Influences of different light sources and light/dark cycles on anthocyanin accumulation and plant growth in Petunia

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Abstract Anthocyanin accumulation and plant growth were examined in petunia (NT and T1 transgenic plants) by determining the effects of different sources of light and varying light/dark cycles. Red light significantly enhanced anthocyanin content of B-peru+mPAP1; however, it had a negative effect on anthocyanin production in RsMYB1 plants. In general, white light was found to be reasonable for anthocyanin accumulation in all plants. In case of light/dark cycles, application of seven days of light:14 days of dark significantly enhanced anthocyanin content. We found that anthocyanin content detected in transgenic plants expressing anthocyanin regulatory transcription factor genes (B-peru+mPAP1 or RsMYB1) was higher than that in NT plants in all treatments. Plant growth was also influenced by the different light sources and dark/light cycles. Taken together, our results suggest that light source and light/dark cycle play an important role in anthocyanin production and plant growth. The choice of the optimal conditions is also important for anthocyanin production and plant growth depending on NT or transgenic plants carrying anthocyanin regulatory transcription factors.

Keywords Anthocyanin content, growth characteristics, transcription factors, transgenic plants

Introduction

Enhancement of anthocyanin accumulation in plants is often found to be a response of plants to different sources of light and photoperiods. The stimulatory effects of light sources on anthocyanin biosynthesis in vegetative organs such as seedlings, leaves, and seeds, and in cell cultures have been demonstrated in some plants, including petunia, Antirrhinum, rose, and apple (Biran and Halevy 1974; Martin et al. 1991; Moscovici et al. 1996; Dong et al. 1998). However, different types of light have been shown to have distinct effects; for instance, blue light was found to be the most effective in anthocyanin biosynthesis (Feng et al. 2010; Chen et al. 2006; Kadomura-Ishikawa et al. 2013), whereas white and red light induced anthocyanin accumulation in tomato seedlings (Bowler et al. 1994). In addition, far-red light also positively affected anthocyanin accumulation in Brassica rapa (Devlin et al. 1992). Hence, the role of different sources of light in anthocyanin biosynthesis is still unclear.

Although the effects of light have been shown to induce anthocyanin biosynthesis in many plant species, anthocyanin biosynthesis in some species, such as Aralia cordata (Sakamoto et al. 1993), Fragaria ananassa (Nakamura et al. 1999), and sweet potato (Konczak-Islam et al. 2000), was achieved in the dark. Chan et al. (2010) claimed that dark/light cycling had a prominent effect on anthocyanin production, in which cells cultured under continuous lighting/darkness did not strongly induce anthocyanin; however, cultures that were exposed to darkness, followed by exposure to light produced high anthocyanin content. Therefore, it is interesting to investigate the effects of different light/dark cycles on anthocyanin biosynthesis.

In our previous work, we produced transgenic petunia expressing anthocyanin-related transcription factors (mPAP1, B-peru, and RsMYB1 genes), which are generally known as anthocyanin regulatory transcription factors (Lim et al. 2012, 2015). However, anthocyanin accumulation in the transgenic plants was weak, particularly in RsMYB1-transformed plants. Based on the literature mentioned above, it was obvious that these factors (light sources and light/dark cycling) singly
affect anthocyanin production in many plant species. However, to our knowledge, interactive effects amongst these factors on anthocyanin production have not been reported in non-transgenic (NT) and transgenic petunia expressing the anthocyanin regulatory transcription factor genes. Thus, in this study, in vitro experiments were conducted to investigate the effects of different sources of light, and light/dark cycling on anthocyanin accumulation in transgenic and NT petunia plants. In addition, plant growth of transgenic and NT plants affected by the factors were also investigated.

Materials and Methods

In vitro seed germination

In our previous work, we produced transgenic petunia cv. ‘Mirage Rose’ expressing the anthocyanin regulatory transcription factor genes B-peru+mPAP1 or RsMYB1, where B-peru from maize, mPAP1 from Arabidopsis, and RsMYB1 from radish were isolated. In this study, T2 seeds of transgenic plants were germinated on Murashige and Skoog (MS) medium containing 1.5 mg/l phosphinothricin (PPT; Duchefa Biochemie, The Netherlands), whereas those of control (non-transgenic) plants were germinated in the same medium without PPT. Germinated seedlings with uniform size were then selected for further experiments.

