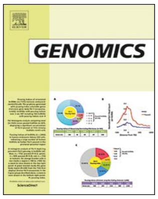
ARTICLE IN PRESS

Genomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno



Transcriptome analysis reveals potential genes involved in flower pigmentation in a red-flowered mutant of white clover (*Trifolium repens* L.)

Heshan Zhang, Hong Tian, Mingxin Chen, Junbo Xiong, Hua Cai, Yang Liu*

Hubei Key Laboratory of Animal Embryo and Molecular Breeding, Institute of Animal Husbandry and Veterinary science, Hubei Academy of Agricultural Sciences, Wuhan 430064, China

ARTICLE INFO

Keywords:
Flower pigmentation
Trifolium repens
Transcriptome analysis
Red-flowered mutant

ABSTRACT

White clover (*Trifolium repens* L.) has been cultivated for ornamental use because of its flowers, leaf marks and creeping habit. Although a mutation in flower color is very infrequent in this species, the red-flowered mutant of white clover was a novel germplasm for ornamental white clover breeding. The mechanism of flower pigmentation in white clover is still limited because of the rarity of mutation materials and the lack of genomic data. In this study, two cDNA libraries from red-flowered white clover mutant between sunlight-exposed plants and shade-treated plants, respectively, were used for transcriptome sequencing. A total of 157,964 unigenes with an average length of 728 bp and a median length of 1346 bp were isolated. A large number of differentially expressed genes (6282) that were potentially involved in multiple biological and metabolic pathways, including anthocyanin flavonoid biosynthetic pathway and flavonoid biosynthetic pathway, were obtained, 70 of which could be identified as putative homologues of color-related genes. Furthermore, eight key candidate genes (*CHS, F3′H, F3′5′H, UFGT, FLS, LAR, ANS*, and *DFR*) in flavonoid biological synthesis pathway were identified by quantitative real-time PCR (qRT-PCR). Mass sequence data obtained by RNA-Seq of white clover and its red-flowered mutant provided basic sequence information and a platform for future molecular biological research on the red flower trait.

1. Introduction

White clover (*Trifolium repens* L.), originally native to the Mediterranean [1], grows in a wide temperature range and in nearly any soil type if provided with sufficient moisture and sunlight. The petals of white clover are generally white. This plant has been called ‘Weiss Klee’, ‘Trefle Blanc’ and ‘Trifoglio bianco’, indicating that the flowers are rarely pigmented [2]. However, flower pigmentation of white clover is occasionally found. For example, a red-flowered mutant of white clover was found at two different sites in 1950 and 1980 [2,3]. For centuries, white clover has been mainly utilized as forage for livestock due to its high protein content [4], but the ornamental use of this species has also been developed because of its ornamental traits, such as flower color, leaf marks, and creeping habit [5]. Several white clover mutants with markedly ornamental characters, including ‘Dark Dancer’, ‘Salsa Dancer’, Dragon's Blood’, ‘Good Luck’, and ‘Purpurescens', have been identified and registered for ornamental use [6]. It was worth noting that two ornamental cultivars with distinct flower color, ‘Dragon's blood’ and ‘Dark Dancer’, are commercially available [5]. Although the flower color of both cultivars was ‘blush’ as described by Brewbaker [2], variation in flowers often attracts consumer attention and are generally of high value in the ornamental market [7]. Thus, the flower color trait, such as a red color, in white clover can be very attractive and have commercial value.

Light is one of the most important environmental factors that influence plant growth and traits, and photoperiod, light intensity, and light quality affect anthocyanin biosynthesis in different ways [8]. High and low light intensity has significantly positive and negative effects on flavonoid accumulation, respectively. For example, light treatment led to significantly higher total anthocyanin levels than those in the dark control in *Fragaria* × *ananassa* [9]. The anthocyanin accumulation in fruit was decreased by fruit bagging, and a high level of anthocyanins reappeared after debagging or the exposure of shaded fruits to sunlight [10].

