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ORIGINAL ARTICLE

Light and abiotic stresses regulate the expression of GDP-L-galactose phosphorylase and levels of ascorbic acid in two kiwifruit genotypes via light-responsive and stress-inducible *cis*-elements in their promoters

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Abstract Ascorbic acid (AsA) plays an essential role in plants by protecting cells against oxidative damage. GDP-L-galactose phosphorylase (*GGP*) is the first committed gene for AsA synthesis. Our research examined AsA levels, regulation of *GGP* gene expression, and how these are related to abiotic stresses in two species of *Actinidia* (kiwifruit). When leaves were subjected to continuous darkness or light, ABA or MeJA, heat, or a hypoxic environment, we found some correlation between the relative levels of *GGP* mRNA and AsA concentrations. In transformed tobacco plants, activity of the *GGP* promoter was induced by all of these treatments. However, the degree of inducibility in the two kiwifruit species differed among the *GGP* promoter deletions. We deduced that the G-box motif, a light-responsive element, may have an important function in regulating *GGP* transcripts under various light conditions in both *A. deliciosa* and *A. eriantha*. Other elements such as ABRE, the CGTCA motif, and HSE might also control the promoter activities of *GGP* in kiwifruit. Altogether, these data suggest that *GGP* expression in the two kiwifruit species is regulated by light or abiotic stress via the relative *cis*-elements in their promoters. Furthermore, *GGP* has a critical role in modulating AsA concentrations in kiwifruit species under abiotic stresses.

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Abbreviations

ABA	Abscisic acid
AsA	Ascorbic acid
GGP	GDP-L-galactose phosphorylase
GUS	β-Glucuronidase
MeJA	Methyl jasmonate
RT-qPCR	Quantitative reverse transcription-polymerase chain reactions
4-MU	4-Methylumbelliferone

Introduction

Ascorbic acid (AsA), commonly known as vitamin C, is an antioxidant and an essential enzyme cofactor in hydroxylation and other reactions (De Tullio and Arrigoni 2004). As such, it is vital for detoxifying the free radicals generated under stress conditions (Conklin and Barth 2004). Additionally, AsA is an critical component of the cellular reduction/oxidation hub, which buffers the production of reactive oxygen species (ROS) while allowing for crucial signaling to regulate plant growth and defenses (Gong et al. 2007; Chaouch et al. 2010). Synthesis of AsA can be induced for protection when plants are exposed to environmental stresses, including ozone (Conklin and Barth 2004), high temperatures (Larkindale et al. 2005; Ma et al. 2008), salt (Huang et al. 2005), and high light intensity (Müller-Moulé et al. 2004). Elucidating the regulatory mechanism that functions under such conditions is important to our understanding of how AsA works in conferring plant resistance to oxidative stress.

GDP-L-galactose phosphorylase (*GGP*: EC 2.7.7.69) catalyzes the conversion of GDP-L-galactose to L-galactose-1-P in the first committed step of the Smirnoff–Wheeler pathway for plant AsA synthesis (Wheeler et al. 1998). The protein product of *GGP* from *Arabidopsis thaliana* is broadly conserved in plants and in animals (Linster et al. 2007). The *vtc2* mutant, encoding *GGP* in *Arabidopsis*, is impaired in AsA synthesis and has less abundant AsA (Linster et al. 2007). *GGP* genes might be a key rate-limiting step for AsA biosynthesis in *Arabidopsis* (Bulley et al. 2009), tobacco (Laing et al. 2007), and apple (Mellidou et al. 2012). Their expression is also correlated with a peak in AsA accumulations in all kiwifruit genotypes; the highest expression is found in *Actinidia eriantha*, the genotype with the greatest amount of AsA (Bulley et al. 2009). The results of *GGP* over-expression via either stable transformation in *Arabidopsis* or transient expression in tobacco leaves have confirmed the hypothesis that these genes catalyze a major control point of AsA biosynthesis through the L-galactose pathway (Bulley et al. 2009).

Evaluations of oxidative stress have demonstrated the relationship between *GGP* and AsA. Stressed plants show increased transcript abundance of all enzymes for the AsA recycling pathway (ascorbate–glutathione system) (Gill and Tuteja 2010), as well as elevated *GGP* mRNA levels and activity (Dowdle et al. 2007). Moreover, *vtc2* mutant plants with reduced AsA concentrations have low resistance to oxidative stress (Müller-Moulé et al. 2004; Dowdle et al. 2007). Previous studies have indicated that the increase in AsA concentrations within *Arabidopsis thaliana* leaves subjected to 24 h of high light is accompanied by enhanced *GGP* expression and activity (Dowdle et al. 2007). Light induction of *GGP* mRNA has also been independently confirmed (Yabuta et al. 2007; Müller-Moulé 2008). In addition, AsA levels in tomato fruits tend to decline under shading, a response that could be linked to a reduction in *GGP* expression (Massot et al. 2012). Recombinant *Chlamydomonas reinhardtii* cells exposed to oxidative stress also show increased levels of *GGP* mRNA and AsA (Urzica et al. 2012). Transcripts of tomato *GGP* are increased dramatically 3 h after plants are transferred from anoxic conditions to air, but no similar change is seen in response to heat stress (Ioannidi et al. 2009). Despite these previous studies, however, it remains unclear how plants control *GGP* expression and synthesize AsA under different environments, especially in the presence of abiotic stress.

To gain a better understanding of the relationship between AsA concentrations and transcriptional levels of two *GGP* genes, and to investigate the regulatory mechanisms for their expression in kiwifruit under stress conditions, we performed a systematic investigation of AsA, mRNA expression, and promoter activities in response to various treatments. For this, we evaluated two species that produce different amounts of AsA.

Materials and methods

Plant materials

Two-year-old plants of kiwifruit seedlings (*Actinidia deliciosa* 'qinmei' and *A. eriantha*; obtained from kiwifruit repository of Northwest A&F University) were placed in plastic pots (250-mm diam.) filled with a 5:1 (v:v) medium of local topsoil and sand and cared for at the Horticultural Experimental Station of Northwest A&F University. Two weeks before the experiment, they were transferred to a controlled-environment growth chamber under a 16-h photoperiod, 65 % relative humidity, and a 25 °C/21 °C (day/night) temperature cycle. At each collection time point, three leaves were sampled from three kiwifruit plants, respectively, total three replications.

Plants of tobacco (*Nicotiana tabacum* 'NC89') were grown in the same controlled environment. The 'NC89' tobacco seeds were provided by Professor Weixing Shan (Northwest A&F University). When the plants were 6 weeks old, they were used for monitoring GUS activity. At each collection time point, three replications were made by three plants, with each involving three leaves from the same plant.

