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QTL-Seq Identifies QTL for Relative Electrical Conductivity Associated with Heat Tolerance in Bottle Gourd (*Lagenaria Siceraria*)

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Heat is a major abiotic stressor seriously affects watermelon produce; however, its effects may be mitigated through grafting onto heat tolerant bottle gourd rootstock. To understand the genetic basis and select reliable markers are necessary in the variety breeding process. In this paper, the relative electric conductivity (REC) was used as a visual indicator for heat stress to study the genetics and SNP marker of heat tolerance in bottle gourd via QTL-seq approach. The results showed that recessive inheritance and a major QTL locus controlled REC related to heat tolerance. Seven heat-tolerant loci (*qHT1.1, qHT2.1, qHT2.2, qHT5.1, qHT6.1, qHT7.1*, and *qHT8.1*) exhibited high Δ(SNP-index) values (ranging from 0.19-0.38), and of these, the greatest value (0.32, $P < 0.01$) and greatest number of detected SNPs (9052) were found in chromosome 2 at *qHT2.1* region (11.03 - 19.25 Mb); *qHT2.1*was taken as a promising major QTL for heat tolerance in bottle gourd. A total of 34 putative candidate genes related to heat stress were detected within the *qHT2.1* region. Three polymorphic SNPs (BG_GLEAN_10022642, BG_GLEAN_10022727, and BG_GLEAN_10022589) that are involved in pollen sterility, intracellular transport, and signal recognition were validated and exhibited significant marker-trait association that could be used in heat tolerant molecular breeding for bottle gourd. The *qHT2.1*region is an important finding can be used for fine mapping and discovering new genes associated with heat tolerance in bottle gourd.

Keywords: Bottle gourd; *Lagenaria siceraria*; Heat tolerance; QTL-Seq; SNP; Relative electric conductivity

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Introduction

Heat stress negatively affected physiological processes, reproduction, and adaptation in plants [1], and changes in the climate have exacerbated such effects [2]. Watermelon (*Citrullus lanatus* var. *lanatus*) dominates the fruit market in the world [3], its growth frequently restricted by high temperatures reach more than 35℃ during summer months [4]. Bottle gourd (*Lagenaria siceraria* (Mol.) Standl.) tended to be the rootstock for watermelon to reduce heat stress and improve performance [5,6]. Phylogenetic tree analysis has demonstrated the close genetic relatedness of bottle gourd and watermelon [7,8], explains the high grafting survival rate and reduction in taste of bottle gourd in grafted watermelon.

Bottle gourd originated from Africa; it is widely distributed across the tropics [9], where long-term exposure to high temperatures has enhanced its tolerance to heat stress, albeit with wide variation among genotypes [10,11]. Understanding the genetics of heat tolerance in bottle gourd and identification of reliable markers are necessary for the development of novel bottle gourd rootstocks for heat adaptation improvement of scion; however, underlying tolerance characteristics remain unclear.

Heat tolerance is believed to be a polygenic trait that renders the introgression of multiple favorable alleles into cultivars problematic [12]. There have been attempts to deconstruct stress tolerance into measurable components for accurate phenotyping, with the aim that QTLs associated with heat tolerance may then be identified and suitable alleles may be introgressed into elite genetic backgrounds [13]. Cell membrane stability, which has been used as an indicator of heat stress [14], may be quantified by relative electric conductivity (REC). REC is highly sensitive to abiotic stress [15] and has been used in studies of abiotic stress tolerance in a range of crops, including salinity-alkalinity tolerance in muskmelon [16], drought tolerance in *Perennial ryegrass* [17], and cold tolerance in alfalfa [18].

Xu et al.[19] first reported partial sequencing of the bottle gourd genome using the 454 GS-FLX Titanium sequencing platform, from which 400 SSR markers were developed. RAD-Seq [20] technology has also been applied to an F_2 bottle gourd population for SNP and insertion-deletions marker development [21,22]. Wu et al.[8] reported a high-quality bottle gourd genome sequence was constructed using de novo genome assembly that reconstructed the most recent common Cucurbitaceae ancestor genome through comparison with available extant modern cucurbit species genome resources [23-25].These studies provide an available reference genome and useful molecular markers for the study of bottle gourd agronomic traits.

Many crop plant traits, such as heat stress tolerance, are quantitative, so identification of quantitative trait loci (QTL) may allow efficient crop breeding through marker-assisted selection (MAS) [26]. However, deriving the molecular markers for genetic linkage analysis, QTL analysis is labor-intensive and

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time-consuming [27]. The bulked-segregant analysis (BSA) provides an effective and simple way to identify molecular markers tightly related to target genes or major QTLs and recent advancements in next-generation sequencing (NGS) technologies provide rapid and efficient QTL identification. For example, Takagi et al. [27] proposed QTL-sequencing (QTL-seq) to detect QTLs that control partial resistance to rice blast using recombinant inbred lines and seedling vigor in F_2 populations; candidate QTLs detected using the QTL-seq method were confirmed using traditional QTL analysis. The QTL-seq approach has been widely used in a range of crops, such as sunflower [28], rice [29], sorghum [30], potato [31], and cucumber [32,33], for the efficient detection of QTLs for complex quantitative traits. The QTL detection for heat tolerance has been studied in rice [34], chickpea [35],wheat [36], and tomato [37]. In this study, we used QTL-seq to identify the major QTL for heat tolerance in bottle gourd, based on REC, from which polymorphic SNPs and putative candidate genes for heat tolerance were identified using available bottle gourd genome sequence information [21].

