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Map-based cloning of a recessive gene v_1 for virescent leaf expression in cotton (*Gossypium spp.*)

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Abstract

Background: Virescence, as a recognizable phenotype in the early development stage of cotton, is not only available for research on chloroplast development and photosynthesis but also for heterosis exploitation in cotton.

Methods: In current study, for fine mapping of virescent-1 (v_1) in cotton, three populations with a total of 5 678 individuals were constructed using T582 which has the virescent trait. Tobacco rattle virus, TRV1 and TRV2 (pYL156), were used as vectors for the virus-induced gene silencing (VIGS) assay.

Results: The v_1 gene was fine-mapped to a 20 kb interval on chromosome 20 of tetraploid cotton. We identified only one candidate gene with four single nucleotide polymorphisms between parents, among which the single nucleotide polymorphism at the position of 1 082 base pair caused the change of amino acid residue from Arg (3–79) to Lys (T582). The relative expression of the candidate gene in virescent plants was extensively lower than that in normal plants. Nullification of the gene by VIGS significantly turned the green leaf of normal cotton plants into yellow. We named this candidate gene as *GhRVL*.

Conclusions: This study will facilitate the further research on virescent formation, and will be useful for breeding of hybrid cottons.

Keywords: Cotton, Virescence, T582, VIGS

Background

The phenotype of the virescent mutant is characterized by yellowish leaves at the early stage, which gradually become normal green leaves at maturity. Virescent mutants have been found in an extensive list of plant species including cotton (Killough and Horlacher 1933), tomato (Richard and Charles 1954), cucumber (Aalders 1959), barley (Jain 1966), peanut (Benedict and Ketring 1972), soybean (Palmer and Mascia 1980), maize (Hopkins and Elfman 1984), tobacco (Archer and Bonnett 1987), rice (Iba et al. 1991), *Arabidopsis* (López-Juez et al. 1998), rape (Zhao et al. 2000) and sugi (Hirao et al. 2009). Many previous researches explored the mechanism of chloroplast development and photosynthesis in virescent mutants (Iba et al. 1991; López-Juez et al. 1998; Benedict and Kohel 1968; Fambrini et al. 2004; Wang et al. 2016a, 2016b), which helped to elucidate the mechanism of chlorophyll (Chl) synthesis and degradation, and to explore the interaction of genes in nucleus and chloroplast (Karaca et al. 2004; Sugimoto et al. 2004) for the complex expression patterns such as temperature-induced virescent mutant (Turcotte and Feaster 1978) and transient or long-lasting virescent mutant (Percival and Kohel 1974; Endrizzi et al. 1984). Further, many other studies on chlorophyll biosynthetic process in *Arabidopsis* and rice provided a critical understanding for virescent variations (Koncz et al. 1990; Falbel and Staehelin 1994; Nakayama et al. 1995; Kruse et al. 1997; Zhang et al. 2006).

The whole pathway of Chl biosynthesis is consisted of 27 genes that encode 15 enzymes from glutamyl-tRNA to Chl b in *Arabidopsis* (Zhu et al. 2014; Wang et al. 2016a, 2016b). In Chl biosynthesis, the initial step is the insertion of Mg^{2+} into protoporphyrin IX. Same as

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in *Arabidopsis*, the mutant *cs* directly involved in Chl biosynthesis by motivating the ATP-dependent binding of Mg^{2+} into protoporphyrin IX (Rissler et al. 2002; Ikegami et al. 2007), exhibiting the yellowish leaf phenotype (Koncz et al. 1990; Kobayashi et al. 2008). While in rice, an oxygenase mutant gene *ygl1* (*osCAO*) led to yellow-green leaves and delayed chloroplast development at seedling stage (Wu et al. 2007). On the other hand, a negative correlation has been found between protoporphyrin and heme biosynthesis in tetrapyrrole biosynthesis, suggesting that excess of heme will cause inhibition of chlorophyll synthesis which resulted in leaf color mutant with lack of chlorophyll (Cao et al. 2009). For instance, elevated accumulation of the *OsHO2* (Heme oxygenase) into Mg-protoporphyrin IX in rice caused the leaf color mutant (Li et al. 2014b).

The virescent trait with a photoperiod-sensitive genetic male sterility in cotton can reduce the cost of hybrid breeding and promote the exploitation of heterosis to overcome the limitations of seed production in the 3-line system (Zhao et al. 2000; Duncan and Pate 1967; Ma et al. 2013). To date, out of twenty virescent genes, seven single recessive nuclear genes from more than 30 mutants of the tetraploid cotton have been mapped in genetic linkage groups (Endrizzi et al. 1984). Previously, Killough and Horlache found that a virescent mutant plant in Mebane varieties of upland cotton controlled by a recessive single nucleus gene v_1 which has been transferred into T582 (Kohel et al. 1965) and mapped into XVII linkage goup (Kohel 1983). Subsequent genetic mapping of F_2 population (T582 × Hai7124) showed that the v_1 gene resided on chromosome 20 of D subgenome with the closest genetic distance of 10.3 cM to CIR094 (Hu and Zhou 2006). Therefore, current study was conducted to clone the v_1 gene with molecular markers which developed from the cotton genome sequence using map-based techniques (Wang et al. 2012; Li et al. 2014a; Li et al. 2015; Lu et al. 2015).

Methods

Plant materials and phenotypic data collection

T582 is a multi-recessive marker stocking with *cu*, *fg*, cl_1, gl_1, and v_1 genes, while 3–79 and Pima 90 are sea island cottons with non-virescent trait. Chi-square tests were performed on three populations, including (i) population I of T582 × (T582 × 3–79) BC_1F_1, (ii) population II consisting of (3–79 × T582) × T582 BC_1F_1, and (iii) population III having BC_1F_1 of (Pima 90 × T582) × T582. A total of 1 200 plants from the population I, 2 193 plants from the population II, and 2 285 plants from the population III were screened for virescent to non-virescent ratio of 1:1, respectively.