Influence of different sources of light, and light/dark cycle on anthocyanin accumulation and plant growth

Germinated seedlings of petunia (non-transgenic and transgenic) with uniform size (~ 10 mm) were transplanted to culture boxes containing MS medium. The seedlings were treated with different sources of light (blue light with 23 μmol m⁻² s⁻¹, red light with 61 μmol m⁻² s⁻¹, or far-red light with 2 μmol m⁻² s⁻¹) for 30 min per day, and the treated culture boxes were placed back under white light (50 μmol m⁻² s⁻¹) used as a control for 16 h at room temperature. Each treatment contained ten seedlings with three replicates. After 21 days of culture, ten plantlets were randomly selected from each treatment for determination of anthocyanin content and plant growth. In addition, to verify effects of light/dark cycles on anthocyanin production, the seedlings were treated with the different light/dark cycles (21 days light, 7 days light/14 days dark, 14 days light/7 days dark, or 7 days light/7 days dark/7 days light), in which white light was used for light condition as it was observed to be a suitable source for anthocyanin production of NT and transgenic plants in above experiment.

Similar to light sources treatments, each treatment contained ten seedlings with three replicates, and ten plantlets were randomly selected from each treatment for determination of anthocyanin content and plant growth after 21 days of culture.

For plant growth, number of leaves per seedling was recorded, and stem elongation as well as root length were measured to the nearest millimeter using an automatic ruler (Absolute Digimatic, Japan).

Analysis of anthocyanin content

Total anthocyanin content of ten plants from each treatment was analyzed according to the protocol described by Naing et al. (2015), with some modifications. Briefly, approximately 500 mg of leaf material excised from the treated plants was crushed. The anthocyanins were extracted by transferring the fine powder to an extraction solution (5 ml) containing a methanol/HCl mixture (99:1 v/v) (Sigma, St. Louis, USA). The mixture was incubated at 4°C for 24 h and then centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was transferred to a fresh tube and the total anthocyanin was determined by measuring the OD at A530 (λmax for anthocyanin) and A657 (peak of absorption for chlorophyll) by using a spectrophotometer (Shimadzu, Kyoto, Japan). Quantification of anthocyanins was performed using the formula: $Q_{anthocyanin} = (A_{530} - 0.25 \times A_{657}) \times M^{-1}$, where $Q_{anthocyanin}$ is the amount of anthocyanins, and M is the weight (g) of the plant material used for extraction, as described by Chu et al. (2013). Error bars indicate the standard error (SE) of the average anthocyanin content.

Statistical Analysis

Data were statistically analyzed using SAS version 11.09 (IBM corporation). The mean separations were carried out using Duncan’s multiple range test (DMRT), and significance was determined at 5% level.

Results

Influence of different sources of light on anthocyanin accumulation and plant growth

When the transgenic and NT plants were treated with different sources of light, anthocyanin content was enhanced by the light sources, however, the enhancement of anthocyanin content by the light sources varied among the tested plants (Fig. 1A). In B-peru+mPAP1-plants, anthocyanin content...
Influence of different light/dark cycles on anthocyanin accumulation and plant growth

According to the results of the above experiments, effects of white light on anthocyanin production and plant growth seemed to be suitable in NT and transgenic plants. Therefore, we selected the white light in the next experiment investigating the effects of different light/dark cycles on anthocyanin production. In this study, different light/dark cycle distinctly influenced anthocyanin production (Fig. 1B). However, anthocyanin contents influenced by the cycles differed among the tested plants as observed in above, meaning that the contents detected in \( B\text{-peru}+mPAP1D \) were found to be the highest followed by \( RsMYB1 \) and NT plants. Among the different treatments, the treatment (7 light/14 dark) significantly enhanced anthocyanin accumulation, and anthocyanin content in NT and transgenic plants enhanced by this cycle were significantly higher than the other light/dark treatments, whereas, effects of the treatments (21 days light and 14 days light/7 days dark) on anthocyanin production were positively higher than the treatment (7 days light/7 days dark/7 days light).