Red colors in flowers are mainly produced by two types of pigments: anthocyanins and betacyanins [11]. Anthocyanins, a type of flavonoid, are the most common flower pigments and are responsible for most pinks, reds, mauves, and blues in flowers and fruits [12]. The biosynthesis of anthocyanin has been extensively investigated [13–17], and much is known about the gene networks that regulate anthocyanin

---

* Corresponding author at: South Lake Road, Hongshan District, Wuhan, Hubei Province, China.
E-mail address: sdzhanghs@hotmail.com (Y. Liu).

http://dx.doi.org/10.1016/j.ygeno.2017.09.011
Received 3 July 2017; Received in revised form 19 September 2017; Accepted 25 September 2017

Please cite this article as: Zhang, H., Genomics (2017), http://dx.doi.org/10.1016/j.ygeno.2017.09.011

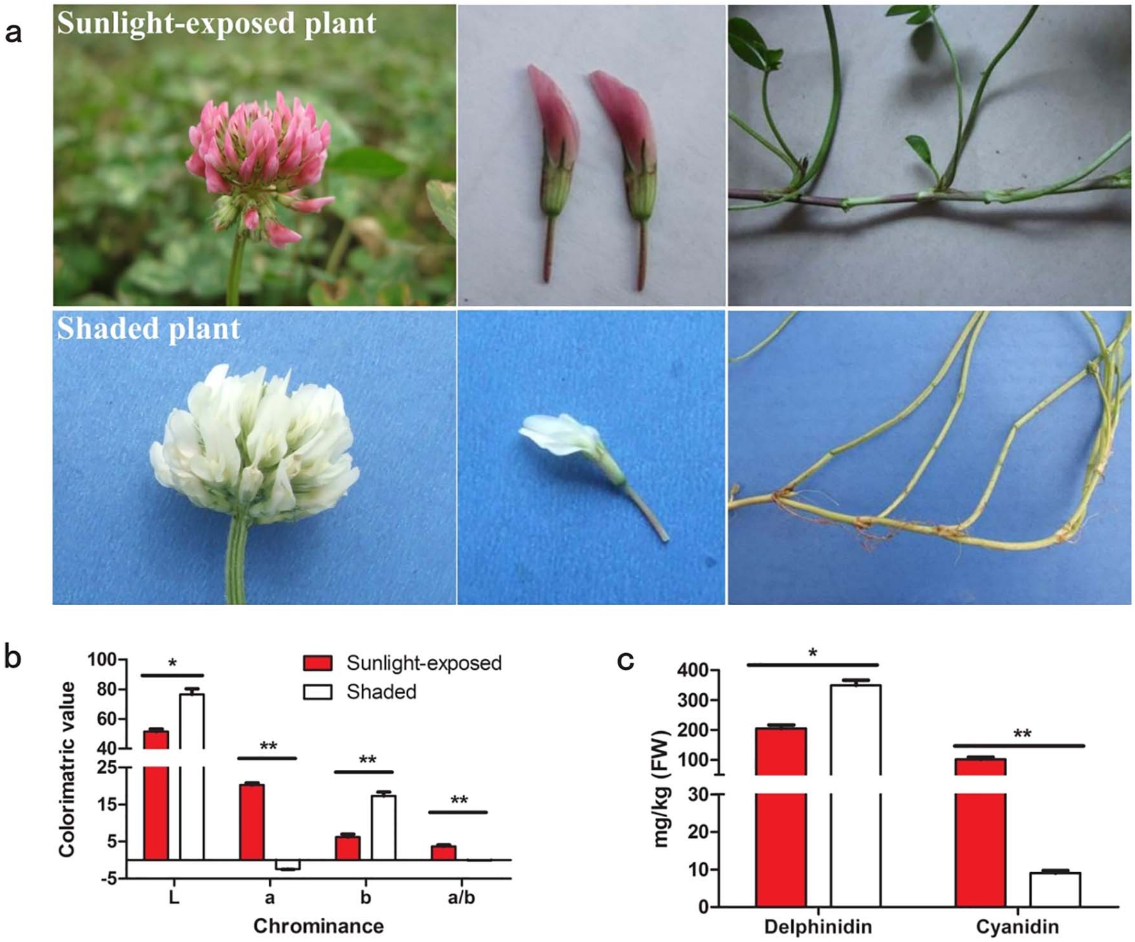


Fig. 1. The difference of pigmentation in sunlight-exposed and shaded plants of white clover. (a) Phenotypic characteristic of sunlight-exposed and shaded plants. (b) Chromatic difference analysis of the petals between sunlight-exposed plants and shaded plants. (c) Anthocyanidin content obtained by UPLC from two different treated samples.

production [17]. Some enzymes involved in the anthocyanin biosynthetic pathway have been identified in various plant species [18–20]. Chalcone synthase (CHS) catalyzes the first reaction of anthocyanin biosynthesis and assists in forming the intermediate chalcone, which is the primary precursor for all classes of flavonoids [21]. Dihydroflavonol 4-reductase (DFR) is the first committed enzyme of anthocyanin biosynthesis in the flavonoid biosynthetic pathway and is responsible for the formation of leucoanthocyanidins which can be converted into colored anthocyanidins by anthocyanidin synthase (ANS). Further modification of anthocyanidins by glycosylation and acylation of aglycons generates a variety of anthocyanins in higher plants [11]. To date, much of the molecular information regarding the regulation and biosynthesis of flower pigments is available in model species such as *Zea mays* [22], *Arabidopsis thaliana* [13,19], *Petunia hybrida* [23] and *Antirrhinum majus* [24]. However, increasing numbers of non-classical plants are providing unique novel insights into the molecular mechanisms of anthocyanin biosynthesis. Therefore, the mechanism of the formation of the red-flowered white clover should be understood, and the key genes should be identified.

