

Detection of Quantitative Trait Loci (QTL) associated with the spring regrowth vigor trait in alfalfa (*Medicago sativa* L.)

Xueqian Jiang

Chinese Academy of Agricultural Sciences Institute of Animal Science

Fan Zhang

Chinese Academy of Agricultural Sciences Institute of Animal Science

Zhen Wang

Chinese Academy of Agricultural Sciences Institute of Animal Science

Ruicai Long

Chinese Academy of Agricultural Sciences Institute of Animal Science

Mingna Li

Chinese Academy of Agricultural Sciences Institute of Animal Science

Fei He

Chinese Academy of Agricultural Sciences Institute of Animal Science

Xijiang Yang

Chinese Academy of Agricultural Sciences Institute of Animal Science

ChangFu Yang

Chinese Academy of Agricultural Sciences Institute of Animal Science

Xu Jiang

Chinese Academy of Agricultural Sciences Institute of Animal Science

Qingchuan Yang

Chinese Academy of Agricultural Sciences Institute of Animal Science

Quanzhen Wang

Northwest Agriculture and Forestry University

Junmei Kang (✉ kangjunmei@caas.cn)

Chinese Academy of Agricultural Sciences

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Abstract

Background: Alfalfa (*Medicago sativa* L.) is a perennial forage legume with a reputation as being the “queen of forage”. Spring regrowth vigor refers to the process of perennial alfalfa returning to growth after winter survival. The objective of this research was to identify candidate genes significantly associated with spring regrowth vigor.

Results: We used a tetraploid alfalfa F1 population comprised of 392 progenies to identify quantitative trait loci (QTL) that control this trait. The F1 population phenotypic data were collected using a total of three environmental phenotypic data. The mapping population was genotyped using Genotyping-by-sequencing (GBS), and linkage maps were developed based on single nucleotide polymorphism (SNP) markers. Fifteen significant QTL for spring regrowth vigor were detected in both parents. Five QTL were identified in the male genetic map, while ten QTL were identified in the female. Four QTL were located on homolog 7D of the male parent, and two QTL were colocalized. Five QTL were mapped on homolog 6D of the female parent, and two QTL were colocalized.

Conclusions: The QTL presented in this study can be used to improve the efficiency of alfalfa breeding programs and provide valuable resources for the genetic improvement of alfalfa using marker-assisted selection (MAS).

β These authors contributed equally to this research.

*Correspondence:

Junmei Kang

kangjunmei@caas.cn

Background

Alfalfa (*Medicago sativa* L.) is a high-value autotetraploid ($2n = 4 \times = 32$) legume forage crop with a genome size of approximately 1000 Mb [1, 2], and has a very wide range of uses, such as in hay, silage and pasture [3]. Alfalfa plants are highly heterozygous and exhibit severe inbreeding depression, precluding the development of inbred lines [4]. Alfalfa breeding programs have focused on forage yield, quality, fall dormancy and resistance to biotic and abiotic stressors. Conventional breeding programs are usually based on simple phenotypic selection, which requires a long breeding cycle and has low efficiency. Modern programs are turning to breeding techniques based on genotyping, including QTL mapping, genome wide association studies (GWAS) and genomic selection (GS), which offer the promise of more rapid breeding cycles and fewer necessary phenotypic evaluations [5].

Spring regrowth vigor refers to the process of perennial alfalfa returning to growth after winter survival. For perennial forage, spring regrowth is an important index to measure its ability to recover growth after fall dormancy and winter hardiness. Moreover, plants with an excellent spring regrowth vigor trait can reduce the pressure of weeds in the spring, which is conducive to growth and has an important impact on the yield

and quality of the first cut of alfalfa. Perennial forage yields are believed to be strongly influenced by the amount of organic reserves developed during the last growing season [6]. These reserves are predominantly nonstructural carbohydrates, including quantities of nitrogenous compounds and accumulate in the roots and crowns of plants [7]. Dhont et al. (2006) reported that an untimely autumn defoliation of alfalfa reduces root accumulation of specific N reserves such as proline, arginine, histidine and vegetative storage proteins that are positively related to the vigor of spring regrowth but poorly related to winter survival [8]. At the same time, spring regrowth is also affected by the last harvest in the previous year. Harvests at full bloom allow for greater spring regrowth than cutting at the late bud stage, possibly because of the accumulation of higher root reserves [9, 10]. For perennial grass, carbohydrate and nitrogen reserves and their remobilization to active growth sites provide substrates for spring regrowth [11].

