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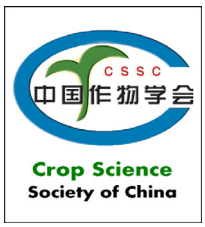

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Red fluorescent protein (DsRed2), an ideal reporter for cotton genetic transformation and molecular breeding

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ABSTRACT

Genes encoding reporter proteins are used as visual marker-assisted tools in genetic transformation as well as plant breeding. In this study, the red fluorescent protein identified in *Discosoma* sp. coral (DsRed2) was successfully used as a visual marker for cotton genetic engineering. DsRed2 was successfully expressed in two cotton cultivars, JIN668 and YZ1, driven by the CaMV-35S promoter via the *Agrobacterium*-mediated transformation. Our results suggest that *DsRed2* expression provides an early-stage selection tool for the transgenic calli via visual observation. Red fluorescence can be detected not only in callus and somatic embryos but also in most tissues and organs of mature plants. The transgenic line Yz-2-*DsRed2* was crossed with four different cotton cultivars to assess the transgene heritability and stability in different genetic backgrounds. The heritability of the red color was highly stable when Yz-2-*DsRed2* was used as a male parent. The *DsRed2* gene expressed 100% in the F_1 hybrids. To investigate the relationship between *DsRed2* transcription and DNA methylation, a methylation-specific PCR approach was applied to the CaMV-35S promoter region. The results showed a negative association between DNA methylation level in the promoter region and the transgene transcription. Taken together, these findings suggest *DsRed2* a visual reporter gene for cotton genetic transformation and molecular breeding programs.

1. Introduction

Cotton is a leading cash crop all over the world and one of major sources of natural fiber and oil worldwide. Development of stable cotton production is the main concern of cotton breeders. Conventional breeding depends only on characteristics that seem phenotypically more promising. By the selection of superior plants, desirable genes can be combined in one genotype. However, the narrow genetic base of cultivated cotton germplasm results in a genetic

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bottleneck and limited yield and quality [1]. Developing new varieties requires wide availability of germplasm. From the early history of plant breeding, visible traits have been used as morphological markers to select desired traits [2]. These markers reflect genetic polymorphisms of the individual that can be identified and manipulated [3]. However, genetic improvement is based on genotyping [4], which is costly and minimizes the usage and utility of superior plants on a larger scale. Using biotechnology, genetic transformation, in breeding programs as an alternative approach to conventional breeding offers remarkable development of cotton agronomic traits, as well as resistance to biotic and abiotic stresses. Although, genetic transformation is commonly used to generate mutated plants, only few transgenic plants are able to regenerate with limited agronomic traits which can not be advantageous for plant production. Earlier evaluation of genetically modified lines would save time, money and energy. The development of new, specific markers and selection tools is a crucial need to permit assessment of the genetic variability and diversity among modified or transgenic plants. Finding a morphological marker can allow the rapid differentiation of transgenic from non-transgenic plants and reduce the work load.

As reporter genes, β-glucuronidase (GUS) and green fluorescent protein (GFP) have been widely used in genetic transformation for a long time in many plant species, including cotton [5–7]. The bacterial enzyme GUS, encoded by the *E. coli gusA* gene, is one of the most widely used transgenic reporter genes. Despite its simplicity, false-positives are difficult to eliminate among the modified plants owing to the foreign GUS activity [8]. The high cost of the substrate (X-Gluc) for GUS staining is another disadvantage of GUS. Whereas GFP from jellyfish is another reporter used widely in many living organisms. The simplicity of its use to measure fluorescence without additional proteins, substrates, or cofactors and its durability to tolerate N and C terminal translational fusions make it a powerful monitoring tool [9]. However, using GFP as a reporter for gene expression studies still has some limitations: its sensitivity is low, the assay may subjected to UV-induced toxicity and photo-bleaching, aggregates of GFP cause cytotoxicity [10, 11], chlorophyll-related autofluorescence is hard to avoid, and GFP, lignin and flavone share emission spectral maxima [12]. DsRed2 is a *DsRed* mutant form of the oral disk of coral (*Discosoma* sp.) and the spectral characteristics are significantly different from those of GFP, with a much higher extinction coefficient and fluorescence quantum yield [13, 14]. DsRed is mostly used in animal imaging. The first successful report of DsRed protein in plants was in transgenic tobacco, where its transient expression and stable transformation did not lead to adverse effects on plant development and morphogenesis [15, 16]. Since then, DsRed has been used in variety of plant transgenic studies, such as in soybean, rice, and walnut [17, 18]. Based on these facts mentioned above, DsRed might be a better alternative reporting marker for plant biotechnology and for molecular breeding as well.

In plant biotechnology, genetic transformation is used as a powerful tool to study gene(s) function and improve plant performance under different stresses. However; transgenes are frequently inactivated by transcriptional and posttranscriptional silencing [19]. Some of these silencing cases are linked with transgene DNA methylation [20]. DNA methylation, one of the processes involved in epigenetic gene regulation, is the addition of a methyl group ($–CH_3$) to the 5th carbon atom of a cytosine ring [21]. DNA methylation plays essential roles in X-chromosome inactivation [22], gene imprinting [23, 24], and foreign DNA transcriptional silencing [25]. While the presence of methylation at or near the gene transcription starter site has been associated with the reduction of gene expression [20, 26]. In higher eukaryotes, the binding of regulatory proteins is inhibited by cytosine methylation [27]. Cytosine DNA methylation in plants is richer and more diverse than that in animals [28]. In plants, transcriptional and posttranscriptional inactivation and methylation also occur in the promoter and coding sequences. Cytosine methylation in the 35S promoter leadsed to gene silencing at the transcriptional level for many genes in diverse plant species such as tobacco [29], petunia [20], gentian [30, 31], *Arabidopsis* [32], and lettuce [33]. However, the effects of DNA methylation on transgene transcription of the transgenic cotton plants still unknown.

In this study, *DsRed2* was used as a visual reporter in cotton genetic transformation for the first time to investigate its ability as a reporting marker for cotton biotechnology and molecular breeding. *DsRed2* expression was detected in the different stages of somatic embryogenesis as well as in almost all organs and tissues of transgenic cotton plants. To determine whether the *DsRed2 gene* can express in different genetic backgrounds, the transgenic plants were crossed with four different cultivars. DNA methylation at the 35S promoter was analyzed to figure out the association between the *DsRed2* expression level and DNA methylation at the 35S promoter. Our study suggests that DsRed2 might be a useful tool for cotton genetic transformation and molecular breeding programs.

