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ORIGINAL ARTICLE

A major quantitative trait locus conferring subgynoecy in cucumber

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Abstract

Key message **A major QTL conditioning high degree of femaleness in cucumber was identified by marker analysis and next generation sequencing.**

Abstract Cucumber (*Cucumis sativus* L.) is a model species for sex determination studies, and its yield is associated with the degree of femaleness. Subgynoecy represents a sex form with a high degree of femaleness for which the genetic basis remains elusive. In this study, genetic analysis in the F_2 and BC_1 populations developed from a cross between subgynoecious S-2-98 and monoecious M95 suggested a quantitative nature of subgynoecy. Application of simple sequence repeat markers between subgynoecious and monoecious bulks constructed from BC_1 plants identified three QTLs: *sg3.1*, *sg6.1*, and *sg6.2*. The major QTL *sg3.1* contributed to 54.6 % of the phenotypic variation, and its presence was confirmed by genome-wide comparison of SNP profiles between parental lines and a subgynoecious bulk constructed from BC_6 plants. Using PCR-based markers developed from the SNP profile, *sg3.1* was further delimited to a genomic region of 799 kb. The genetic basis of subgynoecy revealed here shall shed light on the development of elite cultivars with high yield potential.

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Introduction

Cucumber (*Cucumis sativus* L.) has been a model system to study the biological processes of sex determination (Malepszy et al. 1991), which is of tremendous significance in plant breeding and evolutionary studies. Cucumber can be divided into seven sex types based on the distribution of male, female and bisexual flowers along the main stem. Of these types, the monoecious and gynoecious types are most frequently used in cucumber production. Monoecious cucumber produces unisexual male flowers at the basal part of the stem, followed by both male and female flowers at later growth stages. In contrast, gynoecious cucumber only bears female flowers. The process of sex determination is regulated by the combination of genes, hormones as well as environmental conditions, together resulting in multiple sexual types (Kubicki 1969, 1974; Shifriss 1961; Trebitsh et al. 1987). It has been well established that ethylene is the major regulator of sex determination of cucurbit species (Rudich et al. 1972; Byers et al. 1972; Papadopoulou et al. 2005; Salman-Minkov et al. 2008; Yin and Quinn 1995). Application of ethylene or its precursor ACC promotes the formation of female flowers in monoecious cucumber (Yamasaki et al. 2003), while interfering with ethylene synthesis or signalling using chemical inhibitors induces male flowers in gynoecious cucumber (Takahashi and Jaffe 1983). High endogenous ethylene concentrations as well as elevated expression of the ethylene biosynthesis gene *1-aminocyclopropane-1-carboxylic acid*

synthase 2 (*CsACS2*) are in correlation with the formation of female flower (Saito et al. 2007). In addition, auxin also enhances the formation of female flowers, possibly by induction of ethylene production, whereas gibberellic acid suppresses female flower formation (Trebitsh et al. 1987; Yamasaki et al. 2003). It has been reported that sex determination of cucumber is controlled by three major genes: *F/f*, *M/m*, and *A/a*. The *monoecious* allele (*m*) confers the formation of bisexual flowers, while the dominant *M* allele suppresses stamina development and thus results in female flowers (Kubicki 1969; Li et al. 2009). The *Female* (*F*) locus controls the degree of femaleness, and plants that are homozygous for the *F* allele exhibit gynoecy (Shiber et al. 2008; Trebitsh et al. 1997). The *androecious* locus (*a*) promotes maleness, and plants which are homozygous for both the *a* and *f* allele display androecy (Kubicki 1969; Perl-Treves 1999). Genetic studies revealed that the *M* gene encodes *CsACS2* (Li et al. 2008, 2009) and the *F* locus is genetically associated with another *ACS* gene (*CsACS1G*) (Trebitsh et al. 1997; Mibus and Tatlioglu 2004; Knopf and Trebitsh 2006; Shiber et al. 2008). A genome-wide structural variation study of cucumber reveals that a 30.2-kb duplication involving four genes gave rise to the *F* locus (Zhang et al. 2015). Furthermore, the sex expression of cucumber is modulated by several modifier genes (Galun 1962; Kubicki 1974; Pitrat 1994).

