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Original article – Thematic Issue

# Cloning of the strawberry xanthophyll cycle genes *VDE* and *ZEP* and characterization of their responses to high light stress

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## Summary

**The xanthophyll cycle is associated with the violaxanthin de-epoxidase (VDE)-catalyzed conversion of violaxanthin (V) to zeaxanthin (Z) via antheraxanthin (A) when plants are exposed to excess light energy. Zeaxanthin is reversibly converted to V by zeaxanthin epoxidase (ZEP) under non-saturated light conditions. In this study, the strawberry *VDE* and *ZEP* cDNA sequences were isolated. Additionally, the changes in Z, V, and A contents and *VDE* and *ZEP* expression levels under high light and dark conditions were characterized to investigate the regulation of *VDE* and *ZEP* expression in response to high light stress in strawberry leaves. Under high light conditions, the *VDE* expression level increased considerably during the first 10 min, after which it slowly increased. When leaves were placed in darkness, the *VDE* expression level decreased immediately. The *VDE* expression profile was consistent with the observed changes to the Z content and the de-epoxidation state of the xanthophyll cycle [i.e., (Z + A) / (Z + A + V)]. The *ZEP* transcript level decreased after a 10-min exposure to high light conditions, but then gradually increased. Following a 10-min incubation in darkness, the *ZEP* expression level increased and then decreased to control levels. The changes in *ZEP* expression during the first 10 min of exposure to high light and dark conditions were consistent with the accumulation of V. These results indicate that the rapid responses of the strawberry xanthophyll cycle components to high light stress appears to be regulated by gene expression level changes.**

**Keywords**
*Fragaria* × *ananassa*, gene expression, high light, regulatory mechanism, violaxanthin de-epoxidase, zeaxanthin epoxidase

## Significance of this study

*What is already known on this subject?*

- Leaves of strawberry plants grown in open field are often susceptible to photoinhibition and the xanthophyll cycle is now recognized as an important photoprotective mechanism.

*What are the new findings?*

- In this study, two xanthophyll cycle genes (i.e., *VDE* and *ZEP*) were isolated from strawberry leaves. Their expression levels were related to the interconversion between V and Z. In strawberry leaves, the rapid response of xanthophyll cycle components to high light stress appears to be regulated by changes to gene expression levels.

*What is the expected impact on horticulture?*

- A better understanding of xanthophyll cycle mechanisms in strawberries will provide new ideas for the researches of plant photoprotection and stress physiology, which may be relevant for improved strawberry production and breeding.

## Introduction

Light is the ultimate energy source used by plants during photosynthesis. However, the absorption of excessive amounts of light energy is deleterious to the photosynthetic apparatus as it can enhance the production of reactive oxygen species, permanently decrease photosynthetic efficiency, and even cause photooxidative damage (Barber and Andersson, 1992). Plants have evolved multiple regulatory mechanisms to minimize these types of damages (Li et al., 2009; Niyogi, 2000; Horton et al., 2005). The xanthophyll cycle, which helps dissipate excess light energy as heat (i.e., thermal dissipation), is now recognized as an important photoprotective mechanism (Latowsk et al., 2004; Jahns and Holzwarth, 2012). This cycle includes three xanthophylls, namely violaxanthin (V), zeaxanthin (Z), and antheraxanthin (A). Zeaxanthin plays a central role in the photoprotective mechanism of the xanthophyll cycle (Demmig-Adams, 1990). Antheraxanthin can trap and dissipate surplus excitation energy in PSII, and also has photoprotective effects (Gilmore, 1997; Gilmore and Yamamoto, 2001). Two key enzymes regulate the reversible interconversion of Z, A, and V under different light conditions. When plants are exposed to excessive light, violaxanthin de-epoxidase (VDE) catalyzes the conversion of V to Z, with A serving as an intermediate. Under non-saturated light conditions, Z is reversibly converted to V by zeaxanthin epoxidase (ZEP) (Eskling et al., 1997; Xiong et al., 2012).

Violaxanthin de-epoxidase is a soluble protein present in the thylakoid lumen. It is responsible for the synthesis of Z under high light conditions. Violaxanthin de-epoxidase activity is strictly regulated by thylakoid lumen pH. At neutral pH, VDE is inactive, but when the pH decreases to 6.3, the enzyme attaches to the thylakoid membrane and catalyzes the de-epoxidation of V to Z (Bratt et al., 1995; Arnoux et al., 2009). Furthermore, ascorbic acid, monogalactosyldiacylglycerol, and dithiothreitol also affect VDE activity (Forti et al., 1999; Schaller et al., 2010; Neubauer, 1993). The *VDE* genes have been isolated in various species, and the encoded enzyme may contribute to photoprotection by regulating the Z and non-photochemical quenching (NPQ) levels (Zhang et al., 2003; Huang et al., 2007; Lin et al., 2002). The *Arabidopsis*

*thaliana* mutant *npq1*, which lacks VDE activity, is unable to convert V to Z under high light conditions, and exhibits relatively low NPQ levels (Niyogi et al., 1998). The over-expression of *VDE* in tobacco increases Z levels and enhances the ability of plants to dissipate excess energy under high light conditions. Thus, the sensitivity of photosystems I and II to photoinhibition decreases (Hieber et al., 2002). In contrast, the antisense suppression of *VDE* in tobacco considerably inhibits the de-epoxidation of V and decreases the NPQ level (Chang et al., 2000; Sun et al., 2001).

Zeaxanthin epoxidase is a monooxygenase with dual functions. This enzyme is localized to the thylakoid membrane where it catalyzes the conversion of Z to V. Unlike VDE functions, ZEP activity is thought to be relatively stable. Therefore, the accumulation of Z is dependent on the relative activities of VDE and ZEP (Frommolt et al., 2001). Zeaxanthin epoxidase activity is affected by the availability of the co-substrate NADPH, as well as by oxygen levels, pH, and the cofactor FAD (Hieber et al., 2000; Grotz et al., 1999). Current investigations of ZEP are focused on its role in the xanthophyll cycle and abscisic acid (ABA) biosynthesis in plants exposed to light, drought, and salinity stresses, as well as during seed development (Audran et al., 2001; Seo et al., 2002; North et al., 2005). Although *ZEP* transcripts have been detected in all plant tissues, their abundance differs among various tissues and developmental processes (Audran et al., 1998). In leaves, the accumulation of *ZEP* mRNA follows a day/night cycle (Thompson et al., 2000). Deficiencies in ZEP activity adversely affect photosynthetic performance, resulting in plants that exhibit increased water loss, decreased seed dormancy, and salt sensitivity (Niyogi et al., 1998; Kalituho et al., 2007).