2. Materials and methods

2.1. Vector construction and cotton transformation

The binary vector pCAMBIA2300::35S::DsRed2 containing an enhanced 35S promoter-driven a selectable marker gene (*NPT-II*) and a 35S promoter-driven *DsRed2* gene (protein ID: CAH64889.1) (Fig. 1A) was introduced into the EHA105 *Agrobacterium* strain and then used for in *Agrobacterium*-mediated genetic transformation in cotton seedlings. The cotton cultivars JIN668 and YZ1 were used for *Agrobacterium*-mediated transformation. For the genetic transformation, cotton seeds were sown in half-strength MS medium for germination after seeds decoating and seed's surface sterilization using $HgCl_2$ 0.1% (m/v) for 10 min. Hypocotyls generated from aseptic seedlings were used as explants for *Agrobacterium* transformation, cut into 5–7 mm lengths and then inoculated with *Agrobacterium* harboring the binary vector pCAMBIA2300::35S::DsRed2. The infected hypocotyls were then transferred to MSB co-cultivation medium [MS basal salts, B5 vitamins] [34] supplemented with 3% (m/v) glucose, 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg L^{-1} kinetin, 1 g L^{-1} $MgCl_2$, and 20 mg L^{-1} acetosyringone and solidified with 0.25% (m/v) Phytagel (Sigma, St. Louis, USA), pH 5.8, in the dark at 21 °C for 48 h. Two days later, for callus induction; hypocotyls were

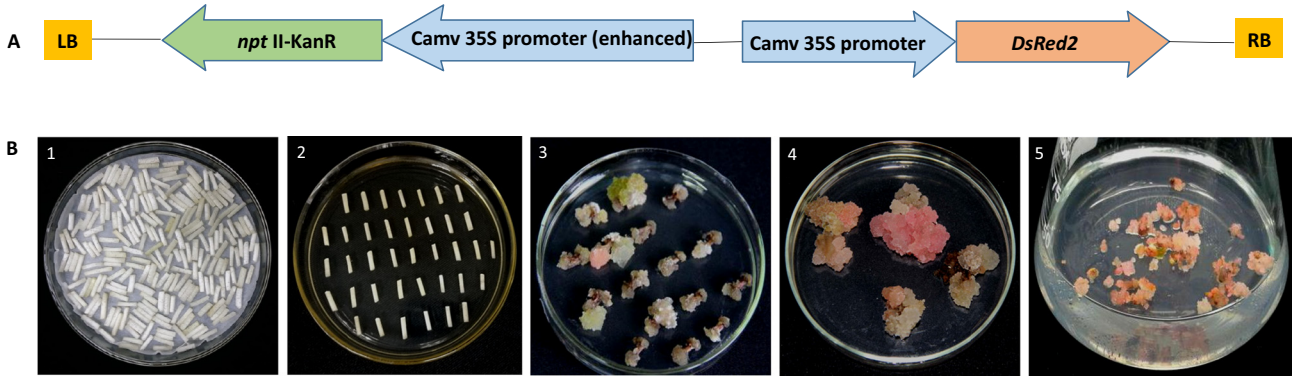

Fig. 1 – *Agrobacterium*-mediated cotton genetic transformation with *DsRed2* as reporter gene. A. T-DNA region of pCAMBIA2300::35S::DsRed2 vector for genetic transformation. B. Agrobacterium-mediated cotton genetic transformation steps. 1. Infected cotton hypocotyls by *Agrobacterium* for 48 h. 2. Callus induction from infected hypocotyls placed in 2,4-D medium two days after *Agrobacterium* infection. 3. Callus induction one and a half month after *Agrobacterium* infection; red color is pronounced in non-embryogenic callus at an early stage. 4. Late stage of non-embryogenic callus derived from the infected ends of hypocotyls; red color is clearer at later stage of callus development (three months). 5. Embryogenic callus with red color.

transferred to Petri dishes containing MSB selection medium supplemented with 3% (m/v) glucose, 0.1 mg L^{-1} 2,4-D, 0.1 mg L^{-1} kinetin, 50 mg L^{-1} kanamycin, and 400 mg L^{-1} cefotaxime and solidified with 0.25% Phytagel, pH 5.8. Three months later, the embryogenic calli that formed on the cut edges (Fig. 1B 3 & 4) were transferred to subculture, differentiation and rooting medium. Somatic embryogenesis and plant regeneration of kanamycin-resistant calli were obtained as described in our previous reports [5, 35–37]. All media mentioned above were adjusted to pH 5.85–5.95 [5].

2.2. DNA extraction, PCR, and southern blot analysis

Genomic DNA was extracted from young leaves of T_0 putative transgenic plantlets and wild-type cotton plants using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) to examine the presence of the *DsRed2* gene. For PCR analysis, *DsRed2* gene primers were used: the forward primer sequence was 5′-ACAGAACTCGCCGT AAAGAC-3′ from the vector backbone sequence and the reverse primer sequence was 5′-CCGTCCTCGAAG TTCATCAC-3′ from a *DsRed2* gene fragment. PCR products were electrophoresed in 1% agarose gel and visualized under UV light. After PCR amplification confirmation, Southern blot analysis was performed to determine the copy number of transgene (T-DNA) insertions. About 20 μg of the extracted genomic DNA from each PCR-positive plant was digested for 72 h at 37 °C with *Hind* III-HF, and the resulting fragments were electrophoresed in 0.8% agarose gel and transferred through a salt bridge to a Hybond N^+ membrane. A fragment of the *NPT-II* gene was used as a hybridization probe. Southern hybridization was performed with a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany). After hybridization, the probe, labeled with digoxigenin-11-dUTP by PCR, was hybridized with the target DNA for 12–16 h and the anti-digoxigenin-AP was used for immuno detection. Finally, CSDP (a chemiluminescence substrate for alkaline phosphatase) was added to display the DNA bands on an X-ray film.

