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ORIGINAL PAPER

Capturing flavors from *Capsicum baccatum* by introgression in sweet pepper

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Abstract

Key message **Biochemical characterization in combination with genetic analyses in BC_2S_1 plants and near-isogenic lines led to the detection and validation of *C. baccatum* loci affecting flavor, terpenoid content and Brix level.**

Abstract The species *Capsicum baccatum* includes the most common hot peppers of the Andean cuisine, known for their rich variation in flavors and aromas. So far the *C. baccatum* genetic variation remained merely concealed for *Capsicum annuum* breeding, due to post-fertilization genetic barriers encountered in interspecific hybridization. However, to exploit the potential flavor wealth of *C. baccatum* we combined interspecific crossing with embryo rescue, resulting in a multi-parent BC_2S_1 population. Volatile and non-volatile compounds plus some physical characters were measured in mature fruits, in combination with taste evaluation by a sensory panel. An enormous variation in biochemical composition and sensory attributes was found, with almost all traits showing transgression. A population-specific genetic linkage map was developed for QTL mapping. BC_2S_1 QTLs were validated in an experiment with near-isogenic lines, resulting in confirmed genetic effects for physical, biochemical and sensory traits. Three findings are described in more detail: (1) A small *C. baccatum* LG3 introgression caused an extraordinary effect on flavor, resulting in significantly higher scores for the attributes aroma, flowers, spices, celery and chives. In an attempt to identify the responsible biochemical compounds few consistently up- and down-regulated metabolites were detected. (2) Two introgressions (LG10.1 and LG1) had major effects on terpenoid content of mature fruits, affecting at least 15 different monoterpenes. (3) A second LG3 fragment resulted in a strong increase in Brix without negative effects on fruit size. The mapping strategy, the potential application of studied traits and perspectives for breeding are discussed.

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Introduction

The genus *Capsicum* originates from South-America and comprises ~25 recognized species (Heiser 1976). Five species of *Capsicum* are cultivated, including the closely related species *C. annuum*, *C. chinense* and *C. frutescens* that belong to the *C. annuum* complex. The other

two domesticated species, *C. baccatum* and *C. pubescens*, are less known and still predominantly confined to Latin America. *Capsicum baccatum* includes the most common hot peppers (both fresh and dried) of the Andean countries and has been domesticated in the highlands of Peru and Bolivia. The species is typically characterized by having flowers with a white corolla with basal green/yellow spots. *C. pubescens* is also a highland species, with thick-walled fleshy fruits, flowers with purple corolla and characteristic black seeds (Pickersgill 1997).

Although the domesticated species are of tropical origin, most *Capsicum* breeding has been carried out in temperate countries, mostly *C. annuum* (Poulos 1994). Some wild species have, however, been used in *C. annuum* breeding programs focusing on (mainly) disease resistance, such as introgression of tomato spotted wilt virus resistance from *C. chinense* (Black et al. 1991) or tobacco mosaic virus resistance from *C. chacoense* (Boukema 1982). The use of the species *C. baccatum* in *C. annuum* breeding programs has been very limited so far, since interspecific hybridization between both species is greatly hampered by post-fertilization genetic barriers (Yoon et al. 2006). Studies with *C. baccatum* focused, therefore, mainly on variation of accessions within the species, showing great variability for fruit quality characteristics, yield, resistances and bioactive compounds (Do Rêgo et al. 2009; Rodriguez-Burruezo et al. 2009; Yoon et al. 2004). Genetic analyses of traits from *C. baccatum* are, however, lacking, with as exception the molecular characterization of resistance to pepper fruit anthracnose derived from *C. baccatum* PI594137 in an intraspecific population (Kim et al. 2010). Thorough genetic analyses of *C. baccatum* (fruit quality) characteristics in interspecific mapping populations are still completely missing.

Here we present the biochemical, sensory, agronomical and molecular characterization of genotypes with *C. baccatum* var. *pendulum* introgressions in *C. annuum* genetic background. To facilitate the molecular mapping, a genetic map was constructed using a multi-parent BC_2 population. QTL mapping in a BC_2S_1 population followed by a validation experiment with near-isogenic lines (NILs) allowed the detection and confirmation of genetic effects for physical, biochemical and sensory traits. We discuss the introgression of several unexpected traits and demonstrate that *C. baccatum* is a valuable source for enrichment of the *C. annuum* breeding pool.

Materials and methods

Plant material

The *Capsicum baccatum* var. *pendulum* accession PEN45, obtained from the Vegetable Crops Research Institute (ZKI, Budapest, Hungary), was used as donor parent for backcrossing (BC) with three cultivated *C. annuum* blocky breeding lines (MT, SM and GNM) provided by the vegetable breeding company Rijk Zwaan. Due to difficulties in interspecific crossing, a multi-parent BC_2 population, consisting of three sub-populations, was generated for linkage map development (Fig. 1). The largest PEN45 BC_2 sub-population with the blocky parents SM and GNM in its pedigree (Fig. 1c) was chosen to study fruit characteristics in more detail. In this population 34 from the in total 54 BC_2 plants gave sufficient inbred seeds to grow BC_2S_1 lines.

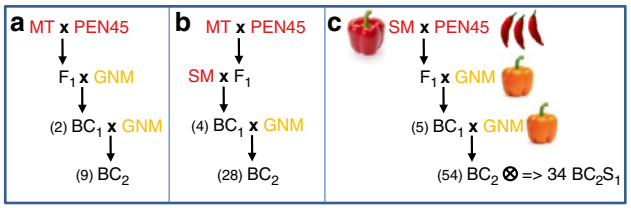

Fig. 1 Three BC_2 sub-populations derived from *C. baccatum* var. *pendulum* PEN45 with *C. annuum* parents MT, SM and GNM. The number of BC-plants per generation are indicated between *brackets*. The different *colors* represent the fruit color of the parents (representative fruits indicated in *c*)

In 2009 the 34 BC_2S_1 lines were grown in plots of 5–9 plants with, when possible, two repetitions (possible for 23 BC_2S_1 lines) in a randomized block design. Plants were grown in soil in a greenhouse at Rijk Zwaan (De Lier, The Netherlands), according to Dutch pepper management conditions with 2.5 plants/m^2. Due to the generation of the material and the presence of two different breeding lines (SM and GNM) in their pedigree, the lines were still segregating for several traits. To grow the BC_2S_1 lines as uniform as possible, plants were pre-selected with a marker based on the Pun1 locus (Stewart et al. 2005) for selection of non-pungent plants and with a marker based on the CCS gene (capsanthin–capsorubin synthase; Lefebvre et al. 1998) to select non-red (i.e. yellow or orange) plants. To compensate for selection against Pun1 or CCS linked PEN45 fragments, with potentially interesting flavor characteristics, two and five BC_2S_1 lines (out of the 34 lines) were used to select plants with homozygous pungent orange fruits and homozygous non-pungent red fruits, respectively. These plants were also grown in two repetitions with plots of five plants. Genotypes SM, GNM and PEN45 were grown as controls in four repetitions. At the time of maturation of the first fruits the BC_2S_1 plots were made phenotypically more uniform by removing the most aberrant, mainly sterile, plants from the plots. In total 25 lines were uniform for orange fruit color; the other nine lines were segregating for plants with either orange or yellow fruits. In the end 250 BC_2S_1 plants remained for QTL mapping. These plants,

of which 160 orange, 61 yellow and 29 red fruited plants, were divided over 69 plots (1-6 plants).

Three BC_2S_1 plants, from different BC_2 plants, were used to develop near-isogenic lines (NILs) by one generation of backcrossing with GNM followed by two selfing steps. Each generation was genotyped with SNPs flanking the original BC_2S_1 introgressions to obtain BC_3S_2 lines with a limited number of introgressions in GNM genetic background. In 2011, 23 NILs and the recurrent parent (GNM) were grown in three repetitions with five plants per plot in a completely randomized setup. Plants were grown under similar conditions as the BC_2S_1 lines in a greenhouse at Rijk Zwaan; however, this time in autumn and on rockwool.

Trait evaluations

Ripe fruits (95–100 % colored) from in general the second fruit set were used for biochemical measurements and sensory evaluation. Fruits of the BC_2S_1 plants were harvested per plot (harvest 22 May) and in case of plots segregating for plants with either orange or yellow fruits, the two colors were bulked separately. 56 BC_2S_1 plots (37 orange, 15 yellow and 4 red) gave sufficient fruits to make representative fruit samples of 5–8 fruits for sensory evaluation. In addition, 32 samples were made of plots and/or individual plants that did not give enough fruits for sensory evaluation or that were pungent. In the NIL experiment (harvest 17 October), 20 NILs and GNM gave sufficient fruits and were evaluated as bulks per plot. In both experiments, fruits were stored after harvesting in a climate room at 20 °C with 80 % relative humidity for 3–4 days to optimize ripening. This is a standard procedure to mimic the Dutch commercial system. During the day of sensory evaluation, fruits were washed with cold, running tap water, dried with a clean towel and cut (top and bottom parts were discarded and seeds were removed) into 1–2 cm pieces. Half of the fruit pieces from each sample were immediately frozen in liquid nitrogen, ground in an electric mill and stored at −80 °C, while the other half was used for flavor evaluation.

A fruit description of all 250 BC_2S_1 plants and controls was made in the first week of July 2009. The shape of the fruits (conical or blocky) was recorded and average length and maximum width (cm; length1 and width1) were estimated by eye from all full grown (ripe and unripe) fruits hanging on the plant, by an experienced breeder using 0.5 cm intervals. In addition, mature (orange/yellow/red) and immature (light green/dark green/pale green) fruit color were recorded. Subsequently the mature fruits were harvested and pooled per plot (76 samples excluding controls). Average weight (gram), length and width (cm; length2 and width2) were measured on five representative fruits. Finally, fruits were cut and roughly evaluated for odor (nasal) intensity (scale 0: no odor—7: a lot of odor, like PEN45) by three untrained persons. From the NILs only the average length and maximum width were estimated by eye of all full grown (ripe and unripe) fruits hanging on the plant, by an experienced breeder using 0.5 cm intervals.