Gynoecy and monoecy are frequently exploited in cucumber breeding. Gynoecious cucumber markedly boosts yield compared to monoecious cucumber; however, optimal growth conditions are required to realize the full fruit production potential of gynoecious cucumber. This presents a challenge for cucumber breeders in China, as it is difficult to maintain such optimal growth conditions. As a result, under the regular growth conditions in China not all female flowers develop into a marketable cucumber, especially when long fruits are produced. Therefore, cultivars with a percentage of female flowers in between that of monoecious and gynoecious cucumber would be more suitable for cultivation. Subgynoecy can be regarded as a special type of monoecy, though with a higher percentage of female flowers. Furthermore, subgynoecious cucumber exclusively develops female flowers at later growth stages. Chen et al. (2011) identified two subgynoecious loci, one acting recessively and another one dominantly, in inbred lines 97-17 and S-2-98, respectively. The inheritance analyses indicated that the subgynoecious trait of S-2-98 is independent of the *F* and *M* genes. However, the genetic basis of the subgynoecious trait in S-2-98 remains unclear.

In this study, we carried out a thorough investigation of the genetic architecture of the subgynoecious trait in S-2-98. Quantitative trait loci (QTLs) were identified by simple sequence repeat (SSR) and sequencing-based analyses. A major QTL was further narrowed down using PCR-based

markers and breeding materials harbouring this QTL were created.

Materials and methods

Plant materials and phenotypic evaluation

A line with high degree of femaleness emerged from the monoecious cultivar "DongHuzao". After nine generations of selfing, it was developed into a stable subgynoecious line, referred to as S-2-98. S-2-98 initially displays a phase in which only male flowers are formed, followed by a phase in which both male and female flowers are formed and terminates into a phase in which sequential female flowers are formed. S-2-98 was used as the maternal parent in the cross with M95. M95 is a monoecious inbred line developed from monoecious variety "JingChun 4". F_1 plants were self-pollinated to produce the F_2 population consisting of 192 plants. To create subgynoecious breeding materials with M95 background, a single plant with the highest degree of femaleness was backcrossed to M95 in each backcross generation. The BC_1 population consisted of 188 plants. Crossing was carried out in the experimental fields in Hunan, China.

Phenotypic evaluation was carried out when the majority of plants in the population developed at least 25 nodes on the main stem. Flower sex type of each node of each plant was recorded. In case flowers were abscised in basal nodes, the flower sex type was determined based on the difference in size between that of the male and female stalks. The percentage of female flowers (R_{female}) of each individual was calculated to determine the degree of femaleness using the formula $R_{female} = N_{female}/(N_{female} + N_{male}) \times 100\%$, where N_{female} stands for the number of nodes bearing female flowers and N_{male} stands for the number of nodes bearing male flowers. Subgynoecious cucumber displays continuous female flower nodes at the later growth stage, a feature differing from monoecious cucumbers. Therefore, the number of continuous female flower nodes at later growth stage, designated N_{cf}, was also taken into account when selecting subgynoecious individuals to carry out backcrossing or to construct bulked DNA pools.

QTL analysis with SSR markers

Two DNA pools, a monoecious pool (M pool) and a subgynoecious pool (S pool), were constructed using BC_1 plants. Genomic DNA was isolated from ten plants with R_{female} ranging from 85 to 95 % for the S pool and ten plants with R_{female} ranging from 1 to 25 % for the M pool. A total of 2112 SSR primers, developed from the whole genome sequence of the cucumber inbred line 9930 (Huang et al.

2009), were screened for polymorphisms in the parental lines and subsequently in the S pool and M pool. Polymorphic markers between the two pools (Supplementary material S1) were subjected to linkage analysis using Join-Map 3.0 software with a Log of odds (LOD) of 3.0 and the Kosambi mapping function (Lander and Botstein 1989). The QTL analysis was performed with Interval Mapping and Multiple-QTL model Mapping (MQM) using MapQTL 4.0 software (Jansen 1993; Jansen and Stam 1994; Van Ooijen 2006).

QTL analysis with next generation sequencing data

In total, 108 BC_6 and 87 BC_7 plants were grown in the autumn of 2012 in the greenhouses in Beijing, China. After phenotypic evaluation, seven plants (BC_6-1, BC_6-2, BC_6-3, BC_6-4, BC_6-5, BC_6-6 and BC_6-7) with high R_{female} and N_{cf} were selected from the BC_6 population (Supplementary material S2). Genomic DNA was isolated from selected individuals using the CTAB method. Equal amounts of genomic DNA of each individual were mixed to construct the subgynoecious BC_6 pool (S_{BC6} pool). Parental lines S-2-98 and M95, as well as S_{BC6} pool were subjected to whole genome sequencing using an Illumina GAIIx Sequencer1. Reads with a length of 100 bp were generated from paired-end sequencing.