2.3. RNA extraction, real-time PCR and real-time quantitative PCR analysis

Total RNA was extracted from young leaves of JIN668 transgenic plants by the modified guanidine thiocyanate method [38]. For each sample, 3 μg of total RNA was transcribed into cDNA using M-MLV reverse transcriptase (Promega, USA). The cDNAs were used as templates to assay the transcription of *DsRed2* in cotton by Real-time PCR (RT-PCR) and Real-time quantitative PCR (qRT-PCR). qRT-PCR using SYBR Green as fluorescent dye, was performed on an ABI Prism 7000 system (Applied Biosystems, Foster City, CA, USA). The cotton *ubiquitin 7* gene (*GhUb7*) (GenBank: DQ116441) was used as an internal control to standardize the gene expression [39]. For each experiment, three technical replicates and a two-step RT-PCR method was used. The relative expression levels of the target gene were calculated by the $2^{-\Delta\Delta Ct}$ method. For RT-PCR and qRT-PCR analysis, the primers were: 5′-CTCCGAGAACGTCATCACCG-3′ and 5′-TGGAGCCGTACTGGAACTGG-3′.

2.4. Fluorescence microscopy

Callus, somatic embryos, tissues and organs of the regenerated plants and the wild-type JIN668 control plants were observed at white light and red light with a fluorescence stereo-microscope. The autofluorescence of wild-type anthers was imaged under white light, red light using a filter set for excitation at 530–550 nm and emission at 575 nm and under blue light using a filter set for excitation wavelengths of 460–490 nm.

2.5. DNA methylation detection in the 35S promoter region

Methylation-specific PCR was applied for DNA methylation analysis of the 35S promoter region driven the *DsRed2* gene. Genomic DNA was extracted from the young leaves of transgenic cotton plants (lines 1–10) using a kit from Takara

Biotechnology Co., Ltd., Dalian, China. One microgram of genomic DNA was subjected to bisulfite treatment using a Clontech EpiXplore methyl detection kit (Clonech Catalog number 631968, USA) and a DNA bisulfite conversion kit (Tiangen Catalog number DP215–02, China) to convert unmethylated cytosines (uC) to thymines (T), and methylated cytosines (mC) to cytosines (C). After the bisulfite treatment, 2 μL DNA was used as a template for PCR to amplify the 35S promoter fragments of the *DsRed2* gene using Takara Epi*Taq* HS (for bisulfite-treated DNA) (Takara, Shiga, Japan). For the first PCR, the primer sequences were 5′-AAAGAT TGGCGAATAGTTTATA-3′ and 5′-CGCACCTTAAAA CGCATAAACTC-3′ and in the second PCR, the primer sequences were 5′-TTACGATTTAATGATAAGAAG-3′ and 5′-TAAACTCGATAATAACGTTCTC-3′. Unmethylated lambda DNA (Promega) was used as a control. The PCR products were then ligated to T-Vector pMD 19 (Simple) (Takara Code 3271) with T4 DNA ligase and then transformed into JM109 competent *E. coli* cells (Takara Code 9052). All positive clones were subjected to Sanger sequencing.

2.6. DNA methylation analysis

For DNA methylation analysis, total thymines (tT) were determined in the bisulfite-treated DNA compared with the reference total thymines (rtT) (unmethylated cytosine sites (uC) = tT − rtT). The total number of cytosine (tC) sites of the reference genome was calculated. Finally, the methylation level was calculated (methylation ratio (%) = (tC − uC) / tC) in the 35S promoter region of the *DsRed2* gene. The average DNA methylation level was calculated in 10-bp bins in the 35S promoter region using a custom Perl script.

2.7. Transgene heritability analysis

Transgenic lines and wild type plants were grown in the greenhouse and field to assess the phenotypic and transgene heritability. The progeny of the T_3 generation of homozygous Yz-2-*DsRed2* line exhibiting strong red color in most tissues and organs were crossed as male parents with four non-transgenic cotton cultivars: SM3, J14, C312, and Z12. One day before flowering, the maternal parents were subjected to artificial emasculation using a tube to protect the stigma while buds of male parents were closed with clips to avoid pollen contamination. During the flowering days, pollen was collected from flowers of Yz-2-*DsRed2* for pollinating. F_1 hybrid plants of YZ-2 ♂ × SM3 ♀ were planted in the greenhouse and F_2 plants were planted in an experimental field.

3. Results

3.1. Effective genetic transformation and stable expression of DsRed2 gene in cotton plants

Cotton plants were transformed with *Agrobacterium* harboring the binary vector pCAMBIA2300::35S::DsRed2 (Fig. 1A). *DsRed2* gene was expressed successfully as a visual reporter gene in almost all tissues and organs. *DsRed2* expression began in the early stage of callus formation and embryo development (Fig. 1B-1, 2). In non-embryogenic callus, one and a half months after *Agrobacterium* infection, red color appeared in some hypocotyls, but was pale (Fig. 1B-3). The red color then became more pronounced with callus age. In the late stage of non-embryogenic callus derived from the infected ends of hypocotyls and in the embryonic callus stage, the red color was stronger and more obvious than the early stage of the non-embryogenic callus (Fig. 1B-4, 5). Callus showing red color developed red plantlets. Once the plantlets began to form, the red color again diminished owing to chlorophyll formation in the green tissues. Although chlorophyll fluorescence obscured the red fluorescence, red color was still present in the green tissues and the transgenic plants could be distinguished from wild-type plants but not as clearly as in non-chlorophyll-containing tissues (Fig. 2). For example, red color in the floral parts of cotton flower was clearer and stronger than in vegetative parts (Fig. 2A–D). Red color in the inner side of the cotton boll was stronger than on the outer side, owing to the accumulation of red fluorescence in the cytoplasm. The more chlorophyll was present, the less was the red fluorescence (Fig. 2E–F).

3.2. Molecular analysis of putative transgenic plants

A total of 33 T_0 plantlets regenerated after *Agrobacterium*-mediated transformation were subjected to kanamycin selection: 24 from JIN668 and nine from YZ1. For molecular analysis, 13 lines were tested by PCR: 10 lines of JIN668 and three lines of YZ1. The PCR results are shown in Fig. 3A. Southern hybridization analysis revealed that all of the T_0 transgenic cotton plants came from 17 independent lines. However; owing to somaclonal variation during callus and embryo culture and damage caused by insects and pathogen attack in the greenhouse, only 13 of the 17 independent transgenic cotton lines reached the next generation. Ten JIN668 transgenic plants were named lines 1–10 and three YZ1 transgenic plants were named Yz-1–Yz-3. The copy numbers of T-DNA integration in the transgenic cotton lines are shown in Fig. 3B. Southern hybridization revealed the numbers of random insertions of T-DNA in the DNA of the transgenic plants harboring the *DsRed2* gene. Of the 13 independent transgenic lines, five (lines 1, 2, 5, 10, and Yz-3) showed a single genomic insertion of T-DNA. Six lines (lines 3, 4, 6, 8, 9, and Yz-2) showed two inserted copies of T-DNA. Two lines (lines 7 and Yz-1) showed multiple genomic insertions of T-DNA.