Nucleic acid extractions, gene cloning, and mRNA expression analysis

Total RNA was isolated from mature leaf samples by the LiCl precipitation method (Asif et al. 2000). To clone the entire cDNA sequence of *GGP* genes *AdGGP* and *AeGGP*, we designed primers *GGP*-F and *GGP*-R (Table 1). Polymerase chain reaction (PCR) products were cloned into the pMD-18T vector (Takara, Dalian, China), and several clones were sequenced for each reaction. Expression was evaluated by quantitative reverse transcription-polymerase chain reactions (RT-qPCR), which were performed on an iQ5.0 instrument (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green qPCR kits (Takara) according to the manufacturer's instructions. Primer specificity (*GGP*-RT-F and *GGP*-RT-R) (Table 1) was determined by RT-PCR and melting-curve analysis. Constitutive *Actin* served as the endogenous control (Li et al. 2010). Data from the individual runs were collated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen 2001), with iQ5 2.0 standard optical system analysis software. Values for mean expression and standard deviation (SD) were calculated from the results of three independent experiments.

Table 1 Primer sequences used in this study

Primer	Sequence (5′–3′)
GGP-F	5′-ATGTTGAAGATCAAGAGGGTTCC-3′
GGP-R	5-TTCTCAGTGCTGAACTAGGCATTCT-3′
GGP-RT-F	5′-AGGCAACAACATACCTTACAAT-3′
GGP-RT-R	5′-CTTCAGACGCCTCCTGATAGT-3′
Actin-F	5′-GCTTACAGAGGCACCACTCAACC-3′
Actin-R	5′- CCGGAATCCAGCACAATACCAG -3′
Ad-F	5′-TCTTCCTGCGGCTGACTCGCATGAA-3′
Ad-R	5′-AATTGGAAACAACAGTCGG-3′
Ae-F	5′-ATGCCATCTTCCTGCGGCTGAC-3′
Ae-R	5′-GCAGGACCTTATCAACACGAAACTC-3′
Ad-DF-1	5′-CCCAAGCTTTCTTCCTGCGGCTGACTCGCATGAA-3′
Ad-DF-2	5′-CCCAAGCTTTCACTCTTCTTTGCCTTCTCGGT-3′
Ad-DF-3	5′–CCCAAGCTTACTGGCGGAATGTTCACGGGGTT-3′
Ad-DF-4	5′-CCCAAGCTTCCAAAGTCTACACAGAATCAACC-3′
Ad-DF-5	5′-CCCAAGCTTACAAGTTGTAGACATCACGGCTA-3′
Ad-DF-6	5′-CCCAAGCTTGGTCACTTCGTTTTCTCCGTCTA-3′
Ad-DR	5′-CCGGAATTCCTCGAACTCAGGAAACGCAAAAACA-3′
Ae-DF-1	5′-CCCAAGCTTATGCCATCTTCCTGCGGCTGAC-3′
Ae-DF-2	5′-CCCAAGCTTCTGAAACCGACACCTTCTGATT-3′
Ae-DF-3	5′-CCCAAGCTTACTGGCGGAATGTTCACGGGGT-3′
Ae-DF-4	5′-CCCAAGCTTCTCACCCCCAAAGTCTACACAG-3′
Ae-DF-5	5′-CCCAAGCTTTAGACATCACGGCTATATTCGG-3′
Ae-DF-6	5′-CCCAAGCTTACTTCGCTTTCTCCGTCTACTA-3′
Ae-DR	5′-CCGGAATTCCTCGAACTCAGAAAACGCAAAAAC-3′

Underlined sequences show restriction enzyme sites

Detection of ascorbic acid concentrations

Frozen and homogenized leaf samples (0.2 g fresh weight) were suspended in 2 ml of cold 6.0 % (v/v) $HClO_4$ and centrifuged at 15,294*g* for 10 min at 4 °C. Their AsA concentrations were determined by the ascorbate oxidase method, essentially as described previously (Tokunaga and Esaka 2007).

Isolation of the *GGP* promoters

Genomic DNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in sterile water. The fragments of the upstream sequences for *GGP*s from *A. deliciosa* and *A. eriantha* were isolated with primers *Ad*-F/*Ad*-R and *Ae*-F/*Ae*-R, respectively (Table 1). PCR products were cloned into the pMD-18T vector, and several clones were sequenced for each reaction. The *GGP* promoter sequences from *A. deliciosa* and *A. eriantha* were named *PadGGP* and *PaeGGP*, respectively. The promoter sequences were analyzed via the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002).

Construction of *PadGGP*::*GUS* and *PaeGGP*::*GUS* fusion vectors

Two expression vectors, pC0390*GUS* and pC35S*GUS* (Xu et al. 2010), were constructed for transient expression assays. A series of nested 5′ deletions of *PadGGP* fragments were generated via PCR amplifications. Six forward primers (*Ad*-DF1 through *Ad*-DF6; Table 1) and one reverse primer (*Ad*-DR; Table 1) were designed to correspond to the −1,112, −879, −741, −487, −327, and −155 sequences of the *AdGGP* promoter. In addition, a series of nested 5′ deletions of *PaeGGP* fragments were generated by PCR amplifications. Six forward primers (*Ae*-DF1 through *Ae*-DF6; Table 1) and one reverse primer (*Ae*-DR; Table 1) were designed to correspond to the −1116, −904, −734, −502, −320, and −152 sequences of the *AeGGP* promoter. A *Hin*dIII restriction enzyme site (underlined sequences) was introduced at the 5′ end of each of the forward primers, while an *Eco*RI site (underlined) was added to the 5′ end of the reverse primer (Table 1). Each of the promoter fragments was double-digested with *Hin*dIII/*Eco*RI, and ligated into the *Hin*dIII/*Eco*RI site of vector pC0390*GUS*. Fusion constructs, verified by sequencing, were introduced

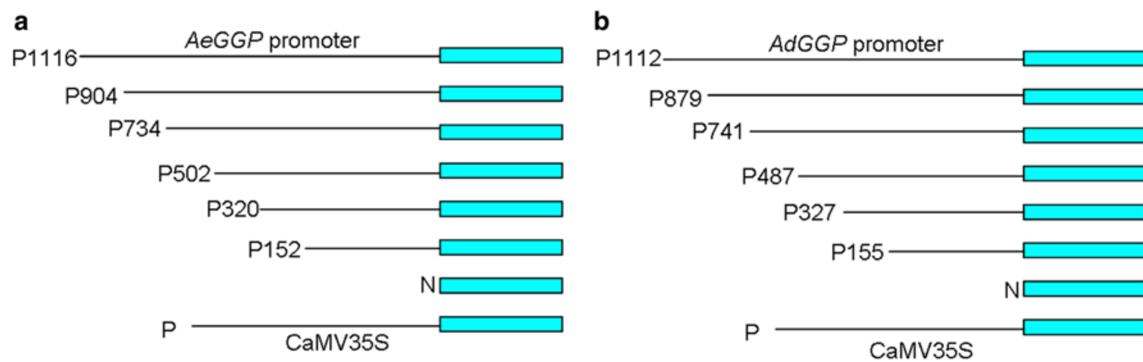

Fig. 1 Schematic diagram of vector constructions for *GGP* promoter deletions in *Actinidia eriantha* (*Ae*, **a**) and *A. deliciosa* (*Ad*, **b**)

into *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method. Schematic of the promoter deletions was shown in Fig. 1.