All reads were aligned to the 9930 reference genome with the BWA software (Li and Durbin 2009). SNP calling was conducted using SAM tools software (Li and Durbin 2009). Because both parents are homozygous lines, only homozygous SNPs in both parents were retained. The Illumina phred-like quality score and mapping score were set to larger than 30. The read depth was set between 3 and 40. The SNP-index was calculated for S_{BC6} pool by QTL-seq and MutMap (Abe et al. 2012; Takagi et al. 2013). The SNP-index was determined by the number of reads showing the same genotype as the parental line S-2-98 divided by the total number of reads mapped to this SNP site. An average SNP-index of SNPs located at a given genomic interval was computed using a sliding window analysis of 1 Mb window size and 10 kb increment. The SNP-index graph for S_{BC6} pool was plotted.

The dCAPS and InDel markers were developed based on the SNP profile. Primers for dCAPS markers were designed using web-based designer software (http://helix.wustl.edu/dcaps/dcaps.html). Primers for InDel markers were designed using Primer 5 program.

Development of near-isogenic subgynoecious lines and fine mapping of the major QTL

Genotypic analysis with dCAPS and InDel markers was undertaken for BC_7 plants. Three plants (BC_7-7, BC_7-16

and BC_7-17) harbouring only the major QTL on chromosome 3 and showing high R_{female} and N_{cf} (Supplementary material S2) were selected from BC_7 populations and back-crossed with male parent M95 to create the BC_8 population. The phenotypic analysis of the three BC_8 populations was carried out in the winter of 2012 in Hainan, China. Five BC_8 plants (NS7-1, NS7-20, NS7-24, NS16-13 and NS17-23) showing high R_{female} and N_{cf} (Supplementary material S2) were self-crossed, producing a total of 496 BC_8S_1 plants, which were subsequently grown in the spring of 2013 in the experimental fields in Beijing, China. Based on genotypic analysis, the 496 BC_8S_1 plants were divided into four groups: homozygous dominant plants, homozygous recessive plants, heterozygous plants and recombinants. The difference in R_{female} among these four groups was used to verify the inheritance pattern of the subgynoecious trait. Genotypic and phenotypic investigation was further carried out for recombinants using PCR-based markers to narrow down the genomic region underlying the major QTL. All the primers for dCAPS and InDel markers used for geno-typing are presented in Supplementary material S4.

Results

Inheritance of the subgynoecious trait in S-2-98

To determine the inheritance pattern of the subgynoecious trait of S-2-98, S-2-98 was crossed to the monoecious parent M95. In the resulting F_1, F_2 and BC_1 populations, the phenotype was scored on the basis of the R_{female}, which corresponds to the percentage of female flowers on the main stem. The R_{female} reached 87 % for maternal parent S-2-98 and 20 % for M95. All F_1 individuals produced sequential female flowers at the later growth stage, similar to maternal parent S-2-98. The average R_{female} of the F_1 population was 71 %, which suggests that the subgynoecious trait of S-2-98 is (semi-) dominant to the monoecious trait of M95. In the BC_1 and F_2 populations the R_{female} displayed a continuous variation, ranging from 7 to 100 % in the BC_1 population and from 3 to 100 % in the F_2 population (Fig. 1), indicative of the polygenic nature of the subgynoecious trait.

Identification of three QTLs through SSR analysis

In order to identify loci underlying the subgynoecious trait of S-2-98, we conducted SSR mapping on the BC_1 population. Among 2112 pairs of SSR markers that were screened, 440 pairs were polymorphic between both parents. Of these 440, 39 were polymorphic between the M pool and the S pool (Supplementary material S1). These 39 markers were subsequently applied to the BC_1 population to map QTLs underlying the subgynoecious trait. Three