Sensory analysis

The descriptive analysis took place in a sensory laboratory at Wageningen UR Greenhouse Horticulture (WUR-GH, Bleiswijk, The Netherlands). Fourteen panelists, who are part of a trained panel with broad experience in sensory evaluations of food products, including pepper, took part in the experiment. In the weeks prior to the test sessions, panelists participated in training sessions with either commercially available pepper genotypes (BC_2S_1 experiment) or with fruits from preselected NILs with divergent tastes (NIL experiment). During the training sessions, panelists agreed on 14 attributes to describe the flavor sensation in the mouth/throat: texture attributes crunchiness, stickiness of the skin, toughness and juiciness, the basic taste attributes sweetness and sourness and the retronasal flavor attributes aroma intensity, grassiness, green bean, carrot, fruity/apple, perfume, petrochemical and musty. During the training session with the preselected NILs the following five attributes were added to the list: flowers, spices (non-pungent), celery, chives and bitter, while the attribute perfume was no longer used. In both experiments the panel did not separately evaluate the odor (nasal) of the fruits, as this was no part of the panel's expertise.

During the BC_2S_1 experiment test sessions, each panelist ($n = 14$) evaluated 21–22 genotypes in a randomized order, split over two subsequent days. On both days, two sessions with 5–6 genotypes and a reference (commercial orange blocky *C. annuum* hybrid) were evaluated per panelist. In this setup each sample was evaluated by 4–5 panelists. For the NIL experiment the panelists ($n = 12$) were divided into three groups of four persons. Each group evaluated the 20 NILs of a single repetition in a randomized order (complete block design), split over two subsequent days. On both days, two sessions with five NILs and GNM as the reference were evaluated per panelist. In both experiments each panelist received five fruit pieces per sample (from multiple fruits) in a ceramic cup and they were asked to mark the intensity of the attributes on a horizontal 100 mm structured line scale on paper, resulting in a scoring between 0 and 100. The pepper fruit pieces were swallowed by the panelists. Between samples, panelists rinsed their mouth with tasteless mineral water to neutralize their palate and were also allowed to eat a small unsalted cracker before rinsing their mouth. No information was provided to the panel about the genotypes.

Metabolic profiling

Biochemical profiling of both experiments was performed as described in Eggink et al. (2012a). In short, the profiling of volatile metabolites was performed using headspace SPME–GC–MS. Derived GC–MS profiles were processed by the MetAlignTM software package (http://www.metalign.nl) for baseline correction, noise estimation and ion-wise mass spectral alignment. MSClust software tool was used to reduce the ion-wise aligned data and to extract structural information of volatile metabolites (Tikunov et al. 2012). Each compound was represented by a single selective ion fragment in the following multivariate data analysis. The compounds (number of fragment ions in a mass spectrum $\geq$5) were then subjected to a tentative identification using the NIST mass spectral library (http://www.nist.gov). Putative identities were assigned to compounds with a mass spectra match factor $\geq$600 and retention index deviation $\leq$30. The compounds which did not meet these criteria were labeled as unknowns.

A lack of suitable authentic chemical standards did not allow determination of the concentrations from the volatiles identified in this study. Volatile compound abundance (intensity) is, therefore, represented as the height of a selective mass peak of a compound detected in chromatograms by MetAlign software. Intensities which were below the detection limit in certain genotypes obtained a random value between 250 and 500.

In both experiments the concentration of sugars (fructose, glucose and sucrose) was measured by enzymatic determination (Velterop and Vos 2001). Anion exchange chromatography was used for citric and malic acid determination based on standard protocols (Dionex Corporation, Sunnyvale, CA; http://www.dionex.com/ Application Note 143 "Determination of Organic Acids in Fruit Juices"). Sugar and acid measurements were completed by pH and by determination of total soluble solids (Brix).

Genotyping and genetic linkage map construction

To genotype the 250 BC_2S_1 plants from the PEN45 BC_2 sub-population with the blocky parents SM and GNM in its pedigree (Fig. 1c), 927 SNP markers were used, of which 239 SNPs were polymorphic in PEN45 versus SM and GNM. Marker phases were set in such a way that the *C. annuum* allele was scored as 'A' and the *C. baccatum* allele as 'B'. When (fruits of) multiple plants were bulked to obtain a plot-phenotype (e.g. in case of sensory evaluations) a corresponding plot-genotype was created. For this purpose an average plot genotype value was calculated with A = 0, H = 0.5 and B = 1 and rounded towards *C. baccatum* following the rules: average plot value <0.25 = A; $\geq$0.25 and <0.75 = H; $\geq$0.75 = B.

In order to develop a population-specific genetic linkage map, all 91 multi-parent BC_2 individuals (Fig. 1) were screened with 412 AFLP markers and the same 927 SNPs. Markers that were polymorphic between PEN45 and the *C. annuum* parents, while not being polymorphic within MT, SM and GNM (138 AFLPs and 199 SNPs) were used for linkage map construction on the combined BC_2 population. The 54 BC_2 plants derived from crosses with SM and GNM (Fig. 1c) were used for mapping a remaining set of 34 SNPs that were segregating in the 250 BC_2S_1 plants. The combined dataset was analyzed in JoinMap 4.0 (Van Ooijen 2006) with the Independence LOD algorithm used for group construction. The regression algorithm was used to calculate marker distances within linkage groups with the (Kosambi 1944) mapping function, and using linkages with recombination frequencies smaller than 0.5 and a LOD larger than 1.0. Linkage groups were named and oriented based on initial knowledge of marker groupings on an unpublished integrated map (IntMap) of Rijk Zwaan, which corresponds with the chromosome numbering and orientation of Wu et al. (2009).

Statistical analysis

The sensory data was analyzed in Genstat version 12 using a linear mixed model REML (residual maximum likelihood) analysis. For the BC_2S_1 experiment a model was used with genotype, replicate and their interaction as fixed terms. Sessions (tasting sessions) within replicate/genotype combinations and panelists within sessions were taken as random terms. For the NIL experiment a model was used with genotype as fixed term. Panelist, the panelist–genotype interaction and repetitions within panelist/genotype combinations were taken as random terms. In this experiment repetition and session were completely confounded with panelist and, therefore, not corrected for as a combined factor. Mean values were calculated for both experiments per genotype per replicate after a correction for session and panelist effects and removal of strong outliers (if the absolute value of a standardized residual was larger than three residual standard deviations).

QTL analysis

The Interval Mapping method within the program MapQTL 6 (Van Ooijen 2009) was used for QTL identification in the BC_2S_1 experiment. A permutation test was applied to each data set (1,000 permutations) to determine the LOD (Logarithm of odds) thresholds. A genome-wide (GW) LOD threshold of 2.7 was used for QTL significance ($p < 0.05$). The chromosomal locations with the highest LOD scores were considered to be the most likely positions of a QTL. Graphics were produced by MapChart software (Voorrips 2002). The NIL experiment was conducted using the non-parametric Kruskal–Wallis test

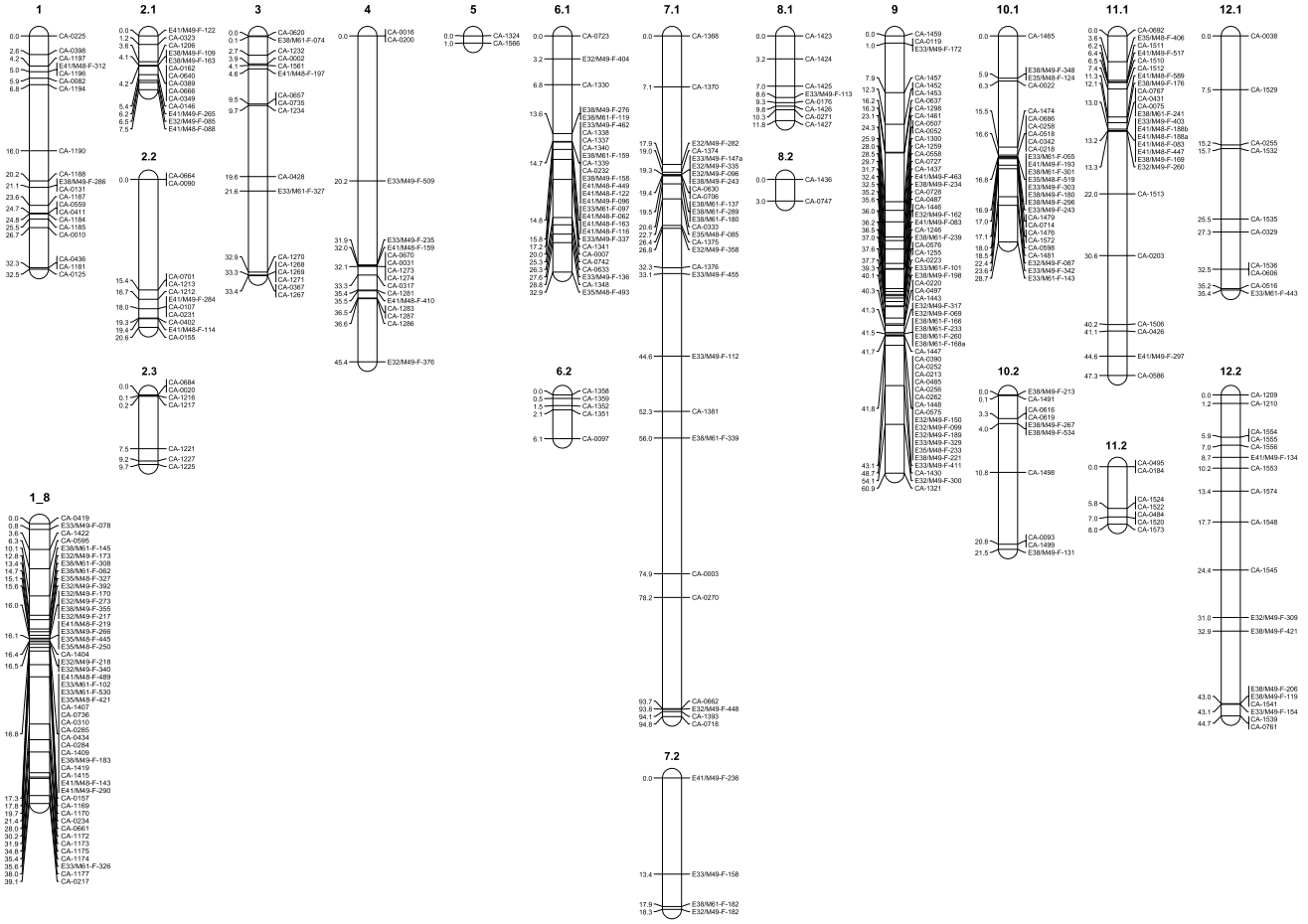

Fig. 2 Genetic map of the C. annuum × C. baccatum BC2 population. Linkage groups were named and oriented based on initial knowledge of marker groupings on an unpublished integrated map of Rijk Zwaan

within MapQTL 6 to identify markers that showed significant ($p < 0.05$) trait associations. The analyses in both experiments were performed with $\log_2$ transformed metabolite data.