Agrobacterium-mediated transient assays and GUS activity analysis in tobacco plants

Agrobacterium-mediated transient assays were performed as described previously (Sparkes et al. 2006). Fully expanded, infiltrated leaves were collected from intact tobacco plants representing each construct and were used to determine GUS activity. Quantitative GUS assays were performed as described previously (Jefferson 1987). Fluorescence of the methylumbelliferone products was quantified with a Hitachi 850 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The total concentration of protein extracts from tested samples was normalized by an established protocol (Bradford 1976). GUS activity was expressed as the nM of 4-methylumbelliferone (4-MU; Sigma-Aldrich) generated per minute per milligram of soluble proteins.

Abiotic treatments with *Actinidia* and *Agro*-infiltrated tobacco plants

For the kiwifruit plants, illumination treatments included exposure at 25 °C for 48 h (continuous dark), 25 °C for 48 h under 200 μmol m^{-2} s^{-1} (continuous light), or 25 °C under a 16-h photoperiod (200 μmol m^{-2} $s^{-1)}$ as the control. For the hormonal treatments, 2-year-old kiwifruit plants were sprayed for 48 h with sterile water (control) or either 100 μM ABA or 100 μM MeJA. The effect of high temperature was tested by exposing infiltrated plants to 42 °C (heat) or 25 °C (control). In the hypoxia (waterlogging) test, the plants were submerged in a large container filled with water. The light regime for the hormonal, heat and waterlogging treatments was the same with the control. For all treatments, samples were taken at 0, 12, 24, 36, and 48 h to determine AsA concentrations and gene expression in kiwifruit plant leaves.

At 48 h after infiltration, tobacco plants were treated for another 24 h with darkness, light (200 μmol m^{-2} s^{-1}), ABA (100 μM), MeJA (100 μM), heat (42 °C), or induced hypoxia. The methods were the same as those applied to the kiwifruit plants. Afterward, leaf samples from all treatments were frozen and homogenized for assessing GUS activity.

Results

Responses by kiwifruit to abiotic stresses as manifested in *GGP* transcript levels and AsA concentrations

As illustrated in Fig. 2a and b, qRT-PCR analyses of the two *Actinidia* species demonstrated that *GGP* expression in the leaves was increased by light but decreased by darkness. Applications of ABA decreased expression of *AdGGP* (Fig. 2c). However, applications of ABA for *A. eriantha* seemed to increase *AeGGP* expression at first before it was decreased (Fig. 2d). A similar expression pattern for *GGP* was seen from the MeJA (Fig. 2c, d) or hypoxia treatment in both kiwifruit species (Fig. 2g, h). Exposing *A. eriantha* plants to heat decreased *AeGGP* transcription and declining to the lowest level by Hour 24, then *AeGGP* expression began to increase (Fig. 2e). However, for *A. deliciosa*, transcript levels showed a slight increase and then began to decrease after such treatment (Fig. 2f).

A similar trend in response to light or dark conditions was found in our results with AsA, compared with the expression of *GGP*. Although levels began to increase gradually after 12 h of continuous light, they were decreased gradually after 12 h of continuous darkness, regardless of species (Fig. 3a, b). Treatment with ABA or MeJA caused AsA concentrations to increase in the leaves, peaking at 24 h before decreasing (Fig. 3c, d), but we found a short

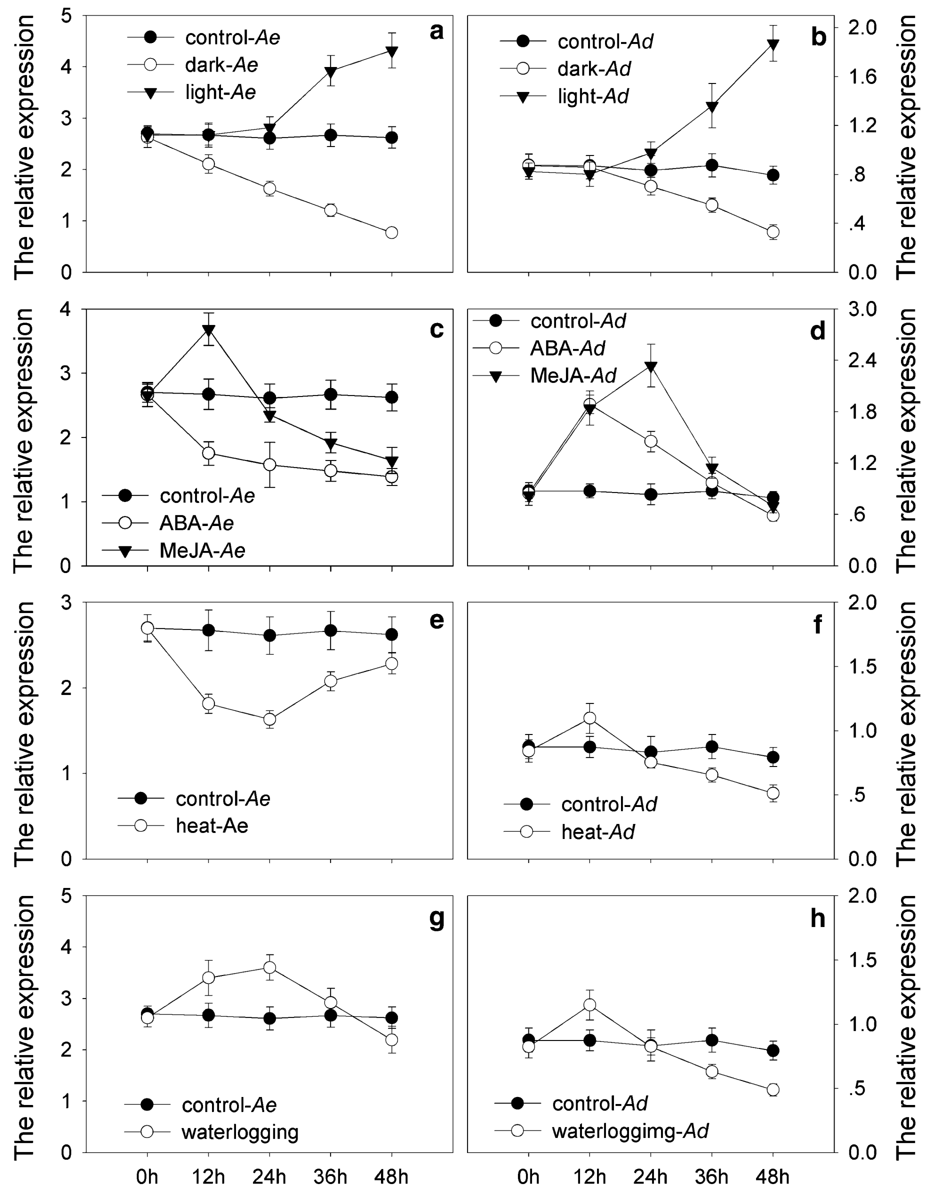

Fig. 2 Relative expression of *GGP* in kiwifruit leaves under abiotic treatments versus normal growing conditions (control). *Ae*, *A. eriantha*; *Ad*, *A. deliciosa*. **a**, **b** Light and dark treatment. **c**, **d** ABA and MeJA treatment. **e**, **f** Heat treatment. **g**, **h** Waterlogging treatment. Light period is 6:00 a.m. to 10:00 p.m. in a day in the chamber. The samples were collected at 8:00 a.m. and 8:00 p.m., respectively. For each sample, transcript levels were normalized relative to those of *Actin*, and the relative expression levels of each gene were obtained using the ddCT method. Values were averaged from three independent experiments. SD is indicated at each point