Materials and Methods

Plant Material and Phenotyping

We selected the heat tolerant inbred line L1 (P17) and sensitive inbred line L6 (P23) as parent material, based on previous work [38], from which F_1 and F_2 segregating populations were created. We germinated three replicates of seed from147, 56 and 60 F_2 individuals of L1, L6 and F_1 plants, respectively. The seedlings were planted in 32-hole plastic plugs (230cm^3/hole) filled with nursery substrate (2:1 mix of turf: vermiculite) and grown in a phytotron growth chamber set to a 16 h light/8 h dark cycle (30,000lux) of 25/18℃, with 80% relative humidity. The first true leaf of the seedlings was collected for DNA extraction, and when the third true leaf began to expand, the plants were moved into a high temperature controlled phytotron (40℃, with 80% relative humidity and 30,000lux) for 6h continuous heat exposure.

The electric conductivity (EC) of leaves was measured before and after the heat treatment to assess cell membrane damage, as described by Zhou and Leul [39] and He et al. [40] with modifications. In brief, sampled leaves were washed using deionized water, cut into 0.5-cm pieces, and immersed in deionized water for 30min; then, conductivity of the solution was measured using a conductivity meter (PHSJ-3F, Jingmi Instruments Co., Ltd., Shanghai, China) and recorded as S1. After boiling the leaf samples for 15 min, EC of the solution at room temperature was measured again and recorded as S2. REC was calculated as S1/ S2×100%.

Leaf relative injury (LRI) was used as a metric of cell membrane damage, where LRI = [(Lt-Lck)/(100- Lck)] ×100% [41-43]; Lt and Lck are the REC values before and after heat exposure, respectively, and greater LRI values reflect a lower degrees of heat tolerance.

We used analysis of variance (ANOVA) and t-tests to determine differences in heat tolerance, as indicated by mean (±SE) LRI, among P_1, P_2 and F_1 plants at $P<0.05$

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using SPSS 13.0 software [16]. For the F_2 population, the frequency distribution of LRI was plotted as a histogram and the normal distribution was fitted using the GaussAmp function in ORIGIN 9.1 software [44].

DNA Sequencing and Analysis

Genome DNA was isolated from young leaves of parental lines L1 and L6, and 147 F_2 plants using the CATB method [45]. For QTL-seq, two DNA pools (heat tolerance: T-pool; heat sensitive: S-pool) were constructed by mixing an equal amount of DNA from nine heat tolerant (LRI = 1-5%) and nine heat sensitive (LRI = 55-77%) individuals from the F_2 population.

A library of around 500 bp insert size was constructed and pair-end sequenced (parent average 15×coverage; progeny average was 20×coverage; read length: 150 bp) on an Illumina Hi-Seq4000 at Shanghai Biozeron Co., Ltd.

Raw sequencing data were generated by Illumina base calling software CASAVA v1.8.2 [46] and raw paired end reads were trimmed and quality controlled by Trimmomatic (http://www.usadellab.org/cms/index.php?page=trimmomatic), with default parameters. The high-quality short reads from T-pool and S-pool were aligned to the bottle gourd (Hangzhou gourd) reference genome (http://cucurbitgenomics.org/organism/13, [21]) using BWA software (http://bio-bwa.sourceforge.net/, [47]). After removing PCR-duplicate reads using Picard Tools (http://picard.sourceforge.net/), the valid BAM file was used to detect SNPs using the GATK "UnifiedGenotyper" function (http://www.broadinstitute.org/gatk/); then, low-quality SNPs, with quality value <20 and read depth coverage <4× or >200×, were excluded using custom perl scripts [48].

To identify candidate regions for heat tolerance QTLs, we calculated and plotted two parameters (SNP-index and Δ(SNP-index) for T-pools and S-pools [32,49]. For the polymorphic SNPs located in the genomic regions harboring the major heat tolerance QTL, further functional annotation was completed using available bottle gourd genome data (http://cucurbitgenomics.org/organism/13) and Uniprot database (www.uniprot.org) using ANNOVAR analysis (http://www.openbioinformatics.org/annovar/) to detect putative candidate genes [21].

SNP Marker Development and Selection

Five candidate SNPs were selected, based on the annotation and classification of putative candidate genes located in the QTL of *qHT2.1* interval; the flank sequences were used for PCR primer development using Primer 6.0 soft (http://www.PromerBiosoft.com). The five SNPs were genotyped for four heat tolerant and four sensitive F_2 individuals using PCR and Sanger sequencing, and sequence results were visualized and checked using SnapGene software. We used trait-marker association to select the promising SNP markers.

Results

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LRI Indicator of Heat Tolerance

There were differences in mean LRI between L1 (P17: 9.88±5.00%) and L6 (P23: 47.26±10.78%) under heat stress conditions (Table 1). Mean LRI of the F_1 was greater (31.98±6.64%) than the mid-parent value (28.32±7.89%), and there was a difference in LRI between F_1 and L1; there was no difference in LRI between F_1 and L6. The LRI of the F_2 progeny ranged from 0.34 to 97.49% (mean: 29.68±8.52) (Table 1). Transgressive segregation was observed on the both sides of the distribution, because distribution of the three F_2 populations was slightly skewed towards L1 (P17) and showed Gaussian segregation (Fig 1A, B, C).

Table 1. The leaf relative injury (LRI) of parental lines, F_1 and three F_2 populations under heat stress conditions.

Type	Code	LRI		Reaction
		Mean±SE	Range	
Tolerance parent	L1 (P17)	9.88±5.00*	4.28-20.25	Tolerant
Sensitive parent	L6 (P23)	47.26±10.78*	33.52-59.32	Sensitive
Mid-parent value	-	28.32±7.89	-	-
F_1population	L1×L6	31.98±6.64b	19.95-43.95	Sensitive
F_2 population	-	29.68±8.52	0.34-97.49	-

*P <0.05.

Fig 1. Frequency distribution of leaf relative injury (LRI) shows phenotypic variation among three F_2 populations with 147 individuals **(A)**, 56 individuals **(B),**and 60 individuals **(C)**.
L1: heat tolerant parent P17; L6: heat sensitive parent P23. Distribution of L1 near the origin of the x-axis indicates negative transgressive segregants, while distribution of L6 indicates positive transgressive segregants under heat-stress conditions. DNA of nine F_2 seedlings was selected from an F_2 population with 147 individuals **(A)** with extreme phenotypes (low and high LRI values) to develop tolerant and sensitive pools.