Major QTL related to yield [12, 13], flowering time [14], water use efficiency [15], fall dormancy and winter-hardiness [16, 17] and leaf rust resistance [18] have been mapped in tetraploid alfalfa. Mccord et al. (2014) identified QTL and markers associated with forage yield, resistance to lodging, and spring vigor in alfalfa [19]. Four QTL for spring vigor were identified, and some of the QTLs were located at the same or similar positions as those related to forage yield, possibly explaining the significant correlation between these traits. Genetic mechanisms of spring regrowth in perennial alfalfa are not well understood due to the quantitative trait of spring regrowth and the complex genetic nature and genomes of most popular perennial plants. Exploration of genetic mechanisms underlying spring regrowth is useful for breeding programs aimed at improving the winter hardiness of perennial forage grasses.

Genetic maps are very important for QTL mapping [20], and single nucleotide polymorphism (SNP) markers can be used to represent the most abundant sources of variation in genomes. SNP markers are ideal for constructing genetic maps because of their large quantity and low cost [21], and SNP-based genetic maps have been developed in several crop species, such as wheat [22], rice [23], and alfalfa [18]. A large number of SNP markers can be obtained by sequencing methods such as genotyping-by-sequencing (GBS), RNA-seq and restriction associated DNA sequencing (RAD-seq). GBS [24], as described by Elshire et al. (2012), is one of the most popular reduced-representation approaches for genomic selection in crop species [5] and utilizes selected restriction enzymes such as methylation-sensitive enzymes to cut the genome at fixed points and then sequences the restriction fragment ends for genotyping [24].

In the present study, we established an F1 population consisting of 392 progeny lines. Genotyping was done using GBS, and linkage maps were constructed by GBS-SNP markers. The objective of this study was to map QTL associated with alfalfa spring regrowth vigor. The identified QTL significantly correlated with the alfalfa spring regrowth vigor trait and will provide important reference information to understand the genetic basis of spring regrowth vigor, and it can also be used in MAS breeding programs of alfalfa.

Results

Phenotypic data

Significant phenotypic differences among the 392 F1 individuals were found in each environment. Additionally, the genotype × year interaction was significant. The F1 population exhibited transgressive segregation (Table 1). The coefficient of variation values in all three environments were above 30%, namely 33.00%, 32.33%, and 31.05% (Table 1). In the normal distribution test, the p values were all < 0.0001 (Table 1), indicating that the data from the three environments do not conform to the normal distribution. The broad sense heritability was obtained by ICIMapping software, and the values of the three environments were 0.80, 0.74, and 0.65 (Table 2).

Table 1
Summary statistics analysis of spring regrowth vigor in the F1 population and parents.

Location	Year	Mean of paternal parent	Mean of maternal parent	F1 population		CV (%)	Skewness	Kurtosis	H ² (%)
				mean	range				
Langfang (LF)	2019	2.33	2.67	1.99	1 ~ 3	33.00	-0.01	-1.14	0.80
Changping (CP)	2018	2.75	1.50	1.84	0.5 ~ 3	32.33	0.23	-0.86	0.74
	2019	2.25	1.75	1.89	1 ~ 3	31.05	0.22	-0.77	0.65

CV, coefficient of variation; H², broad-sense heritability based on the individual mean.