DNA extraction and polymerase chain reaction (PCR) analysis

The genomic DNA of fresh leaf tissues from all populations and their parents was extracted following cetyl trimethyl ammonium bromide (CTAB) method (Paterson et al. 1993). PCR was conducted with a total volume of 10 μL consisting of 1 μL 10 × PCR buffer (TransGen Biotech Co., Ltd., Beijing), 0.5 μL 2.5 $mmol·L^{-1}$ dNTPs (TransGen Biotech Co., Ltd., Beijing), 0.2 μL each 10 $μmol·L^{-1}$ primer (GenScript Co.,Ltd., Nanjing), 30 ng template DNA and 0.1 μL 5 $U·μL^{-1}$ Taq DNA polymerase (TransGen Biotech Co., Ltd., Beijing). The PCR amplification was conducted as follows: 3 min at 94 °C; followed by 27 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. The final extension was done at 72 °C for 5 min. The amplified PCR products were then separated by the 8.0% polyacrylamide gels.

Development of molecular markers for fine mapping

Based on the closest marker (CIR094) to v_1 (Hu and Zhou 2006), we used the SciRoKo34 software to get further SSR(Simple sequence repeats) primers (Kofler et al. 2007). When no polymorphic SSR was found in a fine mapping interval, we explored arbitrary sequence from every 500 base pairs (bp) of the interval through the Primer 5.0 software. To analyze the polymorphism and linkage map of all markers, we used parental plants and six of each virescent and non-virescent individuals from the population I.

Fine mapping of the v_1 gene

We used 1 200 plants (581 virescent and 619 non-virescent) of the population I for the initial mapping by SSR markers. Joinmap 3.0 software was used to analyze linkage between v_1 gene and molecular markers (Van Ooijen and Voorrips 2001). Subsequently, we also used 1 095 virescent plants and 1 098 non-virescent plants from the population II and 1 106 virescent plants and 1 179 non-virescent plants from the population III for the fine mapping of v_1.

Identification and sequence analysis of the v_1 candidate genes

We obtained the sequence of the predicted gene from the Cotton Genome Project database (http://cgp.genomics.org.cn/page/species/index.jsp) and the *G. hirsutum* genome database (http://mascotton.njau.edu.cn/info/1054/1118.htm), and further confirmed by BLAST(Basic local alignment search tool) searches using the EST(Expressed sequence tag) database. The full-length v_1 candidate gene in virescent and non-virescent plants was amplified by the 5′-ATGGCTTCC GTGCTTGGAACCTCAA-3′, 5′-TCAGCTGAAAACCT CATAGAATTTC-3′ primer pair. PCR assays were conducted to amplify the region which was subsequently cloned by One Step Cloning Kit (Vazyme, Nanjing) into PBI121 vector (TaKaRa, Dalian), and sequenced by the Genewiz

(Beijing, China). Sequence alignments were performed with NCBI-BLAST and sequences were analyzed using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

Quantitative reverse transcription-PCR (qRT-PCR) analysis

Total RNA was isolated using EASY spin Plant RNA Kit (Aidlab, Beijing) from fresh leaves of the 3–79 and T582, respectively. Afterwards, PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Dalian) was used to synthesize the first-strand cDNA. qRT-PCR was carried out in a total volume of 20 μL containing 10 μL SYBR® Premix Ex Taq II (2×), 2 μL cDNA, 0.4 μL ROX Reference Dye II (50×), 0.8 μL of 10 $\mu mol{\cdot}L^{-1}$ forward and reverse primers each and 6 μL ddH_2O. PCR was conducted in ABI PRISM@7500-Fast Real-Time PCR system under the following conditions: 30 s at 95 °C; 40 cycles of 5 s at 95 °C and 30 s at 60 °C; followed by 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C (Cheng et al. 2016). qRT-PCR was carried out by the gene-specific primers (5′-ATTGCCACTGTCATCCCCAACTGCT-3′, 5′-TCAGCTGAAAACCTCATAGAATTTC-3′) and *actin* (Genbank ID: AY305733) (5′-ATCCTCCGTCTTGACCTTG-3′, 5′-TGTCCGTCAGGCAACTCAT-3′) was employed as an internal control. Last, relative gene expression was quantified using the $2^{-\Delta\Delta}C_T$method.

VIGS (virus-induced gene silencing) assay

Tobacco rattle virus, TRV1 and TRV2 (pYL156), were used as vectors for the VIGS (Virus-induced gene silencing) assay. The cotton phytoene desaturase (PDS) was used to check the efficiency of VIGS (Tuttle et al. 2008; Pang et al. 2013), since the silencing of PDS gene caused the loss of carotenoids and chlorophyll which resulting in white leaves. The pYL156 was employed as the negative control. A 250 bp fragment of the *GhRVL* gene was amplified from cDNA library of CCRI (China Cotton Research Institute, former name of ICR, CAAS) 12-Dgl leaf tissues by PCR using the primer pair 5′-CTCACATCCTGCTCGGTTTATTCTC-3′, 5′-AGAAGAAAGAGAACTCCTAGCTGAA-3′, and inserted into the vector pYL156 by *Bam*HI and *Kpn*I double digestion to construct pYL156-RVL. Four vectors were transformed into GV3101 strain of *Agrobacterium tumefaciens* by freeze-thaw method. The *Agrobacterium* cultures were grown overnight in LB medium having 25 $\mu g{\cdot}mL^{-1}$ rifampicin, 50 $\mu g{\cdot}mL^{-1}$ kanamycin and gentamycin at 28 °C. The bacteria were harvested at 4 000 $r{\cdot}min^{-1}$ for 5 min and re-suspended in infiltration solution (LB medium having 10 $mmol{\cdot}L^{-1}$ $MgCl_2$, 10 $mmol{\cdot}L^{-1}$ MES and 200 $\mu mol{\cdot}L^{-1}$ acetosyringone). The three different bacterial cultures of pYL156-RVL, pYL156-PDS and pYL156, respectively, mixed with pTRV1 at 1:1 ratio after staying at 25 °C for 4 h. The seedling stage of CCRI 12-Dgl was used for VIGS and the procedures for the infiltration infection were carried out according to the previous description (Gao and Shan 2013). The phenotype of the infiltrated plants was examined 1 week later. Total RNA was isolated from the true leaves of silenced and controlled plants, respectively. qRT-PCR was performed to confirm the silencing of the *GhRVL* gene in the VIGS plants.

