THE CROP JOURNAL XX (2018) XXX–XXX

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

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ARTICLE INFO

Article history:
Received 21 February 2018
Received in revised form 10 April 2018
Accepted 18 May 2018
Available online xxxx

Keywords:
DsRed2
Reporter gene
Transgenic cotton
Molecular breeding
DNA methylation

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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Peer review under responsibility of Crop Science Society of China and Institute of Crop Science, CAAS.
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https://doi.org/10.1016/j.cj.2018.05.002

Please cite this article as: L. Sun, et al., Red fluorescent protein (DsRed2), an ideal reporter for cotton genetic transformation and molecular breeding, The Crop Journal (2018), https://doi.org/10.1016/j.cj.2018.05.002

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

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