Results

Map construction

In total 412 AFLP markers and 927 SNPs were used to genotype the 91 multi-parent BC_2 individuals (Fig. 1). Almost all (366 out of 377) informative markers (138 AFLPs and 233 SNPs) were assigned to 21 linkage groups (Fig. 2), with a total map size of 602.5 cM. More than 12 linkage groups were constructed, due to absence of polymorphic markers connecting the sub-linkage groups of the 12 corresponding pepper chromosomes. Linkage groups could, however, be oriented and assigned to chromosomes based on an unpublished integrated map of Rijk Zwaan. This resulted in 13 linkage groups, as for chromosome one and eight an additional linkage group 1_8 was constructed, to account for the known reciprocal translocation in that region, differentiating the genome of *C. annuum* from that of other *Capsicum* species (Wu et al. 2009).

Distribution of phenotypes

In the BC_2S_1 experiment in total 222 putative volatile compounds were detected of which 22 volatiles were specific to PEN45 (i.e. under detection limit in all BC_2S_1 plants and *C.annuum* parents). Putative identities could be assigned to 178 of these, based on their library match factor and retention indices. In the NIL experiment in total 137 putative volatile compounds were detected. Identities were assigned to 96 of these. In both experiments the sucrose concentrations turned out to be under the detection limit (0.3 g/100 g fresh weight) of our enzymatic determination method.

Most of the characterized biochemical, sensory and physical traits showed continuous variation in the BC_2S_1 population, except for the traits color, shape and pungency which were scored in classes (Table 1). The small fruited PEN45 could be clearly differentiated from the *C. annuum* parents

Table 1 Variation of measured traits in the parents, BC_2S_1 population and NILs

		Parents			BC_2S_1 population[a]					NILs				
Class	**Trait**	**GNM**	**SM**	**PEN45**	**Mean**	**SD**	**Min**	**Max**	**#gen**	**GNM**	**Mean**	**SD**	**Min**	**Max**
	Color ripe[b]	orange	red	red	0.12	0.32	0.0	1.0	250	orange	0.0	0.0	0.0	0.0
	Color unripe[c]	dg	dg	pale	0.77	0.33	0.0	1.0	250	na	na	na	na	na
	Length1 (cm)	8.1	8.3	7.0	7.5	1.3	5.0	10.0	250	8.0	7.4	1.1	4.5	9.0
	Width1 (cm)	8.5	8.3	2.0	5.7	1.5	3.0	9.0	250	8.0	7.1	1.1	4.5	8.0
	Shape[d]	block	block	conical	0.74	0.25	0.5	1.0	250	na	na	na	na	na
	Pungency[e]	nonpun	nonpun	pun	0.012	0.109	0.0	1.0	250	nonpun	0.0	0.0	0.0	0.0
	Length2 (cm)	8.6	7.6	7.4	7.5	1.3	5.0	11.8	76	na	na	na	na	na
Physical characters	Width2 (cm)	8.7	8.1	2.1	6.1	1.3	4.0	8.6	76	na	na	na	na	na
	Weight (g)	209.5	188.0	8.0	92.5	46.3	32.0	224.0	76	na	na	na	na	na
	pH	5.0	5.1	5.0	5.0	0.1	4.8	5.3	88	na	na	na	na	na
	Brix	8.3	8.3	11.0	10.6	2.2	6.8	17.9	88	7.2	8.1	0.7	6.9	10.2
	Glucose[f]	2.8	3.2	4.1	3.7	0.8	1.9	6.4	88	2.4	2.9	0.3	2.4	3.9
	Fructose[f]	3.1	3.0	3.3	3.8	0.9	2.2	6.7	88	2.6	3.1	0.3	2.7	4.0
	Malate[g]	21.9	24.4	79.9	37.4	16.5	14.1	115.4	88	23.5	24.3	11.0	13.0	77.3
	Citrate[g]	364.8	363.9	1037.7	450.9	121.6	210.0	820.0	88	303.6	333.9	64.2	230.0	605.6
	Hexanal	273,101	515,522	29,67,280	832,246	605,386	128,780	27,28,550	88	207,297	232,354	103,477	53,200	458,228
	3-Hepten-2-one	136,745	101,361	7,956	75,650	46,170	10,553	22,4728	88	22,429	22,526	10,495	3,970	51,327
	Linalooloxide	17,585	15,577	16,344	127,721	132,918	7,904	642,158	88	18,537	48,102	71,724	4,047	265,487
	p-Menth-1-en-9-al	30,944	33,239	68,804	290,157	291,680	8,368	13,55,670	88	44,465	109,913	156,677	5,333	502,535
	PAE	5,334	378	410	3,321	5,903	250	25,618	88	na	na	na	na	na
	BAE	356	392	64,326	13,109	1,5888	252	74,676	88	na	na	na	na	na
	Geranylacetone	45,271	82,238	47,450	62,310	38,618	7,313	259,467	88	7,891	10,948	10,059	416	52,915
Biochemical composition	b-Damascenone	84,925	30,845	17,766	62,228	45,086	5,689	259,570	88	10,579	8,965	6,879	400	30,777
	Methoxypyrazine	243,979	259,603	300,809	446,015	318,569	16,194	1,403,980	88	205,412	220,665	114,681	36,227	455,322
	Crunchy	63.9	58.1	na	59.9	7.5	42.4	79.0	56	65.8	68.0	5.9	51.7	79.2
	Sticky	38.0	55.1	na	44.4	14.1	16.6	73.6	56	12.2	19.0	6.5	5.4	35.4
	Tough	23.4	28.0	na	33.6	13.7	9.1	71.6	56	14.9	19.5	5.8	8.9	34.6
	Juicy	63.7	55.5	na	46.1	10.1	21.8	72.2	56	57.9	56.4	7.1	36.5	72.3
	Sweet	34.2	28.6	na	32.9	8.7	16.8	54.3	56	40.2	46.0	6.6	26.5	59.7
	Sour	25.9	20.2	na	22.5	8.0	6.2	41.0	56	31.6	31.8	5.6	21.8	46.5
Sensory attributes	Aroma	38.3	32.7	na	41.3	11.6	16.1	67.6	56	45.3	55.6	10.0	33.0	73.7
	Grass	5.7	10.8	na	8.1	5.9	0.0	30.0	56	9.6	9.1	6.5	0.0	30.1
	Bean	0.3	4.6	na	6.4	5.6	0.0	23.0	56	6.2	8.0	6.7	0.0	31.7
	Carrot	3.3	1.2	na	2.2	2.5	0.0	10.9	56	7.5	4.4	3.6	0.0	14.4
	Fruity	9.9	8.5	na	12.1	8.2	0.1	41.3	56	19.8	15.2	8.2	2.9	36.0
	Perfume	0.4	1.7	na	2.3	2.4	0.0	10.1	56	na	na	na	na	na
	Flowers	na	na	na	na	na	na	na	na	1.8	5.2	4.8	0.0	22.8
	Spices	na	na	na	na	na	na	na	na	3.9	9.4	7.0	0.0	33.4
	Celery	na	na	na	na	na	na	na	na	1.1	3.9	4.4	0.0	18.3
	Chives	na	na	na	na	na	na	na	na	6.0	3.3	5.2	0.0	18.6
	Petrochemical	0.7	0.1	na	2.3	3.1	0.0	14.8	56	2.7	2.9	4.2	0.0	21.2
Sensory attributes	Musty	2.7	1.6	na	1.1	1.6	0.0	8.6	56	4.4	1.7	2.1	0.0	8.5
	Odor	1.8	1.5	7.0	1.9	1.5	0.0	6.0	76	na	na	na	na	na

na not available

[a] The BC_2S_1 population and NILs are described by mean, standard deviation (*SD*) and range (min–max). The number of genotypes (#gen; plots or individual plants) used for phenotyping the BC_2S_1 population is indicated. Metabolite intensities are shown, separated per experiment, in color scale from low (*light red*) to high intensity (*dark red*). For abbreviations of metabolites see Table 2

[b, c, d, e] Class scores were translated numerically in the BC_2S_1 population for the traits color ripe (*red* = 1; *orange* or *yellow* = 0), color unripe [*dark green* (dg) = 1; *light green* = 0.5; *pale* = 0], shape (blocky = 1; conical = 0.5) and pungency [non-pungent (*nonpun*) = 0; pungent (*pun*) = 1]

[f] g/100 g fresh weight

[g] mg/100 g fresh weight

by higher sugar and especially acid concentrations, with up to three times higher malate and citrate levels. A number of selected aroma compounds, representing the major metabolic pathways and known to have an effect on pepper flavor (Table 2) differed also clearly between the parents. Due to its pungency, PEN45 was not included in the sensory evaluations and, therefore, its attribute scores, except for odor, are missing in Table 1. For almost all traits the BC_2S_1 plants or plots showed individual values higher and/or lower than the most extreme parent, a phenomenon known as transgression.