When the same growth parameters investigated in above experiment were examined, the number of leaves produced in the all plants appeared to be higher under the treatments (21 days light or 7 days light/7 days dark/7 days light) as compared those affected by the other treatments (Table 2). However, the number of leaves produced in \( RsMYB1 \) plant treated with 14 days light/7 days dark was the highest. In term of stem elongation, the treatment (21 days light) was found to be the highest while the other periods showed similar effects. Similarly the same treatment (21 days light) produced greater root lengths of the plants as compared to the other treatments. Interestingly, root length of \( RsMYB1 \) plant treated with the period (7 days light/7 days dark/7 days light) was significantly longer than those of other plants.

Discussion

The transcription factor genes such as \( B\text{-peru}, mPAP1 \), and \( RsMYB1 \) have been shown to enhance anthocyanin production in petunia (Ai et al. 2016). However, studies done for effects of different sources of light or light/dark cycle on anthocyanin synthesis in transgenic petunia carrying the anthocyanin regulatory transcription factors are rare and limited.

To determine the response of petunia to anthocyanin accumulation, we cultured seedlings of NT and \( T2 \) transgenic petunia expressing \( B\text{-peru}+mPAP1 \) or \( RsMYB1 \) on the
Table 1  Plant growth of NT and transgenic plants under different light sources

| Light sources | Stem elongation (mm) | Number of leaves | Root elongation (mm) |
|---------------|----------------------|------------------|----------------------|
|               | NT                   | B-Peru + mPAP1   | RsMYB1               |
|               | NT                   | B-Peru + mPAP1   | RsMYB1               |
| White         | 24.32±1.18a          | 21.79±1.51ab     | 20.45±2.32b          |
| Blue          | 15.89±1.63d          | 11.11±0.19i      | 15.18±2.82e          |
| Red           | 13.76±1.42f          | 12.67±0.44g      | 12.68±0.59g          |
| Far-red       | 11.42±0.51h          | 16.43±3.1c       | 14.61±0.43ef         |
| White         | 4.17±0.48c           | 4.33±0.33ab      | 4.50±0.67ab          |
| Blue          | 2.33±0.21hi          | 2.00±0.0i        | 2.67±0.42g           |
| Red           | 3.00±0.52e           | 2.50±0.34h       | 2.83±0.54f           |
| Far-red       | 2.33±0.33hi          | 3.33±0.72d       | 2.83±0.54f           |
|               | 20.45±2.32b          | 20.45±2.32b      | 20.45±2.32b          |
|               | 2.33±0.21hi          | 2.00±0.0i        | 2.67±0.42g           |
|               | 3.00±0.52e           | 2.50±0.34h       | 2.83±0.54f           |
|               | 2.33±0.33hi          | 3.33±0.72d       | 2.83±0.54f           |

Means marked with same letter within each column are not significantly different by DMRT.

Table 2  Plant growth of NT and transgenic plants under different light/dark cycle conditions

| Light/dark cycles | Stem elongation (mm) | Number of leaves | Root elongation (mm) |
|-------------------|----------------------|------------------|----------------------|
|                   | NT                   | B-Peru + mPAP1   | RsMYB1               |
|                   | NT                   | B-Peru + mPAP1   | RsMYB1               |
| T1                | 24.32±1.18a          | 21.78±1.51ab     | 20.41±2.32b          |
| T2                | 15.89±1.63d          | 11.11±0.19i      | 15.18±2.82e          |
| T3                | 13.76±1.42f          | 12.67±0.44g      | 12.68±0.59g          |
| T4                | 11.42±0.51h          | 16.43±3.1c       | 14.61±0.43ef         |
|                   | 4.17±0.48c           | 4.33±0.33ab      | 4.50±0.67ab          |
|                   | 2.33±0.21hi          | 2.00±0.0i        | 2.67±0.42g           |
|                   | 3.00±0.52e           | 2.50±0.34h       | 2.83±0.54f           |
|                   | 2.33±0.33hi          | 3.33±0.72d       | 2.83±0.54f           |
|                   | 36.02±7.51a          | 35.28±7.23a      | 30.85±2.45c          |
|                   | 19.19±6.69g          | 17.30±3.34h      | 29.01±4.29d          |
|                   | 20.91±4.55g          | 19.30±2.94g      | 21.13±6.52fg         |
|                   | 21.81±3.47f          | 31.36±8.95b      | 25.46±4.42c          |

