a, b) suggested that *AcFT1* was not in close linkage with ACP267 (Fig. 3). Whether this association could be with *AcSOC1* remains unknown because this gene could not be mapped in either population. QTL analysis in the non-bolting '2262' progeny from the 2008/9 trial revealed evidence of association of *AcBlt1* with bulb weight. Bulbs homozygous for the ACP267 'CUDH2150' allele had higher mean bulb weight ($T = -1.88$; $P = 0.07$; $df = 23$).

The association detected in 2008/9 on chromosome 3 in the region flanked by markers ACP830 and ACP996 includes the functionally characterised (Vijn et al. 1998) acid invertase locus API89 (NCBI Pr006099770). No bolting plants in the selective genotyping screen were homozygous at API89 for the 'Nasik Red' allele (Chi square = 20.607, df = 1, P value = 0.000). However, API89 genotyping of samples from both families in the 2011/12 validation trials revealed no significant association with bolting frequency (data not shown). Comparison with other onion genetic maps reveals that this region of chromosome 3 corresponds with the QTL reported by Havey et al. (2004) that conditions variability in bulb soluble solids content. Onion acid invertase activity has been shown to be regulated by photoperiod (Lercari 1982), and temperature (Benkeblia et al. 2004), and sucrose is known to be a signal for florigenesis (Matsoukas et al. 2012). Analysis of bulb composition in a sample of 504 non-bolting progeny from the 2008/9 trial (Revanna et al. 2013) revealed that bulbs homozygous for the 'Nasik Red' allele revealed by marker API89-2 (Pr006099770) had significantly higher sucrose levels (T value = −2.49, P value = 0.014, df = 169). Analysis of bulb sucrose composition data from previous studies of inbred families (McCallum et al. 2006a) also revealed the same pattern of recessive inheritance for high sucrose in relation to API89 genotype (Fig. 4). We, therefore, designate this locus *AcSuc*. The marker ACP102 designed

Table 5 Frequencies of bolting observed at harvest in progeny of two F_2 families from the 'CUDH2150 × Nasik Red' cross grown in three environments

Population	Environment	Bolt
2262	Lincoln 2008/9	10 % (50/500[a])
2262	Lincoln 2011/12	31 % (184/598)
G2320	Lincoln 2011/12	10 % (29/277)
2262	Pukekohe 2011/12	25 % (144/578)
G2320	Pukekohe 2011/12	12 % (54/453

