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First genetic linkage map of chilling injury susceptibility in peach (Prunus persica (L.) Batsch) fruit with SSR and SNP markers

Arun Prabhu Dhanapal[1,2*], Pedro J Martínez-García[1], Thomas M Gradziel[1] and Carlos H Crisosto[1]
Correspondence: dhanapala@missouri.edu
[1]Plant Sciences Department, University of California Davis, One Shields Ave, Davis CA 95616, USA.
Present address: [2]Division of Plant Sciences, University of Missouri, Columbia, USA.

Abstract

Peach and nectarine (Prunus persica L) are highly perishable; they ripen and deteriorate quickly at ambient temperature. Storage at low temperature (0–5°C) is a common strategy used to slow the ripening processes and extending shelf life. However, if susceptible varieties are held too long at a low temperature, they will not ripen properly and will develop chilling injury (CI) symptoms like mealiness, flesh browning, and flesh bleeding. Understanding the genetic control of these traits to produce CI resistant cultivars will greatly benefit producers, shippers and consumers. Mapping approach for a set of 40 candidate genes (CGs) obtained after a transcriptomic analysis of peach between high tolerant and sensitivity to CI were used, to identify CI controlling genes in Pop-DG progeny population and CI-susceptible (hermoza) and chilling injury-resistant (oded) peaches. A set of 142 CGs from detailed transcriptomic analysis of two different peach cultivars studied previously and additional 10 CGs nominated from published works and review articles of physiology and transcriptomic study of peach fruit subjected to CI were localized in this study. In present study 12 CGs have been mapped on Pop-DG population with 8 SSR and 26 SNP markers.

Keywords: Chilling injury (CI), mapping, candidate genes (CGs), SNP and SSR

Background

Chilling injury (CI) is the collective term for various disorders that occur during prolonged cold storage and/or after subsequent ripening of stone fruit. Major symptoms of CI include mealiness, flesh browning and red pigmentation (bleeding). Eventhough numerous biochemical and molecular studies identified several factors which may be important in the development of the symptoms, results are often contradictory and therefore we are still lacking complete understanding of the molecular basis for CI. Our present study proposed 12 candidate genes mapped in Pop-DG with SSR and SNP markers along with significant SNP markers for QTLs controlling CI. The elucidation of the inheritance mechanism of the chilling injury will provide a longterm solution of this problem and enable the breeding of new CI-tolerant cultivars. The application of MAS will enable the selection of those CI-tolerant cultivars, diminishing the global peach industry losses due to this postharvest disorder.

Introduction

Peach tree (*Prunus persica*) is a species of Prunus, a genus that also includes nectarine, plum, apricot, cherry, and almond belonging to the subfamily Prunoideae of the family Rosaceae. It is considered one of the genetically most well characterized species in the Rosaceae, and it has distinct advantages that make it suitable as a model genome species for Prunus as well as for other species in the Rosaceae [1,2]. Peach is a diploid with n=8 and has a comparatively small genome currently estimated to be ~220-230 Mbp based upon the peach v1.0 assembly. Peach has a relatively short juvenility period of 2-3 years compared to most other fruit tree species that require 6-10 years. One of the key aspects of fruit ripening is softening and texture of the fruit. Peaches are highly perishable; they ripen and deteriorate quickly at ambient temperature [3]. Therefore, low temperature storage (0–5°C) is used to slow the ripening processes as well as decay development during storage and/or shipment

[3,4]. These temperatures inhibit fruit ripening, thereby extending fruit postharvest life. If susceptible varieties of peach, nectarine, and other stone fruit such as plum and apricot are held too long at a low temperature they will not ripen properly when rewarmed and will develop chilling injury (CI). CI is the collective term for various disorders that occur during prolonged cold storage and/or after subsequent ripening of stone fruit. Major symptoms of CI include mealiness, flesh browning and flesh bleeding. So peaches that are subjected to long periods of cold storage can develop chilling injury symptoms which reduce the postharvest quality of these fruits. The manifestations of CI in peaches and nectarines include defective cell wall disassembly and development of a dry, woolly rather than soft, juicy texture [3]. CI acts as main limiting factor in the shipping of some stone fruits and results in significant economic losses, limiting long distance transport, and affecting peach consumption with frequent complaint by consumers [4,5].

Even though numerous biochemical studies have resulted in the identification of factors which may be important in the development of the symptoms [3,6,7,8], results are often contradictory, and therefore, we are still lacking complete understanding of the molecular basis for mealiness. Some studies claim that mealiness is characterized by loss of juiciness has been associated with abnormal cell wall disassembly during ripening [3,7,9,10]. Normal peach fruit ripening involves a series of cell wall modifications and increased transcription of genes encoding proteins and enzymes associated with the functionality of the endomembrane system [10,11]. There are many enzymes associated with the determination of softening and texture, and several of these enzymes are encoded by multi-gene families [12]. Candidate genes are genes of known biological action involved with the development or physiology of the trait. CGs is of known or presumed function that could correspond to QTLs. These genes may be structural genes or genes in a regulatory or biochemical pathway affecting trait expression. Correlation between the trait understudy and allelic polymorphism at the candidate gene is a strong argument in favour of the candidate gene [13].

Transcriptomic analysis to study different CI symptoms and associating the genes with each specific symptom is getting more popular. Fruit tissue obtained from two full-sib progeny individuals of Pop-DG with contrasting susceptibility to CI and being subjected to various cold storage durations and ripening regimes were used to develop the ChillPeach EST collection, a specialized database (ChillPeach) to target genes expressed during CI development. Genes controlling chilling injury differentially expressed between juicy and woolly fruit were found. In woolly fruit, commonly stress-induced genes, ripening related genes and genes involved in amino acid transport were up-regulated, while HSPs (Heat shock protein) genes were down-regulated, including genes putatively involved in intracellular trafficking and cell wall metabolism that were repressed in woolly fruit [14,10]. A study on peach, found differential expression of genes associated with plastids, mitochondria, endoplasmic membrane and ribosomes when comparing woolly and juicy fruit [15]. There is also some evidence that higher linolenic acid (C18:3) and membrane lipid unsaturation are beneficial for maintaining membrane fluidity, leading to an enhanced tolerance of peach fruit to low temperature [16]. Recent study found that heat shock and cold acclimation treatments induced chilling tolerance of plum fruit, which enhanced the expression of Ps-CII sHSP1 of fruit during subsequent low temperature storage [17].