Based on the description of the sensory panel and the biochemical measurements, three BC_2S_1 plants with fruits that had either an extraordinary flavor resulting from high sweetness, sourness and/or odor scores or a high sugar and acid concentration were chosen for further analysis. These three plants, originating from different BC_2 plants, were used to develop near-isogenic lines (NILs), by one generation of backcrossing with GNM followed by two selfing steps. Each generation was genotyped with SNPs flanking the original BC_2S_1 introgressions to obtain BC_3S_2 lines with one up to

Table 2 Aroma reference volatiles

Metabolite	El. comp[a]	Type	Flavor description/reason
Hexanal	$C_6H_{12}O$	Lipid	Green[b]
3-Hepten-2-one	$C_7H_{12}O$	Lipid	Negative correlation with fruity[c]
Linalooloxide	$C_{10}H_{18}O_2$	Terpenoid	Floral, green bell pepper[d]
p-Menth-1-en-9-al	$C_{10}H_{16}O$	Terpenoid	Positive correlation with fruity[c]
Pentanoic acid, hexyl ester (*PAE*)	$C_{11}H_{22}O_2$	Ester	Fruity, green[e]
Butanoic acid, 2-methyl-hexyl ester (*BAE*)	$C_{11}H_{22}O_2$	Ester	Fruity[b]
Geranylacetone	$C_{13}H_{22}O$	Carotenoid	Sweet, citrus[f]
β-damascenone	$C_{13}H_{18}O$	Carotenoid	Fruity, floral[e]
2-isobutyl-3-methoxypyrazine	$C_9H_{14}N_2O$	Pyrazine	Green pepper[d]

[a] Elemental composition

[b] Rodriguez-Burruezo et al. (2010)

[c] Eggink et al. (2012b)

[d] Luning et al. (1994)

[e] http://www.thegoodscentscompany.com

[f] Tandon et al. (2000)

four homozygous, and in most cases also one or two heterozygous introgressions in GNM genetic background (Fig. 3). NILs were evaluated in comparison with the recurrent parent and again for almost all traits, NILs were found with higher as well as lower traits scores than GNM (Table 1).

QTL analysis in BC_2S_1 population and validation via NILs

The 250 BC_2S_1 plants from the PEN45 BC_2 sub-population having the blocky parents SM and GNM in its pedigree (Fig. 1c) were genotyped with 239 SNPs that were polymorphic in PEN45 versus SM and GNM. Interval mapping, with separate sessions for sensory attributes (14 attributes, 56 plots), metabolites (200 volatiles and six non-volatiles, 88 plots/plants) and several physical fruit characters plus odor (either on 250 plants or 76 plots), allowed identification of QTLs within all trait classes (Table 3).

The traits pungency and red color were used to validate the quality of our QTL mapping results, since both traits have been mapped in other mapping populations previously. CCS (red color) was mapped by Thorup et al. (2000) on chromosome six around 80–100 cM. Blum et al. (2002) mapped the single dominant gene *C* (former name of Pun1), required for capsaicin synthesis, to chr. 2 at 50.1 cM (FA03 map; http://solgenomics.net). Although in our population only three out of the 250 BC_2S_1 plants were pungent, we were able to map the trait pungency with a perfect fit (LOD 99.9; Table 3) to LG2.2 at 15.4 cM, which corresponds to chr. 2, 65.4 cM on our integrated *C. annuum* map (IntMap; unpublished data). Also for red color a QTL was found in the expected CCS region on chr. 6, i.e. marker CA-0097 on LG6.2 at 6.1 cM (Table 3), corresponding to chr. 6, 95.4 cM (IntMap). The percentage of explained variance of the latter QTL (13.5 %) was, however, surprisingly low knowing that a single dominant gene is involved. Taking a closer look at the region around CCS on the IntMap, we noticed that all markers in this region were not used for the population-specific PEN45 BC_2 map, since these markers were polymorphic within the *C. annuum* parents SM (red fruits) and GNM (orange fruits). Due to the multi-parent nature of the BC_2, during map construction we thus automatically selected against markers linked to fruit color. In a subsequent single marker analysis with polymorphic markers within the *C. annuum* parents included, marker CA-0081(chr. 6, 114.7 cM; IntMap) was found with a perfect correlation to red color (data not shown).

For unripe fruit color a very strong QTL (LOD 40.1, 52.8 % explained variance) was found on LG10.1 for the contrast between dark and pale green fruit color. The trait was, however, not measured in the NILs for validation. In addition, several QTLs were found for the physical traits length, width, shape and weight, of which some length and width effects were actually confirmed in the NILs. As expected, there was a big overlap in detected QTLs for the estimated (length1 and width1) and measured (length2 and width2) size QTLs. Both measurements, however, turned out to be valuable as they delivered different QTLs which could be confirmed in the NILs (Table 3).

In the BC_2S_1 population also several QTLs for biochemical compounds were found. For Brix, glucose, fructose, malate as well as citrate a significant effect was found on LG1_8, with the *C. baccatum* allele responsible for an increase in concentration. This QTL, however, coincided with the main fruit size QTL whose width, shape and weight decrease at the *C. baccatum* allele (Table 3). Therefore, the increase in the concentration of these non-volatiles seems to be an effect of smaller fruits rather than an absolute increase in amount. In the NILs this LG1_8 QTL for non-volatiles could not be confirmed. In contrast, a QTL for malate on LG1 could actually be confirmed in the NILs. NIL47 containing this telomeric LG1 *C. baccatum* introgression (Fig. 3) even showed an almost threefold increased concentration of malate compared with the recurrent parent and NILs lacking this introgression (Table 3).

Significant effects which could be confirmed in the NILs were also found for several aroma reference compounds, with a common QTL on LG10.1 for the metabolites linalooloxide, *p*-menth-1-en-9-al, butanoic acid, 2-methyl-hexyl ester (BAE) and β-damascenone (Table 3). A common QTL was found as well for hexanal, 3-hepten-2-one, geranylacetone and methoxypyrazine on LG1_8. The effect of

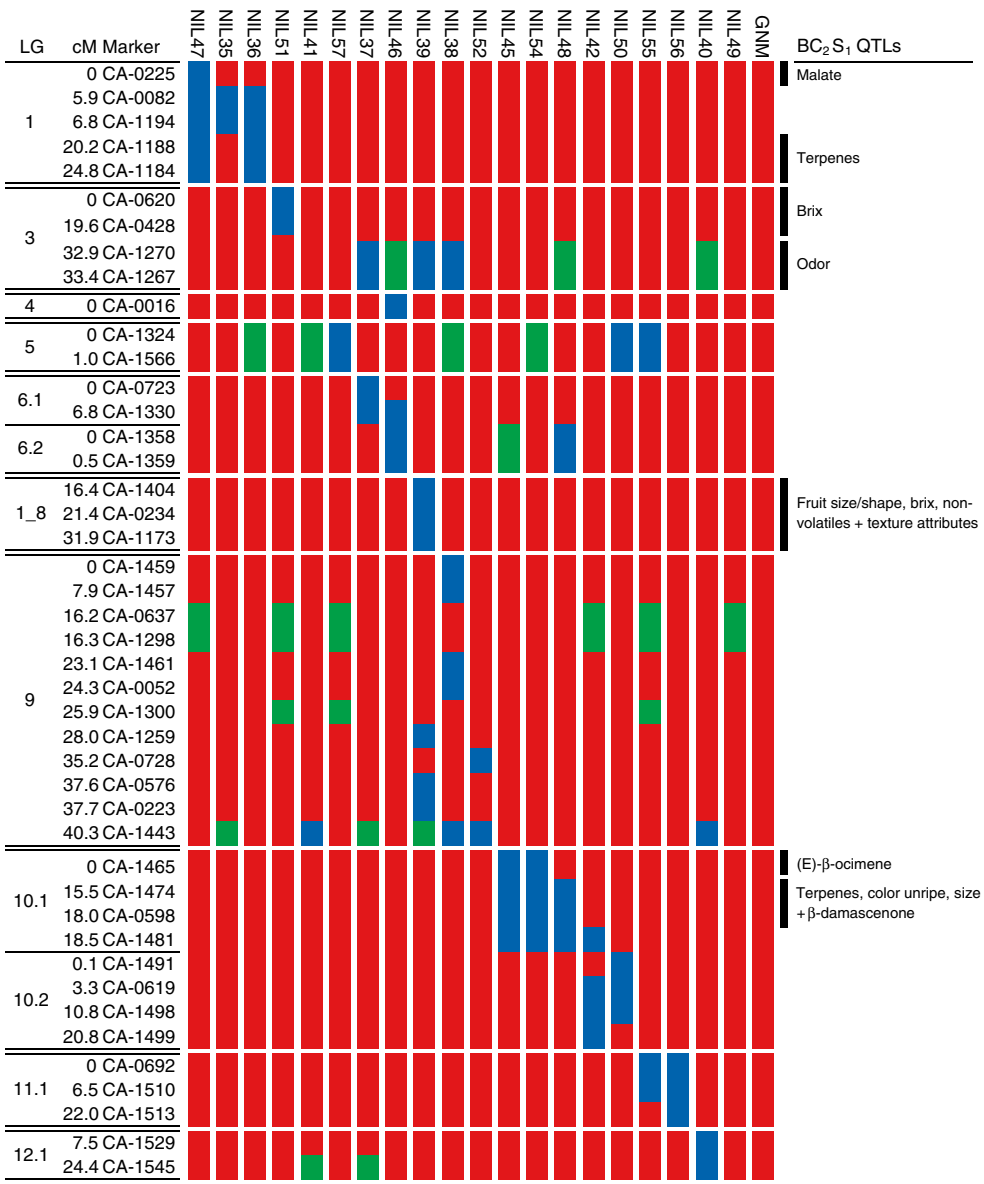

Fig. 3 Graphical representation of the NILs and discussed BC_2S_1 QTLs. Only regions with *C. baccatum* fragments are indicated. Introgressions are visualized with their flanking markers in blue (homozygous) or green (heterozygous, i.e. segregating in the NIL)

this locus could, however, only be confirmed in the NILs for 3-hepten-2-one.

In the BC_2S_1 population a QTL affecting the texture-related attributes toughness, stickiness of the skin and juiciness was found in the same region on LG1_8 that also gave significant effects for the fruit size traits and non-volatiles. Like for the non-volatiles, this QTL could not be confirmed in the NILs (Table 3). Interestingly, a rather strong QTL (LOD 8.0, 38.7 % explained variance) for odor was found on LG3 at 33.3 cM, with the *C. baccatum* allele giving a more intense odor than the *C. annuum* allele (increase of three points on 0–7 scale). Since in the BC_2S_1 population odor was only scored for its intensity and not further specified, in the NILs this trait was separated into multiple attributes, as described below.

Sequences of the flanking markers from the discussed BC_2S_1 QTLs (Table 3; Fig. 3) are given in Supplementary Table 1.