Results

Genetic analysis of virescent traits

The leaves of T582 are virescent at early stage (Fig. 1a), while 3–79 and Pima 90 have green leaves during the entire growth season (Fig. 1b). Among three backcross populations constructing for the fine mapping of the v_1 gene, 581 virescent plants and 619 non-virescent plants derived from the population I (T582 × (T582 × 3–79) BC_1F_1) fit at 1:1 ratio ($\chi^2 = 1.2033$, $P > 0.05$). Similarly, 1:1 ratio ($\chi^2 = 0.0041$, $P > 0.05$) has been found between the 1 095 virescent plants and 1 098 non-virescent plants

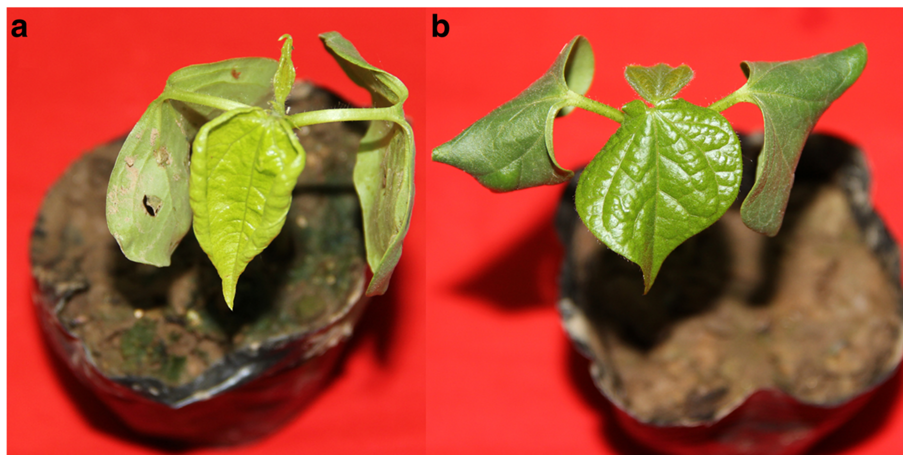

Fig. 1 Comparison of the virescent and non-virescent leaves. **a** Virescent leaf. **b** Non-virescent leaf

of population II (BC_1F_1 segregating plants of (3–79 × T582) × T582). And, the ratio of 1 106 virescent plants to 1 179 non-virescent plants from population III which derived from the BC_1F_1 of (Pima90 × T582) × T582 was 1:1 ($\chi^2 = 2.3322$, $P > 0.05$). These results showed that a recessive nuclear gene v_1 controlled the virescent phenotype in T582, which is also consistent with previous studies (Killough and Horlacher 1933; Kohel et al. 1965).

Primary mapping of the v_1 gene

Previous study showed that CIR094 was identified near the v_1 gene with 10.3 cM distance on chromosome 20 of tetraploid cotton (Hu and Zhou 2006). A linkage marker Dt_chr11–5923 (VS12) was obtained by genome-wide molecular markers (Lu et al. 2015) which is closer to the v_1 gene than BNL2570 and MUSS143 analyzed by 1 200 individuals of population I. There was approximately 5 Mb distance between CIR094 and VS12 markers, which was confirmed by BLAST search in the whole genome sequence of *G. hirsutum* and *G. raimondii* (Wang et al. 2012; Li et al. 2015). Fifteen primer pairs of polymorphic SSR markers which designed by the SciRoKo 34 software were screened to the 5 Mb intervals from *G. hirsutum* genome database. With the linkage analysis by JoinMap 3.0, v_1 was initially mapped to a 275 kb region between VS2 and VS3 (Fig. 2a), which was further lessened to a 100 kb region between VS13 and VS14 (Fig. 2b) with the assistance of virescent and non-virescent plants of the population I.

Fine mapping of the v_1 gene

As no polymorphic SSR is available for the 100 kb interval, the polymorphism of 96 SSR markers from every 500 bp of the 100 kb interval was screened by T582 and 3–79, and obtained five polymorphic arbitrary markers named VS17, VS18, VS19, VS20, and VS21. Then, 1 095 virescent plants and 1 098 non-virescent plants from the population II were used to further refine the range of the v_1 gene, which mapped it approximately 20 kb interval between VS18 and VS19 (Fig. 2c). Meantime, five