[a] Approximate total

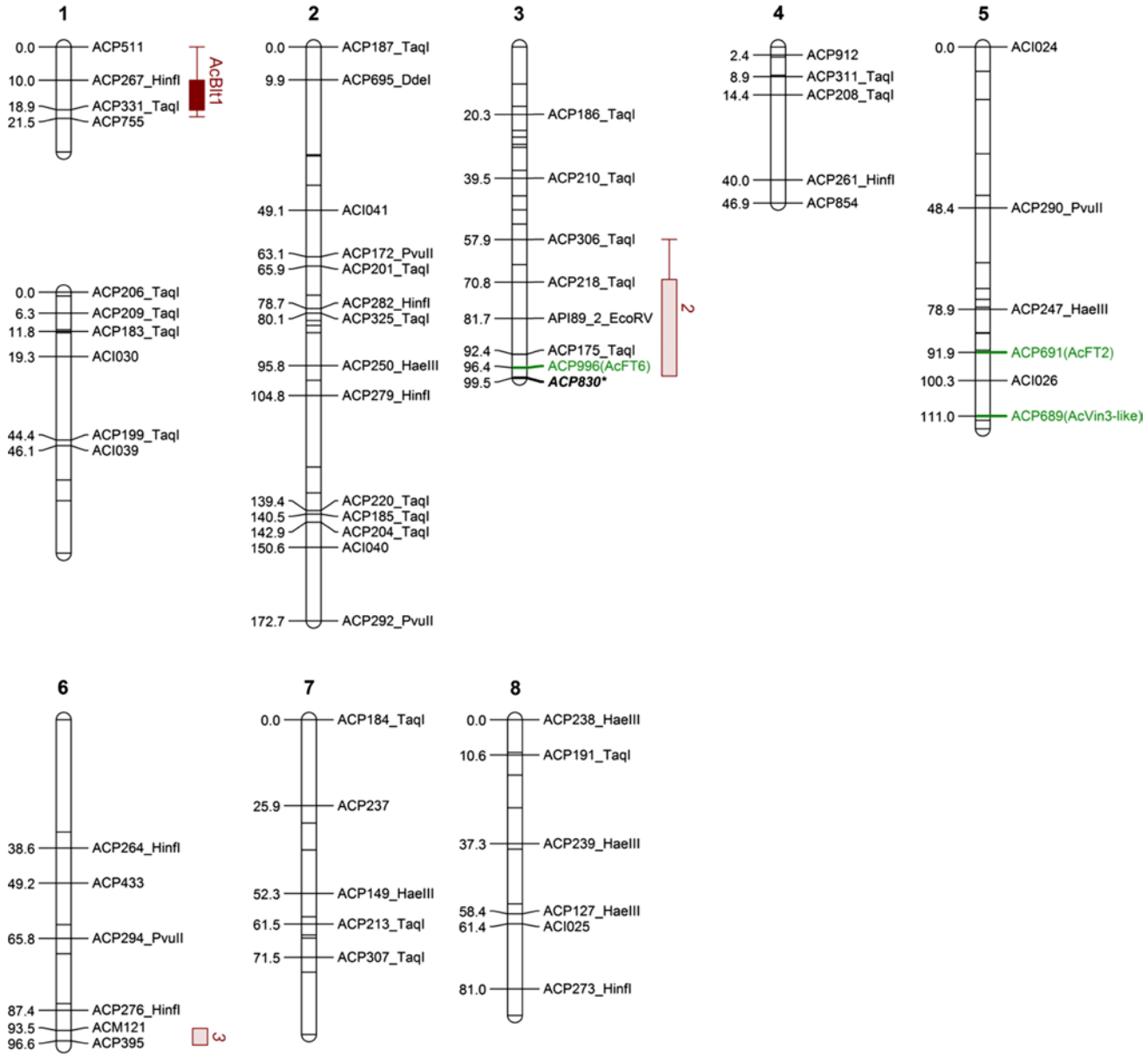

Fig. 2 Chromosomal locations of QTL affecting bolting frequency and candidate gene loci in 'Nasik Red × CUDH2150' families. *Green labels* denote candidate genes and *red bars* denote 1–2 LOD support intervals for QTL revealed by interval mapping (colour figure online)

Table 6 Genetic map positions and estimated variance explained by putative QTL associated with bolting in the '2262' population phenotyped in the 2008/9 season at Lincoln

QTL	Chromosome	Map position	Marker	LOD	% variance explained
1 (*AcBlt1*)	1	10	ACP267	6.56	17
2 (*AcSuc*)	3	96	ACP996	4.50	13
3	6	96	ACP395	3.15	5

The LOD calculations and QTL positions were calculated using the Haley-Knott method and a binary trait model. The variance explained by each QTL was calculated using the multiple QTL mapping functions in R/qtl (Broman et al. 2003)

to an *indeterminate-like* homolog was also located on chromosome 3 although the map position could not be determined.

On chromosome 6, homozygous 'Nasik Red' genotypes for marker ACP395 bolted (14/14). Comparison with the 'W202A × Texas Grano438' linkage map would put this marker closest to the candidate genes *AcCO-like* and *AcFT5*, which would both be distal to the end of the 'Nasik Red × DH2150' chromosome 6 linkage group (Fig. 4). Possible interactions between the loci were detected and tested but the 2008/9 sample size was too limited to draw any accurate conclusions.