decline during the early stages of ABA for *A. eriantha* (Fig. 3c). When the plants were exposed to heat, AsA concentrations first began to rise before declining in *A. deliciosa* (Fig. 3f). However, AsA concentrations decreased in *A. eriantha* through the whole heat treatment time (Fig. 3e). During the early stages of hypoxia for *A. deliciosa*, AsA production was increased and reaching its highest level at Hour 12 before decreasing rapidly (Fig. 3h). By contrast, those levels in *A. eriantha* showed a gradual and continuous decline throughout the hypoxic period (Fig. 3g).

Cloning of *GGP*s and isolation of the promoter regions

The cloned cDNAs of both genes—*AeGGP* (GenBank Accession No. KC146049) and *AdGGP* (GenBank Accession No. GU339036)—encoded a polypeptide of 451 amino acids. Using the MegAlign program of MEGA, we determined that these amino acid sequences shared 97.1 % identity. *GGP* is a member of the GalT/Apa1 nucleoside monophosphate transferase branch of the histidine triad (HIT) protein superfamily (Brenner 2002).

To elucidate whether the differential expression patterns of *AeGGP* and *AdGGP* were correlated with the regulation of their promoters, we compared the genomic sequences upstream of *AeGGP* (GenBank Accession No. KC146047) and *AdGGP* (GenBank Accession No. KC146046) and found that these isolated regions were 1,116 and 1,112 bp long, respectively. Sequence alignment revealed that the *AeGGP* promoter was 90.5 % identical to the *AdGGP* promoter (Fig. 4). A detailed analysis (Supplementary Table

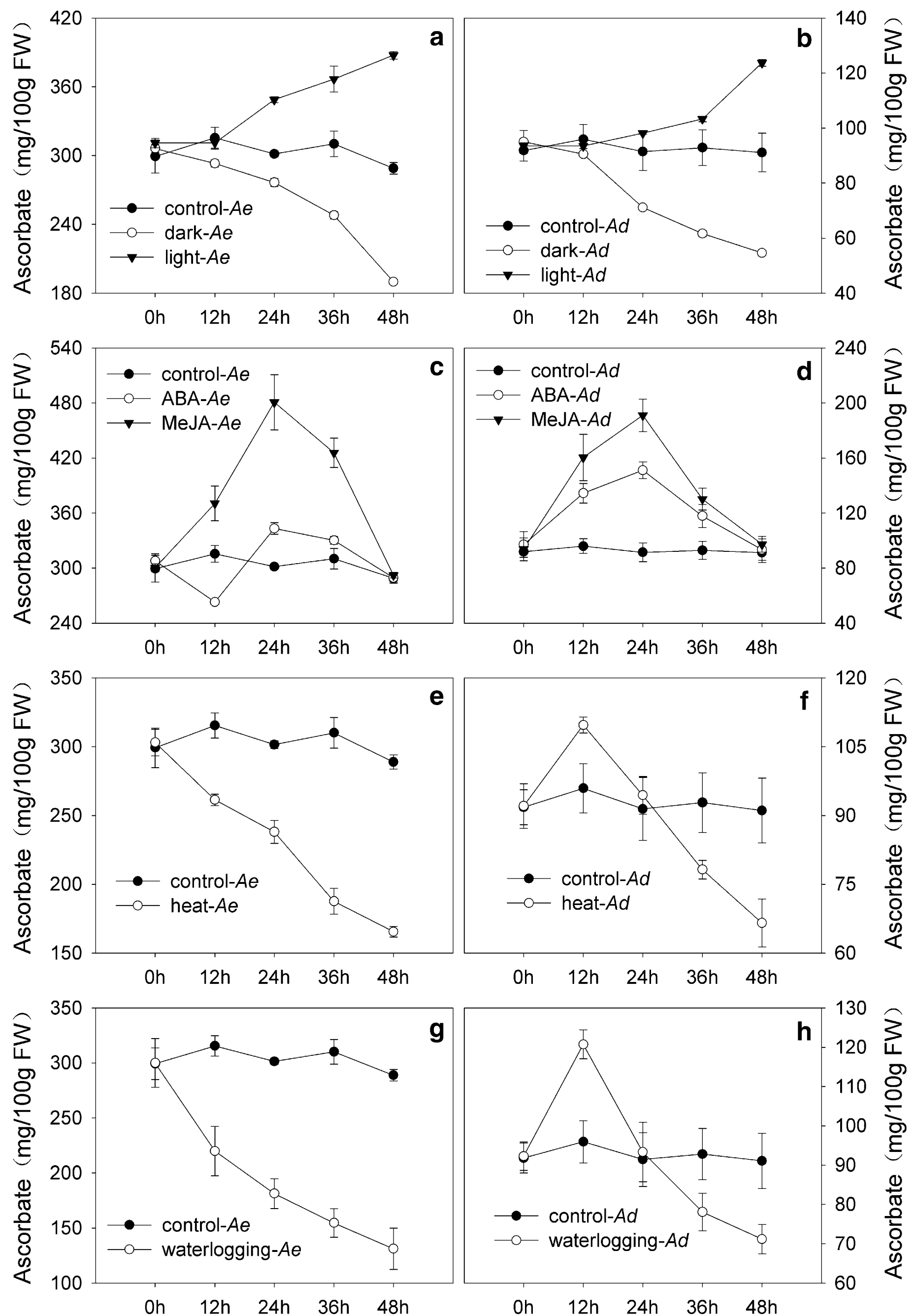

Fig. 3 Ascorbate concentrations in kiwifruit leaves under abiotic treatments versus normal growing conditions (control). *Ae*, *A. eriantha*; *Ad*, *A. deliciosa*. **a**, **b** Light and dark treatment. **c**, **d** ABA and MeJA treatment. **e**, **f** Heat treatment. **g**, **h** Waterlogging treatment. Values were averaged from three independent experiments. SD is indicated at each point