Although many genetics and molecular genetics studies on anthocyanin have been reported for white clover, the pigmentation mechanism of the petals of white clover has not been well understood because of a lack of genomic data. Previous studies on pigmentation in white clover primarily focused on anthocyanin leaf marks. For example, the *R* locus in white clover is associated with four closely linked traits: 'red leaf', (*Rl*), 'red midrib' (*Rm*), 'red fleck' (*Rf*) [25], and 'diffuse red leaf' (*Rld*) [26]. The 'red spot mark' (*Vr2*), 'red halo mark' (*Vh2*) and 'red leaflet' (*Vrl*) traits are associated with the *V* locus [26,27]. Furthermore, some R2R3-MYB family genes regulating the pigmentation phenotypes in the leaflets of white clover have been reported [28], similar to the floral pigmentation of other species [29–31]. In contrast, data regarding anthocyanin accumulation in the petals of white clover are relatively limited. Thus, further study on the red-flowering mutant at the genetic level is essential to germplasm innovation and ornamental breeding in white clover.

The next-generation sequencing technology RNA sequencing (RNA-Seq) provides the opportunity to study the molecular mechanism of non-model organisms that lack a reference genome [32–34]. Several recent studies have exploited this technology to generate transcriptome information for many plant species including *Arabidopsis thaliana* [35], *Capsicum annuum* [36], *Oryza sativa* [37–39], and *Glycine* max [40]. The detailed transcriptome information related to inflorescence in maize was also obtained using RNA-Seq [41–43]. In this paper, a red-flowered white clover mutant was used as the experimental model. We found that the red-flowered white clover mutant could produce white flowers under shaded conditions. Based on this finding, we characterized the transcriptome of flower color in white clover and the putative genes involved in anthocyanin biosynthesis. The present study provides an important molecular basis for further study of red-flowered white clover mutants and is crucial for understanding the color formation mechanism.