Means marked with same letter within each column are not significantly different by DMRT.

medium and treated those with different sources of light followed by with different light/dark cycles. From each experiment, total anthocyanin content and plant growth characteristics corresponding to each plant were evaluated. The present study indicates that the different sources of light could all differentially activate anthocyanin contents along with different plants. In NT and B-peru + mPAP1 plants, blue light had more of a negative effect than white light assumed as control, which was not consistent with the findings of Feng et al. (2010) and Chen et al. (2006), who claimed that blue light could effectively regulate anthocyanin biosynthesis. Here, we found that red light was the best source for anthocyanin accumulation in B-peru + mPAP1 plants but not in NT and RsMYB1 plants. Therefore, the best source of light depended on anthocyanin contents or transcription factors over-expressed in plants. For instance, white or red was found to be the best source of light for NT and RsMYB1 or B-peru + mPAP1 plants. In a study done by Bowler et al. (1994), white and red light induced anthocyanin accumulation in tomato seedlings. Under different light sources conditions, total anthocyanin content of transgenic plants carrying B-peru + mPAP1 or RsMYB1 was higher than that of NT plants. It might be due to overexpression of the anthocyanin-regulatory transcription factor in petunia. However, it was observed that amount of total anthocyanin induced by the transcription factors under influence of the light sources.

Anthocyanin content detected under 7 days light/14 days dark condition was significantly higher than those under other light/dark treatments. However, when the treatment was inversely changed (14 light/7 dark), total anthocyanin content dramatically decreased, which was similar in the treatment (21 days light), while the content detected in the treatment (7 light/7 dark/7 light) was the lowest. It seemed that long dark period is required for anthocyanin production in this species, which was similar to the report of Nakamura et al. (1999), who reported that darkness is required for high anthocyanin production from high anthocyanin-producing cell lines of Fragaria ananassa (strawberry). How et al. (2003) also reported that maximum anthocyanin production can be obtained from sub-culturing the callus of Ajugu in the dark for 2 or 3 weeks, followed by transferring to a light condition for a few days. Hence, based on our experiments, we can conclude that the optimum cycle plays important role in high anthocyanin accumulation.

Under the different light conditions, generally, effect on plant growth of the different sources of light varied; however, according to mean results presented in Figure 1bcd, white light significantly promoted plant growth of NT and transgenic plants. Although Lee et al. (2010) and Azad et al (2011) claimed that red light increased growth characteristics in
Lactuca sativa and Capsicum annuum, in this study, it appeared to have an inhibitory effect on plant growth. Blue light was effective in the formation of chlorophyll (Senger, 1982), chloroplast development (Akoyunoglou and Anni, 1984), stomatal opening (Zeiger, 1984) and enzyme synthesis (Senger, 1982), which are important for plant growth; however, plant growth of RsMVI plants only under blue light was significantly promoted in this study. Thus, it seemed that the advantages bestowed by blue light may depend on other factors. Overall, this study suggested that different sources of light differently affected plant growth under different experimental conditions, and superiority of plant growth under white light might be due to optimum plant photosynthesis given by it.

Investigation of plant growth affected by light/dark cycles has not been well documented in petunia. The present study revealed significant effects of light/dark cycles on plant growth of petunia. Generally, growth characteristics responded by the light/dark cycles varied; for instance, the light/dark period (light/dark/light) responded better in terms of number of leaves per plant, whereas continuous light conditions showed better response in terms of all parameters. The variation in plant growth characteristics by different light/dark cycles may be due to anthocyanin content induced under the different conditions.

In conclusion, this study indicated that culture of NT and transgenic plants on the medium with 7% sucrose under white light was effective in the formation of chlorophyll (Senger, 1982), chloroplast development (Akoyunoglou and Anni, 1984), stomatal opening (Zeiger, 1984) and enzyme synthesis (Senger, 1982), which are important for plant growth; however, plant growth of RsMVI plants only under blue light was significantly promoted in this study. Thus, it seemed that the advantages bestowed by blue light may depend on other factors. Overall, this study suggested that different sources of light differently affected plant growth under different experimental conditions, and superiority of plant growth under white light might be due to optimum plant photosynthesis given by it.

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