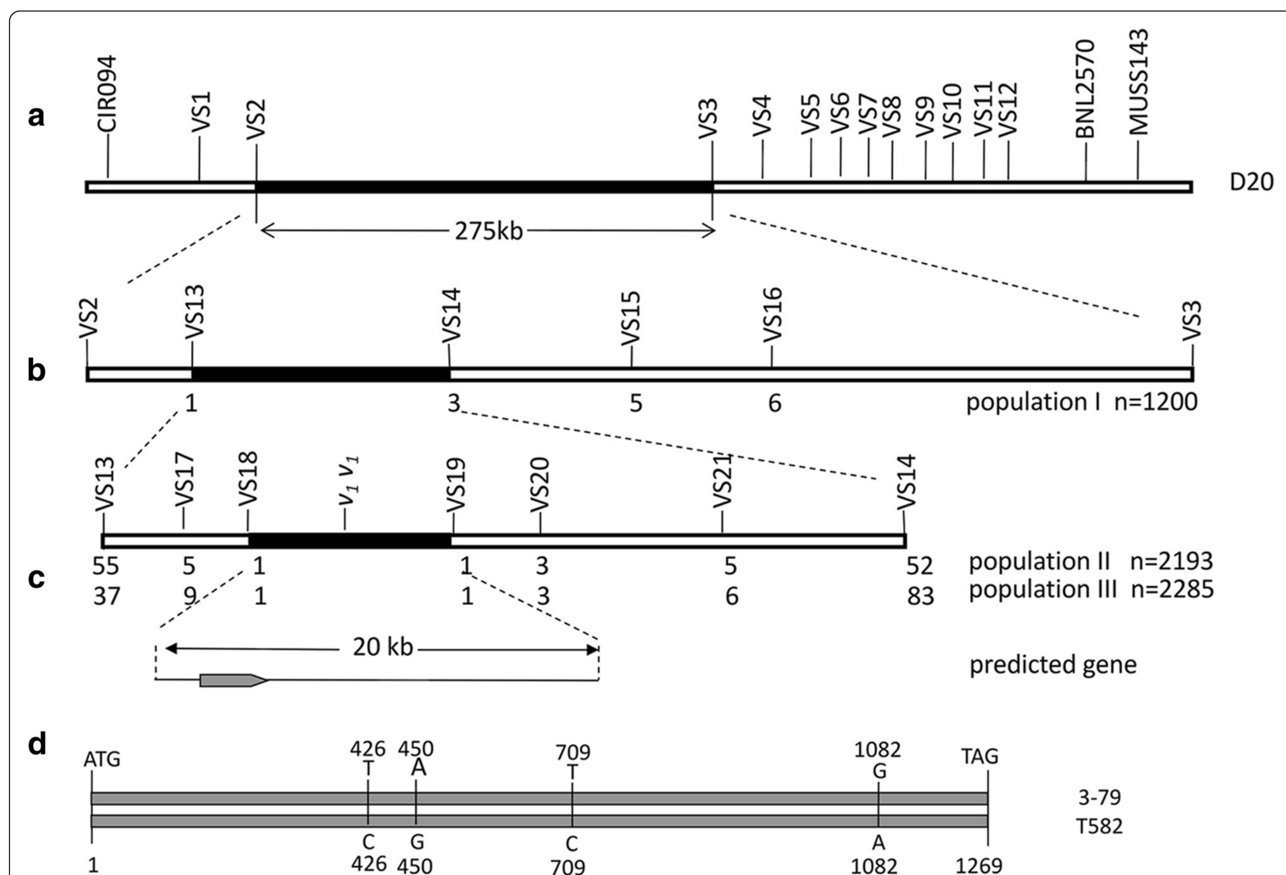

Fig. 2 Genetic and physical maps of the v_1 gene and sequence analysis of the candidate gene on chromosome 20 of the D subgenome in cotton. **a** Linkage map of the scaffold assembled using 1 200 selected individuals from population I. v_1 was mapped between VS2 and VS3 markers at around 275 kb. **b** Additional mapping of v_1. v_1 was further mapped to a 100 kb interval by VS13 and VS14 with the recombinants in population II. **c** Candidate region for v_1 and predicted gene. Candidate region for v_1 was identified between VS18 and VS19 at about 20 kb. One gene was found in that region. Numbers of recombinants between v_1 and markers were presented under the linkage map. **d** Sequence comparisons of the candidate gene in 3–79 and T582. SNPs at the sequence positions of 426, 450, 709 and 1 082 were shared by 3–79 and T582

polymorphic arbitrary markers were verified between the virescent and non-virescent plants of population III, which were consistent with the fine mapping results of population II (Fig. 2c). As no polymorphic SSR and recombinant individuals were found in all three mapping populations, it was concluded that v_1 gene was located in the 20 kb interval between VS18 and VS19. Information about all markers was listed in Table 1.

Identification and sequence analysis of the v_1 candidate gene

One candidate gene *Gh_D10G0283* which was identified in the 20 kb mapping interval based on genome annotation databases (http://cgp.genomics.org.cn/page/species/index.jsp and http://mascotton.njau.edu.cn//info/1054/1118.htm) and BLAST search result with EST and Unigene databases in NCBI. The length of open reading frame (ORF) was 1 269 bp encoding 422 amino acids (Fig. 3). The candidate gene was homologous to the magnesium chelatase I gene (ChlI, AAM98163) of *Arabidopsis*, which derived the ATP-dependent insertion of Mg^{2+} into protoporphyrin IX with yellowish leaf phenotype (Rissler et al. 2002; Ikegami et al. 2007). Sequence alignment between 3-79 and T582 showed 4 single nucleotide polymorphisms (SNPs) differences at 426, 450, 709, and 1 082 positions, respectively (Fig. 2d). According to protein sequence alignment, the SNP at position of 1 082 caused amino acid residue mutant from Arg (3–79) to Lys (T582), while the other SNPs had synonymous substitution. Furthermore, qRT-PCR showed that the relative expression of the candidate gene in T582 was significant lower than that of 3–79 (Fig. 4a), suggesting that the candidate gene may cause the formation of virescent leaf.

Silencing of the v_1 candidate gene leading to yellow leaf

Functional analysis of the v_1 gene was performed in CCRI 12-Dgl (Cheng et al. 2016) using VIGS to validate its role in the formation of virescent leaf in cotton. The 250 bp interference fragment was inserted into a TRV2 (tobacco rattle virus) vector to construct the VIGS vector. One week after the *Agrobacterium*-mediated infection, the mutant phenotypes of the VIGS-treated plants started to emerge. The plants injected with pYL156-PDS revealed a photo-bleaching of leaves (Fig. 5c), while yellow-green leaves of the plants infiltrated with pYL156-RVL were observed (Fig. 5b). Meanwhile, the

Table 1 List of polymorphic molecular markers for mapping of the recessive v_1 gene