Sequencing Data

Sequencing yielded 31.9, 32.7, 40.9, and 40.9 M 150-bp paired-end raw reads from L1, L6, T-pool, and S-pool, respectively. After trimming and filtering, more than 80% of reads were mapped to the bottle gourd reference genome (Table 2). Aligned to the bottle gourd reference genome (313.4 Mb), we found 23.0, 24.4, 29.0, and 29.5 M short reads were mapped to L1 (10.10×depth coverage or 92.75% coverage), L6 (10.85×depth coverage or 92.85% coverage), T-pool (12.71×depth coverage or 93.25% coverage) and S-pool (12.92×depth coverage or 93.25% coverage), respectively. Based on unique mapped reads, we identified 184,531 and 188,304 SNPs from the T-pool and S-pool, respectively.

Table 2. Sequencing and SNP detection.

Sample	Number of raw reads	Number of trimmed and filtered reads	Uniquely mapped reads				Number of SNPs		
			Number	%	Average Depth	Coverage (%)[a]	Total	Homozygous	Heterozygous
L1	31,863,484	28,368,754	22,974,995	80.99	10.10	92.75	476,940	345,242	131,698
L6	32,686,118	29,026,164	24,419,983	84.13	10.85	92.85	513,552	418,749	94,803
T-pool	40,647,374	35,340,578	29,026,863	82.13	12.71	93.25	543,798	359,267	184,531
S-pool	40,930,720	35,936,306	29,457,284	81.97	12.92	93.25	549,415	361,111	188,304

[a]size of the bottle gourd reference genome is 313,397,697 bp (Xu et al. 2014).

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QTL Detection for Heat Tolerance by QTL-seq

The SNP-index graphs were generated for the T-pool (Fig 2A) and S-pool (Fig 2B), and Δ(SNP-index) was plotted against the genome positions (Fig 2C). By examining the Δ(SNP-index) plot, we identified seven genomic positions that exhibited high Δ(SNP-index) values (Table 3) and comprised regions on chromosome 1, from 26.3 to 27.3 Mb (Δ(SNP-index) = 0.25; P < 0.01); chromosome 2, from 11.03 to 19.25 Mb (Δ(SNP-index) = 0.32; P < 0.01) and from 19.4 to 21.0 Mb (Δ(SNP-index) = 0.19; P < 0.01); chromosome 5, from 39.4 to 40.4 Mb (Δ(SNP-index) = 0.38; P< 0.01); chromosome 6, from 7.5 to 8.5 Mb (Δ(SNP-index) = 0.21; P<0.01); chromosome 7, from 23.2 to 24.2 Mb (Δ(SNP-index) = 0.22; P< 0.01); and, chromosome 8, from 24.9 to 25.9 Mb (Δ(SNP-index) = 0.21; P< 0.01). The number of SNPs in the two chromosomes with the greatest Δ(SNP-index) values was greater in Chr2 (11.03-19.25 Mb; 9052) than Chr5 (2) (Table 3) and the SNP-index in the T-pool was lower (0.31) than in the corresponding region of the S-pool (0.63), appearing as a clear mirror image (Δ(SNP-index) = 0.32; P < 0.01; Fig 2). Thus, a promising major QTL controlling heat tolerance appeared to be harbored in the 11.03 to 19.25 Mb regions on chromosome 2 and was designated as *qHT2.1*.

Fig 2. SNP-index plots of T-pool**(A)**and S-pool **(B)** and Δ(SNP-index) plot **(C)** from QTL-seq analysis.

The x-axis represents the position of eleven chromosomes and the y-axis represents SNP-index. The Δ(SNP-index) plot (**C**) shows confidence intervals under the null hypothesis of no QTL (P < 0.01). The promising genomic region identified for LRI associated with heat tolerance is shaded at 11.03-19.25 Mb on Chromosome 2.

Identification of Heat Tolerance Candidate Genes in *qHT2.1* Intervals

The full intervals of the regions covered by the seven major Δ(SNP-index) peaks were wide and ranged 8.22 Mb for *qHT*2.1 (Table 3). The 9052 SNPs in this region comprised 527 genes, of which 279 were annotated by BLAST analysis against the non-redundant protein database (S1Table, [50]) to leave 62 nonsynonymous and stoploss type of SNPs with Δ(SNP-index) ≥0.5 (S2 Table). Gene ontology classification revealed that these 62 genes were mainly associated with biological processes (Fig 3), such as metabolism, cellular components, such as the cell membrane, and molecular function, such as catalytic activity. The biological processes of the 62 candidate genes were retrieved from the available bottle gourd genome data and Uniprot database, 34 genes related to heath stress were determined, along with their corresponding biological processes, comprising pollen and flower sterility, oxidative stress response, autophagy, and abiotic stress response (Table 4).

Table 3. QTLs (QTL-seq) that conferred heat tolerance in bottle gourd.

Region	QTL	SNP Number	SNP-index		Δ (SNP-index)	P-value	Interval
			T-pool	S-pool			
Chr01:26,260,000-27,279,999	qHT1.1	12	0.3713	0.622	0.25	0.000413811	1,019,999
Chr02:11,030,000-19,249,999	qHT2.1	9052	0.3092	0.6285	0.32	9.79E-06	8,219,999
Chr02:19,410,000-20,989,999	qHT2.2	1355	0.3579	0.5463	0.19	0.006172464	1,579,999
Chr05:39,390,000-40,419,999	qHT5.1	2	0.45	0.8333	0.38	1.42E-07	1,029,999
Chr06:7,460,000-8,459,999	qHT6.1	934	0.5917	0.379	-0.21	0.001783751	999,999
Chr07:23,220,000-24,229,999	qHT7.1	5	0.3648	0.5812	0.22	0.001986976	1,009,999
Chr08:24,880,000-25,889,999	qHT8.1	4	0.1853	0.3988	0.21	0.002248176	1,009,999

Fig 3. GO results for 62 candidate genes located in the *qHT2.1* region.