Strawberry (*Fragaria* × *ananassa* Duch.) is one kind of shade-adapted plant, which is fond of warmth and could not endure high temperature and light. However, the main planting mode of strawberries in China is still open-field cultivation. So during the summer growing season, leaves of strawberry plants grown in open field are often susceptible to photoinhibition due to prolonged exposure to high light conditions, which seriously affects strawberry yield and quality (Duan et al., 2009; Zhang et al., 2004). Previous studies regarding the regulation of the xanthophyll cycle in strawberry leaves suggest that this cycle may be important for plant defenses against photodamage (Zhen, 2005; Xu et al., 2005). However, these studies were limited to morphological and physiological analyses, and little is known about the molecular mechanism underlying the xanthophyll cycle as yet, especially the regulation of VDE and ZEP. In this study, the *VDE* and *ZEP* cDNA sequences were isolated from strawberry leaves. Additionally, changes in the accumulation of Z, V, and A as well as the *VDE* and *ZEP* expression levels under high light and dark conditions were characterized to clarify the role of *VDE* and *ZEP* in responses to high light stress in strawberry. Future genetic engineering of the xanthophyll cycle may lead to an increased tolerance of strawberry plants to environmental stresses. Therefore, a better understanding of xanthophyll cycle mechanisms in strawberries will provide new ideas for the research of plant photoprotection and stress physiology, which may be relevant for improved strawberry production and breeding.

## Materials and methods

### Plant materials and growth conditions

Seedlings of the strawberry cultivar 'Fu Mei Yi Hao' that exhibited uniform and strong growth were transplanted at the three-leaf and half-leaf stages to 15 cm × 15 cm plastic pots containing a loam:peat:coarse sand (7:3:2) growth substrate. The seedlings were grown in a phytotron with a 10-h light (600 μmol $m^{-2}$ $s^{-1}$)/14-h dark photoperiod. The phytotron was maintained at 25°C (light)/15°C (dark). Plants with five or six true leaves were treated as described in the following sections.

### High light and dark treatments

The fourth functional leaves from the top were used. They were cut in the petiole level and placed in soaked filter papers, then exposed to high light conditions (2,000 μmol $m^{-2}$ $s^{-1}$) for 4 h and then kept in darkness for another 4 h for recovery. Untreated leaves (i.e., not exposed to high light conditions and darkness) were used as controls. All leaf samples were immediately frozen in liquid $N_2$ for pigments and RNA extraction.

The treatments were conducted in a RTE-211 thermostatic flume (NESLAB, USA) in which the strong irradiance was provided by a metal halide lamp. A heat filter containing the circulating water was placed between the lamp and leaf to ensure the temperature remained roughly constant (25°C).

### Pigment extraction and UPLC analysis

Three pigments (i.e., V, A, and Z) were extracted from leaves according to the method described in a previous study (Chen et al., 2016). Briefly, 0.1 g ground leaf material was dissolved in 5 mL cold acetone. After mixing, the extract was incubated at 4°C for 5 min and then centrifuged (12,000 ×*g*, 4°C, 10 min). The supernatant was dried in a centrifugal evaporator. The dried extract was dissolved in 5 mL acetone and filtered through a nylon membrane (pore size: 0.22 μm) prior to being analyzed by ultra-performance liquid chromatography (UPLC). Pigments were separated on an ACQUITY BEH $C_{18}$ column (2.1 mm × 100 mm, 1.7 μm) (Waters, USA) and identified using a photodiode array detector. The chromatography was completed using an acetonitrile:methanol (9:1) mobile phase, a column temperature of 30°C, a flow rate of 0.5 mL $min^{-1}$, and a wavelength of 445 nm. Additionally, the epoxidation state of the xanthophyll cycle [i.e., (Z + A) / (Z + A + V)] and the xanthophyll cycle pool size (i.e., A + Z + V) were estimated.

### RNA extraction and gene cloning

Total RNA was extracted from strawberry leaves using the Plant Total RNA Mini Kit (Bioteke Corporation, China). Single-stranded cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Partial *ZEP* and *VDE* sequences were amplified using their respective degenerate primers (Supplemental Information – Table S1). The polymerase chain reaction (PCR) program was as follows: 94°C for 3 min; 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 90 s; 72°C for 7 min. The 5′ and 3′ cDNA ends of *ZEP* and *VDE* were obtained using the SMART™ RACE cDNA Amplification Kit (Clontech, USA) with gene-specific primers (Supplemental Information – Table S1). All PCR products were cloned into the pMD-18T vector (TaKaRa, Japan) and sequenced. The full-length *ZEP* and *VDE* cDNA sequences were assembled according to the conserved sequences as well as the 5′ and 3′ sequences (Supplemental Information – Table S1).

### Sequence analysis

Homologous sequences were aligned using the Basic Lo-

**Supplemental Information – Table S1.** Primers used for cloning and RqRT-PCR of *VDE* and *ZEP* related to experimental procedures.

| Experiment | | Forward primer | Reverse primer | Accession nr. |
|---|---|---|---|---|
| cDNA fragment | *VDE* | 5'- GATGCTCTCAARACATGYACTTGC -3' | 5'-CCHCCATCCCATGCRTCATT-3' | |
| | *ZEP* | 5'- TCAAGTTTGATACNTTCACTCCWGC -3' | 5'-TCCTCAATVGCCATGCABTCC -3' | |
| 3'-RACE | *VDE* | 5'- CTGCTCTTGTTCAAAGCTTTGATATGGA -3' | 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT -3' | |
| | *ZEP* | 5'-ACAGAGGAAGATGCAATTCTACGA-3' | 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT -3' | |
| 5'-RACE | *VDE* | 5'-TCATCCACGACACTGTTCTCAA-3'<br>5'-TCTCAAACAGGTCTCCGCATT-3' | 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT -3' | |
| | *ZEP* | 5'- CGATACCCTACAGAACTAATG-3'<br>5'-ACATTCGGCTTATGACTCTG-3' | 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT -3' | |
| Full-length | *VDE* | 5'-CTCTTTGCCTGCGTCGTCCT-3' | 5'- GATGCTCAGAAACAAAAGTAGAAG -3' | |
| | *ZEP* | 5'- ATATGATACCACCTCAATTTTCC-3' | 5'-AGTCAAAATCTATATATTCACATTG-3' | |
| RqRT-PCR | *VDE* | 5'-ATGGAAGCAACCGAAGTAGAAAA-3' | 5'-CTTTCAGTGTCGGACTACCTTAGCT-3' | JN979373 |
| | *ZEP* | 5'-TGCTACTGTTTTCTCAAGGACCAA-3' | 5'-TGGAACATAAGGTGAAATCTCAACTG-3' | JN979372 |
| | *18S* | 5'-GTAGTCATATGCTTGTCT-3' | 5'-GAATGATGCGTCGCCAGCACAAAGG-3' | X15590.1 |