Table 2
Variance analysis of spring regrowth vigor in the F1 population.

	df	Type III SS	Mean square	F value	P value
Genotype	391	853.10	2.18	7.92	< .0001
Environment	2	8.70	4.35	15.79	< .0001
Replication	3	14.03	4.68	16.97	< .0001
Genotype*environment	748	283.10	0.38	1.37	< .0001

Qtl Mapping For Spring Regrowth Vigor

The linkage groups (LG) were constructed as described in our previous study (Zhang et al., 2019 accepted). Briefly, SNPs from both parents were assembled into 32 linkage groups using a minimum logarithm of odds (LOD) score of 7 for the male parent and 16 for the female parent. The 32 linkage groups (LG) of the male parent consisting of 944 SNP markers were assembled in a linkage map spanning approximately 4,088.70 cM with an average marker density of 4.33 cM/SNP. The total length of the female linkage groups was 4,229, 15 cM with 2,874 SNP markers. The average marker density was 1.5 cM/SNP.

A total of 15 QTL were identified, and each QTL explained approximately 3.1 to 48.8% of the phenotypic variation (Table 3, Fig. 1). In the three environments, we detected five QTL (qCP2018 ~ 1, qCP2018 ~ 2, qCP2018 ~ 3, qCP2018 ~ 4, qCP2018 ~ 5) in CP2018, four QTL (qCP2019 ~ 1, qCP2019 ~ 2, qCP2019 ~ 3, qCP2019 ~ 4) in CP2019 and six QTL (qLF2019 ~ 1, qLF2019 ~ 2, qLF2019 ~ 3, qLF2019 ~ 4, qLF2019 ~ 5, qLF2019 ~ 6) in LF2019. In the male parent, we identified no QTL in the CP2019 environment. Among these 15 QTL, there were eight QTL with phenotypic variation explained (PVE) > 10%. Three of eight (qLF2019 ~ 4, qCP2018 ~ 2, qCP2019 ~ 1) were detected on 6D in the female parent. The other five QTL (qLF2019 ~ 5, qLF2019 ~ 6, qCP2018 ~ 3, qCP2018 ~ 4, qCP2018 ~ 5) were identified in the male parent, and they all explained > 10% of the phenotypic variation (Table 3).

Table 3
Quantitative trait loci (QTL) for spring regrowth vigor in an F1 population.

Parents	QTL code	LG	Position	Left markers	Right markers	Interval (cM)	LOD	PVE (%)	Add
Male parent	qLF2019 ~ 5	3B	47	TP92280	TP91785	44.5 ~ 48.5	3.1	10.42	0.1
Male parent	qLF2019 ~ 6	7D	58	TP20704	TP97357	57.5 ~ 58.5	13.1	46.04	0.2
Male parent	qCP2018 ~ 3	7D	36	TP98026	TP41213	35.5 ~ 36.5	7.9	13.44	0.2
Male parent	qCP2018 ~ 4	7D	45	TP16589	TP80096	44.5 ~ 45.5	6.4	10.67	0.1
Male parent	qCP2018 ~ 5	7D	58	TP20704	TP97357	57.5 ~ 58.5	14.2	23.98	0.2
Female parent	qLF2019 ~ 1	4A	22	TP93383	TP63484	20.5 ~ 22.5	3.5	2.61	0.1
Female parent	qLF2019 ~ 2	5B	82	TP68584	TP27954	81.5 ~ 82.5	3.9	3.9	-0.1
Female parent	qLF2019 ~ 3	6D	16	TP88872	TP62169	15.5 ~ 17.5	7.9	6.61	-0.1
Female parent	qLF2019 ~ 4	6D	26	TP48491	TP53281	25.5 ~ 26.5	50.1	48.8	-0.4
Female parent	qCP2018 ~ 1	6D	3	TP42676	TP74220	0 ~ 5.5	5.4	7.13	-0.2
Female parent	qCP2018 ~ 2	6D	29	TP29932	TP58410	28.5 ~ 29.5	30	33.84	-0.3
Female parent	qCP2019 ~ 1	6D	25	TP72418	TP48491	24.5 ~ 26.5	19.3	22.71	-0.3
Female parent	qCP2019 ~ 2	7B	102	TP38045	TP46685	100.5 ~ 102.5	3.9	4.99	0.1
Female parent	qCP2019 ~ 3	8C	28	TP52104	TP53137	25.5 ~ 29.5	3.5	4.45	-0.1
Female parent	qCP2019 ~ 4	8C	103	TP36099	TP5247	101.5 ~ 105.5	3.8	3.96	0.1

Five QTL for male parents and ten QTL for female parents were mapped on genetic linkage maps for the phenotypic datasets. Left markers, marks at the left of the LOD peak; right markers, marks at the right of the LOD peak; LG, linkage group; interval (cM), 1-LOD support interval; LOD, the logarithm of the odds; PVE, the percentage of the phenotypic variation explained by QTL; Add: the additive effects of the QTL.