Table 4. Function annotation of candidate genes.

Code	Gene	Start	End	Gene annotation	GO results	Gene function	Reference	Classification
1	BG_GLEAN_10022767	18202876	18208313	ARM repeat superfamily protein		gametogenesis	[51]	Pollen and flower sterility
2	BG_GLEAN_10022642	16351903	16356748	annexin 5	pollen tube growth calcium-dependent	pollen development	[52]	
3	BG_GLEAN_10022513	14863003	14864202	beta-galactosidase 7		pollen development	[53]	
4	BG_GLEAN_10022514	14864272	14865477					
5	BG_GLEAN_10022346	12947058	12948329	ORF158	metal ion binding	Ogura cytoplasmic male sterility	[54]	
6	BG_GLEAN_10022530	15056845	15057528					
7	BG_GLEAN_10022457	14198082	14198549	CRK8	cellular metabolic process	protect against oxidative stress	[55]	Defying oxidative and signal recognition
8	BG_GLEAN_10022458	14202592	14203758					
	BG_GLEAN_10022533	15143557	15144330					
10	BG_GLEAN_10022534	15150418	15153009					
11	BG_GLEAN_10022556	15364584	15366248					
12	BG_GLEAN_10022653	16479728	16480867					
13	BG_GLEAN_10022511	14855903	14856478	DEAD/DEAH box RNA helicase family protein		perform essential roles in stress response	[56]	
14	BG_GLEAN_10022502	14706104	14708853	serine-type peptidase activity		thermal tolerance	[57]	
15	BG_GLEAN_10022339	12878315	12879195	PQL1, PQL2	single-organism carbohydrate metabolic process	photosystem II oxygen evolving complex/calcium ion binding	[58]	

16	BG_GLEAN_10022589	15709012	15709407	serine/threonine-protein kinase SRK2C-like (Cucumissativus)		fungus infection signal recognition and transduction	[59]	
17	BG_GLEAN_10022369	13153956	13158125	ATG18	single-organism cellular process/cytoplasmic part	autophagy-related genesresponses to stress	[60]	Autophagy
18	BG_GLEAN_10022459	14206108	14206779	Retrotransposon gag protein		retrotransposon	[61]	Transcription factor
19	BG_GLEAN_10022500	14679103	14679579					
20	BG_GLEAN_10022504	14723300	14723737					
21	BG_GLEAN_10022508	14767873	14768121					
22	BG_GLEAN_10022531	15058961	15059329					
23	BG_GLEAN_10022553	15346449	15347135					
24	BG_GLEAN_10022555	15349247	15349756					
25	BG_GLEAN_10022720	17357350	17357901	Transposase, MuDR, plant		transposase	[62]	DNA-binding
26	BG_GLEAN_10022734	17634365	17635519	MuDR family transposase	binding			
27	BG_GLEAN_10022402	13576671	13577819	Zinc finger, CCHC-type	binding	DNA-binding protein	[63]	
28	BG_GLEAN_10022652	16477031	16477843					
29	BG_GLEAN_10022770	18248778	18250028					
30	BG_GLEAN_10022515	14929579	14930304	gag/pol protein		intracellular transport	[64]	Intracellular

31	BG_GLEAN_10022727	17456571	17458331	Clathrin adaptor complex small chain family protein	intracellular protein transport AP-3 adaptor complex		[65]	transport
32	BG_GLEAN_10022776	18292930	18294105	RNA-directed DNA polymerase (reverse transcriptase)-related family protein				
33	BG_GLEAN_10022777	18295459	18295665	DNAse I-like superfamily protein		catalysis	[66]	Catalysisenzyme
34	BG_GLEAN_10022276	12078272	12083573	pyridoxal phosphate-dependent enzyme	metabolic process/pyridoxal phosphate binding/Cys or Met metabolism	oxidative biocatalysis	[67]	

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SNP Marker Development and Selection

Based on the 34 candidate genes, five SNPs were selected for primer design (Table 5), comprising one related to pollen and flower sterility (SNP 2: BG_GLEAN_10022642, annexin 5), two related to signal recognition (SNP 15: BG_GLEAN_10022339, PQL1 and PQL2; and, SNP 16: BG_GLEAN_10022589, serine/threonine-protein kinase), and two related to intracellular transport (SNP 26: BG_GLEAN_10022734, MuDR; and, SNP 31: BG_GLEAN_10022727, AP-3 adaptor complex). Genotyping of four heat tolerant and four sensitive F_2 individuals (Fig 4, Table 5) showed two of the five SNPs (SNP 15 and SNP 26) were similar between the two heat sensitivity groups, while SNPs 2 and 31were homozygous in heat tolerant individuals (CC and TT, respectively) and homozygous and heterozygous in heat sensitive individuals (AA and AC for SNP 2, and AA and AT for SNP 31). The only homozygous SNP for heat tolerance and heat sensitive individuals was SNP16 (BG_GLEAN_10022589; GG and AA, respectively. Three polymorphic markers (SNPs 2, 31, and 16) showed good marker-trait association, where SNPs 2 and 31may be dominant markers, and SNP 16 may be a co-dominant marker for heat tolerance.

Table 5. Primer design and Sanger sequencing verification of five candidate loci in four tolerant and sensitive F_2 individuals.