Fig. 1 Distribution of R_{female} of BC$_1$ and F$_2$ populations. *Arrows* point the R_{female} of S-2-98 and M95
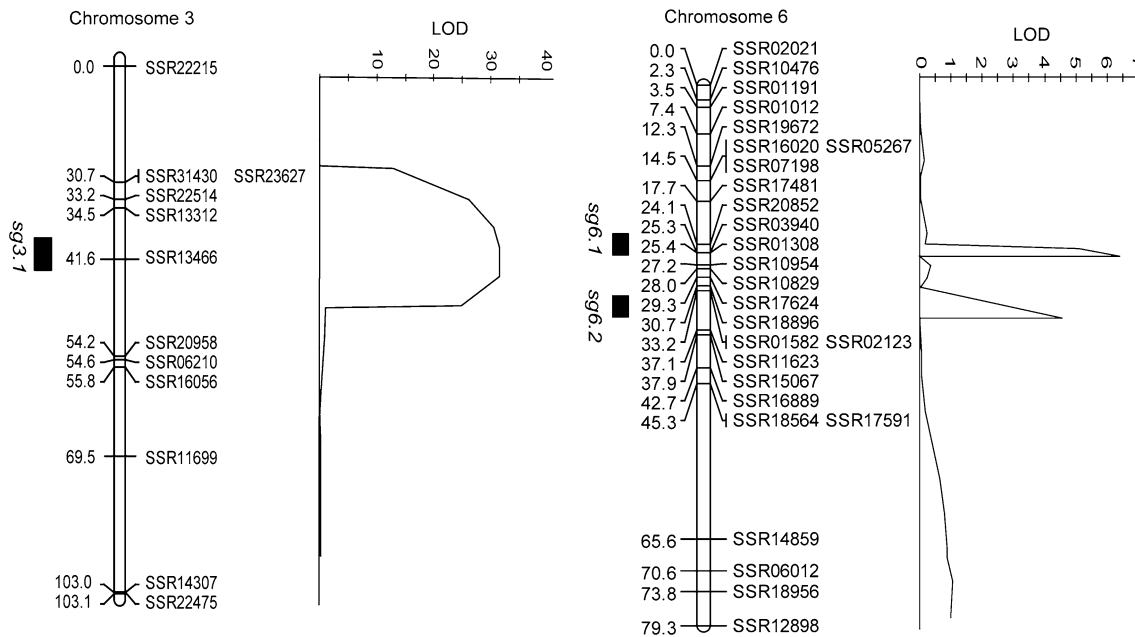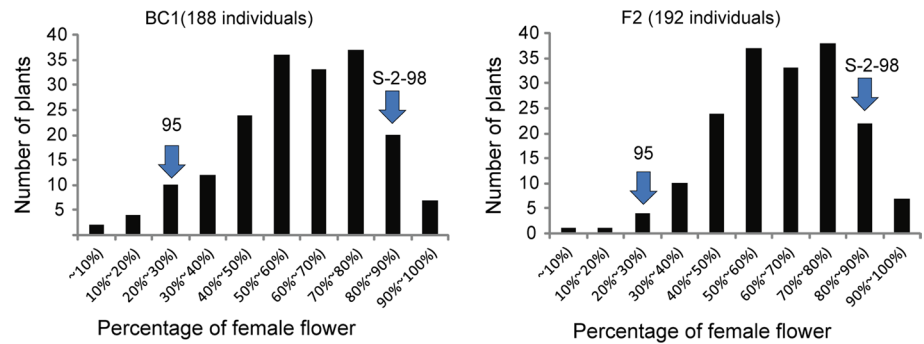
Fig. 2 Identification of three QTLs (*sg3.1*, *g6.1*, and s*g6.2*) by SSR analysis in BC$_1$ population

Table 1 QTL analysis of subgynoecious trait in BC$_1$ population

| QTL	LOD	Phenotypic variation (%)	Additive effect	Tigthly linked SSR marker	Position of SSR marker (bp)[a]
sg3.1	35.53	54.6	0.30	SSR13466	12,529,771
sg6.1	6.13	6.3	0.10	SSR01308	8,191,323
sg6.2	4.21	4.2	−0.12	SSR02123	20,793,026

[a] Position is presented based on the cucumber inbred line 9930 draft genome (Huang et al. 2009)

QTLs were identified; *sg3.1* located on chromosome 3, which is tightly linked to SSR13466, and *sg6.1* and *sg6.2* both located on chromosome 6, which are tightly linked to SSR01308 and SSR02123, respectively (Fig. 2; Table 1). These three QTLs together explained 62.9 % of the phenotypic variation, of which 54.6 % was explained by *sg3.1* alone (Table 1). These results indicated that the subgynoecious trait of S-2-98 was controlled by a major QTL (*sg3.1*) and two minor QTLs.