To understand the genetic control of CI and their molecular basis of sensitivity or tolerance to CI, a peach linkage map and a candidate gene approach based on current physiological information have been used [14,18,19,20]. In our previous study one major quantitative trait locus (QTL) and a few minor QTLs have been localized for mealiness, browning and bleeding using the Pop-DG map [19]. A gene encoding a cell wall modifying enzyme, endopolygalacturonase (endoPG) co-localized with the major QTL affecting mealiness [18,21]. Another gene in the anthocyanin biosynthesis pathway, leucoanthocyanidin dioxygenase (PpLDOX), mapped to the same genomic region where the major QTL controlling browning was identified [20]. The application of next generation sequencing technologies and bioinformatic scripts to generate high frequency SNPs distributed throughout the peach genome for use in genome mapping and phenotype selection and development of high density genetic linkage maps using SNP markers were constructed for two breeding populations, Pop-DF ('Dr. Davis' x 'F8, 1-42') with 117 progeny and Pop-DG ('Dr. Davis' x 'Georgia Belle') with 64 progeny have been developed [22]. Transcriptomic analyses of two peach cultivars namely Oded and Hermoza, which differ in their resistance to chilling injury, were examined after two weeks of cold storage at 5°C by using ChillPeach cDNA microarray platform and identified 102 CGs proposed to be involved in CI [23].

A combination of new genomic tools: a Chillpeach microarray [14,23] and the Pop DG **('**Dr. Davis'×'Georgia Belle') peach population [20] segregating for CI, in a bulk segregant gene expression analysis approach to investigate the changes in the peach fruit transcriptome and to reveal the genes underlying differential response to cold storage of sensitive and tolerant peach fruit. The results of the study proposed potential candidate genes involved in CI. Among the possible strategies used to identify genes in general, the "Candidate Gene" (CGs) approach to genetic mapping and QTL analysis still appears to be the simplest one for *Prunus*. The purpose of this study was to map potential candidate genes and to propose mapped CGs of Pop- DG as potential marker for CI (Mealiness, flesh bleeding and fresh browning) and for use in marker assisted selection. In addition to the present objective of this study 102 CGs

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identified [23] have been localized in scaffolds of peach genome with at least one SNP flanking marker based on physical position.

Materials and methods

Plant materials

Pop-DG' a peach intraspecific cross between 'Dr. Davis' (female parent) and 'Georgia Belle' (pollen parent) was used in this study. 'Dr.Davis' is a modern canning peach cultivar and 'Georgia Belle' is a century-old fresh market peach cultivar which contrasts for many fruit quality and other chilling injury related traits (**Table 3**). 'Pop-DG' was created and managed at Kearney Agricultural Center (Parlier, CA, USA). This orchard was established in 1998 containing 51 verified hybrids. Each progeny genotype was represented by two trees in the orchard; the leaves were collected from parents and 51 Pop-DG populations, any one of the orchard tree in April 2010 and frozen in liquid nitrogen and stored at -80°C until used.

DNA extraction

DNA was extracted from leaves of 'Dr. Davis', 'Georgia Belle', and their progeny population. The plant tissue consisted of 2–6 expanding leaves of three cm length or less. Fresh leaf tissue of ~5g was ground into fine powder using liquid nitrogen. Ten ml of CTAB buffer [100 ml of 1 M Tris HCl pH 8.0, 280 ml of 5 M NaCl, 40 ml of 0.5 M EDTA and 20 g of CTAB (cetyltrimethyl ammonium bromide) with total volume to 1 L with ddH_2O] was taken in 50 ml falcone tube. Ground samples were put into buffer and 20 µl of 2-mercaptoethanol was added and mixed well. This setup was left in water bath at 60 ºC for 30 minutes. Samples were taken and left outside to cool for 10 minutes before chlofoform-Isoamyl alcohol (24:1) was added until 30 ml of the 50 ml falcone tube and tilted well until the layer/phases mixed well (2 to 5 times for five to 10 minutes). Tubes were taken to centrifuge for 30 minutes at 4000 rpm and were taken out carefully without disturbing the layers (two separate layers). Pasteur-pipette was used to obtain the upper phase to one new labeled falcone tube. Ice-cold Isopropanol (-20ºC) was added until 45 ml of the 50 ml falcone tube and mixed gently, the supernatant was discarded without disturbing the loose pellet-DNA. Twenty ml of wash-buffer (30 ml of 3 M sodium acetate and 970 ml of 100 % ethanol) was added and mixed gently. This step was repeated 2-3 times until the DNA pellet was very clean. Final wash was made with 70 % ethanol and let it dry until ethanol was completely evaporated. About 2-3 ml of 1X TE was added to each falcone tubes.

Candidate Gene selection for Pop-DG

Fruit mesocarp samples of individual from the progeny peach trees of the Pop-DG mapping population were used. Genotypes selected represent siblings with extremes of susceptibility (one resistant T, one susceptible S) to mealiness and internal browning in Pop-DG. The CI phenotype of the different genotypes was evaluated and confirmed similar to our previous study [14]. Fruit from the different genotypes were forced-air cooled at 0–2°C within 6 h of harvest and then stored at 5°C with 90% relative humidity. At 1, 2 and 3 weeks after cold storage (with or without shelf life), observations were made on the mesocarp for mealiness and browning and after this fruit were cut in halves through the suture plane.

Samples representing each at least 6 fruit from each of the genotypes with different treatments during harvesting, cold storage and ripening were bulked as described [14], and immediately frozen in liquid nitrogen before storing at –80°C until used. The samples were later used for RNA extraction for gene expression studies and select potential candidate genes as described [23]. The genes selected from above microarray analysis were subjected to validation with 96.96 dynamic arrays results over the same pools of susceptible (S) and tolerant (T) fruits and further analyzed by qRT PCR. Finally, 40 genes have been nominated as genes expressed during chilling injury process. A total of 40 candidate genes were selected based on their annotation according to their potential role in CI with functional category (1) Cell wall structure and secondary metabolism, (2) Response to stress and cellular homeostasis, (3) Response to RNA transcription and regulation, (4) Protein degradation and signal transduction pathway.