3.3. Relative relationship between the phenotype and the transcription level of the DsRed2 gene in the DsRed2 transformed cotton plants

Visually, DsRed2 was expressed efficiently in transgenic callus, embryogenic calli, embryos, tissues and organs of regenerated plants. Interestingly, callus displayed red color 2–3 months after the co-cultivation stage. Of 134 kanamycin-resistant calli, 45 showed clear red phenotypes. Red callus then developed red embryos, red petals, red stamens, red pistils, and red stems. More importantly, the red color phenotype in T_0 plants was stably transmitted to the F_1 and F_2 generations. Heterogeneity in phenotypes was observed in

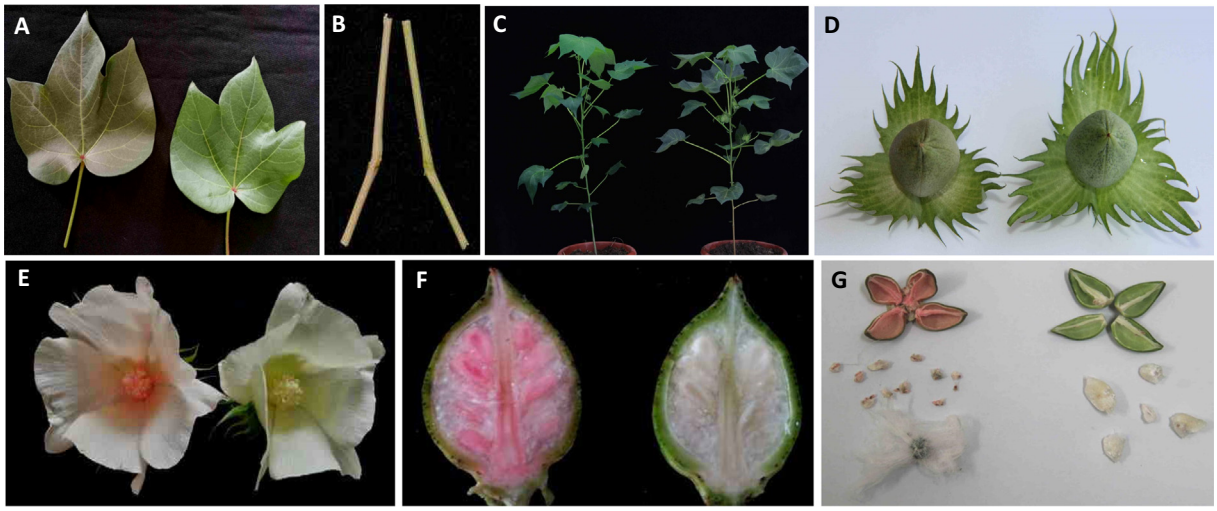

Fig. 2 – *DsRed2* gene expression in different tissues and organs in transgenic and wild-type cotton plants under white light. A–D. Red color in the greenish tissue is pale and weak owing to the presence of chlorophyll, but the difference between the *DsRed2* transgene plants and the wild-type is readily seen. E–F. Red color in the floral parts of the cotton flower is clearer and stronger than in the vegetative parts. F. Red color in the inner side of the cotton boll is stronger than on the outer side owing to the accumulation of red fluorescence in the cytoplasm. The more chlorophyll present, the lower the red fluorescence is.

JIN668-*DsRed2* transgenic lines. Although some lines harbored the *DsRed2* gene and T-DNA in their genome, they showed no red phenotype. Only four of the 10 positive lines (lines 7–10), showed a red phenotype in almost all plant organs, whereas the other six lines showed no red phenotype despite the presence of *DsRed2* gene in their DNA. Accordingly, the transcription intensity of the *DsRed2* gene in the transgenic lines was analyzed by RT-PCR and results are shown in Fig. 4A. The results supported the phenotyping assessment: the transcription level of *DsRed2* gene in the lines showing the red phenotype was higher than that in the lines without the red phenotype. Increased transcription levels of *DsRed2* were associated with more intense red color in plant tissues (Fig. 4B).