Flavor effect of *C. baccatum* LG3 introgression

During the sensory evaluation of the fruits in the BC_2S_1 experiment it became clear that the vocabulary (i.e. predefined attributes) of the trained panel was not sufficient to cover all the flavor variation, which resulted in remarks on the evaluation sheets like ‘presence of tropical fruit flavor’

Table 3 QTLs detected in the BC_2S_1 population and genotype means of the NILs

Class	Trait	BC_2S_1 population										NILs				
		LG	cM	Marker	LOD	%EV	μA	μH	μB	add.	A/H/B	Signif[a]	mA	mH	mB	A/H/B[b]
Physical characters	Pungency	2.2	15.4	CA-0701	99.9	100.0	0.0	0.5	1.0	−0.50	247/0/3	–	–	–	–	no var
	Color ripe	12.2	0.0	CA-1209	8.1	13.9	0.1	0.8	1.5	−0.67	242/4/1	–	–	–	–	no var
		2.1	1.2	CA-0323	8.0	13.8	0.1	0.7	1.2	−0.57	244/3/2	–	–	–	–	no var
		6.2	6.1	CA-0097	7.8	13.5	0.1	0.4	0.8	−0.35	235/9/5	–	–	–	–	no var
		12.1	0.0	CA-0038	4.6	8.3	0.1	0.4	0.6	−0.26	231/14/5	–	–	–	–	no var
	Color unripe	10.1	18.5	CA-1481	40.1	52.8	0.9	0.6	0.2	0.36	147/64/24	–	–	–	–	not meas
		4	0.0	CA-0200	2.9	5.2	0.8	0.7	0.6	0.11	170/49/24	–	–	–	–	not meas
		7.1	93.7	CA-0662	2.8	5.1	0.8	0.9	1.1	−0.16	221/16/11	–	–	–	–	not meas
	Length1	7.1	78.2	CA-0270	5.3	10.5	7.7	6.7	5.7	0.96	219/14/11	–	–	–	–	no introgr
		10.1	16.6	CA-0686	5.1	10.2	7.8	7.2	6.6	0.60	146/75/28	**0.0001**	**7.63**	–	**5.50**	**57/0/6**
		1	0.0	CA-0225	4.0	7.9	7.8	7.3	6.8	0.50	132/75/39	ns	7.4	–	8.0	60/0/3
		3	2.7	CA-1232	3.7	7.3	7.4	8.2	9.0	−0.81	213/29/6	ns	7.4	–	8.0	60/0/3
	Length2	10.1	16.6	CA-0686	3.6	20.5	8.1	7.0	6.0	1.00	39/32/4	**0.0001**	**7.63**	–	**5.50**	**57/0/6**
	Width1	1_8	19.7	CA-1170	19.8	33.9	6.4	4.8	3.2	1.62	146/96/4	**0.05**	**7.23**	**–**	**6.00**	**60/0/3**
		10.1	15.5	CA-1474	8.9	16.9	6.2	5.3	4.4	0.88	143/78/28	**0.0001**	**7.50**	**–**	**5.17**	**54/0/9**
		9	37.6	CA-0576	7.8	15.1	5.9	4.4	2.8	1.55	214/31/2	**0.05**	**7.23**	**–**	**6.00**	**60/0/3**
		8.1	0.0	CA-1423	6.7	12.9	6.0	4.6	3.2	1.38	202/42/0	–	–	–	–	no introgr
		11.1	7.4	CA-1512	6.1	11.9	6.1	5.3	4.4	0.83	158/73/0	ns	7.2	–	7.0	60/0/3
		5	1.0	CA-1566	4.4	8.8	5.9	5.2	4.5	0.74	197/36/16	**0.005**	**7.11**	**6.75**	**8.00**	**42/12/9**
		4	36.5	CA-1283	3.6	7.3	5.9	5.0	4.0	0.93	202/41/4	ns	–	–	–	no introgr
	Width2	1_8	16.8	CA-1409	8.2	40.4	7.0	6.0	5.0	0.98	27/25/24	**0.05**	**7.23**	**–**	**6.00**	**60/0/3**
		9	40.3	CA-0220	3.9	21.7	6.4	5.0	3.6	1.40	47/16/0	ns	7.2	7.0	7.3	42/9/12
		11.1	13.0	CA-0767	3.2	18.3	6.5	5.4	4.3	1.11	46/28/0	ns	7.2	7.0	7.3	42/9/12
	Shape	1_8	16.8	CA-1409	23.1	38.0	0.9	0.7	0.6	0.16	150/3/97	–	–	–	–	not meas
		11.1	7.4	CA-1512	7.2	13.7	0.8	0.7	0.5	0.15	158/73/0	–	–	–	–	not meas
		9	37.6	CA-0576	5.0	9.9	0.8	0.6	0.3	0.22	214/31/2	–	–	–	–	not meas
		5	1.0	CA-1566	3.9	7.8	0.8	0.7	0.5	0.12	197/36/16	–	–	–	–	not meas
		8.1	0.0	CA-1423	3.0	6.0	0.8	0.6	0.4	0.16	202/42/0	–	–	–	–	not meas
	Weight	1_8	16.8	CA-1409	7.8	38.9	125.4	90.7	55.9	34.71	27/25/24	–	–	–	–	not meas
		10.1	15.5	CA-1474	3.2	18.2	109.6	76.9	44.2	32.68	39/33/4	–	–	–	–	not meas
		9	40.3	CA-0220	3.0	17.1	103.0	58.1	13.2	44.88	47/16/0	–	–	–	–	not meas
Biochemical composition	Brix	1_8	16.8	CA-1409	5.6	25.3	9.4	10.7	12.0	−1.31	37/24/27	ns	8.0	–	8.0	60/0/3
	Glucose	1_8	16.8	CA-1409	3.5	16.8	3.4	3.8	4.2	−0.40	37/24/27	ns	2.9	–	3.1	60/0/3
		9	16.2	CA-0637	2.9	14.2	3.6	4.6	5.5	−0.94	81/6/1	ns	2.9	3.0	–	45/18/0

Table 3 continued

Class	Trait	BC_2S_1 population										NILs				
		LG	cM	Marker	LOD	%EV	µA	µH	µB	add.	A/H/B	Signif[a]	mA	mH	mB	A/H/B[b]
	Fructose	9	16.2	CA-0637	3.6	17.1	3.7	4.8	5.9	−1.08	81/6/1	ns	3.1	–	3.1	60/0/3
		1_8	16.8	CA-1409	3.5	16.7	3.5	3.9	4.3	−0.41	37/24/27	ns	3.1	–	3.2	60/0/3
	Malaat	1_8	16.8	CA-1409	3.6	17.2	30.3	38.3	46.4	−8.02	37/24/27	ns	24.2	–	25.2	60/0/3
		1	2.6	CA-0398	3.0	14.5	31.6	40.9	50.3	−9.39	42/37/9	**0.005**	**22.3**	–	**64.3**	**60/0/3**
		9	28.5	CA-0558	2.7	13.2	35.3	51.1	67.0	−15.85	77/10/1	ns	24.2	–	25.2	60/0/3
	Citrate	1_8	16.8	CA-1409	5.1	23.2	389.9	458.8	527.8	−68.94	37/24/27	ns	333.9	–	303.8	60/0/3
		12.2	17.7	CA-1548	3.3	15.9	441.9	707.7	973.4	−265.75	85/3/0	ns	331.6	–	348.6	60/0/3
		8.2	0.0	CA-1436	3.1	15.1	444.2	643.3	842.4	−199.09	86/1/1	–	–	–	–	no introgr
	Hexanal	1_8	16.8	CA-1409	12.1	46.8	18.5	19.4	20.2	−0.85	37/24/27	ns	17.6	–	17.4	60/0/3
		4	35.4	CA-1281	3.5	16.6	19.1	20.1	21.2	−1.03	73/14/1	–	–	–	–	no introgr
	3-Hepten-2-one	1_8	16.8	CA-1409	5.9	26.5	16.4	15.9	15.3	0.59	37/24/27	**0.05**	**14.3**	**–**	**13.4**	**60/0/3**
	Linalooloxide	10.1	16.6	CA-0218	11.1	44.1	15.0	17.3	19.6	−2.29	47/41/0	**0.0001**	**13.8**	–	**17.1**	**54/0/9**
	p-Menth-1-en-9-al	10.1	16.6	CA-0218	10.2	41.4	16.3	18.5	20.7	−2.24	47/41/0	**0.0001**	**14.9**	–	**18.3**	**54/0/9**
		1	20.2	CA-1188	4.1	19.1	16.6	18.0	19.3	−1.35	47/38/3	**0.0005**	**15.0**	–	**18.6**	**57/0/6**
	PAE	9	24.3	CA-0507	2.9	14.2	9.5	13.0	16.5	−3.54	83/5/0	–	–	–	–	no comp
	BAE	10.1	17.0	CA-1479	8.1	34.6	10.2	13.3	16.4	−3.08	50/35/3	–	–	–	–	no comp
	Geranylacetone	1_8	16.4	CA-1404	4.1	19.1	15.3	16.1	16.8	−0.76	45/43/0	ns	12.9	–	13.2	60/0/3
		10.1	16.6	CA-0342	0.6[1]	3.0	15.6	15.8	16.1	−0.25	47/36/5	**0.0001**	**12.7**	–	**14.5**	**54/0/9**
	β-damascenone	10.1	16.6	CA-0218	1.3[1]	6.5	15.8	15.2	14.6	0.58	47/41/0	**0.0001**	**13.1**	–	**9.5**	**54/0/9**
	Methoxypyrazine	1_8	16.8	CA-1409	3.4	16.4	17.8	18.4	19.0	−0.58	37/24/27	ns	17.6	–	16.1	60/0/3
		3	33.3	CA1268	3.2	15.5	18.6	17.8	17.1	0.78	66/15/7	**0.0001**	**17.8**	**17.6**	**16.0**	**45/9/9**
Sensory attributes	Crunchy	6.1	14.7	CA-1338	2.8	20.2	61.7	53.8	45.9	7.89	43/13/0	–	–	–	–	no introgr
	Sticky	1_8	17.8	CA-1169	5.6	36.9	37.1	52.2	67.2	−15.03	31/23/2	ns	18.6	–	19.5	60/0/3
	Tough	11.1	22.0	CA-1513	3.7	26.0	28.9	41.5	54.0	−12.56	37/17/2	ns	19.2	–	21.6	60/0/3
		1_8	16.8	CA-1409	2.8	20.4	27.3	35.1	42.9	−7.80	24/19/13	ns	19.2	–	21.3	60/0/3
	Juicy	1_8	17.8	CA-1169	4.5	31.1	50.8	41.0	31.1	9.84	31/23/2	ns	56.5	–	54.9	60/0/3
	Perfume	3	33.3	CA-1268	2.9	21.1	1.7	3.6	5.4	−1.84	42/10/4	–	–	–	–	split up
	Musty	7.1	0.0	CA-1368	5.0	33.4	0.7	2.6	4.5	−1.87	46/8/2	–	–	–	–	no introgr
	Odor	3	33.3	CA-1268	8.0	38.7	1.4	2.9	4.4	−1.50	56/14/6	–	–	–	–	split up
		9	24.3	CA-0507	4.9	26.0	1.7	4.8	7.9	−3.06	71/5/0	–	–	–	–	split up