QTL-seq identified four QTLs, including a new QTL

To refine the regions harbouring the QTLs identified through SSR mapping, QTL-seq was conducted to compare the SNP profiles of both parental lines and the S$_{BC6}$ pool on a genome-wide scale. The S$_{BC6}$ pool consisted of seven plants (from BC$_6$-1 to BC$_6$-7) selected from the BC$_6$ population on the basis of an R_{female} ranging from 50 to 81 % and an N_{cf} ranging from 10 to 17 (Supplementary

Fig. 3 Identification of four QTLs by QTL-seq in BC_6 population. X-axis represents the position of seven chromosomes and Y-axis represents the SNP-index. The grey line represents the lowest SNP-index value within statistical confidence intervals under the null hypothesis of no QTL ($P < 0.05$)
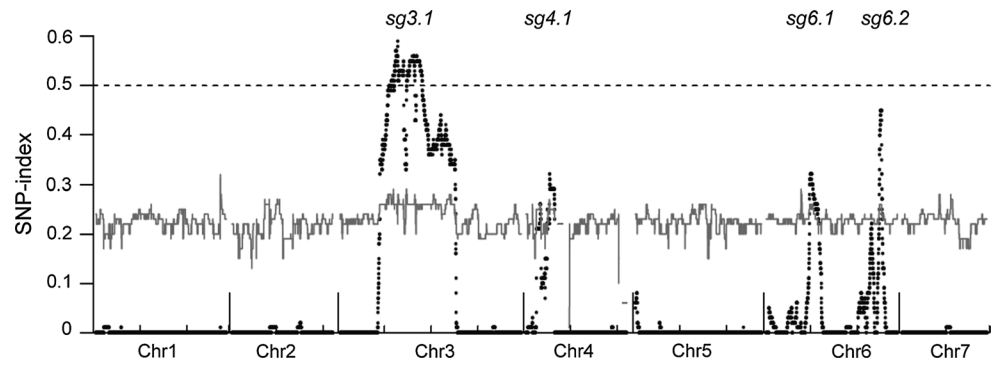
material S2). The SNP-index plot of the S_{BC6} pool revealed four regions harbouring QTLs on chromosomes 3, 4 and 6 (Fig. 3). One region corresponding to the major QTL *sg3.1* was located at the interval between 8.4 and 24.7 Mb on chromosome 3. Two other regions corresponding to the two minor QTLs *sg6.1* and *sg6.2* were located at the intervals of 9.2–11.3 Mb and 24.2–24.8 Mb on chromosome 6, respectively. The fourth region, referred to *sg4.1*, was situated at the interval between 2.9 and 6.1 Mb on chromosome 4. QTL *sg4.1* was not identified through SSR mapping probably owing to the shortage of polymorphic SSR markers on chromosome 4 between the parental lines (Supplementary material S3). SSR markers were populated at a density of 13 per Mb on chromosome 4, whereas across other chromosomes the density ranged from 16 to 34 per Mb. Compared with chromosomes 3, 4 and 6, the SNP-index of chromosomes 1, 2, 5 and 7 nearly approached 0, indicating that these chromosomes were rich in alleles from the paternal parent M95.