Molecular marker and Genotyping

40 SSR primer pairs were designed for 40 CGs resulted from Granell study (unpublished data), designed using Primer3 software [24]. Fragment size polymorphism of targeted gene fragments was observed as reproducible marker polymorphism on the PAGE profiles of the CGs PCR products. PCR reaction mixture included 10 ng/µl genomic DNA, 10 mM dNTPs 0.5ul (dNTP Mix PCR grade, Qiagen), 5 units/µl of Taq 0.1 µl (Taq DNA polymerase, Qiagen), 0.03 µl of forward, 0.112 µl of IRD 700 (IRDye 700 phosphoramidite, LI-COR® Biosciences), IRD 800 (IRDye 800 phosphoramidite, LI-COR® Biosciences), 0.112 µl of reverse primers (10 µM per each), 10X buffer 1.4 µl and ddH2O was used to bring final volume of 10 µl. PCR condition included initial denaturation step at 94°C for 5 min, then 30 cycles of (94 °C for 30 sec, 57°C for 35 sec, 72 °C for 45 sec) and then 8 cycles of (94°C for 30 sec, 57°C for 35 sec, 72°C for 45 sec) and final elongation at 72°C for 30 min and 4°C forever. Only polymorphic primers and SSR markers used for genetic mapping are shown (**Table 1**).

PAGE analysis

The 1 µl of PCR product was mixed with 4 µl of LiCOR dye (LI-COR, Inc. Lincoln, NB) A 6 % polyacrylamide gel [21 ml of 7M UREA solution, 4 ml of 30 % Bis- acryl amide, 150 ml of 10 % APS (0.1 g Ammonium per sulphate to 1.0 ml deionized water in a small test tube) and 15 ul of TEMED before pouring the gel] was used. From 5 µl of sample

Table 1. Name of candidate genes along with their primer sequence, amplicon size and SSRs marker information for Pop-DG

S No.	Primer Name	Forward (5'-3')	Reverse (5'-3')	Temp (°C)	Amplicon Size (bp)	Polymorphism type
1	CG1	GCTGATTACATGTAAGTACTCAAGG	GTAGCCTCACTGCAAAGGTAT	57	291	SSR
2	CG2	AACCTCTCCAGAATACCACTC	TATTTCAAGAGCTGAGTTTGG	57	218	SSR
3	CG5	TAGAGACAGCAAACAGAGGAA	AAACCCATCTTCTGCTTCTAC	57	129	SSR
4	CG6	TTCTGGTACCGAACGAAA	AGCACTAATCAGCTCCTAA	57	138	SSR
5	CG7	GGCGCTTCCTCCTTATACAAC	GATCTGGCCGAGACTGAATG	57	201	SSR
6	CG12	ATGAAGAGTTTGTGGCAAGG	CCACTTCATTCACAATCACG	57	211	SSR
7	CG14	GAGGTGTTGGAACCATCAAG	GGTAGTTGCTGGTGCTCTTT	57	219	SSR
8	CG16	CGGTCAATCTTCCGATATTC	TTCCTCGCTCATATTGGACT	57	205	SSR
9	CG18	AGTCCCTGGATTCCTCAAA	ACACACGAGTGACCAGCA	57	257	SSR
10	CG19	CGTGAAGAAGCCTCACAGA	TCCTCAAACAACCCAACAA	57	216	SSR
11	CG30	ATTGCAACGGAAACCAACT	AATGCGTGGATCGTTCTTT	57	248	SSR
12	CG31	AAGGGTCATGTGACCTGCT	AGTCGAAGCCATTAATGCAG	57	248	SSR
13	CG36	CTAAGAGACCCGAGGTTGAAG	AGCTTCATGTCTGTCAAGTGG	57	242	SSR
14	CG37	GACAGCAAAAACGAAGGTTG	TACGGCTCTTGTTCTTGTCC	57	288	SSR
15	CG38	AGTTCTTCAGATGCCAACCAT	TTGTCCCTGTCTTCATCCAT	57	299	SSR
16	CG40	GAAGCCTGCCATTGATTCTA	ATCTCGCGTAATGTCTCCAC	57	248	SSR
	SSRs	Forward (5'-3')	Reverse (5'-3')	Temp (°C)	Amplicon Size (bp)	Polymorphism type
1	BPPCT-021	TGCATGAGAAACTTGTGGC	CCAAGAGCCTGACAAAGC	57	288	SSR
2	BPPCT-020	CGTGGATGGTCAAGATGC	ATTGACGTGGACTTACAGGTG	57	216	SSR
3	BPPCT-021B	TGCATGAGAAACTTGTGGC	CCAAGAGCCTGACAAAGC	57	248	SSR
4	UCDCH15	TGCATGAGAAACTTGTGGC	CCAAGAGCCTGACAAAGC	57	248	SSR
5	BPPCT-036	AAGCAAAGTCCATAAAAACGC	GGACGAAGACGCTCCATT	57	248	SSR
6	BPPCT-015	ATGGAAGGGAAGAGAAATCG	GTCATCTCAGTCAACTTTTCCG	57	129	SSR
7	BPPCT-026	ATACCTTTGCCACTTGCG	TGAGTTGGAAGAAAACGTAACA	57	138	SSR
8	BPPCT-017	TTAAGAGTTTGTGATGGGAACC	AAGCATAATTTAGCATAACCAAGC	57	218	SSR

only 0.5 µl of PCR product was loaded in gel. The LI-COR 4200 Series instrument was used to run gel according to manufacturer instructions (LI-COR, Inc. Lincoln, NB). Gel scoring was done both manually and by using Gel buddy software [25].

Candidate gene selection from CI-susceptible 'hermoza' and CI-resistant 'oded' peaches

Transcriptomic analyses [23] conducted on two peach cultivars, which differ in their resistance to chilling injury, were examined after two weeks of cold storage at 5°C by using ChillPeach cDNA microarray platform. One hundred and two CGs (**Supplementary table 1**) were obtained from this transcriptomic analysis of two peach cultivars which were white and melting-flesh, however 'Oded' (*Prunus persica* cv. Oded), is a cling-stone, early season peach while 'Hermoza' (*P. persica* cv. Hermoza), is a free-stone, mid-season cultivar.