Table 7 Genotype counts and Chi-square goodness-of-fit tests for marker ACP267 linked to the QTL *AcBlt1* over seasons and populations

Season	Population	Environment	Bolt					Non-bolting				
			Genotype counts			χ^2 2 *df*	*P* value	Genotype counts			χ^2 2*df*	*P* value
			'Nasik red' homozygote	Heterozygote	'CUDH2150' homozygote			'Nasik red' homozygote	Heterozygote	'CUDH2150' homozygote		
2008/9	2262	Lincoln	23	26	1	19.44	0.000	4	25	14	5.79	0.055
2011/12	2262	Lincoln	55	36	9	50.16	0.000	15	36	24	2.28	0.320
2011/12	2262	Pukekohe	69	72	36	18.46	0.000	5	19	25	18.8	0.000
2011/12	2320	Pukekohe	39	76	17	10.36	0.006	17	61	46	13.6	0.000

Discussion

Based on expression and complementation studies, we previously concluded that there is strong evidence of a functional role in regulation of bulbing and flowering of three of the six *FT-like* genes we identified in onion, which we designated *AcFT1*, *AcFT2* and *AcFT4* (Lee et al. 2013). We hypothesised that *AcFT1* and *AcFT4,* respectively, promote and repress bulbing, and that *AcFT2* may be the key signal for florigenesis induced in vernalized meristems. Interactions among these *FT-like* genes may condition the developmental transitions among the juvenile, 'thermophase', and 'competition' phases of onion floral development described in the classical model of Van Kampen (1970). The evidence of population differentiation identified for *AcSOC1* with the re-sequencing data is interesting as this could not be placed on the 'Nasik Red × DH2150' linkage map but was physically located to chromosome 1 and hence, remains a candidate for the *AcBlt1* locus. *SOC1* is one of six flowering time genes within the *days to heading* QTL (*dth1.1*) interval on chromosome 1 in rice (Thomson et al. 2006). MAS was used to produce sub-introgression lines (SILs) containing one or more of these six candidate genes from *Oryza rufipogon* in an *O. Sativa* background. Only the SIL with an introgression containing *SOC1* and a *Flowering time locus T* (*FT-L8*) was consistently early in all experiments (Maas et al. 2010). In onion, the association of *AcBlt1*with bulb weight in the 2008/9 trial suggests that *AcBlt1* may condition variability in the 'competition' phase of floral development (Brewster 2008; Van Kampen 1970), so that bulb formation is favoured over inflorescence development.

Our observation that the *AcSuc* locus conditions variation in bulb sucrose levels and that it may be associated with bolting in some environments suggests that further investigation of its role in adaptive physiology is worthwhile. Physiological studies have demonstrated that acid invertase activity in onion is very strongly regulated by environment. Photoperiodic regulation of acid and neutral invertases was reported by Lercari (1982), and Benkeblia et al. (2004) reported that acid invertase activity in bulbs of *cv* 'Tienshan' is higher at 10 °C storage compared to 20 °C. Further indirect support for a functional role of acid invertase in bolting is suggested by the origins of the tulip acid invertase cDNA clone used by Vijn et al. (1998) to clone the onion gene corresponding to API89. This clone was shown to encode an acid invertase that was strongly up-regulated by cold and shown to be involved in driving flower stalk elongation (Balk and de Boer 1999). Correlation between carbon physiology traits including invertase activities and flowering date have been reported in maize (Thévenot et al. 2005). Based on the reported effects of environmental factors on onion acid invertase,