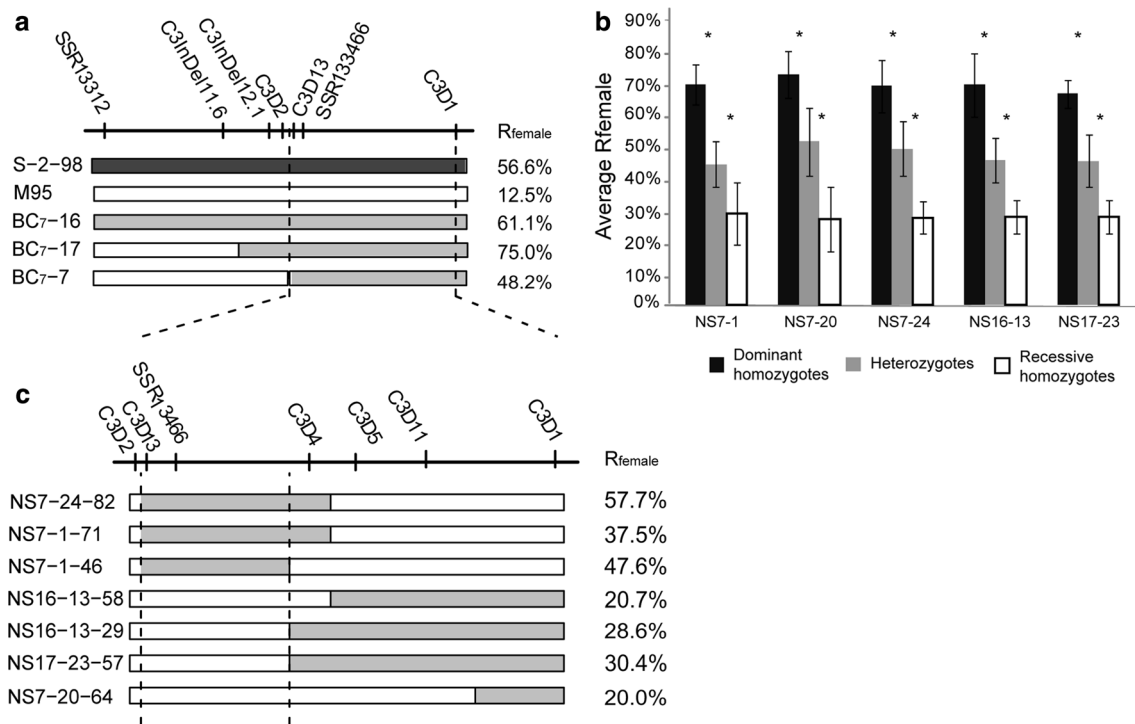### Refinement of the major QTL *sg3.1* to a 799-kb interval

To speed up the application of the subgynoecious trait in breeding as well as fine mapping of the major QTL, we isolated plants harbouring only the major QTL *sg3.1* using marker-assisted selection. In the BC_7 population, three individuals (BC_7-7, BC_7-16 and BC_7-17) harbouring the major QTL *sg3.1* (Fig. 4a) were used to create advanced lines by one round of backcrossing to M95, followed by one round of selfing. The resulting BC_8S_1 families (NS7-1, NS7-20, NS7-24, NS16-13 and NS17-23) consisted of 496 individuals (Fig. 4b). These individuals were grouped into four types according to genotype: homozygous dominant, homozygous recessive, hetereozygous and recombinants. The average R_{female} was 68.9 % for homozygous dominant plants and 46.9 % for heterozygous plants, both of which were significantly higher than the average R_{female} (27.6 %) for homozygous recessive plants (Fig. 4b). This evidence further supported that the subgynoecious trait of S-2-98 was (semi-) dominant and mainly controlled by *sg3.1*.

Selfing was performed for homozygous dominant plants to create near isogenic lines aiming at high yield.

To narrow down the major QTL, recombinants from BC_7 and BC_8S_1 populations were analysed using dCAPs markers. BC_7-17 and BC_7-7 had high R_{female} (75.0 and 48.2 %, respectively) indicating the presence of the S-2-98 allele, which pinpointed *sg3.1* to the heterozygous genomic region on the right side of marker C3D2. Recombinant NS7-20-64 showed low R_{female} (20.0 %) and was homozygous recessive at the left side of marker C3D1. This, together with the data from BC_7-17 and BC_7-7, delimits *sg3.1* to the interval between C3D2 and C3D1. Recombinants NS7-24-82 and NS7-1-71 showed a moderately high R_{female} (57.7 % and 37.5 %, respectively) indicating the presence of the S-2-98 allele, which pinpointed *sg3.1* to the left side of marker C3D5. This delimited interval is supported by the data from NS16-13-58, which showed low R_{female} (20.7 %) and was homozygous recessive at the left side of marker C3D5. NS7-1-46 depicted a moderately high R_{female} (47.6 %) indicating the presence of the S-2-98 allele, which pinpointed *sg3.1* to the left side of marker C3D4. This delimited region was supported by the data from NS16-13-29 and NS17-23-57, which showed low R_{female} (28.6 and 30.4 %, respectively) and were homozygous recessive at the left side of C3D4. Altogether, combination of the genotypic and phenotypic data from these recombinants delimited *sg3.1* to a 799-kb genomic region between markers C3D2 and C3D4. (Fig. 4c; Supplementary material S4).