Code	Gene	Mutation	Primer-F	Tm-R	Primer-R	Tm-F	Heat tolerant				Heat sensitive			
							1	2	3	4	1	2	3	4
15	BG_GLEAN_10022339	C->T	TACTCCTCGGCGTCTAACCA	60.035	TGAGAAGCAGCCTCTCGTTG	60.038	C	C	C	C	C	C	C	C
26	BG_GLEAN_10022734	A->G	AGGTAGGCCCGAAGACTCAT	60.032	GGGAGGGCAGCTTGTTACTT	59.962	AG	AG	AG	AG	AG	AG	AG	AG
2	BG_GLEAN_10022642	C->A	CGCGCTATGAAGGTCCAGAA	60.179	CCCAGGATTCTCAGCACACA	59.674	C	C	C	C	A	AC	AC	A
31	BG_GLEAN_10022727	T->A	CGTCTATGGGGGTTGGCAAT	60.107	CGAGAGTCCTGCAGCAGAAA	60.038	T	T	T	T	A	AT	AT	A
16	BG_GLEAN_10022589	A->G	GGGGATAGACGTTGCGAGAG	59.969	TTTGACTCGCTCCTCCACAC	59.966	G	G	G	G	A	A	A	A

Fig 4. Genotyping of four heat F_2 tolerant (**A**, **C**, **E**, **G**, **I**) and four heat sensitive F_2 (**B**, **D**, **F**, **H**, **J**) individuals derived from five candidate SNPs.
(AB) SNP15: BG_GLEAN_10022339. **(CD)** SNP26: BG_GLEAN_10022734. **(EF)** SNP 2: BG_GLEAN_10022642. **(GH)** SNP 31: BG_GLEAN_10022727. **(IJ)** SNP 16: BG_GLEAN_10022589. The SNP loci are shaded and indicated with a red arrow.

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Discussion

Genetics of Heat Tolerance in Bottle Gourd

Plants have evolved a range of metabolic responses, such as antioxidant activity, membrane lipid unsaturation, protein stability, gene expression and translation, and accumulation of compatible solutes [68], to cope with heat stress through the activation of stress-response genes [69]. Heat tolerance, which is characterized by a series of phenotypic and physiological traits, may be summarized as the quantitative trait [70]. Poly genes are known to play a critical role in the control of heat tolerance in crop plants, such as wheat [71,72], rice [34], and broccoli [73,74], although there are reports monogenic and oligogenic responses to heat tolerance [75,76]. Previous understanding of inheritance patterns of heat tolerance in bottle gourd was limited, and our study is the first to report on the recessive inheritance and a major QTL locus for heat tolerance in this crop plant, based on REC using a QTL-seq approach.

The plants in the F_1 progeny of the L1 × L6 cross were heat sensitive, indicating that heat tolerance is a recessive trait in bottle gourd, as confirmed through genotyping of the candidate marker SNPs 2 (BG_GLEAN_10022642) and 31 (BG_GLEAN_10022727) as homozygous CC or TT, respectively, that indicate heat tolerance, and A_ (AA, AC or AT) that indicate heat sensitivity. These results also infer the presence of a dominant locus of AA in heat sensitive parent material.

The distribution of the F_2 population was skewed towards L1, indicating that a major QTL locus controlled heat tolerance-related LRI. According to the principle of QTL-seq, described by Takagi et al. [27], the SNP-index graphs of the T-pool and S-pool were expected to be identical for the genomic regions, without phenotypic differences (heat tolerance), whereas the genomic region(s) harboring heat tolerance QTL(s) were expected to exhibit unequal contributions from P_1 and P_2 parental genomes. Furthermore, the SNP-indexes of these regions for T- and S-pools should appear as a mirror image around SNP-index = 0.5. Although seven QTLs were identified using QTL-seq, only *qHT2.1* was identified as the most promising major QTL for heat tolerance, because of the greatest Δ(SNP-index) value (0.32, $P < 0.01$) and number of detected SNPs (9052), and clear mirroring of SNP-index plots. Similar results have been reported for African rice (*Oryza glaberrima* Steud.), in which a major QTL that controlled survival rate of seedlings exposed to high temperatures was governed by the Thermo tolerance 1 gene encoded in the 23S proteasome subunit, which degrades ubiquitinated cytotoxic denatured proteins that are formed due to high temperature stress [77]. Major QTL control of complex traits, such as heat tolerance, may be explained by inheritance patterns of the trait [13] and action of major regulator genes that may switch off subordinate genes, if a key gene is mutated [78].

Annotation of Heat Tolerance Candidate Genes

Understanding of functional genomics in plants facilitates the elucidation of candidate genes and their relationship with traits [35]. Heat stress affected pollen and flower sterility [79]. Of the 34 candidate genes, four genes were identified to have key roles

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in gametogenesis [51], pollen development [52,53] or Ogura cytoplasmic male sterility[54]. Heat-stress also caused cellular damage by oxidative stress and toxicity due to the reactive oxygen species (ROS) formation [80,81]. Antioxidants scavenge reactive oxygen species to mitigate oxidative. Four putative candidate genes were found to have a function in defying oxidative stress and recovering plants from heat-stress damage. Many transcription factors, binding factors, intracellular transporters and enzyme responsible for abiotic tolerance in different crops were reported earlier [35]. In addition, signalling molecule [59], autophagy [60] play important roles in plant responses to stress.

Annotation of Candidate SNP

In this study, three SNPs with contrasting function annotation were found to be associated with heat tolerance in bottle gourd, including SNP2 (BG_GLEAN_10022642; annexin 5) associated with pollen and flower sterility; SNP 31 (BG_GLEAN_10022727; AP-3 adaptor complex) related to intracellular transport; and, SNP 16 (BG_GLEAN_10022589; serine/threonine-protein kinase) involved in signal recognition; which exhibited significant marker-trait association and could be attractive option to identify the heat tolerance bottle gourd genotypes.