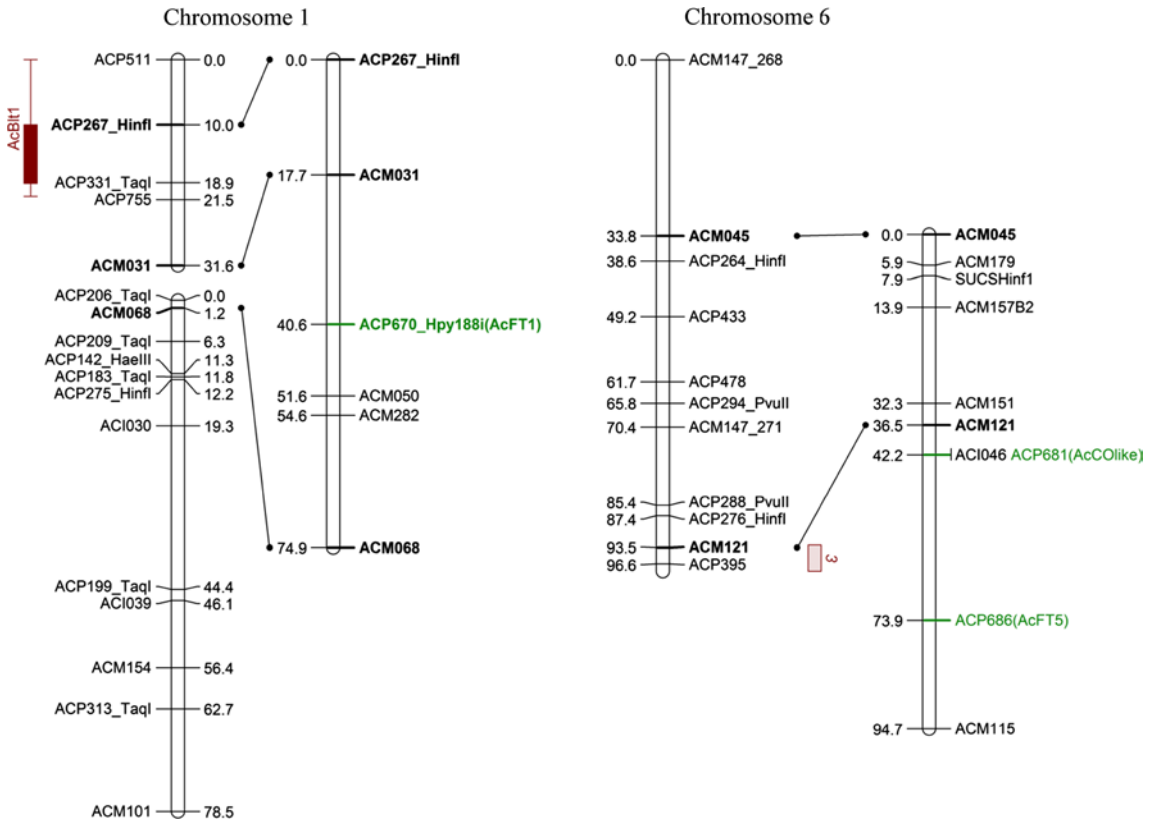

Fig. 3 Alignment of linkage groups for onion chromosome 1 and 6 from the onion populations 'Nasik Red × CUDH2150' (L) and 'W202A × Texas Grano 438' (R), showing relationships between putative QTL and candidate gene markers (*green*) (colour figure online)