cal Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/). Additionally, multiple amino acid sequences, including *ZEP* and *VDE* sequences, were aligned using the DNAMAN program. Physicochemical analyses of the amino acid sequences were completed with the ProtParam online tool (http://web.expasy.org/protparam/). The protein membrane-spanning domains, signal peptides, and transmembrane regions were predicted using SMART (http://smart.embl-heidelberg.de/), the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), and the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. The phosphorylation and phosphate kinase sites were predicted with DISPHOS (http://www.dabi.temple.edu/disphos/) and the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/). The secondary and tertiary protein structures were predicted using CFSSP (http://www.biogem.org/tool/chou-fasman/) and SWISS-MODEL (https://swissmodel.expasy.org/interactive). Phylogenetic trees and protein structures were constructed using the MEGA 5.2 and IBS1.0.1 programs, respectively.

### Quantitative real-time PCR

The *ZEP* and *VDE* expression levels in various tissues (i.e., roots, stems, leaves, flowers, and fruits) and under different light conditions were analyzed by quantitative real-time PCR (qRT-PCR; relative quantification). The qRT-PCR primers were designed based on available *ZEP*, *VDE*, and *18S* sequences (Supplemental Information – Table S1). The PCR samples included 2.5 µL template cDNA, 0.12 µL each primer, 12.5 µL Power SYBR Green PCR Master Mix (Applied Biosystems), and 9.76 µL double-distilled $H_2O$ for a final volume of 25 µL. The PCR program was as follows: 50°C for 2 min and 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s; one cycle of 60°C for 60 s, 95°C for 30 s, and 60°C for 15 s. A blank control that used double-distilled $H_2O$ as the template was also analyzed. The PCR products were analyzed using the ABI 7500 SDS software.

### Statistical analysis

The UPLC and qRT-PCR experiments were conducted using three replicates, and values for each sample are presented as the mean ± standard deviation. Significant differences between means were measured with Duncan's multiple range tests ($p < 0.05$) using the SPSS 17.0 program.

## Results

### Isolation of *VDE* and *ZEP* from strawberry

Partial *VDE* and *ZEP* sequences (644 bp and 615 bp, respectively; Figure 1) were amplified using their respective degenerate primers. The 5′ and 3′ cDNA ends were obtained by rapid amplification of cDNA ends and reverse transcription PCR based on the fragment sequences. The 5′ and 3′ cDNA ends of *VDE* consisted of 1,080 bp and 963 bp, respectively, while the 5′ and 3′ cDNA ends of *ZEP* comprised 1,630 bp and 1,159 bp, respectively (Figure 1). The full-length *ZEP* and *VDE* cDNA sequences were assembled using the abovementioned three fragments (Figure 1) (*ZEP* GenBank accession number: JN979372; *VDE* GenBank accession number: JN979373). Sequencing results revealed that *VDE* was 1,842 bp long, which included a 206-bp 5′ untranslated region (UTR), a 169-bp 3′ UTR, and a 1,467-bp open reading frame (ORF). The encoded protein consisted of 489 amino acids, with a predicted molecular weight of 55.44 kDa and a hypothetical isoelectric point of 5.79. The deduced VDE amino acid sequence was 81%, 81%, 76%, and 76% homologous with plum, peach, apple, and citrus sequences, respectively. The full-length *ZEP* cDNA sequence was 2,325 bp long, with a 135-bp 5′ UTR and a 210-bp 3′ UTR. The sequence also included a 1,980-bp ORF that encoded a 660-amino acid protein with a predicted molecular weight of 72.22 kDa and a hypothetical isoelectric point of 6.78. The ZEP amino acid sequence was 82%, 82%, 80%, and 72%, identical to apricot, peach, apple, and citrus sequences.

### Sequence, structure, and phylogenetic analysis

The *VDE* and *ZEP* amino acid sequences were aligned with sequences from different species using the DNAMAN program. The *VDE* and *ZEP* sequences were highly conserved and homologous to *VDE*s and *ZEP*s from other plant species (Figure 2). The conserved C- and N-terminal sequences of *VDE* were rich in glutamic acid and cysteine residues, respectively. These regions likely formed the *VDE* active site.

Schematic diagrams of the *VDE* and *ZEP* structures were drawn using the IBS1.0.1 program (Figure 3). The *VDE* sequence contained three domains (i.e., Cys-rich domain, lipocalin domain, and Glu-rich domain) and many kinase sites (i.e., three threonine kinase sites, six serine kinase sites, and one tyrosine kinase site). The *ZEP* protein mainly consisted of the FAD_binding_3 and FHA domains along with four thre-

onine kinase sites, five serine kinase sites, and one tyrosine kinase site. These two proteins lacked signal peptides and membrane-spanning domains. The secondary and tertiary protein structures were predicted (Figure 4). The *ZEP* protein consisted of 7.3% α-helices, 39.38% extended strands, and 53.32% random coils, while *VDE* contained 39.88% α-helices, 17.79% extended strands, and 42.33% random coils.

Two phylogenetic trees were constructed to investigate the genetic relationships of *VDE* and *ZEP* among various plant species (Figure 5). The strawberry *ZEP* belonged to the same group as the *ZEP*s from *Malus domestica*, *Prunus mume*, *Prunus persica*, and *Prunus armeniaca*, and was most closely related to the *Malus domestica ZEP*. Additionally, it was distantly related to the *Morus notabilis ZEP*. Similarly, the strawberry *VDE* was grouped together with the *VDE*s of *Rosaceae* species (i.e., *Prunus mume*, *Prunus persica*, and *Malus domestica*). It was more closely related to the *VDE* from *Malus domestica* than that from *Eucalyptus grandis*.