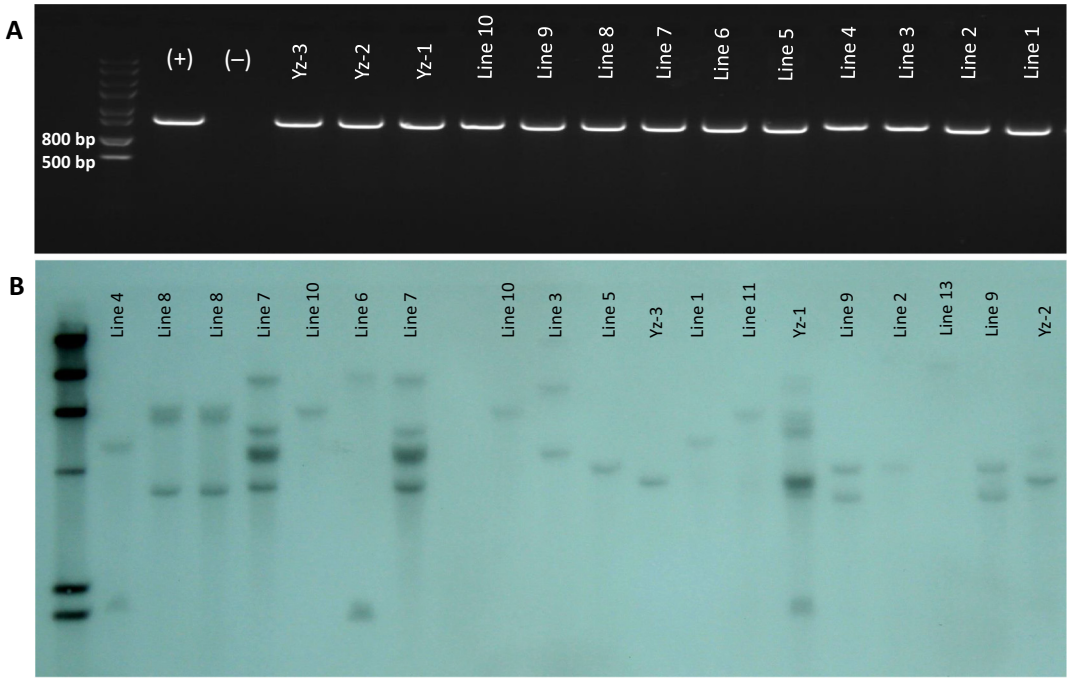

Fig. 3 – PCR and Southern blot analysis of putative transgenic plants. A. PCR amplification of *DsRed2* transgenic plantlets: lines 1–10 and Yz-1–3, and wild-type plants (–). B. Southern blot analysis of the *DsRed2* gene in transformed cotton plantlets lines 1–10 and Yz-1–3, showing the number of transgene integrations in each line using PCR products.

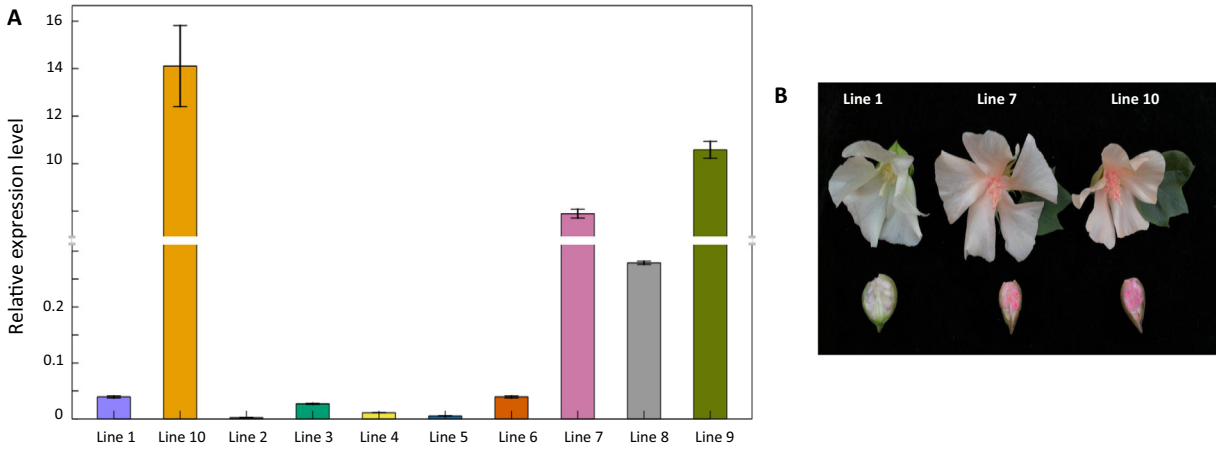

Fig. 4 – Association between phenotype and transcriptional level of the *DsRed2* gene in transgenic cotton lines. A. The expression level of *DsRed2* in transgenic lines 1–10. **Lines 7–10 showed red color in almost all plant organs, whereas lines 1–6 showed no red color.** B. Red fluorescence intensity in different *DsRed2* transgenic lines. **Line 1 showed no red phenotype and a low expression level of *DsRed2*, whereas line 7 showed red fluorescence but with less intense red color than seen in lines with higher expression of *DsRed2*. Line 10 showed the strongest red fluorescence and the highest expression of *DsRed2*.**

3.4. *Negative association between DNA methylation level in the 35S promoter region and transgene transcription*

JIN668-*DsRed2* transgenic lines showed heterogeneity in their phenotypes, with six of 10 of these transgenic lines showing no red phenotype. The expression level of these lines was lower than that of plants showing red color. To investigate this heterogeneity in the phenotype of JIN668-*DsRed2* cotton plants, the DNA methylation level in the 35S promoter region of the *DsRed2* gene was measured. The methylation level at the 35S promoter site was revealed by the methylation-specific PCR approach and varied markedly among the lines (Fig. 5A, B). Most lines (lines 1, 2, 4, 5, and 6) with low expression levels of the *DsRed2* gene showed high levels of DNA methylation in the 35S promoter of *DsRed2*, whereas in lines 8–10, showing high expression of *DsRed2*, showed a low level of DNA methylation in the 35S promoter. The phenotype of the transgene was clearly associated with DNA methylation at the 35S promoter site. Our results suggest a negative association between DNA methylation level in the 35S promoter region and the transcription of *DsRed2* in transgenic cotton plants.

3.5. *Red fluorescence and autofluorescence exhibition with different light wavelengths using fluorescence stereomicroscope*

Red fluorescence was detected under the fluorescence stereomicroscope in several organs of *DsRed2* plants and the wild-type plants and was detected under white light as well. In almost all organs of *DsRed2* plants, red fluorescence was clearly visible under the red light, whereas no red fluorescence was visible in the wild type under the red light. Under white light, the red fluorescence of *DsRed2* plants was clear to the naked eye in almost all plant organs with no additional calibration. Red fluorescence intensity under the white light as well as under the red light varied from one organ to another. Non-embryonic callus exhibited faint red fluorescence compared with embryogenetic callus and embryos, and red fluorescence was more obvious in the non-chlorophyllic organs than those organs with higher chlorophyll content (Fig. 6).

In addition, the autofluorescence in the anthers of cotton wild-type plants were detected under blue light, light is used to detect GFP fluorescence, and red light, light is used to detect RFP fluorescents. Autofluorescence detection was pronounced clearly when subjected to blue light and was much similar to the green fluorescence, whereas no autofluorescence was detected under red light in the anthers of the wild-type (Fig. 7). This finding suggests that RFP detection is much easier to distinguish and visualize than GFP due to the faint autofluorescence exhibited under red light, making it a preferable option for use in plant genetic transformation as a reporter gene.