Five QTL for spring regrowth vigor were identified in the male parent, and all of them had a positive effect on trait value (Table 3). Four of the five QTL (qLF2019 ~ 6, qCP2018 ~ 3, qCP2018 ~ 4, qCP2018 ~ 5) were detected on homolog 7D. The QTL qLF2019 ~ 6 and qCP2018 ~ 5 were in the same genomic region (57.5cM ~ 58.5 cM) (Table 3, Fig. 1). The PVE percentage of these two QTL were 46.04% and 23.98%, and their LOD values were 13.1 and 14.2, respectively. This outcome suggests that this chromosome is important for the spring regrowth vigor trait. Another QTL (qLF2019 ~ 5) was located on LG 3B at a position from 44.5 cM to 48.5 cM, and the PVE was 10.42% (Table 3, Fig. 1). We identified 10 QTL in the female parent. Two QTL (qLF2019 ~ 4 and qCP2019 ~ 1) were detected on homolog 6D and were located at 25.5 cM ~ 26.5 cM and 24.5 cM ~ 26.5 cM, respectively. They have an overlapping interval. The LOD values were 50.1 and 19.3, and the PVEs were 48.80% and 22.71%, respectively (Table 3, Fig. 2). Another significant QTL was qCP2018 ~ 2, which explained 33.84% of the phenotypic variation. In addition to these 3 QTL on 6D, other potential QTL (qLF2019 ~ 3, qCP2018 ~ 1) were detected on this homolog, suggesting that this homolog is important for the spring regrowth vigor trait. All QTL detected on 6D had negative effects on trait value. We also identified 2 QTL (qCP2019 ~ 3, qCP2019 ~ 4) on 8C. Furthermore, another three QTL, qLF2019 ~ 1, qLF2019 ~ 2 and qCP2019 ~ 2, were also detected on the homologs of female parent chromosomes: 4, 5, and 7, respectively. The percentage of PVE by these 5 QTL varied from 2.61–4.99% (Table 3, Fig. 2).

Discussion

In alfalfa, no consensus DNA map exists, despite that a limited number of genetic maps were published, and genomic resources are very scarce. Before the development of third-generation sequencing, obtaining a large number of markers was very difficult. Genetic maps were constructed by SSR, RFLP, and other marker types not based on large-scale genome sequencing [5]. An early reported tetraploid alfalfa linkage map only had seven linkage groups with 443 cM [28]. With the development of sequencing technology, SNP markers are widely used in genetic maps. SNP markers can be generated using many sequencing methods such as RNA-seq [29] and RAD-Seq [13], and we used GBS to sequence the 392 alfalfa individuals. Using the GBS sequencing method, genetic linkage maps have been constructed for rice[30], barley and wheat [20], olive [31, 32], alfalfa [26] and other plant species.

Spring regrowth vigor is a complex trait that is controlled by multiple genes. While it has been studied in perennial ryegrass [29]. However, genetic studies of spring regrowth vigor are limited, and genes controlling spring regrowth vigor have not been elucidated. Since spring regrowth is complex and involves a large number of genes, some key genes likely have only a small phenotypic variation. This notion indicates that QTL mapping analysis of complex quantitative traits in alfalfa could be challenging and that large populations will be needed [33]. Compared with other QTL mapping populations in alfalfa [12, 17] and other species [34, 35], the population in our study was sufficiently large for the QTL mapping of spring regrowth vigor traits.