### Transcript levels of *VDE* and *ZEP* in different strawberry tissues

A qRT-PCR experiment was used to determine the *VDE* and *ZEP* expression levels in different strawberry tissues. The *VDE* and *ZEP* transcripts were detected in all analyzed tissues (Figure 6). The *VDE* transcripts were more abundant in the leaves, fruits, and stems than in the flowers and roots. The *VDE* expression level was 36-fold higher in the leaves than in the roots. In contrast, *ZEP* was more highly expressed in the leaves and fruits than in the stems, flowers, and roots, with *ZEP* transcript abundance 85-fold higher in the leaves than in the roots.

### Accumulation of Z, V, and A under high light and dark conditions

The changes in the accumulation of Z, V, and A under high light and dark conditions were characterized to investigate the responses of the xanthophyll cycle pigments to excess light in strawberry. The changes of the xanthophyll cycle components (Z, V, and A) are presented in Figure 7. The untreated leaves contained high V contents (80.15 μg $g^{-1}$), followed by Z (49.96 μg $g^{-1}$) and A (24.03 μg $g^{-1}$). Exposure to high light conditions increased the Z level, with a sharp increase observed at 10 min. The Z level clearly decreased during a 10-min incubation in darkness, and was lowest after 4 h. Unlike the changes to the Z level, the abundance of V decreased under high light conditions, but increased in darkness. Additionally, the two most obvious changes in the V levels occurred after 10 min under high light conditions and after 10 min in darkness. Regarding the changes to the A contents, there were no significant changes to the A levels under high light, while it decreased in dark conditions.

We also investigated the changes to the xanthophyll cycle pool size (i.e., A + Z + V) and the de-epoxidation state of the xanthophyll cycle [i.e., (Z + A) / (Z + A + V)] in response to different light conditions to confirm the photoprotective role of the xanthophyll cycle pigments (Figure 7). The xanthophyll cycle pool size increased after a 10-min exposure to

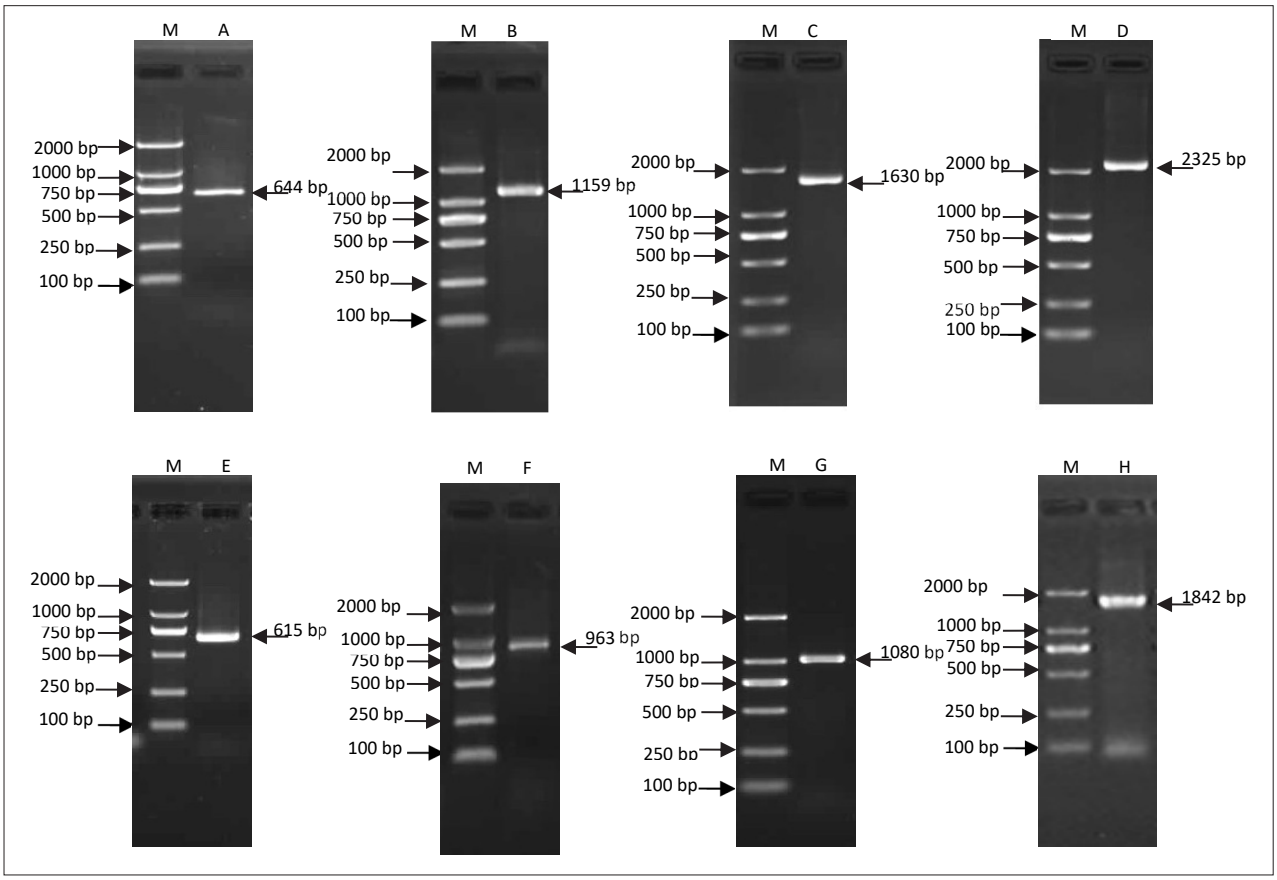



**Figure 1.** The PCR amplification results of *ZEP* and *VDE* from strawberry. M was DL2000 DNA marker. A, B, C and D were conserved region product, 3′-RACE product, 5′-RACE product and the full length product of *ZEP*, respectively. E, F, G and H were conserved region product, 3′-RACE product t, 5′-RACE product and the full length product of *VDE*, respectively.