A common response of crop plants to temperature stress is significant yield loss, as a result of effects on spikelet fertility in rice [82], pot set in lentil [78], and pollen sterility in canola [79]. Annexins belong to a ubiquitous family of proteins present in eukaryotic organisms [83] localized in various subcellular compartments [84], and are known to be involved in a variety of cellular processes, such as maintenance of vesicular trafficking, cellular redox homeostasis, actin binding, and ion transport [85], due to their calcium- and membrane-binding capacity. Annexin 5 is involved in pollen grain development and germination, and pollen tube growth through the promotion of calcium-modulated endo-membrane trafficking [86]. For example, down-regulation of Arabidopsis annexin5 in transgenic Ann5-RNAi lines has been shown to cause sterility in pollen grains [52].

Adaptor proteins that are involved in protein trafficking and sorting [87-89] may recognize cargo and coat proteins during vesicle formation [90]. AP-3 was first identified in mammalian cells and likely functions as a clathrin adaptor [91]; losses in AP-3 function reduce seed germination potential [92,93], due to mistargeted protein S-ACYL transferase10 that is critical for pollen tube growth during dynamic vacuolar organization in Arabidopsis [94].

Serine/threonine-protein kinases (STKs) are involved in signal transduction networks to coordinate growth and differentiation of cell responses to extracellular stimuli [95]. In a range of smut pathogenesis-related biological processes, Huang et al. [59] reported that STKs may act as receptors or signaling factors, such as in Ca^{2+} signaling, that then activate defense responses. Umezawa et al. [96] reported that SRK2C is annotated as a putative and osmotic-stress-activated STK in Arabidopsis; it may mediate signal transduction and regulate a series of drought stress-response genes

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that enhance expression of 35S:SRK2C-GFP to improve drought tolerance in plants.

Conclusion

To understand genetic basis and select reliable markers are important in the variety breeding process for bottle gourd. The accurate QTL-seq results have clearly shown that the recessive heat tolerance inheritance and three polymorphic SNPs involved in pollen sterility, intracellular transport, and signal recognition were exhibited significant marker-trait association. Intensity of global warming will increase in next two to three decades and will exacerbate the negative effects on Cucurbitaceae plants. It will help in identification of heat tolerance genotypes in breeding program of bottle gourd for further heat tolerance strengthening of other Cucurbitaceae plants by grafting, such as those for watermelon. The results also revealed the novel region (from 11.03 to 19.25 Mb on chromosome 2) harbored *qHT2.1*, which first provide the promising direction for further exploration on heat tolerance in bottle gourd for fine mapping to discover the new gene and understanding the complex mechanisms.

Supporting information

S1 Table. Predicted and annotated SNPs in the *qHT2.1* region.

S2 Table. Details of 62 nonsynonymous and stoploss type of SNPs in the *qHT2.1* region.