3.6. *Stability in hybrid progeny*

Red callus generated from *Agrobacterium*-mediated transformation, containing the *DsRed2* gene, developed red plants and red seeds as well. First-generation plants produced from T_0 red seeds showed red color with no segregation in the red fluorescence trait (Fig. 8). To test the inheritance and stability of the red fluorescence in further generations, Yz-2-*DsRed2* plants generated by three generations of self-pollination were crossed with some cotton cultivars. The first generation of the hybrids (F_1) showed red phenotype in all plants and these red plants produced red seeds (Fig. 9A). F_2 plants generated from F_1 hybrid plants of Yz-2-*DsRed2* and SM3 were grown consecutively in the field. Plants of the second generation showed variation in phenotype. Out of 98 plants, 71 plants showed a clear red phenotype in the field, whereas 27 plants showed normal phenotype, similar with the wild-type phenotype. Only plants with the red phenotype produced red seeds. These hybrid red seeds could be identified as potential hybrids when a Yz-2-*DsRed2* homozygous transgenic line was used as a male parent comparing with those seeds produced by using Yz-2-*DsRed2* line as a female parent in the cross

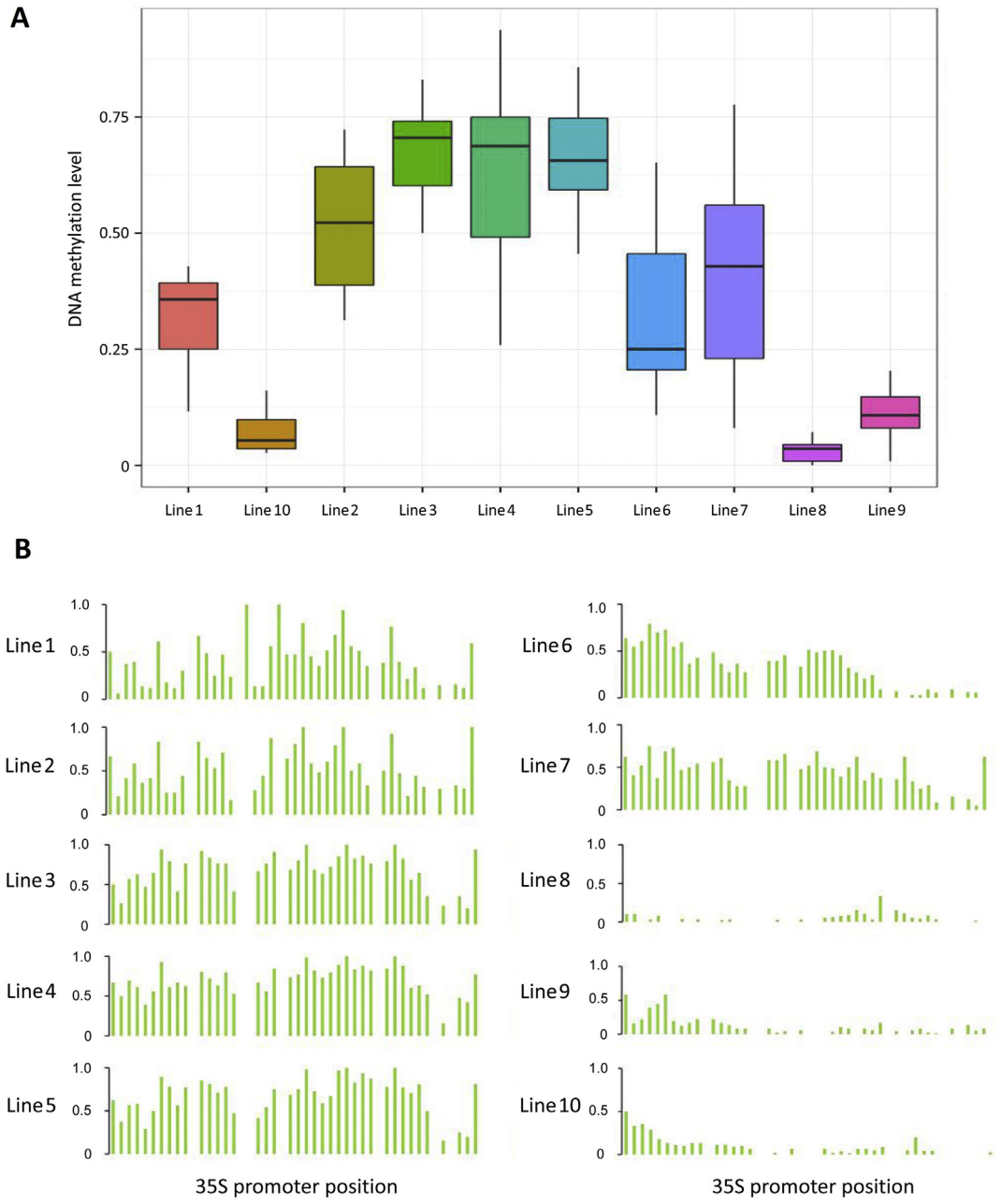

Fig. 5 – DNA methylation analysis of JIN668-*DsRed2* transgenic lines in the 35S promoter site. A. Methylation level of transgenic lines 1–10 in the 35S promoter site. B. Distribution of methylation sites in the 35S promoter in transgenic lines 1–10.

(Fig. 9B). Thus, our results revealed that the red phenotype in transgenic cotton plants was faithfully transmitted from one generation to another and the expression of *DsRed2* gene was genetically stable in cotton plants with diverse genetic background and in further generations as well.

4. Discussion

DsRed is a red fluorescent protein that is responsible for the red coloration in the oral disk of corals of the *Discosoma* genus. Recently, it has attracted great interest as a possible expression reporter that would be better as an alternative to the analogous reporter, the green fluorescent protein from *Aequorea*. It has been used successfully in animals [40], fungi [41, 42], and plants [15, 18, 43]. In the present study, due to its simplicity and capability to be detected in an early stage of plant genetic transformation; the viability of *DsRed2* gene was evaluated in cotton genetic transformation as an alternative reporter gene to GFP. *DsRed2* expressed effectively in cotton plants and red color was clearly visible in almost all tissues and organs. The red color was easily monitored from an early stage of callus development up to the last stages of plant development without the need of any chemical staining. Our results in agreement with the respective