S1) of the *cis*-regulatory elements within their promoters enabled us to classify them into three functional groups: light-responsive elements (LREs), hormone-responsive elements (HREs), and defense and stress-responsive elements (DSREs). The LREs consisted of ATC motif (Phillips et al. 1999), Box I (Arguello-Astorga and Herrera-Estrella 1996), chs-CMA2b (Arguello-Astorga and Herrera-Estrella 1996), G-box (Foster et al. 1994), etc. The HREs included abscisic acid (ABA)-responsive elements (ABRE) (Baker et al. 1994), MeJA-responsive elements (CGTCA motif and TGACG motif) (Fink et al. 1988), gibberellin (GA_3)-responsive elements (GARE motif and P-box) (Pastuglia et al. 1997), auxin-responsive elements (TGA-box) (Liu et al. 1994), and elements involved in the regulation of zein metabolism (O_2 site) (Wu et al. 1998). The DSREs consisted of anaerobic-responsive element (ARE) (Walker et al. 1987), hypoxia-responsive element (GC motif) (Dolferus et al. 1994), heat stress-responsive element (HSE) (Pastuglia et al. 1997), MYB binding site (MBS) (Nash et al. 1990), defense and stress-responsive element (TC-rich repeats) (Diaz-De-Leon et al. 1993), high transcription level-related element (5UTR Py-rich stretch)

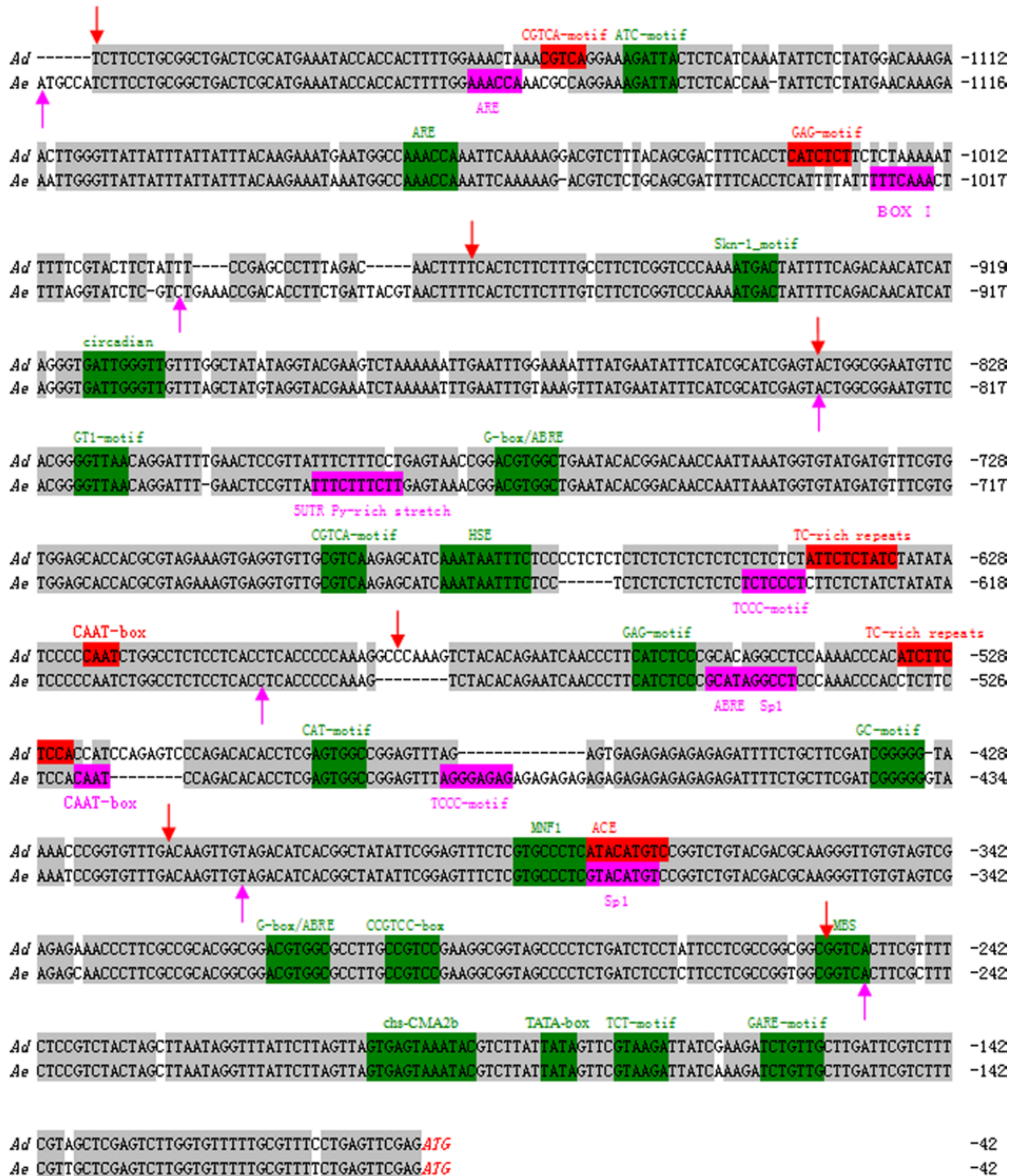

Fig. 4 Comparison of *GGP* promoter sequences between two kiwifruit species. Translational start sites (+1) are shown in *red italic type*. *Coordinates* indicate nucleotide positions relative to translation start (ATG) site. Motifs with significant similarity to previously identified *cis*-acting elements in both species are shaded in *green*; *names above elements* are in *green* color. Motifs found only in *AeGGP* or *AdGGP* promoters are shaded in *pink* or *red*, respectively. *Arrowheads* represent start point of 5′-deleted derivatives. *Ae*, *A. eriantha*; *Ad*, *A. deliciosa*

(Daraselia et al. 1996). Further comparison of the *cis*-regulatory elements in these two species revealed that *AeGGP* had more 5UTR Py-rich stretch elements in its upstream regions (13, versus 8 for *AdGGP*). The promoter of *AeGGP* gene also had other LREs, such as SP1 and the TCCC motif (Arguello-Astorga and Herrera-Estrella 1996). Of

particular interest with *AdGGP* were two TC-rich repeats that were unique to its promoter sequence.

Responsiveness of *GGP* promoters to signaling molecules and abiotic stress

To test the inducible activities of the *AeGGP* and *AdGGP* promoters and locate the responsive *cis*-regulatory region, we prepared a series of deletions of those promoters and fused them to the promoterless *GUS* reporter gene. Each construct was introduced into tobacco leaves and tested for GUS activity after various treatments. When the plants were growing under normal conditions, GUS activity was highest throughout the P1116 of the *AeGGP* promoter, rather than in the entire P1112 of the *AdGGP* promoter.

Compared with the control (16-h photoperiod), GUS activities for all *GGP* promoter deletions were lowest after 24 h of darkness (Fig. 5a, b). Only promoter P152 for *AeGGP* and P155 for *AdGGP* failed to show significant declines compared with the controls when under a normal photoperiod. After 24 h of continuous light, GUS activities were significantly induced in the overwhelming majority of deletions. For the promoter deletions of *AeGGP*, GUS activity in the promoter region from −1,116 to −320 was significantly induced when compared with that of the control (Fig. 5c). For the promoter deletions of *AdGGP*, GUS activity in the promoter region from −1,112 to −327 was significantly induced over that of the control (Fig. 5d). An exception, however, was found with the P487 construct.