Among the 15 QTL identified in our study, 9 QTL were located on 7D of the male parent and on 6D of the female parent. These nine QTL explained the high phenotypic variation (PVE > 10%). Specifically, the two QTL, qLF2019 ~ 6 and qLF2019 ~ 4, explained 46.04% and 48.80% of the phenotypic variation, and the LOD values were 13.1 and 50.1, respectively. These QTL on the two chromosomes are relatively concentrated, especially on the 6D chromosome of the female parent, where four QTL are concentrated in the 14 cM interval range of 15.5–29.5 cM. Because the interval in these two chromosomes has been identified as containing QTL for spring regrowth vigor in different environments (locations and years), all of the QTL have a high PVE. This is a strong indication that these two chromosomes contain stable, effective loci for spring regrowth vigor.

A previous study by Mccord et al. (2014) reported QTL for alfalfa spring regrowth vigor [19]. Unfortunately, no QTL in our study were located at the same or similar positions as that reported in their study [19]. They reported a significant correlation between forage yield and spring vigor because of colocalized QTL that were identified for these traits. In the present study, two QTL (qLF2019 ~ 5, qCP2019 ~ 4) were located at similar positions to the QTL associated with yield in the study [19]. At the same time, QTL detected on chromosome 6 (qLF2019 ~ 4, qCP2019 ~ 1) in this study were located at similar genomic locations as reported in previous studies [12]. The QTL were used to BLAST search against the *M. truncatula* genome [18, 36], and flanking markers were used for comparative analysis of the genomic region. However, *M. truncatula* is an annual plant that has little regrowth ability [37]. Sakiroglu and Brummer (2017) found that no SNPs associated with spring regrowth were mapped to the *M. truncatula* genome [38]. In our study, we only found some genes (MTR_7g118350, MTR_7g118120, and MTR_6g069870) that were associated with forage yield in our QTL region (QTL name). These results further indicate that there is a significant correlation between alfalfa spring vigor and forage yield.

The QTL analysis performed in this study revealed that some of the QTL associated with spring vigor were located at a similar region as those associated with fall dormancy and winter injury in previous studies [16, 17]. Adhikari et al. (2018) mapped four QTL associated with winter injury on chromosome 6 in the 18–40 cM, and our study found four QTL in the 15.5–29.5 cM region of chromosome 6 [17]. Unfortunately, there was no QTL located in the same or similar interval to the QTL associated with fall dormancy in their study [17]. Li et al. (2015) identified six QTL associated with fall dormancy on chromosomes 4, 6 and 7, and five QTL associated with winter injury on chromosomes 3 and 7 [16]. These QTL were located in the same or similar intervals as the QTL (qLF2019 ~ 5, qCP2018 ~ 3, qLF2019 ~ 1, qLF2019 ~ 4, qCP2019 ~ 1) associated with spring regrowth vigor in our study. However, due to differences in mapping populations and methods used in the research process, further investigation of the association between these traits in alfalfa is warranted.

The traits related to QTL and SNPs that were stably present in the different environments and had a higher percentage of the phenotypic variation can be used to accelerate alfalfa breeding programs. However, the QTL identified in this paper were not sufficient for use in direct molecular breeding because a single quantitative trait locus may contain hundreds of genes [39], and some QTL may only exist in a specific population [40]. Therefore, it is necessary to verify the reported QTL in different genetic backgrounds and multiple environments.

Conclusion

We have established an F1 population of 392 individuals, and QTL mapping analysis was performed using the genetic linkage maps that were constructed in a previous study. We identified 15 QTL associated with spring regrowth vigor in the F1 population. Four QTL were located on LG 7D of the male parent, and five QTL were located on LG 6D of the female parent. Both male and female parents had one colocalized quantitative trait locus on LG 7D and LG 6D, respectively. A correlation was observed between spring regrowth vigor and forage yield. These results suggest that there is a significant correlation between alfalfa spring vigor and forage yield. Our results will provide useful information for molecular breeding of alfalfa. These QTL will be a valuable genomic resource for the development of alfalfa spring regenerative vigor by MAS.