Physical position of the CGs and SNPs markers

SNPs markers were obtained from both parents from our previous work [26]. The peach "GS0012410-OPA.opa" consisted of 1,536 SNPs and was used to genotype Pop-DG population. The selection of high quality SNPs and genotyping of Pop-DG was carried out and high quality SNP map was created [22]. The SNPs and the two set of CGs obtained from the two different sources were ordered by reference to their position using the 'Lovel' using "peach v1.0" reference genome (released by the International Peach Genome Inititative (IPGI) in 2010) . Additional to 40 CGs from our first source (Granell, unpublished) and 102 CGs from

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Table 2. Features of candidate genes with their functional annotation of Pop-DG. The list shows name of 50 CGs with their unigene functional annotation and their position in scaffolds of peach genome. 40 CGs were from transcriptomic analysis of Pop-DG. 10 CGs (41-50) were from other published sources

S.No.	Name of CGs in mapping and scaffolds	Name of Candidate Genes from transcriptomic analysis	Source	Similar Prunus persica DNA sequence ID	Unigene Functional Annotation	Position in scaffolds of peach genome
1	CG1	PPLDOX	Chill Peach	ABX89943	Leucoanthocyanidin dioxygenase gene	scaffold_5:9,817,818..9,819,338
2	CG2	PPN011D08	Chill Peach	BU044303	Phi-1 protein	scaffold_1:18,494,503..18,502,877
3	CG3	PPN059B10	Chill Peach	NA	Os01g0186900 protein	scaffold_7:19,378,715..19,379,406
4	CG4	PPN038D06	Chill Peach	DW341257	Glycosyltransferase QUASIMODO1	scaffold_8:18,126,089..18,127,289
5	CG5	PP1004C04	Chill Peach	DN676686	Mannan endo-1,4-beta-mannosidase	scaffold_1:21,828,585..21,829,753
6	CG6	PPN040G09	Chill Peach	NA	Cell cycle checkpoint protein RAD17	scaffold_7:17,694,654..17,695,645
7	CG7	PP1002E07	Chill Peach	AJ824111	Omega-6 fatty acid desaturase	scaffold_7:10,931,319..10,934,687
8	CG8	PP1001G06	Chill Peach	P43297	Cysteine protease CP1	scaffold_1:22,788,451..22,790,193
9	CG9	PP1001A05	Chill Peach	P51846	Pyruvate decarboxylase 1	scaffold_6:24,372,690..24,377,696
10	CG10	PP1001C07	Chill Peach	DY649642	Mitochondrial pyruvate dehydrogenase kinase isoform 2	scaffold_1:8,042,187..8,043,567
11	CG11	PP1003F01	Chill Peach	DY641887	4-coumarate-CoA ligase-like protein	scaffold_5:10,637,165..10,637
12	CG12	PPN004H06	Chill Peach	AB044662.1	1-aminocyclopropane-1-carboxylate synthase 1	scaffold_2:18,502,578..18,502,877
13	CG13	PP1001C04	Chill Peach	DY643195	Ring zinc finger protein	scaffold_6:5,374,851..5,376,021
14	CG14	PPN045B08	Chill Peach	BU043492	Major cherry allergen Pru av 1.0203	scaffold_1:9,560,967..9,566,560
15	CG15	PP1004E08	Chill Peach	DY650607	AT3g15350/K7L4_15	scaffold_4:10,707,438..10,709,198
16	CG16	PPN009B03	Chill Peach	NA	Chlorohydrolase family protein	scaffold_1:22,614,349..22,615,237
17	CG17	PPN025B02	Chill Peach	BU040021	similar to Cyclic nucleotide-gated ion channel 1	scaffold_8:12,221,208..12,222,294
18	CG18	PPN070C12	Chill Peach	DW346523	PP_LEc0008N19f Peach shoot Prunus persica	scaffold_3:9,394,101..9,394,881
19	CG19	PPN032H11	Chill Peach	BU041375	Histone protein Hist2h3c1	scaffold_1:22,024,422..22,025,550
20	CG20	PP1002D01	Chill Peach	AJ822461	Putative ripening-related protein	scaffold_6:23,110,781..23,111,935
21	CG21	PPN012B10	Chill Peach	DY644352	Putative ripening-related protein	scaffold_6:23,110,786..23,112,557
22	CG22	PP1001B03	Chill Peach	DY639769	Os04g0623400 protein	scaffold_6:26288000..26289999
23	CG23	PP1005B08	Chill Peach	NA	Unknown	scaffold_4:17436741..17438740
24	CG24	PPN075H08	Chill Peach	DY642462	Os01g0818000 protein	scaffold_6:25353012..25355011
25	CG25	PPN032A07	Chill Peach	DW347694	T23O15.3/T23O15.3	scaffold_3:15336089..15338088
26	CG26	PPN078F09	Chill Peach	DY638908	Protease-associated PA	scaffold_1:22011177..22025550
27	CG27	PPN054B03	Chill Peach	DY639769	CBF1	scaffold_2:24800744..24802743
28	CG28	PPN054B07	Chill Peach	DY640636	Calcium-binding EF-hand	scaffold_4:11650422..11652421
29	CG29	PPN003H11	Chill Peach	DY641715	Cinnamoyl-CoA reductase-like protein	scaffold_1:7494927..7496926
30	CG30	PP1002E04	Chill Peach	AF362990.1	Alpha-L-arabinofuranosidase / beta-D-xylosidase	scaffold_1:9204523..9206522
31	CG31	PP1005H08	Chill Peach	NA	No annotation available	scaffold_7:15559769..15564768
32	CG32	PPN005G05	Chill Peach	DY653721	Anther ethylene-upregulated protein ER	scaffold_1:8242276..8244275
33	CG33	PPN039H11	Chill Peach	DY637895	Glutathione S-transferase	scaffold_3:95408..97407
34	CG34	PPN070G08	Chill Peach	DY634402	Beta-galactosidase precursor (EC 3.2.1.23) (Lactase)	scaffold_7:19628203..19630202
35	CG35	PP1009D05	Chill Peach	DY645444	Amino acid transporter	scaffold_1:30527322..30529321
36	CG36	PPN023G06	Chill Peach	DY646993	Mi-2 autoantigen-like protein	scaffold_4:6182101..6184100
37	CG37	PPN029B05	Chill Peach	NA	No annotation available	scaffold_1:30527234..30529233
38	CG38	PPN021B10	Chill Peach	DY648396	similar to Location of EST 206I21T7	scaffold_5:12724586..12726585
39	CG39	PPN021G05	Chill Peach	NA	Armadillo	scaffold_4:1774113..1776112
40	CG40	PPN037E06	Chill Peach	AJ827029	RSI-1 protein precursor	scaffold_3:12708896..12710895
S.No.	**Name of CGs in mapping and scaffolds**	**Name of Candidate Genes from other sources**	**Source**	**Similar Prunus persica DNA sequence ID**	**Unigene Functional Annotation**	**Position in scaffolds of peach genome**
41	CG41	PPN003H07-1	Chill Peach	AF362987	Thaumatin-like 1 protein	scaffold_7:8543779..8545778
42	CG42	PPN003H07-2	Chill Peach	AF362988	Thaumatin-like 2 protein	scaffold_3:9873109..9875108
43	CG43	CL1439Contig1	Chill Peach	DQ251187	major allergen Pru p 1	scaffold_1:9586936..9597544
44	CG44	PP1001B08	Chill Peach	AF367443	NADP-dependent isocitrate dehydrogenase	scaffold_3:242975..247974
45	CG45	CL417Contig1	Chill Peach	BU043362	Pectate lyase	scaffold_6:22023991..22025990
46	CG46	PPN002G04	Chill Peach	X95991	Pectinesterase	scaffold_7:18644917..18650259
47	CG47	PP1006B11-1	Chill Peach	AF367459	PpExp1, expansin 1	scaffold_1:22788899..22790898
48	CG48	PP1006B11-2	Chill Peach	AB047518	PpExp2, expansin 2	scaffold_1:27209405..27211404
49	CG49	CL971Contig1	Chill Peach	AB047519	PpExp3, expansin 3	scaffold_6:5141253..5143970
50	CG50	PP1006B11-3	Chill Peach	AB054319	PpExp4, expansin 4	scaffold_3:19591237..19593236