When compared with results from the sterile water-only treatment, both ABA and MeJA induced activities by the *GGP* promoters. For *AeGGP*, ABA-inducible promoter activities were detected in tobacco leaves harboring the P1116, P734, and P502 constructs (Fig. 6a). In contrast, MeJA-inducible promoter activities were detected only in the P734 deletion (Fig. 6c). For *AdGGP*, ABA significantly induced GUS activities in the P741, P487, and P327 constructs (Fig. 6b), whereas MeJA induced GUS activities in P1112, P879, and P741 (Fig. 6d).

With regard to heat or waterlogging treatments, GUS activities were induced to various degrees. For the promoter deletions of *AeGGP*, a rise in temperature resulted in an increase in activity in construct P734 (Fig. 7a), whereas waterlogging induced activity in P1116, P904, P734, and P502 (Fig. 7c). For *AdGGP*, constructs P741 and P487 showed significant induction when heat was applied (Fig. 7b). Constructs P1112, P741, and P487 were significantly induced when plants were under hypoxic conditions (Fig. 7d).

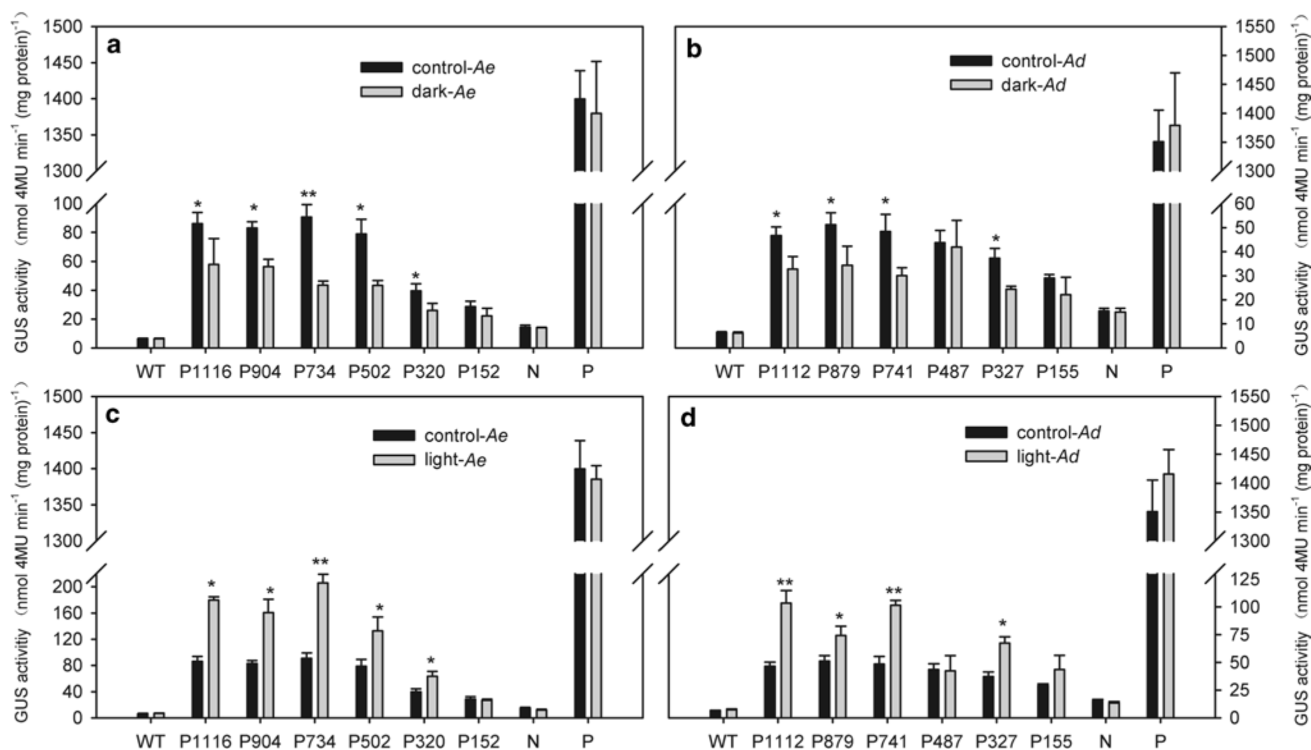

Fig. 5 Analysis of GUS activity from promoters in transiently transformed tobacco leaves in response to dark or light treatment. Dark-*Ae* (**a**), dark-*Ad* (**b**), light-*Ae* (**c**), and light-*Ad* (**d**). *Ae*, *A. eriantha*; *Ad*, *A. deliciosa*. Mean activity was averaged from three independent experiments. SD is indicated on each *bar*. Significant difference between treatment and control was assessed by one-sided paired *t* tests (**$P < 0.01$, *$P < 0.05$). *WT* wild type (no expression), *N* negative control (no promoter), *P* positive control (CaMV 35S promoter)