The marker with the highest LOD score in the BC_2S_1 population is indicated including its linkage group (*LG*) and position in cM. Additionally, percentage of explained variance (% EV) at the marker position, estimated (µ, Van Ooijen 2009) or direct means (*m*), genotype distribution (A/H/B) and estimated additive effect (*add.*) are given

[a] not significant (*ns*)

[b] no variation for trait (*no var*), trait not measured (*not meas*), introgression not present (*no introgr*), compound not detected (*no compound*) and split up in multiple attributes (split up). For abbreviations of metabolites see Table 2. Metabolite values represent $\log_2$ values of peak intensities. Significant NIL values are printed bold

Table 4 LG3 volatile and flavor QTLs

Compound/attribute	BC_2S_1 population						NILs				
	LOD	%EV	μA	μH	μB	Add.	Signif.	mA	mH	mB	A/H/B
Aroma	na	na	na	na	na	na	0.0001	51.4	60.8	68.1	45/9/9
Grassiness	na	na	na	na	na	na	0.01	10.6	5.6	5.2	45/9/9
Flowers	na	na	na	na	na	na	0.01	3.8	7.8	8.9	45/9/9
Spices (non-pungent)	na	na	na	na	na	na	0.005	7.0	13.7	15.2	45/9/9
Celery	na	na	na	na	na	na	0.0001	2.2	6.3	8.9	45/9/9
Chives	na	na	na	na	na	na	0.0001	1.4	6.6	10.5	45/9/9
4-Mercapto-4-methyl-2-pentanone	4.3	20.3	11.9	13.8	15.7	−1.89	–	nd	nd	nd	nd
6-Methyl-4-oxo-5-heptenal	16.1	56.9	9.3	13.3	17.2	−3.96	0.0001	10.3	15.0	14.0	45/9/9
unknown_5871	8.1	34.5	13.1	13.7	14.4	−0.64	–	nd	nd	nd	nd
(Z)-Butanoic acid 3-hexenyl ester	3.9	18.3	14.6	13.0	11.3	1.68	0.0001	14.1	13.9	12.0	45/9/9
2-Isobutyl-3-methoxypyrazine	3.2	15.5	18.6	17.8	17.1	0.78	0.0001	17.8	17.6	16.0	45/9/9
(E)-3,6-dihydroxy-2-methyl-1,4-benzoquinone-4-methoxyimine-N-oxide	21.7	67.8	8.7	11.7	14.6	−2.94	–	nd	nd	nd	nd
unknown_8805	17.1	59.2	8.9	11.8	14.7	−2.89	0.0001	8.9	12.5	12.0	45/9/9
unknown_8832	15.2	54.9	8.7	10.7	12.7	−1.98	–	nd	nd	nd	nd
unknown_9961	21.3	67.2	8.7	11.1	13.5	−2.37	–	nd	nd	nd	nd

LG3 refers to the *C. baccatum* introgression of 32.9–33.4 cM from linkage group 3. Percentage of explained variance (*% EV*), estimated (μ, Van Ooijen 2009) or direct means (*m*), estimated additive effect (*add.*) and genotype distribution (A/H/B) are given. Metabolite values represent $\log_2$ values of peak intensities. *na* not available, *nd* not detected

or 'similar to papaya taste'. This was caused by the fact that the panelists participated in training sessions with only commercially available genotypes, lacking these (unexpected) flavors. For the NIL experiment the test panel was, therefore, also trained with fruits from preselected NILs having more extreme flavors than currently available in the Dutch commercial segment. This resulted in an expansion of the panel's vocabulary with the attributes flowers, spices (non-pungent), celery, chives and bitter.

Analysis of the sensory data from the NIL experiment using the non-parametric Kruskal–Wallis test showed that NILs having either a heterozygous (NIL40, 46 and 48) or homozygous (NIL37, 38 and 39) LG3 introgression containing the BC_2S_1 odor QTL (Table 3; Fig. 3) have significantly higher scores for the attributes aroma, flowers, spices, celery and chives (Table 4). This confirmed that the small *C. baccatum* introgression on LG3 from 32.9 to 33.4 cM causes an extraordinary effect on flavor. Subsequently we checked for which metabolites there are QTLs in this LG3 region in the BC_2S_1 population, which resulted in nine volatiles (Table 4). Out of these nine metabolites, four were also detected and confirmed in the NILs. The compounds 6-methyl-4-oxo-5-heptenal and unknown_8805 showed a strong increase in intensity in the presence of the *C. baccatum* allele, while (Z)-butanoic acid 3-hexenyl ester and 2-isobutyl-3-methoxypyrazine were decreased in NILs having the introgression (Table 4). The confirmed up-regulated compounds were also checked for their direct relation to odor in the BC_2S_1 population resulting in a correlation of 0.53 for 6-methyl-4-oxo-5-heptenal and 0.43 for unknown_8805.

LG10.1 and LG1 terpenoid QTLs

An initial analysis of the 137 metabolites detected in the NILs by principal components analysis made clear that a large part of the metabolic variation (46.1 %, data not shown) between the genotypes is caused by a group of terpenoids, of which the intensity levels between parents and offspring varied enormously. For the two terpenoids linalooloxide and *p*-menth-1-en-9-al reported in Table 1, the maximum intensities found in the BC_2S_1 population were up to 39.3 and 19.7 times higher, respectively, than in the PEN45 donor parent. For these terpenoids a common major QTL (LOD >10) on LG10.1 and a *p*-menth-1-en-9-al specific QTL (LOD 4.1) on LG1 were found (Table 3). Taking a closer look at the NILs having these LG10.1 (NIL45, 48 and 54) or LG1 (NIL36 and 47) introgression revealed a group of at least 15 terpenoids that are affected (Table 5). In most cases, both introgressions resulted in up-regulation of the compounds. α-terpineol, e.g., was more than ten times increased in NILs with the LG10.1 introgression compared to lines without any of these two introgressions. For hotrienol the effect of the introgressions was even larger with a 27.1-fold increase for LG10.1 and a 16.6-fold increase for the LG1 fragment. Some terpenoids were specifically

Table 5 LG10.1 and LG1 terpenoid QTLs

Compound	El. comp[a]	LG[b]	BC_2S_1 population						NILs			
			LOD	%EV	μA	μH	μB	Add.	Signif.	mA	mB	A/B
α-Terpinene	$C_{10}H_{16}$	10.1	4.3	20.2	13.9	14.8	15.7	−0.88	0.0001	10.7	13.2	54/9
		1	0.0[ns]	0.0	14.3	14.3	14.3	0.02	0.001	10.9	13.2	57/6
γ-Terpinene	$C_{10}H_{16}$	10.1	8.3	35.3	12.0	13.7	15.3	−1.62	0.0001	10.4	12.8	54/9
		1	2.3[ns]	11.0	12.4	13.4	14.3	−0.94	0.001	10.5	12.9	57/6
Terpinolene	$C_{10}H_{16}$	10.1	10.3	41.7	14.7	16.7	18.7	−2.00	0.0001	12.4	15.3	54/9
		1	2.3[ns]	11.1	15.2	16.3	17.3	−1.07	0.001	12.6	15.2	57/6
Limonene	$C_{10}H_{16}$	10.1	10.4	42.0	15.3	16.6	18.0	−1.35	0.0001	13.6	16.4	54/9
		1	0.8[ns]	3.9	15.8	16.2	16.6	−0.43	0.005	13.8	16.1	57/6
Myrcene	$C_{10}H_{16}$	10.1	7.3	31.8	12.0	15.1	18.3	−3.18	0.0001	11.7	14.7	54/9
		1	1.7[ns]	8.5	12.7	14.2	15.6	−1.45	0.001	11.8	14.8	57/6
(E)-β-Ocimene	$C_{10}H_{16}$	10.1[c]	2.9	14.1	14.3	15.5	16.8	−1.23	0.0005	12.5	15.1	57/6
Hotrienol	$C_{10}H_{16}O$	10.1	5.9	26.5	14.2	17.2	20.2	−2.98	0.0001	9.7	14.6	54/9
		1	3.0	14.6	14.7	16.6	18.6	−1.96	0.005	10.0	14.0	57/6
p-Menth-1-en-9-al	$C_{10}H_{16}O$	10.1	10.2	41.4	16.3	18.5	20.7	−2.24	0.0001	14.9	18.3	54/9
		1	4.1	19.1	16.6	18.0	19.3	−1.35	0.0005	15.0	18.6	57/6
Geranic-oxide	$C_{10}H_{18}O$	10.1	10.6	42.6	15.8	16.9	18.0	−1.10	0.0005	13.6	14.6	54/9
		1	0.4[ns]	2.1	16.2	16.5	16.7	−0.26	0.005	13.7	14.4	57/6
Myrcenol	$C_{10}H_{18}O$	10.1	10.5	42.2	11.5	13.8	16.1	−2.31	0.0001	9.8	13.2	54/9
		1	1.6[ns]	8.0	12.1	13.2	14.2	−1.04	0.005	10.0	12.8	57/6
α-Terpineol	$C_{10}H_{18}O$	10.1	10.7	42.8	17.9	20.2	22.7	−2.44	0.0001	16.4	19.3	54/9
		1	2.9	14.2	18.3	19.5	20.8	−1.24	0.005	16.6	19.2	57/6
Linalool	$C_{10}H_{18}O$	10.1	9.5	39.3	16.9	19.5	22.1	−2.62	0.0001	16.0	18.1	54/9
		1	2.6[ns]	12.7	17.4	18.7	20.1	−1.31	0.005	16.2	17.8	57/6
Cineole	$C_{10}H_{18}O$	1	6.8	29.8	12.0	14.7	17.3	−2.62	0.0001	9.2	14.6	57/6
(E)-Linalooloxide	$C_{10}H_{18}O_2$	10.1	11.0	43.9	16.7	19.1	21.5	−2.41	0.0001	15.2	18.7	54/9
		1	2.7	13.0	17.2	18.4	19.5	−1.16	0.001	15.3	18.8	57/6
Linalooloxide	$C_{10}H_{18}O_2$	10.1	11.1	44.1	15.0	17.3	19.6	−2.29	0.0001	13.8	17.1	54/9
		1	2.5[ns]	12.2	15.6	16.6	17.7	−1.07	0.001	13.9	17.3	57/6

ns not significant

[a] Elemental composition

[b] LG10.1 and LG1 refer to markers CA-0218 at 16.6 cM and CA-1188 at 20.2 cM, respectively, on the corresponding linkage groups