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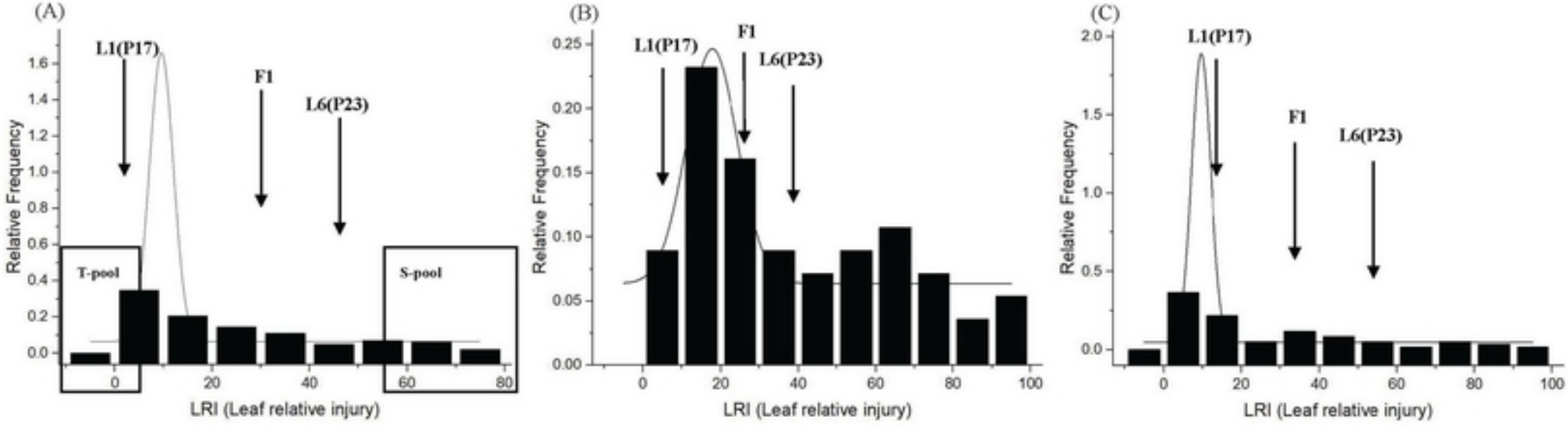
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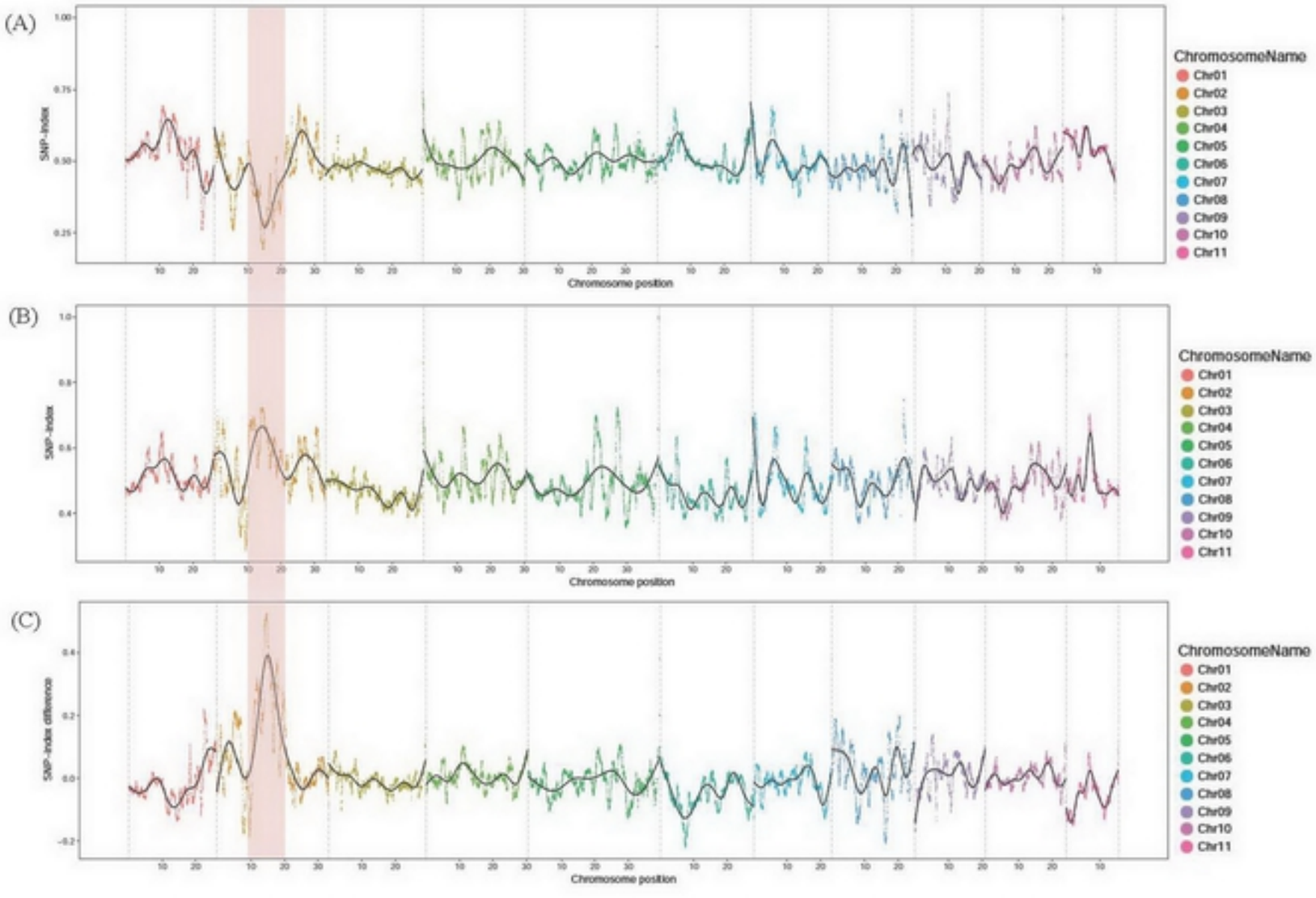
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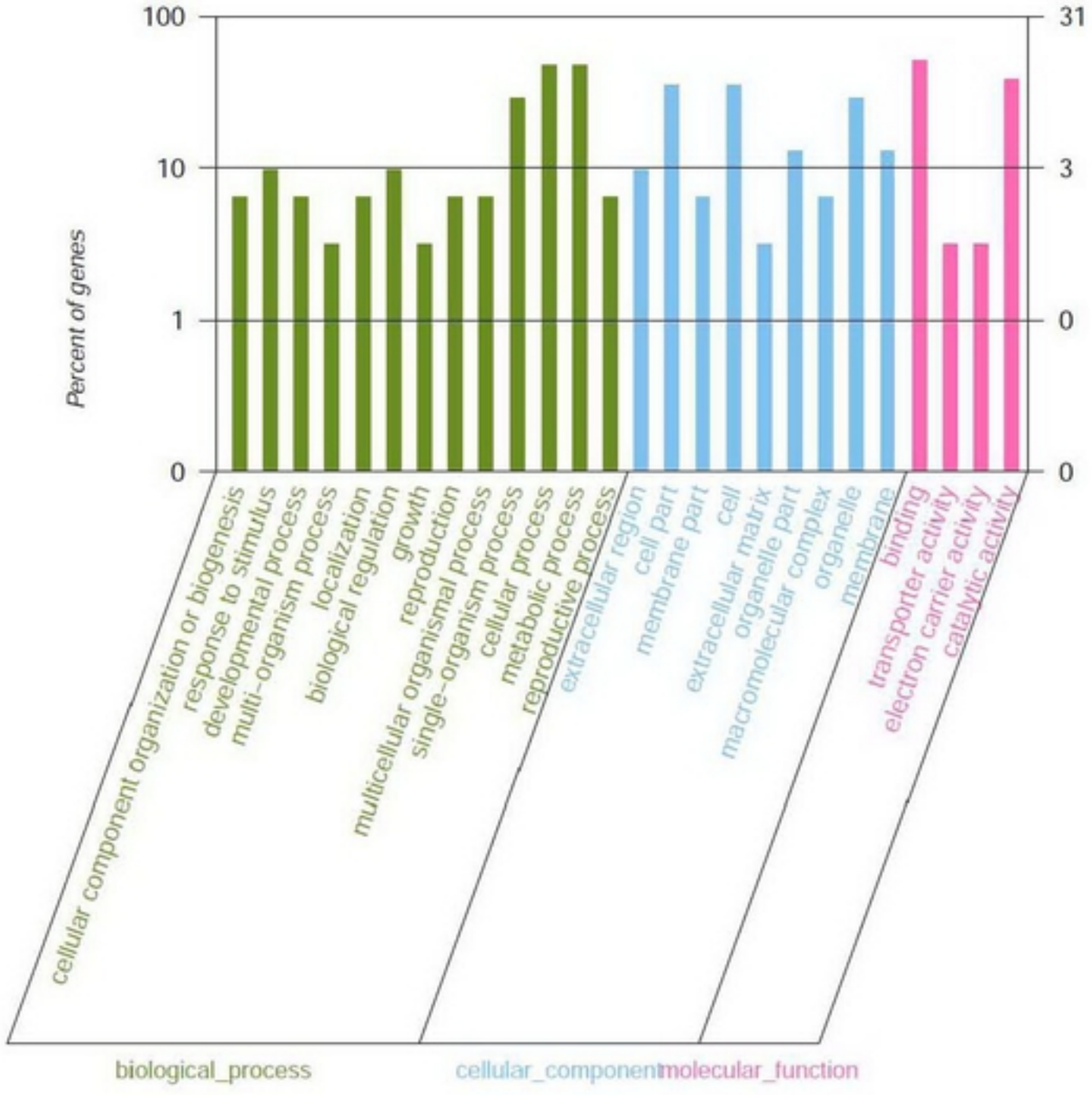
SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in *Arabidopsis thaliana*. Proc Natl Acad Sci USA. 2004; 101: 17306–17311. doi: 10.1073pnas.0407758101