Marker	Product size /bp	Forward primer (5′-3′)	Reverse primer (5′-3′)
CIR094	80	ATACCTCCTTTGGCATC	ATTCAGCAACTTCACACA
VS1	140	TCAATATTGGTGGGCTGAAA	GCCCATAGATTTGCCTTCAA
VS2	223	ATCTCGGCGGCCTATTAGTT	CCTAGGCTCCTCAGCTTCCT
VS3	193	CCTAATGTGGGTGGATTTGG	TCAACTCAACCGGACACAAC
VS4	222	GATGACGATGACGATGATGG	CGCTATTGAATGATGATGTGC
VS5	213	TCTTCATCAATTCTCCTCCTCC	CGTTATCAGTGGGTTCGAATG
VS6	220	TGGGTTCTTGAATCGTGTCAT	TACCCGAACCCTCCCATATT
VS7	174	CAGCGGTGGTGTAAAGATCA	TGAAGCACAAATGCCTCATC
VS8	248	GAACCCAAGAGACAACGTATCA	TTTGGAGGAAACAAGCCAAT
VS9	191	AAGAAATAGGCAGCGCAATG	CACGACTGCCACTTGAGAAA
VS10	250	TCCTTATCCAACACTCACCAAG	TGAGGCATGTCACTGATTCAA
VS11	242	TTAGAAGAATGGTTGAATTAAGCTC	AAATAAACTTGATCTCCATGTAACAAA
VS12	230	TAATTTGCTCAAATGCGCTC	ATTCTTTGGACTACAGCACCA
BNL2570	236	TTCTACAAAAAAAGAAAAAATGGG	AAATACGGATGGGACCAACC
Muss143	173	AGATAAAGCTCCCACTTCCTCC	CTTCAGGATCCTTCCAAGAGG
VS13	480	TGGCACAAGTGCTGACTGAT	TCAGACTCGAACCCGGAAAC
VS14	183	TGTATTCAAATGCACAGTCCAA	GGTTATGCTTGATGACATGGG
VS15	240	CCAAGTAATGGAGCACCAACT	AACGCCCTAACGATTATGTCA
VS16	136	AAATAAATTCGGATTGACTCACTTT	GCCGACAGAGTGTGGATCTT
VS17	417	CTGAGGTTGCACCGCATTTT	TTTAGCGGATGAAGGCGTTG
VS18	318	GCCCACACATGCATTTCACT	ACGGTAGGTCAACGAAGTAGC
VS19	493	TTGCTTGACTCAGCTCGACA	TTGCATGAGCTGCACTACCA
VS20	425	CAAACCATCGCTGCAGTTCC	ACTTTGCCAGCATGGGTACA
VS21	472	GCAATGCGATTTGGCTTCCT	ATCCAAACGCCGCTAAAAGC

ATGGCTTCCGTGCTTGGAACCTCAACCGCAATCTTAGCTTCTCGGTCCCTCGTTTCTCCTTCGTTAAAGCCTGCCTTTCCCTCTATCTCCATTAAC
CCAGGACAGGGTTATGGGAGGAAACTGTATGGAGGAATTGGGATTCAGGGAAGAAAGGGGAGGCCTCAATTTCATATTGCAGTAACCAAT
GTCGCCACTGAAATTAACTCTGTTGAACAGGCTCAGAAGGTTGCCGCTAAAGAAAGTCAAAGACCAGTATTTCCATTTGCTGCTATAGTAGGA
CAAGATGAGATGAAATTGTGTCTCCTGTTGAATGTGATTGATCCCAAGATTGGTGGTGTTATGATAATGGGTGATAGGGGAACTGGGAAGTCC
ACAACTGTTAGGTCCTTGGTTGATTTATTGCCTGAAATCAGGGTGGTCTTCGGCGACCCTTATAACTCGGACCCGGAGGATCCGGAATCAATG
GGCATAGAAGTTAGAGAAAAGGTTGTAAATGGAGAGGAACTGACTATTACAATGACTAAAATCAACATGGTGGATTTGCCATTAGGTGCTAC
TGAAGATAGGGTCTGTGGAACCATTGACATTGAGAAAGCCCTCACTGAGGGTGTCAAGGCATTTGAGCCTGGACTTCTAGCTAAAGCTAATC
GAGGGATTCTTTACGTCGATGAAGTTAACCTTTTAGATGACCATTTGGTGGATGTTCTTCTGGATTCCGCTGCATCAGGATGGAACACTGTTG
AGAGGGAAGGTATTTCGATCTCACATCCTGCTCGGTTTATTCTCATCGGCTCAGGTAATCCAGAAGAAGGAGAGCTTAGACCACAGCTTCTTG
ATCGATTCGGAATGCATGCTCAAGTCGGGACAGTGAGGGACGCTGAGCTTAGAGTGAAGATTGTGGAGGAAAGAGCACGGTTTGATAAAA
ACCCAAAAGAATTCCGTGATTCTTACAAGGCAGAGCAAGAGAAGCTCCAACAGCAGATTGCTTCAGCTAGGAGTTCTCTTTCTTCTGTTCAG
ATTGATCAAGACCTAAAGGTTAAAATATCAAAGGTTTGCGCTGAGTTGAATGTTGATGGATTGAAAGGAGATATTGTCACTAATAGAGCTGCA
AAAGCTCTTGCAGCTCTTAAAGGAAGAGATAAAGTCATTGCAGAAGATATTGCCACTGTCATCCCCAACTGCTTGAGACACCGTCTTCGCAAG
GATCCTTTGGAGTCTATCGACTCCGGTTTACTCGTTATCGAGAAATTCTATGAGGTTTTCAGCTGA

Fig. 3 The sequence of the v_1 candidate gene

plants infiltrated with pYL156 had no effect on non-virescent leaf (Fig. 5a). To check the silencing efficiency, RNA was extracted from leaves of VIGS plants for qRT-PCR. Expression of the candidate gene in the plants infected by pYL156-RVL which was reduced largely as comparing with the plants infected by pYL156 (Fig. 4b). Therefore, we concluded that silencing of the candidate v_1 gene caused yellow leaves, and the candidate gene was subsequently named as *GhRVL* (*Gossypium hirsutum* regulator of virescent leaf) gene.

Discussion

T582 serves as a basic tool for scientific study into the mechanism of cotton metabolism, inheritance and development due to its multiple recessive marker stocking with *cu*, *fg*, cl_1, gl_1, and v_1 (Kohel et al. 1965). The candidate gene of the pigment glands which related gene gl_1 would provide the prospects of fabricating gossypol-free cotton seeds (Cheng et al. 2016). In addition, many important genes were subdivided into different linkage groups through T582 (Percival and Kohel 1974; Endrizzi et al. 1984). Especially, seven of the 20 virescent genes were reported at one recessive locus in the tetraploid cotton species, which further have been mapped by linkage analysis (Duncan and Pate 1967; Endrizzi et al. 1984). Genes v_1 and v_7 were found to be homoeoallelic, which are functionally similar and located on homoeologous chromosomes (Turcotte and Feaste 1973). Therefore, cloning of gene v_1 is helpful to clone the other gene v_7 and to analyze their interaction in cotton.