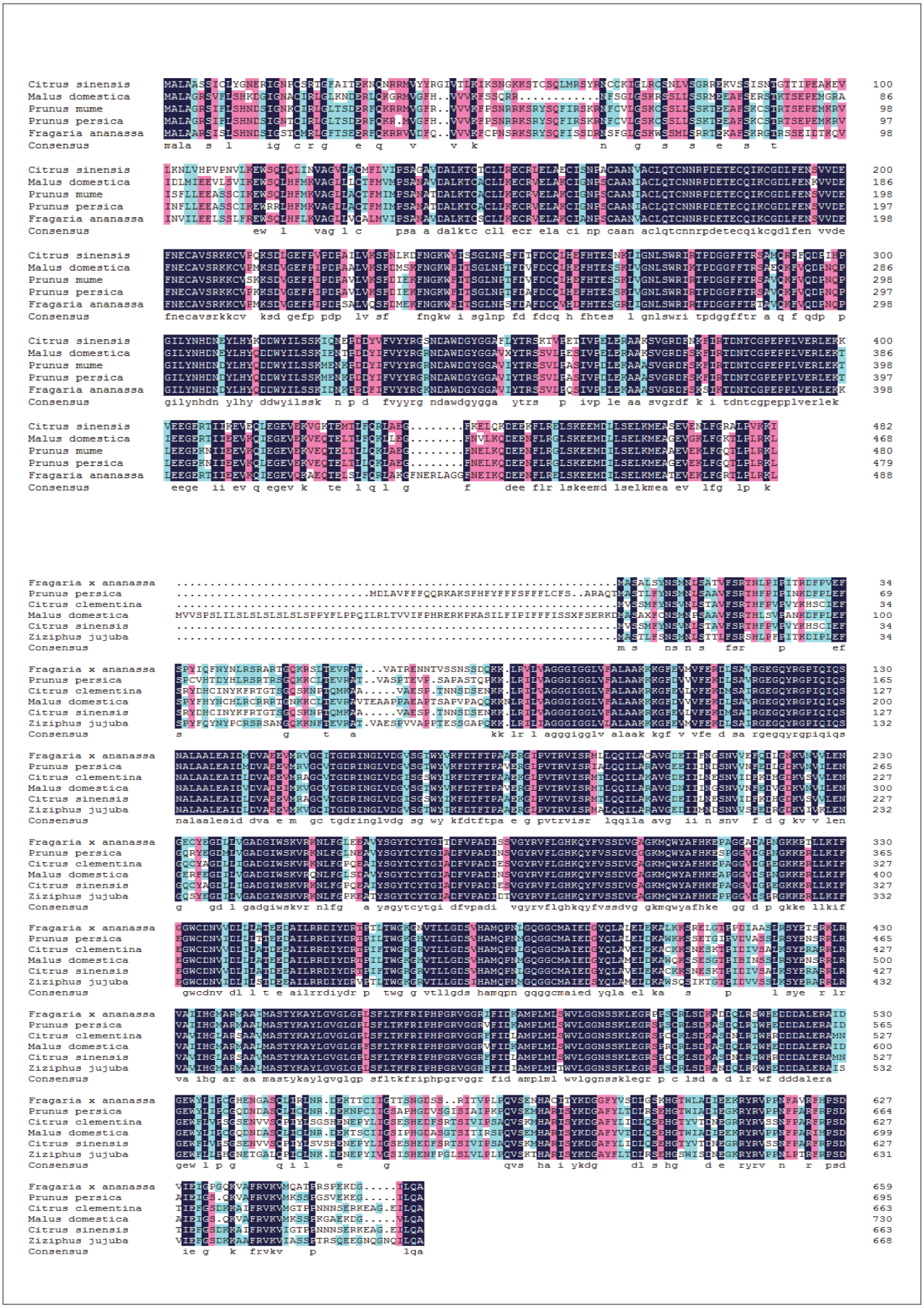



**FIGURE 2.** The above was the alignment of VDE from strawberry with other VDE proteins, and the below was the alignment of ZEP from strawberry with other ZEP proteins.

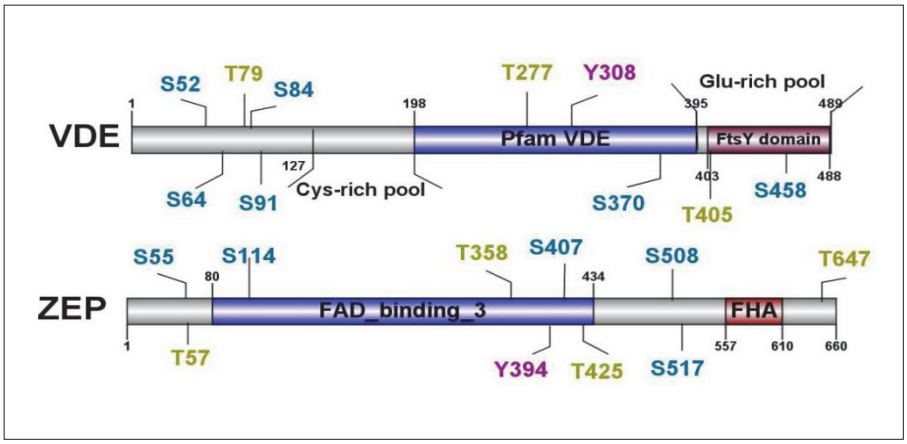



**Figure 3.** The above was the structure of the deduced VDE protein and the below was the structure of the deduced ZEP protein. VDE contained six Serine kinase sites (S52, S64, S84, S91, S370, S458), three Threonine kinase sites (T79, T277, T405) and one Tyrosine kinase site (Y308). ZEP contained five Serine kinase sites (S55, S114, S407, S508, S517), four Threonine kinase sites (T57, T358, T425, T647) and one Tyrosine kinase site (Y394).