Discussion

The resource-limited cultivation practice in China impedes the expansion of gynoecious cucumbers. Subgynoecious cucumbers bearing a higher percentage of female flowers can enhance fruit production at low cost. Therefore, it is important to investigate the genetic basis of subgynoecious traits and create desirable breeding materials. In this study, we conducted genetic analysis of the subgynoecious trait of S-2-98 and identified four QTLs underlying this trait.

Fig. 4 Refinement of the major QTL *sg3.1*. **a** Three BC$_7$ individuals harbouring the major QTL *sg3.1*. **b** The average R_{female} of dominant homozygous, heterozygous and recessive homozygous plants of five BC$_8$S$_1$ families for recombinant screening. *Asterisks* represent significant difference between average R_{female} of plants with different genotypes ($P < 0.05$). **c** The major QTL *sg3.1* was narrowed down to a 799-kb interval between dCAPs markers C3D2 and C3D4. R_{female} higher than 30 % was a criterion to assess whether the region harboured *sg3.1*. The *grey region* carries heterozygous alleles. The *white region* carries recessive homozygous alleles as M95. The joints of *grey* and *white* regions mean that a crossover occurs somewhere between the neighbouring markers

Furthermore, we applied marker-assisted selection and created subgynoecious near isogenic lines of the major QTL *sg3.1*.

Four subgynoecious QTLs were identified in this study, and the major QTL *sg3.1* explaining 54.6 % of the phenotypic variation was further delimited to a 799-kb genomic region on chromosome 3. Fazio et al. (2003) reported three QTLs (*sex1.1*, *sex1.2* and *sex6.1*) associated with the number of female nodes on the main stem. The peak marker for *sex6.1* was positioned at 63.8 cM on chromosome 6, adjacent to the marker SSR02123 tightly linked to *sg6.2* identified in this study. Yuan et al. (2008) identified three QTLs for female flower ratio, two located on chromosome 2 and one located on chromosome 6 distant from either *sex6.1* or *sg6.2*. The major QTL on chromosome 2 contributed to over 40 % of the phenotypic variation, and has not been fine-mapped. Of note, the major QTL on chromosome 2 was not found in Fazio et al. (2003) or our mapping results, likely because cucumber materials were derived from different backgrounds.

A total of 110 putative genes were predicted in the 799-kb region underlying QTL *sg3.1*. Genes *Csa3g180260* and *Csa3g180265* both contain one copy of AP2/ERF domain found in members of the AP2/ERF family. In Arabidopsis, the AP2/ERF gene family is divided into three groups (AP2, ERF and RAV) based on sequence similarity and the number of AP2/ERF domains (Sakuma 2002; Nakano et al. 2006). The AP2 class, containing two copies of the AP2 domain, is required for regulating temporal and spatial expression of flower homeotic genes (Drews et al. 1991; Jofuku et al. 1994). Genes belonging to the other two groups contain only one copy of the AP2 domain. They function in biotic or abiotic stress signalling by regulating the expression of downstream ethylene responsive genes by binding to the GCC box found in ethylene response elements (Lee et al. 2015; Okamuro et al. 1997). Ethylene is a major regulator of sex determination in cucumber. *Csa3g180260* and *Csa3g180265* might be involved in regulating sex determination of S-2-98 via interacting with ethylene-responsive genes. Another gene, *Csa3g179160* is a potential homolog of an Arabidopsis *SOC1*-like gene, *AGL42*. *SOC1* is a classic MIKC-type MADS box gene, which plays an important role in the regulation of flower induction and development by integrating multiple flowering pathways (Lee and Lee 2010). *AGL42* is involved in floral transition and acts through a gibberellin-dependent

pathway (Dorca-Fornell et al. 2011). To examine whether these genes were associated with the subgynoecious trait, we performed an association study using 115 cucumber accessions (containing 9 gynoecious and 106 monoecious accessions) focusing on the genomic interval of *sg3.1*. However, no significant associations were found in this region (data not shown). This might be explained by the absence of subgynoecious accessions with the same feature as S-2-98 among the 115 accessions used in this analysis. To elucidate the genetic basis of the subgynoecious trait, further effort is needed to map the major QTL *sg3.1* at high resolution and functionally characterize candidate genes.

Author contribution statement The experiment was conceived by SH. FB and QS performed molecular experiments. HC performed the phenotypic assay. QZ and ZZ conducted the bioinformatics analysis. DG, FB and SH wrote the paper.

Compliance with ethical guidelines

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

Ethical standards The authors declare that all experiments described in this manuscript comply with the current laws in China.

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