second source [23], 10 more CGs (**Table 2**) were nominated from published works and review articles of physiology and transcriptomic study of peach fruit subjected to CI.

Linkage analysis

The two classes of marker data (SSR and SNP) that showed mendelian segregation were used for linkage analysis using JoinMap® 4 [24]. The marker data type was coded as cross pollination in JoinMap® 4 and maps were calculated without adding the marker order into the scaffolds. Linkage parameters were set as 3.0 minimum LOD and 0.45 maximum recombination fraction were used as thresholds [20]. The Kosambi mapping function [27] was used to convert recombination fraction to map distances in centimorgans (cM). Graphical presentation and their alignment were performed using Map chart 2.2 [28].

Results

Primer design and marker polymorphism

Among the 40 SSR primer pairs designed (**Table 1**) for 40 CGs resulted from the transcriptomic analysis, only polymorphic primers were used in the present study for CGs mapping. SSRs were first studied in the parents, the six plants of the progeny to detect polymorphism. Polymorphic and well amplified markers were later run with the whole population of 51 progeny. Sixteen primers were polymorphic between the parents and also segregated in the progeny. Seven primers showed polymorphism in parents, but not in the progeny. The remaining 17 primers showed monomorphic bands and not segregated in the progeny. The proportion of SSRs giving polymorphism for CI was lower than expected from our previous study [20].

For the 16 CGs having polymorphism, were taken for mapping in F1 population in the Pop-DG. Four candidate genes were not mapped. The Pop-DG intraspecific peach linkage analysis resulted in a total of 12 CGs with 17 SSR and 31 SNPs flanking and QTL significant makers distributed over five linkage groups corresponding to the haploid chromosome number of peach (**Figure 1**). The common SSR markers enabled the determination of linkage group orientation and assignment of linkage group numbers for the Pop-DG map. Among the twelve CGs, seven were localized on Pop DG linkage group LG1 (CG2, CG14, CG5, CG16, CG30, CG37 and CG19), one on LG3 (CG18), one on LG4 (CG36), two on LG5 (CG1 and CG38) and one on LG7 (CG7). Previously identified QTLs significant markers [22] for mealiness (qML1 and qML4.1), flesh bleeding (qBLa, qBLb, qBLc and qBL4) and browning (qBrL5) were in LG1, LG4 and LG5 respectively. The most significant markers for all above traits were mapped along with candidate genes identified in this study (**Figure 1**).

Association of CGs with SNP linkage map of Pop-DG

Genetic linkage analysis using SNP markers constructed [22] for Pop-DG ('Dr. Davis' x 'Georgia Belle') was used for association of CGs localized in this study with selected significant markers identified previously. Both sets of genotyping data were loaded into Join-Map® 4 and map was constructed with CGs and SNP markers together (data not shown). When nearby flanking markers and CI QTL significant markers were identified, the final map was constructed only with CGs and nearby flanking markers. For each candidate genes on the linkage group at least two flanking SNP markers were identified.

Sequence blast and localization in Prunus genome

Table 3. Different fruit quality characteristics of Pop-DG parental cultivars, 'Dr. Davis' and 'Georgia Belle'

S.No.	Trait/Character	'Dr. Davis'	'Georgia Belle'
1	Ripening date	Later	Earlier
2	Skin color	Orange ground, with blush	Green/yellow, no blush
3	Flesh color	Yellow -orange	White-cream
4	Stone adhesion	Clingstone	Freestone
5	Flesh texture	Firm, non meting flesh	Soft, melting flesh
6	Aroma	Bland	Sharp
7	Sweetness (SSC)	11.5	13.0
8	Acidity (TA)	High	Low
9	Mealiness	None	High susceptibility
10	Browning	Medium susceptibility	High susceptibility
11	Bleeding	High susceptibility	Low susceptibility