Previous study reported that gene v_1 was located in the interval between CIR094 and BNL2570 markers on

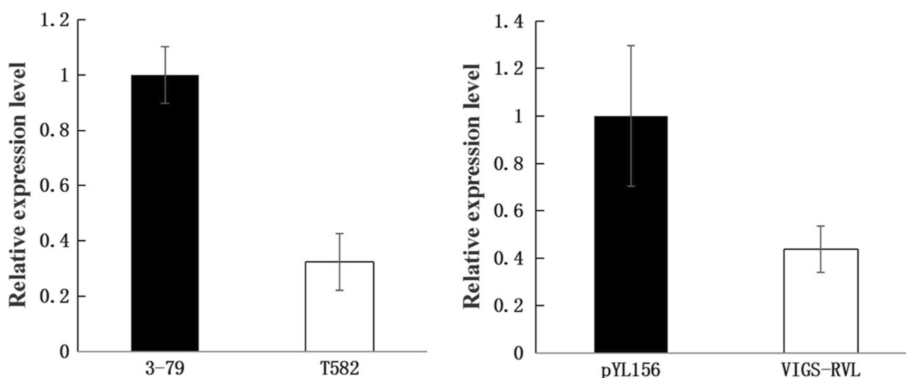

Fig. 4 Results of qRT-PCR. **a** Expression analysis of the candidate gene in 3–79 and T582, respectively. Y-axes indicates the relative expression level of the gene and X-axes represents the candidate gene in the 3–79 and T582, respectively. b: The results of the pYL156 and VIGS-RVL in CCRI 12-Dgl, respectively. Y-axes indicates the relative expression level of the gene and X-axes represents the pYL156 and VIGS-RVL in the CCRI 12-Dgl, respectively

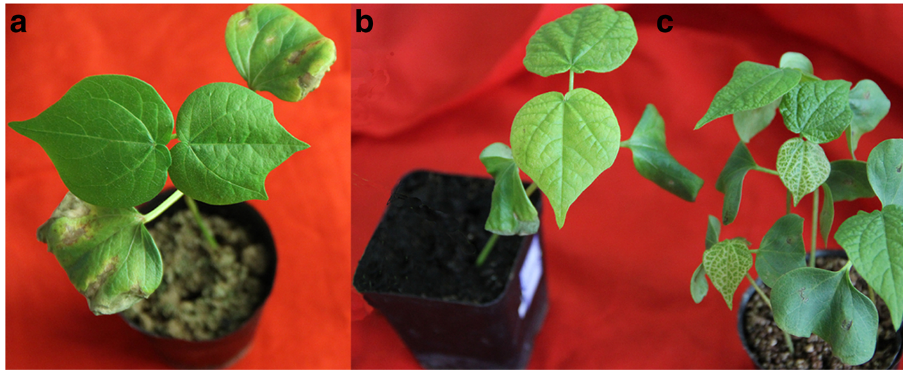

Fig. 5 Silencing of the v_1 candidate gene by VIGS resulted in CCRI 12-Dgl. pYL156 and pYL156-PDS were used as negative and positive controls, respectively. Leaves of VIGS plants displayed mutant phenotype. **a** Normal green phenotype in negative control plant; **b** yellowish phenotype in the plant infected by pYL156-RVL; **c** Photo-bleaching phenotype in positive control plant

chromosome 20 of the D sub-genome (Hu and Zhou 2006). In this study, we took advantage of the databases of cotton genome and genome-wide molecular markers (Wang et al. 2012; Li et al. 2014a, ; Li et al. 2015; Lu et al. 2015) to develop new SSR markers and arbitrary sequences for fine mapping of the v_1 gene. The polymorphism and recombination events were detected in three populations, which identified one candidate gene in a 20 kb interval between VS18 and VS19 markers. Results showed that v_1 is a single recessive gene in cotton which is homologous to the magnesium chelatase I (*ChlI*) gene, contains a P-loop NTPase domain and is a member of the AAA+ protein family (Fodje et al. 2001; Iyer et al. 2004). It plays an important role in chlorophyll biosynthesis by motivating the inclusion of Mg^{2+} into protoporphyrin IX. Chlorina mutant aci5–3 in *Arabidopsis* and one semi-dominant *Oil yellow 1* (*Oy1*) mutants in maize are caused by missense mutations in the highly conserved AAA+ domain of *ChlI* subunits (Soldatova et al. 2005; Sawers et al. 2006). However, *CHLI* is encoded by two genes in *Arabidopsis* compared with a single copy gene of barley and tobacco, which shows 82% similarity between *CHLI1* and *CHLI2* (Kobayashi et al. 2008). The expression level of *CHLI2* which contributes to the assembly of the Mg-chelatase complex is much lower than that of *CHLI1* (Kobayashi et al. 2008). But, a transgene of *CHLI2* motivated by the promoter of *CHLI1* can be functionally equivalent to *CHLI1* (Huang and Li 2009). In transgenic tobacco, either a decreased or increased expression of the *CHLI* subunit would diminish Mg chelatase activity and significantly reduce chlorophyll content (Papenbrock et al. 2000). In current study, the candidate gene *GhRVL* was homologous to the magnesium chelatase I gene (ChlI, AAM98163) in *Arabidopsis*. The candidate gene *GhRVL* just has single base change at 1 082 bp position which caused the change of the 361st amino acid residue from Arg (3–79) to Lys (T582). And the results of qRT-PCR showed that the relative expression level of GhRVL in virescent plants was much lower than that in non-virescent plants. We hypothesized that the different phenotype of the *virescent-1* mutant in T582 compared with normal plant in 3–79 is probably due to the promoter difference between them (Zhu et al. 2017; Mao et al. 2018).

Virescent character is a useful morphological indicator which is controlled by recessive genes (Benedict et al. 1972). However, more than 30 virescent mutants were not found their target genes in cotton (Song et al. 2012). The virescent gene v_1 can serve as a valuable resource for heterosis utilization (Duncan and Pate 1967; Ma et al. 2013), for exploring the mechanism of photosynthesis as well as for a better understanding of genetic interactions.

Conclusions

This report reveals fine mapping and cloning of the candidate gene *GhRVL* of virescence in cotton which significantly turned the green leaf color of normal cotton plants into yellow by VIGS.