Methods

Mapping Population

An F1 population consisting of 392 progeny lines was described in our previous study (Zhang et al, 2019 accepted). In 2015, the seeds of the F1 population were planted in the greenhouse at the Chinese Academy of Agricultural Sciences (CAAS) in Langfang, Hebei Province, China. Clones were propagated from each individual by stem cuttings. During the early branching stage (2016-4-12), the cloned plants were removed from the propagation flats and transplanted to Langfang (LF), Hebei Province and Changping (CP), Beijing Province. The experimental design at each location was a randomized complete block, with three replications in LF and four replications in CP. Every replicate contained 392 individuals with one cloned plant. The plants were spaced 80 cm apart within each row, and the rows were spaced 100 cm from each other. The plants at the two locations grew naturally, other than with weed control, without irrigation and without fertilization.

Data Analysis

Details of the genotype data analysis in this study were described previously (Zhang et al., 2019 accepted). Briefly, DNA was extracted using the CWBIO Plant Genomic DNA Kit (CoWin Biosciences, Beijing, China) according to the manufacturer's protocol. DNA concentrations were adjusted to 50 ng/ μ L and subsequently used for GBS library preparation. The DNA was digested using the EcoT221 restriction enzyme, and libraries were sequenced on a Hi-seq2000 (Illumina) system using 100-base paired-end sequencing at the Cornell University Sequencing Facility (Ithaca, NY, USA). The Tassel 3.0 Universal Network Enabled Analysis Kit (UNEAK) pipeline was used for SNP calling and genotyping [25]. Single-dose alleles (SDA, AAAB x AAAA) were calculated according to the method of Li et al. (2014) [26]. QTL IciMapping was used to identify QTL with the functionality of BIP by inclusive composite interval mapping with an additive effect (ICIM-ADD) [27]. A LOD threshold of 3.0 was used for declaring significant QTL and other parameters remained at the default value. The QTL detected for each parental map were indicated on linkage maps using MapChart.

Alfalfa phenotypic data were collected as regrowth height and plant density after fall dormancy and winter hardiness in the spring. To ensure consistency between the plants at the two locations, the last clipping

was performed simultaneously. We collected phenotypic data at LF (2018) and CP (2018, 2019). We have comprehensively considered the plants height and cover density. Specifically, spring regrowth vigor was rated on April 25 at LF and on April 27 at CP using a 0–3 scale in which 0, 1, 2, and 3 represented completely dead plants without recovery, and low, moderate, or high recovery of green tissues, respectively. Phenotypic data for the trait were analyzed using SAS9.4. The broad sense heritability (H^2) was calculated using the function AOV of QTL IciMapping [27]. The mean data for each environment (CP2018, CP2019, LF2018) were used for QTL analysis.

Abbreviations

QTL

Quantitative trait loci; GBS:Genotyping by sequencing; SNP:Single nucleotide polymorphism; MAS:marker-assisted selection; GWAS:Genome-wide association studies; GS:genomic selection; RAD-seq:Restriction associated DNA sequencing; LG:Linkage group; LOD:logarithm of odds; PVE:Phenotypic variation explained by QTL; Add:Estimated additive effect of the marker; UNEAK:Universal network enable analysis kit; ICIM-ADD:Composite interval mapping with additive effect.

Declarations

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Availability of data and materials

All GBS data were upload to NCBI with the BioProject ID of PRJNA522887. (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA522887>). The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

JK conceived and designed the experiments; XJ, FZ, RL, YS, ML, ZW, FH, XY and CY performed the experiments; XJ and FZ analyzed the data; XJ and FZ wrote the manuscript; all the authors revised the manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