[c] Refers to marker CA-0022 at 6.3 cM on LG10.1. Again percentage of explained variance (*% EV*), estimated (μ, Van Ooijen 2009) or direct means (*m*), estimated additive effect (*add.*) and genotype distribution (A/B) are given. Metabolite values represent $\log_2$ values of peak intensities

affected by one of the introgressions. For cineole only the LG1 introgression was effective and for (E)-β-ocimene the up-regulation was specific to the telomeric LG10.1 introgression present in NIL45 and NIL54 and absent in NIL48 (Fig. 3). In the BC_2S_1 population the LG1 locus was not significant for ten of the terpenoids, while the introgression in the NILs always resulted in a significant effect. On the other hand, the effect of the LG10.1 introgression was always supported by a significant QTL in the BC_2S_1 population (Table 5).

Non-volatile effects in the NILs

As mentioned above, a QTL for malate was found in the BC_2S_1 population of which the effect was confirmed in the NILs. For the other non-volatile compounds glucose, fructose and citrate and the total soluble solids measure Brix, some QTLs were found in the BC_2S_1, but none of these effects could be confirmed in the NILs (Table 3). However, in the NILs themselves we observed some very interesting non-volatile effects, relating to the *C. baccatum* introgression on LG3 of 0–19.6 cM and, to a lesser extent, to the LG10.1 introgression of 15.5–18.0 cM. Brix and the concentration of glucose, fructose and citrate were significantly increased in NILs having these fragments, while in both cases the malate concentration was not significantly affected (Table 6).

The LG3 introgression gave the strongest effects (Table 6), resulting in, e.g. a Brix increase of 1.76° compared with all NILs lacking the fragment and even of 2.47° compared with the recurrent parent GNM. Most interestingly, this effect seemed to be unrelated to fruit size, as the fruits of NIL51 had a similar size (8 × 7 cm; length × width) as GNM (8 × 8 cm). The LG10.1 introgression, on the other hand, coincided with confirmed length and width QTLs, resulting in fruits of on average 5 × 5 cm.

Table 6 Brix, sugar and acid effects in the NILs

Trait	LG3				LG10.1			
	Signif.	mA	mB	A/B	Signif.	mA	mB	A/B
Brix	0.005	7.94	9.70	60/3	0.0005	7.89	8.81	54/9
Glucose	0.005	2.87	3.71	60/3	0.005	2.87	3.16	54/9
Fructose	0.01	3.07	3.72	60/3	0.0005	3.05	3.37	54/9
Citrate	0.005	323.40	513.43	60/3	0.005	326.98	365.28	54/9
Malate	ns	24.48	19.79	60/3	ns	25.22	18.53	54/9

LG3 and LG10.1 refer to the *C. baccatum* introgressions of 0–19.6 and 15.5–18.0 cM, respectively, on the corresponding linkage groups. Direct means (*m*) and genotype distribution (A/B) are given

ns not significant

Discussion

Challenges due to interspecific crossing

A few studies addressed variation in biochemical compounds and agronomical traits in the species *C. baccatum* (e.g. Do Rêgo et al. 2009; Rodriguez-Burruezo et al. 2009; Wahyuni et al. 2013) and ample suggestions for improvement of genotypes within the species have been made. In this study we have investigated the possibility to use *C. baccatum* for enrichment of the *C. annuum* gene pool. For this purpose the *C. baccatum* var. *pendulum* accession PEN45 was used, as it was shown previously to contain potentially interesting volatile and non-volatile variation (Eggink et al. 2010). Interspecific crossing in combination with embryo rescue was performed to overcome the post-fertilization genetic barriers as described in Yoon et al. (2006). Due to difficulties experienced during interspecific crossing, however, we were not able to generate a single bi-parental mapping population of sufficient size, but instead developed the described multi-parent BC_2 and BC_2S_1 populations, which made the genetic analyses more complex.

The total size of the map developed in this study (602.5 cM) is small compared with the previously published pepper map sizes (Wu et al. 2009; 1,613 cM) or (Barchi et al. 2007; 1,857 cM). This is, however, as expected, since our map is based on a BC_2 population with a limited number of effective recombinations and potentially suppressed recombination, due to its interspecific nature, causing clustering of markers. Chromosome five specifically, turned out to be rather underrepresented as the corresponding linkage group consists of only two markers (Fig. 2). Five other markers originating from chromosome five are, however, present on the map, but positioned on other linkage groups [LG3 (1), LG6.1 (1) and LG9 (3)]. An explanation for this could be chromosome five specific translocations, but it seems more likely to be caused by absence of enough polymorphic markers that can be connected in one linkage group. Overall, the available integrated map turned out to be indispensable for orientation and assignment to pepper chromosomes.

Combined QTL detection and validation approach

For the initial experiment 250 BC_2S_1 plants were used originating from 34 BC_2 plants. Depending on the phenotyping protocol plants were measured individually or per plot leading to different numbers of phenotypes per trait (ranging from 56 to 250; Table 1). Consequently, plot genotypes had to be created in cases when (fruits of) multiple plants were combined in the measurements. In this procedure we not only assumed that the *C. baccatum* effects were dominant, but also took the number of plants in a plot into account. So, e.g. in case a plot contained four plants of which two scored A and two scored H, the plot score would be H (dominance of *C. baccatum* allele), but in case the plot would contain three A scores and one H, the plot score would be A (higher number of A plants). This procedure turned out to work very well as the control traits pungency and red color could be perfectly mapped to the positions at which they were mapped earlier by (Thorup et al. 2000; red color) and (Blum et al. 2002; pungency). In the initial Interval Mapping analysis for red color, however, four QTLs with limited effect were found (Table 3), of which only the QTL on LG6.2 fell in the expected CCS region. Knowing that a single dominant gene is involved the other three QTLs had to be false positive signals. These artifacts could be explained by accidental co-segregation of the PEN45 allele from markers CA-0323 (LG2.1), CA-0038 (LG12.1) and CA-1209 (LG12.2) with marker CA-0097 from LG6.2 in 5 to 7 BC_2S_1 plants (data not shown), caused by the limited number of BC_2 plants from which the BC_2S_1 plants originate. In addition we showed that due to the multi-parent nature of the BC_2, during map construction, markers linked to fruit color genes were selected against, demonstrating another limitation of our used mapping population. Finally, pre-selection of the BC_2S_1 plants for pungency, color and fertility led to extremely skewed segregations

of markers at certain linkage groups, exemplified by the pungency locus at LG2.2 with 247 plants homozygous *C. annuum* versus only three plants homozygous *C. baccatum* (Table 3). It should be noted, therefore, that these limitations might have resulted in other false-positive, or even missed QTLs in the BC_2S_1 population. Nevertheless, there is a vast amount of BC_2S_1 QTLs that were confirmed in the NILs for physical, biochemical as well as sensory traits, proving the consistency of such QTLs and indicating the strength of our combined approach in multi-parent interspecific mapping populations.

Unexpected flavor variation

As the *C. baccatum* PEN45 accession is pungent, it was not included in the sensory evaluations of the expert panel. To base the choice for this accession, as donor parent for backcrossing into *C. annuum*, not only on the volatile and non-volatile variation found (Eggink et al. 2010). Fruits of PEN45 were therefore tasted by a group of untrained persons accustomed to eat pungent food. This group described the taste of PEN45 as fruity, not very aromatic and sour. This rather conservative description made the much larger variation in flavors found in the BC_2S_1 experiment, including remarks like 'tropical flavor', 'chives taste' or 'similar to papaya', very unexpected. Especially the flavor effect of the small introgression on LG3 is evident and an enrichment of the taste variation within *C. annuum*. The size of the fragment, 0.5 cM based on the genetic map developed within this study, corresponds to a region of 8.5 cM on the integrated map. This size of an introgression, in combination with the availability of in-fragment markers and absence of (clear) linkage drag, makes it very interesting for breeding. In the BC_2S_1 population an additional odor QTL was found on LG9 at 24.3 cM (Table 3). In the NIL experiment we could, however, neither confirm, nor completely rule out an effect of this locus alone, as this introgression was only present in NIL38, which also contains the LG3 fragment. Given the population structure, most likely it is a false-positive signal due to accidental co-segregation in the BC_2S_1.

We not only investigated the genetics of the observed flavor variation, but also made an attempt to identify the responsible biochemical compounds. The metabolites 6-methyl-4-oxo-5-heptenal and unknown_8805 were confirmed to be specifically up-regulated in the LG3 containing NILs and BC_2S_1 plants, at the same time showing a strong correlation with odor. Given the complex nature of flavor, however, it is unlikely that they are the only responsible compounds. Having, therefore, a closer look at the six LG3 containing NILs it turned out that the intensity of the two down-regulated compounds, (Z)-butanoic acid 3-hexenyl ester and 2-isobutyl-3-methoxypyrazine, is only decreased in the homozygous NILs (NIL37, 38 and 39) and not in the heterozygous NILs (NIL40, 46 and 48; Table 4). It could be true, therefore, that these compounds interact with, or even partially mask the effect of, the up-regulated compounds resulting in a more intense aroma in the homozygous NILs (68.11; Table 4) and a slightly suppressed aroma intensity (60.81) in the heterozygous lines, while the concentrations of 6-methyl-4-oxo-5-heptenal and unknown_8805 are even slightly higher in the heterozygous versus the homozygous NILs. Such a suppression effect might also be expected from 2-isobutyl-3-methoxypyrazine, as it is commonly described in sniffing port analyses as characteristic (green) bell pepper aroma (Luning et al. 1994; Van Ruth et al. 1995; Rodriguez-Burruezo et al. 2010), while the LG3 NILs are especially described as having non-typical pepper aroma, with use of the attributes flowers, spices, celery and chives. An effect of 2-isobutyl-3-methoxypyrazine alone on aroma was not found, as NILs with a significantly decreased (NIL47 and 56) or increased (NIL45 and 54) intensity were unaffected for aroma intensity. In another study addressing Andean peppers (Kollmannsberger et al. 2011), however, 2-isobutyl-3-methoxypyrazine, with 2-heptanethiol, was identified by sniffing port analysis as the volatile with the largest contribution to the aroma of two *C. baccatum* accessions. This contrasting result might be caused by a different sensitivity of sniffing versus taste evaluations, as suggested previously (Eggink et al. 2012a) or by variation between the (limited number of) *C. baccatum* accessions tested. A follow-up experiment with sub-NILs having either LG3 or LG9 and the combination of both would serve validation of the genetics and at the same time further elucidation of the biochemical relations.