TA = Titratable acidity, SSC = soluble solids content

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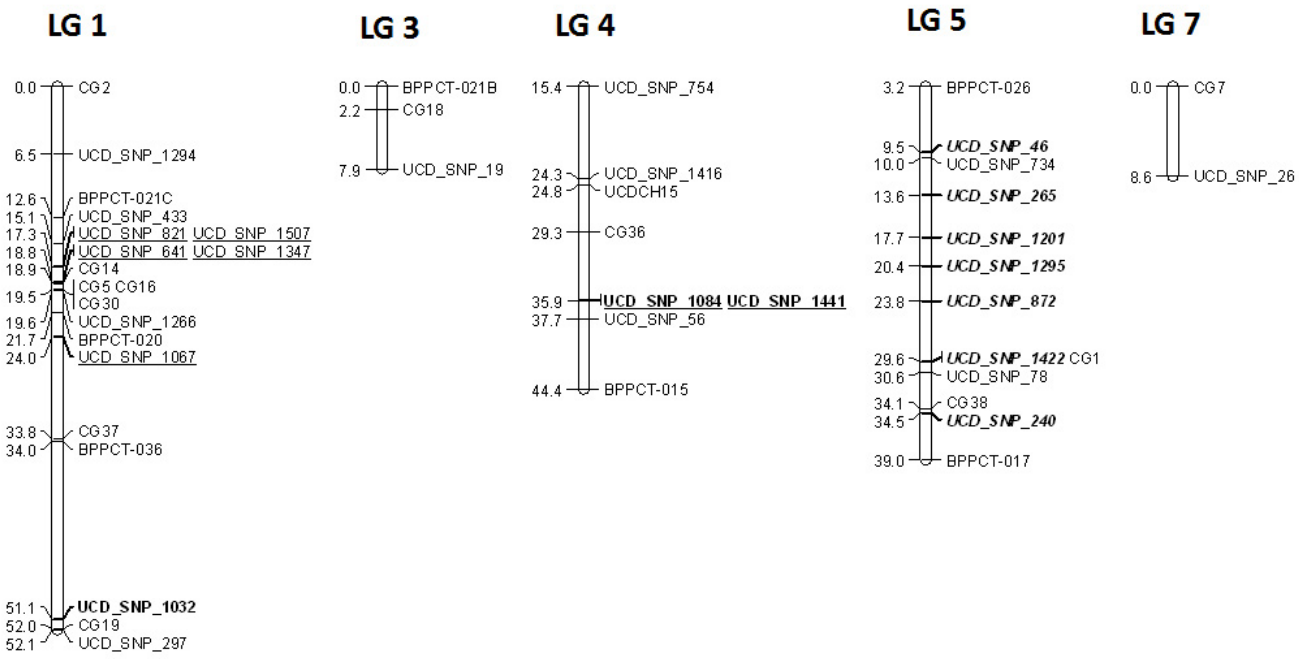

Figure 1. Candidate Gene map of Pop-DG with flanking SSRs, SNPs and QTLs significant SNP markers constructed using JoinMap ®4.0. The Genetic distance in the Pop-DG map is shown in centimorgans (cM) on left side. The location of all candidate genes mapped in Pop-DG agreed with the positions of their homologous sequences in corresponding peach genome scaffolds. Significant markers for various CI traits are mapped. Underlined font denotes flesh bleeding; bold font for mealiness; bold and underlined for both mealiness and flesh bleeding; bold italic font denotes flesh browning.

Sequences of the 40 CGs from first source of study, 102 CGs and other 10 nominated CGs were blasted against peach genome v1.0 scaffolds (http://www.phytozome.net/search.php?show=blast&method=Org_Ppersica), and the resulting homolog sequences were located in the scaffolds (corresponding to the linkage groups of Prunus genetic maps) using the GBrowse function in website http://www.phytozome.net/cgi-bin/gbrowse/peach/. The position of each CGs on scaffold only with nearest SNP marker is shown in **Figure 2** and **Figure 3**.

Discussion

Influence of year and genetic factors on CI susceptibility in peach have been well reviewed in our previous work [**29**]. Thus CI resistance is thus a viable long-term strategy to reduce losses in the fresh and processed peach and nectarine industries. We have mapped CGs and created a preliminary CI linkage map for Peach. We have 26 SNPs and 8 SSRs flanking markers with 12 CGs mapped on Pop-DG using SSR marker. Simple sequence repeats (SSRs) have proven to be highly polymorphic, easily reproducible, codominant markers. However, developing an SSR map is very time-consuming and expensive atleast for candidate genes. A number of SSR markers for *Prunus persica* and other species of the same genus are available for different purposes [**30**-**33**].

Data from study in Pop-DG population for fruit quality gene map of prunus showed that the polymorphism was ~50 % [**34**], lower than the observed in T×E (~85%), but higher than the observed for Pop-DG (~25%). The lower rate of polymorphism observed in 'Venus' × 'Big Top' (V×BT) and Pop-DG compared to T×E could be explained since T×E is a F2 population from an inter-specific cross. A genetic linkage map of linkage group 4 (LG4) was constructed with SSR and candidate genes from a segregating population developed using the cross 'Venus' × 'BigTop'. Significant quantitative trait loci (QTLs) for mealiness, graininess, leatheriness and bleeding were found in this linkage group, validating QTLs for CI symptoms previously reported in this linkage group from an unrelated progeny population [**20**,**35**].

The first source of 40 candidate genes was from transcriptomic analysis conducted on Pop-DG population. SSR primers designed for 40 candidate genes resulted in only 16 polymorphic markers and among them only 12 were mapped in five linkage groups, 7 were localized on LG1. The blast search of 40 CGs and resulting homologous sequence showed that these genes were scattered all over the eight scaffolds of peach genome with scaffold 1 harboring 13 CGs.