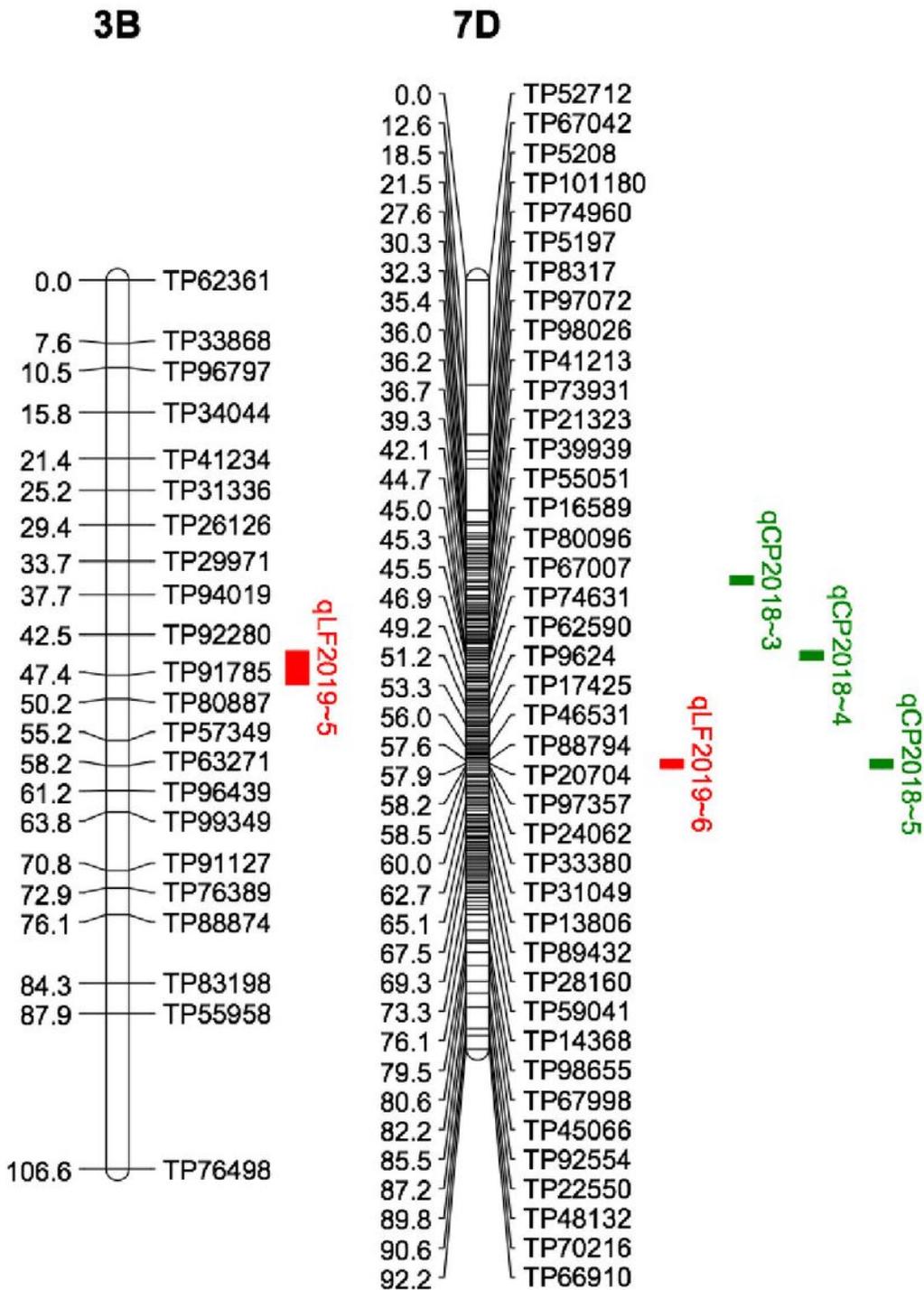


Figure 1

Figure 1. Spring regrowth vigor QTL mapped on LGs of homolog 3B (left) and 7D (right) for the male parent. QTL are depicted as colored vertical bars to the right of the linkage groups. Different colors represent different environments. The color of the QTL (red and green) represents the LF2019 and CP2018. Groups with no detected QTL are not shown.