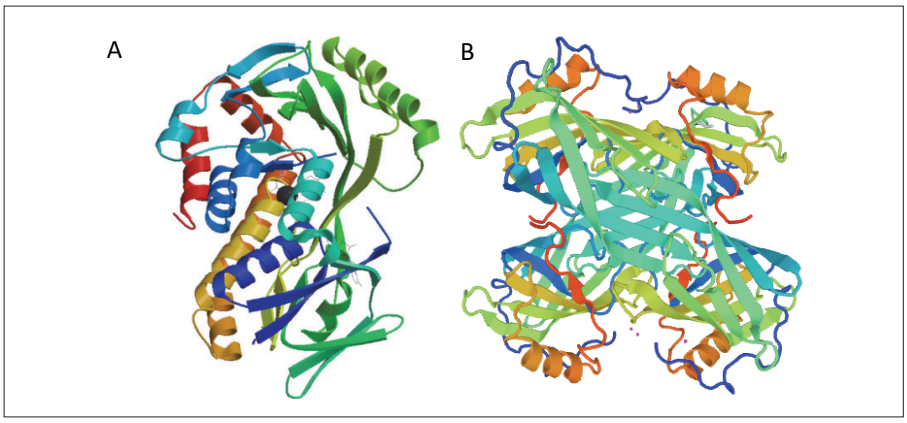



**Figure 4.** Three levels of protein structure of the deduced VDE (A) and ZEP (B).

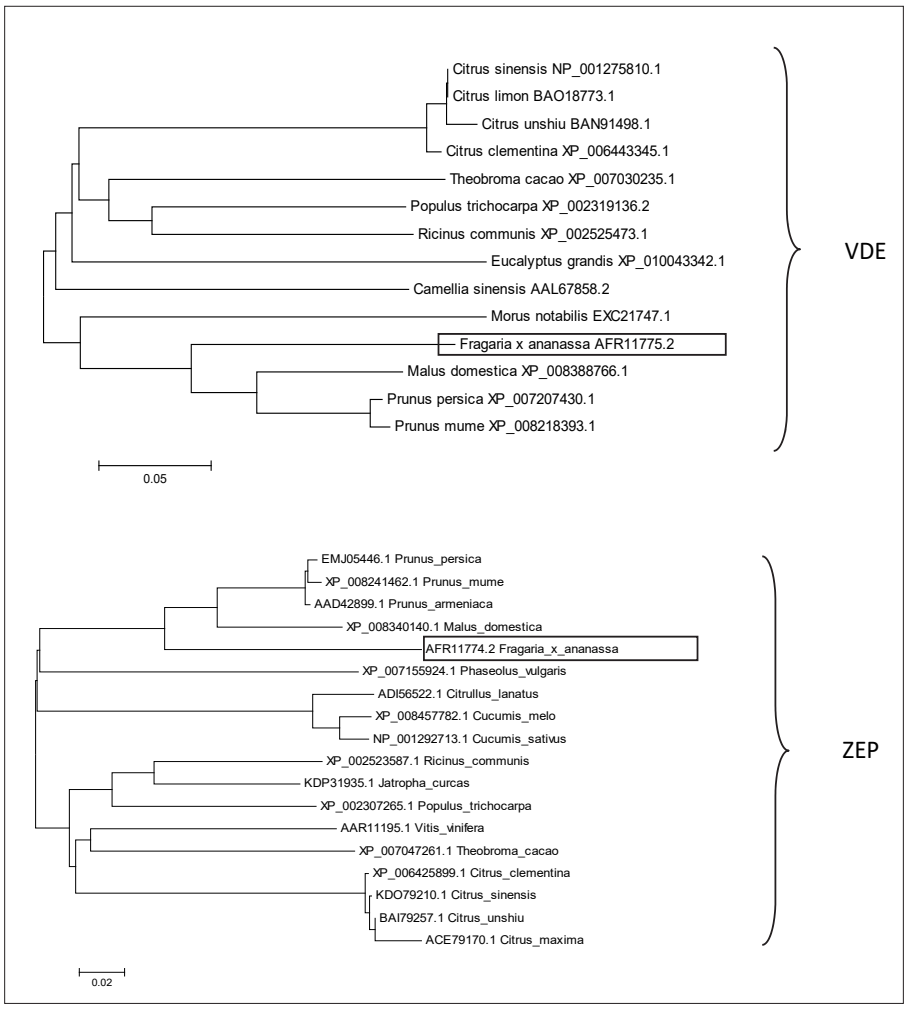



**Figure 5.** Phylogenetic trees of VDE and ZEP proteins. The strawberry proteins have been highlighted in black box.

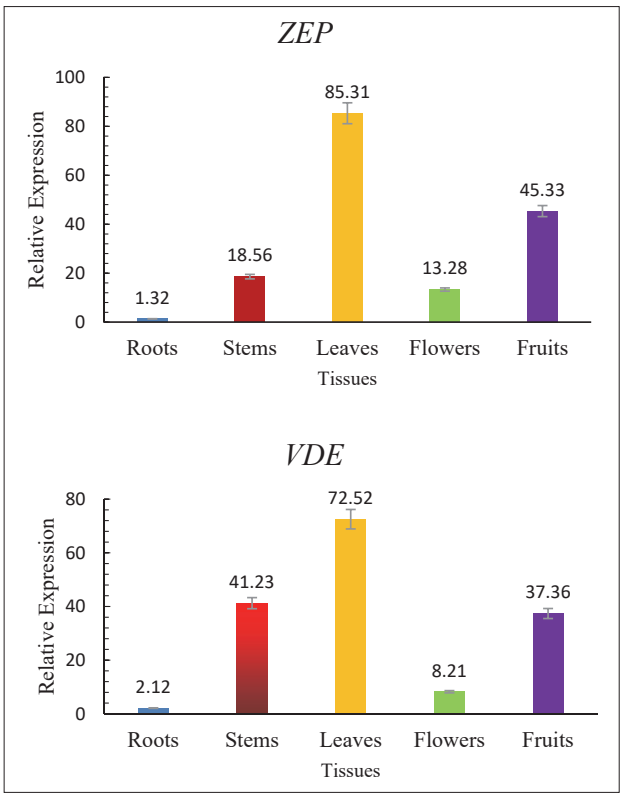



**FIGURE 6.** The expression of *VDE* and *ZEP* in different tissues of strawberry by Real-time PCR.

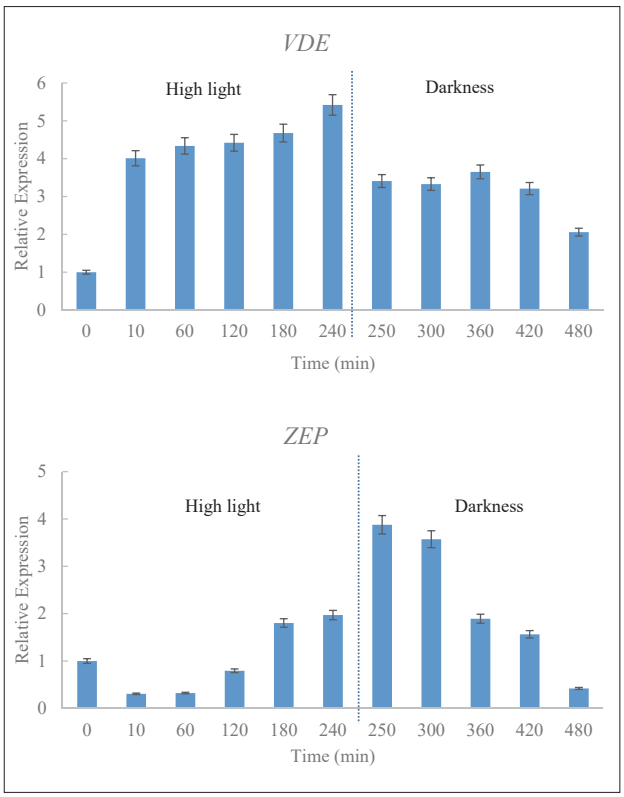



**FIGURE 7.** The expression of *VDE* and *ZEP* under high light and dark conditions in strawberries by Real-time PCR.

high light conditions, then remained high, while it decreased when samples were placed in darkness. The epoxidation state of the xanthophyll cycle exhibited a marked increase in high light conditions, but an obvious decrease in darkness. The biggest changes for the high light and dark conditions occurred during the first 10 min after treatment.

**Expression profiles of *VDE* and *ZEP* under high light and dark conditions**

The *VDE* and *ZEP* expression levels under the high light and dark conditions were characterized by qRT-PCR (Figure 8). The *VDE* expression level increased dramatically after 10 min of high light exposure, after which it continued to slowly increase over the duration of the high light treatment. When the high light stress was removed (i.e., samples were placed in darkness), the *VDE* expression level decreased immediately, with the most obvious decrease occurring after 10 min in darkness. The *ZEP* expression profile differed from that of *VDE* under high light and dark conditions. The high light treatment resulted in a decline in the *ZEP* expression level after 1 h, but it increased over the next 3 h under high light conditions. Interestingly, the highest *ZEP* expression level was observed during the first 10 min in darkness. The expression level then gradually decreased to the control level after 4 h in darkness.