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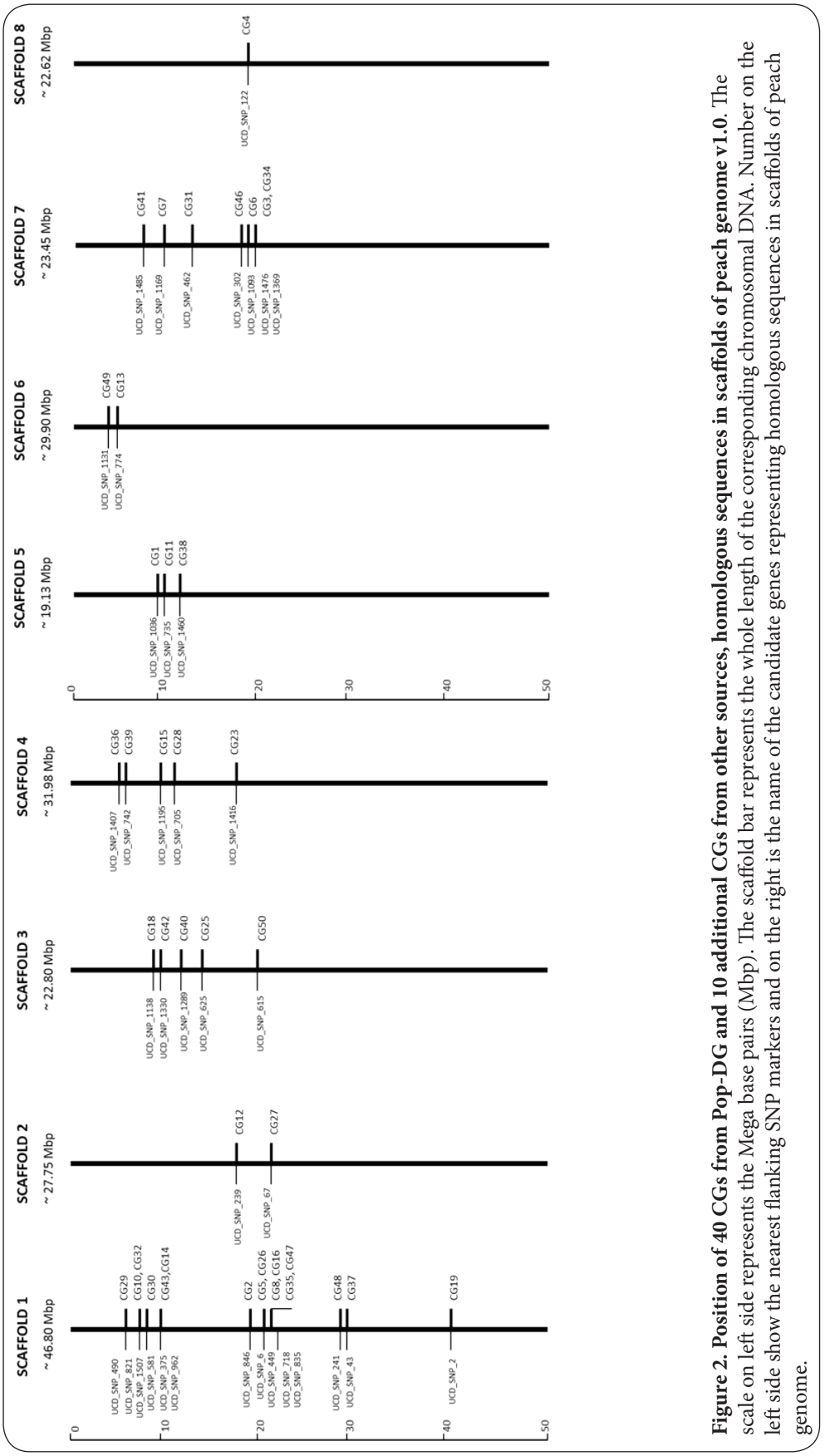

Figure 2. Position of 40 CGs from Pop-DG and 10 additional CGs from other sources, homologous sequences in scaffolds of peach genome v1.0. The scale on left side represents the Mega base pairs (Mbp). The scaffold bar represents the whole length of the corresponding chromosomal DNA. Number on the left side show the nearest flanking SNP markers and on the right is the name of the candidate genes representing homologous sequences in scaffolds of peach genome.

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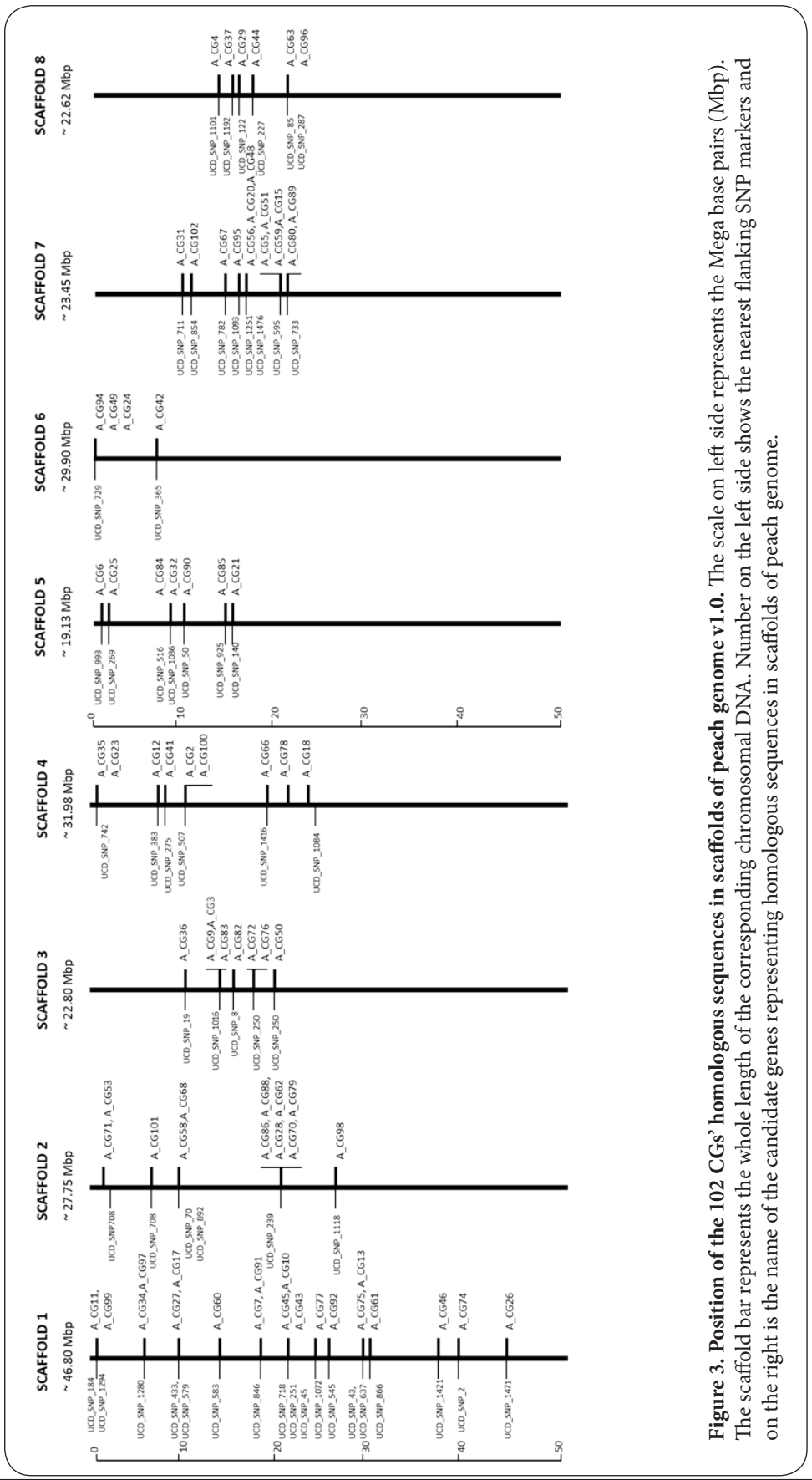