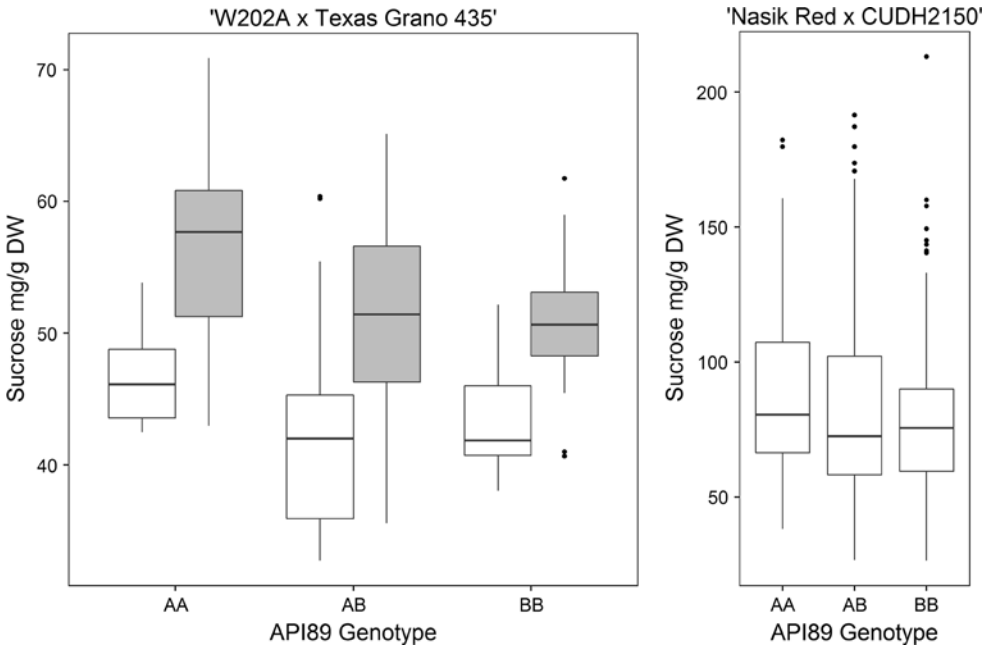

Fig. 4 *Box plots* showing distribution of bulb sucrose content in relation to genotype at the chromosome 3 acid invertase locus API89 (NCBI Pr006099770) in inbred $F_{2:3}$ families 'W202A × Texas Grano 438' evaluated in two environments (McCallum et al. 2006a) and in individual F_2 bulbs from 'Nasik Red × CUDH2150'. *Box centre-line* and *bounds* denote median, 25th and 75th percentiles, respectively. *Whiskers* denote 1.5 times the interquartile distance. *AA* denotes homozygous for the high solids parent ('Nasik Red' and 'W202A', respectively)

we would expect that our strategy of using large, non-replicated progenies would be inefficient for validating the effects of any locus conditioning genetic variation in this activity. This is emphasised by the large variability for sucrose content observed among F_2 bulbs of 'Nasik Red × CUDH2150'.

The QTL *AcBlt1* had the largest estimated effect on bolting explaining 17 % of the additive variation and this is likely to have been over-estimated given the selective genotyping strategy used in this study. Buckler et al. (2009) detected 29–59 additive QTL for flowering time traits in maize. It was suggested that this could be explained by the out-crossing nature of maize compared to the other self-pollinating model species such as *Arabidopsis* and rice, and that small-effect QTLs may have been advantageous to ensure that offspring where likely to have overlapping flowering times. Interestingly the putative QTL coinciding with *AcSuc* on chromosome 3 had a dominant effect. This would be difficult to fix in out-breeding crops such as onion without MAS.

The 'Nasik Red × CUDH2150' populations employed in this research are the products of a cross between genetically distant (Baldwin et al. 2012a; McCallum et al. 2008) and divergently adapted parent lines from SD and LD backgrounds. Generating F_2 onion populations by mass-pollination of F1 bulbils, induced spontaneously or by cytokinin treatment (Andrew 1951; Thomas 1972), allows evaluation of large samples across multiple environments. The principal disadvantage of this strategy is that it does not permit comparison of replicated combinations of the same allelic combinations over environments, such as those conducted in studies of bulb composition (McCallum et al. 2006b). However, developing a sufficient number of inbred or backcross lines from such a cross would be highly challenging due to the severe impact of adaptive loci on bulb storage and seed production. The more limited population sizes practical using inbred lines limit the power to detect epistatic interactions, and also dominance variance, which is likely to be of great significance in adaptive and yield traits (Wardyn et al. 2007). Availability of parent lines from public sources, gene-based maps and falling costs of genotyping mean that our findings may be reproduced and tested by other researchers. Developing a deeper understanding of the genetic architecture of onion adaptation will require use of many such crosses among landrace and domesticated germplasm, as well as diverse panels phenotyped across environments such as that previously conducted in the tropic regions by Currah and Proctor (1990).

Comparative mapping with the 'W202A × Texas Grano' population map and the placement of candidates on this map are important as these lines are from genetic backgrounds widely used in breeding. An obvious extension to this research would be an association mapping strategy using the candidate gene sequences and markers identified from the QTL analysis in more diverse SD and LD germplasm. The action of *AcBlt1* and *AcSuc* in developmental physiology could be determined by analyzing sets of progeny classified by QTL-linked marker genotype and then subjected to different environmental regimes under controlled environments or different planting dates. Given the difference in flowering habits across the vegetable Alliums, comparative mapping with shallot (generally asexually propagated) and closely related *Allium* such as Japanese bunching onion (*A. fistulosum*) and garlic (clonally propagated) would also help further understand the major genes involved in controlling flowering in the Asparagales.

Utilisation of exotic germplasm for breeding and selection by breeders to introduce novel traits is severely hampered by bolting. Although it is an easy trait to visually select, dominance could mask inferior genotypes and slow the adaptation. MAS for a recessive trait would be highly desirable for breeders trying to utilise crosses between maladapted and elite, well-adapted lines. The application of the marker associations detected in this research could be tested in larger breeding collections or genebanks to assess the predictive values for MAS programmes.

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Conflict of interest The authors declare that they have no conflict of interest.

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