## Discussion

In strawberries, most studies about the xanthophyll cycle focused on the relationship among the changes of Z, V, and A contents, thermal dissipation and fluorescence parameters, but the isolation, identification and expression of the key enzyme genes, *ZEP* and *VDE*, have not been reported prior to this study. Thus, very little was known about the molecular mechanism of the xanthophyll cycle in strawberry. In this study, *VDE* and *ZEP* were isolated from strawberry leaves for the first time, then the sequences and genes expression levels were analyzed. Highly conserved DNA sequences are thought to have functional value. The VDE sequence consisted of three highly conserved regions (i.e., Cys-rich regions, lipocalin regions, and Glu-rich regions), suggesting that they likely formed the VDE active site (Figures 2 and 3). Namely, the lipocalin regions may contain the binding sites of the VDE de-epoxidation of substrates V and MGDG in thylakoid membrane (Hieber et al., 2000), the rich glutamic acid in N-terminal can make VDE protein acidic, which accelerates the combination of VDE and thylakoid membrane to make a reaction with the substrate (Yamamoto and Higashi, 1978), and Cys-rich regions is probably not directly related to the activity of VDE, but it can reduce the inhibition of VDE inhibitor DTT on the enzyme activity (Yamamoto and Kamite, 1972; Wei et al., 2002). In addition, we observed that the genetic relationship of the ZEPs and VDEs from the same family was closer than that from the different family. As shown in Figure 5, the ZEP and VDE from strawberry were grouped together with the ZEPs and VDEs of *Rosaceae* species. Similarly, the ZEPs and VDEs belonging to *Cucurbitaceae* and *Citrus* also formed groups in phylogenetic trees respectively. It implies that they may have similar physiological and biochemical characteristics in the process of evolution. The results of a qRT-PCR analysis indicated that the *VDE* and *ZEP* transcripts were present in diverse tissues, and both genes were most highly expressed in the leaves. The lowest expression levels were observed in the roots. These findings are consistent with the results of earlier studies in wheat and cucumber (Zhang et al., 2003; Li et al., 2013). The tissue-specific expression of *VDE* and *ZEP* in strawberry plants is likely related to their roles in the xanthophyll cycle.