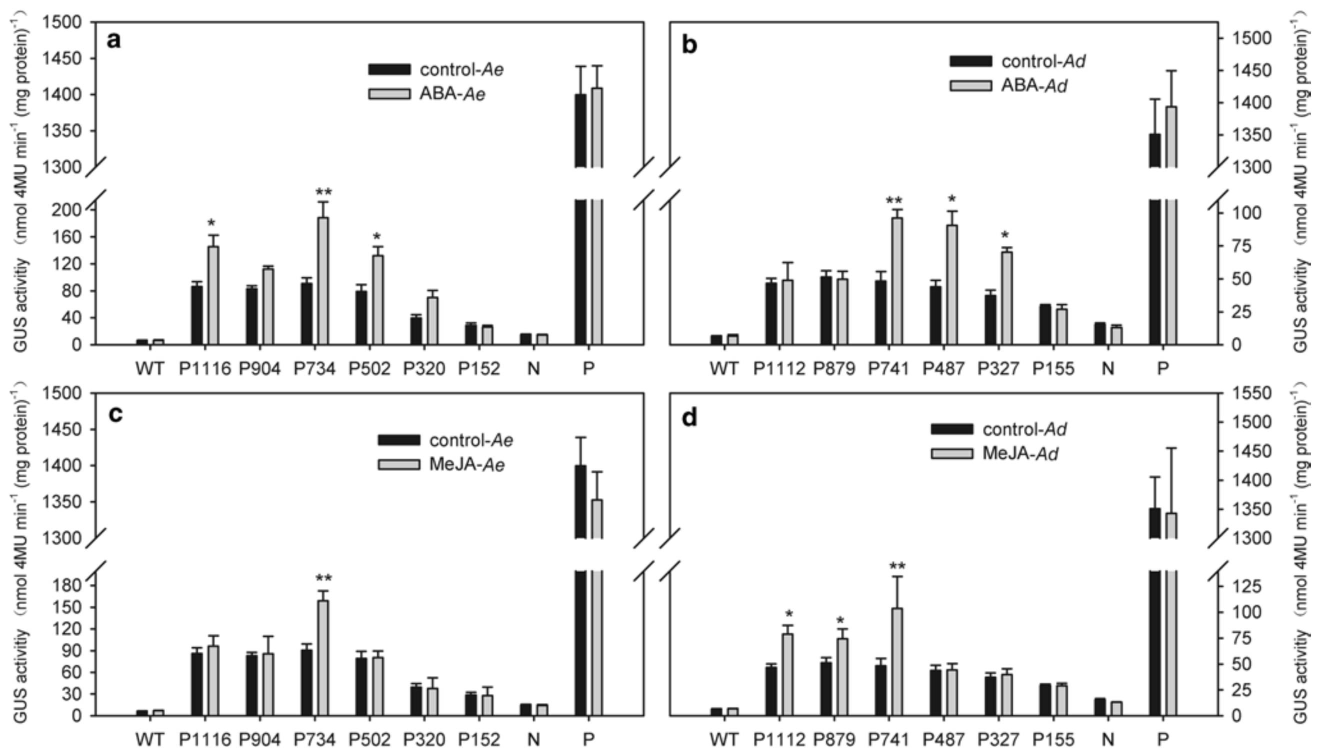

Fig. 6 Analysis of GUS activity from promoters in transiently transformed tobacco leaves in response to ABA or MeJA treatment. ABA-*Ae* (**a**), ABA-*Ad* (**b**), MeJA-*Ae* (**c**), and MeJA-*Ad* (**d**). *Ae*, *A. eriantha*; *Ad*, *A. deliciosa.* Mean activity was averaged from three independent experiments. SD is indicated on each *bar*. Significant difference between treatment and control was assessed by one-sided paired *t* tests (**$P < 0.01$, *$P < 0.05$). *WT* wild type (no expression), *N* negative control (no promoter), *P* positive control (CaMV 35S promoter)

Discussion

In this study, we compared the expression of *GGP* genes and their promoter activities in two kiwifruit species, *A. eriantha* and *A. deliciosa*. Analysis of expression patterns and activities, combined with measurements of AsA levels under stress conditions, allowed us to obtain functional characteristics for two genes. *GGP* is thought to be the rate-limiting step for AsA biosynthesis in plants (Linster and Clarke 2008; Bulley et al. 2009, 2012; Mellidou et al. 2012; Urzica et al. 2012). Our results here provide support for the conclusions from those earlier reports at the transcriptional level. Moreover, *GGP* expression in the leaves examined here were in keeping with the trends in AsA levels that we determined under normal growing conditions. Transcripts were more abundant in *A. eriantha* than in *A. deliciosa.* Likewise, GUS activity was greater for the entire *AeGGP* promoter than for that of *AdGGP* under control conditions. One of the reasons for this may have been that *A. eriantha* had more 5UTR Py-rich stretch *cis*-elements (13 versus 8), which conferred higher levels of transcription (Daraselia et al. 1996).

RT-qPCR results with our kiwifruit leaves revealed that expression of the *GGP* genes was induced by light and abiotic treatments and was clearly correlated with AsA concentrations. Previous studies have indicated that *GGP* mRNA levels are increased in *Arabidopsis thaliana* seedlings grown in the light when compared with those exposed to continuous darkness (Dowdle et al. 2007; Yabuta et al. 2007; Müller-Moulé 2008). *GGP* expression has a circadian rhythm and highly regulated by light levels in *Arabidopsis* (Dowdle et al. 2007). However, we did not observe circadian rhythm in kiwifruit. This may result from the lower light density in chamber which was much lower for kiwifruit plants than that grown under natural conditions. Maybe, *GGP* expression is not highly controlled by circadian rhythm in day in kiwifruit. Further study was needed to explain this point. To elucidate the functional organization of the *GGP* promoters and dissect the promoter elements involved in this dark or light induction, we created 5′-serially deleted *GGP* promoter constructs fused to *GUS*. In both kiwifruit species, GUS activities by most of those deletions decreased under continuous dark treatment but increased in response to continuous light. This implied that some essential light-responsive regulatory sequences are present in the promoter regions. In fact, the predictions from PlantCARE had demonstrated that the *GGP* promoters of these genes from both kiwifruit species contain several LREs, including chs-CMA2b, TCT motif, MNF1, GAG motif, G-Box, and GT1 motif. Based on the GUS