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Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Authors' contributions

Song GL managed the project and designed the research. Zhang YP, Wang QL, Zuo DY, Cheng HL, Liu K, Ashraf J, Li SM, Feng XX, and Yu JZ performed the experiments and prepared figures and tables. Zhang YP, Song GL and Wang QL wrote and revised the paper. All authors reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
Aalders LE. 'Yellow cotyledon', a new cucumber mutation. Can J Genet Cytol. 1959;1(1):10–2.
Archer EK, Bonnett HT. Characterization of a virescent chloroplast mutant of tobacco. Plant Physiol. 1987;83(4):920–5.
Benedict CR, Ketring DL. Nuclear gene affecting greening in virescent peanut leaves. Plant Physiol. 1972;49(6):972–6.
Benedict CR, Kohel RJ. Characteristics of a virescent cotton mutant. Plant Physiol. 1968;43(10):1611–6.
Benedict CR, Mccree KJ, Kohel RJ. High photosynthetic rate of a chlorophyll mutant of cotton. Plant Physiol. 1972;49(6):968–71.
Cao YY, Duan H, Yang LN, et al. Effect of high temperature during heading and early grain filling on grain yield of indica rice cultivars differing in heat-tolerance and its physiological mechanism. Acta Agron Sin. 2009; 35(3):512–21.
Cheng HL, Lu CR, John ZY, et al. Fine mapping and candidate gene analysis of the dominant glandless gene *Gl2e* in cotton (*Gossypium* spp.). Theor Appl Genet. 2016;129(7):1347–55.
Duncan EN, Pate JB. Inheritance and use of golden crown virescence in cotton: and its relationship to other virescent stocks. J Hered. 1967;58(5):237–9.
Endrizzi JE, Turcotte EL, Kohel RJ. Qualitative genetics, cytology, and cytogenetics. Cotton. 1984;4:81–129.
Falbel TG, Staehelin LA. Characterization of a family of chlorophyll-deficient wheat (*Triticum*) and barley (*Hordeum vulgare*) mutants with defects in the magnesium-insertion step of chlorophyll biosynthesis. Plant Physiol. 1994; 104(2):639–48.
Fambrini M, Castagna A, Vecchia DF, et al. Characterization of a pigment-deficient mutant of sunflower (*Helianthus annuus* L.) with abnormal chloroplast biogenesis, reduced PSII activity and low endogenous level of abscisic acid. Plant Sci. 2004;167(1):79–89.
Fodje MN, Hansson A, Hansson M, et al. Interplay between an AAA module and an integrin I domain may regulate the function of magnesium chelatase1. J Mol Biol. 2001;311(1):111–22.
Gao X, Shan L. Functional genomic analysis of cotton genes with agrobacterium-mediated virus-induced gene silencing. Methods Mol Biol. 2013;975:157–65.
Hirao T, Watanabe A, Kurita M, et al. A frameshift mutation of the chloroplast *mat*K coding region is associated with chlorophyll deficiency in the *Cryptomeria japonica* virescent mutant *Wogon-Sugi*. Curr Genet. 2009;55(3): 311–21.
Hopkins WG, Elfman B. Temperature-induced chloroplast ribosome deficiency in virescent maize. J Hered. 1984;75(3):207–11.
Hu FP, Zhou ZH. Molecuar marker and genetic mapping of five mutant genes in upland cotton. Molecular Plant Breeding. 2006;4:680–4.
Huang YS, Li HM. Arabidopsis CHLI2 can substitute for CHLI1. Plant Physiol. 2009; 150(2):636–45.
Iba K, Takamiya K, Toh Y, et al. Formation of functionally active chloroplasts is determined at a limited stage of leaf development in virescent mutants of rice. Dev Genet. 1991;12(5):342–8.
Ikegami A, Yoshimura N, Motohashi K, et al. The CHLI1 subunit of *Arabidopsis thaliana* magnesium chelatase is a target protein of the chloroplast thioredoxin. J Biol Chem. 2007;282(27):19282–91.
Iyer LM, Leipe DD, Koonin EV, et al. Evolutionary history and higher order classification of AAA+ ATPases. J Struct Biol. 2004;146(1/2):11–31.
Jain ML. Biochemical definition of yellow-virescent and light-green suppressor mutations in barley. Genetics. 1966;54(3):813–8.
Karaca M, Saha S, Callahan FE, et al. Molecular and cytological characterization of a cytoplasmic-specific mutant in pima cotton (*Gossypium barbadense* L.). Euphytica. 2004;139(3):187–97.
Killough DT, Horlacher WR. The inheritance of virescent yellow and red plant characters in cotton. Genetics. 1933;18(4):329–34.
Kobayashi K, Mochizuki N, Yoshimura N, et al. Functional analysis of *Arabidopsis thaliana* isoforms of the mg-chelatase CHLI subunit. Photochem Photobiol Sci. 2008;7(10):1188–95.
Kofler R, Schlötterer C, Lelley T. SciRoKo: a new tool for whole genome microsatellite search and investigation. Bioinformatics. 2007;23(13):1683–5.
Kohel RJ. Genetic analyses of the yellow-veins mutant in cotton. Crop Sci. 1983; 23(2):291–3.
Kohel RJ, Lewis CF, Richmond TR. Linkage tests in upland cotton, *Gossypium hirsutum* L. Crop Sci. 1965;5(6):582–5.
Koncz CS, Mayerhofer R, Koncz KZ, et al. Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in *Arabidopsis thaliana*. EMBO J. 1990; 9(5):1337–46.
Kruse EL, Mock HP, Grimm B. Isolation and characterisation of tobacco (*Nicotiana tabacum*) cDNA clones encoding proteins involved in magnesium chelation into protoporphyrin IX. Plant Mol Biol. 1997;35(6):1053–6.
Li FG, Fan GY, Wang KB, et al. Genome sequence of the cultivated cotton *Gossypium arboreum*. Nat Genet. 2014a;46(6):567–72.
Li QZ, Zhu FY, Gao XL, et al. Young leaf chlorosis 2 encodes the stroma-localized heme oxygenase 2 which is required for normal tetrapyrrole biosynthesis in rice. Planta. 2014b;240(4):701–12.
Li FG, Fan GY, Lu CR, et al. Genome sequence of cultivated upland cotton (*Gossypium hirsutum* TM-1) provides insights into genome evolution. Nat Biotechnol. 2015;33(5):524–30.
López-Juez E, Jarvis RP, Takeuchi A, et al. New Arabidopsis *cue* mutants suggest a close connection between plastid and phytochrome regulation of nuclear gene expression. Plant Physiol. 1998;118(3):803–15.
Lu CR, Zou CS, Zhang YP, et al. Development of chromosome-specific markers with high polymorphism for allotetraploid cotton based on genome-wide characterization of simple sequence repeats in diploid cottons (*Gossypium arboreum* L. and *Gossypium raimondii* Ulbrich). BMC Genomics. 2015;16(1):55.
Ma JH, Wei HL, Liu J, et al. Selection and characterization of a novel photoperiod-sensitive male sterile line in upland cotton. J Integr Plant Biol. 2013;55(7):608–18.
Mao GZ, Ma Q, Wei HL, et al. Fine mapping and candidate gene analysis of the virescent gene v_1 in upland cotton (*Gossypium hirsutum*). Mol Gen Genomics. 2018;293(1):249–64.
Nakayama M, Masuda T, Sato N, et al. Cloning, subcellular localization and expression of CHLI, a subunit of magnesiumchelatase in soybean. Biochem Biophys Res Commun. 1995;215(1):422–8.
Palmer RG, Mascia PN. Genetics and ultrastructure of a cytoplasmically inherited yellow mutant in soybeans. Genetics. 1980;95(4):985–1000.
Pang JH, Zhu Y, Li Q, et al. Development of agrobacterium-mediated virus-induced gene silencing and performance evaluation of four marker genes in *Gossypium barbadense*. PLoS One. 2013;8(9):e73211.
Papenbrock J, Pfündel E, Mock HP, et al. Decreased and increased expression of the subunit CHL I diminishes mg chelatase activity and reduces chlorophyll synthesis in transgenic tobacco plant. Plant J. 2000;22(2):155–64.
Paterson AH, Brubaker CL, Wendel JF. A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. Plant Mol Biol Report. 1993;11(2):122–7.
Percival AE, Kohel RJ. Genetic analysis of virescent mutants in cotton. Crop Sci. 1974;14(3):439–40.
Richard WR, Charles MR. New tomato seedling characters and their linkage relationships. J Hered. 1954;45(5):241–8.
Rissler HM, Collakova E, DellaPenna D, et al. Chlorophyll biosynthesis. Expression of a second chl I gene of magnesium chelatase in Arabidopsis supports only limited chlorophyll synthesis. Plant Physiol. 2002;128(2):770–9.
Sawers RJH, Viney J, Farmer PR, et al. The maize *Oil yellow1* (*Oy1*) gene encodes the I subunit of magnesium chelatase. Plant Mol Biol. 2006;60(1):95–106.