Terpenoids and their potential application

In both mapping populations a large variation in terpenoid levels was found, with for some terpenes a maximum concentration, which was almost 40-fold higher than detected in the most extreme parent. The described PEN45 LG10.1 and LG1 introgressions had major effects on the terpenoid content of the mature fruits, affecting at least 15 different terpenoids (Table 5). These compounds, derived from precursors IPP and DMAPP, belong to a large class of terpenoid metabolites that serve multiple roles in plants and which are sub-divided into the main groups monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20). In our study, the QTLs on LG10.1 and LG1 affected the accumulation of monoterpenes only, whereas sesquiterpenes and diterpenes were unaffected by these two introgressions. In the BC_2S_1 population, 12 sesquiterpenes were detected however, of which four compounds (γ-himachalene, α-cuprenene, δ-amorphene and α-muurolene) gave significant QTLs on LG1_8 and LG8 (data not shown). Unfortunately, neither of these

two regions was represented in the NILs and could, therefore, not be validated.

The backbones of mono-, sesqui- and diterpenes are synthesized by enzymes that belong to the structurally related terpene synthase (TPS) family (Bohlmann et al. 1998). The genome of cultivated tomato (*Solanum lycopersicum*) contains 44 of such TPS genes, including 29 that are functional or potentially functional. Many of the tomato TPS genes are found in clusters on chromosomes 1, 2, 6, 8 and 10 (Falara et al. 2011). Chromosomes 1 and 10 of pepper are highly syntenic to those of tomato (e.g. Wu et al. 2009), which make the tomato TPS genes in the chromosome 1 and 10 clusters interesting candidates for the monoterpenoid concentration increase caused by our PEN45 introgressions. Furthermore, this matches with the observation that the majority of the TPS genes in the tomato chromosome 1 and 10 clusters encode monoterpene synthases (Falara et al. 2011).

Although some monoterpenes are well-known flavor compounds (e.g. linalool and β-ocimene; Luning et al. 1994), in our study there was no co-localization of terpenes and any taste attribute. This is in agreement with the findings of Kollmannsberger et al. (2011), in which odor-contributing volatiles (OCVs) were identified and it is concluded that terpenoids make only a minor contribution to the aroma of (two) *C. baccatum* accessions, despite the large range of terpenoids identified in the species. Still, the increase in monoterpenoid concentration can be relevant for pepper, since terpenoids have been shown to play a role in the interactions of plants with their environment. More specifically, they can be active as direct defense compounds, containing antifungal or antibacterial properties (reviewed by Kalemba and Kunicka 2003). In addition, volatile terpenoids can function as indirect defense compounds by attracting predators or parasitoids of the attacking insect (reviewed by Walling 2000). For some of the elevated monoterpenes in this study (Table 5) specific relations with relevant pepper pathogens have already been described. E.g. in cucumber (*Cucumis sativus*), although in vegetative organs instead of fruits, a positive correlation was found between the attraction of predatory mites (*Phytoseiulus persimilis*) and the amount of emitted (E)-β-ocimene, after infestation of the plants with herbivorous spider mites (*Tetranychus urticae*; Kappers et al. 2011). In addition, antimicrobial properties related to monoterpenes have been reported in several studies of essential oils. (Pérez-Sánchez et al. 2007), e.g. reported a clear growth inhibition of the pathogenic fungi *Colletotrichum acutatum* and *Fusarium oxysporum* (causing anthracnose and internal fruit rot, respectively, in pepper), which showed the highest correlation with the concentration of the monoterpene α-terpinene, extracted from the oil of *Thymus zygis*. These examples indicate that it will be very interesting to study the behavior of the LG10.1 and LG1 containing NILs in relation to pathogen infestation.

Like for the LG3 flavor introgression, the size of the LG10.1 and LG1 fragments is limited, i.e. 2.5 and 4.6 cM, respectively, corresponding to 17.2 and 10.3 cM on the integrated map. It should be noted, however, that the LG10.1 introgression also contains QTLs affecting length, width and unripe fruit color, resulting in fruits which are smaller (on average 5 × 5 cm) than the recurrent parent GNM (8 × 8 cm) and having a pale green color. In addition, NILs with the LG10.1 introgression show a decreased concentration of β-damascenone, a product of β-carotene degradation, while geranylacetone, which is produced from open chain carotenoids, precursors of β-carotene, shows up-regulation at that locus (Table 3). Brand et al. (2012) also described a QTL for unripe fruit color at the top of chromosome 10 in a cross between a dark-green *C. annuum* and a pale green *C. chinense* accession. They showed that the unripe fruit color intensity reflects the content of chlorophyll and other metabolites associated with the chloroplast at immature fruit stage, and is likely due to the increased chloroplast number and chloroplast compartment size observed in dark-green genotypes, as found in high pigment tomato accessions (Cookson et al. 2003). Although dark-green genotypes also had more and larger chromoplasts at ripe stage, levels of the major carotenoids accumulating at ripe stage seemed not to be affected. On the one hand, we cannot rule out the possibility that chloroplast abundance and morphology rather than variation in biosynthetic pathway genes may be the cause of the QTLs for terpenoids and carotenoid-derived volatiles in our LG10.1 NILs, since these compounds are synthesized in plastids (Nagegowda 2010). On the other hand, as mentioned, it is known that the upper part of tomato chromosome 10 contains a cluster of monoterpene synthase genes (Falara et al. 2011), which would also be likely candidates for the observed variation in terpenoids. The availability of a pepper genome sequence will certainly be of great help to address which candidate genes could be involved in controlling the traits on LG10.1.

In contrast to the LG10.1 NILs, fruits of NIL36 and 47, containing the LG1 introgression affecting terpenoid content, have a similar size and fruit color as the recurrent parent, making them amenable for direct use in breeding.

Fruit size independent Brix increase

For the non-volatile compounds glucose, fructose, malate and citrate and the total soluble solids measure Brix a common QTL with a positive effect from the *C. baccatum* allele was found on LG1_8, which co-localized with a QTL affecting the texture-related attributes toughness, stickiness of the skin and juiciness (Table 3). As these QTLs

coincided as well with the main fruit size QTL, which led to severely reduced fruit width and conical shape in the BC_2S_1 population, it seems plausible that the increase in the concentrations of the non-volatiles resulted directly from smaller fruit size, rather than an absolute increase in amount, a negative relation, that is well known in, e.g. tomato, where small size cherry tomatoes have higher sugar concentrations than large-fruited beef tomatoes. The same could be argued for the texture-related attributes, as smaller conical fruits had generally more rigid fruit walls leading to less juicy and tougher fruits. Both seem to be in line as well, with the observation that in the NILs, where the LG1_8 QTL had only a minor effect on fruit width, the non-volatiles and texture attributes were not significantly affected anymore (Table 3).

In the NILs, however, we did find some interesting regions with an effect on non-volatile concentrations, as the *C. baccatum* introgression at the top of LG3 in NIL51 and, to a lesser extent, the LG10.1 fragment of 15.5–18.0 cM in NIL45, 48 and 54 resulted in an enormous increase in Brix and underlying soluble solids, glucose, fructose and citrate, compounds which are known to play an essential role in flavor. In tomato, e.g. taste intensity is mainly attributed to reducing sugars and organic acids (Stevens et al. 1977; Krumbein and Auerswald 1998; Bucheli et al. 1999) and overall liking, as determined by consumer panels, is found to be strongly correlated with Brix levels in both pepper and tomato (Verkerke and Kersten 2000). Often, however, a negative relationship between Brix and fruit size and/or yield is found (Grandillo et al. 1999; Georgelis et al. 2004). Also in the LG10.1 NILs such a negative effect on fruit size was found, making them less interesting for breeding. It is remarkable, therefore, that the LG3 fragment with the largest effect on total soluble solids content did not show this negative relation, as NIL51 fruits were similar in size as those from the recurrent parent. Unfortunately, we did not measure fruit weight or moisture content in the NILs to study their relation with the increased Brix level. A next step, therefore, would be to perform a solid yield experiment to study the effect of the LG3 introgression on total harvest, individual fruit weight and moisture content.

It is not completely clear why in the BC_2S_1 population we did not find any Brix QTL on LG3, while the effect in the NIL population is evident. A plausible explanation could, however, be that in the BC_2S_1 population still multiple genetic factors influencing Brix levels, like sugar and acid synthesis, transport and conversion, but also factors like fruit size, moisture content and fruit set, segregated simultaneously, which, in combination with aforementioned limitations of our mapping population, made mapping of individual effects impossible. It anyhow proves, that next to the validation possibilities, the NILs were indispensable for dissecting a complex trait like Brix.

Conclusion

This study demonstrates that *C. baccatum* is a valuable source for enrichment of the *C. annuum* genetic pool. In several cases unexpected traits were introgressed in *C. annuum*, as shown by the wide flavor variation, transgressive terpenoid levels and the fruit size unrelated Brix effect. The combination of developed populations allowed not only the mapping of simple morphological traits, but also genetic dissection of quantitative traits with complex inheritance patterns. The current *C. annuum* genome sequence knowledge in combination with candidate genes from syntenic crops, like tomato, will allow further elucidation of our studied traits. Furthermore, the availability of NILs containing a limited amount of introgressions of restricted size make flavor enhancement and/or potential (in) direct defense applications in commercial breeding programs directly possible.

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

Ethical standards The experiments comply with the current laws of the Netherlands.

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