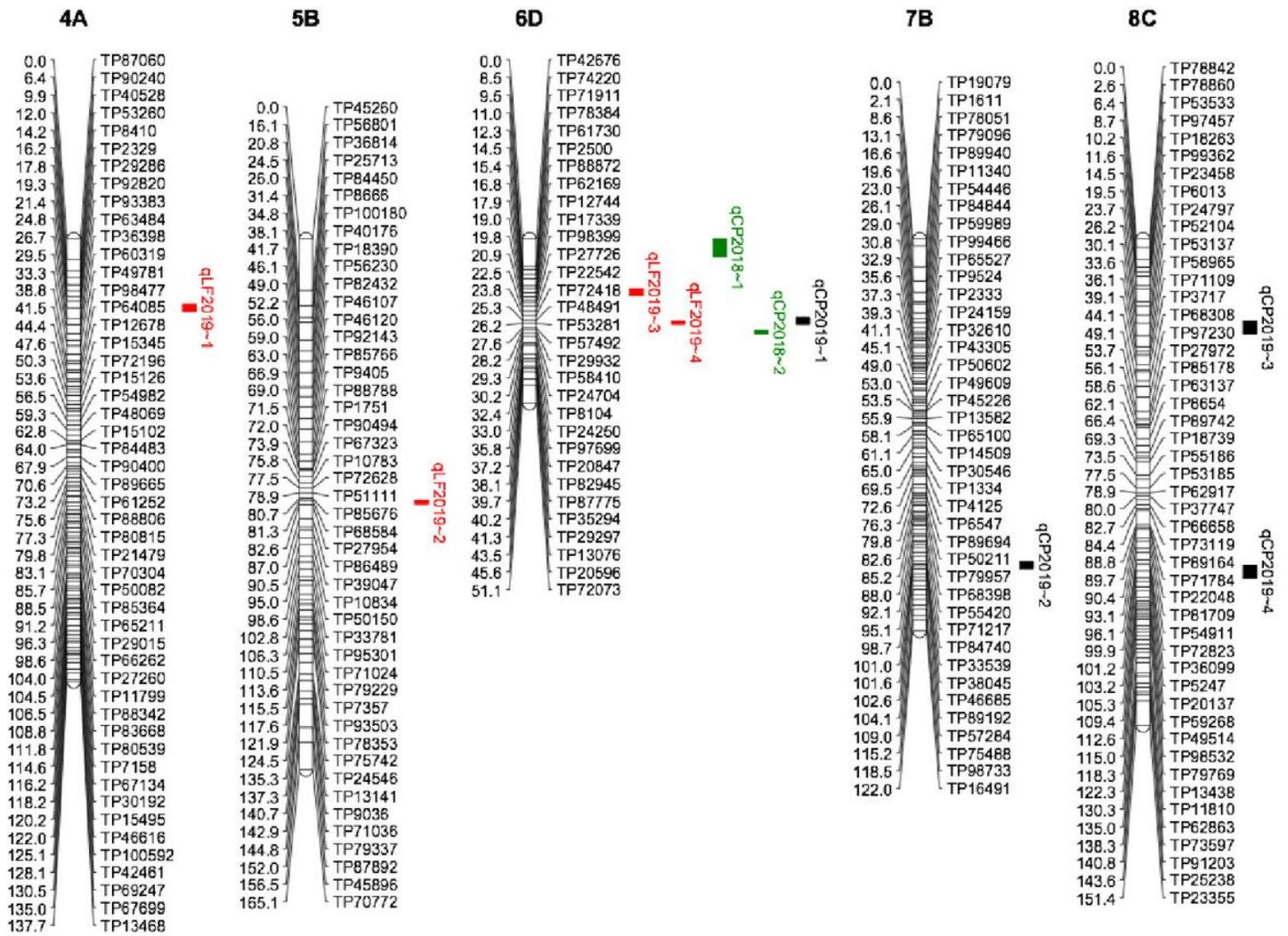


Figure 2

Figure 2. Spring regrowth vigor QTL mapped on LGs for the female parent. QTL are depicted as colored vertical bars to the right of the linkage groups. Different colors represent different environments. The colors of the QTL (red, green and black) represent the LF2019, CP2018 and CP2019. Groups with no detected QTL are not shown.