Soldatova O, Apchelimov A, Radukina N, et al. An Arabidopsis mutant that is resistant to the protoporphyrinogen oxidase inhibitor acifluorfen shows regulatory changes in tetrapyrrole biosynthesis. Mol Gen Genomics. 2005; 273(4):311–8.
Song MZ, Yang ZG, Fan SL, et al. Cytological and genetic analysis of a virescent mutant in upland cotton (*Gossypium hirsutum* L.). Euphytica. 2012;187(2):235–45.
Sugimoto H, Kusumi K, Tozawa Y, et al. The virescent-2 mutation inhibits translation of plastid transcripts for the plastid genetic system at an early stage of chloroplast differentiation. Plant Cell Physiol. 2004;45(8):985–96.
Turcotte EL, Feaste CV. The interaction of two genes for yellow foliage in cotton. J Hered. 1973;64(4):231–2.
Turcotte EL, Feaster CV. Inheritance of three genes for plant color in American pima cotton. Crop Sci. 1978;18(1):149–50.
Tuttle JR, Idris AM, Brown JK, et al. Geminivirus-mediated gene silencing from cotton leaf crumple virus is enhanced by low temperature in cotton. Plant Physiol. 2008;148(1):41–50.
Van Ooijen JW, Voorrips RE. JoinMap® 3.0, software for the calculation of genetic linkage maps. Wageningen: Plant Research International. 2001. p. 1–51.
Wang KB, Wang ZW, Li FG, et al. The draft genome of a diploid cotton *Gossypium raimondii*. Nat Genet. 2012;44(10):1098–103.
Wang YK, He YJ, Yang M, et al. Fine mapping of a dominant gene conferring chlorophyll-deficiency in *Brassica napus*. Sci Rep. 2016a;6:31419.
Wang YL, Wang CM, Zheng M, et al. WHITE PANICLE1, a Val-tRNA synthetase regulating chloroplast ribosome biogenesis in rice, is essential for early chloroplast development. Plant Physiol. 2016b;170:2110–23.
Wu ZM, Zhang X, He B, et al. A chlorophyll-deficient rice mutant with impaired chlorophyllide esterification in chlorophyll biosynthesis. Plant Physiol. 2007; 145(1):29–40.
Zhang HT, Li JJ, Yoo JH, et al. Rice Chlorina-1 and Chlorina-9 encode ChlD and ChlI subunits of mg-chelatase, a key enzyme for chlorophyll synthesis and chloroplast development. Plant Mol Biol. 2006;62(3):325–37.
Zhao Y, Wang ML, Zhang YZ, et al. A chlorophyll-reduced seeding mutant in oilseed rape, *Brassica napus*, for utilization in F_1 hybrid production. Plant Breed. 2000;119(2):131–5.
Zhu LX, Zeng XH, Chen YL, et al. Genetic characterisation and fine mapping of a chlorophyll-deficient mutant. Mol Breed. 2014;34(2):603–14.
Zhu JK, Chen JD, Gao FK, et al. Rapid mapping and cloning of the virescent-1 gene in cotton by bulked segregant analysis–next generation sequencing and virus-induced gene silencing strategies. J Exp Bot. 2017;68(15):4125–35.

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