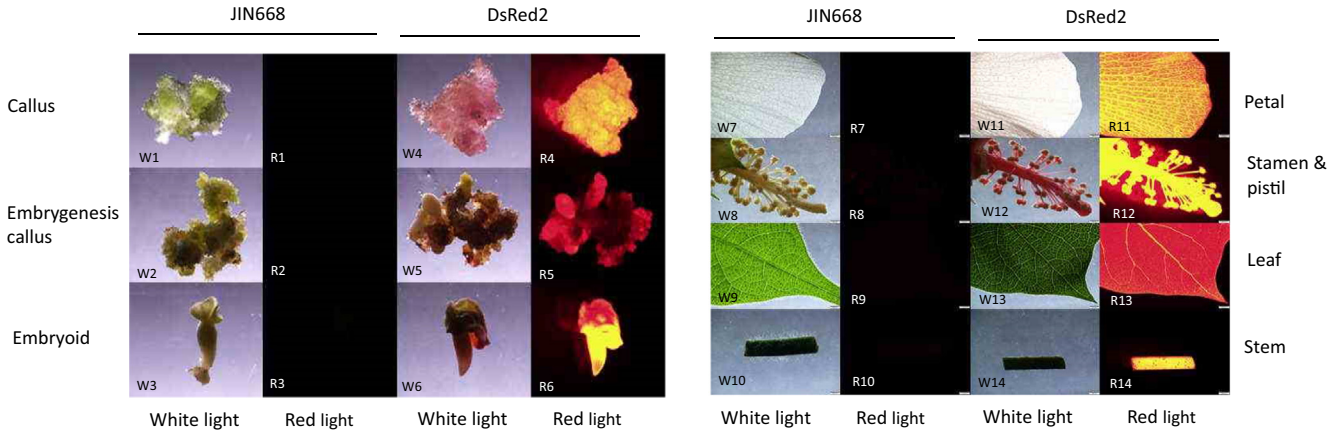

Fig. 6 – Different tissues of wild-type JIN668 plants and *DsRed2* plantlets under white light and red light using fluorescence microscopy. W1–3. Image showing no red color in callus, embryogenetic callus, and embryoids of the wild type under white light. R1–3. Red fluorescence image of different tissues of wild-type plants showing no red color. W4–6. Red fluorescence image of *DsRed2* transgenic plants under white light showing clear red fluorescence in different tissues. R4–6. Different tissues of *DsRed2* transgenic plants under red light showing red color. W7–10. Image of wild-type under white light showing no red color in petal, stamen, pistil, leaf and stem tissues. R7–10. Red fluorescence image of petal, stamen, pistil, leaf, and stem tissues of wild-type plants exhibiting no red fluorescence. W11–14. Red fluorescence was shown clearly in petal, stamen, pistil, leaf, and stem of *DsRed2* transgenic plants under white light. R4–6. Red fluorescence of petal, stamen, pistil, leaf, and stem of *DsRed2* transgenic plants under red light, showing red fluorescence.

findings of Jach [15] and Zhang [18] that red fluorescence protein is an effective visual reporter gene for genetic transformation of tobacco and walnut plants, respectively.

Strangely, JIN668-*DsRed2* transgenic lines showed heterogeneity in phenotypes, with some of these transgenic lines showing no red phenotype. The expression level of these lines was apparently low comparing with those plants showing red color. This phenomenon corresponded to the DNA methylation level in the 35S promoter region of *DsRed2*. A negative association between DNA methylation level in the 35S promoter site and transgene transcription was identified. This observation is in agreement with those of Mishiba [30] and Yamasaki [44], who reported that cytosine methylation occurring in the 35S promoter can cause transcriptional gene silencing. It seems that DNA methylation suppressed the expression of *DsRed2* gene, this is in agreement with the finding of Okumura [33] when reported that gene silencing is accompanied by DNA methylation.

Red fluorescence was readily detected not only under fluorescence microscopy but also under normal conditions, white light, in all tissues of the transgenic cotton plants, similar finding was observed in tobacco [15] and walnut [18] plants. Additionally, when the autofluorescence was detected in the wild-type anthers under blue light, autofluorescence was clear and much comparable to green fluorescence, whereas no autofluorescence was detected under red light. Unlike GFP, RFP detection is much easier to be distinguished and visualized due to the faint autofluorescence exhibited under red light. In

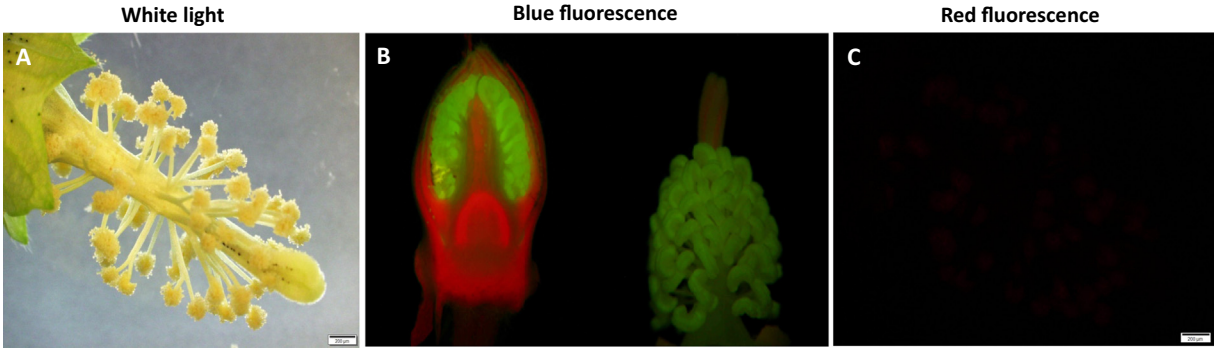

Fig. 7 – Autofluorescence of the anther of JIN668 wild-type cotton plant under white light, blue light and red light. A. Anther autofluorescence in the wild type under white light. B. Anther autofluorescence in the wild type under blue light field at an excitation wavelength of 460–490 nm, exhibiting strong autofluorescence. C. Anther autofluorescence in the wild type under red light field at an excitation wavelength of 530–550 nm exhibiting no autofluorescence.