(A)
L1(P17)
F1
L6(P23)
T-pool
S-pool
Relative Frequency
LRI (Leaf relative injury)
(B)
L1(P17)
F1
L6(P23)
Relative Frequency
LRI (Leaf relative injury)
(C)
L1(P17)
F1
L6(P23)
Relative Frequency
LRI (Leaf relative injury)

Figure

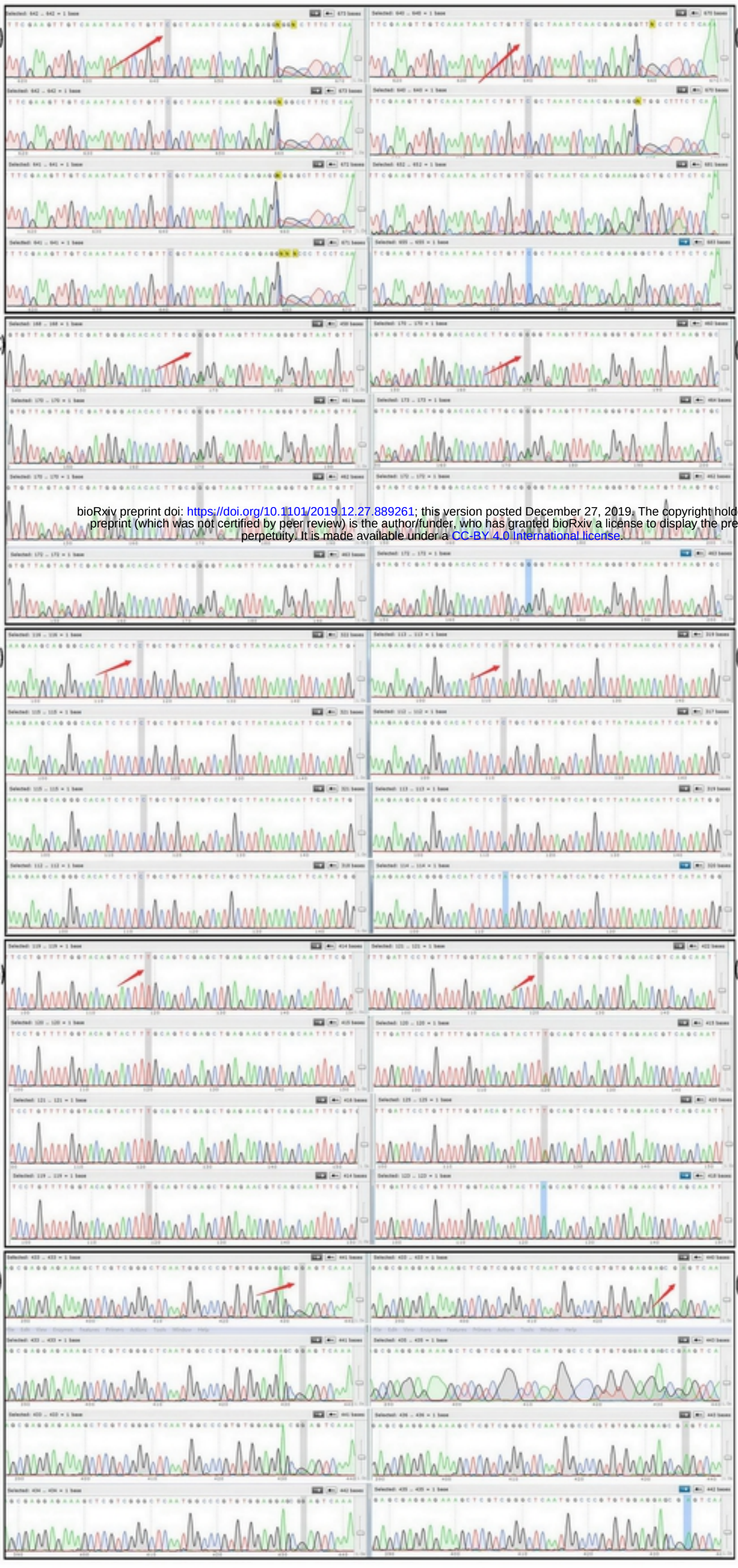

(A)
SNP-index
Chromosome position
(B)
SNP-index
Chromosome position
(C)
SNP-index difference
Chromosome position
ChromosomeName
Chr01
Chr02
Chr03
Chr04
Chr05
Chr06
Chr07
Chr08
Chr09
Chr10
Chr11

Figure


100
10
1
0
31
3
0
0
Percent of genes
cellular component organization or biogenesis
response to stimulus
developmental process
multi-organism process
localization
biological regulation
growth
reproduction
multicellular organismal process
single-organism process
cellular process
metabolic process
reproductive process
extracellular region
cell part
membrane part
cell
extracellular matrix
organelle part
macromolecular complex
organelle
membrane
binding
transporter activity
electron carrier activity
catalytic activity
biological_process
cellular_component
molecular_function

Figure

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Figure