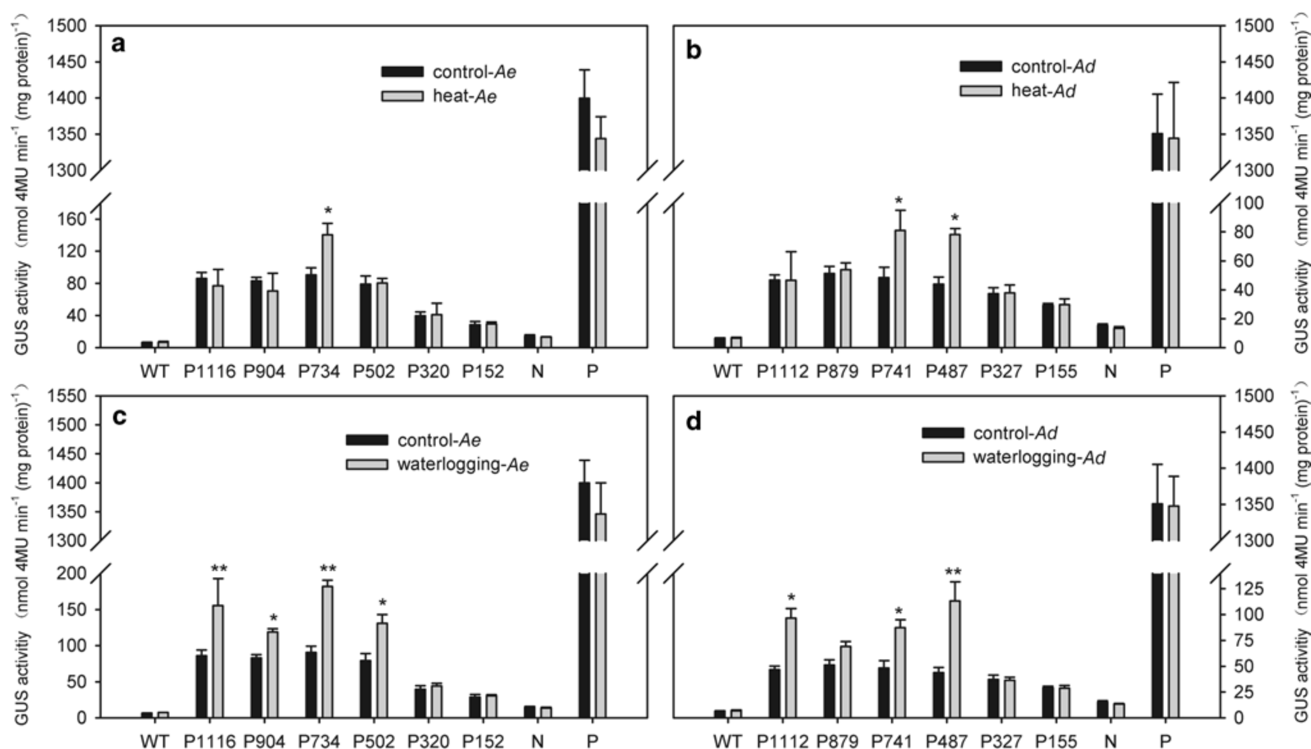

Fig. 7 Analysis of GUS activity from promoters in transiently transformed tobacco leaves in response to heat or waterlogging (hypoxic) treatment. Heat-*Ae* (**a**), heat-*Ad* (**b**), waterlogging-*Ae* (**c**), and waterlogging-*Ad* (**d**). *Ae*, *A. eriantha*; *Ad*, *A. deliciosa.* Mean activity was averaged from three independent experiments. SD is indicated on each *bar*. Significant difference between treatment and control was assessed by one-sided paired *t* tests (***P* < 0.01, **P* < 0.05). *WT* wild type (no expression), *N* negative control (no promoter), *P* positive control (CaMV 35S promoter)

activities for *AeGGP* and *AdGGP* promoter deletions after dark or light treatment, as well as the distribution of LREs in those promoters, we could conclude that the G-box motif, which is in the −320 to −152 region for the *AeGGP* promoter and in the −327 to −155 region for the *AdGGP* promoter, has an important role in regulating *GGP* transcripts under various light conditions in both *A. deliciosa* and *A. eriantha* (Fig. 4). That particular motif is one of the best characterized *cis*-regulatory elements in plants and has been identified in the promoters of a diverse set of unrelated genes, including those controlled by visible and UV light (Hartmann et al. 1998). A family of plant basic leucine zipper (bZIP) proteins also has been identified that interacts with G-box elements to confer high promoter activity (Foster et al. 1994; Martínez-Hernández et al. 2002). Results from a transient expression assay with −397 bp of the *GalUR* promoter, fused to the *LUC* reporter gene, have suggested that the G-box is important for the expression of *GalUR* in strawberry fruit under different types of illumination (Agius et al. 2005).

Levels of AsA increase after applications of ABA, implying that the cellular antioxidative status has a fundamental role in this process (Jimenez et al. 2002). ABA-responsive elements (ABRE) are also important for increasing the transcriptional levels of *GGP* and the GUS activities of *GGP* promoters. For example, we found that that, except for construct P904, GUS activity for the *AeGGP* promoter (containing the region from −1116 to −502) was significantly induced by ABA. We deduced that the ABRE, at position −445, may play an important role in *A. eriantha*. However, there may be some negative element in the region from −904 to −734 of the *AeGGP* promoter that offsets ABRE induction in that species. In addition, GUS activity for the *AdGGP* promoter (containing the region from −741 to −327) was significantly induced by ABA, leading us to conclude that the ABRE at −210 (Fig. 4) may have an important role in that promoter.

Methyl jasmonate (MeJA) is a naturally occurring regulator of development in higher plants, as well as a compound that controls gene expression and responses to external stimuli (Creelman and Mullet 1995). In our study, MeJA enhanced promoter activities and induced *GGP* expression in parallel with an increase in AsA levels in the two kiwifruit species. We noted the presence of the CGTCA motif in the *GGP* promoter sequences, which are involved in MeJA responsiveness. After MeJA treatment, GUS activity was induced only in P734 of the *AeGGP* promoter deletions. We also found a CGTCA motif at

position −582 in that promoter (Fig. 4). GUS activity for the *AdGGP* promoter (−1,112 to −741) was significantly induced by MeJA. We deduced that the CGTCA motif at −1,059 and −592 has an important role in that promoter (Fig. 4). Therefore, these results demonstrate that this motif possibly assists in regulating the expression of *GGP* when MeJA acts as a signaling molecule. In addition, this responsiveness to MeJA may be combined with other effector pathways to determine the conditions under which genes respond to this hormone (Creelman and Mullet 1995).

Elevated levels of AsA might protect plants from injury upon exposure to heat (Ma et al. 2008). Our results showed that expression of *AeGGP* and *AdGGP* was slightly increased by this stimulus. By contrast, expression of the *GGP* gene from tomato is not altered by heat stress (Ioannidi et al. 2009). However, some *GGP* promoter deletions for our two kiwifruit species were significantly induced by heat in the transformed tobacco leaves. For *AeGGP*, only P734 was significantly induced. Interestingly, HSE, a *cis*-acting element involved in heat stress responsiveness, was found at position −566 in that promoter (Fig. 4). For *AdGGP*, GUS activities by P741 and P487 were significantly induced by heat. Except for one HSE element at −576, two TC-rich repeat motifs (Fig. 4), both *cis*-acting elements that are involved in defenses and stress responsiveness, were found at positions −535 and −428 in the *AdGGP* promoter.

Waterlogging creates hypoxic conditions for plants. In the early stages of our hypoxia treatments, AsA concentrations decreased to various extents in both kiwifruit species, possibly as a means for protecting those plants against damage related to oxidative stress. Their enhanced *GGP* transcripts may have served to rescue AsA concentrations in such an environment. Recombinant *C. reinhardtii* cells exposed to oxidative stress show increased levels of *GGP* mRNA and AsA (Urzica et al. 2012). Furthermore, transcripts of tomato *GGP* rise dramatically at 3 h after being transferred from anoxia treatment to air (Ioannidi et al. 2009). Here, GUS activities for our *GGP* promoter deletions were partially induced by hypoxia. The ARE motifs are *cis*-acting regulatory elements that are essential for anaerobic induction. Although we found one and two such motifs in the *AdGGP* and *AeGGP* promoters, respectively, the existence of their deletions implied that ARE may not play an important role in hypoxia stress. We believe this because the GUS activities of some deletions without the ARE element were also significantly induced by waterlogging. We speculated that large chunks of the promoter region have been removed which could have multiple effects on gene expression. Therefore, we cannot exclude the possibility that other interactions, such as between transcription factors and *cis*-regulatory elements, might activate the promoter to up-regulate *GUS* expression in tobacco.

In summary, differential gene expression was detected within two kiwifruit species in response to light or abiotic stresses. This finding expands our understanding of the regulatory mechanisms for *GGP* expression and confirms the important role that this gene has in determining AsA levels. Comparative analysis of *GGP*s and the promoter region in these two species demonstrated that the genes are conserved, but their non-coded upstream regions have diverged, providing us with a new clue for investigating the mechanism for gene regulation. In addition, our data support the hypothesis that *GGP* has a critical function in controlling AsA concentrations in kiwifruit species.

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