Figure 3. Position of the 102 CGs' homologous sequences in scaffolds of peach genome v1.0. The scale on left side represents the Mega base pairs (Mbp). The scaffold bar represents the whole length of the corresponding chromosomal DNA. Number on the left side shows the nearest flanking SNP markers and on the right is the name of the candidate genes representing homologous sequences in scaffolds of peach genome.

Among 10 additionally selected CGs three of them have their homologous sequence in scaffold 1. This result shows CGs mapped on Pop-DG and CGs localized on peach scaffolds, among which major number of genes were mapped and localized on LG1 and scaffold 1. For total of 50 CGs, 41 CGs were localized in all eight scaffolds of peach genome with at least one flanking SNP marker based on their scaffold position (**Figure 2**).

The 102 CGs from transcriptomic analyses of two peach cultivars Oded and Hermoza, which differ in their resistance to chilling injury, formed the second source of CGs. Blast search of their homologous sequence showed that all the 102 CGs were scattered all over the 8 scaffolds of peach genome (**Supplementary table 2**). Among the genes localized, about 20 CGs were localized in scaffold 1. In scaffold 2 SNP marker UCD_SNP_239 was the flanking marker for six CGs. In total, of 102 CGs, 79 CGs were localized in 8 scaffolds of peach genome with at least one flanking SNP marker based on their scaffold position (**Figure 3**). Even though in our present study among the 40 SSR markers designed for CGs only, 16 SSRs were polymorphic. Result of our present study shows that other markers systems such as Single nucleotide polymorphism (SNPs) should be used for CGs mapping in order to increase the number of polymorphic markers and get other CGs mapped.

The previously identified QTL for browning on LG5 [**36**] was compared to location of candidate gene CG1 (PpLDOX) and this co-location implies the control of QTL by that particular gene and SNP marker UCD_SNP_78 was closely linked to this CG1. Previously identified CGs [**18**,**20**,**36**-**38**] for mealiness and flesh bleeding on LG1 and LG4 and flesh browning in LG5 and most significant markers [**22**], identified were mapped along with CGs in our present study. QTLs identified for mealiness (qML1; LOD score 4.18) at 51.06 cM in LG1 was close to presently mapped CG19 at 52.0 cM with flanking marker UCD_SNP_297. Another QTL for mealiness (qML4.1;LOD score 8.74) at 35.89 cM in LG4 was 6.6 cM away from CG36. Four QTLs identified for flesh bleeding in which three in LG1 [qBLa (17.28cM); LOD score 3.86, qBLb (18.80cM); LOD score 3.19, and qBLc (23.96cM); LOD score 3.22] were close to CG14 (18.9cM), CG5 (19.5cM), CG16 (19.5cM) and CG30 (19.5cM). One QTL identified in LG4 (qBL4; LOD score 4.45) at 35.89 cM was 6.5 cM away from CG36 [**22**].

More than one significant marker associated with QTL was identified for flesh browning in LG5 (qBrL5) in previous study [**22**]. CG1 was at same position of significant marker identified (UCD_SNP_1422) for flesh browning and CG38 was 4.5 cM away from qBrL5. The major QTL for mealiness was validated in V×BT population and QTL for browning not found on our previous work on LG4 [**35**] was found in Pop-DG [**22**]. Location of candidate gene Thaumatin-like protein 1 precursor from our previous study [**36**] was compared to the scaffold location of CG41 from the present study and this implies its co-location of this gene. Recent comparative study of melting and non-melting flesh peach cultivars reveals that during fruit ripening endo-PG is mainly involved in pericarp textural changes, not in firmness reduction [**39**].

Conclusion

Results showed that in total of 152 CGs, (40+102+10) major numbers of CGs were localized on scaffold 1. The markers localized in this manner may provide additional information for peach physical mapping efforts. Although this is a preliminary data showing the location of CGs in scaffolds of peach genome, detailed study of this CGs in mapping population of peach will determine their applicability as potential CGs for marker assisted breeding (MAB). Knowledge of the genetic basis of CI traits and their linkage with SSR and SNP markers permit a more realistic estimate of the effort needed to produce a new cultivar with CI resistance. Such information also reduces the labor and time required to develop cultivars and improves the accuracy of marker-assisted selection (MAS). Field evaluation is limited to trees containing the genes of interest, significantly reducing the costs associated with maintaining undesirable trees to maturity. The CGs mapped in Pop-DG in this study can be used as potential markers to preselect seedlings for CI fruit traits such as mealiness, flesh bleeding and flesh browning, while also speeds the development of commercially acceptable cultivars with minimum deterioration or no deterioration. Our future efforts will be to map all the CGs from both sources by SNP or SSCP based marker system.

Additional files

Supplementary table 1
Supplementary table 2

Competing interests

The Author's declare that they have no competing interests.

Authors' contributions

APD organized the plant materials, designed experiments, carried out molecular genetic studies and drafted the manuscript. APD performed all data analysis along with PMG. TMG provided plant materials. APD and CHC revised critically the manuscript. CHC provided financial support to the study. All authors read, discussed and approved the final manuscript.

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