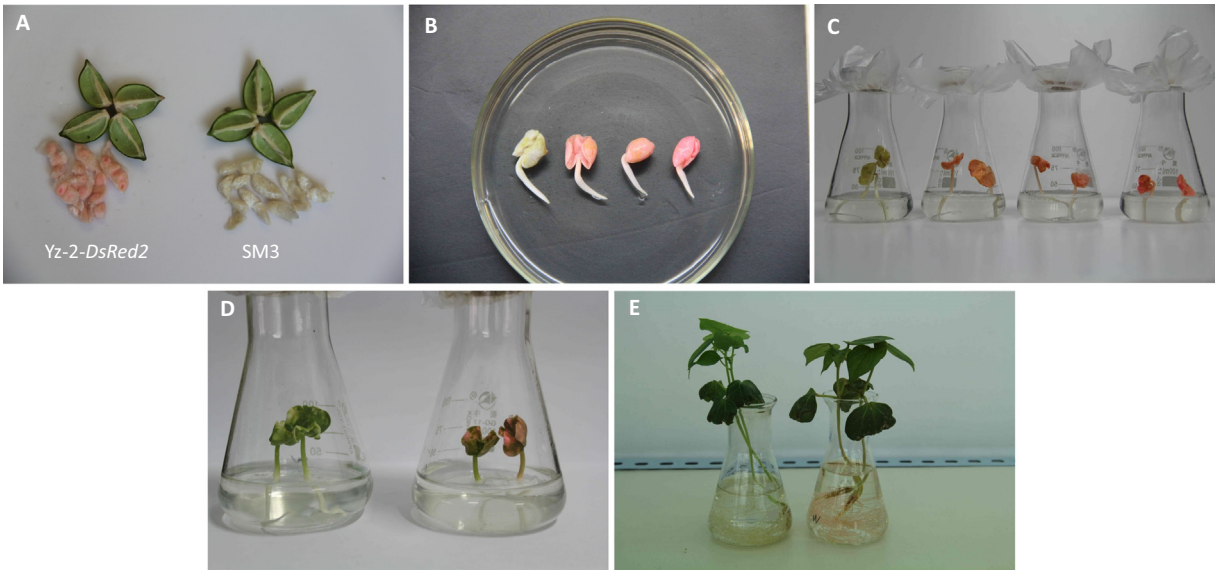

Fig. 8 – T_0 seeds grown for regeneration under light, red color is transmitted to T_1 generation. A. **T_0 seeds were used for hybridization; seeds with red color are transgenic Yz-2-*DsRed2* and seeds with white color are of the SM3 cotton cultivar.** B. **One-day-old seedlings showing clear red fluorescence.** C. **Two-day-old seedlings with lower red fluorescence at the initiation stage of first-leaf formation.** D. **Three-day-old seedlings showing pale red fluorescence due to chlorophyll formation.** E. **Two-week-old seedlings showing the lowest red fluorescence but higher in roots due to the lack of chlorophyll.**

contrast, autofluorescence often interferes with GFP marker detection, especially in green tissues, due to the high autofluorescence of chlorophyll [45, 46]. These results indicate that RFP is preferable than GFP as a reporter gene in cotton biotechnology and molecular breeding. Plantlets with red phenotype stably transmitted the red fluorescence to their progeny, indicating the high heritability of the *DsRed2* gene. Yz-2-*DsRed2* performed efficiently as a male parent and produced a potential hybrid harboring the *DsRed2* gene as a visible marker able to identify genuine hybrids among a large F_1 population. Forner and Binder [47] reported that the red fluorescence was stably transmitted to subsequent generations, making it fully compatible in dual-labeling experiments.

5. Conclusions

The DsRed2 protein is an ideal morphological reporter for plant breeding, as it can visually distinguish transgenic from non-transgenic cotton. It expressed in almost all plant tissues

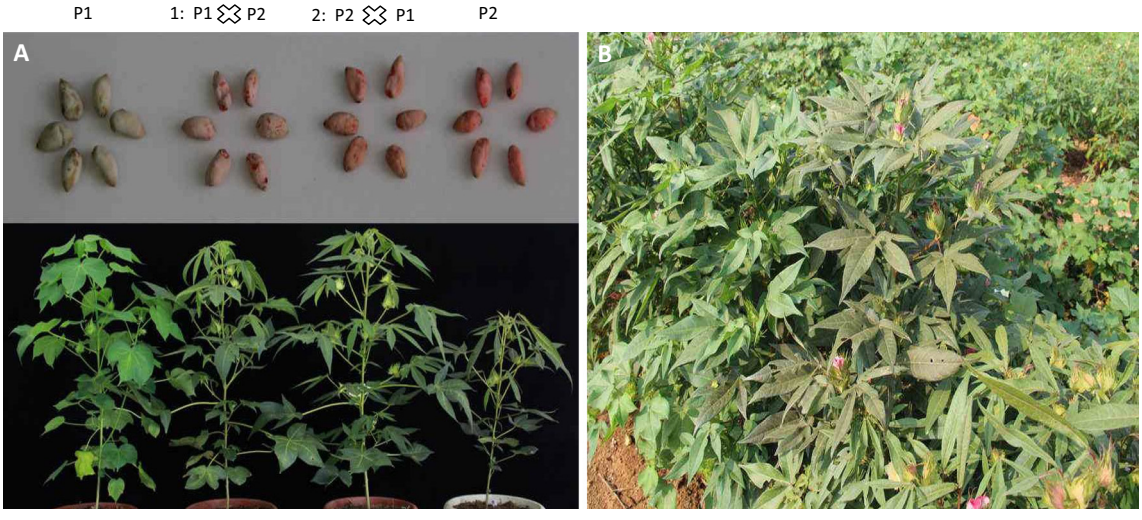

Fig. 9 – F_1 and F_2 hybrids generated from a cross between *DsRed2* transgenic plants and MS3 cultivar. A. **F_1 generation using Yz-2-*DsRed2* and SM3 as parents grown in the greenhouse.** P1 **is cotton cultivar** SM3**;** P2 **is the** Yz-2-*DsRed2* **mutant**. 1. MS3 **used as a male parent and** Yz-2-*DsRed2* **as a female parent.** 2. Yz-2-*DsRed2* **used as a male parent and** MS3 **as a female parent.** B. **F_2 generation using** Yz-2-*DsRed2s* **as male parent and** SM3 **as a female parent, grown in the field. Red fluorescence is pronounced.**

and provided a reliable visual marker that can identify transformed cells earlier than any other marker. DNA methylation in the 35S promoter was negatively associated with transgene transcription, which resulted in *DsRed2* gene silencing in some transgenic lines. In addition, red fluorescence can be stably transmitted to further generation and *DsRed2* homozygous transgenic lines can be used as valuable male parents in hybridization breeding of cotton.

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