To investigate the responses of the xanthophyll cycle pig-

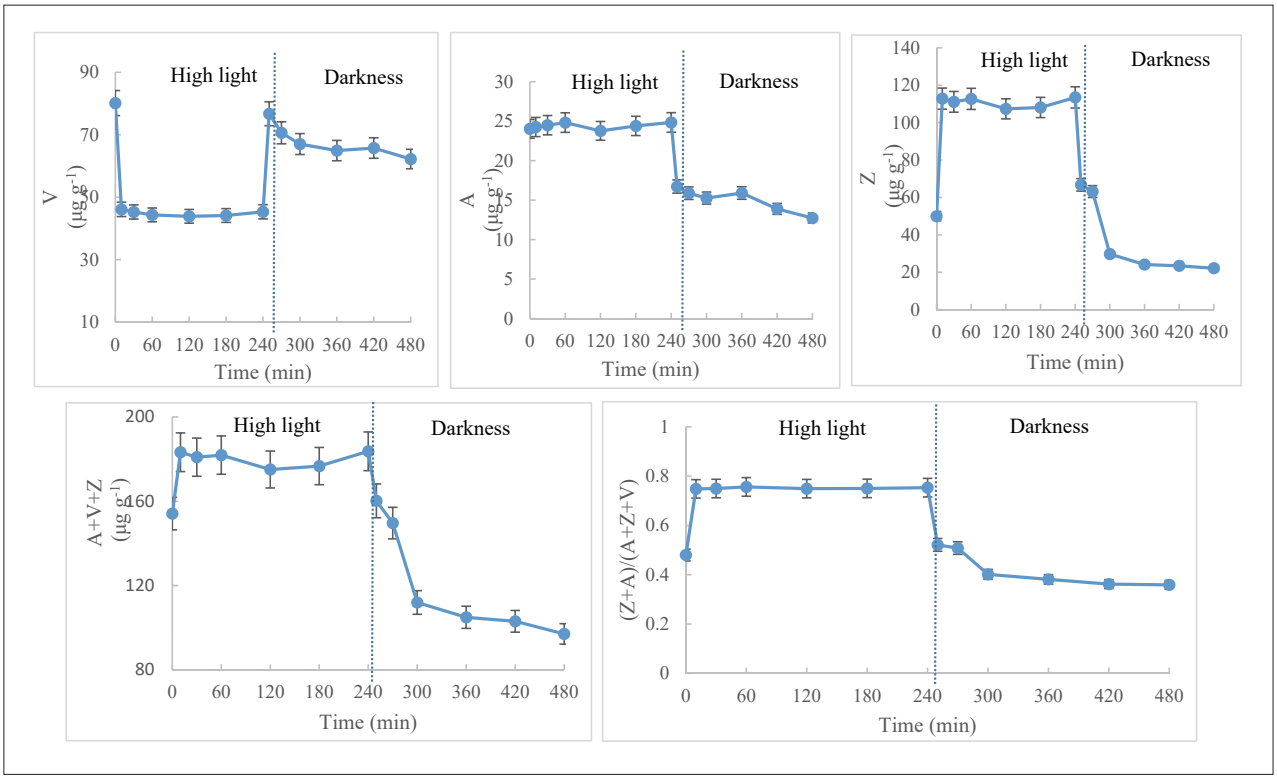



**Figure 8.** The changes of xanthophyll cycle components in strawberries under high light and dark conditions. V: violaxanthin; A: antheraxanthin; Z: zeaxanthin.

ments to high light stress in strawberry leaves, the changes in Z, V, and A contents under high light and dark conditions were characterized. Figure 7 showed that exposure to high light conditions increased Z levels, but incubation in darkness resulted in an immediate decrease in Z contents. In contrast, V levels decreased under high light conditions, but increased in darkness. Thus, the V and Z xanthophyll cycle pigments are likely interconverted under high light and dark conditions. Specifically, V is converted to Z under high light conditions, while Z is re-converted to V in darkness. Moreover, the xanthophyll cycle pool size and the epoxidation state of the xanthophyll cycle were observed to be tightly associated with the accumulation of Z. These observations implied that the mechanism of xanthophyll cycle was started up in high light stress and Z may be important for the photoprotection of strawberry leaves. As an increase in Z has been thought to be an energy dissipation mechanism that protects the photosynthetic apparatus against excess light (Demmig-Adams, 1990). Recent studies have shown that Z also quenched the excited chlorophyll in the antenna of photosystem II (PSII), and functions as an antioxidant that protects membrane lipids from peroxidation (Sujak et al., 1999; Amarie et al., 2009; Johnson et al., 2007). Interestingly, during the 4-hour high light or dark treatments, the largest changes to V and Z levels as well as the de-epoxidation state of the xanthophyll cycle [i.e., (Z + A) / (Z + A + V)] both occurred during the first 10 min, suggesting that the xanthophyll cycle of strawberry leaves may have a fast response time for photoinhibition and its recovery. This finding is consistent with the results of a previous study by Xu et al. (2005), who studied the response of strawberry leaves photosynthesis to strong light and concluded that the photoinhibition of the strawberry leaves was rapid and reversible, which is mainly related to the non-radiative energy dissipation of non-photochemical quenching. Therefore, our results demonstrated the important role of xanthophyll cycle in the photoinhibition of strawberry.

It is known that the VDE enzyme catalyzes the conversion of V to Z via A under high light conditions. Many previous studies revealed that *VDE* expression in leaves was associated with the accumulation of Z and A (Han et al., 2010; Huang et al., 2007; Lin et al., 2002). In this study, the *VDE* expression level increased dramatically after a 10-min exposure to high light conditions, after which it slowly increased for the duration of the high light treatment (Figure 8). When samples were placed in darkness, the *VDE* expression level decreased immediately, especially during the first 10 min. A strong correlation between *VDE* expression and Z accumulation suggests *VDE* may be important for Z production. In contrast, the *ZEP* expression level decreased after a 1-h exposure to high light conditions, while it increased over the following 3 h. After a 10-min incubation in darkness, the *ZEP* expression level increased, after which it gradually decreased to the control level after 4 h. The *ZEP* expression level changes during the first 10 min of a high light or dark treatment were consistent with the observed accumulation of V. The subsequent *ZEP* expression patterns were consistent with the increases in Z content. These findings suggest that the regulation of *ZEP* may be related to the accumulation of V and Z in strawberry leaves. The function of *ZEP* gene in xanthophyll cycle was also confirmed in tobacco, *Arabidopsis thaliana* and tomato (Audran et al., 1998; Niyogi, 2000; Wang et al., 2008). Above all, *VDE* and *ZEP* may be the key genes in regulating the accumulation of the xanthophyll cycle pigments (i.e., A, Z and V). Under high light stress, the xanthophyll cycle mechanism was started up. *VDE* quickly controlled the conversion of V to Z, and Z contents and the de-epoxidation state of the xanthophyll cycle [i.e., (Z + A) / (Z + A + V)] increased, which accelerated the dissipation of excess light energy in

order to protect against photoinhibition. While when leaves were placed in darkness, the photoinhibition alleviated, Z is reversibly converted to V by the regulation of ZEP.

The photoinhibition of photosynthesis in strawberry leaves in the field conditions was obvious. Moderate shade is helpful for solving such problems in actual cultivation. However, shading on photosynthesis of strawberry has its advantages and disadvantages. A previous study of Liu et al. (2006) showed that shading indeed had a protective effect on PSII reaction center and alleviated photosynthetic photoinhibition, but it also lead to a decline in the carboxylation efficiency (CE) and a rise in $CO_2$ compensation point. Chi et al. (2001) found that shading also reduced the net photosynthetic rate (Pn), the electron transport activity of PSII and 1.5-diphosphate ribulose carboxylase activity. So improving the plant adversity-resistant ability seems to be more crucial. In this study, we demonstrated the important role of xanthophyll cycle in photoinhibition of strawberry leaves and preliminarily clarified the molecular mechanism of the cycle. The results may be relevant for clarifying the photoprotection mechanism and stress physiology in strawberry.

## Conclusions

In this study, we observed that the xanthophyll cycle pigments were interconverted under high light and dark conditions. Specifically, V was converted to Z in leaves exposed to high light conditions. When samples were placed in darkness, Z was converted back to V. Two xanthophyll cycle genes (i.e., *VDE* and *ZEP*) were isolated from strawberry leaves. Their expression levels were related to the interconversion between V and Z. In strawberry leaves, the rapid response of xanthophyll cycle components to high light stress appears to be regulated by changes to gene expression levels.

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